Virus Research 143 (2009) 61–67

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

'Kwanzan Stunting' syndrome: Detection and molecular characterization of an Italian isolate of Little cherry virus 1

Slavica Matic[a,*], Angelantonio Minafra[a], Jesús A. Sánchez-Navarro[b], Vicente Pallás[b], Arben Myrta[c], Giovanni P. Martelli[a]

[a] Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale, Sezione di Bari, Via Amendola 165/A, 70126 Bari, Italy
[b] Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas-UPV, CPI 8E, C/ Ingeniero Fausto Elio s/n, 46022 València, Spain
[c] Certis Europe, Via A. Guaragna 3, 21047 Saronno (VA), Italy

ARTICLE INFO

Article history:
Received 13 November 2008
Received in revised form 2 March 2009
Accepted 9 March 2009
Available online 21 March 2009

Keywords:
Closteroviridae
Stone fruits
Identification
Sequence analysis

ABSTRACT

Evident stunting was observed for the first time on *Prunus serrulata* 'Kwanzan' indicator trees in Southern Italy during the indexing of two sour cherry accessions from cultivars 'Marasca di Verona' and 'Spanska'. Bud break and shooting were delayed and the developing leaves remained small. During the third year many Kwanzan plants died, regardless of the indexed cultivar. Electrophoretic analysis showed the presence of dsRNA pattern in extracts of stunted Kwanzan with a similar size to that of viruses of the family *Closteroviridae*. An identical pattern of more abundant dsRNA bands was obtained from GF305 seedlings grafted with the same sour cherry accessions. Observations by electron microscopy revealed the presence of long flexuous virus particles in both indicators (Kwanzan and GF305), characteristic of closteroviruses. Subsequent cloning work, starting from the dsRNA extracts of cultivar Marasca di Verona grafted on GF305 indicator, yielded 7 different clones, all showing high identity to the Little cherry virus 1 genome. Full sequencing of this virus isolate (ITMAR) was then done resulting in a complete genome composed of 16,936 nt. Primers designed on the obtained sequences for RT-PCR detection confirmed the presence of Little cherry virus 1 in Kwanzan and GF305 trees, inoculated with both sour cherry cultivars. Phylogenetic analysis of the minor coat protein grouped virus isolates into two clusters: one including Italian isolates of sweet cherry, Japanese plum, peach and almond, together with German sweet cherry UW1 isolate, and a second one containing the Italian isolates of sour cherry (ITMAR and ITSPA), that were found associated with strong symptoms of 'Kwanzan Stunting'.

1. Introduction

Flowering cherry *Prunus serrulata* 'Kwanzan' is the recommended indicator for the presence of Cherry green ring mottle virus (CGRMV) in cherry (ISHS, 2004), in which CGRMV causes leaf epinasty and vein necrosis 2-3 months after graft-inoculation. This indicator is commonly used in the indexing program for the certification of stone fruits in the Apulia region (Southern Italy). During indexing of two sour cherry accessions from the cultivars Marasca di Verona and Spanska, strong stunting was observed on Kwanzan, 2-3 months after budwood grafting in the greenhouse. Besides Kwanzan, peach GF305 was used contemporaneously for indexing as an indicator for the presence of *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV), *Apple chlorotic leaf spot virus* (ACLSV) and some others less common stone fruit viruses found in Italy (Myrta et al., 2003).

There were no previous records of severe stunting on Kwanzan indicator. However, on Shirofugen, a different cultivar (cv.) of *P. serrulata* also used as indicator plant for virus-like diseases, similar stunting was reported by Kunze (1983). During indexing sour cherries, 'Shirofugen' indicator trees formed extremely short shoots, leaves of reduced size, and delayed bud break. At the end declined trees died, while their mazzard rootstocks grew strongly. Shirofugen stunting was caused by a graft-transmissible agent of supposed virus etiology, that was not mechanically transmissible to *Cucumis sativus*, *Chenopodium quinoa* and *Chenopodium amaranticolor*.

Here, we present the results of transmission trials of two accessions from the cvs. Marasca di Verona and Spanska to woody (Kwanzan and peach GF305) and diverse herbaceous indicators, and the subsequent molecular and electron microscope assays undertaken in order to identify and characterize the graft-transmissible agent strictly associated to the syndrome.

* Corresponding author. Present address: International Centre for Genetic Engineering and Biotechnology, Via Piovega 23, 31056 Ca' Tron di Roncade, Treviso, Italy Tel.: +39 080 5442538; fax: +39 080 5443608.
E-mail address: matic@icgeb.org (S. Matic).

0168-1702/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2009.03.005

Table 1
List of LChV-1 primers used to acquire the genome of LChV-1 ITMAR isolate.

Primer (5′-3′)	Target region	Amplified fragment (bp)
IN1for: (CGTTTTTATCTCCCAGCTTTGTGCCT) sense IN1rev: (CGAAAGTGGGTATGATCTTCCC) antisense	ORF1a	1774
IN2for: (CTCTTAACCGCAGCGATGGGCG) sense IN2rev: (GGATTGACGTCGCAACCAATCG) antisense	ORF1a	2093
4for1: (TGCGATTGTGGGAAAGCTAGC) sense 5rev2: (CATCATCATCAGATTGCTCCCC) antisense	ORF1a	1600
M1for: (GCCGATTAAAGAGTACATCG) sense M1rev:(AGAAGCCGGAGAGTATTCGG) antisense	ORF1a/1b	2192
M2for: (GGAACCAAGCCGTTTGGCTG) sense M2rev: (TGATACCCCAGGAGTGACAG) antisense	ORF1b/ORF2/ HSP70	2010
M3for: (GGCTTTGGCCGAGTTTATCTC) sense 1rev: (CGGGTAGTAGCAAGTATATCAGGC) antisense	HSP70/ORF4	1845
1for: (TAGCTGTGGCGCTGACTCTAG) sense 3rev: (CTTCTACCGCGACGTGGTCCC) antisense	ORF4/CP	1748
3for: (GCACTAGTGCCATAATAAAGTCACC) sense 6rev: (TGGGTTTCGACTCGACTGGTG) antisense	CPm	1047
6for: (CCTCTCTGAGGTGAATGGAGTG) sense 2rev: (CCCAATCACTTCGTGCTCTTGAG) antisense	CPm	670
Ffor: (TAGGATGGGTACGTGGAGTC) sense Frev: (GACTAGAGAAGGTAAGCGG) antisense	CPm/ORF7/ ORF8	1737

2. Materials and methods

2.1. Biological assays

In September 2004, two sour cherry accessions of cvs. Marasca di Verona and Spanska were budwood grafted on five repetitions of healthy, *in vitro* propagated Kwanzan and GF305 seedlings and maintained for 4 weeks for bud uptake in an acclimatised greenhouse. Later, the plants were maintained in a screenhouse so as to have the necessary chilling requirements during winter. At the end of winter, the plants were transferred again in an acclimatised greenhouse, cut above the grafting point, and observed for symptoms for several months. After 6 months, the indicator plants were transplanted to an open field in the experimental farm of the Mediterranean Agronomic Institute of Bari, Southern Italy. Symptoms were scored for at yearly intervals at any growing season after transplanting.

Leaf tissue from graft inoculated Kwanzan and GF305 plants (both from the greenhouse and open field) was ground in 0.1 M phosphate buffer pH 7.2, containing 2% nicotine and gently rubbed onto carborundum-dusted leaves of *Nicotiana benthamiana, N. occidentalis, N. clevelandii, Cucumis sativus*, *Chenopodium quinoa* and *Chenopodium amaranticolor*. Scoring for symptoms began 4–7 days after mechanical inoculation.

2.2. Nucleic acid extraction

Double-stranded RNA (dsRNA) extraction was done from 30 g of cortical scrapings or leaves of Kwanzan and GF305 graft inoculated with cvs. Marasca di Verona and Spanska as described by Dodds (1993). Additional dsRNA purification was done by nuclease digestion according to Saldarelli et al. (1994). A 10% aliquot of extracted dsRNA was electrophoresed in a 6% polyacrylamide gel stained by silver nitrate. Total nucleic acid (TNA) was extracted from

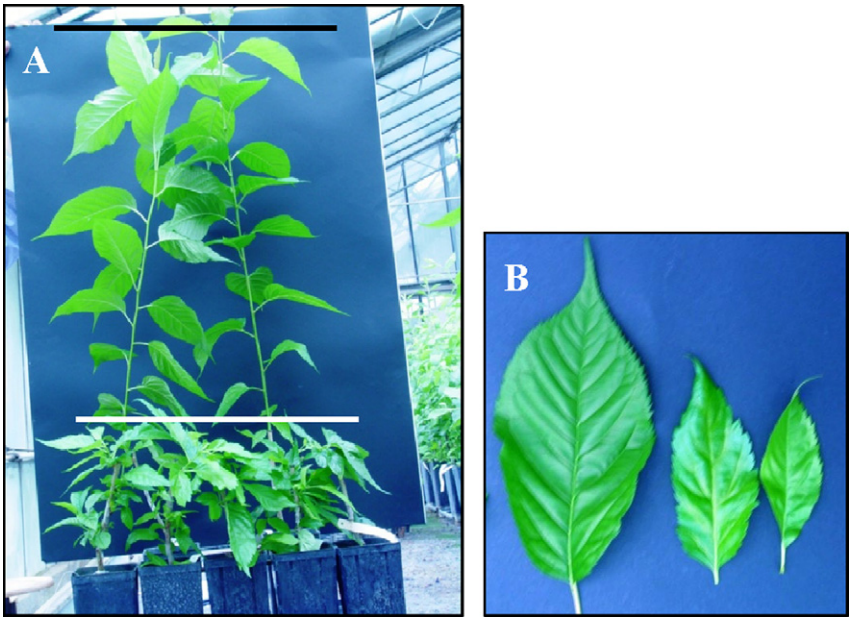

Fig. 1. Symptoms associated with the presence of 'Kwanzan Stunting' in the greenhouse: (A) stunted Kwanzan indicator plants (white color marks plant tops) in comparison with healthy ones (black color marks plant tops); (B) small leaves of stunted Kwanzan compared with normal leaves of healthy Kwanzan.