Investigation of virus occurrence in different tissues throughout the year and sequence variability of *Apple stem pitting virus*

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Abstract

The occurrence of *Apple stem pitting virus* (ASPV) isolate PB 66 in three different types of tissue of four different apple varieties throughout the year was determined. Reliable virus detection in phloem tissue was observed in all four apple varieties investigated, at all sampling dates during the year. The complete nucleotide sequence of ASPV isolate PB 66 was determined and compared to ASPV isolate PA 66. The isolates show 80 % sequence identity. Comparison of the ASPV PA 66 coat protein amino acids sequence with 16 other ASPV isolates from different hosts revealed an insertion event of 18 amino acids.

Keywords: *Apple stem pitting virus*, Foveavirus, Flexiviridae

Introduction

In commercial apple cultivars infection with latent RNA viruses cause yield losses up to 60%. *Apple stem pitting virus* (ASPV) is one of the most common latent viruses in apple worldwide. It is classified as a *Foveavirus* and belongs to the family *Flexiviridae* (Adams et al., 2004; Martelli and Jelkmann 1998). Viruses belonging to this family have flexuous filamentous virions with a size of 12 -13 nm in diameter and monopartite, positive sense ssRNA genomes with a 3'-polyA tail. ASPV consists of five open reading frames (ORF) of which the first ORF encodes the replication related proteins. ORF 2-4 encode the triple gene block which is necessary for virus movement in the plant. The coat protein is encoded by ORF 5. High sequence variability of different ASPV isolates was reported previously (Yoshikawa et al., 2001).

ASPV is transmitted by grafting, root contact and mechanical inoculation. To avoid spreading of ASPV it is necessary to use virus free material for propagation. For testing/re-testing virus free propagation material it is important to know which tissue of apple trees provides reliable results and if this depends on the sampling time during the year. Investigation of virus occurrence in different tissues throughout the year was done previously for Apple stem grooving virus (Kundu et al., 2003).

In this report we present the results of sequence comparison between the two ASPV isolates PA 66, PB 66 and other isolates in Genbank, also the results of virus detection in different tissues at different times throughout the year.

Material and methods

The ASPV isolate PB 66 was maintained in four different apple varieties (*Malus domesticus* 'Gloster', 'Golden Delicious', 'Cox' and 'Egremont Russet') in the field and in *Nicotiana occidentalis* 'Wheeler' 37B in the greenhouse. For the time course investigation total RNA was extracted at least once a month throughout the year from leaves, phloem from stem tissue, and phloem from root tissue, using the silica capture method (Rott and Jelkmann 2001). cDNA was synthesized from viral RNA template using random hexanucleotide primers (Invitrogen) and M-MuLV reverse transcriptase (Fermentas). Virus detection was performed with the primers described by Menzel et al. (Menzel et al., 2002). For the determination of the sequence of isolate PB 66 the virus RNA was extracted with RNeasy Plant Kit (Qiagen). First strand cDNA synthesis was performed with SuperScript reverse transcriptase II (Invitrogen) and Oligo(dT) Primer (Fermentas). Second strand DNA synthesis was performed with the Phusion Taq- Polymerase (Biocat). Amplification of the 5'- end was done with RACE- PCR. In both cases the amplified products were analyzed in 1% agarose gels in 1x TAE buffer for 50 min at 100V, and stained with EZ Vision (AMRESCO). The PCR products for sequence determination were cloned using the pJet1.2 vector (Fermentas) and sequenced by Seqlab (Göttingen). Alignments and analysis of sequences were performed with Lasergene programs (DNASTAR).

Results

For the investigations on time course studies for virus detection total RNA was extracted 16 times per year from leaves and phloem from stem tissue, and 13 times from the phloem of roots, because the frozen ground prevented extractions from roots in the wintertime. In the apple varieties 'Egremont Russet' and 'Gloster' ASPV could be detected by PCR during the whole year, in all three tissue types (Table 1). In the two apple varieties 'Golden Delicious' and 'Cox' PCR results were positive, except for August in leaves and in December in the phloem from roots. Apart from these exceptions, reliable virus detection was observed in all four apple varieties throughout the year.

Tab. 1 Results of *Apple stem pitting virus* occurrence in four apple varieties in different tissues throughout the year

Apple variety	Tissue	Positive results
Egremont Russet	leaf/bud	16/16
	phloem	16/16
	phloem of roots	13/13
Golden Delicious	leaf/bud	15/16
	phloem	16/16
	phloem of roots	12/13
Gloster	leaf/bud	16/16
	phloem	16/16
	phloem of roots	13/13
Cox	leaf/bud	15/16
	phloem	16/16
	phloem of roots	12/13
Golden Delicious	leaf/bud	16/16
	phloem	16/16
	phloem of roots	13/13

The complete nucleotide sequence of ASPV isolate PB 66 was determined. The molecule is 9363 nucleotides (nt) excluding the polyA- tail and has a GC- content of 43,5 %. It consists of five open reading frames (ORFs). Comparison of isolate PA 66 (Jelkmann 1994) and isolate PB 66 revealed 80 % sequence identity (Table 2).

Tab. 2 Comparison between the genomes of *Apple stem pitting virus* isolates PA 66 and PB 66 (ORF: Open reading frame; nt : nucleotide; aa : amino acid; UTR : untranslated region)

	PA 66		PB 66		nt sequence	amino acid
	nt	aa	nt	aa	identity (in %)	similarity (in %)
genome size	9332	-	9363	-	80	-
5' End UTR	59	-	60	-	96	-
ORF 1	6549	2183	6549	2183	79	90
UTR between ORF 1 and ORF 2	99	-	175	-	76	-
ORF 2	669	223	669	223	83	95
UTR between ORF 2 and 3	1	-	1	-	-	-
ORF 3	360	120	363	121	87	91
overlapping Region ORF 3/ ORF 4	91	-	94	-	-	-
ORF 4	210	70	228	76	85	90
UTR between ORF 4 und ORF 5	88	-	75	-	75	-
ORF 5	1242	414	1188	396	82	81
3' End UTR	132	-	135	-	90	-

The first ORF (M_r 246810) which encodes the replication related proteins is identical in size between both isolates. It has 79 % nt identity and 90 % amino acid (aa) identity. ORF 1 has the lowest nt identity of all coding regions. ORF 2 – 4 encode the triple gene block. ORF 2 (M_r 25242) has 83 % nt identity and 95 % aa identity between these two isolates. The ORF 2 of both isolates encodes a putative protein 223 aa in length. ORF 3 (M_r 12916) is one aa longer in isolate PB 66 and has a sequence identity of 87 % and an aa identity of 91 %. ORF 4 (M_r 8057) has a nt identity of 85 % and an aa identity of 90 %. It is 6 aa longer in isolate PB 66. ORF 5 (M_r 42144) encodes the coat protein and has a nt identity of 82 % and an aa identity of 81 %. It has the lowest aa identity of all ORFs and is 18 aa shorter in isolate PB 66. Figure 1 shows an alignment of 17 ASPV coat protein sequences between the start codon and position 65 related to isolate PA 66. Only isolate PA 66 and the isolate associated with pear vein yellows (Jelkmann, 1994) have an insertion of 18 aa (from pos. 28 to 45). The 5'- end untranslated region (UTR) shows 96 % nt identity and is one nt longer in isolate PB 66. The 3'- end UTR has 90 % sequence identity and is three nt longer in PB 66. The other UTRs between the ORFs show sequence identities under 80 %.

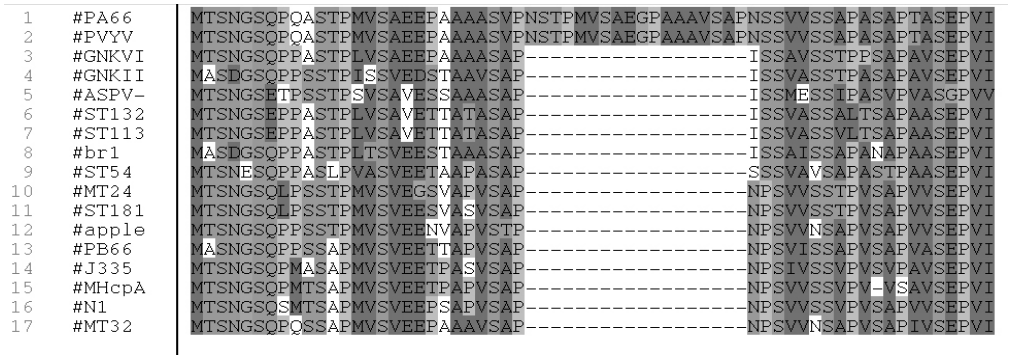

Fig. 1 Aligment with ClustalX of a partial coat protein amino acid sequence of different ASPV isolates. Gaps indicated by tabs.

Discussion

The complete genomic sequence of ASPV isolate PB 66 consists of 5 ORFs with a total length of 9363 nt excluding the polyA-tail. In comparison to isolate PA 66 it is 30 nt longer. Both isolates have a sequence identity of 80 %. The 5'-UTR with 96 % and the 3'- UTR with 90% sequence identity are highly conserved among these two isolates. This underlines their importance for the infectivity of the virus. The first ORF which encodes the replication related proteins shows the lowest nt identity, but has 90 % aa similarity thus demonstrating a high rate of silent mutations. The triple gene block seems to be more conserved among ASPV isolates. The fourth ORF is 6 aa longer in PB 66 than in PA 66. Comparison with the two other complete ASPV isolate sequences from Genbank show a deletion of 5 aa (QGVSV) at the C-terminus in ASPV isolate PA 66 and one additional aa isolate PB 66 (Q). The effect of this sequence variation in the triple gene block protein for virus movement has not yet been investigated. The coat protein shows very low nt identity with 82 % and also low aa identity at 81 %. The isolate PA 66 has an insertion of 18 aa in the coat protein. Comparison with other isolates from the database show that only the isolate PA 66 and a pear vein yellows isolate have the insertion of 18 aa in the coat protein. It was suggested that an insertion in the coat protein sequence can influence the geographical distribution of virus isolates (Viswanathan, Karuppaiah et al. 2009), host specialization of certain isolates (Galipienso et al., 2009) or the virus accumulation in the host plant (Szathmary et al., 2009).

Detection of ASPV is possible during the whole year. In summer the virus titer seems to decrease in the leaves of all four varieties, which was also reported previously for *Apple stem grooving virus* (Kundu et al., 2003). For the detection of ASPV throughout the year phloem is a reliable tissue in all four apple varieties. The detection primers (Menzel et al., 2002) which were used for the investigation on virus occurrence in different tissues are located in the coat protein coding region. In the case of ASPV isolate PB 66 the forward primer has no mismatches or gaps. The reverse primer has a mismatch and a base exchange at the 3'- end. These isolates show high variability in the coat protein coding sequence indicating a possibility that there could be isolates that are not detected with the published detection primers.

Literature

Adams, M. J., Antoniw, J. F., Bar-Joseph, M., Brunt, A. A., Candresse, T., Foster, G. D., Martelli, G. P., Milne, R. G., and Fauquet, C. M., 2004: The new plant virus family *Flexiviridae* and assessment of molecular criteria for species demarcation. Arch Virol **149**, 1045-1060.

Galipienso, L., Rubio, L., Lopez, C., Soler, S., and Aramburu, J., 2009: Complete nucleotide sequence of a Spanish isolate of Parietaria mottle virus infecting tomato. Virus Genes **39**, 256-260.

Jelkmann, W., 1994: Nucleotide sequences of apple stem pitting virus and of the coat protein gene of a similar virus from pear associated with vein yellows disease and their relationship with potex- and carlaviruses. Journal of General Virology **75**, 1535-1542.

Kundu, J. K., Svoboda, J., and Polák, J., 2003: Detection of *Apple Stem Grooving Virus* in Different Tissues of Apple Trees throughout the Year. Plant Protect.Sci. **39**, 93-96.

Martelli, G. P. and Jelkmann, W., 1998: *Foveavirus*, a new plant virus genus. Arch Virol **143**, 1245-1249.

Menzel, W., Jelkmann, W., and Maiss, E., 2002: Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. Journal of Virological Methods **99**, 81-92.

Rott, M. E. and Jelkmann, W., 2001: Characterization and detection of several filamentous viruses of cherry: Adaption of an alternative cloning method (DOP-PCR), and modification of an RNA extraction protocol. European Journal of Plant pathology **107**, 411-420.

Szathmary, E., Nadudvari, J. N., Szabo, L., Tobias, I., Balazs, E., and Palkovics, L., 2009: Characterization of a natural Plum pox virus isolate bearing a truncated coat protein. Archives of Virology **154**, 141-145.

Viswanathan, R., Karuppaiah, R., and Balamuralikrishnan, M., 2009: Identification of new variants of SCMV causing sugarcane mosaic in India and assessing their genetic diversity in relation to SCMV type strains. Virus Genes **39**, 375-386.

Yoshikawa, N., Matsuda, H., Oda, Y., Isogai, M., and Takahashi, T., 2001: Genome Heterogeneity of Apple Stem Pitting Virus in apple trees. In: M. F. Clark (ed.), Acta Hort., 285-290.