Molecular characterization of a novel *Sunflower chlorotic mottle virus* (SuCMoV) strain

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ABSTRACT

Sunflower plants showing chlorotic ring spot symptoms were observed during the 2005/2006 crop season in the southeast of the Province of Buenos Aires in Argentina. Preliminary studies, including host range symptoms, serological tests and electron microscopy, had identified this virus isolate as a potyvirus closely related to *Sunflower chlorotic mottle virus* (SuCMoV). The nucleotide sequence of the genomic 3´ terminal region of this potyvirus was determined and characterized. The sequence consisted of 1304 nucleotides (nt) includingthe C-terminal region of the nuclear inclusion b protein gene (NIb), the capsid protein gene (CP) and the 3´non-coding region (3´-NCR). The partial putative NIb gene (240 nt) encoded a protein of 80 amino acids (aa) residues and the CP gene (807 nt) encoded a protein of 269 aa residues. The 3´-NCR was 257 nt in length excluding the poly (A) tract. Sequence comparisons of the predicted CP aa and 3´-NCR were analyzed separately in order to determine the relationship between this potyvirus and SuCMoV, and other reported potyviruses. The CP of this potyvirus isolated from sunflower shared 94.8% aa identity with SuCMoV (Argentina) and 89.2% with SuCMoV-Zi (Brazil). The 3´-NCR shared 94.2% nt sequence identity with SuCMoV. These data indicate that the potyvirus causing chlorotic ring spot (CRS) symptoms in sunflower is closely related to SuCMoV and it is provisionally referred to as SuCMoV strain CRS.

Key words: coat protein sequence – molecular assays – SuCMoV – sunflower – strain.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops in Argentina, with a total planted area of 2,380,000 ha and a total yield of 3,500 million tons in the 2006/2007 growing season. It has been a strategic revenue crop for this country since Argentina is the main exporter of edible sunflower oil in the world. *Sunflower chlorotic mottle virus* (SuCMoV) is one of the most widely distributed potyviruses on cultivated and wild sunflowers in this country and was reported in several provinces. Achene yield was significantly reduced by SuCMoV infections occurring at early ontogenetic stages (Lenardon et al., 2001). Recently, SuCMoV has been recognized as a new PVY strain by the ICTV (Dujovny et al., 2000; Berger et al., 2005), however some inconsistencies of its taxonomic status need to be clarified. During the 2005/2006 growing season, SuCMoV broke out in commercial sunflower hybrids in the southeast of the province of Buenos Aires with an unusual increase in disease incidence. At the same time, a whole commercial sunflower field showing chlorotic ring spot symptoms (CRS) on leaf blades (Fig. 1) was detected in the same geographical area. Preliminary biological, serological experiments, and electron microscopy studies had previously identified the virus as a potyvirus related to SuCMoV (Lenardon et al., 2005).

Fig. 1. Sunflower leaves showing chlorotic ringspot symptoms.

The genus Potyvirus, in the plant family Potyviridae, is by far, the largest virus genus known in the plant kingdom, with nearly 200 members, which accounts for almost 25% of known plant viruses. Its members share similar morphology, particle structure, host range, and modes of transmission (Berger et al., 2005). The virions contain a single molecular linear, positive-sense 8.2 to 9.7 kb ssRNA that has a VPg structure at its 5´terminus and a poly (A) tract at its 3´terminus. The coding ORF is translated to one polyprotein, which is subsequently processed into 10 different proteins by virus-encoded proteinases (Allison et al., 1986). The potyvirus genomic RNA is encapsidated by a single type of coat protein (CP). Genomic sequence data have become useful for demarcating virus strains and species (Fauquet et al., 2003; Berger et al., 2005). Sequences within the 3´ proximal portion of the genome are commonly used for species demarcation (Shukla et al., 1994; Adams et al., 2005), including the nucleotide or amino acid sequences of CP and the nucleotide sequence of the 3´ non-coding region (3´-NCR).

This report was undertaken to determine molecular properties (genome organization, amino acid sequence and phylogenetic analyses) of SuCMoV-CRS.

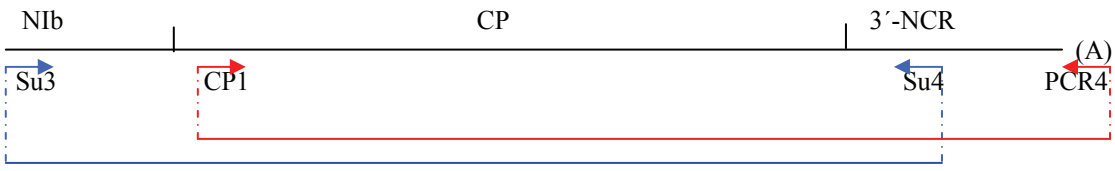
MATERIALS AND METHODS

Virus source and maintenance

Sunflower plants showing leaf blades with isolated and confluent chlorotic ring spots were collected from commercial sunflower fields located in Pieres county, in the southeast of the province of Buenos Aires. Single chlorotic ring spots were mechanically inoculated onto sunflower hybrid CF-7 and *Nicotiana occidentalis* L., in which they were maintained. The mechanical inoculations were conducted according to our standard procedure: leaves from infected plants were ground in 0.01M phosphate buffer, pH 7, containing 0.1% Na_2SO_3 (1:5 w/v) with a mortar and pestle. Extracted juice was mixed with 600 mesh carborundum before being rub-inoculated on the hosts and plants were kept under greenhouse conditions (22ºC + 5ºC) for symptom expression.

RT-PCR

Total RNA was extracted from sunflower fresh leaf tissue (100 mg) using the RNeasy Plant Mini Kit (Qiagen, California, USA) according to the manufacturer's instructions. To clone the 3´ end of the NIb and the CP cistrons, DNA was synthesized using the Access RT-PCR system (Promega, Wisconsin, USA) and specific primers Su3 and Su4 (Fig. 2). To clone the CP cistron and the 3´-NCR a first strand cDNA was made with M-MLVreverse transcriptase (Promega) and Eco/Not as initial primer (Tsuneyoshi et al., 1998). PCR was carried out using Taq DNA Polymerase (Promega) and the primers CP1, and PCR4 (Fig. 2). The amplified products were visualized by electrophoresis on a 1.4% agarose gel stained with ethidium bromide.



Su3: 5´-GAGGCGTGGGGCTATCC-3´
Su4: 5´-AAAAGTAGTACAGGAAAAGCC-3´
CP1: 5´-GGTGACAACATAGATGCAGG-3´
PCR4: (Tsuneyoshi et al., 1998)

Fig. 2. Cloning strategy of the SuCMoV-CRS 3´end. The position of the different PCR-generated cDNA clones is shown below the viral genomic map.

Cloning and sequencing of PCR products
PCR products were cloned using the pGEM T-easy vector system (Promega), following the manufacturer's instructions and subsequently subjected to DNA sequencing at Macrogen Inc. (Seoul, Korea).

Sequence analysis
The nucleotide (nt) and predicted amino acid (aa) sequences of the whole CP coding region and the nt sequence corresponding to the 3´- NCR of the SuCMoV-CRS were compared with 38 potyvirus sequences deposited in GenBank, EMBL, DDBJ and PDB databases using pair-wise Align program (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 Neighbor-Joining method using the software MEGA version 4 (Tamura et al., 2007). Statistical significance was estimated by performing 500 replications of bootstrap resampling of the original alignment using the bootstrap option of the phylogenetic tree menu.

RESULTS

Following mechanical inoculation sunflower and *N. occidentalis* plants became infected, developing symptoms similar to those seem in the field collected plants.

A 1304 nt fragment of the 3´ terminal region genome of the SuCMoV-CRS was cloned and sequenced (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. This sequence covered part of the NIb coding region (nt 1 to 240), the whole CP coding region (nt 241 to 1047) and the 3´-NCR (nt 1048 to 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 269 aa residues with the Asp-Ala-Gly (DAG) motif presented at the N-terminus of CP (4 aa from the cleavage site). Also, the following consensus motifs have been found in the putative CP: MVWCIENGTSP, AFDF, QMKAAAL at 117, 200 and 220 aa from the cleavage site. The 3´-NCR consisted of 257 nt excluding the polyadenylated tract.

The percentage of identity between the nucleotides of the SuCMoV-CRS CP and selected potyviruses ranged from 39.8% to 87.3%. Comparisons of the predicted CP aa ranged from 47.6% to 94.8% identity (Table 1). SuCMoV-CRS shared 94.8% aa identity with the CP of an Argentinian SuCMoV isolate which caused chlorotic mottling on sunflower, 89.2% aa identity with a Brazilian SuCMoV-Zi isolated reported from zinnia plants and 84.1% with *Bidens mosaic virus* (BiMV) (Table 1). Comparisons of the CP core aa (Lys^{32} to $Prol^{184}$) between this virus and SuCMoV (Argentina) showed identity of 96.1%. Additionally, the identity of the CP of SuCMoV-CRS was less related with other potyviruses-infecting sunflower such as *Sunflower chlorotic spot virus* (syn *Bidens mottle virus*) (72.8%) and *Sunflower mosaic virus* (66.7%) (Table 1).

Nucleotide comparisons of the 3´-NCR between SuCMoV-CRS and several potyvirus species showed the highest degree of sequence identity with SuCMoV (94.2%) and BiMV (76.8%). The other potyviruses showed a lower degree of sequence identity ranging from 34.4% (TEV) to 57.9% (PepMoV) (Table 1).

In the phylogenetic analysis the high bootstrap values confirmed grouping of the SuCMoV-CRS with SuCMoV, SuCMoV-Zi and BiMV, which are clustered with high confidence to PVY isolates and PepSMV (Fig. 3).

Table 1. Percentage of nucleotide and amino acid identities between the coat protein and the 3´NCR of SuCMoV - CRS with those of selected potyviruses, respectively

Virus acronym	Accession number	CP nucleotide % identity	CP amino acid % identity	3´-NCR nucleotide % identity
SuCMoV	AF255677	**87.3**	**94.8**	**94.2**
SuCMoV-Zi	AY344048	82.4	**89.2**	without data
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
PepMoV	AY748921	70.0	76.6	**57.9**
PepYMV	EF488081	70.0	74.5	56.8
PTV	AJ437280	69.9	73.9	41.8
SuCSV	AF538686	69.3	**72.8**	48.4
WPMV	AJ437279	70.6	72.2	40.4
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
ChiVMV	AJ237843	54.3	59.6	40.7
ZYMV	AB369279	59.6	59.6	41.7
CDV	AM113761	59.3	59.5	45.8
PetFMV	AF030689	47.6	59.5	45.8
SMV	D00717	58.7	59.1	42.3
PRSV	AY162218	60.0	59.0	37.6
PVA	Z21670	59.7	58.9	47.3
BCMV	AM258976	59.1	56.6	44.4
MDMV	D00949	58.9	55.3	45.9
TVMV	U38621	**39.8**	54.1	38.3
ClYVV	AB011819	58.0	53.0	40.6
PPV	NC_001445	55.1	51.4	45.0
SCMV	EU196455	**52.1**	**47.6**	45.4

DISCUSSION

Molecular characteristics of the sunflower-potyvirus inducing CRS, such as nucleotides, predicted amino acid sequence identities and the arrangement of the 3´ end of the genome clearly showed that it is closely related to SuCMoV. This research confirms previous findings based on biological and serological properties and virion morphology (Lenardon et al., 2005).

The 3´-NCR of SuCMoV-CRS showed a higher sequence identity with SuCMoV (94.2%) and BiMV (76.8%) and a lower one with other potyvirus members than usually reported for strains (34.4% to 57.9%). According to Frenkel et al. (1989), the nt sequence of the 3´-NCR of potyvirus strains is highly conserved (83-99%), while distinct potyviruses have only 30-53% nt sequence similarity. Furthermore, Shukla et al. (1994) proposed that species of the same virus should have a 3´-NCR sequence identity of >75%. Considering this criterion, SuCMoV-CRS should remain as a SuCMoV strain.

The CP of SuCMoV-CRS showed a sequence identity of 94.8 % with SuCMoV and most of its differences in aa residues between them were confined to the N-terminal part of this protein. This region is known to be highly variable and to contain mayor virus specific epitopes, due to its localization at the surface of the virions (Shukla et al., 1988). The essential DAG triplet for aphid transmission in the potyvirus genus was found in the N-terminal region together with other consensus aa motifs for CP coding regions common in other potyviruses (Shukla et al., 1994; Atreya et al., 1990, 1991).

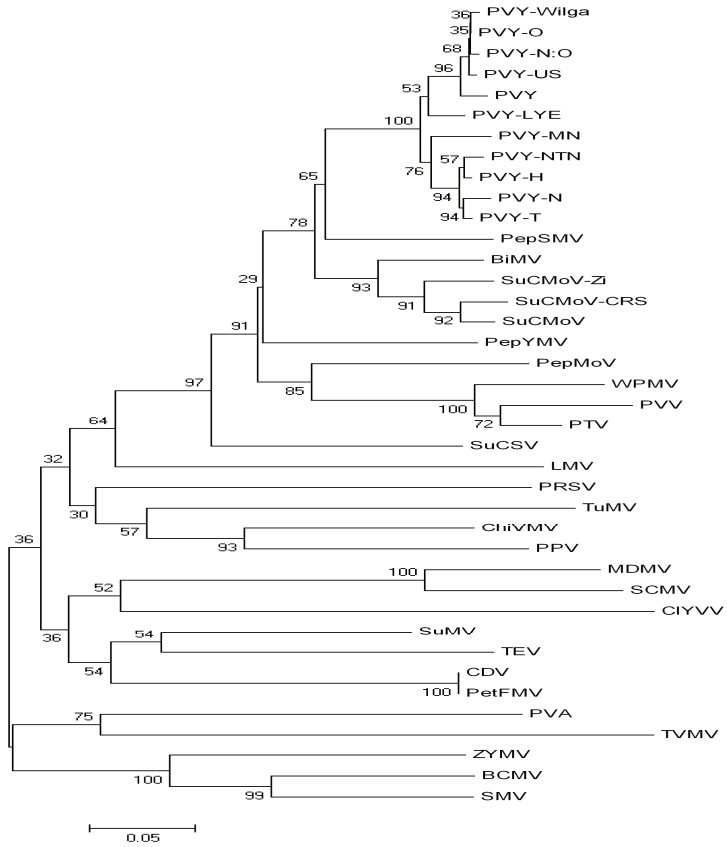


Fig. 3. Phylogenetic tree illustrating the position of the SuCMoV-CRS among the members of the *Potyviridae* family. Neighbor-joining trees were constructed by the program Mega-4 from multiple sequence alignments of CP aa using Clustal W. The bootstrap values of 500 replications are shown in each node.

Comparisons within the SMV/BCMV and the SCMV subgroups demonstrated that the discrimination between strains of the same species and isolates of different virus species occurred at about 83% aa identity (Chen et al., 2004), whereas Adams et al. (2005) consider that a value of 82% aa identity would reliably distinguish between most species except for the PPV, WPMV and PTV group. More recently, species demarcation criteria for the genus *Potyvirus* included CP nt identity of less than 76% and aa identity of less than about 80% (Berger et al., 2005) based on earlier studies of several virus species and strains.

The comparisons between the CP aa among potyviruses have shown that SuCMoV is regarded as a PVY strain (Berger et al., 2005), and BiMV is also considered a strain of PVY (Inoue-Nagata et al., 2006). Nevertheless, other propositions were made recently for species demarcation based on nt and aa identity within ORFs and the CI gene have been proposed as being the best region for diagnosis and taxonomy studies if only a sub portion of the genome is to be sequenced, rather than the CP usually used, because it most accurately reflects the taxonomic status according to the complete ORF (Adams et al., 2005).

The phylogenetic analysis based on the CP aa sequence identities confirmed the taxonomic relatedness of this sunflower-potyvirus inducing chlorotic ring spot symptoms to SuCMoV, SuCMoV-Zi and BiMV, which could be clustered into a new subgroup among PVY isolates.

The denomination of SuCMoV- (strain) CRS is proposed for this potyvirus closely related with SuCMoV in the light of its association with the symptoms on naturally-infecting sunflower and its ability to reproduce the same systemic symptoms on healthy sunflower plants mechanically inoculated under greenhouse conditions. Research about the identification of the viral genomic region involved in symptom expression is under way and may provide insight of the virus-host interaction.

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