REVIEW

PLUM POX VIRUS VARIABILITY DETECTED BY THE ADVANCED ANALYTICAL METHODS

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Summary. – Plum pox virus (PPV) infects stone-fruit trees with important economical impact mainly in Europe and Mediterranean region. The data about PPV intra-species variability accumulated markedly in the last two decades. Six PPV strains have been recognized using different approaches including serology, protein analysis, specific amplification, and genome sequencing. Reliable and sensitive diagnostics is the most important requirement for application of early control and safety measures. Therefore, many techniques and their modifications have been adapted to detect PPV and its different forms. Here, we review the improvement of the PPV detection and variability analysis in the context of progress in laboratory methods since the virus discovery till today.

Key words: PPV strains; serotypes; biological indexing; RT-PCR; sequencing, capsid protein

Contents

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Abbreviations: CP = capsid protein; EPPO = European Plant Protection Organization; HC-Pro = helper component proteinase; IC-RT-PCR = immunocapture RT-PCR; MAb(s) = monoclonal antibody(ies); NASBA = nucleic acid sequence-based assay; PPV = Plum pox virus; RFLP = restriction fragments length polymorphism

1. Introduction

The findings of Atanassoff (1932) based on the observation of damages on plum trees in Bulgaria is considered the first documented report on the occurrence of the sharka disease, called according to Bulgarian term for pox. This disease caused by the PPV has been subsequently observed in most of Mediterranean and central European countries and later in several localities outside the Europe (Table 1). The expansion of sharka depended

Table 1. The occurence of PPV outside the Europe

PPV strain	Locality	Reference
D	Chile	Reyes *et al*. (2001); Herrera *et al*. (1998)
D	Argentina	Dal Zotto *et al*. (2006)
D	USA	Levy *et al*. (2000b)
D, W	Canada	James *et al*. (2003)
unidentified	Azores	Mendonça *et al*. (1997)
EA	Egypt	Wetzel *et al*. (1991a)
D	Tunisia	Boulila *et al*. (2004)
M, D	Cyprus	Papayiannis *et al*. (2007)
M, Rec, atypical	Turkey	Candresse *et al*. (2007); Glasa and Candresse (2005); Elibüyük (2003); Sahtiyanci (1969)
M	Syria/Jordan	Al-Rwahnih *et al*. (2001); Dunez (1986)
D, Rec	Pakistan	Kollerová *et al*. (2006)
unidentified	India	Bhardwaj *et al*. (1995)
D	Kazakhstan	Spiegel *et al*. (2004)
D	China	Navrátil *et al*. (2005)

evidently on the commercial transport of horticultural material and insufficient implementation of quarantine measures. Natural transmission of PPV through several aphid species, although important for local dissemination plays a subordinate role at the massive virus spread covering large distances (Levy *et al*., 2000a). Also, the seed transmission has not been confirmed (Milusheva *et al*., 2007; Glasa *et al*., 1999; Schimanski *et al*., 1988).

The economic importance of PPV lies in the symptom severity, epidemic character of the disease in many countries, as well as in synergistic effects in mixed infections with other viruses (Németh, 1994). The symptoms in infected trees vary and depend on the host sensitivity and physiological status, season, and weather. PPV causes chlorotic diffuse or ring-shape spots on the leaves of susceptible genotypes. Vein chlorosis and leaf deformation are common in peaches (Fig. 1a,b). The leaf symptoms become less expressive in summer (Németh, 1986). The fruits are deformed, darker, with light rings on the skin and stones, with lower mass and sugar content. They often fall away before ripening what markedly lowers the yields.

The natural host range of PPV includes all cultivated stone-fruit crops (*Prunus spp*.) (Kölber *et al*., 2003). Transmissibility by at least 20 aphid species has been experimentally demonstrated, but only few of them are important natural vectors (*Brachycaudus cardui, B. helichrysi, Myzus persicae, Phorodon humuli, Aphis spiraecola*). The virus transmission proceeds in a non-persistent manner and does not depend on the ability of aphids to colonize trees. It occurs commonly by short test sucking during vector migration from the primary to the secondary host species and vice versa in spring and autumn (Labonne *et al*., 1995). Epidemiologically important secondary (herbaceous or non-*Prunus*) aphid hosts that would be also hosts for PPV have not been hitherto detected, although the virus was experimentally transmitted on relatively broad range of herbaceous plants. On the other hand, significant source of the virus constitute wild and ornamental *Prunus* plants (Sebestyén *et al*., 2007; Labonne *et al*., 2004; Polák, 2004).

Although, it is possible to prove the PPV presence in the different plant parts (leaves, flowers, bark, phloem, roots, fruit skin) the distribution of the virus in the tree is very uneven (Bodin-Ferri *et al*., 2002; Adams *et al*., 1998; Korschineck *et al*., 1991). This fact imposes big demands on visual inspection of symptoms in orchards. Using sensitive diagnostic methods, the virus may be detected even in dormant trees in winter. However, the best choice for effective diagnostics is to collect samples (from several parts of each suspicious tree) after unfolding of leaves on new sprouts in the spring (May-June), when the replication activity of PPV culminates (Gentit, 2006). Sampling in that season lowers the danger of false negative results and leads

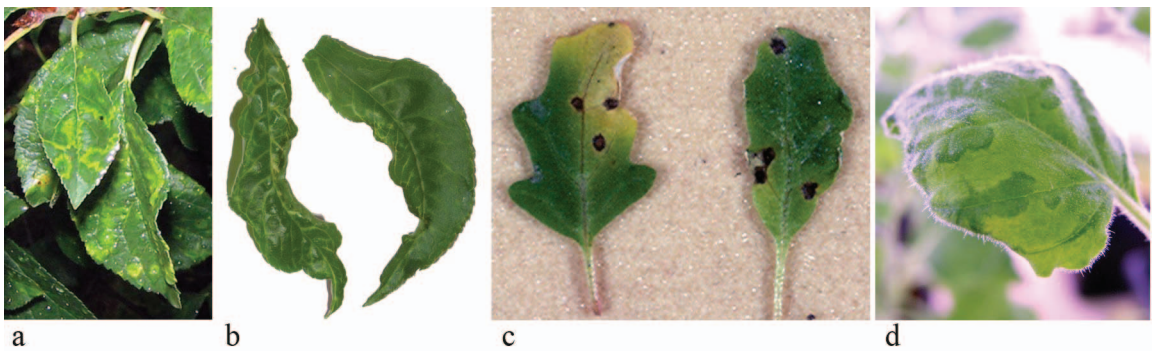

Fig. 1

PPV symptoms in plum (a), peach GF305 (b), *Ch. foetidum* (c), and *N. benthamiana* (d)

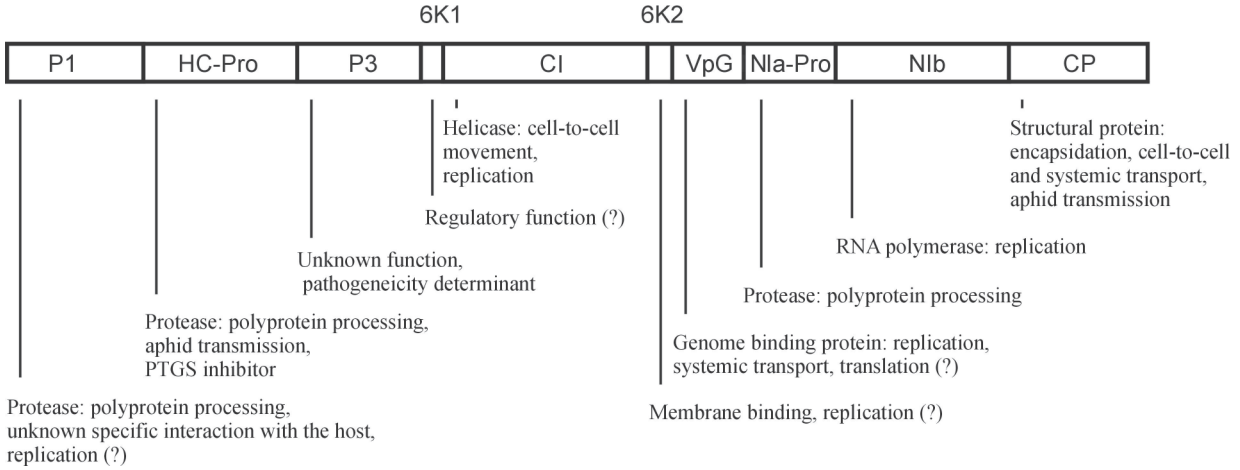

Fig. 2

Structure of the PPV polyprotein together with identified or hypothetic functions of each protein

to best correlation between results obtained by different detection methods (Olmos *et al*., 2007).

Techniques applicable to the PPV detection and analysis developed intensively in the last time. They can be divided to methods for detection of viral infectivity (biological tests), virus particles and inclusions (microscopy), proteins (serology, mass spectrometry, PAGE), and RNA (hybridization, amplification, sequencing). The goal of this review is to summarize the application of particular procedures for detection and research of PPV and characterization of PPV strain variability.

2. PPV taxonomy and strain variability

PPV belongs to the genus *Potyvirus*, the family *Potyviridae*. The virions are flexible filaments of about 750 x 15 nm. The genomic (+)ssRNA of approximately 9,800 nts is encapsidated by multiple copies of a single type of capsid protein (CP). Potyviruses belong to the picorna-like supergroup of plant viruses concerning the genome structure and replication strategy. Viral RNA is polyadenylated on the 3'-terminus and contains a covalently bound virus-coded protein VpG on the 5'-terminus (Riechmann *et al*., 1989; Laín *et al*., 1988). Viral genome serves as a messenger for the expression of polyprotein from a single ORF. The polyprotein is co- and post-translationally digested to reach 10 final polypeptide chains in the infected cells. The proteolytic activity is associated with 3 viral domains: P1, HC-Pro, and NIa-Pro (Ravelonandro *et al*., 1993; García *et al*., 1989a,b). The roles of viral proteins in the infection are to some extent identified, some of them are hypothesized (Fig. 2). Probably, most of the proteins are multifunctional. In addition to final polypeptides, also their partial cleavage products are present in the infected cell. These shorter polyproteins may be active and have probably regulatory functions (Salvador *et al*., 2006; García *et al*., 1994). For instance, the cleavage between domains NIa-VpG and NIa-pro is relatively slow and may relate to the switch between different functions of viral proteins in the course of replication cycle (Merits *et al*., 2002).

Six strains of PPV are currently recognized and 3 of them are epidemiologically important – PPV-D, PPV-M, and PPV-Rec (Candresse and Cambra, 2006). PPV-D named according the isolate Dideron is considered as the major strain occurring throughout the Europe and Americas (Kerlan and Dunez, 1979). PPV-D infects mainly plums and apricots, rarely peaches (Dallot *et al*., 1998). PPV-M named according the isolate Marcus is epidemically spread mainly in eastern Mediterranean, central and south-eastern Europe (Kerlan and Dunez, 1979). Generally, this strain is linked to the severe epidemics of peaches. PPV-Rec was derived from a homologous recombination between strains PPV-M and PPV-D. PPV-Rec is widespread in several central and eastern European countries almost exclusively in plums (Glasa *et al*., 2004). Appearance of other strains is geographically or host-limited. Strain PPV-EA (El Amar) was found only in Egypt, PPV-W (Winona) only in North America (James *et al*., 2003; Wetzel *et al*., 1991a). The strain PPV-C (cherry) was sporadically detected in some European countries exclusively on sweet or sour cherries without important economical impact. Natural infection of cherry trees by other PPV strains has not been recorded (Crescenzi *et al*., 1997a).

3. Techniques used for the detection of strain variability of PPV

3.1. Biological tests

Despite some disadvantages related to time and space requirements or subjectivity of evaluation, biological tests remain still necessary in plant virus research for detection of infectious viruses and for observation of their biological behavior on the host plant (pathotypization).

In the case of PPV, several woody indicators are used, particularly the peach cultivar GF305 (Németh, 1986). They can be experimentally infected either by mechanical inoculation of very young seedlings, by controlled aphid transmission, or mainly by chip-budding using small rind slices from suspicious trees. The symptoms include vein chlorosis and leaf distortion on young shoots (Fig. 1b). They occur usually 2–8 weeks after budding according to sample infectivity and experimental conditions. The intensity of symptoms may be to some extent strain-dependent, particularly PPV-M is usually efficiently detectable. It may relate to the higher adaptation of this strain to peaches (Moreno *et al*., 2007; Šubr *et al*., 2006). Generally, PPV-M causes more intensive symptoms on peach cultivar GF305 than PPV-D. On the contrary, PPV-Rec and PPV-C develop the weakest (frequently latent) reaction (Gentit, 2006; Glasa *et al*., 1997).

The myrobalan hybrid GF31 reacts to the PPV infection by rusty bark necrosis in young sprouts (Németh and Kölber, 1981). The plants *Prunus tomentosa* (IR473 x IR474 hybrid) express a leaf necrosis or chlorotic spots. This pathotypization appeared partially to correlate with the strain specification – PPV-M was necrotic and PPV-D chlorotic (Damsteegt *et al*., 1997; Ranković, 1980).

Successful transmission to herbaceous host plants was an important milestone in PPV research. Local necrotic or chlorotic lesions are produced on mechanically infected leaves of *Chenopodium foetidum* approximately 1 weak post inoculation (Fig. 1c). So-called "intermediate" virus isolates induced chlorotic lesions with necrotic centre (Paulechová, 1981).

Some pea cultivars (Colmo, Gloria) react to PPV infection by a mild mosaic. Various *Nicotiana spp.* are important artificial PPV hosts, especially *N. clevelandii* and *N. benthamiana* that develop mosaic symptoms of a various severity (Fig. 1d). They are used for the preparative virus propagation in laboratory settings, because of fast growth and systemic accumulation of PPV. High levels of the virus are present in all plant parts including roots (Šubr *et al*., 2007). The actual virus titer depends on the plant species and PPV isolate. Necrotic and "intermediate" isolates usually propagated more effectively than chlorotic isolates. However, systemic herbaceous hosts cannot be generally used for strain-specific diagnostics.

The PPV aphid transmissibility is enabled by the affinity of virions to the inner vector stylet surface mediated by a viral protein HC-Pro (helper component proteinase). Amino acids essential for this binding are localized in the N-terminal region of the CP molecule (López-Moya *et al*., 1999). Mechanical passages in plants may lead to generation of aphid-non-transmissible virus forms in consequence of point mutations or deletions in the CP gene. Deleterious PPV-D isolate NAT is a well-known example (Breyel *et al*., 1986).

This raises the problem of quality of the sample material, what often influences the reliability of isolate characterization. In order to obtain as realistic picture of the isolate variability as possible, it is essential to use original biological material (virus source) for the typing and phylogenetic/epidemiologic studies, in order to avoid the accumulation of artificial mutations during propagation in experimental herbaceous hosts.

In addition to indexing in indicator plants, the biological experiments may include testing of the host range or vector transmission. Grüntzig and Fuchs (1986) divided PPV isolates in two groups according to their ability to infect several *Chenopodium* species.

Problematic evaluation of the biological tests should be highlighted in several examples. Widely used peach cultivar GF305 seems to be not suitable for PPV-Rec indexing, although one PPV-Rec isolate was able to cause marked symptoms in this indicator (Glasa *et al*., 2004, 2005). This raises the problem of relatively wide intra-strain biological variability. Clear-cut frontiers between different strains cannot be drawn by biological methods. Although strains PPV-M and PPV-D did not infect cherries naturally, several isolates could be artificially transmitted to cherry or *Prunus mahaleb* rootstocks. However, they could not establish systemic infection and disappeared from the trees after time (Dosba *et al*., 1987). On the contrary, naturally cherry-infecting PPV-C could systemically infect plums and display broader host range than other strains (Bodin *et al*., 2003). In conclusion, in absence of strain-specific experimental hosts, the labor- and time-consuming biological tests with problematic evaluation have no practical application in the PPV strain typing.

3.2. Immunochemical methods

Serological methods for detection of polypeptides are commonly and widely used despite some sensitivity limitation. Quantitative comparison of results obtained with different antibodies or even different batches of one antibody may be problematic (Cambra *et al*., 2006). On the other hand, serological techniques are fast, cheep, without considerable demands on material or equipment, suitable for parallel analysis of many samples, and usually sufficiently reproducible. Therefore, they preserve an important place in practical diagnostics.

Different form of antigens may be used for immunization, e.g. purified virus, inclusion bodies, and proteins obtained

from gel after electrophoresis or from heterologous expression in *Escherichia coli.* Antibodies against various non-structural proteins of PPV have been used mainly for their intracellular localization using electron microscopy. A single chain variable fragment of a monoclonal antibody (MAb) against the NIb protein (replicase) was able to bind this protein in plants (Esteban *et al*., 2003). This fragment could be applied in the research, as well as in antivirus therapy (Gil *et al*., 2004). Antibodies against several non-structural proteins of potyviruses have potential for virus-specific diagnostics. However, the CP is the most frequent target of commonly used serological methods. High level of CP in infected cells is guaranteed by its virus genome-protecting function. Other viral products may be degraded or modified in the course of infection. Moreover, virus particles are easy to purify for the immunization purpose.

PPV is a good immunogen and the polyclonal antibodies of high quality may be prepared. Commercial preparations of antibodies with satisfactory sensitivity are available. The strains PPV-M and PPV-D were originally distinguished as serotypes using double-immunodiffusion of formaldehyde-treated purified viruses (Kerlan and Dunez, 1979). It is possible to prepare broad-specific antibodies recognizing many potyviruses, as well as antibodies with narrow virus or strain specificity (Richter *et al*., 1995; Jordan and Hammond, 1991). Synthetic peptides based on known sequence have been used to produce antibodies reacting specifically with PPV-C (Crescenzi *et al*., 1997b). Potential risk of cross-reactivity is the main disadvantage of the polyclonal antisera.

However, the application of MAbs solves this problem. Each MAb recognizes a single epitope on the complex protein. MAbs specific for PPV as well as strain-specific MAbs for PPV-M, PPV-D, PPV-C, and PPV-EA were prepared (Myrta *et al*., 1998, 2000; Boscia *et al*., 1997; Cambra *et al*., 1994; Hilgert *et al*., 1993). They are reliable for the detection of a broad range of isolates. However, some PPV isolates are able to escape the detection with particular MAbs, because the affinity of MAbs may be influenced even by the single amino acid change in the recognized epitope (Candresse *et al*., 1998a). Therefore, the detailed serotyping of PPV using MAbs may result in a confusing picture of the virus variability (Matic *et al*., 2007; Myrta *et al.*, 2001). By now, MAb 5B-IVIA against PPV CP is the only known MAb able to detect reliably isolates of all PPV strains (Cambra *et al*., 1994, 2006). Currently, the preparation of MAbs for special detection of PPV-W is going on, using recombinant peptides as antigens (Croft *et al*., 2007). Specific MAbs against PPV-Rec are still not available.

In terms of topology and stability, the epitopes may be divided to neotopes (surface-located, denaturation-sensitive), cryptotopes (internal, uncovered by denaturation), and metatopes (surface-located, denaturation-resistant) (van Regenmortel, 1982). The type of recognized epitope determines the techniques applicable with particular MAb. Himmler *et al*. (1988) prepared MAbs against all epitope types in PPV. Candresse *et al*. (1998b) mapped the epitopes in the PPV CP molecule using expression of cloned peptides of the various lengths in *E. coli*. The PEPSCAN test based on detection of short overlapping peptides synthesized *in vitro* according known protein sequence enables exact epitope localization (Esteban *et al*., 2003).

The core region of the potyviral CP is localized in the internal part of mature virion and interacts with viral RNA. Both terminal regions are exposed on the surface of virus particles. They may be removed by mild trypsinolysis and after this treatment the virions preserve their shape, dimensions, as well as infectivity (Shukla *et al*., 1988; Allison *et al*., 1985). Both core and the C-terminal part of CP are highly conserved, while the N-terminal part is the most variable region of PPV genome (Fig. 3). Strain-specific epitopes are localized in this

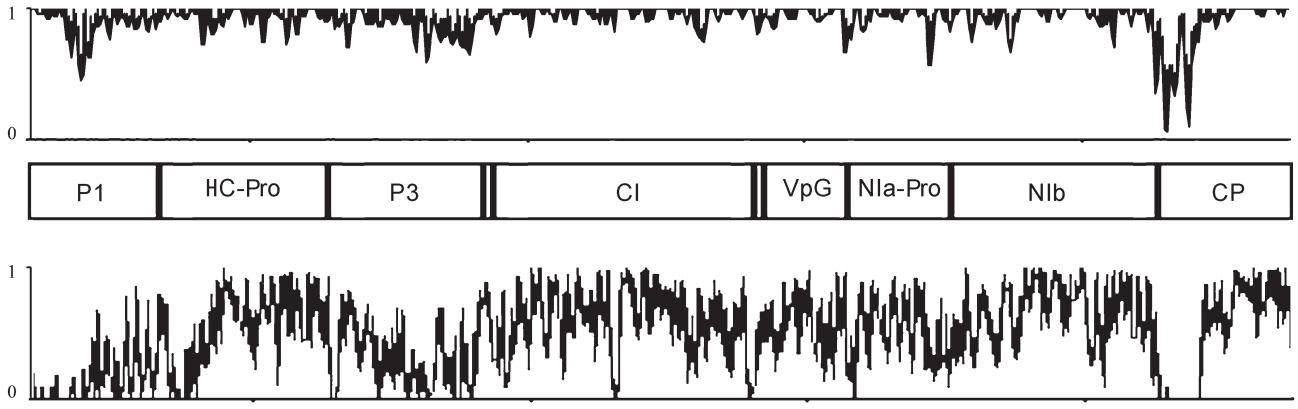

Fig. 3

Genome variability of the PPV (top) and of the genus *Potyvirus* (bottom)

Sequence homology of the polyprotein regions (rectangles) is demonstrated in the range 0 to 1.

immunodominant region, but the antibodies with broader specificity react rather to the core CP (Shukla *et al*., 1989).

ELISA is the most common immunochemical test that allows parallel analysis of many samples with sufficient sensitivity (Clark and Adams, 1977). This characteristic together with a low expense make ELISA suitable for wide-scale application also in developing countries. ELISA comes in various arrangements, among them the direct double-antibody sandwich ELISA is the most common. In other arrangement, the wells of the microplate can be coated directly by the virus from the sample, but it may result in partial steric deformation of the antigen and consequently in affinity alteration of some MAbs.

Immunodetection may be performed on membranes after tissue printing or as slot immunobinding assay with sensitivity comparable to ELISA (Hoffmann *et al*., 1997; Dicenta and Audergon, 1995). Currently, there are available one-step immunochromatographic carriers for fast and simple PPV detection (Oberhänsli *et al*., 2007; Mumford *et al*., 2006). The test is based on the use of polyclonal antibodies and its sensitivity is relatively low, but it is a good means for fast preliminary analysis of suspicious samples directly in the field conditions. The sample moves by a capillary action through the carrier with immobilized antibody and the positive reaction is visualized in a few minutes. On the contrary, there are highly modern instrumental methods based on the serology, e.g. the suspension array technology, where the immunocomplexes immobilized on the surface of special microspheres are detected by a two-laser Bio-Plex system (Croft *et al*., 2007).

Serological methods are applied mostly to detection of proteins, however, other biopolymers as polysaccharides and polynucleotides have antigenic properties too. Indirect ELISA with the antibody against polyinosine/polycytidine acid was used to detect double-stranded RNA of PPV and achieved a higher sensitivity than in the CP detection (Aramburu and Moreno, 1994). However, this approach has no practical diagnostic applications for the reason of its low specificity.

Antibodies are often used in electrophoretic, microscopic, or amplification techniques, as described in corresponding sections. For the MAbs, one have to consider their applicability in particular analysis, e.g. MAbs recognizing denaturation-stable (linear) epitopes are required for immunoblotting, or MAbs recognizing the surface epitopes are desired in immunoelectron microscopy.

3.3. Microscopic techniques

Electron microscopy of potyvirus-infected cells enabled to observe the viral particles inside the cell as well as various inclusion bodies. The microscopy itself has a weak diagnostic information value. Particle morphology and dimensions as well as inclusion type and shape do not allow the differentiation among various PPV strains or among different potyviruses. However, intracellular localization of viral proteins is an important tool for the research of infection mechanisms on molecular and cellular level. The immunoelectron microscopy is appropriate, when the antibodies serve either for specific antigen enrichment or for its depiction (Noel *et al*., 1978).

As a consequence of the potyvirus replication strategy, all viral proteins are produced in equimolar amounts. Some of non-structural proteins are compounds of cell inclusions in the cytoplasm (CI) or nucleus (NIa, NIb), others have been observed in the cytoplasmatic diffuse electrodense material or in association with the nuclear inclusions (HC-Pro, P1, P3, 6K2) as detected by specific antibodies (Riedel *et al*., 1998; Martin and Gelie, 1997; Adamolle *et al*., 1994; Restrepo *et al*., 1990). Intracellular translocation of viral proteins plays probably a regulatory role in potyvirus replication cycle. The nuclear inclusions are expected to originate by transport of abundant NIa and NIb molecules from the cytoplasm, where they are active in replication and proteolysis (Riechmann *et al*., 1992; Restrepo *et al*., 1990). It is not known, if the protein complex of nuclear inclusions directly influences the host replication or transcription in the nucleus, although this cannot be excluded. Recently a DNase activity of the NIa protease has been demonstrated (Anindya a Savithri, 2004).

Cytoplasmic cylindric inclusions are in fact functional bodies with complex pinwheel-shaped structure. They are composed of viral CI protein with helicase and ATPase activity and probably directly participate in the cell-to-cell movement of the virus (Roberts *et al*., 1998; Laín *et al.*, 1990). The presence of this type of inclusions serves as a broad diagnostic marker as they are typical for the family *Potyviridae.*

More precise specification, e.g. differentiation of respective PPV strains is enabled by decoration of virions using strain-specific antibodies conjugated with colloidal gold particles (Crescenzi *et al*., 1997b; Kerlan *et al*., 1981). Immunoelectron microscopy was used for screening of hybridomas for MAb production (Himmler *et al*., 1988). Particular epitopes were immediately characterized by the antibody coupling with intact virions or with the CP outside the virions.

3.4. Electrophoretic methods

CP of different PPV isolates did not show the same mobilities in SDS-PAGE, as described by several authors (Boulila *et al*., 2004; López-Moya *et al*., 1994). Electrophoretic profile of a purified PPV usually included additional shorter fragments resulting from CP proteolytic degradation, depending on the host plant (Šubr *et al*., 1998;

Laín *et al*., 1988). However, the major band of each isolate preserved its mobility irrespective of the host and of the sample type (crude plant sap, purified virus) (Šubr and Glasa, 1999).

Ranković and Veliković (1983) first detected the correlation between the CP mobility and strain affiliation of PPV isolates. CP of PPV-M migrated faster than CP of PPV-D. CP of PPV-Rec in most cases migrated in the form of a double-band (Šubr and Glasa, 1999). The slower band was less intensive, but never fully disappeared. This double-band pattern was originally attributed to mixed infections or considered to be a laboratory artefact. However, later it has been shown as reproducible for PPV-Rec (Šubr *et al*., 2007; Navrátil *et al*., 1998).

SDS-PAGE is generally used for approximate estimation of Mr of polypeptides. Overestimation of Mr for glycosylated proteins by SDS-PAGE is common, since their carbohydrate content does not bind SDS, what results in a lower charge density of the molecule (Werner *et al*., 1993). Theoretical Mr value calculated from the known CP sequence of particular PPV isolates does not correlate with corresponding electrophoretic mobility. Thus, the SDS-PAGE profiles of PPV isolates reflected rather difference in CP posttranslational modifications that was confirmed also by CP Ferguson plot analysis (Kollerová *et al*., 2007; Z. Šubr, unpublished results). The PPV CP has been found glycosylated and phosphorylated by the mass spectrometry. O-glycosylation by N-acetylglucosamine was localized in the N-terminal CP domain, which was in agreement with the fact that core CP of different PPV isolates migrated equally in SDS-PAGE (Fernández-Fernández *et al*., 2002; Šubr and Glasa, 1999). On the other hand, the phosphorylation was distributed along the CP including the core region (Kollerová *et al*., 2007). The phosphate residues have an effect on the double-band pattern of PPV-Rec as well (Šubr *et al*., 2007). So far, no data about possible function of glycosylation and phosphorylation of the CP and their cooperation *in vivo* are available.

PPV isolates with the deletions in the CP gene are effectively detected by SDS-PAGE and several PPV isolates have been discovered in this way. Some of them resulted from repeated mechanical passaging, others were isolated from natural sources (Palkovics *et al*., 2004; Navrátil *et al*., 1998; Maiss *et al*., 1989).

SDS-PAGE is often combined with immunoblotting that generally enhances the specificity and sensitivity and is essential for analysis of non-purified (crude) samples as well. Application of strain-specific antibodies may increase the information value of immunoblotting analysis (Crescenzi *et al*., 1997b). Regarding the different CP mobility of major PPV strains (M, D, Rec), immunoblotting may be used under some circumstances for fast differentiation among them even in mixed infections (Šubr *et al*., 2006). However, some exceptions have been reported**:** CP of some PPV-M and PPV-D isolates migrated atypically and in addition, a few PPV-Rec isolates with a single-band profile have been recorded (Šubr *et al*., 2007; Dallot *et al*., 1998). Because of this limitation the SDS-PAGE/immunoblotting has to be combined with other techniques for PPV strain typing.

A protocol for native electrophoresis of PPV in agarose or agarose-polyacrylamide composite gels combined with immunoblotting has been published, but it found no broader application (Manoussopoulos *et al*., 2000).

3.5. Hybridization

Dot-blot hybridization was the first technique of molecular biology applied for the detection of PPV. cDNA of the PPV-D CP gene or RNA obtained by *in vitro* transcription served as radioactively labeled probes. The PPV-D isolates were recognized slightly better, but the probes reacted also with PPV-M (Varveri *et al*., 1987, 1988). Additionally, the probes derived from several non-structural protein coding regions did not show any inter-strain differences. The detection limit depended on the length of used probes. Generally speaking, hybridization is not suitable for strain discrimination, because the highly variable sequence stretches are relatively short compared to the length of hybridization probes. The hybridization technique allowed identification of the PPV genes also in 20% of ELISA-negative symptomless samples (Wetzel *et al*., 1990).

Non-radioactively digoxigenin-labeled probes were also applied for the detection of PPV genes on the membrane or microtitration plate (Olmos *et al*., 2002, 2007). Their sensitivity has been shown comparable with radioactively-labeled probes and the protocols avoid the manipulation with isotopes. Palkovics *et al*. (1994) optimized sandwich hybridization for the PPV detection. A biotinylated RNA probe was bound to the microwell plate through streptavidine. Then the viral RNA was specifically captured from the sample and next, the digoxigenin-labeled probe was hybridized to viral RNA. The sandwich was finally detected by incubation with enzyme-conjugated anti-digoxigenin antibody.

The hybridization technique as a micromethod led to the development of a microarray technology that has been also applied to PPV detection. The procedure looks like inverted standard hybridization – the probes are immobilized on the surface of the solid phase and the samples are fluorescently labeled. The main advantage of microarray is a parallel testing of one sample with the large number of probes. This principle is very helpful and convenient especially in human genetics. In diagnostics of plant viruses, it enables to test samples for presence of multiple pathogens at the same time (Mumford *et al*., 2006; Hadidi *et al*., 2004).

3.6. Amplification methods

Amplification detection methods are considerably more sensitive in comparison to the immunochemical and hybridization techniques. Since PPV is an RNA virus, the cDNA has to be prepared by reverse transcription prior to the PCR. This test can be optimized to a high specificity according to known sequence data of various genome regions of PPV, namely 3' non-coding region, CP gene, P3-6K1-CI, and NIb region (Laimer *et al*., 2003; Glasa *et al*., 2002b; Levy and Hadidi, 1994; Wetzel *et al*., 1991b).

Wetzel *et al*. (1991b) amplified a 243 bp fragment from the 3' part of the CP gene. The primers P1 and P2 were designed according to the sequences of several PPV-D isolates. Nevertheless, the reactivity was not strain-specific for the reason of the high homology of this genome part. The protocol enabled the universal PPV detection, despite few mismatches between the P2 primer and PPV-EA sequence. The strains PPV-D, -M, and -EA recognized at that time, could be partially differentiated by restriction fragment length polymorphism (RFLP) of the produced amplimers (Candresse *et al*., 1994). The products of PPV-M and PPV-EA, but not PPV-D contained a recognition site for the restriction enzyme *Rsa*I. RFLP enabled also the differentiation of PPV-C by the absence of an *Alu*I restriction site in this region compared to other mentioned strains (Nemchinov and Hadidi, 1996). Because the RFLP is sensitive for the point mutations in particular restriction sites, it is not completely safe for the strain-typing of the isolates (Laimer *et al*., 2003). Nevertheless, the combination of P1/P2 primer pair-based RT-PCR/RFLP spread fast and was adopted as a standard technique by many research laboratories worldwide.

RT-PCR/RFLP analysis was optimized also for the P3-6K1 genome region. The comparison of results obtained from different genome parts contributed to the discovery of PPV-Rec (Glasa *et al*., 2001, 2002a). Previously, the isolates of this strain have been typed as PPV-M according to the serological tests and CP gene polymorphism, although the major part of their genome was highly homologous with PPV-D.

Primers binding to regions with high degree of inter-strain heterogeneity may be also used for PPV typing. Although such tests did not give absolutely consistent results (primarily regarding PPV-EA and PPV-C), their reliability was higher than reliability of tests using specific MAbs (Candresse *et al*., 1995, 1998a). Differentiation of PPV-M, PPV-D, PPV-Rec, and PPV-C have been optimized by strain-specific primers (Šubr *et al*., 2004a; Glasa *et al*., 2002b; Nemchinov and Hadidi, 1998). Primers leading to the products of different lengths according to the PPV strain could be used for simultaneous detection of strains M, D, and Rec in multiplex RT-PCR by simple visual evaluation of gels (Šubr *et al*., 2004a,b).

The sensitivity of RT-PCR increased about 100-times by application of two primer pairs in separate steps (nested or seminested PCR), or in a single reaction (co-operational PCR) (Olmos *et al*., 2002; Candresse *et al*., 1998a). Universal PPV primers were used in the first step of nested arrangements, followed by application of strain-specific primers (Olmos *et al*., 1997). Szemes *et al*. (2001) were able to differentiate among strains M, D, C, and EA including double- and triple-mixed infections using degenerate primers in the first step of nested RT-PCR in the CP gene region.

PPV has been detected also by loop-mediated isothermal amplification using three primer pairs (Varga and James, 2006b). This method does not require a thermocycler, but the high sensitivity may lead to a false-positive result due to a contamination. Nucleic acid sequence-based assay (NASBA) is another isothermal method, where amplification results from cooperation of three enzymes (reverse transcriptase, ribonuclease and T7 DNA polymerase). Briefly, NASBA is a transcription *in vitro* from the T7 promoter involved in one of used primers (Compton, 1991). In combination with flow-through hybridization, it constitutes a highly sensitive detection method suitable also for analysis of samples out of the active vegetation period (Olmos *et al*., 2007).

Common RT-PCR also consists of several enzyme reactions (reverse transcription, DNA synthesis, in nested PCR two separate polymerization steps). Several protocols promoting all reactions in one tube have been developed on behalf of minimizing the number of steps for manipulation with biological material and minimizing the risk of sample contamination (Olmos *et al*., 1999, 2002).

The virus binding on the surface of solid phase by antibodies is used in immunocapture RT-PCR (IC-RT-PCR). This method was about 1000-times more sensitive than ELISA and allowed the PPV detection also in dormant woody material (Adams *et al*., 1999; Candresse *et al*., 1994; Wetzel *et al*., 1992). Two synergistic factors are included: specific enrichment of the sample and removing of potential enzyme inhibitors present in crude extracts (Olmos *et al*., 2006). Samples blotted on a piece of filter paper may be analyzed as was successfully demonstrated by PPV detection in the single aphids. The blots were stable and storable at laboratory temperature and PPV was released into the solution for the IC-RT-PCR by Triton X-100 (Olmos *et al*., 1996, 1997).

Other combined methods should increase the detection sensitivity. Korschineck *et al*. (1991) applied the amplimer obtained by a biotinylated primer on the surface of nitrocellulose membrane with subsequent detection by a streptavidine-conjugated alkaline phosphatase (PCR Spot Assay). Similarly, a digoxigenin-labeled nucleotide was incorporated into the amplification product, allowing its detection by anti-digoxigenin antibodies in PCR-ELISA

(Olmos *et al*., 1997; Schönfelder *et al*., 1995). A strain-specific biotinylated probe hybridized with the PCR product was used for binding to the streptavidin-activated microtitration plate and specific detection of PPV-D and PPV-M (Poggi Pollini *et al*., 1997). Youssef *et al*. (2002) applied this approach for a multiplex detection of PPV and Prune dwarf virus (family *Bromoviridae*, genus *Ilarvirus*) – two most common economically important stone-fruit tree viruses. The reaction was evaluated spectrophotometrically and could be properly quantified unlike to the PCR Spot Assay. There is no need to perform the electrophoresis, what is a common advantage of these combined methods. However, the protocols are relatively complicated regarding the number of steps.

The advantage of amplification analysis in the solution is fully utilized in a real-time RT-PCR. In the last years this technique spread widely thanks to increasing commercial accessibility of required technology. Real-time RT-PCR enables fast parallel analysis of many samples, as well as their direct quantification. Schneider *et al*. (2004) applied this method for the NIb and core CP regions and detected four PPV strains (M, D, EA, C) in various plant parts with differentiation of PPV from a related, serologically cross-reacting potyvirus. Olmos *et al*. (2005) used real-time RT-PCR for precise detection and quantification of PPV in the individual aphids. Comparable occurrence and virus levels in different aphid species including natural vectors and non-vectors were demonstrated.

Real-time RT-PCR with both most common detection strategies has been applied also for the PPV strain discrimination. Varga and James (2005, 2006a) used the non-specific detection of the product by SYBR Green and were able to differentiate between strains M, D, EA, C, and W by melting curve analysis. The amplified region from the 3'-end of the CP gene was 74 bp long. A longer amplimer (155 bp) did not allow the discrimination between PPV-W and PPV-EA. Capote *et al*. (2006) applied the TaqMan technology with specific minor groove-binding probes to distinguish between PPV-M and PPV-D. As universal PPV primers were used, one of strains could escape the detection in mixed infections with high disproportion of respective compounds (more than 1:1000).

Weekly, the real-time RT-PCR allowes to analyze several thousands of samples using 384-well plates and automatic pipettors. The need for previous sample preparation is the limiting factor like in the other molecular methods (Mumford *et al*., 2006). A crude plant extract may be sometimes analyzed preferably captured to a solid phase as in Spot real-time PCR (Capote *et al*., 2007; Varveri, 2004). However, most of standard applications require isolated RNA. Therefore, an adequate attention should be paid to this step (Faggioli *et al*., 1998). Especially the extracts from woody sources may contain substances with inhibitory effects (MacKenzie *et al*., 1997). Despite a considerable development and commercial availability of kits for RNA isolation, this step remains the disadvantage of the molecular methods especially when large numbers of samples are analyzed.

3.7. Sequence analysis

Amplification of the genome domains is used also for the purpose of sequence analysis. Routine DNA sequencing allowed an accumulation of relatively high number of sequence data accessible in the public databases. The improvement of analytical software facilitates the application of the data for the predictions of structure and function of viral proteins, as well as for the general genotype specification. Sequence comparisons and construction of the phylogenetic trees provide the best depiction of the relationships among isolates (Candresse and Cambra, 2006). Since the viruses are genetically simple, the results of such comparisons cannot be referred to the real model of evolutionary history. Convergent and divergent processes are hardly distinguishable for single-base exchanges. Moreover, recombination plays a significant role in the potyvirus evolution (Worobey and Holmes, 1999; Roossnick, 1997).

Unlike other potyviruses, PPV was for a long time regarded as a virus with relatively high population homogeneity and stability, as a consequence of limited number of available sequence data and their composition (Revers *et al*., 1996). Later, data on PPV variability considerably accumulated including identification of several recombination events in the evolutionary history of PPV (James and Glasa, 2006).

The first mention about recombination in PPV is dated from the work of Cervera *et al*. (1993). Based on the sequence data, the isolate o6 originated from the recombination between PPV-D and PPV-M near the 3'-terminal part of the NIb gene. This finding was regarded as atypical and unimportant, but recently natural occurrence of such virus in many European and Asian countries has been detected (Candresse *et al*., 2007; Jevremović *et al*., 2007; Kajić *et al*., 2007; Navrátil *et al*., 2007; Zagrai *et al*., 2007; Zindović *et al*., 2007; Szathmáry *et al*., 2006; Myrta *et al*., 2005; Glasa *et al*., 2001, 2005). Thereafter, a separate strain PPV-Rec was proposed for these isolates because of their population homogeneity and wide spreading (Glasa *et al*., 2004). The experiments with inter-strain mixed infections in woody and herbaceous hosts did not lead to the recombinations *de novo* (Capote *et al*., 2006; Šubr *et al*., 2006). Therefore, the expansion of PPV-Rec is rather a consequence of its high competitiveness than a „hot-spot“ recombination in the PPV genome. The sign of recombination is also in the 5'-proximal genome regions of PPV-M, D, and Rec, as shown by the sequence

Table 2. Inter- and intra-strain homology of PPV

Strain[#]	D	Rec	M	W	EA	C
D	**98,9** ***99,1***	95,4	87,5	75,8	75,6	72,3
Rec	*97,9*	**98,5** ***99,3***	89,3	75,9	75,8	72,7
M	*96,3*	*97,2*	**98,2** ***98,6***	75,4	75,4	73,1
W	*89,3*	*89,6*	*89,3*	*	72,4	76,2
EA	*91,2*	*91,4*	*91,1*	*88,9*	**96,1**** ***97,6***	71,1
C	*87,6*	*87,8*	*87,6*	*88,7*	*87,3*	**99,1** ***98,3***

[#]All data (in %) were obtained from full-length sequences in GenBank. Above the diagonal are nucleotide sequences, under the diagonal (italic script) amino acid sequences. The numbers on the diagonal (boldface) are values of intra-strain homology. *Only one sequence available. **Two subisolates coming from one natural isolate.

analysis of the complete genomes (Glasa *et al*., 2004). Recently, a partial sequencing revealed a new Turkish isolate different from PPV-Rec that could be a recombinant of PPV-M with an unknown ancestor in the HC-Pro-P3 region (Glasa and Candresse, 2005). Similarly, the PPV-W seems to undergo several recombinations with PPV-M and PPV-C in its evolutionary history (Myrta *et al*., 2006; James and Varga, 2005).

The variability of PPV differs significantly for particular genome parts. The homology of some regions (CI gene) is markedly higher on the amino acid level than on the nucleotide level (Myrta *et al*., 2006). Most variable is the 5'-terminus of CP that may be used in the partial sequencing for strain-typing (Fig. 3). However, for this purpose a comparison of complete sequences is most reliable. The first completely sequenced PPV genome belonged to the PPV-D isolate Rankovic (Laín *et al*., 1989). Nowadays, the complete sequences of several isolates belonging to all six strains are available. The strain PPV-C shows the most differences in comparison with other strains (Table 2). Especially high divergence was recorded for the genes P1 and CP, but also for the gene P3 usually highly conserved in frame of a potyvirus species (Fanigliulo *et al*., 2003).

Sequence data fully correspond to the classification of PPV into six strains (Fig. 4). Sequences of respective strains are more or less colinear and the length variability is very low. The isolate Dideron contains 1 aa longer HC-Pro, in several PPV isolates deletions were localized in the N-terminal region of CP (Palkovics *et al*., 2004; Maiss *et al*., 1989). Compared to other strains, polyproteins of PPV-W,

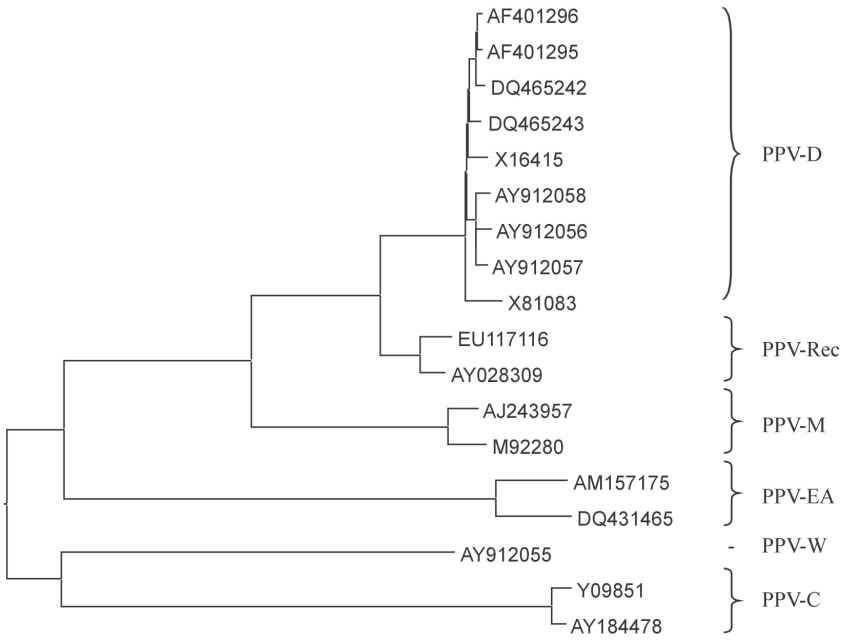

Fig. 4

Phylogenetic tree of PPV isolates based on the complete nucleotide sequences

The PPV isolates are represented by accession numbers.

PPV-EA and PPV-C contain the chain longer for 1, 2, and 3 aa, respectively. These additional aa are localized in the CP and NIa-Pro region.

The intra-strain variability of nucleotide sequences among natural isolates is below 2%, the inter-strain homology is 71–95%, and similarity of PPV with other potyviruses is 48–58%. The genomes are usually more conserved on the amino acid level (Table 2). The PPV strains are well-marked groups of isolates with high intra-strain and significantly lower inter-strain similarities. This status may be a result of relatively short evolution in *Prunus* hosts, where the virus was several times separately transferred from a more variable non-horticultural source (Candresse and Cambra, 2006).

Different selection pressure may influence the quasi-species variability of the virus population in various host plants or transmission mode (Eigen, 1996). More efficient mechanical passaging of PPV in pea plants leads to a substantial increasing of the mutation rate in comparison with equal number of passages by the aphid transmission (Wallis *et al*., 2007). The sequence of the original PPV-EA differed from its mechanically passaged subisolate as much as 3.9% and 2.4% on the nt and aa levels (Glasa *et al*., 2006; Myrta *et al*., 2006).

Relatively long deletions in the 5' non-coding region significantly influence the competitiveness with full-length PPV. This region was not essential, but enhanced substantially the virus fitness (Simón-Buela *et al*., 1997b). Two conserved potyviral motifs located in the 5' non-coding region are probably functional in translation or replication of viral RNA (James and Varga, 2005; Fanigliulo *et al*., 2003). Interestingly, the first initiation codon is skipped at the translation and the ribosomes bind to the next one by a leaky scanning mechanism (Simón-Buela *et al*., 1997a).

The proteolytic cleavage sites in the polyprotein are identical in all PPV isolates (Salvador *et al*., 2006). In particular polyprotein parts, several conserved amino acid motifs are recognized that are essential for the aphid transmission (KITC and PTK in HC-Pro, DAG in N-terminal CP region), for RNA polymerization (GDD in NIb) or other functions in the viral replication cycle (Glasa and Šubr, 2005).

Various results have been published considering the regional origin of virus isolation. The sequence differences of PPV-M, PPV-D, and PPV-Rec comprising the genome region P3-6K1-CI did not correlate with the geographical origin of isolates (Glasa *et al*., 2002a). In the context of the recent PPV outbreak in Northern America, the PPV-D isolates from USA and Canada were observed to cluster separately according the NIb sequences. It could indicate two separate disease foci originating from different import events (James and Varga, 2005).

Overall, 28 full-length, 7 nearly-full-length (apart from 25 and 61 terminal nts of the non-coding regions) and more than 150 partial sequences of about 150 different PPV isolates have been deposited in the GenBank before the end of 2007. Most of partial sequences (124) referred to the CP gene or to a longer part of the 3'-terminal genome region. Only about one sixth of the data related to the 5'-proximal or middle genome regions (Table 3). The highest number of full sequences belongs to the isolates of strain PPV-D. However, only about one half of them refer to independent natural isolates, the other data are concerning sub-isolates from the laboratory passages. The full-length sequences could not be so far effectively used for complex evaluation of PPV variability in broad epidemiological context, since they are imbalanced and non-representative. Thus, the complete sequences of a larger number of isolates from various host plants and from different geographic origin across the PPV strain spectrum have to be collected and evaluated for these purposes.

Table 3. PPV sequences deposited in GenBank until the end of 2007

Sequenced part of genome	No. of deposited sequences
P1-HC	1
P1-HC-P3-6K1	1
HC/HC-P3	10
P3-6K1-CI	14
NIa	1
CI-6K2-NIa-NIb-CP	1
NIb-CP	77
CP	47
Genome without terminal regions	7
Complete genome	28
Total	187

4. Present contribution and perspectives of the detection methods

A reliable diagnostic is the base for the virus spread control. The only possibility of crop protection in the regions with the epidemiological PPV occurrence is serial exchange of susceptible or tolerant genotypes by resistant cultivars. A consistent control of imported growing materials and early eradication of the potential infection foci is needed to prevent the virus extension in the new localities (Hennig *et al*., 2004). It requires a qualified application of fast and sensitive detection methods.

Scientists and producers do not underestimate the importance of sharka disease. A standard PPV detection protocol has been elaborated by the European and Mediterranean Plant Protection Organization (EPPO) and is continuously actualized according the newest knowledge (OEPP/EPPO, 2004). A tradition of the PPV experts' meetings was found in 1994 (Prague 1994, Langelois 1995,

Budapest 1996, Smolenice 1998, Pitesti 2001, Rogow 2004, Pula 2007). Original central European merit of these meetings turned to a world-wide significance. The aim is to keep the interested community informed about results of the PPV research and about the resistance against it. Contributions dealing with the new and improved virus detection methods are of special importance. Many of them are cited in this article.

We can conclude that two tests are the most important for the sharka diagnostics in a long run – ELISA and RT-PCR (standard or real-time). They probably will not be replaced by any other method in the near future (Cambra *et al*., 2006). A progress in the technology recorded a strong increase in the detection sensitivity. While ELISA may detect tenths of millions viral particles in 1 ml of extract, in hybridization and RT-PCR the sensitivity raises to millions particles in 1 ml, and in IC-RT-PCR to even thousands particles in 1 ml. The choice of detection method depends on the analysis goal. Protocols based on reactions in solutions may be used for virus quantification in the sample (ELISA and related techniques, real-time RT-PCR). Particular method types are focused on different aspects of viral infection. Immunochemistry proves usually the presence of CP, molecular methods detect the presence of viral RNA (even in the absence of CP or its presence under the detection limit), biological tests identify the virus able to replicate. The intensity of symptoms is not directly proportional to the concentration of PPV or presence of CP in the infected cells. The symptoms are rather caused by specific interactions with some host factors than by a general plant stress (Whitham and Wang, 2004; Almási *et al*., 1996). Some non-structural proteins like HC-Pro, P3 and 6K1 are obviously the pathogenicity determinants and their action is probably dependent on the host plant (Dallot *et al*., 2001; Saenz *et al*., 2000).

Pathotypization using *Ch. foetidum* was the first attempt in the history of intra-species classification of PPV (Sutic *et al*., 1971). The chlorotic and necrotic isolates could correspond to different strains according to the actual systematics, but this hypothesis was not confirmed. As shown by Glasa *et al*. (1997), the type of symptoms induced by an isolate may vary in dependence on the inoculum source (virus donor species). The early results of biological typing indicated the PPV variability. However, they do not make possible a clear and consistent intra-species classification, because of the environmental influences and subjectivity of evaluation (Candresse and Cambra, 2006). Although the PPV strains differ partially by the biological properties, detailed studies show that the virulence and competitiveness of isolates often do not correlate with their strain affiliation according to the sequence data (Salvador *et al*., 2006). Previously, PPV-M has been declared as more aggressive and more efficiently aphid-transmissible than PPV-D (Quiot *et al*., 1995). Some PPV-M isolates were really found more virulent in the experiments with consecutive or simultaneous mixed infections in herbaceous and woody plants, but there is no reason for a generalization (Capote *et al*., 2006; Šubr *et al*., 2006). The results are partially misrepresented by common use of biological tests on GF305 plants that seem to be more sensitive to PPV-M. Moreover, long-time overlooked PPV-Rec isolates (serologically interchangeable with PPV-M) could in the past considerably misrepresent the view on the epidemiology of PPV-M (Glasa *et al*., 2005).

The host adaptation plays a significant role in the PPV epidemiology. The geographic distribution of PPV strains is related to the history of growing particular crops (species, varieties) in different regions and to the importance attributed to them. Recent introduction of PPV into the New World involved exclusively the „less virulent“ strain PPV-D, what confirmed the key role of the trade on the long-distance spread of the virus.

Discovery of two serotypes M and D that were later confirmed also by SDS-PAGE and RT-PCR/RFLP stood at the birth of modern PPV strain typing (Quiot *et al*., 1995; Bousalem *et al*., 1994; Kerlan and Dunez, 1979). It led to the conclusion about two major groups of PPV isolates. Increasing interest in PPV research resulted in finding of further virus forms. Ten years ago, four strains of PPV were known: M, D, EA, and C (Pasquini and Barba, 1997). Additional two strains PPV-Rec and PPV-W were discovered in the first years of the new millennium (James *et al*., 2003; Glasa *et al*., 2001). It is probable that greater diversity within PPV will be observed in the future, especially by characterization of PPV in newly contaminated or inaccessible regions.

Concerning the strain discrimination, MAbs with the specificity to five of the six PPV strains have been prepared (Myrta *et al*., 2000; Boscia *et al*., 1997; Cambra *et al*., 1994). However, inconsistence of the serological and molecular tests for some PPV isolates let us to state that a definitive criterion for strain classification can be only the genome sequence analysis (Candresse and Cambra, 2006; Candresse *et al*., 1998a). According to the actual knowledge, the most reliable method of typing is the amplification of the NIb-CP genome region followed by the sequencing. This segment involves the most variable region of the genome, as well as the recombination point in the strain PPV-Rec. Of course, one cannot exclude escaping of the new virus forms from this typing like recent discovery of the isolate AbTk (Glasa and Candresse, 2005). Therefore, several parallel tests focused on the different genome parts are essential for the reliable typing.

The molecular PPV variability is considerably higher than expected a few years ago and a discovery of unknown virus forms in the near future could be anticipated. The pathogen evolution may be stimulated and accelerated also by growing

of the new plant cultivars with increased resistance against PPV causing selection of a new more virulent virus form. Monitoring of endangered regions using the detection methods of high specificity, sensitivity, and cost availability remains an important and effective tool in this arms race.

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