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Bean leafroll virus (BLRV) in Argentina: molecular characterization and detection in alfalfa fields

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Abstract Alfalfa (*Medicago sativa* L.) is the main forage crop in Argentina and plays an essential role in the animal production chain, but it can be negatively affected by many factors, such as viral diseases. Here, we report the presence of *Bean leafroll virus* infecting alfalfa (BLRV-Arg), its distribution on alfalfa fields and its complete genome sequence. BLRV-Arg showed a prevalence of over 50 % and a wide distribution in Argentine alfalfa fields. The BLRV genome of the Manfredi isolate (Manfredi BLRV-Arg) is 5884 nucleotides in length, with a genome organization similar to that already reported for this virus. The complete nucleotide sequence or selected genes were subjected to sequence comparisons. Manfredi BLRV-Arg had more than 96 % nucleotide and amino acid sequence identities, both for complete genome sequence and ORFs, with those of the two full-length genome sequences available in GenBank. The complete CP sequences of Manfredi BLRV-Arg showed an identity percentage over 98 % at nt and deduced aa level when were compared with those of the other CP complete sequences available in GenBank. This is the first evidence for the presence of BLRV in Argentina, its geographical distribution in alfalfa production areas and the complete genome sequence of an isolate from alfalfa as natural host.

Keywords *Medicago sativa* · *Luteovirus* · Complete sequence · Field detection

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Alfalfa (*Medicago sativa* L.) is the most important forage species in Argentina and an essential component of the bovine meat and milk production. The species is well adapted to a large area of the landscape in Argentina due to its good salinity and drought resistance as well as its efficient atmospheric nitrogen fixing capacity (Basigalup 2007). At present, alfalfa is one of the crops with largest cultivated area (over 4 million hectares) in the country, along with maize (*Zea mays* L.), both after soybean [*Glycine max* (L.) Merr.] (SIIA 2015; TodoAgro 2014). Being a perennial crop, alfalfa is exposed to the damage of numerous pests and diseases, which has a direct negative effect on the animal production chain. A viral disease affecting alfalfa was reported in Argentina by Lenardon et al. (2010). The symptoms observed in plants were generalized chlorosis and a bushy appearance produced by internode shortening, deformations and chlorosis at the margins of the leaflet; varying-sized vein enations on its abaxial surfaces were also reported (Trucco et al. 2014). Plants with the described symptoms were naturally co-infected with a rhabdovirus named Alfalfa dwarf virus-ADV (Bejerman et al. 2011; Bejerman et al. 2015) and *Alfalfa mosaic virus*-AMV; also some plants showed spherical viral particles resembling those of luteoviruses (Trucco

et al. 2014). Thus, the alfalfa crop in Argentina is affected by at least three viruses: this fact highlights the importance of performing molecular characterization as a tool to identify which of these pathogens are involved in symptom development, and how they contribute to symptom expression. So far, we have obtained the complete nucleotide sequence of ADV (Bejerman et al. 2015) and AMV (Trucco et al. 2014).

Bean leafroll virus (BLRV) is a member of the genus *Luteovirus* within the family *Luteoviridae*. BLRV is a phloem-limited virus that is present at a very low concentration and is transmitted by aphids in a persistent manner. *Acyrthosiphon pisum* is known to be the main vector, but it is not transmitted by mechanical inoculation of sap or dodder (*Cuscuta* spp.) (Ashby 1984). BLRV was first reported in pea (*Pisum sativum* L.) and broad bean (*Vicia faba* L.) in Germany by Quantz and Völk (1954) and has been frequently reported infecting numerous species of legumes in Europe, India, Middle East, North Africa, Australia and the USA (Ashby 1984; Domier et al. 2002; van Leur and Kumari 2011). BLRV infection in some leguminous species may cause general stunting, yellowing and leaf rolling, with yield losses up to 86 % having been reported in field beans (*Vicia faba* L.) in Europe, depending on the sowing dates (Ashby 1984; Heathcote and Gibbs 1962). In addition, BLRV affects alfalfa, red clover (*Trifolium pratense* L.) and white clover (*T. repens* L.), usually inducing asymptomatic infections (Cockbain and Gibbs 1973; Ashby 1984).

The luteovirus genome contains a single molecule of infectious, linear and positive-sense single-stranded RNA (ssRNA). Viral particles are organized in non-enveloped, icosahedral particles of 25 to 30 nm in diameter. The BLRV genome consists of five open reading frames (ORFs) designated ORF 1 through ORF 5. ORF 1 encodes a 42 kDa protein and is overlapped with ORF 2, which encodes a 62 kDa protein. ORF 1 and 2 encode the replication-related proteins (tombusvirus-like RNA-dependent RNA polymerase); ORF 2 is predicted to be expressed via a translational frameshift from ORF 1. ORF 3 encodes a 22 kDa coat protein (CP) and is followed in frame by ORF 5 and separated from ORF 2 by an intergenic region. ORF 4 is completely overlapped with ORF 3 and encodes a 16 kDa protein (probably a movement protein), and ORF 5 encodes a 59 kDa protein (possibly an aphid transmission and virus particle stability factor) (Domier et al. 2002; Domier 2012). So far, only two complete genome sequences of BLRV are available in the GenBank database obtained from broad bean (AF441393) and pea (HM439776) in USA.

Here we provide the first evidence of BLRV infecting alfalfa in Argentina, its geographical distribution in the main alfalfa production areas of the country and the full-length genome sequence of the Manfredi isolate.

Sampling was conducted between 2010 and 2014. Five diseased alfalfa samples showing the described symptoms were collected from each of 91 commercial fields distributed in 16 of 23 provinces of Argentina (Buenos Aires, Catamarca, Chaco, Córdoba, Entre Ríos, Jujuy, La Pampa, Mendoza, Neuquén, Río Negro, Salta, San Juan, San Luis, Santa Fe, Santiago del Estero and Tucumán), pooled, lyophilized and conserved at −20 °C until use. BLRV infections on alfalfa in Argentina (BLRV-Arg) were serologically determined by triple antibody sandwich, enzyme-linked immunosorbent assay (TAS-ELISA) using commercial test kits (DSMZ GmbH), according to the manufacturer's instructions. Following incubation, optical densities (ODs) at 405 nm were read using a MRX II microplate reader (DYNEX Technologies Inc., USA). Samples were considered infected when their OD readings exceeded the mean plus three standard deviations of the six healthy controls. BLRV-Arg prevalence was determined as the ratio between the number of fields where the BLRV-Arg was present and the total number of sampled fields (Nutter et al. 1991). DIVA-GIS software (version 7.4.0.1) was used to generate a map showing the BLRV distribution data obtained so far from different alfalfa fields of Argentina.

For RT-PCR, Immunosorbent Electron Microscopy (ISEM) and complete genome characterization studies, two pools one from five diseased and the other from five healthy alfalfa plants were used. The diseased plants (cv. "Costera SP INTA") located adjacent to each other and showing the symptoms previously described, were collected from a 5-years-old field of the rural area of Manfredi (Córdoba province), pooled and conserved to −70 °C (Trucco et al. 2014). For RT-PCR assay, total RNA was extracted in duplicate from the diseased and healthy alfalfa plant pools using RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Primers for the amplification of full-length CP gene were designed based on the consensus sequence of the two available complete genome sequences. BLRV sense and antisense primers were 5'TAGGTTCCTTCGATTACAAG3' and 5'CTTCAATATTCGTCCAGTTC3', respectively, and these primers amplified a DNA fragment of 955 bp. cDNA was synthesized at 42 °C for 1 h using ~700 ng

of total RNA, 1 μl of each primer (2.5 μM) and 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega, Madison, WI, USA) in a final volume of 25 μl, following the manufacturer's instructions. Polymerase chain reactions (PCRs) were performed using 2 μl of cDNA in a total volume of 25 μl of reaction with 2.5 μM each of sense and anti-sense primers and GoTaq DNA polymerase (Promega), according to the manufacturer's instructions. The PCR conditions were as follow: denaturation step at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min using a T3000 Thermocycler (Biometra GmbH, Germany). The amplified PCR products were electrophoresed on 2 % agarose gels, stained with ethidium bromide and visualized on a UV transilluminator (BioRad Laboratories Inc., USA).

In ISEM tests, sap extracts were obtained by homogenization of the diseased and healthy alfalfa plant pools using the phosphate buffer (0.01 M $NaPO_4$, pH 7.0) in a ratio of 1:4 (sample weight: buffer volume). Briefly, microscope grids were incubated on drops of diluted BLRV-antiserum (1/100, *v*/v) (Catalogue no. RT-0142-0227/1, Leibniz-Institut DSMZ GmbH, Braunschweig, Germany) with phosphate buffer at room temperature for 30 min, rinsed with phosphate buffer and dried on filter paper. Then, the antiserum-coated grids were incubated on sap extracts for 15 min, washed with distilled water and negatively stained with 2 % of uranyl acetate solution (Milne and Lesemann 1984). Grids were observed using a JEOL 1200 EX II transmission electron microscope.

The complete genome of the BLRV isolate from Manfredi (Manfredi BLRV-Arg) was determined by deep sequencing of small RNAs. Total RNA was extracted from the diseased alfalfa plant pool using Trizol reagent (Life Technologies) according to the manufacturer's instructions. The quality and amount of the RNA were checked by agarose gel electrophoresis and an ND 1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA). A sample of 120 ng/μl of total RNA was sent to Fasteris Life Sciences SA (Plan-les-Ouates, Switzerland), where the bands located between 20 and 30 bp were excised, purified, processed and sequenced on an Illumina HiSEq 2000 Genome Analyzer. The raw data were processed using the pipeline ngs_backbone 1.4.0 (Blanca et al. 2011) in order to eliminate adapters and low-quality regions. The cleaned sequences were *de novo* assembled with VELVET v0.6.04 software package (Zerbino and Birney 2008). The K-mer (hash) length was 17. The individual contigs were identified by BLASTn and BLASTx algorithms. Then, the cleaned reads were mapped against the BLRV genome sequence of the pea isolate, the closest full-length match available from GenBank, using the software ngs_backbone 1.4.0. The whole-genome consensus sequence of the Manfredi BLRV-Arg isolate was determined with the SAMtools mpileup command (Li et al. 2009) and the 5′ and 3′ ends were also confirmed by comparing them to the respective end of the known complete genome sequences of BLRV. The obtained bam files were visualized and analyzed with the Tablet software (Milne et al. 2013). The molecular weights of each protein were determined using the free trial of Geneius 8.1 software (Biomatters Ltd). The percentages of nucleotide (nt) and amino acid (aa) sequence identity of the Manfredi BLRV-Arg isolate with available BLRV sequences were calculated using ClustalW (Thompson et al. 1994) and implemented in the BioEdit 7.0.9.0 software (Hall 1999).

BLRV was serologically tested in the samples collected from different regions of Argentina and detected in 51 of the 91 fields, revealing a prevalence of 56 % and a wide distribution in the country, since it was found in 15 of the 16 provinces analyzed (Fig. 1). As BLRV is not seed-transmitted, its occurrence in wide and diverse geographical regions of Argentina may be largely attributable to behavior of aphid vectors and the possible existence of other BLRV-susceptible hosts associated with this crop. In Argentina, besides the main vector *Acyrthosiphon pisum* (Ashby 1984), the following aphid species were detected in alfalfa fields: *Aphis craccivora*, *A. fabae* and *Myzus persicae*, which were mentioned as less efficient vectors of BLRV (Ashby 1984; Imwinkelried et al. 2013). These aphid vectors are found in different species of the family *Fabaceae* (Blackman and Eastop 2006) and they could contribute to the spread of BLRV from alfalfa to other susceptible crops economically important for Argentina, like soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), lentil (*Lens culinaris*), pea (*Pisum sativum*), and faba bean (*Vicia faba*). Since BLRV can occur at high incidences and cause significant losses in some of these crops (Aftab and Freeman 2006; Heathcote and Gibbs 1962), it could become a potential threat for legume production in Argentina.

The first evidence of the presence of a possible luteovirus in Manfredi alfalfa samples was obtained by electron microscopy and the identification of this virus as BLRV was made by RT-PCR, ISEM and genome sequencing as shown below. The RT-PCR products of

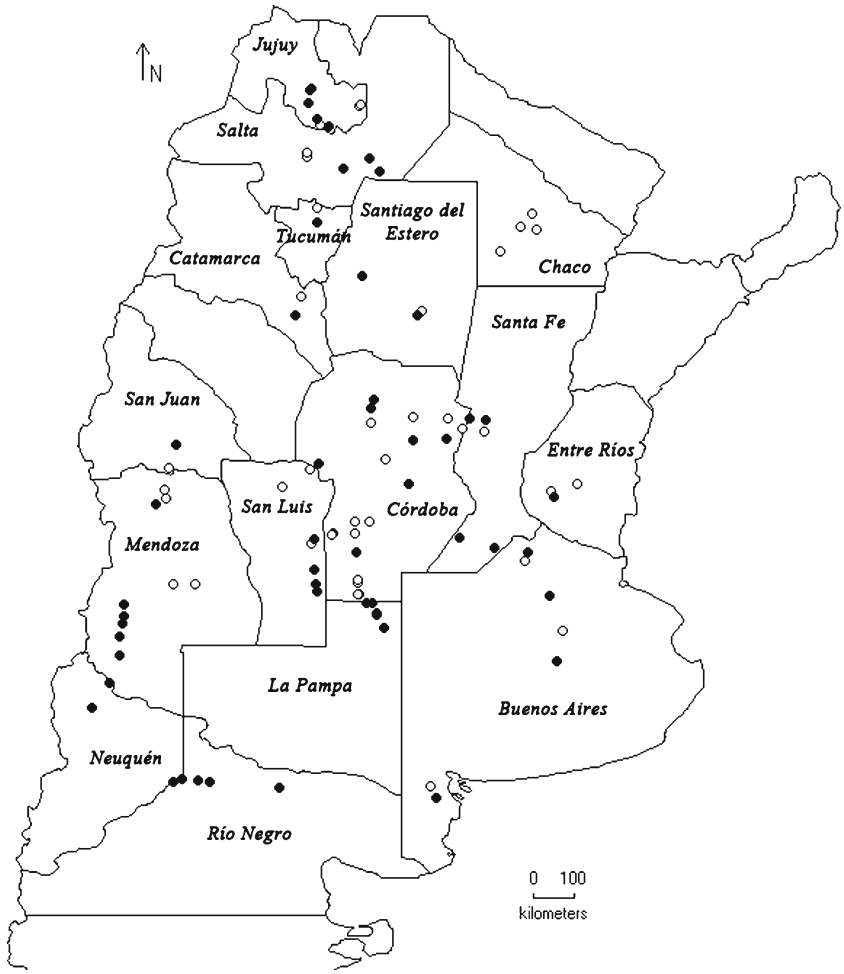

Fig. 1 Sampling sites of diseased alfalfa in Argentina. Black and white circles indicate the positive and negative detection of BLRV, respectively

the expected size (955 bp) were amplified from diseased samples, and no reactions were obtained in samples from healthy plants (data not shown). The ISEM technique showed isometric virus-like particles (VLPs) with a diameter of about 30 nm that were trapped from diseased leaf extract on grids coated with antibody specific for BLRV (Fig. 2), and no VLPs were observed in grids prepared with healthy plant extract. In these tests, few VLPs were observed probably due to the low concentration of BLRV-Arg in the samples.

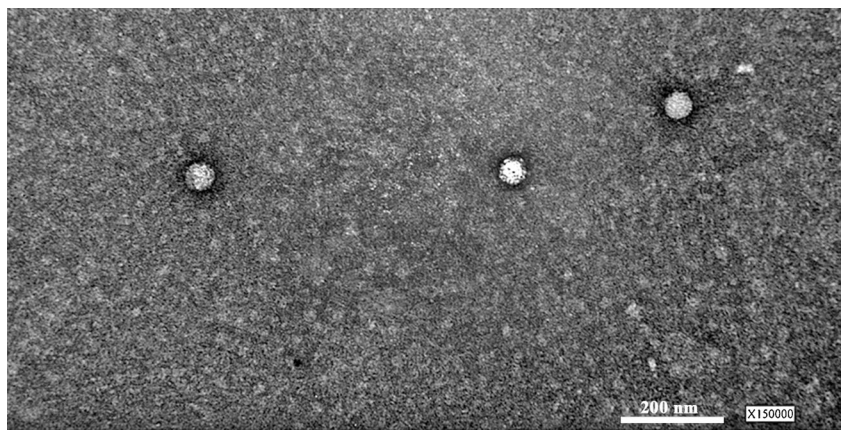

Fig. 2 Electron micrograph of viral particles from infected alfalfa sap in ISEM preparations with BLRV-specific serum

Table 1 Comparison of identity percentages of nucleotide (nt) and deduced amino acid (aa) sequences of the complete genome and individual ORFs of Manfredi BLRV-Arg with those of the complete genome and ORF 3 sequences of BLRV available in GenBank

GenBank accession number	Complete genome sequence	ORF 1		ORF 2		ORF 3		ORF 4		ORF 5	
	nt %	nt %	aa %	nt %	aa %	nt %	aa %	nt %	aa %	nt %	aa %
HM439776 (pea)	98.5	98.9	99.4	98.7	98.1	99.6	100	99.7	99.2	97.4	97.7
AF441393 (broad bean)	96.3	97.7	100	97.1	98.5	98.8	99.4	99.3	98.5	96.9	98.2
GQ404380	—					98.6	98.9				
U15978	—					99.3	99.4				
X53865	—					99.3	100				

We obtained the entire genome of Manfredi BLRV-Arg by deep-sequencing of small RNAs, a useful tool to get the complete genome of plant viruses (Kreuze et al. 2009; Seguin et al. 2014). We obtained 6,591,308 cleaned reads with a sequence quality average of 38, and 1,777,115 of them were *de novo* assembled originating 3507 contigs in total, including those of the BLRV, with a median coverage depth of 10.93. The BLAST results confirmed the presence of both AMV and ADV in the sample, as previously reported (Trucco et al. 2014; Bejerman et al. 2011), and showed that the BLRV contigs presented the highest sequence identity with the BLRV genome sequence of pea isolate. Contigs assembly covered over 50 % of the Manfredi BLRV-Arg genome sequence, which was completely assembled by mapping the cleaned reads to the pea isolate genome; the reference-guided assembly approach have already been reported (Zhang et al. 2014). The whole genome sequence of Manfredi BLRV-Arg was generated from a total of 56,935 reads with an average length of 20 bp (mean coverage of 193). The complete Manfredi BLRV-Arg genome was 5884 nt in length (GenBank accession number KR261610) and revealed a similar genomic organization to that previously described for the broad bean isolate (Domier et al. 2002). Manfredi BLRV-Arg genome contains five ORFs: ORF 1 of 1107 nt, ORF 2 of 1629 nt, ORF 3 of 591 nt, ORF 4 of 429 nt and ORF 5 of 1578 nt, encoding putative proteins of ~42 kDa, ~62 kDa, ~22 kDa (CP), ~16 kDa and ~59 kDa, respectively (Fig. 3). The complete genome sequence of Manfredi BLRV-Arg and each ORF were compared to those of broad bean and pea; the complete genome sequence exhibited nt sequence identity of 96.3 % and 98.5 %, respectively, and the identity percentages between ORFs ranged from 96.9 % to 99.7 % at nt level and from 97.7 % to 100 % at deduced aa level, for both cases (Table 1). The analysis of the CP sequence identity with the five complete CP sequences available in GenBank showed values ranging from 98.6 % to 99.6 % at nt level, and from 98.9 % to 100 % at aa level, exhibiting the highest values with isolates from USA (pea isolate) and Germany (X53865) (Table 1). Thus, these high values of identity percentages among the CP sequences (over 98 % for both nt and aa sequences) show a low level of sequence variation among these isolates.

So far, three viruses have been found in the diseased alfalfa plants from Argentina: ADV (Bejerman et al. 2011, 2015), AMV (Trucco et al. 2014) and BLRV. The viral complex causing the described symptoms remains unclear

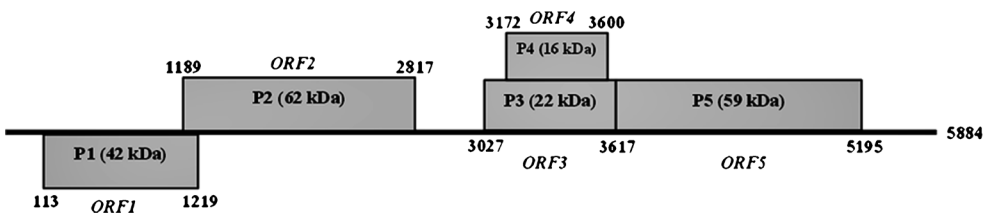

Fig. 3 Schematic representation of Manfredi BLRV-Arg genome organization. The bold line represents the ssRNA and boxes represent the ORFs. P1 and P2: proteins involved in replication (RNA-dependent RNA polymerase). P3: coat protein. P4: movement protein. P5: protein involved in aphid transmission. The nt positions where each protein begins and ends are indicated. The molecular weight of proteins encoded by each ORF is shown in parentheses

because, besides ADV and AMV, BLRV was found affecting this crop, and we cannot discard the possibility of other viruses involved in the complex.

To our knowledge, this is the first evidence for BLRV infecting alfalfa and of its geographical distribution in Argentina, being the first complete genome sequence obtained in South America and from alfalfa as the natural host.

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