Genetic Diversity of Begomoviruses Infecting Soybean, Bean and Associated Weeds in Northwestern Argentina

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(Accepted for publication 04/08/06)

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Rodríguez-Pardina, P.E., Zerbini, F.M. & Ducasse, D.A. Genetic diversity of begomoviruses infecting soybean, bean and associated weeds in Northwestern Argentina. Fitopatologia Brasileira 31:342-348. 2006.

ABSTRACT

The subtropical Northwestern region of Argentina (provinces of Tucumán, Salta, Jujuy, Santiago del Estero and Catamarca) suffers from a high incidence of the whitefly *Bemisia tabaci*, and the detection of begomoviruses is also common. The Northwest is the main bean-growing region of the country, and approximately 10% of Argentina's soybean crop is grown in this area. We have used a PCR-based assay to establish the identity and genetic diversity of begomoviruses associated with bean and soybean crops in Northwestern Argentina. Universal begomovirus primers were used to direct the amplification of a fragment encompassing the 5' portion of the capsid protein gene. Amplified fragments were cloned, sequenced and subjected to phylogenetic analysis to determine the sequence identity to known begomoviruses. The data indicated the presence of four distinct begomoviruses, all related to other New World begomoviruses. The prevalent virus, which was present in 94% of bean and soybean samples and also in two weed species, is closely related to *Sida mottle virus* (SiMoV). A virus with high sequence identity with *Bean golden mosaic virus* (BGMV) was found in beans. The two remaining viruses displayed less than 89% identity with other known begomoviruses, indicating that they may constitute novel species. One of these putative novel viruses was detected in bean, soybean and tomato samples.

Additional keywords: *Sida mottle virus*, *Bean golden mosaic virus*, geminivirus.

RESUMO

Diversidade genética de begomovírus associados às culturas da soja e feijoeiro na região Noroeste da Argentina.

A região Noroeste da Argentina (províncias de Tucumán, Salta, Jujuy, Santiago del Estero e Catamarca), de clima subtropical, apresenta uma alta incidência da mosca-branca *Bemisia tabaci* e a detecção de begomovírus também é freqüente. O Noroeste é a principal região produtora de feijão do país e produz aproximadamente 10% da soja da Argentina. A identidade e diversidade genética de begomovírus associados à soja e ao feijoeiro no Noroeste da Argentina foram estudadas com base na amplificação de fragmentos do genoma viral via PCR, utilizando oligonucleotídeos universais para o gênero *Begomovirus* que amplificam um fragmento correspondente à região 5' do gene da proteína capsidial. Os fragmentos amplificados foram clonados e seqüenciados, e as seqüências foram submetidas à análise filogenética. Os resultados indicam a presença de quatro espécies de begomovírus, todas relacionadas às espécies do Novo Mundo. O vírus prevalente, detectado em 94% das amostras de feijoeiro e soja e em duas amostras de plantas daninhas, apresentou alta identidade de sequência e relacionamento filogenético com o *Sida mottle virus* (SiMoV). Um vírus com alta identidade de seqüência com o *Bean golden mosaic virus* (BGMV) foi detectado em feijoeiro. As duas outras espécies apresentaram menos de 89% de identidade com os demais begomovírus, sugerindo-se tratar de novas espécies. Uma dessas possíveis novas espécies foi detectada em plantas de feijoeiro, soja e tomateiro.

Palavras-chave adicionais: *Sida mottle virus*, *Bean golden mosaic virus*, geminivírus.

INTRODUCTION

Geminiviruses represent one of the major plant pathogens in tropical, subtropical and, to a more limited extent, temperate regions (Morales & Anderson, 2001). Geminiviruses are characterized by their twinned icosahedral particles and circular single-stranded DNA genomes. The family *Geminiviridae* is divided into four genera (*Mastrevirus*, *Curtovirus, Topocuvirus* and *Begomovirus*), based on genome structure and phylogeny, type of insect vector and host range (Stanley *et al.*, 2005). Begomoviruses are transmitted by whiteflies (*Bemisia tabaci*) to dicotyledonous plants. Most of the species in this genus have bipartite genomes consisting of two ssDNA molecules, referred to as DNA-A and DNA-B. Genes in DNA-A encode proteins responsible for viral replication (Rep and REn), regulation of gene expression (TrAP) and particle encapsidation (CP). Genes in DNA-B encode for two proteins (MP and NSP)

involved in cell-to-cell movement within the plant, host range and symptom modulation. A region of approximately 200 nucleotides common to both genomic components contains *cis*-acting signals required for DNA replication and transcription (reviewed by Hanley-Bowdoin *et al.*, 1999).

The subtropical Northwestern (NW) region of Argentina (provinces of Tucumán, Salta, Jujuy, Santiago del Estero and Catamarca) suffers from a high incidence of the whitefly *Bemisia tabaci,* and the detection of begomoviruses is also common (Vizcarret, 1999). This region is the main bean-growing area of the country, and approximately 10% of Argentina's soybean crop is grown in this area. Two begomoviruses have already been reported in bean crops in Argentina, *Bean golden mosaic virus* (BGMV) and *Bean dwarf mosaic virus* (BDMV) (Morales *et al.*, 1990; Vizgarra, 1995; Zumelzu & Docampo, 1984) but to our knowledge no molecular studies have been carried out to characterize these viruses. The occurrence of a begomovirus in soybean crops in NW Argentina was first reported in 1997 (Sakai *et al.*, 1997). Molecular characterization showed that a virus related to *Tomato golden mosaic virus* (TGMV) was involved in the disease (Rodríguez-Pardina *et al.*, 1998), but further evidence showed that more than one virus could be involved.

The objective of this study was to establish the identity and genetic diversity of begomoviruses associated with bean and soybean crops in Northwestern Argentina.

MATERIALS AND METHODS

Collection of plant samples. Leaf samples of soybean and bean plants showing typical begomovirus symptoms, such as severe yellow or golden mosaic, chlorotic mottling, blistering, leaf distortion and dwarfing (Figure 1), were collected in several fields located in the provinces of Catamarca, Tucumán, Salta and Jujuy, in Northwestern Argentina. Additional soybean samples were collected in fields located in the Chaco province (Northeast), and north of the city of Córdoba, where soybean plants showing typical begomovirus symptoms were first observed during the 2003 growing season. A small number of tomato and weed samples showing golden mosaic symptoms were also collected. Weeds were often found bordering the crops or as undergrowth within them. All samples were freeze-dried and stored at 4ºC until used for DNA extraction.

FIG. 1 - Symptoms of severe mosaic, blisters and leaf distortion in a soybean sample collected at Horcones, province of Salta, and PCR-positive for begomovirus infection.

Confirmation of begomovirus infection. The presence of begomoviruses was initially confirmed by PCR, using primers PAL1v1978/PARc496 (Rojas *et al.*, 1993) or MP16/MP82 (Umaharan *et al.*, 1998), or by ELISA using the 3F7 antiserum (Agdia). For PCR, DNA was extracted using a CTAB protocol (Doyle & Doyle, 1987). Reactions were carried out with puRe*Taq* Ready-To-Go PCR Beads (Amersham Biosciences), prepared in a 25 µl volume containing 3 µl of template DNA and a final primer concentration of 0.25 µM (PAL1v1978/PARc496) or 0.75 µM (MP16/MP82). Reactions were submitted to an initial denaturing step at 94ºC for 3 min, followed by 30 cycles of denaturing at 94ºC for 1 min, annealing at 50ºC (PAL1v1978/PARc496) or 55ºC (MP16/MP82) for 1 min, and extension at 72ºC for 2 min, and a final extension at 72ºC for 10 min. Double-antibody sandwich ELISA was carried out using a commercial kit (Agdia) according to manufacturer's recommendations. Leaf samples were ground in four volumes of PBS (1×) containing 0.5 ml/l Tween 20 and 20 g/l PVP and incubated for 18 h at 4ºC in ELISA Nunc 96-well Immunoplates. IgG and conjugate were each incubated for 4 h at 37ºC. After adding the substrate the reaction was quantified using a Dynatech MR 4000 ELISA plate spectrophotometer. Samples were considered positive when A_{450} values were higher than the mean plus three times the standard deviation of the values for healthy controls.

Cloning and sequencing procedures. DNA fragments from selected samples were amplified by PCR using primers MP16/MP82, which direct the amplification of a ~450 bp fragment encompassing the conserved nonanucleotide TAATATTAC at the origin of replication and the nucleotide sequence encoding eight conserved amino acids (CEGPCKVQ) at the 5' region of the capsid protein gene. PCR reactions were performed as described above. Amplification products were cloned using the TOPO TA Cloning kit (Invitrogen). The sequence of one to three clones from each sample was completely determined in both orientations using an ABI PRISM 377 automatic sequencer (Applied Biosystems).

Sequence analysis. Sequences were assembled and checked using DNAMan ver. 4.0 (Lynnon Biosoft). Database searches were carried out with Blast*n* (Altschul *et al.*, 1990). Multiple sequence alignments of nucleotide sequences of the entire cloned fragment and the deduced

amino acid sequences of the N-terminal region of the CP were performed with Clustal W (www.ebi.ac.uk/clustalw). Phylogenetic trees were generated with MEGA 3.1 (Kumar *et al.*, 2004) using the UPGMA method. Tree branches were bootstrapped with 2000 replications.

RESULTS

A total of 117 soybean and 124 bean samples with typical begomovirus symptoms were collected from several locations in Northwestern Argentina from 1993 to 2003. Forty-three (37%) soybean and 32 (26%) bean samples tested positive for the presence of begomoviruses, either by ELISA or PCR. In the province of Tucumán, 24 out of 62 soybean samples (38%) were positive for begomovirus infection. Eight out of 16 soybean samples (50%) collected in the province of Salta tested positive, while in Catamarca nine out of 35 soybean samples (26%) were positive. Three soybean samples were collected in Córdoba and one of them (33%) was positive for begomovirus infection. The single soybean sample collected in Chaco province was also positive. Eighty-four bean samples were collected in Salta province, and 20 of them (24%) tested positive for the presence of a begomovirus. In Santiago del Estero, 11 out of 36 bean samples (30%) were infected. Four bean samples were collected in Jujuy province and one of them (25%) tested positive. A total of 22 tomato samples were collected, all from the same field in Tucumán, and eight (36%) were positive. Four weed samples (two of *Leonurus sibiricus* and one of *Malvastrum coromandelianum* collected in Salta, and one of *Sida rhombifolia* collected in Santiago del Estero) were positive for begomovirus infection.

DNA fragments amplified from 12 soybean and eight bean samples were selected for cloning, based primarily on the location where samples were collected and on the year of collection, in order to have samples from the largest possible number of locations and years of collection (Table 1). DNA fragments amplified from two tomato and three weed samples were also cloned (Table 1).

Fifty-six out of the 65 clones analyzed displayed greater than 90% sequence identity with *Sida mottle virus* (SiMoV, GenBank accession number NC_004637) for the first 69 amino acids of the capsid protein (Table 2). When the nucleotide sequence of the complete fragment

Table 1 - Plant species and geographic distribution of field collected samples selected for begomovirus molecular characterization. Only a fraction of the total number of samples tested for begomovirus infection is listed

Sample code	Plant species	Field location	Province	Year	Number of clones sequenced
L1	*Leonurus sibiricus*	Metán	Salta	2003	3
M2	*Malvastrum coromandelianum*	El Tandíl	Salta	2002	2
B3	*Phaseolus vulgaris* (bean)	Isca Yacu	Santiago del Estero	2002	3
B4	bean	Isca Yacu	S. del Estero	2002	3
B5	bean	Pichanal	Salta	2002	3
B6	bean	La Junta	Salta	2002	3
B7	bean	Gral Ballivian	Salta	2002	1
B8	bean	Padre Lozano	Salta	2003	2
B9	bean	Padre Lozano	Salta	2003	3
B10	bean	Arrayanal	Jujuy	2003	3
Si11	*Sida rhombifolia*	Pozo Hondo	S. del Estero	2002	3
S12	*Glycine max* (soybean)	Las Cañas	Salta	1993	3
S13	soybean	Horcones	Salta	1995	3
S14	soybean	Las Bajadas	Salta	1998	1
S15	soybean	Las Breñas	Chaco	1999	3
S16	soybean	R. de la Frontera	Salta	2002	3
S17	soybean	Cañetes	Tucumán	2002	2
S18	soybean	La Virginia	Tucumán	2002	1
S19	soybean	7 de Abril	Tucumán	2002	2
S20	soybean	El Tandíl	Salta	2002	3
S21	soybean	Metán	Salta	2003	3
S22	soybean	El Abra	Catamarca	2003	3
S23	soybean	Totoral	Córdoba	2003	3
T24	*Lycopersicon esculentum* (tomato)	Vipos	Tucum án	2000	3
T25	(tomato)	Vipos	Tucumán	2000	3

Table 2 - Amino acid sequence identity of the N-terminal region of the capsid protein (before the slash) and nucleotide sequence identity of the entire amplified fragment (after the slash), between the different clones and the species with higher identity

Clone[a]	Species with higher identity[b]	Clone	Species with higher identity	Clone	Species with higher identity
L1-1	SiMoV (91/91)	B9-3	SiMoV (92/89)	S18-1	SiMoV (92/90)
L1-2	SiMoV (92/91)	B10-1	SiMoV (92/89)	S19-1	SiMoV (92/88)
L1-3	SiMoV (92/91)	B10-2	SiMoV (92/89)	S19-2	SiMoV (92/91)
M2-1	SiMoV (90/87)	B10-3	SiMoV (91/89)	S20-1	SiMoV (92/90)
M2-2	SiMoV (89/87)	Si11-1	SiMoV (90/85)	S20-2	SiMoV (92/89)
B3-1	SiMoV (92/89)	Si11-2	SiMoV (91/85)	S20-3	SiMoV (91/88)
B3-2	SiMoV (92/89)	Si11-3	SiMoV (92/85)	S21-1	SiMoV (92/91)
B3-3	TGMV (87/86)	S12-1	SiMoV (91/88)	S21-2	SiMoV (92/91)
B4-1	SiMoV (92/89)	S12-2	SiMoV (92/89)	S21-3	SiMoV (92/91)
B4-2	SiMoV (92/89)	S12-3	SiMoV (91/89)	S22-1	SiMoV (91/89)
B4-3	SiMoV (92/89)	S13-1	TGMV (89/87)	S22-2	SiMoV (90/90)
B5-1	SiMoV (92/89)	S13-2	SiMoV (90/92)	S22-3	SiMoV (91/89)
B5-2	BGMV (97/92)	S13-3	SiMoV (94/90)	S23-1	SiMoV (92/89)
B5-3	SiMoV (90/90)	S14-1	SiMoV (92/89)	S23-2	SiMoV (92/89)
B6-1	SiMoV (92/89)	S15-1	SiMoV (94/89)	S23-3	SiMoV (91/90)
B6-2	SiMoV (92/89)	S15-2	SiMoV (94/90)	T24-1	WGMV (84/78)
B6-3	SiMoV (91/89)	S15-3	SiMoV (94/90)	T24-2	WGMV (83/76)
B7-1	SiMoV (91/89)	S16-1	SiMoV (94/91)	T24-3	WGMV (75/74)
B8-1	SiMoV (92/89)	S16-2	SiMoV (94/90)	T25-1	TGMV (87/83)
B8-2	SiMoV (92/89)	S16-3	SiMoV (92/91)	T25-2	TGMV (86/84)
B9-1	SiMoV (91/89)	S17-1	SiMoV (92/89)	T25-3	TGMV (87/84)
B9-2	SiMoV (92/89)	S17-2	SiMoV (91/89)		

[a] Letters and numbers before the dash refer to the sample code in Table 1, and numbers after the dash refer to different clones obtained from each sample.
[b] SiMoV, *Sida mottle virus*; BGMV, *Bean golden mosaic virus*; TGMV, *Tomato golden mosaic virus*; WGMV, *Wissadula golden mosaic virus.*

was compared, including 200 bp of the common region, maximum identities were 89% and 87% with two SiMoV isolates (NC_004637 and AJ557450), indicating that these clones could represent SiMoV isolates (Table 2). These 56 clones include clones obtained from all soybean, bean and weed samples analyzed, but none of the tomato samples.

Clone B5-2 displayed 97% sequence identity with *Bean golden mosaic virus* (BGMV, NC_004042) for the first 69 amino acids of the capsid protein, and 92% identity at the nucleotide level for the complete fragment (Table 2). This result indicates that this clone may represent an isolate of BGMV, and that sample B5 had a mixed infection with SiMoV and BGMV, since clones B5-1 and B5-3 displayed 89% and 90% nucleotide sequence identity with SiMoV for the entire fragment, respectively (Table 2).

The remaining eight clones displayed less than 89% amino acid sequence identity to all begomovirus sequences currently deposited in the GenBank, indicating that these clones could represent novel species (Table 2). Two variants were found based on phylogenetic analysis (Figure 2). Variant A, showing the highest identity with *Tomato golden mosaic virus* (TGMV), was detected in soybean, bean and tomato samples (clones B3-3, S13-1, T25-1, T25-2 and T25-3), and variant B (close to *Wissadula golden mosaic virus*) was detected only in tomato (clones T24-1, T24-2 and T24-3). Mixed infections were detected in soybean and bean plants. In soybean, SiMoV isolates were detected co-existing with variant A isolates (sample S13), whereas in bean, SiMoV was found with variant A (sample B3) and BGMV (sample B5).

Results of phylogenetic analysis demonstrated that most of the clones with higher sequence identity with SiMoV (*eg* B4-2, B8-1, L1-1, S12-2, S15-3, S23-1, and Si11-1) clustered with the original SiMoV isolate and with SiMoV-A1 (incorrectly named as SimMV in GenBank) (Figure 2). An exception was clone M2-1, which clustered with SimMV-A2, indicating that it could be an isolate of this species. The single clone identified as BGMV (B5-2) clustered with the original BGMV isolate. Clones classified as "variant A" (*eg* B3-3, S13-1 and T25-1) clustered with *Tomato golden mosaic virus*. Clones classified as "variant B" (*eg*, T24-1) did not form any evident cluster with known begomovirus species (Figure 2).

DISCUSSION

Begomovirus infection of beans has been reported in Argentina since the 1980's and attributed to BGMV (Morales & Anderson, 2001), a virus which is widespread in the neighboring country of Brazil (Faria & Maxwell, 1999).

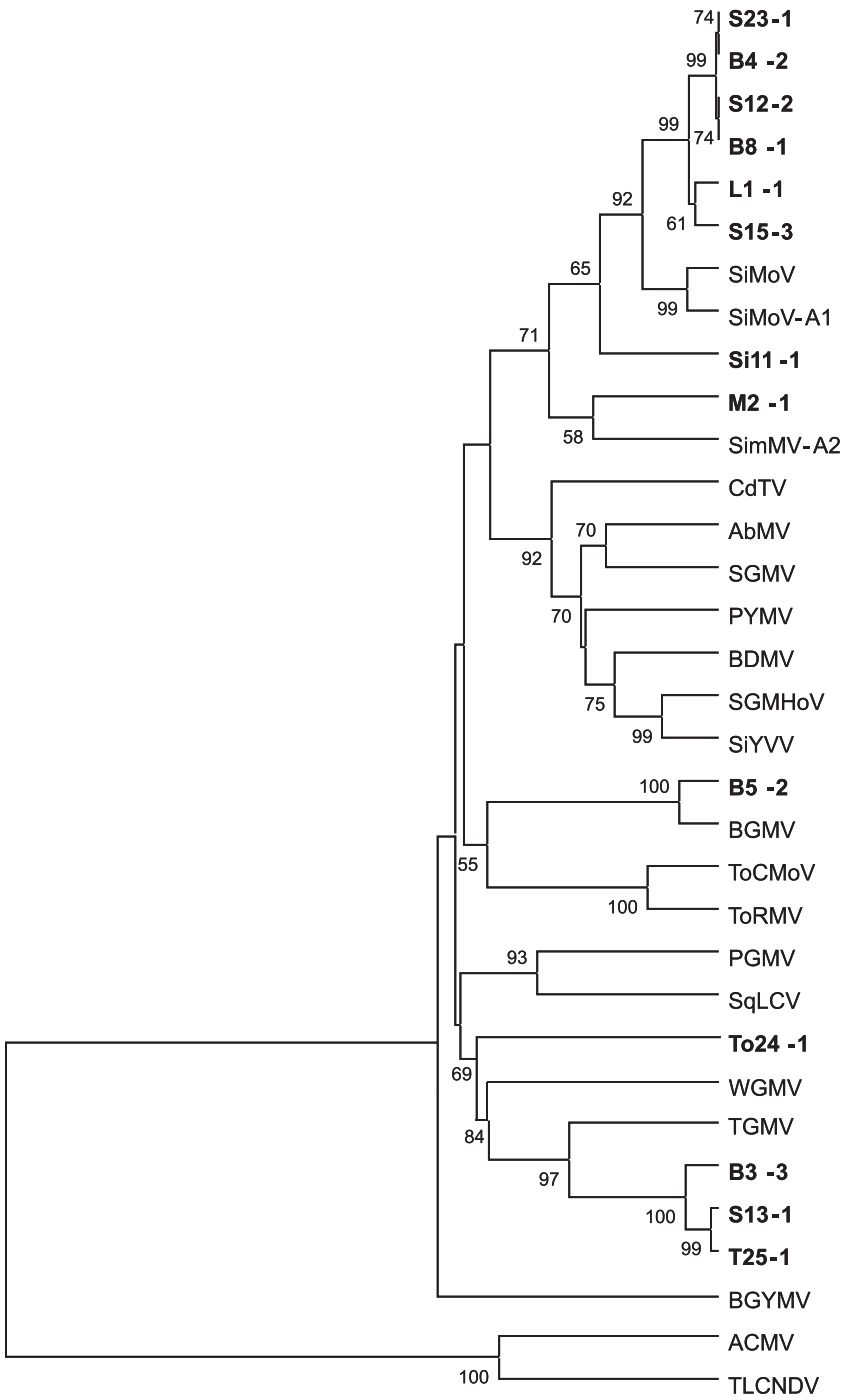

FIG. 2 - Phylogenetic tree based on the nucleotide sequence identity of the entire amplified fragment, generated using the UPGMA method. Branches were bootstrapped with 2000 replications (only values greater than 50% are indicated). AbMV, *Abutilon mosaic virus* (GenBank accession number NC_001928); ACMV, *African cassava mosaic virus* (NC_001467); BDMV, *Bean dwarf mosaic virus* (NC_001931); BGMV, *Bean golden mosaic virus* (NC_004042); BGYMV, *Bean golden yellow mosaic virus* (NC_001439); CdTV, *Chino del tomate virus*, (NC_003830); PGMV, *Pepper golden mosaic virus* (NC_004101); PYMV, *Potato yellow mosaic virus* (NC_001934); SGMV, *Sida golden mosaic virus* (NC_002046); SGMHoV, *Sida golden mosaic Honduras virus* (NC_004659); SiMoV-A1, *Sida mottle virus* isolate A1 (AJ557450); SimMV-A2, *Sida micrantha mosaic virus* isolate A2 (NC_005330); SiMoV, *Sida mottle virus* (NC_004637); SiYVV, *Sida yellow vein virus* (NC_004661); SqLCV, *Squash leaf curl virus* (NC_001936); TGMV, *Tomato golden mosaic virus* (NC_001507); ToCMoV, *Tomato chlorotic mottle virus* (NC_003664); TLCNDV, *Tomato leaf curl New Delhi virus* (NC_004611); ToRMV, *Tomato rugose mosaic virus* (NC_002555); WGMV, *Wissadula golden mosaic virus* (U69280).

Although bean-infecting begomoviruses are disseminated throughout the Americas, soybean-infecting begomoviruses are much less common. In fact, the first reports of soybean infection by begomoviruses in Argentina and Brazil took place only recently (Mello *et al.*, 2000; Mello *et al.*, 2002; Rodríguez-Pardina *et al.*, 1998), and these remain as the only countries in the Western hemisphere where soybean-infecting begomoviruses have been reported.

Here, we attempted to characterize the begomoviruses infecting soybean and bean in Northwestern Argentina, the main bean-growing region of the country and an important soybean-growing area as well. Samples were collected in all provinces of NW Argentina, over a period of ten years. However, most of the samples (90%) were collected in the years 2002 to 2003. Overall, approximately 37% and 26% of soybean and bean samples, respectively, tested positive for the presence of a begomovirus. In soybean, 38, 50 and 26% of the samples collected in the provinces of Tucumán, Salta and Catamarca, respectively, tested positive for the presence of a begomovirus. In bean, 24, 25 and 30% of the samples collected in the provinces of Salta, Jujuy and Santiago del Estero were positive. Although it is not possible to correlate these data with the incidence of begomovirus infection in the field (since only samples with obvious virus-like symptoms were collected), these results indicate a high relative incidence of begomoviruses compared to the other viruses which infect soybean and bean.

Although the portion of the viral genome sequenced was small, it included 200 nucleotides (nt) at the 5' region of the *cp* gene, which is highly variable and has been proposed as an informative region for predicting taxonomic relationships within the genus *Begomovirus* (Brown *et al.*, 2001; Padidam *et al.*, 1995). Definitive species assignment requires sequencing of the complete DNA-A, especially considering the high recombination rate of begomovirus genomes (Preiss & Jeske, 2003). We cannot rule out the presence of recombinant viruses in the field, which would not be detected using our strategy. However, considering the high degree of identity among most isolates and previously characterized begomoviruses, tentative species assignments are possibly quite reliable.

A virus with high sequence identity and phylogenetic relationship with SiMoV was found in 100% of bean and soybean samples, and was also detected in *Leonurus sibiricus* and *Malvastrum coromandelianum*. SiMoV was first reported in Brazil infecting *Sida rhombifolia* (Fernandes *et al.*, 1999), and three years later it was detected in soybean samples also from Brazil (Mello *et al.*, 2002). This is the first report of this virus (or a closely related new virus) in Argentina, and appears to be its first report infecting *L. sibiricus*, *M. coromandelianum* and bean. Surprisingly, from the eight bean samples analyzed, only one had BGMV, and still this sample was co-infected with the SiMoV-like virus. In all other areas of the world where BGMV or *Bean golden yellow mosaic virus* (BGYMV) are present, they are by far the most prevalent viruses in this crop. Thus, NW Argentina seems to be an exception to this rule. The reasons for this prevalence are unknown, but it could have important epidemiological consequences considering that this virus has also been detected in soybean in Brazil. If the conditions that allowed this virus to be prevalent in Argentina are somehow reproduced in Brazil, it is not unreasonable to suppose that SiMoV could eventually replace BGMV as the main bean-infecting begomovirus in that country.

The virus detected in soybean, bean and tomato samples, named "variant A", displayed a maximum sequence identity (87%) with TGMV, and is probably the same isolate found in soybean crops in earlier studies (Rodríguez-Pardina *et al.*, 1998) and tentatively identified as TGMV at that time based on partial sequence comparisons. It has a wide host range in terms of economically important crop species (soybean, bean and tomato), which makes it attractive in terms of its biological properties and host interactions.

"Variant B" was found at a very low incidence. It was detected in a single tomato sample. Further studies have to be carried out in order to establish its real incidence in tomato crops. However, its detection is an indicative of the diversity among the native begomovirus flora of Argentina. This isolate could be a source of additional variation that could drive begomovirus evolution by means of recombination and pseudorecombination.

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