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# Influence of *Apple stem grooving virus* on *Malus sieboldii*-derived apple proliferation resistant rootstocks

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## Abstract

*Apple stem grooving virus* (ASGV, Capillovirus) is widely spread in apple growing regions. As it causes no symptoms on most cultivated apple varieties and rootstocks it is considered latent in *Malus* x *domestica*. In Asia, however, ASGV has been found associated with topworking disease of apple rootstocks originating from *Malus sieboldii*. Recently, *M. sieboldii* and its hybrids have gained new interest in Europe as they confer resistance to apple proliferation (AP) disease. A new breeding program aiming to develop AP-resistant rootstocks of agronomic value for modern apple culture, reported unexpected tree decline which was to be associated with ASGV. As little information is available on the variability of ASGV isolates in Germany, the complete genome of a German isolate of ASGV associated with tree decline was cloned and sequenced. Sequence comparisons with available ASGV isolates revealed two regions of high variability in the genome. The genetic variability of additional isolates from Germany and other countries were collected and the variable areas characterised. In addition ASGV was successfully maintained in micropropagated apple trees and could be transmitted by *in vitro* grafting to various genotypes, making it possible to study *in vitro* the effect of the virus and virus/phytoplama combination on *M. sieboldii*-derived genotypes.

Keywords: Latent apple viruses, Candidatus *Phytoplasma mali*, micropropagation, *in vitro* grafting, genetic variability.

## Introduction

Apple proliferation (AP) disease is one of the most devastating fruit tree diseases in Europe. The disease is caused by Candidatus *Phytoplasma mali* and causes significant economic loss by rendering the fruit unmarketable. Symptoms include "witches brooms", enlarged stipules, growth reduction and undersized fruits (Seemüller et al., 2008). The planting of healthy material and elimination of diseased trees are not sufficient in controlling the spread of the disease because of the difficulty controlling the two psyllid vectors, *Cacopsylla picta* and *C. melanoneura* (Frisinghelli et al., 2000; Tedeschi and Alma 2004). The breeding of apple proliferation resistant rootstocks is considered to be the most promising solution to control apple proliferation disease. Natural resistance was discovered in the wild *Malus* species *M. sieboldii* making it a prime candidate for breeding experiments to develop apple proliferation resistant rootstocks (Kartte and Seemüller, 1988, 1991; Jarausch et al., 2008; Seemüller et al., 1992, 2007). During the screening of *M. sieboldii* and its hybrids for AP-resistance, unexpected tree decline was observed and found to be associated with latent apple viruses (Seemüller et al., 2008). An AP-resistance screening system has also been established *in vitro* (Bisognin et al., 2008) which is based on *in vitro* graft inoculation of *Ca.* P. mali (Jarausch et al., 1999). After the report of Seemüller et al. (2008) about virus-associated tree decline in the breeding progeny a similar decline could be observed in the *in vit*ro resistance screening. In this case the decline was associated with *Apple stem grooving virus* (ASGV) alone.

ASGV is widely spread in apple growing regions. As it causes no symptoms on most cultivated apple varieties and rootstocks it is considered latent in *Malus* x *domestica*. In Asia, however, ASGV has been found associated with topworking disease of apple rootstocks originating from *M. sieboldii* (Yanase, 1974; 1981). *Citrus tatter leaf virus*, a strain of ASGV, causes bud union incompatibility and necrosis when grafted on sensitive citrus material (Calavan et al., 1963; Miyakawa and Matsui, 1977; Miyakawa and Ito, 2000). ASGV has a positive sense single stranded genome consisting of 6496bp (excluding the poly A tail) containing two overlapping open reading frames (ORF's) (Yoshikawa & Takahashi, 1988; Yanase et al., 1990; Yoshikawa et al., 1992), with two areas of high variability,V1 from amino acid (aa) 530-570 and V2 from aa 1583-1868 (Tatineni et al., 2009). The natural transmission is unknown. It is transmitted through grafting of infected material.

The objective of this study is to understand the influence of latent viruses on phytoplasma resistant genotypes. We established an *in vitro* test system to better understand the virus and the virus isolates associated with the rapid decline in phytoplasma resistant plants.

## Materials and methods

Healthy, ASGV and/or Ca. *P. mali* infected Malus shoot cultures were propagated in a growth chamber on modified Murashige & Skoog medium as described (Jarausch et al., 1996, 1999; Ciccotti et al., 2008). The plantlets were subcultured every 6 to 8 weeks. ASGV and/or Ca. *P. mali* infected lines were developed by grafting ASGV and/or Ca. *P. mali* infected material on healthy Malus plantlets (Jarausch et al., 1999). Healthy genotypes were *M.* x *domestica* cv. Golden Delicious and *Malus sieboldii.* The graft contact was maintained for 6-8 weeks and then, graft tips were removed and tested for infection. Total nucleic acid from the inoculated rootstocks was extracted using the CTAB method described in Jarausch et al. (2004) and tested with RT-PCR and PCR using pathogen specific primers for ASGV and Ca. *P. mali*, respectively (Massart et al., 2008; Jarausch et al., 1994).

The complete genome of a German isolate of ASGV was amplified cloned and sequenced according to standard procedures. ASGV variability analysis was performed on isolates received form Germany, Canada, France, Australia and Austria. The two variable regions were amplified and the generated fragments cloned and sequenced. BioEdit (Ver. 7.0.4) (Hall, 1999) was used to perform sequence editing and compilation. Generated ASGV nucleotide sequences were compared to ASGV sequences downloaded from GenBank, using the ClustalW (Ver. 1.4) alignment function embedded in the BioEdit software.

## Results

As little information is available on the variability of ASGV isolates in Germany, the complete genome of a German isolate of ASGV (ASGV-AC) associated with tree decline was cloned and sequenced. The 6496bp (excluding the poly A tail) generated ASGV-AC sequence showed 80.6-82.5 % identities when compared to available ASGV sequences from GenBank. Sequence comparisons also revealed two regions of high variability in the genome, consistent with the findings of Tatineni et al., (2009). The genetic variability of additional isolates from Germany, Canada, France, Australia and Austria were collected and the two variable areas characterised. Phylogenetic analysis showed that all the studied German isolates grouped together.

Tissue culture lines of *M.* x *domestica* cv. Rubinette infected with ASGV or Ca. *P. mali* have been established previously in the lab. In this work, repeated molecular analysis of the viral infection status has been used to select and maintain homogenous culture lines infected with the specific ASGV-AC isolate. This culture line was used to successfully transmit ASGV by *in vitro* grafting to *M. sieboldii* and *M.* x *domestica* cv. Golden Delicious yielding an overall transmission rate of 32 %. These grafting experiments were performed to reproduce *in vitro* the putative hypersensitive reaction of *M. sieboldii* to ASGV in comparison to the reaction of the virus tolerant cultivar Golden Delicious. And indeed, *M. sieboldii* showed a high incidence of graft union necrosis when grafted with ASGV infected material. No necrosis was observed on the Golden Delicious controls. These data are preliminary and need confirmation, but they show that the *in vitro* system can be used to study the interactions associated with the rapid decline observed in *M. sieboldii* derived AP-resistant genotypes.

## Conclusion

Tissue culture lines were developed and established making it possible to reproduce symptom expression observed in the field for the system ASGV – Ca. *P. mali – M. sieboldii*. The tissue culture system allows us to monitor and enhance symptom expression under controlled conditions. As the interaction between virus and phytoplasma is poorly understood, this system provides a means to analyse the effect of these two pathogens on each other's titres, symptom expression and transmission capabilities. To understand these interactions more knowledge is required on the different Ca. *P. mali* strains, virus genome functions and variability and host reactions to the pathogens. The German ASGV-AC isolate was successfully cloned and sequenced and will be used to construct an infectious clone.

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