An Isolate of *Apple stem grooving virus* Associated with Cleopatra Mandarin Fruit Intumescence

Osvaldo Lovisolo[1], Gian Paolo Accotto[1], Vera Masenga[1], Addolorata Colariccio[2]

[1]Istituto di Virologia Vegetale del CNR, Strada delle Cacce 73, CAP 10135, Torino, Italia, e-mail: o.lovisolo@ifa.to.cnr.it;
[2]Centro de Sanidade Vegetal, Instituto Biologico de São Paulo, Av. Conselheiro Rodrigues Alves, 1252, CEP 04014-002, São Paulo, SP, e-mail: colariccio@biologico.br

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Corresponding author: Addolorata Colariccio

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ABSTRACT

A citrus tatter leaf isolate (CTLV-Cl) of *Apple stem grooving virus* (ASGV) has been found to be associated with a fruit rind intumescence in Cleopatra mandarin (*Citrus reshni*) in Limeira (SP). The CTLV-Cl was mechanically transmitted to the main experimental herbaceous hosts of CTLV. *Chenopodium quinoa* and *C. amaranticolor* reacted with local lesions and systemic symptoms while other test plants reacted somewhat differently than what is reported for CTLV. A pair of primers designed for specific detection of ASGV and CTLV amplified the expected 801 bp fragment from the CTLV-Cl-infected plants. Typical capillovirus-like particles were observed by the electron microscope in experimentally infected *C. quinoa* and *C. amaranticolor* leaves.

Additional key words: Citrus tatter leaf virus, *Capillovirus*, RT-PCR.

RESUMO

Isolado do *Apple stem grooving virus* associado a intumescências em frutos de tangerina Cleópatra

A presença de intumescências em frutos de tangerina Cleópatra (*Citrus reshni*) observadas em Limeira (SP), foram associadas ao Citrus tatter leaf (CTLV-Cl), um isolado do virus do acanalamento do lenho da maceira (*Apple stem grooving virus*, ASGV). O CTLV-Cl foi transmitido mecanicamente para as principais hospedera experimentais do CTLV. *Chenopodium quinoa* e *C. amaranticolor* reagiram com lesões locais e sintomas sistêmicos, enquanto as demais reagiram ligeiramente diferente do relatado para CTLV. Um par de primers desenhados para CTLV e ASGV amplificaram um fragmento de 801 pb, conforme o esperado, a parteir de plantas herbaceas infetadas com o CTLV-Cl. Particulas do tipo capillovirus foram observadas ao microscópio eletrônico, em folhas de *Chenopodium quinoa* e *C. amaranticolor* experimentalmente infetadas.

INTRODUCTION

The virus formerly known as Citrus tatter leaf virus (CTLV) has now been included within the species *Apple stem grooving virus* (ASGV) family *Closteroviridae*, genus *Capillovirus* (van Regenmortel *et al*., 2000). However, as the literature on the two viruses is largely separate and under the two different names, the acronyms CTLV and ASGV will be used to refer to these viruses.

While the genome properties of CTLV are well known (Ohira *et al*., 1995), the biological proprieties are still obscure and difficult to completely clarify, mainly because the diseases in citrus (*Citrus* spp.) have a long incubation period and are mainly associated with bud-union incompatibility. The reaction of experimental herbaceous hosts is also rather variable, and few strain comparisons have been done. Citrange stunt (Wallace & Drake, 1968) was shown to be caused by isolates of ASGV that vary somewhat in biological properties (Roistacher *et al*., 2000; Miyakawa & Ito, 2000).

CTLV was first reported in latently infected Meyer lemon [*Citrus limon* (L.) Burn.f.], a supposed hybrid of *Citrus limon*, introduced to California from China in 1908 (Wallace & Drake, 1962). CTLV latently infects most cultivated citrus when grown on their own roots. Disease appears when symptomless infected plants are grafted on trifoliate orange [*Poncirus trifoliata* (L.) Raf.] or trifoliate orange hybrids, such as Rusk, Troyer or Carrizo citrange (*P. trifoliata x C. sinensis*) or Citremon (*P. trifoliata x C. limon*). Grafted trees show stunting, chlorosis and bud-union incompatibility, resulting in bud-union creases and trunk fluting of the rootstock. Typical tatter leaf and chlorotic leaf symptoms are produced in *C. excelsa* Webster, Mexican lime [*C. aurantifolia* (Christm.) Swing.] and Rusk citrange, when experimentally graft-inoculated, one to six months after inoculation (Frison & Taher, 1991; Roistacher, 1991; Miyakawa & Ito, 2000).

Fruit rind intumescence was found in Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) during surveys for *Citrus leprosis virus* (CiLV) a putative family *Rhabdoviridae* in Limeira, SP (Lovisolo *et al*., 2000). It was also found to be associated with an isolate of ASGV nominated CTLV-Cl. We report field observations and investigations on mechanical

transmission to herbaceous test plants, electron microscopy, RT-PCR amplification and analysis with CTLV primers.

MATERIALS AND METHODS

Mechanical transmission

The virus was isolated as described for CiLV (Colariccio *et al*., 1995). Symptomatic parts of the fruit rinds of Cleopatra mandarin (Figure 1d, e) were inoculated into a series of test plants of CiLV and other citrus viruses. The inoculum was obtained by grinding the fruit rinds in liquid nitrogen and adding, during thawing, from 0.5 to 1% activated carbon and 0.05 M phosphate buffer pH 7, containing 0.005 M Na-Dieca, 0.001 M Na-EDTA, and 0.005 M Na-thioglycolate (about one part of tissue in four parts of buffer, w/v). Further transmission tests to investigate host range were done, omitting the grinding in liquid nitrogen, in insect-proof greenhouses kept at a mean temperature of 21-24 °C.

Electron microscopy

Samples of systemically infected leaves of *Chenopodium amaranticolor* Coste & Reyn and *C. quinoa* Willd were homogenized in cold 0.1 M phosphate buffer pH 7 containing 2% (w/v) polyvinylpyrrolidone. Then they were negatively stained in 1% aqueous uranyl acetate and viewed in a Philips CM 10 electron microscope.

RT-PCR

A pair of primers was designed based on the CTLV sequence (GenBank acc. No. D16681), which amplified an 801bp DNA fragment in the region of the CP gene. They were: CTL5607 (+) 5'- CCCTCTCAGCTAGAATTGAA -3' and CTL6407 (-) 5'- CAGGCATGTCAACCTGCAAG -3'. This pair of primers is also homologous to the ASGV sequence (acc. No. D14995), except for a single mismatch (G in ASGV, A in CTLV) at the eighth nucleotide of CTL5607 (+). *Chenopodium quinoa* leaves with local lesions collected ten days post inoculation, and *C. quinoa* and *C. amaranticolor* leaves with systemic infection collected 30-40 days post inoculation were used for RNA extraction. Total RNA was extracted from 0.1 g using the RNAWIZ reagent (Ambion) and diluted to 0.1-0.5 µg/µl. One µl was used for RT-PCR with the One Step RT-PCR System (Life Technologies/Invitrogen), according to Louro *et al*. (2000). Briefly, following RNA denaturation (5 min at 65 °C), the reaction mix was reverse-transcribed (30 min at 50 °C), denatured (30 s at 95 °C), and 35 cycles of amplification (15 s at 95 °C, 30 s at 50 °C, 1 min at 72 °C) were performed. An aliquot of the reaction was finally analyzed on 1.2% agarose gel.

RESULTS

Field observations

The fruit rind intumescence in Cleopatra mandarin (Figure 1d, e) occurred on plants also showing CiLV symptoms on the leaves. In previous surveys, Cleopatra mandarin was found to be a new natural host of CiLV with symptoms on the leaves, less frequently on the stems, but never on the fruits (Lovisolo *et al.*, 2000).

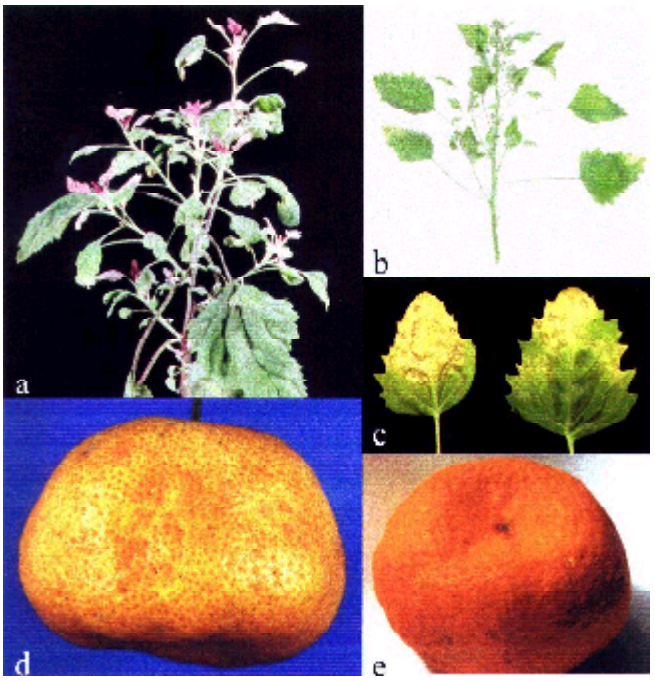

FIG. 1 - Symptoms caused or associated to CTLV-Cl: a) Systemic symptoms on *Chenopodium amaranticolor*, about two months after inoculation; b) Systemic on *Chenopodium quinoa*, about two months after inoculation; c) Local lesions on *C. quinoa*, ten days after inoculation; d) Intumescent areas on a fruits of Cleopatra mandarin (*Citrus reshni*), in May; e) Intumescent areas in September.

The intumescent areas on the fruits were large, covering half or more of the fruit surface; their color was paler than the normal orange of healthy fruits. No association was found between the intumescent areas, CiLV infection, fungus attacks, or arthropod feeding injuries.

The infected plants were Cleopatra mandarin rootstocks on which Murcott tangor (*Citrus reticulata* x *C. sinensis*) scions had been grafted, and then cut back, leaving the Cleopatra mandarin trees.

Mechanical transmission to herbaceous hosts

CTLV-Cl was isolated from the symptomatic fruit rind through mechanical inoculation to herbaceous test plants. The virus was mechanically transmitted to the herbaceous plants (Table 1), in which the type of symptoms, the minimum incubation period (days for symptoms appearance) and the results of back inoculation are listed.

The most useful test plants were *C. quinoa* and *C. amaranticolor*, on which the virus caused local lesions (Figure 1c) and systemic symptoms (Figure 1a, b), particularly severe with leaf deformation and general growth reduction. Infected plants tended to recover two-three months after infection.

TABLE 1 - Experimental host reactions of *Citrus tatter leaf virus* (CTLV-Cl) isolated from Cleopatra mandarin (*Citrus reshni*)

Test plant	Local symptoms	Systemic symptoms	Days for symptoms appearance	Back inoculation to *C. quinoa* from local/systemic symptoms
Amaranthaceae				
Amaranthus tricolor L.	0	0		
Gomphrena globosa L.	er.n.l.le.	0	9	+/+
Apocynaceae				
Catharanthus roseus (L.) G. Don	0	0		-
Chenopodiaceae				
Chenopodium amaranticolor	er.s.l.n.le.	ap.	7	+/+
C. quinoa	l.c./n.rs.le.	mo.ap.	5	+/+
Cucurbitaceae				
Citrullus lunatus (Thumb.) Mansf.	0	0		-
Cucumis sativus L.	0	0		-
Cucurbita pepo L.	0	0		+
Labiatae				
Ocimum basilicum L.	0	0		
Leguminosae				
Glycine max (L.) Merr	0	0		
Phaseolus vulgaris 'Saxa'	s.l.n.le.	0	8	+/-
Vigna unguiculata 'Black'	s.l.n.le.	0	10	+/+
Myrtaceae				
Psidium guajava L.	0	0		
Pedaliaceae				
Sesamum indicum L.	0	0		
Solanaceae				
Datura stramonium L.	0	0		-
Nicotiana benthamiana	0	0		-
N. clevelandii A. Gray	0	0		+
N. megalosiphon Henrck & Muell	0	0		-
N. occidentalis	0	0		-
N. rústica L.	0	0		-
N. tabacum 'White Burley' L.	0	0		-
Petunia x hybrida Vilm.	0	0		-
Tetragoniaceae				
Tetragonia tetragonioides (Pall.) Ktze	0	0		-

Symbols used: ap. = apical shoot proliferation, leaf malformation and/or stunt; c. = chlorosis; er. = erratic; l. = local; le. = lesion; mo = mottle; n. = necrosis; rs. = ringspot; s. = small; 0 = no symptom; + = infected; - = not infected.

Erratic local symptoms were observed on C. *amaranticolor* and *Gomphrena globosa* L. *Phaseolus vulgaris* L. 'Saxa' and *Vigna unguiculata* (L.) Walp. 'Black' reacted with necrotic local lesions rather irregularly. Systemic symptoms were never seen, but the virus could be re-isolated from uninoculated young leaves of *V. unguiculata*.

Electron microscopy

Few capillovirus-like particles were seen in the homogenized leaf tissues of *C. amaranticolor and* C. *quinoa* (Figure 2a). Most of these particles appeared broken or trapped in host material.

RT-PCR

The CTLV-Cl specific 801 bp DNA fragment was amplified from total RNA extracted from both *C. quinoa* (local and systemic infection) and *C. amaranticolor* (systemic infection), as well as from the ASGV positive control (Figure 2b). No extra bands were visible.

DISCUSSION

Our results on host range and symptoms, RT-PCR detection of a specific DNA fragment, and virus morphology, show that the virus isolated from Cleopatra fruit with rind intumescence (CTLV-Cl) is an isolate of ASGV. CTLV has never been reported in Cleopatra mandarin, but Garnsey (1999) in a table on "Susceptibility to systemic diseases in citrus trees on different rootstocks" lists Cleopatra mandarin in the category of "Tolerant or resistant" to CTLV. Fruit rind intumescence, if experimentally reproduced, would be the first symptom reported for CTLV-infected Cleopatra mandarin. CTLV was isolated from fruit rind of Meyer lemon, but no symptoms were reported (Yarwood, 1963).

Both CTLV-Cl and the previously known CTLV isolates infected *C. quinoa* and *C. amaranticolor* with rather similar local and systemic symptoms, while other test plants reacted somewhat differently from what is reported for CTLV. The main differences between these isolates were observed

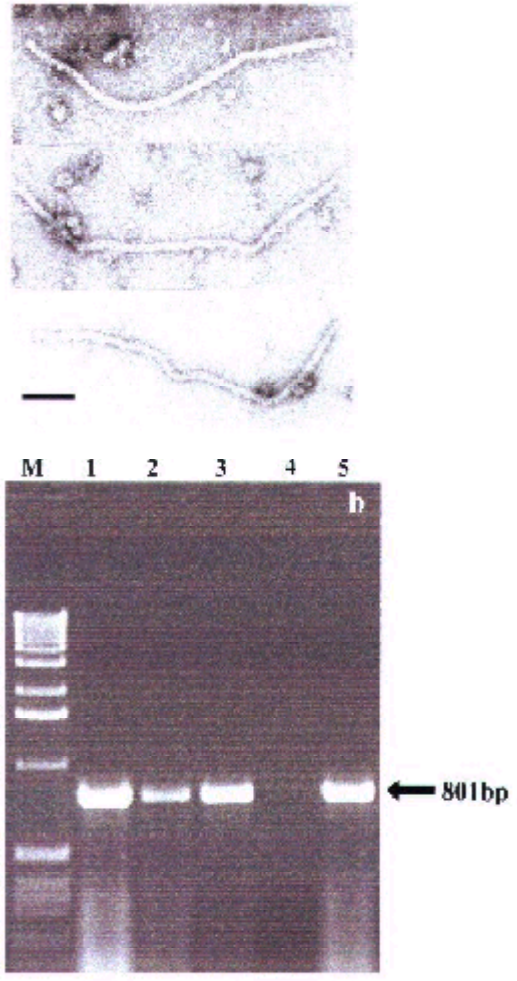

FIG. 2 - a) Capillovirus-like particles in homogenized leaf tissues of *Chenopodium quinoa* stained with 1% uranyl acetate. Bar =100 nm; b) Agarose gel electrophoresis of DNA fragments obtained by RT-PCR from locally (lane 1) or systemically (lane 2) infected *C. quinoa*, systemically infected *C. amaranticolor* (lane 3), uninoculated (lane 4) and *Apple stem groving virus* (ASGV)-infected (lane 5) *C. quinoa*. Lane M contains size markers (1Kb DNA ladder, Life Technologies). The arrow indicates the position of the ASGV/CTLV- specific 801 bp DNA fragment.

in *V. unguiculata* and *P. vulgaris*. CTLV-Cl caused small local lesions in *V. unguiculata* and *P. vulgaris* French bean, which were different from and less numerous than the ones illustrated by Semancik & Weathers (1965) and Roistacher (1991) for CTLV. The isolate described by Semancik & Weathers (1965) induced more rapid and severe reactions in some hosts than CTLV-Cl did, but it is known that several factors may influence the symptoms expression of viruses. Roistacher (1991) mentions that warm temperatures may mask symptom development of CTLV.

The one-step RT-PCR procedure reported here was only applied for total RNA extracted from mechanically inoculated experimental hosts. However, the method appeared reliable and robust, and will probably be useful for rapid screening of field citrus samples, as reported by Hailstones *et al.* (2000).

CTLV is widespread in China, Taiwan, Japan, and has also been reported in Korea, South Africa, Australia and the USA, where it was first found in Meyer lemon (Wallace & Drake, 1962; OEPP/EPPO, 1992). CTLV could have been introduced into other countries where Meyer lemons (symptomless hosts) have been imported, but that does not mean that the virus underwent secondary spread. This may be the case of Morocco where CTLV was found by Wallace (1975), but never reported again.

The geographic distribution of isolates of ASGV infecting Rosaceae is wider than that of the former CTLV isolates. According to Lister (1970; 1996), ASGV has been reported in Australia, China, India, Italy, Japan, the Netherlands, New Zealand, North America, Portugal, the UK, and is probably present worldwide in apples. ASGV was detected in Brazil (Betti *et al.*, 1995) in samples of apple Virginia Crab (*Mallus sylvestris* Mill.) from commercial plantations in Angatuba and Paranapanema in the State of São Paulo, using woody indicator hosts. Recently, Nickel *et al.* (1999; 2001), using RT-PCR and ISEM assays were able to detected ASGV in apple mother stocks and cultivars introduced to Southern Brazil. The origins of the infected plants (20% in a restricted survey) were São Joaquin (RS, Brazil), the Netherlands, Japan, and Caçador (SC, Brazil).

CTLV was previously reported in Meyer lemon fruits (Yarwood, 1963), and now in Cleopatra mandarin fruits. Further investigations will be necessary to find out if CTLV is seed-transmissible in citrus, as is the case for lily (*Lilium candidum* L.) seeds and some herbaceous test plants (Inouye *et al.*, 1979). CTLV is also known to be easily sap transmissible through knife cuts from citron (*Citrus medica* L.) to citron (Roistacher, 1991). Nothing is known about the natural transfer of ASGV from Rosaceae to citrus plants and to lily, or vice-versa, but agronomic contamination of citrus from apple or pear (*Pyrus communis* L.) could have occurred via grafting knives (Herron & Skaria, 2000). CTLV can not be eliminated with shoot-tip grafting (STG) alone, but needs the combination of thermotherapy and STG (Song *et al.*, 1999). CTLV is included in the list of the viruses that should be checked for the safe movement of citrus germplasm (Frison & Taher, 1991).

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