J Phytopathol

doi: 10.1111/j.1439-0434.2009.01656.x

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Identification and Characterization of a New Strain of Sunflower chlorotic mottle virus, a Potyvirus Infecting Asteraceae in Argentina

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Received July 17, 2009; accepted November 9, 2009

Keywords: sunflower, Sunflower chlorotic mottle virus, chlorotic ringspot (CRS), capsid protein, Argentina

Abstract

Sunflower (*Helianthus annuus* L.) plants with chlorotic ringspot symptoms were observed during the 2005–2006 crop season in the Southeast of the province of Buenos Aires, Argentina. Symptomatic plants were tested by biological, serological and molecular assays, and the virus isolated was identified as a potyvirus closely related to Sunflower chlorotic mottle virus, common isolate (SuCMoV-C), the most prevalent virus in sunflower crops in the country. Infected plants were serologically positive when probed with a SuCMoV-C antiserum. In the 3′-terminus region, 1304 nucleotides (nt) were sequenced, and it includes the C-terminal region of the nuclear inclusion b protein (NIb) gene (240 nt), the whole capsid protein (CP) gene (807 nt) and a 3′-non-coding region (3′-NCR) with 257 nt excluding the poly (A) tail. The CP of the Sunflower potyvirus causing chlorotic ringspot (CRS) shared 94.8% aa identity with SuCMoV-C and 89.2% with SuCMoV-Zi. The 3′-NCR shared 94.2% nt sequence identity with SuCMoV-C. A RT-PCR/RFLP assay with PvuII and EcoRV restriction enzymes successfully differentiated SuCMoV-C and the virus isolate causing CRS symptoms. This potyvirus was identified as a new SuCMoV strain, provisionally designated SuCMoV-CRS.

Introduction

Sunflower is an economically important oilseed crop in Argentina with a planted area of 2 622 346 ha and a total yield of 4 646 065 tons in the 2007/2008 season. Sunflower viruses have been reported as secondary diseases worldwide, but in India, an uncommon necrosis disease, which has become increasingly important, has been associated with an *Ilarvirus* (Bhat et al. 2002; Ramiah et al. 2001). Recently, in Uganda, a virus-like disease showing more that 40% incidence has been reported in commercial sunflower fields (Aritua 2006).

In Argentina, a sunflower virus disease was described in the early 1980s in commercial hybrid plots in the Southeast of the province of Buenos Aires. The disease was termed 'necochense mosaic' and its incidence was up to 28% (Kiehr-Delhey and Delhey 1985). At the same time, potyvirus-like particles were associated to infected sunflowers from the province of Córdoba (Muñoz et al. 1981).

Later, several virus-like symptoms were described on cultivated sunflowers (Lenardon 1994). One of the viruses causing chlorotic mottling was characterized and named 'Sunflower chlorotic mottle virus' (SuCMoV) (Dujovny et al. 1998, 2000). SuCMoV field crop symptoms initially appear as chlorotic yellow bright pin points (mottling) on leaves, that later coalesce as yellowing patches, and infected plants become stunted (Dujovny et al. 1998; Lenardon 1994). This isolate has been named 'SuCMoV-C' (common). This virus is a potential threat to sunflower production because it can significantly reduce plant height, stem and capitulum diameters, yield and weight of seeds and achene size (Lenardon et al. 2001).

SuCMoV-C has been identified in several provinces (Entre Ríos, Santa Fé, Córdoba and Buenos Aires) infecting mainly cultivated and wild sunflowers. It belongs to the *Potato virus Y* (PVY) subgroup in the *Potyvirus* genus within the *Potyviridae* family (Dujovny et al. 1998, 2000), and recently it has been recognized as a PVY strain by the ICTV (Berger et al. 2005).

During the 2005–2006 crop season, a SuCMoV-C epidemic breakout with an unusual increase of disease prevalence (95%) and incidence (5–25%) developed in sunflower fields in the Southeast of the Buenos Aires province (Lenardon and Giolitti 2007). At the same time, a commercial sunflower field showing 27% incidence of systemic chlorotic ringspot (CRS) symptoms (Fig. 1) was detected in Pieres, a town located in the same geographical area. In this study, we identified the

Fig. 1 Sunflower leaves showing chlorotic ringspot symptoms

potyvirus causing CRS symptoms as a new SuCMoV strain, hereby termed 'SuCMoV-CRS', and a diagnostic assay to differentiate both strains was developed. Preliminary reports have been published in conference proceedings (Bejerman et al. 2008).

Materials and Methods

Virus source and maintenance

An isolate from plants showing systemic chlorotic ringspots was obtained in Pieres (Southeast of Buenos Aires province), as described by Dujovny et al. (1998). The isolate was mechanically inoculated onto healthy sunflower Contiflor 17 DRM hybrid and *Nicotiana occidentalis* L. according to our standard procedure: leaves from infected plants were ground with a mortar and pestle in 0.01 M phosphate buffer, pH 7, containing 0.1% Na_2SO_3 (1 : 5 w/v). Extracted sap was mixed with 600 mesh carborundum before being rub-inoculated on the hosts and plants were kept in a greenhouse (22 ± 5°C) for symptom expression. Control samples with SuCMoV-C and Sunflower mild mosaic virus (an unrelated potyvirus) were collected in the rural area of Paraná, Entre Ríos province (Lenardon 1994).

Serological tests

Double Antibody Sandwich – Enzyme-linked Immunosorbent Assay (DAS-ELISA)

Sixteen field samples showing systemic chlorotic ringspots were tested by DAS-ELISA. SuCMoV-C trapping IgGs and conjugate detecting antibodies were diluted to 1 : 2000 (Dujovny et al. 1998). ELISA plates were read with an MR XII microplate reader (DYMEX Technologies, Chantilly, VA, USA) at 405 nm; positive controls reached an OD value of 1.0 before the final reading. Healthy and SuCMoV-C infected sunflowers were used as negative and positive controls, respectively. Samples were considered positive when A_{405} readings were higher than the mean of the healthy controls plus three standard deviations.

Western blots

Sunflower leaf samples were ground in TBS (0.02 M Tris, 0.15 M NaCl, 0.02% NaN_3, pH 7.5, +0.05% Tween 20 and 0.01 M diethyldithiocarbamic acid) 1 : 5 (w/v). The slurry was centrifuged at 2230 ***g*** at 4°C for 5 min, and the supernatant was saved. Eight microlitres of each suspension were mixed with 2 μl of protein sample buffer (Laemmi 1970) and heated at 100°C for 5 min. Prestained SDS–PAGE Standards, Low Range (Bio-Rad Laboratories, Hercules, CA, USA) were treated as earlier. Samples were subject to SDS–PAGE with a Mini-PROTEAN 3 cell (Bio-Rad Laboratories) in a 5–15% discontinuous gel and then transferred to nitrocellulose membranes with a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories). Membranes were treated as described by Dujovny et al. (1998) and then developed with an antiserum against SuCMoV-C at a 1 : 10 000 dilution and with a monoclonal antibody common to aphid-transmitted potyviruses (Agdia SRA 27200, Elkhart, IN, USA) at a 1 : 200 dilution. Tests were performed on samples from SuCMoV-C and CRS-infected plants, on plants infected with Sunflower mild mosaic virus (Giolitti et al. 2007; Giolitti and Lenardon 2008) and on a healthy control.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from sunflower fresh leaf tissue showing CRS symptoms (100 mg) using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. To clone the 3′-end of the NIb and the CP genes, DNA was synthesized by a one-step RT-PCR, using the Access RT-PCR system (Promega, Madison, WI, USA) and specific primers Su3 (GAGGCGTGGGGCTATCC) and Su4 (AAAAGTAGTACAGGAAAAGCC), which were designed from SuCMoV-C specific sequences of 3′ region of NIb and 5′ region of 3′-NCR, respectively. The RT-PCR was performed in a T3 thermo block (Whatman Biometra, Göttingen, Germany), to give one step at 48°C (50 min); one step at 94°C (4 min); 35 cycles at 94°C (1 min), 53°C (1 min) and 68°C (1 min); and a final step at 68°C (10 min). To clone 3′-NCR and confirm the CP gene sequence, a first-strand cDNA was made with M-MLV reverse transcriptase (Promega) and *Not*1-d$(T)_{18}$ as initial primer (Tsuneyoshi et al. 1998). PCR was carried out using Taq DNA polymerase (Promega) and the primers CP1 (GGTGACAACATAGATGCAGG) and PC-R4 (Tsuneyoshi et al. 1998), in a T3 thermo block (Whatman Biometra), to give one step at 94°C (4 min); 35 cycles at 94°C (1 min), 55°C (1 min) and 72°C (1 min); and a final step at 72°C (10 min). The amplified products were visualized by electrophoresis on a 1.4% agarose gel stained with ethidium bromide.

Molecular cloning and sequencing

The amplified fragments were cloned using the p-Gem T easy vector system (Promega). For each fragment, three clones were sequenced in both directions at Macrogen Inc. (Seoul, Korea).

Sequence analysis

The relationship between both SuCMoV isolates (-CRS and -C) and other species of the *Potyvirus* genus was assessed by comparing the nucleotide (nt) and predicted amino acid (aa) sequences of the whole CP-coding region and the nt sequence corresponding to the 3′-NCR using the Needleman–Wunsch algorithm, implemented in the EMBOSS needle program (European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK; http://www.ebi.ac.uk/Tools/emboss/align/index.html). Complete genomic sequences of 29 potyviruses used for comparative analyses were retrieved from the GenBank (Table 1).

Sequence assembly and analysis were performed utilizing the Lasergene software package, including Editseq, Seq Man and MegAlign programs (DNASTAR, Inc., Madison, WI, USA). Multiple sequence alignments produced by Clustal W algorithm were used as input data for reconstructing phylogenetic trees by the neighbour-joining method using the software MEGA version 4 (Tamura et al. 2007). Statistical significance was estimated by performing 1000 replications of bootstrap resampling of the original alignment using the bootstrap option of the phylogenetic tree menu.

RT-PCR/Restriction Fragment Length Polymorphism (RFLP)

CP nt sequences of SuCMoV, -C and -CRS isolates were analysed using the DNAMAN 6.0 software (Lynnon Corp., Pointe-Claire, QC, Canada), looking for restriction endonucleases that cut both viruses differentially. Two restriction endonucleases were chosen: PvuII and EcoRV (Promega); the first recognized one cleavage site in the -C isolate, but none in the -CRS isolate, whereas the second recognized one cleavage site in the -CRS isolate, but none in the other isolate. RT-PCRs were performed to amplify the CP gene, using the Access RT-PCR system (Promega) and specific primers CP1 and CP2 (5′ACATGTTACGAACCCCAAGC3′) in a T3 thermo block (Whatman Biometra), to give one step at 48°C (50 min); one step at 94°C (4 min); 35 cycles at 94°C (1 min), 55°C (1 min) and 68°C (1 min); and a final step at 68°C (10 min); after that, the RFLP assays were performed. Ten microlitre aliquots of RT-PCR products were digested with 10 U of PvuII or EcoRV and incubated overnight at 37°C. Restriction products were subject to electrophoresis on a 2% agarose gel and visualized by ethidium bromide.

Results

Following mechanical inoculation, sunflower plants showed systemic chlorotic ringspots, which never coalesce, similar to those seen in the original samples collected in the field. The 16 field samples showing typical CRS symptoms reacted strongly when tested by ELISA against a SuCMoV-C antiserum (data not shown). In Western blots, membranes containing the capsid proteins from the -CRS and -C isolates reacted when were probed with the SuCMoV-C antiserum (Fig. 2). -CRS isolate reacted as a double band and -C isolate as a single band. The higher band of -CRS isolate present a lightly superior molecular weight to -C isolate band. The lower band of -CRS isolate is probably a degradation product of the higher band (Fig. 2).

Table 1
Nucleotide and amino acid identity between the -CRS isolate and selected potyviruses for the coat protein and the 3′-NCR

Virus acronym	Accession number	CP nucleotide % identity	CP amino acid % identity	3′-NCR nucleotide % identity
SuCMoV-C	AF255677	87.3	94.8	94.2
SuCMoV-Zi	AY344048	82.4	89.2	Without date
BiMV	AY960151	77.4	84.1	76.8
PVY-LYE	AJ439545	77.0	80.4	57.7
PVY-H	M95491	76.8	80.1	54.8
PVY-NTN	AJ890347	76.5	80.0	56.2
PVY-T	D12570	77.6	79.6	51.7
PVY-O	EF026074	75.5	79.3	56.2
PVY-US	M81435	76.7	79.3	55.3
PVY-MN	AF463399	76.7	79.2	56.5
PVY	U09509	77.0	78.9	55.3
PVY-N	D00441	77.4	78.9	51.7
PVY-N:O	EF026076	75.9	78.9	56.8
PVY-Wilga	AJ889867	75.6	78.8	56.3
PepSMV	NC_008393	72.5	77.4	40.1
AlsMV	AB158522	72.7	76.8	44.7
PepMoV	AY748921	70.0	76.6	57.9
PepYMV	EF488081	70.0	74.5	56.8
ALiMV	AB158523	69.0	74.4	47.0
PTV	AJ437280	69.9	73.9	41.8
BiMoV	AF538686	69.3	72.8	48.4
WPMV	AJ437279	70.6	72.2	40.4
AmLMV	AJ580021	68.3	72.2	57.6
PVV	AJ243766	68.4	71.1	44.6
SuMV	AF465545	60.9	66.7	37.8
LMV	AJ278854	61.9	63.7	45.4
TEV	M11458	63.4	63.2	34.4
TuMV	AB105134	61.0	61.1	41.7
PVA	Z21670	59.7	58.9	47.3

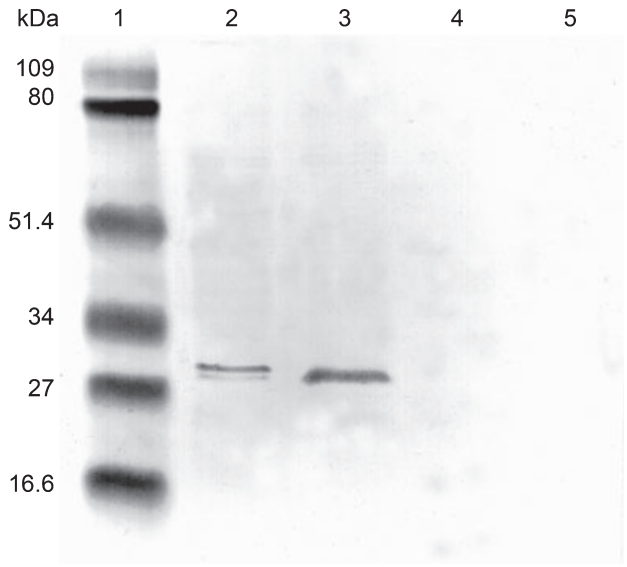

Fig. 2 Western blot of sunflower leaf extracts probed with SuCMoV-C antiserum; lane 1: prestained low-range molecular weight marker; lane 2: leaves infected with -CRS isolate; lane 3: leaves infected with SuCMoV-C; lane 4: leaves with Sunflower mild mosaic virus; lane 5: healthy sunflower

A membrane with both isolates also reacted with the monoclonal antibody common to aphid-transmitted potyviruses showing one band in each case (data not shown).

A 1304 nt fragment of the 3′-terminal region genome of the -CRS isolate was cloned and sequenced, and the nt and aa sequences were submitted to the GenBank (accession number EU418771). Sequence analysis of this virus genome portion revealed putative proteins and a 3′-NCR similar in size and arrangement to those of representative potyviruses. Sequence analysis showed an uninterrupted reading frame of 349 aa and covered part of the NIb-coding region (nt 1–240), the whole CP-coding region (nt 241–1047) and the 3′-NCR (nt 1048–1304). The first predicted 80 aa belonged to the C-terminus of the NIb and the dipeptide at the putative NIb/CP junction was Q/G. The CP gene encoded a protein of 269 aa, and it has the same number of residues as SuCMoV-C (Dujovny et al. 2000), with the Asp-Ala-Gly (DAG) motif presented at the N-terminus of CP (4 aa from the cleavage site). In addition, the consensus motifs MVWCIENGTSP, AFDF and QMKAAAL have been found in the putative CP at 117, 200 and 220 aa from the cleavage site, respectively. The 3′-NCR consisted of 257 nt excluding the polyadenylated tail.

CP nt sequence identities of the -CRS isolate with those of selected potyviruses ranged from 59.7 to 87.3%, while the CP deduced aa sequence identities ranged from 58.9 to 94.8% (Table 1). The deduced aa sequence showed that the -CRS isolate shared 94.8% aa identity with the -C isolate, 89.2% aa identity with a Brazilian SuCMoV-Zi reported from zinnia (*Zinnia elegans* Jacq.) and 84.1% with Bidens mosaic virus (BiMV) (Table 1). Comparisons of the CP core aa (Lys^{32} to $Prol^{184}$) between -C and -CRS isolates showed identity of 96.1%, and comparisons of the N-terminal part of the CP, showed 77.4% identity. Additionally, the identity of the whole CP of the -CRS isolate was less closely related to other potyviruses infecting sunflower such as *Bidens mottle virus*, BiMoV (referred as Sunflower chlorotic spot virus) (72.8%) and *Sunflower mosaic virus*, SuMV (66.7%) (Table 1). Comparisons with PVY strains showed aa sequence identities ranging from 78.8 to 80.4% (Table 1).

Nucleotide sequence identities of the 3′-NCR of the -CRS isolate with those of selected potyviruses ranged from 94.2 to 34.4%. The higher identity degree values correspond to SuCMoV-C (94.2%) and BiMV (76.8%) (Table 1).

In the phylogenetic analysis, the high bootstrap values confirmed grouping of the -CRS isolate with SuCMoV-C, SuCMoV-Zi and BiMV, which are clustered with high confidence to PVY isolates, *Alstroemeria mosaic virus* (AlsMV), *Pepper severe mosaic virus* (PepSMV), *Pepper yellow mosaic virus* (PepYMV), Amazon lily mosaic virus (ALiMV), *Amaranthus leaf mottle virus* (AmLMV), *Pepper mottle virus* (PepMoV), *Wild potato mosaic virus* (WPMV), *Potato virus V* (PVV) and *Peru tomato mosaic virus* (PTV) (Fig. 3).

Electrophoresis of the RT-PCR/RFLP products showed one band of 807 nt for the undigested -C and -CRS isolates. The C isolate digested with PvuII yielded two bands (673 and 134 nt) but was not cut with EcoRV. The -CRS isolate was not digested with PvuII, whereas it yielded two bands (564 and 243 nt) when treated with EcoRV (Fig. 4).

Discussion

The symptoms induced in sunflower by the -C and -CRS isolates are clearly different. The term 'strain' defines a collection of isolates causing the same symptoms (Barnett 1992), supporting clearly that both these isolates should be regarded as different strains of SuCMoV. Therefore, the new potyvirus isolated from sunflower causing CRS symptoms should be considered as a new SuCMoV strain. The denomination of SuCMoV-CRS is proposed for this potyvirus due to its close relationship to SuCMoV-C by its association with the ringspot symptoms on naturally infected sunflowers, and its ability to reproduce the same systemic symptoms on mechanically inoculated healthy sunflower plants under greenhouse conditions. A RT-PCR/RFLP assay successfully differentiated both SuCMoV isolates, and it can be used to separate both viruses quickly and efficiently.

Serological assays indicated that the -CRS isolate is closely related to -C isolate because it strongly reacted against the SuCMoV-C antiserum. When the molecular analysis was performed, most of the differences found in the aa residues of the CP between both SuCMoV isolates were within the N-terminal part of the CP (7 of 14). This region is known to be highly variable and to contain major virus-specific epitopes (Shukla et al. 1988). The DAG triplet for aphid transmission in the *Potyvirus* genus was found in the N-terminal region

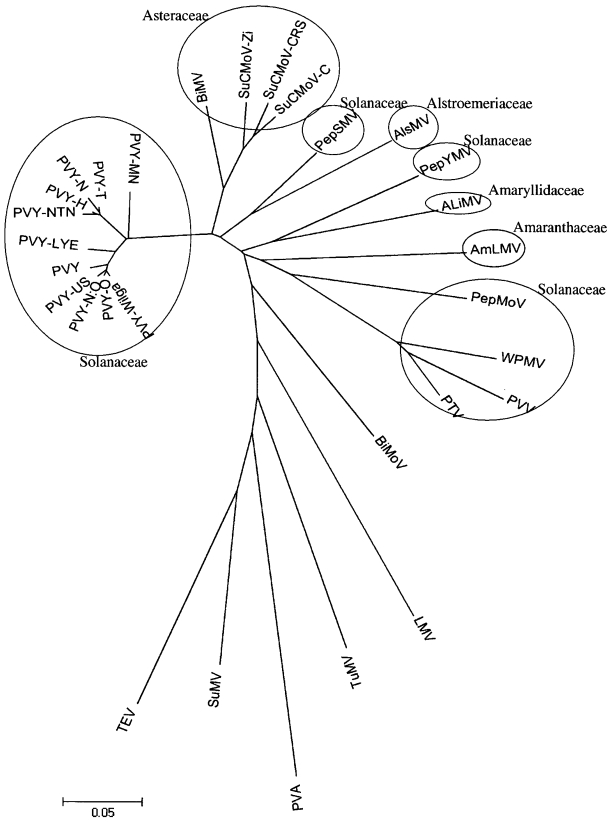

Fig. 3 Phylogenetic tree illustrating the position of the -CRS isolate among the members of the *Potyviridae*. Circles indicate subgroups of potyviruses. Plant families to which subgroups acquired infectivity are also indicated. Neighbour-joining trees were constructed by the program Mega-4 from multiple sequence alignments of CP aa using Clustal W

together with other consensus aa motifs for CP-coding regions, common in other potyviruses (Atreya et al. 1990, 1991; Shukla et al. 1994).

Comparisons of the whole CP aa sequences within the *Soybean mosaic virus* (SMV), *Bean common mosaic virus* (BCMV) and the *Sugarcane mosaic virus* (SCMV) subgroups demonstrated that discrimination between strains of the same species and isolates of different virus species occurred at approximately 83% aa identity (Chen et al. 2004) and Adams et al. (2005) considered that a value of 82% aa identity would reliably distinguish between most species except for the *Plum pox virus* (PPV), WPMV and PTV group. Also, other species demarcation criteria for the genus *Potyvirus* suggested a limit of about 80% aa identity (Berger et al. 2005).

SuCMoV-C is regarded as a PVY strain (Berger et al. 2005); however, CP aa identity values between SuCMoV-C and PVY strains are so close to 80%, that it is just in the limit to be considered as a strain of the PVY. The latest ICTV report (Berger et al. 2005) classified PTV and WPMV as distinct species, despite 85% aa identity in CP sequences, so the SuCMoV should be regarded as a new potyvirus species belonging to the PVY 'subgroup'.

The phylogenetic analysis based on the CP aa sequence identities confirmed the taxonomic relatedness of the -CRS and -C isolates, with viruses belonging to the PVY species complex. These viruses most probably evolved from a common ancestor that was adapted to infecting Solanaceous plants (the optimal hosts for most of the viruses in this group). Members from this aphid-transmitted virus group generally infect *Solanaceae*, but other natural hosts have been discovered in the *Alstroemeriaceae* (Fuji et al. 2004), *Amaranthaceae* (Segundo et al. 2007), *Amaryllidaceae* (Fuji et al. 2004) and *Asteraceae* (Dujovny et al. 1998), and the lineage that eventually gave rise to SuCMoV subsequently became better adapted to infect members of the family *Asteraceae*. Interestingly, the origins and/or first reported regions of the PVY subgroup's natural host plants are in South America (Inoue-Nagata et al. 2006). SuCMoV was geographically isolated in a nearby potato field and was positioned adjacent to PVY in the phylogenetic tree. These results suggest that SuCMoV could be a pioneer species descending from PVY to acquire infectivity to other plant families.

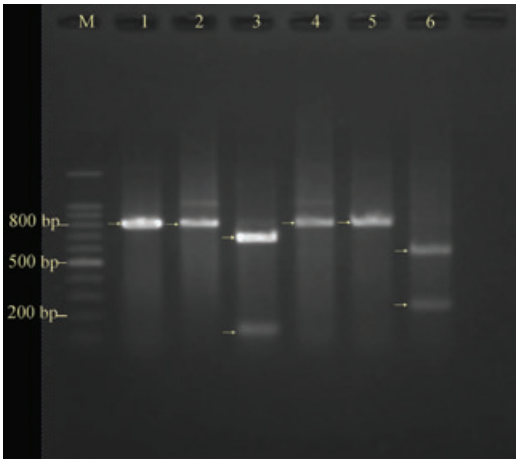

Fig. 4 RT-PCR/RFLP products from the -C and the -CRS isolates of SuCMoV. M, 100 bp DNA Ladder (Promega); 1, SuCMoV-C without digestion; 2, -CRS isolate without digestion; 3, SuCMoV-C digested with PvuII; 4, SuCMoV-C digested with EcoRV; 5, -CRS isolate digested with PvuII; 6, -CRS isolate digested with EcoRV

Acknowledgements

The authors thank FONCyT (PICT2004-25982) for financial support of this work, and they are grateful to Dr E. Taleisnik for checking the manuscript. F. Giolitti and N. Bejerman are doctoral students at the Doctorado en Ciencias Biológicas of the Universidad Nacional de Córdoba, Córdoba, Argentina.

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