CHAPTER 8

Apple stem pitting virus

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Introduction

Apple stem pitting virus (ASPV) is a latent virus of apple, common in commercial cultivars. It is found worldwide wherever infected apples are grown. It frequently occurs in combination with *Apple chlorotic leafspot virus* (ACLSV) and other latent viruses of apple. Apple stem pitting disease was first described as incompatibility between *Malus sylvestris* 'Virginia crab' and certain apple scion cultivars in the United States of America in the 1940s (Jelkmann, 1997; Kundu and Yoshikawa, 2008; Nemeth, 1986; Smith, 1954; Stouffer, 1989). Guengerich and Millikan (1956) were the first to demonstrate by bud inoculation that the causal agent was an infectious, graft-transmissible agent. Subsequently, the same authors observed the symptoms of 'Spy 227' epinasty and decline when this rootstock was graft inoculated with buds from diseased trees. They suggested that the causal agent was identical with the previous one (Guengerich and Millikan, 1959; Millikan and Guengerich, 1959).

Indexing of infected apple, pear, and quince on woody indicator plants indicated that different syndromes such as pear vein yellows (Posnette, 1957), pear red mottle (Posnette, 1963a), pear necrotic spot (Kishi et al., 1976; Takanashi et al., 1983), quince sooty ring spot (Cropley, 1968; Refatti and Osler, 1973), quince fruit deformation (Paunovic, 1995; Scaramuzzi, 1957), and pear stony pit (Kienholz, 1939; Thomsen, 1989) were probably induced by the same causal agent of 'Spy 227' epinasty and decline and apple stem pitting (Desvignes and Savio, 1975; Desvignes, 1971; Kegler et al., 1976; Refatti and Osler, 1975).

Hibino and Schneider (1971) demonstrated the presence of filamentous virus particles in the leaves of pear trees with vein yellows by electron microscopy. Van der Meer (1986) reported for the first time successful transmission of an apple stem pitting-related virus from apple and from vein yellows and stony pit diseased pear to *Niccotiana occidentalis* '37B.' The same types of symptoms produced by the isolates from apple and pear suggested that the diseases were caused either by identical or similar viruses. Yanase et al. (1989) confirmed the presence of sap-transmissible, 12–15-nm wide, and 800-nm long filamentous virus particles associated with pear necrotic spot, pear vein yellows, and apple stem pitting diseased plants. Purification of these virus particles from *Nicotiana occidentalis* and their morphological characterization was described by Koganezawa and Yanase (1990). Final biological evidence that apple stem pitting and pear vein yellows diseases are caused by the same virus was achieved by back-transmission of ASPV from herbaceous plants to apple seedlings and subsequent reproduction of expected syndromes on woody indicator plants (Leone et al., 1995; Leone et al., 1998).

Molecular characterization of ASPV was initially achieved by dsRNA extraction, followed by cDNA cloning and sequence analysis (Jelkmann et al., 1992). The extraction of dsRNA from leaves of apple stem pitting, pear vein yellows, pear stony pit, quince sooty ring spot, and fruit deformation diseased pome fruit cultivars (Jelkmann et al., 1992; Jelkmann, 1994; Paunovic, 1995; Paunovic et al., 1999) further suggested infection with identical or closely related viruses. The major advance in the knowledge of genome characteristics of ASPV has been reported by Jelkmann (1994) and Yoshikawa et al. (2001). The ASPV genome, together with a considerable number of partial sequences of strains from around the world, has enabled development of less time-consuming detection methods. Using these methods, ASPV was detected in latently infected apple and pear with vein yellows symptoms (Batlle et al., 2004; Kummert et al., 1998; Kundu, 2003, 2008; Malinowski et al., 1998; Menzel et al., 2002; Nemchinov et al., 1998; Paunovic and Jevremovic, 2004). The association of ASPV in infected pear with stony pit disease, quince with sooty ring spot, and quince fruit deformation diseases was confirmed by reverse transcription and polymerase chain reaction (RT-PCR) systems (Menzel et al., 2003; Paunovic et al., 1999; Schwarz and Jelkmann, 1998).

Taxonomic Position and Nucleotide Sequence

Apple stem pitting virus is the type species of genus *Foveavirus* (Martelli and Jelkmann, 1998). The genus was recently assigned to the newly established family *Betaflexiviridae* (Adams et al., 2004; Martelli et al., 2007). Other members in the genus are *Apricot latent virus* ApLV and *Rupestris stem pitting-associated virus* (RSPaV) and *Asian prunus viruses*. ASPV consists of flexuous, filamentous particles approximately 800 nm in length and 12–15 nm in width, with helical symmetry. The length distribution of the particles in dip preparations showed the formation of end-to-end aggregations (Giunchedi and Poggi Pollini, 1992; Jelkmann et al., 1992; Jelkmann, 1997; Koganezawa and Yanase, 1990). In cytopathological studies of infected *N. occidentalis* plants, flexuous filamentous virus particles can be seen in cytoplasm of mesophyll parenchyma cells, usually arranged in long bundles (Giunchedi and Poggi Pollini, 1992). Infected cells displayed higher vacuolization but no other obvious cytopathological alterations. In thin sections, Kundu et al. (2006) found alterations of cellular membrane including proliferation of the endoplasmic reticulum and formation of vesicles.

The ASPV genome is a monopartite, positive, single-stranded RNA (ssRNA) of 9,332 nt excluding the poly A tail. The genome comprises five open reading frames (ORFs) encoding the

replication-related protein (ORF1), the putative movement proteins (MPs, encoded as a triple gene block: ORFs 2 to 4), and the coat protein-CP (ORF5). The replication-related protein contains motifs associated with methyltransferase, helicase, and RNA-dependent RNA polymerase activities. The ORFs 2 to 4 represent the triple gene block (TGB) typically found in *Potexvirus, Mandarivirus, Allexivirus,* and *Carlavirus* (Martelli et al., 2007). Coat protein subunits are of one type, about 42–44 kDa in size (Jelkmann, 1994). The replication of ASPV is likely to take place with a strategy comparable to that of *Potexviruses,* based on direct expression of the 5′-proximal ORF and expression of downstream ORFs through subgenomic RNAs (Martelli and Jelkmann, 1998).

The complete nucleotide sequences of two ASPV isolates (PA66 and IF38) from apple are available in the GeneBank nucleotide sequence databases (D21829 and AB045731) (Jelkmann, 1994; Yoshikawa et al., 2001). The complete PA66 genome consists of 9,332 nucleotides, excluding the 3′ poly (A) tail. At the 5′ terminus, 59 non-coding nucleotides were identified. The putative polypeptides encoded by different ORFs are as follows: ORF1 (nt positions 34 [as resolved in database accession no. D21829] to 6,582) M_r 247 K; ORF2 (nt 6,685 to 7,353) M_r 25 K; ORF3 (nt 7,358 to 7,717) M_r 12 K; ORF4 (nt 7,629 to 7,838) M_r 7 K; ORF5 (nt 7,930 to 9,171) approximate M_r 44 K. The 3′-noncoding region consists of 135 nucleotides, excluding the poly (A) tail (Jelkmann, 1994; unpublished results). Recently, a second genotype has been identified from the PA66 source, showing less than 80% nucleotide sequence homology with the original sequence (Jelkmann, unpublished results).

The complete nucleotide sequence of the genome of isolate IF38 reported by Yoshikawa et al. (2001) shows high variability as compared with the PA66 genome. The IF38 genome consists of 9,237 nts excluding the 3′ poly (A) tail. At the 5′ end, it contains 60 non-coding nucleotides. The 3′-non-coding region consists of 132 nucleotides, excluding poly (A) tail. As demonstrated for the CP of isolate PA66 (Jelkmann et al., 1992; Jelkmann and Keim-Konrad, 1997), the 42 K protein expressed in *E. coli* reacted with antiserum against ASPV and coelectrophoresed with the ASPV CP from infected tissues, indicating that this 42 K protein is indeed the ASPV-CP. The comparison of nucleotide sequences of the PA66 and IF38 genomes shows only 76% identity between the two isolates. Comparison of amino acid sequences of the five encoded proteins indicates identity values of 87%, 94%, 87%, 77%, and 81% for the ORFs from 1 to 5, respectively. The 25 K (TGB1) protein is most conserved between the two isolates. Another remarkable variability feature between IF38 and PA66 is found in the N-terminal region of CP in which there are many deletions of IF38 as compared with PA66, which is 18aa smaller as a result.

The results obtained by Schwarz and Jelkmann (1998) and Nemchinov et al. (1998) also showed genetic variability of the nucleotide sequences of the CP coding regions of several ASPV isolates originating from pear and apple, as well as deletions in the N-terminal part of the coat protein gene in some of those isolates. Using restriction length polymorphism (RFLP) and sequence analysis of a 600 nt fragment of nine ASPV isolates Yoshikawa et al. (2001) found identity levels of between 64.8 to 89.7%. Up to three sequence variants were found in one isolate.

Economic Impact

Prior to the introduction of thermotherapy and certification systems, most commercial pome fruit cultivars and rootstocks were latently infected with ASPV, often in mixed infection with the two apple latent viruses *Apple stem grooving virus* (ASGV) and *Apple chlorotic leaf spot virus* (ACLSV), as well as *Apple mosaic virus* (ApMV). Several studies have shown significant effect of these viruses on cropping and growth characteristics of infected trees (Campbell, 1963; Li et al., 1993; Nemeth, 1986; Posnette, 1960, 1963b). In addition to decline of infected apple scions grafted on 'Virginia Crab,' the cultivar 'Charden' showed incompatibility on Malling rootstocks due to ASPV infection (Lemoine, 1977). However, since virus detection in the previous reports was based on symptomatology on woody indicators, and often mixed infections occurred, it was difficult to assess the effect of ASPV on its own.

Pear cultivars infected only with ASPV showed slightly lower percentage of bud taking (5%) in nursery than virus-free ones, 15–50% lower cropping, which varied by cultivar, and a 10–15% decrease in tree vigor (Lemoine and Michelesi, 1990, 1995). The virus infection may reduce stem girth up to 18% and shoot growth 29–55% relative to the controls, depending on year and cultivar (Cropley and Posnette, 1973; Thomsen, 1975).

Presence of the ASPV associated with pear stony pit and quince fruit deformation can significantly reduce fruit quality and yield (Paunovic, 1995; Thomsen, 1989). The ratio of damaged fruits (between 18–94%) may change considerably from year to year on the same tree.

Symptoms

Most of commercially grown apple cultivars remain symptomless after infection with ASPV, but susceptible cultivars of different pome fruit species and indicator plants do react with a variety of symptoms. Characteristic symptoms on susceptible apple cultivars 'Charden' and 'Reinette Clochard' (Desvignes et al., 1999) and on woody indicator plants *M. pumila* 'Virginia crab' are xylem pitting in the stem (Fig. 8.1). The configura-

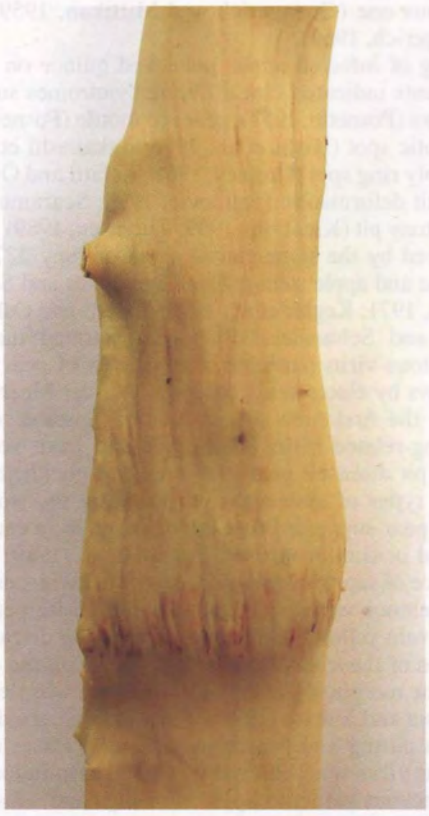

Fig. 8.1. Stem pitting caused by ASPV on 'Virginia crab.'

tion of the pits and the severity of wood pitting are variable. On 'Virginia crab,' symptoms appear on the stem above the union line. The virus was named after the symptoms caused on 'Virginia crab.' Fruits of 'Virginia crab' are shorter than normal. Another characteristic syndrome produced by infection with ASPV is epinasty and decline on apple indicator plants *M. pumila* 'Spy 227' (Fig. 8.2). ASPV causes a severe topworking disease in Japan and induces lethal decline in apple trees grown on sensitive *M. sieboldii* rootstocks (Koganezawa and Yanase, 1990).

The symptoms on susceptible pear cultivars and pear indicators of *P. communis,* i.e., cvs 'Beurre Hardy', 'Nouveau Poiteau' and 'Jules d'Airroles,' are expressed as in narrow chlorotic bending of the secondary and tertiary veins and parts of adjacent tissue and red mottling (Fig. 8.3). The leaf symptoms develop in spring under cool weather conditions and become most conspicuous by midsummer. Many commercial pear cultivars develop symptoms only during the first few years of growth, whereas older trees can be symptomless. Some pear cultivars express symptoms of red mottling and flecking along both side of finer veins or necrotic spotting on the leaves (Cameron, 1989). The fruits of some sensitive cultivars and indicator plant 'Beurre Bosc' can express symptoms of bumps and distortions with sclerenchymatous cells in the flesh, well-known as stony pit (Fig. 8.4). On *Pyronia veitchii,* ASPV induces chlorotic spots on leaves (Fig. 8.5), epinasty, and deformation of leaves, as well as longitudinal grooving on the xylem.

Fig. 8.2. Spy epinasty and decline (three plants at left; healthy at right).

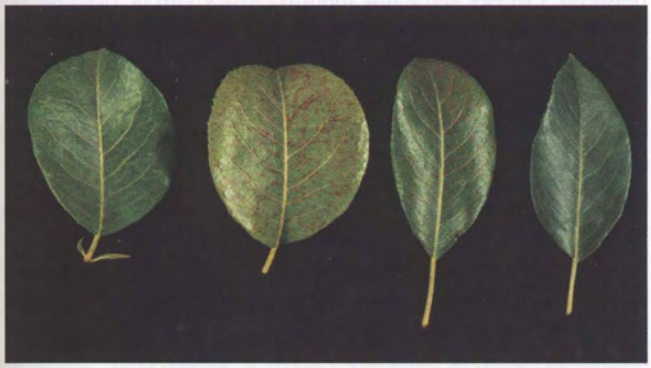

Fig. 8.3. Vein yellows and red mottle on *Pyronia veitchii,* healthy at right.

The symptoms associated with ASPV infection on susceptible quince cultivars and indicator plants are characteristic black sooty lines and rings bordering veins, pale yellow spots on leaves, and fruit malformations with numerous depressions and bumps (Fig. 8.6) (Desvignes, 1971; Paunovic, 1995).

ASPV induces small necrotic local lesions 4–7 days after inoculation on *N. occidentalis* subsp. *obliqua,* followed by systemic vein yellows, vein necrosis, and necrosis of leaf parts or

Fig. 8.4. Pitted and deformed fruit caused by ASPV on Beurré Bosc at top left; healthy fruit at top right; longitudinal sections of ASPV -infected Beurré Bosc fruit at bottom left showing sclerenchyma cells beneath or surrounding the pits; section of healthy fruit control at bottom right.

Fig. 8.5. Symptoms of chlorotic leaf spots on *Pyronia veitchii* caused by ASPV.

Fig. 8.6. Quince fruit deformation of cv. 'Leskovacka' associated with ASPV infection.

Fig. 8.7. ASPV symptoms on *Nicotiana occidentalis* subsp. *obliqua;* necrotic local lesions at left; systemic vein yellowing at right. Healthy leaf in the middle.

whole leaves (Fig. 8.7), whereas systemic vein yellows and necrosis of the finest veins occur on *N. occidentalis* '37B'.

Host Range

The natural host range of ASPV is restricted to a few hosts such as *Malus* spp., *Pyrus communis* (European pear), *P. serotinia* var. culta (Japanese pear), *P. ussuriensis* (Chinese pear), *Cydonia oblonga* (quince), *Crategus* spp., and *Sorbus* spp. (Nemeth, 1986). Experimental hosts are *N. occidentalis* Wheeler '37B' (van der Meer, 1986) and *N. occidentalis* subsp. obliqua (Koganezawa and Yanase, 1990).

Transmission

ASPV has no known vector. The virus is transmitted by grafting, budding, and through infected clonal rootstocks. ASPV from infected apple trees with stem pitting as well as from pear trees with vein yellows, necrotic spot, and stony pit is mechanically transmissible, although with some difficulty, to the most sensitive test plants: *N. occidentalis* subsp. *ovbliqua* and *N. occidentalis* Wheeler '37B' (Koganezawa and Yanase, 1990; Nemeth, 1986; van der Meer, 1986; Yanase et al., 1989). A successful back-transmission from *N. occidentalis* to apple and pear was achieved by Leone et al. (1995). Mechanical transmission of ASPV isolates has resulted in different leaf symptoms on *Chenopodium quinoa, C. murale, Sesamum indicum, Celosia cristata, Gomphrena globosa, N. clevelandii, Beta vulgaris* subsp. *cicla, Physalis floridana, Cucumis sativus,* and *Tetragonia expansa*. However, virus particles have only been reported from *N. occidentalis* (Koganezawa and Yanase, 1990), so it is not clear whether the symptoms in these other hosts were caused by ASPV or by other co-infecting viruses. ASPV isolates associated with quince fruit deformation and quince sooty ring spot diseases have not been mechanically transmitted to herbaceous plants (Paunovic and Rankovic, 1998).

Geographical Distribution and Epidemiology

Apple stem pitting virus is widespread, occurring worldwide wherever infected apple, pear, and quince are grown. Disease reports from all continents, based on woody indexing, are reviewed in Cameron (1989), Nemeth (1986), Stouffer (1989), Takanashi (1989), and Thomsen (1989). With the availability of molecular diagnostic techniques in the early 1990s, APSV isolates from apple and pear have been increasingly reported from all continents, thus confirming earlier disease reports.

Because ASPV has no natural vectors, the main way of spreading is by infected plant propagation material, using either infected rootstocks or buds, or both. Transmission by root contact has been reported. Therefore, isolation distances between certified propagation material and nursery plants of lower health status are required (EPPO, 1999). Despite the availability of certified virus-free plants, use of propagation material of lower health status or uncontrolled exchange of infected propagation material remains a source of virus spread.

Detection

Field inoculation of woody indicator plants is still the standard biological indexing procedure for ASPV (EPPO, 1999; Jelkmann, 2001; Nemeth, 1986). *M. pumila* cvs 'Virginia crab' and 'Spy 227', *Pyrus communis* cvs 'Jules d' Airolles', 'Beurrre Bosc' and 'Doyenné du Comice,' and *Pyronia veitchii* are standard indicator plants for testing for the presence of ASPV in pome fruit. Two to three years are needed for the expression of symptoms on some of these indicator plants, and an even longer period is necessary for the occurrence of symptoms on indicator fruits (EPPO, 1999; Stouffer and Fridlund, 1989).

Due to some disadvantages of field indexing, such as time and space requirements, as well as variability or lack of symptoms from year to year as the result of uncontrolled environmental conditions, indexing may be conducted in a greenhouse under controlled conditions (Fridlund, 1980; Gilles and Bormans, 1989). Greenhouse testing can significantly increase accuracy, efficiency, and economy because the average time for symptom development can be drastically reduced from 1–4 years to between 3 weeks and a few months. ASPV can be detected on *M. pumila* cvs 'Virginia crab,' 'Radient,' 'Kola,' 'Sparckler,' 'Spy 227,' and from pear and quince hosts on *Pyrus communis* 'Nouveau Poiteau,' 'Beurré Bosc,' or 'Passe Crassane.' The symptoms can be expressed after 4–10 weeks (with cutting back of indicator shoot after 4 weeks to 7–8 cm and complete defoliations) at constant temperatures of 22–26°C.

ASPV isolates from apple and pear can be detected with low sensitivity by mechanical inoculation of *N. occidentalis* subsp. *obliqua* and *N. occidentalis* '37B' (van der Meer, 1986).

Laboratory techniques such as immunosorbent electron microscopy (IEM), plate trapped enzyme-linked immunosorbent assay (ELISA), and Western blot analysis were possible after production of polyclonal antibodies for experimentally purposes (Jelkmann et al., 1992; Jelkmann and Keim-Konrad, 1997; Paunovic et al., 1999). Recently, monoclonal antibodies have been produced for reliable routine detection of ASPV in double-antibody-sandwich ELISA (DAS-ELISA) in older and

intermediate apple and pear leaves (Gugerli and Ramel, 2004). These monoclonal antibodies have been commercially available since 2005.

Upon successful nucleotide sequencing of the ASPV genome (Jelkmann, 1994), specific, rapid, reliable, and the most sensitive laboratory detection of this virus is possible by several RT-PCR based methods (Jelkmann and Keim-Konrad, 1997; Kummert et al., 1998; Malinowski et al., 1998; Nemchinov et al., 1998; Schwarz and Jelkmann, 1998; Kundu, 2003; Paunovic et al., 1999; Paunovic and Jevremovic, 2004). In addition, rapid and sensitive gel-free detection system (AmpliDet RNA) of ASPV has been developed through RNA amplification and probing with fluorescent molecular beacons (Klerks et al., 2001). Roussel et al. (2005) have developed real-time RT-PCR assays using fluorogenic-3′ minor groove binder DNA probes for detection of ASPV in bark tissues of dormant wood. ASPV may also be detected by multiplex RT-PCR simultaneously with *Apple mosaic virus* (ApMV) (Menzel et al., 2002; Menzel et al., 2003) and with one-tube pentaplex RT-PCR simultaneously with the following pome fruit viruses: ASGV, ACLSV, and ApMV (Hassan et al., 2006). Thanks to the availability of commercial RNA extraction kits and the development of methods circumventing enzyme reaction-interfering components such as polyphenols and polysaccharides (Kundu, 2003; Rott and Jelkmann, 2001), RT-PCR has become a reliable virus detection method for woody plants. In light of the progress in extraction technology together with several studies on variability of ASPV strains, RT-PCR has shown to be the superior detection method for ASPV compared with woody indexing (Menzel et al., 2003).

Control

Because ASPV is transmitted only by grafting, it can be controlled by planting pathogen-tested material of pome fruit cultivars and rootstocks produced by application of certification schemes, such as those recommended in IR-2/NRSP-5 (now known as NCPN-FT or the National Clean Plant Network for Fruit Trees) in the United States (Fridlund, 1976; Mink, 1998), or by EPPO (1999) in European countries. Virus-free plants can be propagated from tips after application of thermotherapy at 37°C for 60–80 days (Mink et al., 1998; Nemeth, 1986).

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