

# OCCURRENCE OF APPLE STEM GROOVING CAPILLOVIRUS IN SANTA CATARINA, BRAZIL, DETECTED BY RT-PCR

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## ABSTRACT

Apple stem grooving capillovirus (ASGV) was detected in apple plants in Fraiburgo, Santa Catarina State. The virus was isolated from the cultivars 'Royal Gala' and 'Fuji' grafted onto 'Maruba-kaido', with an MM106 interstock between rootstock and canopy. Two nurseries involving several thousand plants were heavily affected by the virus and had to be destroyed. Declining plants showed tissue necrosis in the 'Maruba-kaido' region around the stem. The

virus was readily transmitted to *Chenopodium quinoa* from flower petals and young leaves of affected plants. In mechanically inoculated *Nicotiana occidentalis* 37B infection was latent. Virus identification was performed by RT-PCR and ISEM.

Key words: stem grooving, apple, identification, PCR, silica capture.

## RESUMO

### Ocorrência de apple stem grooving capillovirus em Santa Catarina, Brasil, detectada por RT-PCR

Apple stem grooving capillovirus (ASGV) foi detectado em macieiras cv. 'Royal Gala' e 'Fuji' sobre porta-enxerto 'Maruba-kaido' com um inter-enxerto de MM 106 entre a copa e o porta-enxerto. Dois viveiros envolvendo milhares de plantas foram afetados severamente e tiveram que ser destruídos. Macieiras em declínio apresentavam ne-

crose da casca no porta-enxerto 'Maruba' ao redor do tronco. O vírus foi facilmente transmitido mecanicamente de pétalas e folhas jovens de macieiras afetadas para *C. quinoa* e desta para *N. occidentalis* cv. 37B, na qual o isolado produziu reação latente. A identificação do vírus foi feita por RT-PCR e ISEM.

Apple stem grooving capillovirus ASGV is disseminated worldwide in *Rosaceae* fruit trees such as apple (Yanase, 1983), pear (Waterworth, 1971), apricot (Nemeth, 1986) and cherry (Kinard *et al.*, 1996), usually as a latent infection in most commercial cultivars. ASGV, the type-species of the genus *Capillovirus*, has flexuous, filamentous particles of approximately 600 to 700 nm and a ssRNA genome with 6496 nucleotides and coat protein of 27 kDa (Yoshikawa & Takahashi, 1988). It has been reported previously in São Paulo, Brazil (Sablowski *et al.*, 1988). Although usually latent in commercial varieties, it induces tree decline on 'Mitsuba-kaido' (*Malus sieboldii*) and 'Kobanozumi' (*M. sieboldii* var. *arborescens*) (Yanase, 1983) and several symptoms on a number of sensitive varieties such as Virginia Crab apple, which develops long grooves on the stem, usually apparent when the bark is removed. Here, ASGV was found infecting apple plants of the cultivars Royal Gala and Fuji grafted onto 'Maruba-kaido' rootstock (*M. prunifolia* var. *ringo*) with MM106 acting as an interstock between the canopy and the roots.

Symptoms in apple plants include severe phloem necrosis in the Maruba-kaido rootstock and dieback of the canopy. The scion variety and the filter did not show wood symptoms. Necrotic tissues were restricted to the rootstocks. Plants grafted with infected material usually die in the nurs-

eries in the first year. However in two orchards, plants aged 4-9 years, grafted onto Maruka-kaido rootstock, were sampled with symptoms that resemble those caused by ASGV; these inocula are under study. In the meantime, ASGV has been detected in two more regions in Santa Catarina and Rio Grande do Sul. The virus causes total loss of whole blocks in established orchards or nurseries.

The ASGV isolate reported here was recorded as isolate UV01 and established in the virus collection of Embrapa Uva e Vinho by grafting. Symptoms in 'Maruba-kaido' were characterized by a severe phloem necrosis with longitudinal bark cracks, pits in the wood with gum-like and necrotic deposits and brown staining of the wood of the rootstocks. The foliage of affected plants was sparse. Inoculation was done from apple petals and young leaves to *C. quinoa* and from this species onto *Cucumis sativus*, *Cucurbita maxima*, *C. pepo*, *Phaseolus vulgaris*, *Nicotiana occidentalis* 37B, *Vigna unguiculata* and *N. glutinosa* mechanically in the presence of 0.1 M phosphate buffer pH 7.5. Leaf extracts were decorated with antisera (AS) against ACLSV (Apple chlorotic leafspot virus) and ASGV for ISEM (immunosorbent electron microscopy). Seeds from infected *C. quinoa* plants were collected and sown to investigate seed transmission of the virus.

Total RNA was extracted from *C. quinoa*, *N. occidentalis* 37B and from apple leaves using silica capture (M. Rott, BBA, Dossenheim, Germany, personal communication). Briefly, 100 mg of leaf tissue were ground in liquid nitrogen or in the presence of grinding buffer (1:10, wt/v; 4 M guanidine thiocyanate, 0.2 M sodium acetate pH 5.2, 25 mM EDTA, 1 M potassium acetate pH 5.2, 2.5 % wt/vol polyvinylpyrrolidone 40, 1% 2-mercaptoethanol), followed by the addition of n-lauryl sulphate to a final concentration of 1.6% and incubation at 70 °C for 10 minutes. After centrifugation for 10 minutes at 13 K, supernatants were incubated at room temperature with a slurry of silica particles (commercial formulation of SiO<sub>2</sub>, 80% 1-5 microns), 20% ethanol and 2.3 M sodium iodide (final concentrations) for 10 minutes. After washing (10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 50 mM NaCl, 50% ethanol), incubation at 70 °C for 5 minutes and centrifugation at 13 K for 3 minutes, the supernatant with nucleic acids was used immediately for reverse transcription.

DNA sequences for the synthesis of amplification primers (ASGV6396r, 5' CTG CAA GAC CGC GAC CAA GTT T 3' and ASGV5873, 5' CCC GCT GTT GGA TTT GAT ACA CCT C 3') were those designed by Mackenzie *et al.* (1997) based on previously published sequences (Yoshikawa *et al.*, 1992).

Since symptoms on 'Maruba-kaido' rootstocks of dying plants resembled those of "topworking disease" caused by ACLSV (Yanase, 1983), the following primer sequences, designed according to published nucleotide sequences of ACLSV were also synthesised (Jelkmann, 1994): ACLSV7518 5' GTA GTA AAA TAT TTA AAA GTC TAC AGG C 3' and ACLSV7288 5' CTT TTA CAG ACT GAA TTT GCA 3' and used to prime extracts of *N. occidentalis* 37B with ASGV UV01 as well as total RNA extracts of positive control plants infected with ACLSV.

Total RNA (5 µl) for reverse transcription (RT) was denatured for 10 minutes at 70 °C. The RT cocktail (20 µl) contained 0.5 µM oligonucleotide ASGV6396r, 5 mM DTT, the four dNTP (0.125 mM each), and the first strand buffer. After the addition of 200 U M-MLV reverse transcriptase, the complete RT mixture was incubated for 50 minutes at 42 °C and 15 minutes at 70 °C. The PCR reaction was performed in 50 µl reaction volume containing the following: 2 µl of the cDNA reaction mix, 2.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 0.2 µM of primers ASGV6396r and ASGV5873, 10x PCR reaction buffer and 5 U Taq DNA polymerase. Samples were submitted to the following amplification steps in a thermocycler: 2 minutes at 94 °C, 35 cycles of 40 seconds at 94 °C, 40 seconds at 50 °C, and 60 seconds at 72 °C, for denaturing, annealing and chain extension, respectively, and a final extension cycle of 5 minutes at 72 °C. Amplification products were analysed by electrophoresis (90 Volt) in 1% agarose gels submerged in 1x Tris hydroxymethyl-amino-methane-EDTA-buffer (TAE).

ASGV isolate UV01 was readily transmitted mechanically from infected apple leaves and petals to *Chenopodium quinoa* causing a systemic infection. Symptoms were noticeable approximately 1 week after inoculation. They are initially characterized by chlorotic spotting and mottling, and later by leaf distortion and dark green leaf epinasty, as well

as fairly noticeable stem curvatures and reduction in growth. In advanced stages of infection, leaves formed after the establishment of infection on the whole plant are very small. ASGV isolate UV01 also infected *Nicotiana occidentalis* 37B in which it was latent. Immunosorbent electron microscopy (ISEM) of extracts of inoculated but nonsymptomatic *N. occidentalis* 37B showed strong decoration (not shown) of particles with an antiserum (AS) against a German isolate of ASGV while showing no reaction to an AS against a German isolate of ACLSV. Symptoms of ASGV in *C. quinoa* observed here agree with reports described by other authors (Uyemoto & Gilmer, 1971). *N. glutinosa*, *Phaseolus vulgaris*, *Vigna unguiculata*, and *Cucumis sativus* showed no visible reactions to inoculation with the Brazilian isolate of ASGV while *Cucurbita pepo* e *C. maxima* showed weak mottling and weak chlorotic spotting in preliminary tests. Studies concerning the reaction of herbaceous hosts are being continued. Preliminary experimental evidence of seed transmission of ASGV isolate UV01 was confirmed in three different trials, ranging from 8 to 15% of the plants raised from seeds of diseased *C. quinoa*.

Using total RNA purified from fresh leaves of *N. occidentalis* 37B and of *C. quinoa* infected with ASGV isolate UV01 as template for RT-PCR, a PCR product of 523 bp could be amplified as expected (figure 1). When samples

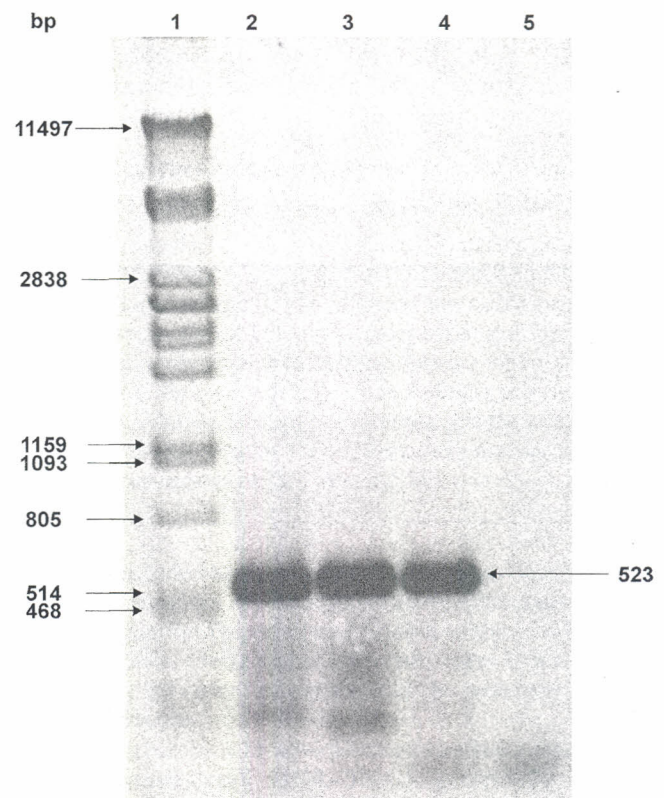


FIG. 1 - Analysis of PCR products by gel electrophoresis in 1 % agarose. Lane 1, MW marker, PstI digested  $\lambda$ ; lanes 2 and 3, RNA from partially purified ASGV-UV01; lane 4, total RNA from *C. quinoa* inoculated with extracts from diseased apples; lane 5, total RNA from healthy control samples.

with ASGV UV01 were submitted to PCR with primers that amplify fragments of several European ACLSV strains, there was no amplification (not shown). Also there was no amplification in extracts of healthy tissues. The successful amplification of Brazilian and German isolates of ASGV with primers designed for an American strain indicates that these isolates are quite closely related to each other, and that ACLSV does not occur in the analysed ASGV samples.

This is the first experimental proof of the occurrence of ASGV in Santa Catarina and of the identification of the agent that killed several thousand plants in 1997-98. Further monitoring of nurseries in Santa Catarina and Rio Grande do Sul has shown that ASGV is more widely spread as the causal agent of heavy plant losses than previously thought.

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