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Short communication

Pome fruit viruses at the Canadian Clonal Genebank and molecular characterization of *Apple chlorotic leaf spot virus* isolates

L.P. Wang[a,b,c], N. Hong[a,c], S. Matić[d], A. Myrta[e], Y.S. Song[a,c], R. Michelutti[b,*], G.P. Wang[a,c,**]

[a] National Key Lab. of Agromicrobiology, Huazhong Agricultural University, Wuhan, Hubei 430070, PR China
[b] Greenhouse and Processing Crops Research Centre, Agriculture and Agri-Food Canada (AAFC), 2585 County Road 20, Harrow, Ontario, N0R 1G0, Canada
[c] College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, PR China
[d] Istituto di Virologia Vegetale, CNR, Strada delle Cacce 73, 10135 Torino, Italy
[e] Certis Europe, Via A. Guaragna 3, 21047 Saronno (VA), Italy

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ABSTRACT

A survey for apple and pear viruses was carried out at the Canadian Clonal Genebank (CCG), Harrow, Ontario, Canada, during the fall/winter of 2007 and spring of 2008. Leaves and/or dormant cuttings were randomly collected from 438 to 122 accessions of apple and pear, respectively. Samples were tested by Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay (DAS-ELISA) for the presence of *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV) and *Apple mosaic virus* (ApMV). Infection rates for apples were ACLSV (48.1%), ASGV (10%), ASPV (6.6%) and ApMV (7.1%), and for pears ACLSV (42.6%). ACLSV was detected and characterization by multiplex RT-PCR with primers targeting a fragment of 677 bp corresponding to the partial coat protein (CP), movement protein (MP) and untranslated (3′UTR) region in 22 accessions of apple and pear. Multiplex RT-PCR showed a higher sensitivity over the ELISA test. The nucleotide and amino acid deduced partial CP identities ranged from 82.6–100% to 91–100%, respectively, while partial MP identities was 62.5–100% at aa level based on the amplified fragment appropriate for partial MP using a frame shift, among 22 ACLSV isolates. Phylogenetic analyses based on the partial CP region clustered CCG ACLSV isolates in two different groups, while those based on the partial MP region embraced CCG ACLSV isolates in two sub-clusters within the same group. This is the first report on the detection of ACLSV, ASPV, ASGV and ApMV at CCG, and the molecular characterization of ACLSV isolates in apple and pear plants from worldwide countries to deduce possible heterogeneity and evolution.

1. Introduction

Apple (*Malus domestica* (L.) Borkh) and pear (*Pyrus communis* L.) are the most widely planted fruit trees in Canada. Apple is a major fruit tree crop, grown on 20,271 ha with a total annual national production of 340,183 t. Ontario is the major producer of apple, possessing 42% of total apple growing area. Ontario and British Columbia are the largest producers of pear in Canada with an annual production of 7000 t. *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV) and *Apple mosaic virus* (ApMV) are the main viruses affecting pome fruit trees. These viruses frequently occur in mixed infections on nursery and commercial fruit trees, and are associated with economic losses of 30–40% (Campbell, 1963; Cembali et al., 2003).

All certification and quarantine programs include the testing of mother materials as an important step by using sensitive and reliable methods like reverse transcription polymerase chain reaction (RT-PCR) (Spiegel et al., 2006). RT-PCR has shown to be more sensitive than ELISA for the detection of pome fruit viruses, and is used as an important tool for result confirmation (MacKenzie et al., 1997; Menzel et al., 2003; Spiegel et al., 2005). Recently, a multiplex RT-PCR assay has been developed for the simultaneous detection of ACLSV, ASPV and ASGV, which is significantly time saving and cost effective (James, 1999; Menzel et al., 2002; Hassan et al., 2006; Jarošová and Kundu, 2010).

ACLSV is the type species of the genus *Trichovirus* (Martelli et al., 1994; Adams et al., 2004), the family *Betaflexiviridae* (Carstens, 2010). ACLSV is an important virus due to worldwide occurrence and wide range of hosts. ACLSV exists as numerous strains producing different symptoms in sensitive fruit trees cultivars, whereas other strains are latent in many apple and pear cultivars. The severity of symptoms caused by ACLSV is highly dependent on plant

* Corresponding author. Tel.: +1 519 738 1289; fax: +1 519 738 2929.
** Corresponding author at: National Key Lab. of Agromicrobiology, Huazhong Agricultural University, Wuhan, Hubei 430070, PR China. Tel.: +86 027 87283278; fax: +86 027 87384670.
E-mail addresses: micheluttir@agr.gc.ca (R. Michelutti), gpwang@mail.hzau.edu.cn, wanglp1999@gmail.com (G.P. Wang).

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species and virus strains (Németh, 1986). ACLSV virions are flexuous filaments, >640–760 × 12 nm in size. The complete genomic sequences of ACLSV isolates from plum, apple, cherry and peach are available (German et al., 1990, 1997; Sato et al., 1993; Yaegashi et al., 2007; Marini et al., 2008). The genome of ACLSV contains three open reading frames (ORFs) encoding, respectively, a 216.5 kDa protein (ORF1) involved in genome replication, a 50.4 kDa movement protein (MP) (ORF2), and the 21.4 kDa coat protein (CP) (ORF3).

Several studies on the molecular characterization in CP genes of different ACLSV isolates from European countries (Candresse et al., 1995; Malinowski et al., 1998; Pasquini et al., 1998; Krizbai et al., 2001; Al Rwahnih et al., 2004; Cieślińska et al., 2007), Egypt (Farrag et al., 2004); India (Rana et al., 2009, 2010), Japan (Yaegashi et al., 2007) and China (Cai et al., 2005; Zheng et al., 2007) on apple and stone fruit tree hosts have been reported. Little information on the molecular characterization was obtained for Sand pear (*Pyrus pyrifolia*) from China (Song et al., 2011), European pear (*Pyrus communis*) from Italy (Al Rwahnih et al., 2004) and India (Rana et al., 2010). Previous reports showed that ACLSV isolates have high variability at the N-terminal end of CP region partially overlapped by the MP region, although the CP was relatively conserved among ACLSV strains (German et al., 1997; Yaegashi et al., 2007).

The Canadian Clonal Genebank (CCG) is a part of the national crop genetic resource system for Agriculture Agri-Food Canada (AAFC). CCG has approximately 3300 unique accessions, including around 800 apple and 130 pear accessions. CCG aims to preserve the genetic diversity of Canadian fruit crops and their wild species, and to prevent import of virus-infected plant materials. Since no reports on pome fruit viruses exist at the CCG, the objective of the work was to assess apple and pear accessions at the CCG for four economically important and widespread viruses of the ACLSV, ASPV, ASGV and ApMV, and to characterize the phylogenetic relationships and molecular variability of CCG ACLSV isolates in European pear and apple hosts from domestic and imported origins in Canada based on the impact of ACLSV high infection rates. The results will contribute to further define possible evolution relationship for ACLSV isolates from different origins.

2. Materials and methods

2.1. Plant material collection

During the fall/winter of 2007 and spring of 2008, leaves (about twenty leaves per sample) and dormant cuttings were randomly collected from 438 to 122 accessions of apple and pear, respectively, at the Greenhouse and Processing Crops Research Center (GPCRC) in Harrow, Ontario, Canada. Virus-like symptoms observed in apple plants consisted of chlorotic ring-like spots in leaves and little leaves, while symptoms in pear leaves were ring pattern mosaic, chlorosis, vein yellowing and red mottle. However, most of the apple and pear accessions exhibit no symptoms. Leaf samples collected were stored at 4 °C and −70 °C for Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay (DAS-ELISA) and nucleic acid tests, respectively.

2.2. DAS-ELISA

DAS-ELISA (Clark and Adams, 1977) was performed for the detection of ACLSV, ASPV, ASGV and ApMV using commercial kits (Bioreba, Switzerland) as the manufacturer's instructions. Samples with DAS-ELISA readings three times higher than the healthy control were considered positive for the presence of any of the virus tested.

2.3. Isolation of total RNA

Total RNA was extracted from healthy and infected leaf tissues or phloem scraping with cetyltrimethylammonium bromide (CTAB) (Li and Mock, 2005) with a slight modification. Plant tissues (100 mg) cooled in a −30 °C freezer was processed in 1 ml of CTAB (2% CTAB, 2% polyvinylpyrrolidone (PVP) (Sigma, USA), 100 mM Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, and 0.2% 2-mecaptoethanol) in the FastPrep Instrument (MP Biomedicals, North America) for 60 s. The homogenate was incubated at 65 °C for 15–60 min and centrifuged at 12,000 × *g* for 12 min. The supernatant (650 μl) was transferred to a 1.5-ml microcentrifuge tube and mixed with an equal volume of 24:1 (v/v) chloroform–isoamylalcohol. The mixture was centrifuged at 12,000 × *g* for 15 min. This step was repeated for three times. The supernatant (500 μl) was transferred to a 1.5-ml microcentrifuge tube before adding 350 μl isopropanol. The mixture was incubated on ice for 10 min and centrifuged at 12,000 × *g* for 10 min. The pellet was washed with 70% ethanol and centrifuged at 7500 × *g* for 5 min. The pellet was air dried and dissolved in 50 μl of sterile distilled water. The quality and quantity of isolated RNA were evaluated by spectrophotometry (Ultrospec 2100 pro, Amersham BioScience) at the wavelength of 260, 230, and 280 nm.

2.4. One-step RT-PCR

A multiplex RT-PCR was performed using Qiagen® one-step RT-PCR kit (Qiagen, Hilden, Germany) in combination with the primers for ACLSV targeting a fragment of 677 bp corresponding to the partial CP, MP and untranslated (3′UTR) region, ASPV targeting a fragment of 370 bp to the partial CP region, and ASGV targeting a fragment of 273 bp to the partial CP region (Menzel et al., 2002). For the ApMV, the primers amplified a fragment of 450 bp for the region flanking the CP gene (Hassan et al., 2006). The NADH dehydrogenase subunit 5 (nad5) genes were used as internal control. The RT-PCR reactions were conducted in a thermocycler (Model PTC-200, MJ Research, USA) following cycling conditions as described by Hassan et al. (2006). PCR products were separated in 2% agarose gel stained with SYBR safe (Invitrogen, Carlsbad, USA).

A simple one-step RT-PCR was carried out for ACLSV by using SuperScript™ III one-step RT-PCR kit (Qiagen), and primers as described by Menzel et al. (2002). The total RNA template (2 μl) was added to 48 μl of the PCR mixture consisting of 25 μl 2× Reaction Mix, 1 μl of each 10 μM primer, 1 μl of SuperScript™ III RT/Platinum Taq High Fidelity Enzyme Mix, and sterile water to a final volume 50 μl. Final cycling conditions were according to Hassan et al. (2006) with a little modification: reverse transcription step for 30 min at 50 °C, activation of the hotstart Taq polymerase at 95 °C for 15 min followed by 40 cycles of at 94 °C for 30 s, 62 °C for 45 s, and 72 °C for 1 min, and by final extension step at 72 °C for 7 min.

2.5. Cloning, sequencing and sequence analysis of the CP/MP genes of ACLSV

The PCR products were purified with the QIAquick PCR purification Kit (Invitrogen), and then cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA). Three independent cDNA clones of each isolate were sequenced bi-directionally by automatic sequencing at Robarts Research Institute, Ontario, Canada. Database search was performed with BLAST (Blast local alignment search tool) programs at the National Center for Biotechnology Information (NCBI). Multiple alignments of nucleotide sequences of ACLSV MP/CP/3′UTR and deduced amino acid sequences of ACLSV CP and MP were obtained using the program Clustal X 1.8 (Thompson et al., 1997). Phylogenetic analyses were done