

## Pathogen profile

**Plum pox virus and sharka: a model potyvirus and a major disease**

JUAN ANTONIO GARCÍA<sup>1,\*</sup>, MIROSLAV GLASA<sup>2,\*</sup>, MARIANO CAMBRA<sup>3,\*</sup> AND THIERRY CANDRESSE<sup>4,\*</sup>

<sup>1</sup>Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología (CNB-CSIC), Campus Universidad Autónoma de Madrid, 28049 Madrid, Spain

<sup>2</sup>Institute of Virology, Slovak Academy of Sciences, Dubravska cesta 9, 84505 Bratislava, Slovakia

<sup>3</sup>Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera de Moncada-Nàquera km-5, 46113 Moncada, Valencia, Spain

<sup>4</sup>Equipe de Virologie, UMR 1332 Biologie du Fruit et Pathologie, INRA, Université de Bordeaux, CS 20032, 33882 Villenave d'Ornon Cedex, France

**SUMMARY**

**Taxonomic relationships:** *Plum pox virus* (PPV) is a member of the genus *Potyvirus* in the family *Potyviridae*. PPV diversity is structured into at least eight monophyletic strains.

**Geographical distribution:** First discovered in Bulgaria, PPV is nowadays present in most of continental Europe (with an endemic status in many central and southern European countries) and has progressively spread to many countries on other continents.

**Genomic structure:** Typical of potyviruses, the PPV genome is a positive-sense single-stranded RNA (ssRNA), with a protein linked to its 5' end and a 3'-terminal poly A tail. It is encapsidated by a single type of capsid protein (CP) in flexuous rod particles and is translated into a large polyprotein which is proteolytically processed in at least 10 final products: P1, HCPro, P3, 6K1, CI, 6K2, VPg, NIap, NIb and CP. In addition, P3N-PIPO is predicted to be produced by a translational frameshift.

**Pathogenicity features:** PPV causes sharka, the most damaging viral disease of stone fruit trees. It also infects wild and ornamental *Prunus* trees and has a large experimental host range in herbaceous species. PPV spreads over long distances by uncontrolled movement of plant material, and many species of aphid transmit the virus locally in a nonpersistent manner.

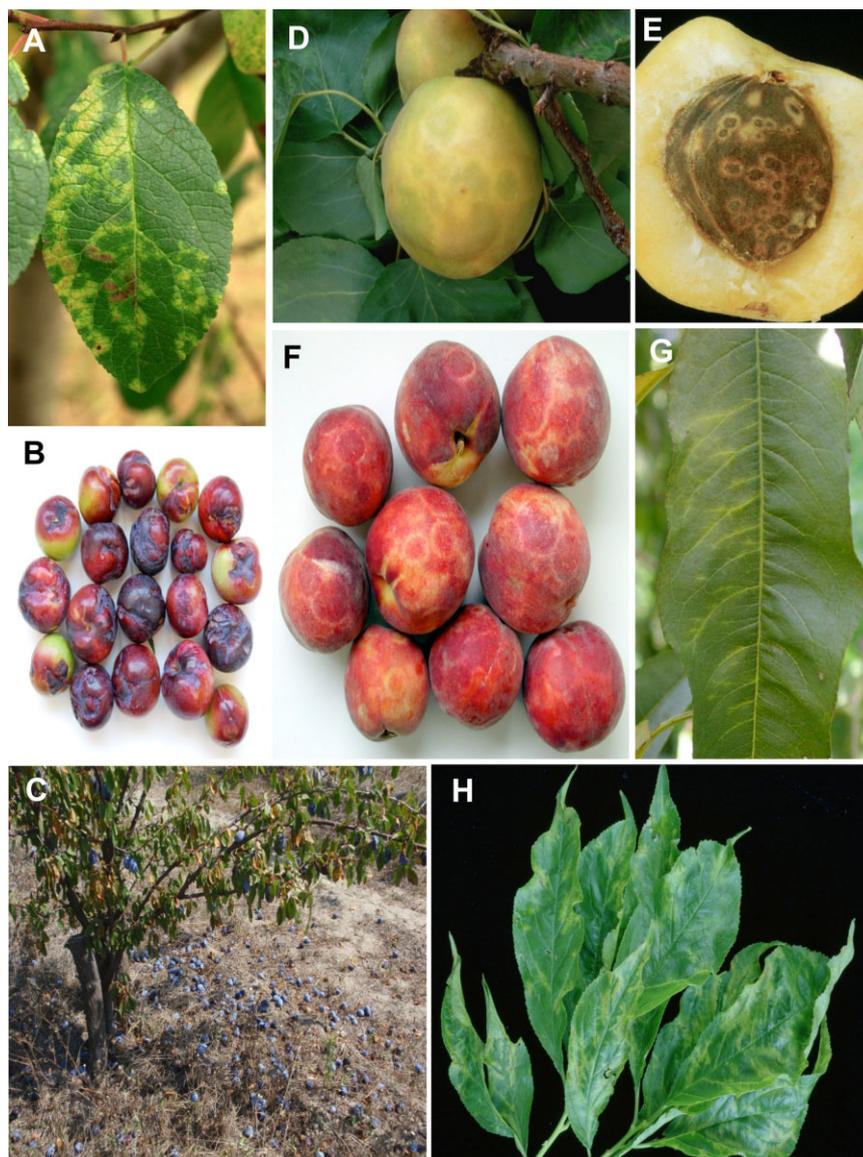
**Sources of resistance:** A few natural sources of resistance to PPV have been found so far in *Prunus* species, which are being used in classical breeding programmes. Different genetic engineering approaches are being used to generate resistance to PPV, and a transgenic plum, 'HoneySweet', transformed with the viral CP gene, has demonstrated high resistance to PPV in field tests in several countries and has obtained regulatory approval in the USA.

**INTRODUCTION**

Sharka (plum pox), caused by *Plum pox virus* (PPV), is the most serious viral disease for the stone fruit industry, particularly because it causes severe losses in susceptible cultivars and is spread efficiently by aphids. As a result of domestic and international regulations, the presence of the pathogen in an area greatly complicates stone fruit production and the multiplication and trade of nursery plants. Sharka was first reported in plum trees in Bulgaria in 1917–1918 and was recognized as a viral disease by Atanasoff (1932). Since then, the virus has spread progressively to most of Europe, around the Mediterranean basin and the Near and Middle East. It has also spread to South and North America and Asia (Barba *et al.*, 2011). Despite considerable efforts and quarantine regulations in many countries, sharka has been reported in most of the important *Prunus* industries worldwide, and is occasionally intercepted in internationally traded *Prunus* planting material. The disease has not been reported to date in California (USA), Australia, New Zealand and South Africa [European and Mediterranean Plant Protection Organization (EPPO), 2013].

Under natural conditions, the disease affects plants of the genus *Prunus*, used as commercial cultivars as well as rootstocks: *P. armeniaca*, *P. cerasifera*, *P. davidiana*, *P. domestica*, *P. mahaleb*, *P. marianna*, *P. mume*, *P. persica*, *P. salicina* and interspecific hybrids between these species. *Prunus avium*, *P. cerasus* and *P. dulcis* may be infected occasionally or only by specific PPV strains. In addition, several ornamental and wild *Prunus* species have been identified as natural or experimental hosts of PPV (Damsteegt *et al.*, 2007; James and Thompson, 2006). Sharka is particularly detrimental in apricots, European plums, peaches and Japanese plums because it can seriously reduce yield and fruit quality. Losses in susceptible cultivars may reach 100% in some cases (Kegler and Hartmann, 1998; Németh, 1994). The alcohol and spirits produced from diseased fruits also see their yield and quality reduced. PPV symptoms may appear on leaves, shoots, bark, petals, fruits and even stones (Fig. 1). They are usually distinct on leaves early in the growing season and include mild light-green discoloration, chlorotic spots, bands or rings, vein clearing or yellowing and leaf deformation. Flower symptoms can

\*Correspondence: Email: jagarcia@cnb.csic.es, Miroslav.Glasa@savba.sk, mcambra@ivia.es, candress@bordeaux.inra.fr



**Fig. 1** Typical symptoms induced by *Plum pox virus* on a domestic plum leaf (A), domestic plum fruits (B), premature domestic plum fruit drop (C), an apricot fruit (D), an apricot stone (E), peach fruits (F), a peach leaf (G) and Japanese plum leaves (H). (A, B, D, E and F) were kindly supplied by Dr M. A. Cambra, Centro de Protección Vegetal y Certificación, Diputación General de Aragón, Montañana-Zaragoza, Spain.

occur on petals (discoloration) of some cultivars. Infected fruits show chlorotic spots or lightly pigmented yellow rings or line patterns. Fruits may become deformed or irregular in shape, and may develop brown or necrotic areas under the discoloured rings. European plums and apricots may also show premature fruit drop, whereas Japanese plums and peaches show ring spotting on fruits. The stones from diseased apricots show typical pale rings or spots. Sweet and sour cherry fruits undergo fruit deformations and premature drop. Infected almond trees generally show no or inconspicuous leaf symptoms. Generally, the fruits of early maturing cultivars of all susceptible species show more marked symptoms than those of late maturing cultivars. PPV also experimentally infects a number of herbaceous hosts (Llácer, 2006; Polák, 2006). Further information about PPV and sharka disease, including illustrations of disease symptoms, can be found in Barba

*et al.* (2011), CABI (2013), EPPO (2004, 2006), García and Cambra (2007), PaDIL (2013) and Sochor *et al.* (2012).

The costs associated with the disease in many countries involve not only direct losses related to yield and quality losses, quarantine, eradication and compensatory measures, but also indirect costs related to preventative measures, inspections, diagnostics and their impact on foreign and domestic trade (Barba *et al.*, 2011). It is estimated that the costs of managing sharka worldwide since the 1970s have exceeded 10 000 million euros (Cambra *et al.*, 2006c).

## EPIDEMIOLOGY AND TRANSMISSION

The illegal traffic and insufficiently controlled exchanges of plant material in a global market are the main pathways for PPV

spread over long distances. The introduction of infected propagative plant material is followed by natural and local spread by aphids. PPV is graft transmitted and the vegetative multiplication of infected plants greatly contributes to the spread of the virus from infected areas if certified virus-free material is not used. Once PPV has become established in an orchard, a number of aphid species with a worldwide distribution may transmit the virus locally in a noncirculative, nonpersistent manner (Ng and Falk, 2006), with *Myzus persicae*, *Aphis spiricola* and *Hyalopterus pruni* being the main vector species (Cambra *et al.*, 2006b; Gildow *et al.*, 2004; Labonne and Dallot, 2006). A single probe of a viruliferous aphid is sufficient to inoculate about 26 000 PPV RNA molecules in a receptor GF305 peach seedling, with a 20% chance of resulting in a systemic infection (Moreno *et al.*, 2009).

The efficiency of natural transmission by aphids and the spatial pattern of spread of sharka may differ for different PPV isolates and host cultivars (Dallot *et al.*, 2003; Sutic *et al.*, 1976). In southern Europe and North America, preferential movement of viruliferous aphids to trees several tree spaces away was observed (Gottwald, 2006; Gottwald *et al.*, 1995). Other virus–host combinations showed a compound contagion process with long-range (up to 150 m) and short-range to adjacent tree movements in Spain (Capote *et al.*, 2010). In France, 90% of diseased trees were found within 200 m of previously infected ones, but natural dissemination at distances over 600 m has also been recorded (Labonne and Dallot, 2006). Infections starting with a completely random spatial pattern which finally reaches a uniform distribution in the orchard have also been reported (Varveri, 2006). The application of horticultural mineral oil has been shown to be an efficient control strategy to reduce PPV incidence in nursery plots (Vidal *et al.*, 2013).

Several weed species can be infected with PPV, but the significance of weeds in the epidemiology of the disease is considered to be negligible (Llácer, 2006). There is no confirmed evidence for seed or pollen transmission of PPV in any of its *Prunus* hosts (Pasquini and Barba, 2006).

## DETECTION AND IDENTIFICATION

To avoid PPV spread over long distances by the movement of plant material, reliable detection methods are needed for the accurate detection of the virus in symptomless nursery plants and propagative material. Two official and validated international protocols for the detection and characterization of PPV strains have been developed [EPPO, 2004; International Plant Protection Convention-Food and Agriculture Organization (IPPC-FAO), 2012]. An update of these protocols is currently being prepared by EPPO. The recommended methods include biological indexing, serological and molecular assays, as well as sampling, reagents and detailed protocols for each technique.

The choice of the most appropriate PPV detection method is crucial and must be adapted to the purpose of the analysis and to the expected prevalence of the disease (Vidal *et al.*, 2012a, b).

Biological indexing based on graft inoculation of GF305 (*P. persica* seedlings), Nemaguard (*P. persica* × *P. davidiana*, hybrid seedling) and/or *P. tomentosa* is best performed according to Damsteegt *et al.* (1997) and Gentit (2006). Serological enzyme-linked immunosorbent analyses (ELISAs) based on the PPV-specific monoclonal antibody 5B-IVIA/AMR or on polyclonal antibodies have been used extensively for the universal detection of PPV isolates (Cambra *et al.*, 2006a, 2011). Molecular techniques based on reverse transcription-polymerase chain reaction (RT-PCR) assays were first reported for the detection of PPV by Wetzel *et al.* (1991b). In subsequent years, other RT-PCR systems, as well as variants based on hemi-nested, nested RT-PCR in a single closed tube and co-operational-PCR techniques, have been developed to increase sensitivity (García and Cambra, 2007). Nowadays, the technique of choice for nucleic acid-based PPV detection is real-time RT-PCR (Olmos *et al.*, 2005; Schneider *et al.*, 2004), but loop-mediated isothermal amplification (LAMP) has also been developed into an interesting option (Varga and James, 2006b). Protocols are available for the direct use of plant crude extracts or immobilized tissue prints of plant samples feasible as PCR targets, instead of purified RNA (Capote *et al.*, 2009). Reviews of these user-friendly methods are available (De Boer and Lopez, 2012; Moreno *et al.*, 2009). In order to estimate diagnostic parameters, such as sensitivity, specificity and likelihood ratios, of different PPV detection methods, latent class models using maximum likelihood functions and a Bayesian approach have been employed by Vidal *et al.* (2012a). The basic conclusions were as follows: (i) ELISA (5B-IVIA/AMR based) is highly specific and is recommended when low prevalence of PPV is expected; moreover, it is sufficiently sensitive to consistently detect PPV in composite samples of four plants in spring and summer; and (ii) the highly sensitive spot real-time RT-PCR can be successfully used to detect PPV in composite samples (up to 10) in any season of the year, and to assess the PPV-free status of key material because of its high negative predictive values. The use of sensitive real-time RT-PCR is recommended when more than 10% PPV prevalence is expected. The combination of both techniques reaches 100% accuracy in any season of the year (Olmos *et al.*, 2008).

Strain-specific monoclonal antibodies (Cambra *et al.*, 2006a, 2011; Candresse *et al.*, 1998, 2011) or molecular methods based on RT-PCR amplification and sequencing (Capote *et al.*, 2006; Glasa *et al.*, 2011, 2013; Olmos *et al.*, 1997, 2002; Šubr *et al.*, 2004; Varga and James, 2005, 2006a) can be used for the identification or characterization of PPV strains. These methods are summarized in the IPPC-FAO (2012) protocol for PPV diagnosis.

## CAUSATIVE AGENT: GENOME AND EXPRESSION

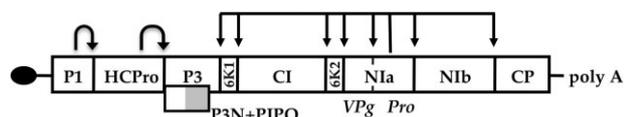
### Genome and capsid structure

PPV is a member of the genus *Potyvirus* of the family *Potyviridae* (Adams *et al.*, 2012; López-Moya and García, 2008). Its genome consists of a positive-sense single-stranded RNA (ssRNA) of 9741–9795 nucleotides (Faniigliulo *et al.*, 2003; Glasa and Šubr, 2005; Glasa *et al.*, 2011, 2013; James and Varga, 2005; Laín *et al.*, 1989; Maejima *et al.*, 2011; Maiss *et al.*, 1989; Myrta *et al.*, 2006; Palkovics *et al.*, 1993; Schneider *et al.*, 2011; Teycheney *et al.*, 1989; Ulubaş Serçe *et al.*, 2009; SharCo database, <http://w3.pierroton.inra.fr:8060/>).

The PPV genomic RNA has a protein (viral protein genome-linked, VPg) linked to its 5' end and a 3'-terminal poly A tail (Riechmann *et al.*, 1989), and is encapsidated by a single type of capsid protein (CP) subunit. However, detectable levels of another viral protein, helper component proteinase (HCPro), have been found to be associated with PPV virions (Manoussopoulos *et al.*, 2000). This association could be related to the ability of HCPro to act as a bridge between virus particles and the stylet of aphids which specifically transmit the virus (Blanc *et al.*, 1997; López-Moya *et al.*, 1995; Roudet-Tavert *et al.*, 2002). However, roles unrelated to aphid transmission have also been suggested for interactions between HCPro and CP (Roudet-Tavert *et al.*, 2002).

### RNA translation and proteolytic processing

Most of the genomic RNA encodes a long open reading frame (ORF) which is translated into a polyprotein of about 355 kDa, starting from its second AUG codon (nucleotides 147–149) (Riechmann *et al.*, 1991) probably by a leaky scanning mechanism (Simón-Buela *et al.*, 1997a). This polyprotein is processed by three virus-encoded proteinases to produce at least 10 mature protein products: P1, HCPro, P3, 6K1, CI, 6K2, VPg, NIap, NIb and CP (Fig. 2). As reported for other potyviruses (Chung *et al.*, 2008), another PPV protein, P3N-PIPO, is predicted to be produced by a frameshift into a short ORF embedded within the P3 coding sequence.



**Fig. 2** Genomic map of *Plum pox virus*. The long open reading frame (ORF) is represented by a rectangular box divided into viral products by solid black lines. PIPO ORF, translatable with a frameshift, is indicated by a grey box below the P3 region. Cleavage sites recognized by the indicated proteinases are signalled by arrows. The terminal protein (VPg) is represented as a black ellipse.

The N-terminal region of the PPV polyprotein is processed by the serine proteinase P1 and the cysteine proteinase HCPro, which cleave at their respective C-termini (García *et al.*, 1993; Ravelonandro *et al.*, 1993). The proteolytic activity of the C-terminal catalytic domain of the P1 protease requires the contribution of a host factor present in wheat germ, but not in rabbit reticulocyte lysate (Rodamilans *et al.*, 2013).

NIap is the protease involved in the cleavage of the central and C-terminal regions of the PPV polyprotein (García *et al.*, 1989b). It is linked to the protein VPg in the NIa product, which, together with the protein NIb, forms crystalline inclusions, mainly located in the nucleus, but also detected in the cytoplasm of PPV-infected cells (Martín *et al.*, 1992; van Oosten and van Bakel, 1970). Processing by NIap takes place at sites characterized by a consensus sequence  $e/q-x-V-x-H-Q/e\downarrow_s$ , and appears to be highly regulated, allowing partially processed products to play functional roles (García *et al.*, 1989a, 1990, 1992). For instance, although mature 6K1 has been detected in PPV-infected cells (Waltermann and Maiss, 2006), a main functional role has been suggested for the unprocessed P3 + 6K1 protein (Riechmann *et al.*, 1995).

### RNA replication, movement and counteraction of host defences

As is a general rule for plus-strand RNA viruses (Grangeon *et al.*, 2012), PPV RNA replication takes place in association with intracellular membranes (Martín and García, 1991). Leaf extracts in which PPV RNA is synthesized *in vitro* are enriched in endoplasmic reticulum and tonoplast vesicles, but no *in vivo* information is available about the PPV replication complexes (Martín *et al.*, 1995). However, they should not differ very much from the membrane vesicles and large perinuclear ring-like structures in which RNA replication of other potyviruses has been shown to occur (Cotton *et al.*, 2009; Grangeon *et al.*, 2010, 2012; Wei and Wang, 2008; Wei *et al.*, 2010b). In these structures, the potyviral RNA is replicated by the RNA-dependent RNA polymerase NIb (Hong and Hunt, 1996), using as primer VPg uridylylated by the same polymerase (Anindya *et al.*, 2005; Puustinen and Mäkinen, 2004). Another viral factor required for PPV replication is the CI protein (Fernández *et al.*, 1997), which forms the pinwheel-shaped inclusions typical of potyviral infections (Martín *et al.*, 1992), and has NTPase and RNA helicase activities (Fernández *et al.*, 1995; Laín *et al.*, 1990, 1991).

Several studies with different potyviruses, including PPV, have shown that the CI protein is also involved in virus movement (Carrington *et al.*, 1998; Gómez de Cedrón *et al.*, 2006). As expected from its ability to form inclusion bodies, PPV CI is able to self-interact (López *et al.*, 2001); however, CI–CI interactions required for RNA replication and virus movement appear to be different to some extent (Gómez de Cedrón *et al.*, 2006).

Results obtained with *Turnip mosaic virus* suggest that, together with P3N-PIPO, CI coordinates the formation of conical structures at plasmodesmata for cell-to-cell spread (Wei *et al.*, 2010a). Specific interactions of CI with virus particles might be important for virus movement, but also for RNA uncoating and translation initiation (Gabrenaite-Verkhovskaya *et al.*, 2008).

To be amplified in a host plant, the virus not only has to complete the processes of replication and movement, but also needs to escape the plant antiviral defences. Thus, the proteinase HCPro of PPV is not only required for aphid transmission, but is also essential to counteract antiviral RNA silencing (Tenllado *et al.*, 2003; Varrelmann *et al.*, 2007).

### Post-translational modifications

Given the limited size of the genome of plus-strand RNA viruses, it is not surprising that many proteins of these viruses are multifunctional and their activities require a meticulous regulation. Post-translational modifications could contribute to this regulation. The CP protein, which is expected to be involved in the control of the genomic RNA allocated for translation, replication and propagation during potyviral infection (Ivanov *et al.*, 2001), is phosphorylated (Fernández-Fernández *et al.*, 2002a; Šubr *et al.*, 2007) and *O*-*N*-acetylglucosylated (*O*-GlcNAcylated) by the *O*-GlcNAc transferase SECRET AGENT (Chen *et al.*, 2005; Fernández-Fernández *et al.*, 2002a; Scott *et al.*, 2006). Specific sites of *O*-GlcNAc modification (Kim *et al.*, 2011; Pérez *et al.*, 2006, 2013) and a single amino acid mutation that appears to alter the phosphorylation status of the protein (Šubr *et al.*, 2010) have been mapped to the N-terminal region of PPV CP. Although *O*-GlcNAcylation of CP is not essential for PPV infectivity, it plays a relevant role in the infection process (Chen *et al.*, 2005; Pérez *et al.*, 2013).

### PPV DIVERSITY

Given its economic importance, much effort has been devoted to the study of the biological, serological and molecular variability of PPV. This effort has revealed that the diversity of PPV is structured into individual monophyletic ensembles of closely related isolates, which have been designated as strains. Currently, eight strains are recognized for PPV, which may be more than for any other potyvirus.

Initially, the existence of two different PPV serotypes, named M (Marcus) and D (Dideron), was reported by Kerlan and Dunez (1979). With the advent of molecular biology, these two serotypes have been confirmed to represent two molecularly distinct strains based on their genome sequences (Lain *et al.*, 1989; Maiss *et al.*, 1989; Palkovics *et al.*, 1993; Teycheney *et al.*, 1989).

PPV-D is widespread in Europe, whereas PPV-M is found mainly in southern and central European countries. PPV-D is also respon-

sible for most outbreaks outside of Europe (Damsteegt *et al.*, 2001; Maejima *et al.*, 2011; Reyes *et al.*, 2003). Although widely present on apricots and plums, this strain is less frequently associated with peach under natural conditions. The PPV-M strain can be split into two subgroups that show partial geographical separation, but so far have not been reported from outside Europe (Dallot *et al.*, 2011; Myrta *et al.*, 2001). PPV-M isolates are efficiently aphid transmitted, causing fast epidemics, mainly in peach orchards (Capote *et al.*, 2010; Dallot *et al.*, 2003).

In addition to the two major PPV-D and PPV-M strains, two minor strains were identified in the 1990s. The substantial divergence in the genomic sequence of the Egyptian El Amar isolate has led to its classification into the distinct PPV-EA strain (Glasa *et al.*, 2006; Myrta *et al.*, 2006; Wetzel *et al.*, 1991a), which remains geographically limited to Egypt, where additional isolates have been found on apricot, peach and Japanese plum (Matic *et al.*, 2011; Youssef and Shalaby, 2006).

PPV isolates naturally infecting sour cherries in Moldova were classified into a new, PPV-C (cherry), strain (Kalashyan *et al.*, 1994; Nemchinov *et al.*, 1996). Later, occasional findings of molecularly similar PPV isolates in sour and sweet cherries were reported from Italy (Crescenzi *et al.*, 1997; Fanigliulo *et al.*, 2003), Hungary (Nemchinov *et al.*, 1998), Belarus (Malinowski *et al.*, 2012) and Croatia (Kajic *et al.*, 2012). Given its restricted natural host range, the actual epidemiological impact of PPV-C seems to be lower than that of the major PPV strains.

The picture of PPV genetic diversity has changed further in the past 10 years. The development of detection tools targeting different parts of the genome (Glasa *et al.*, 2002) has led to the discovery of a homogeneous group of isolates deriving from a recombination between PPV-M and PPV-D. These isolates were classified as the PPV-Rec (Recombinant) strain and have been found in several European countries, as well as outside Europe, mainly infecting plum and apricot trees (Candresse *et al.*, 2007; Glasa *et al.*, 2002, 2004; Matic *et al.*, 2006; Thompson *et al.*, 2009). The efficient aphid transmission of PPV-Rec isolates has been demonstrated (Glasa *et al.*, 2004). Given its wide distribution and prevalence, PPV-Rec is now considered as the third major PPV strain. As the first reported PPV recombinant isolate originated from Serbia (Cervera *et al.*, 1993), the Balkans have been suggested to be the centre of origin of PPV-Rec, which then spread to other areas through the exchange of infected propagation material of tolerant plum genotypes (Glasa *et al.*, 2005).

A divergent PPV-W3174 isolate was originally detected in 2003 in a plum tree in Canada (James and Varga, 2005) and, based on its molecular distinctiveness, was assigned to a new strain, PPV-W (Winona). Later, PPV-W isolates were recorded in Latvia, Ukraine and Russia (Glasa *et al.*, 2011; Mavrodieva *et al.*, 2013; Sheveleva *et al.*, 2012), confirming the suggestion that the origin of this strain may be found in eastern Europe. Moreover, these new

PPV-W isolates differed from the W3174 Canadian isolate in not being affected by the two recombination events detected in the W3174 genome (Glasa *et al.*, 2011). The W strain has been found in the field on plum, blackthorn, Canadian plum, cherry plum and downy cherry (Mavrodieva *et al.*, 2013). The analysis of partial and complete genome sequences indicates that PPV-W diversity is greater than that of the other PPV strains (Glasa *et al.*, 2012; Mavrodieva *et al.*, 2013; Sheveleva *et al.*, 2012).

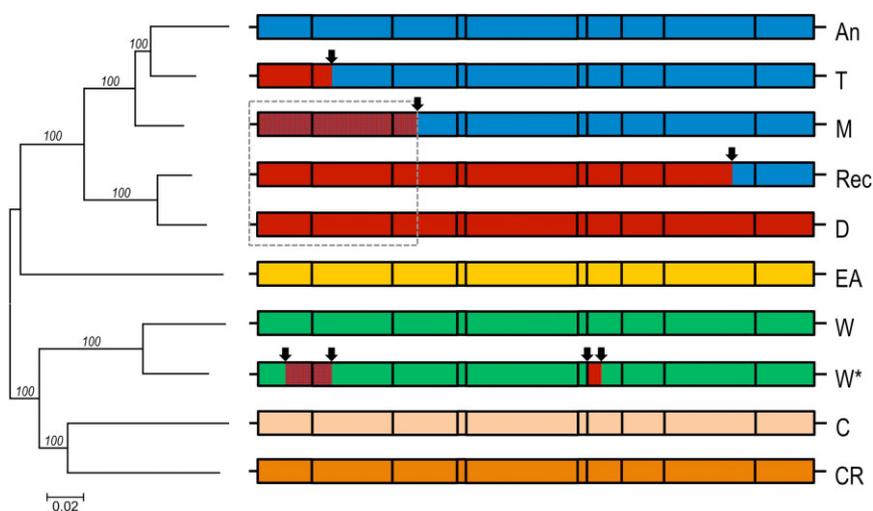
Genome characterization of the atypical Turkish Ab-Tk isolate has revealed a recombination event affecting its 5' genomic region (Glasa and Candresse, 2005; Ulubaş Serçe *et al.*, 2009). Further surveys have confirmed the occurrence of closely related isolates in the Ankara region in Turkey, which have been classified into a new strain, PPV-T (Turkey) (Ulubaş Serçe *et al.*, 2009). PPV-T isolates have been found to be widely distributed in apricots, peaches and plums in Turkey, and an occasional finding of PPV-T has been recorded from Albania (unpublished results of the European SharCo FP7 project).

Very recently, unusual PPV isolates recovered from naturally infected sour cherries in the Volga river basin (Russia) have been characterized and proposed to form a second cherry-adapted strain, PPV-CR (Cherry Russian) (Glasa *et al.*, 2013). The spread of similar isolates was confirmed in old sour cherry trees in the Moscow region (Chirkov *et al.*, 2013). The epidemiology of this strain remains to be determined.

An additional putative PPV strain (PPV-An) could be represented by a recently identified isolate from eastern Albania (Palmisano *et al.*, 2012). The full-length genomic sequence of this isolate fulfils the features of an ancestral PPV-M isolate previously hypothesized in the PPV evolutionary scenario (Glasa and Candresse, 2005; Fig. 3).

Full-length genomic sequences have been determined for PPV isolates representing each of the recognized strains, providing a clear picture of the phylogenetic relationship between strains and of the PPV evolutionary history. PPV strains are characterized by relatively low intrastrain diversity (reaching 1.1%–3.9% at the nucleotide level for full-length genomes, except for PPV-W, where the divergence reaches 7.9%) and by comparatively high between-strain diversity (4.4%–22.8%; Glasa *et al.*, 2012). Despite the extensive exchanges of *Prunus* propagation material, PPV strains still show, for at least some of them, a partial or complete geographical structure. The analysis of PPV diversity has also provided the first indications that recombination plays a role in the evolution of potyviruses (Cervera *et al.*, 1993). Although forming monophyletic groups, PPV-M, PPV-D, PPV-Rec, PPV-T and PPV-W are evolutionarily linked by recombination events, including an ancestral recombination affecting the 5' part of PPV-M, PPV-D and PPV-Rec strains (Fig. 3).

The possibility that future surveys of PPV variability, in particular in poorly explored areas such as Asia, or employing new unbiased



**Fig. 3** Phylogenetic and recombination analysis of *Plum pox virus* (PPV) strains. Phylogenetic tree of representative PPV isolates belonging to the known PPV strains (left). The genomic organization and recombination history of the corresponding PPV strains are shown on the right. The phylogenetic tree was reconstructed with the neighbour-joining technique from full-length nucleotide sequences and bootstrap (1000 replicates) was used to evaluate branch validity. The following sequences were used: PPV-An (unpublished sequence; Palmisano *et al.*, 2012); PPV-T (EU734794); PPV-M (AJ243957); PPV-Rec (AY028309); PPV-D (AY912057); PPV-EA (DQ431465); PPV-C (HQ840518); PPV-CR (KC020126). For PPV-W, two isolates were used to reflect differences in recombination history between members of this strain: LV-145bt (HQ670748) and W3174 (AY912055), which is marked by an asterisk. For the right panel, strains are colour coded and arrows mark the recombination breakpoints identified. The 5' genome portion in PPV-M, PPV-D and PPV-Rec, affected by an ancestral recombination, is boxed and the colour of the affected region in PPV-M is modified from that of the parental PPV-D to reflect its divergence posterior to the recombination event.

and high-throughput next-generation sequencing technologies, may reveal further new, unusual or emerging forms of PPV in the future, cannot be excluded.

### PATHOGENICITY AND HOST RANGE DETERMINANTS

Although PPV strains are entities clearly differentiated from molecular, serological and evolutionary perspectives, it is much less clear whether they show specific biological features, such as pathogenicity, host range and epidemiological behaviour (Candresse and Cambra, 2006). Under field conditions, PPV-Rec isolates are rarely found in peach, and experimental transmission to the peach seedling indicator GF305 results in very mild symptoms, suggesting that PPV-Rec could be poorly adapted to peach (Candresse and Cambra, 2006; Glasa *et al.*, 2004). Moreover, as mentioned above, PPV isolates of strain M seem to spread more readily to peach than isolates of strain D, which is generally considered to be poorly epidemic in peach (Candresse and Cambra, 2006; Llácer and Cambra, 2006). However, this conclusion is challenged by the existence of atypical PPV-D isolates that efficiently spread in peach, suggesting that some pathogenicity properties could be more dependent on isolate-specific traits, rather than on strain-specific ones (Dallot *et al.*, 1998; Glasa *et al.*, 2010; Levy *et al.*, 2000).

The most conspicuous strain-specific pathogenicity feature of PPV is the ability to infect cherry trees of isolates of the PPV-C and PPV-CR strains (Chirkov *et al.*, 2013; Crescenzi *et al.*, 1997; Glasa *et al.*, 2013; Nemchinov and Hadidi, 1996; Nemchinov *et al.*, 1996). However, although PPV-C isolates appear to be specifically adapted to cherry, they are also able to infect other *Prunus* species under experimental conditions (Bodin *et al.*, 2003; Crescenzi *et al.*, 1997; Nemchinov and Hadidi, 1996).

The characterization of molecular determinants of specific pathogenicity traits of PPV isolates in the field has been hampered by several factors, such as high within-strain variability and the differential epidemiological behaviour of an isolate depending on the *Prunus* host or on local agroecological conditions, etc. In addition, a substantial amount of intra-isolate variability is observed within single *Prunus* trees, demonstrating the dynamic structure and heterogeneous nature of PPV populations (Jridi *et al.*, 2006; Predajňa *et al.*, 2012b).

However, some information is available about the determinants of pathogenicity and host range of PPV in experimental conditions, mainly in herbaceous plants. Using a collection of *Arabidopsis thaliana* accessions, it has been shown that multiple specific interactions between virus and host factors control PPV infection (Decroocq *et al.*, 2006). PPV isolates of the C strain are unable to infect systemically any of the *Arabidopsis* ecotypes, whereas some *Arabidopsis* ecotypes are specifically infected by particular PPV isolates. Thus, isolates of the PPV-EA and PPV-M

strains were able to systemically spread only in *Arabidopsis* ecotypes or mutants with a dysfunctional resistance to *Tobacco etch virus* movement (RTM) system, and the viral determinant to overcome the RTM resistance was mapped to the N-terminal region of the CP (Decroocq *et al.*, 2009).

The analysis of chimeras between PPV isolates of different strains (Dallot *et al.*, 2001; Sáenz *et al.*, 2000) or of the same strain (Salvador *et al.*, 2008a) with diverse biological characteristics has shown that determinants for these properties are largely spread throughout the viral genome, and that, in some cases, optimal adaptation to *P. persica* or *Nicotiana clevelandii* is mutually exclusive. In particular, a pathogenicity determinant for infection in herbaceous (Sáenz *et al.*, 2000) and woody (Dallot *et al.*, 2001) hosts was localized in the P3 + 6K1 region. In agreement with this, nucleotide changes in the P3 and 6K1 coding sequences have been associated with adaptation to *N. clevelandii* (Salvador *et al.*, 2008a). Nucleotide changes in the P1 (Salvador *et al.*, 2008a) and CP (Carbonell *et al.*, 2013) coding sequences have also been detected during adaptation to this host, and a specific mutation occurred consistently when a peach PPV isolate was adapted to pea (Wallis *et al.*, 2007).

The P1 protein appears to be especially relevant for host adaptation (Valli *et al.*, 2007). Replacement of the PPV P1 coding sequence by the corresponding sequence of another potyvirus, *Tobacco vein mottling virus*, abolished infectivity in *P. persica*, but enhanced virus competence in *N. clevelandii* (Salvador *et al.*, 2008b). Moreover, point mutations in the P1 gene causing effects on infectivity, virus accumulation and symptom severity were detected in virus variants that coexisted in a PPV population (Maliogka *et al.*, 2012). Also supporting the importance of P1 for PPV pathogenicity, the 3' proximal part of the P1 gene was shown to determine the symptomatology of interstrain PPV chimeras (Nagyová *et al.*, 2012). Interestingly, long sequences of the 5' noncoding region of PPV that are not essential for viral infectivity also contribute to viral competitiveness and pathogenesis (Simón-Buela *et al.*, 1997b).

HCPPro is a known potyviral pathogenicity factor, as a consequence of its ability to suppress RNA silencing (Kasschau *et al.*, 2003) and, probably, because of interactions with other host processes (Eggenberger *et al.*, 2008; Mlotshwa *et al.*, 2005). A contribution of HCPPro to PPV pathogenicity in *N. clevelandii* has also been reported (Sáenz *et al.*, 2001); HCPPro defects have been shown to contribute to the restriction of PPV systemic spread in *N. tabacum* (Sáenz *et al.*, 2002). Moreover, synergistic interactions of PPV HCPPro with another virus, *Potato virus X*, have also been described (González-Jara *et al.*, 2005; Pacheco *et al.*, 2012).

Information about the biochemical basis of viral symptoms is very scarce. However, results suggest that imbalance in antioxidant systems and increased generation of reactive oxygen species might contribute to the deleterious effects of PPV infection (Díaz-Vivancos *et al.*, 2006, 2008; Hernández *et al.*, 2006).

## INTERACTOME STUDIES AND THE IDENTIFICATION OF HOST FACTORS CONTRIBUTING TO PPV INFECTION

Our understanding of the many ways in which potyviruses interact with their host plants has dramatically progressed in recent years, thanks to the convergence of a range of strategies, including biochemical, molecular, genomic and genetic approaches. Although probably still far from complete, our current view of the potyviral interactome is thus far more complex today than it was only a decade ago (Elena and Rodrigo, 2012; Revers *et al.*, 1999). Every single protein encoded by the potyviral genome has several identified viral or host interactors if potyviruses are considered collectively (Elena and Rodrigo, 2012). Although some of these interactions are likely to be virus specific, in many other cases a level of generality is probably associated with these findings. A good example is the finding that all potyviruses analysed to date require (and interact with) one or more isoforms of translation initiation factor 4E (eIF4E), but that different potyviruses may interact with different isoforms (Nicaise *et al.*, 2007). Although PPV is not the most prominent *Potyvirus* in interactomic studies, PPV research has allowed us to fill several blanks in our growing knowledge of the potyviral interactome.

Systematic efforts have demonstrated the existence of 52 of 100 possible interactions between the various PPV proteins (including self-interactions) (Zilian and Maiss, 2011), making PPV the best or second best known potyvirus from this perspective and a clear model for other genus members. When it comes to the identification of host plant interactors, work on PPV has allowed the identification of two plant proteins physically interacting with viral proteins. The *Arabidopsis* RH8 helicase interacts with VPg (Huang *et al.*, 2010) and the *Nicotiana benthamiana* photosystem I PSI-K protein interacts with the CI helicase (Jiménez *et al.*, 2006). Reduction of the accumulation of RH8 has a negative effect on PPV infection, demonstrating that it behaves as a susceptibility factor. In contrast, the down-regulation of PSI-K leads to higher PPV accumulation, suggesting that it has an antiviral role. The fact that the co-expression of PPV CI causes a decrease in the accumulation of PSI-K transiently expressed in *N. benthamiana* suggests that CI could be involved in counteracting the defensive role of PSI-K.

Although the physical interactions involved have not been studied in detail, both eIF(iso)4E and eIF(iso)4G1 have been shown to be absolutely required for successful PPV infection in *Arabidopsis* (Decroocq *et al.*, 2006; Nicaise *et al.*, 2007), a situation that parallels that observed for many other potyviruses. In the specific cases of PPV and *Turnip mosaic virus*, two further proteins have been shown to partially affect viral accumulation, probably through their effect on eIF(iso)4E accumulation: the DNA-binding protein phosphatase AtDBP1 (Castelló *et al.*, 2010) and a small interactor of AtDBP1, DIP2 (Castelló *et al.*, 2011).

Although these studies have so far not resulted directly in the identification of further host plant interactors, it is worth noting that PPV is one of the best studied potyviruses when it comes to both transcriptomic studies (Babu *et al.*, 2008; Dardick, 2007; Schurdi-Levraud Escalettes *et al.*, 2006; Wang *et al.*, 2005) and the genetic dissection of host determinants of the interaction in *Arabidopsis* (Decroocq *et al.*, 2006; Pagny *et al.*, 2012; Sicard *et al.*, 2008). The latter has allowed the demonstration that PPV is among the potyviruses controlled by the RTM resistance system (Decroocq *et al.*, 2009), and the identification and mapping of various host resistance determinants, including recessive ones, is likely to correspond to susceptibility factors (Pagny *et al.*, 2012). These studies, and the physical mapping of a major resistance locus of *P. armeniaca* cultivars, suggest that MATH domain proteins could be involved in the control of PPV long-distance movement (Pagny *et al.*, 2012; Zuriaga *et al.*, 2013).

## APPROACHES TO GENERATE RESISTANCE AGAINST PPV

### Conventional breeding

The identification of natural resistance in *Prunus* germplasm and its introduction into commercial cultivars by conventional breeding is one of the main strategies to control PPV, especially in areas of endemicity (Decroocq *et al.*, 2011). First reports on resistant *Prunus* genotypes, based on field observations under natural infection pressure, date from the 1940s (Christoff, 1947; Jordovic, 1968; Syrgiannidis, 1980). Later experimental evaluations of *Prunus* for resistance involved artificial inoculations by grafting, chip-budding or aphids in the field (Bivol *et al.*, 1987; Minoiu, 1973; Trifonov, 1975; Zawadzka, 1981) or under controlled conditions (Dosba *et al.*, 1991; Martínez-Gomez and Dicenta, 1999). However, limitations in the reliability of detection methods and differences in the evaluation protocols, PPV isolates used and agroclimatic context resulted in conflicting results in some cases (Kegler *et al.*, 1998).

In spite of many years of extensive efforts, very few natural sources of resistance have been identified so far in *Prunus* species (Kegler *et al.*, 1998; Martínez-Gómez *et al.*, 2000). Resistant apricot genotypes (mainly of North American origin) have been used in several breeding programmes (Badenes and Llácer, 2006; Krška *et al.*, 2006). PPV resistance in apricots is believed to be a complex trait controlled by at least two genes (Guillet-Bellanger and Audergon, 2001; Moustafa *et al.*, 2001; Vilanova *et al.*, 2003). No known source of resistance has been identified in peach, but resistance has been identified in the wild relative *P. davidiana*, in almond (*P. amygdalus*) and in almond × peach hybrids (Kegler *et al.*, 1998; Pascal *et al.*, 2002; Rubio *et al.*, 2003).

In the absence of resistant cultivars in domestic plum, tolerant cultivars that do not display fruit symptoms, but do not restrict

PPV multiplication and movement, have been used in southern and central Europe (Kegler *et al.*, 1998; Ogašanic *et al.*, 1994). The hypersensitive response (Kegler *et al.*, 1991, 2001), an active defence response resulting in localized cell death, has been found to be an effective resistance mechanism against PPV under natural or artificial inoculation, and has been used in plum breeding programmes (Hartmann, 1998), although, in rare cases, the response was found to be partial, depending on the PPV isolate (Polák *et al.*, 2005).

Marker-assisted selection, based on molecular markers associated with resistance, has been used to streamline the lengthy breeding and selection of resistant genotypes (Lalli *et al.*, 2005; Vilanova *et al.*, 2003). In apricot, linkage groups 1 and 3 have been highlighted as bearing PPV resistance quantitative trait loci (QTLs) (Marandel *et al.*, 2009).

### Genetic engineering

Given the economic importance of PPV, it is no surprise that, following the initial construction of virus-resistant transgenic plants, several laboratories embarked on this quest. It was a particularly ambitious goal as this implied both the development of the technology for PPV and the generation of transgenic woody plants. Following initial efforts at genome characterization, early constructs allowed the expression of the PPV CP gene in transgenic herbaceous (Ravelonandro *et al.*, 1992; Regner *et al.*, 1992) and *Prunus* (Laimer da Camara Machado *et al.*, 1992; Scorza *et al.*, 1994) hosts. Remarkably, among the plum trees produced during these early efforts, one transgenic line, C5, was shown to be highly resistant to PPV (Ravelonandro *et al.*, 1997) as a result of post-transcriptional gene silencing (PTGS) (Hily *et al.*, 2005; Scorza *et al.*, 2001). The resistance of this C5 clone, later renamed 'HoneySweet', has been validated extensively in long-term field trials in a range of countries and agronomical conditions (Hily *et al.*, 2004; Malinowski *et al.*, 2006; Polák *et al.*, 2008). The biosafety of this transgenic plum line has also been evaluated extensively, in both field and laboratory experiments, in particular within the framework of a collaborative European Union-funded project (Fuchs *et al.*, 2007). Particular attention was paid to the possibility of the emergence of recombinants between an infecting virus and the transgene (Capote *et al.*, 2008; Zagrai *et al.*, 2011) and to resistance stability after infection with heterologous viruses (Zagrai *et al.*, 2008), but many other aspects were also analysed, culminating in the regulatory approval of the HoneySweet plum in the USA (Scorza *et al.*, 2013). As a consequence of these detailed studies, the HoneySweet plum is probably one of the best studied virus-resistant transgenic plants (Collinge *et al.*, 2010; Gottula and Fuchs, 2009; Simón-Mateo and García, 2011).

Efforts to develop PPV-resistant transgenic plants have by no means been limited to the CP expression strategy. Over the years,

a wide range of other approaches have been evaluated, with variable success. Given that the HoneySweet plum resistance is PTGS based, it is no surprise that the expression of a range of other PPV genome regions, in wild-type or mutated form, has been shown to confer resistance, probably through the same mechanism (Barajas *et al.*, 2004; Guo and García, 1997; Guo *et al.*, 1998a, 1999; Jacquet *et al.*, 1998; Taver-Roudet *et al.*, 1998; Wittner *et al.*, 1998). Similarly, the effectiveness of PTGS-inducing, hairpin-containing viral transgenes has been confirmed in a wide range of studies (Di Nicola-Negri *et al.*, 2005; Hily *et al.*, 2007; Pandolfini *et al.*, 2003; Tenllado *et al.*, 2003; Zhang *et al.*, 2006). A potential limitation of resistance conferred by the expression of viral genomic sequences is the possibility that it could be suppressed by infection with a heterologous virus (Simón-Mateo *et al.*, 2003). The susceptibility of engineered PPV chimeras to endogenous microRNAs suggests that the expression of artificial microRNAs might also be an effective option (Simón-Mateo and García, 2006). However, the fact that PPV rapidly escaped the silencing mechanism through the accumulation of point mutations poses caution on this antiviral approach.

A wide range of other strategies have been envisioned in an effort to develop virus-resistant transgenic plants (Prins *et al.*, 2008), but so far these nonconventional approaches have met with only limited success in the case of PPV (Liu *et al.*, 2000; Wen *et al.*, 2004), with the possible exception of the transgenic expression of single-chain antibodies targeting the viral NIb replicase (Esteban *et al.*, 2003; Gil *et al.*, 2011).

The most recent strategy evaluated with success against PPV brings together interactomics or genetic studies aimed at the identification of host susceptibility factors (see above). In theory, the inactivation of such genes could result in resistance to viral infection, as was demonstrated in *Arabidopsis* in the case of eIF(iso)4E for several potyviruses (Duprat *et al.*, 2002), including PPV (Decroocq *et al.*, 2006). Several transgenic plum lines in which eIF(iso)4E expression had been knocked down through RNA silencing showed 100% PPV infection evasion, even after two successive vegetative cycles (Wang *et al.*, 2013), demonstrating that this strategy can be used in stone fruits against PPV. In the long run, to avoid public reluctance (at least in Europe) against transgenic plants, the use of this strategy without the need for transgenesis could even be envisioned, either through the targeted screening of the *Prunus* diversity for suitable null or mutant eIF(iso)4E alleles or through the selection of mutant alleles using TILLING (Targeting Induced Local Lesions IN Genomes) technology (Piron *et al.*, 2010).

### PPV AS A TOOL IN BIOTECHNOLOGY

Plant viruses are the object of interest not only because of the harm they cause to crops. Viral infections can enhance the aesthetic value of ornamental plants (Garber, 1989; Saunders *et al.*,

2003) and viruses may even establish interactions mutually beneficial to the virus and the host (Roossinck, 2005). Although there are no reports indicative of the beneficial effects of PPV, genetic engineering has allowed us to modify and use PPV, or parts of it, as a valuable biotechnological tool.

The availability of functional full-length cDNAs of the PPV genome (López-Moya and García, 2000; Maiss *et al.*, 1992; Predajňa *et al.*, 2012a; Riechmann *et al.*, 1990; Szathmary *et al.*, 2009) has facilitated the development of PPV-based vectors to express either small peptides fused to the viral CP or independent proteins (García *et al.*, 2006). Several vectors developed to express epitopes of foreign agents at the surface of PPV virions differed in their tolerance to inserted sequences and in the antigenicity and immunogenicity of the expressed epitopes (Fernández-Fernández *et al.*, 1998, 2002b).

PPV-based vectors allowing the expression of whole independent proteins have also been constructed, using as insertion site the P1/HCP or NIb/CP junction (García *et al.*, 2006). These vectors have been used to express reporters that facilitate monitoring of the viral infection (Dietrich and Maiss, 2003; Guo *et al.*, 1998b; Ion-Nagy *et al.*, 2006; Lansac *et al.*, 2005), but also antigenic proteins to produce recombinant vaccines (Fernández-Fernández *et al.*, 2001).

Viral vectors can be expressed in transgenic plants transformed with full-length cDNA copies of the viral genome. These amplicons combine the genetic stability of transgenic plants with the elevated replication rate of viruses. PPV amplicons have been developed in *N. benthamiana*, but show important constraints that limit their utility (Calvo *et al.*, 2010). A PPV amplicon has been used to design a method to control virus expression by regulating the temperature during plant transformation and its subsequent culture, which could help to reduce such limitations (Dujovny *et al.*, 2009).

The protease domain of the NIa protein of PPV has demonstrated a notable biotechnological interest as its high efficiency and specificity make it very attractive for the processing of fusion proteins both *in vitro* (Pérez-Martín *et al.*, 1997; Zheng *et al.*, 2008) and *in vivo* (Zheng *et al.*, 2012).

## CONCLUSION

For several decades now PPV has been among a handful of intensively studied potyviruses and, as a consequence, is among the most studied and best understood viruses in this vast, widespread and highly damaging genus. The high visibility of PPV is no doubt a consequence of both its high socioeconomic impact in the affected *Prunus* crops and its quarantine regulatory status in many countries. These factors have contributed to its inclusion in a recent list of the 10 most significant viruses in molecular plant pathology (Scholthof *et al.*, 2011). Research efforts on PPV have been particularly active and trend-setting in several areas, includ-

ing the development of advanced diagnostic and detection techniques (to support quarantine, eradication and certification control strategies), efforts in epidemiology and modelling of disease spread, plant–virus interaction studies and the development of classical or transgenic resistance. In the past few years, many of these research lines have converged under the auspices of the SharCo project supported by the European Union, leading to an exemplary collaborative translational research effort to better control the devastating sharka disease. Further collaborative inputs of the same magnitude are needed today to capitalize on the progress made in our understanding of this virus and to provide the fruit industry with a range of control options, including panels of varieties with high-level and durable resistance to PPV for all the major affected *Prunus* crops.

## ACKNOWLEDGEMENTS

We are grateful to F. Palmisano, A. Minafra, D. Boscia, V. Savino and A. Myrta for providing unpublished information about PPV-An. We acknowledge the support of the European Union for the authors within the framework of the FP7 KBBE-204429 SharCo project. The research of the authors was also supported by grants BIO2010-18541 from Ministerio de Economía y Competitividad (MINECO) (JAG), APVV-0042-10 and APVV-0174-12 from the Slovak Research and Development Agency (MG), AGL2009-07531 from MINECO (MC), and EU-FP7 Marie Curie STONE and French FranceAgriMer 2009 0076 020 104 and 2011 0038 012 104 (TC). We would like to apologize to those individuals whose relevant publications could not be cited because of space constraints.

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