Arch Virol (2010) 155:423–428
DOI 10.1007/s00705-010-0590-7

BRIEF REPORT

A distinct tospovirus causing necrotic streak on *Alstroemeria* sp. in Colombia

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Received: 13 October 2009 / Accepted: 14 December 2009 / Published online: 12 February 2010

Abstract A tospovirus causing necrotic streaks on leaves was isolated from *Alstroemeria* sp. in Colombia. Infected samples reacted positively with tomato spotted wilt virus (TSWV) antiserum during preliminary serological tests. Further analysis revealed a close serological relationship to tomato chlorotic spot virus (TCSV) and groundnut ringspot virus (GRSV). A major part of the S-RNA segment, encompassing the nucleocapsid (N) protein gene, the 5′ untranslated region and a part of the intergenic region 3′ of the N gene, was cloned and sequenced. The deduced N protein sequence showed highest amino acid identity (82%) to that of TCSV, indicating that the virus represents a new tospovirus species, for which the name Alstroemeria necrotic streak virus (ANSV) is coined. Phylogenetic analysis based on the N protein sequence revealed that this Alstroemeria-infecting tospovirus clustered with tospoviruses from the American continent. *Frankliniella occidentalis* was identified as potential vector species for ANSV.

R. Goldbach: Deceased.

The nucleotide sequence data reported in this manuscript have been deposited at the NCBI/GenBank under accession no. GQ478668.

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Colombia represents one of the most important countries for production and export of various ranges of cut flowers, with annual sales over 475 million US $ [25]. Worldwide, Colombia has the largest Alstroemeria cultivation, with a production area exceeding 200 ha (Könst Alstroemeria BV). Native to South America, Alstroemeria is becoming an important ornamental plant worldwide [32]. So far, at least 12 viruses, belonging to the genera *Carlavirus*, *Cucumovirus*, *Fabavirus*, *Nepovirus*, *Potexvirus*, *Potyvirus*, *Tobamovirus*, *Tobravirus* and *Tospovirus* have been reported to infect *Alstroemeria* sp. [13].

The genus *Tospovirus* contains the plant-infecting members of the *Bunyaviridae*, a family of primarily animal-infecting viruses [12]. The type member of this genus is tomato spotted wilt virus (TSWV), which has been studied extensively because of its economic impact and broad host range [14, 15].

Tospovirus particles are quasi-spherical, 80–120 nm in diameter and enveloped by a lipid membrane. They are propagatively transmitted by thrips (*Thysanoptera*, *Thripidae*) [12], of which the western flower thrips, *Frankliniella occidentalis* (Pergande) is the most important vector species of the 13 thrips species identified as a tospovirus vector [9, 26, 28, 34].

Tospoviruses contain a single-stranded, tripartite RNA genome [10, 11, 20] of which the small (S) RNA segment encodes the nucleocapsid (N) protein and a nonstructural NS_S protein in an ambisense gene arrangement.

So far, 19 tospovirus species are recognised [3] based on nucleocapsid (N) protein sequence identity (90% threshold) and vector specificity [12]. Members of a few of them, i.e.

Iris yellow spot virus (IYSV), Impatiens necrotic spot virus (INSV), Chrysanthemum stem necrosis virus (CSNV), and Calla lily chlorotic spot virus (CCSV) were initially isolated from ornamentals [2, 6, 21, 22]. Up to now, members of four tospovirus species, i.e. TSWV, INSV, IYSV [1, 13] and most recently tomato yellow ring virus (TYRV) [17, unpublished data] have been identified to infect *Alstroemeria* sp. Reports on TSWV-infected *Alstroemeria* sp. cv. Rosario have described negative effects on stem growth and both quality and quantity of the inflorescences [16].

In 2008, a putative tospovirus was isolated from the Alstroemeria crop in Colombia. Infected plants displayed necrotic streak symptoms on leaves and stems (Fig. 1). Initial serological analysis using TSWV-specific antisera revealed a weak but clearly positive reaction in double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). To assess whether the Alstroemeria virus was a mixture of TSWV, GRSV and/or TCSV, the Alstroemeria isolate was inoculated onto three transgenic *N. benthamiana* seedlings harbouring a chimeric cassette containing partial N gene sequences of these three viruses and two additional Asian tospoviruses to confer multiple resistance based on RNA silencing [18]. The Alstroemeria isolate was able to infect the transgenic plants, suggesting that this isolate clearly diverged from TSWV, GRSV and TCSV. Plants that were singly inoculated with TSWV, GRSV or TCSV did not show any infection, as confirmed by DAS-ELISA. Since the Alstroemeria virus propagated on the transgenic lines was free from TSWV, GRSV and TCSV, leaf material from these plants was used as a source of Alstroemeria virus for further experiments. Here, we describe the identification and characterisation of this newly isolated tospovirus.

The Alstroemeria isolate and tospoviruses TSWV (BR-01), GRSV (SA-05), TCSV (BR-03), and INSV (NL-07), which was used as a reference [8], were mechanically inoculated and serially passaged on *N. benthamiana*. To analyse its host range, at least three plants of several species were selected and mechanically inoculated with leaf extracts of infected *N. benthamiana* source material and scored for infection both visually and serologically (Table 1). The virus, like the reference tospoviruses, infected petunia and cucumber only locally, while vegetables like pepper and tomato became systemically infected. In contrast to TSWV, TCSV and GRSV, the Alstroemeria isolate caused severe top necrosis on *N. benthamiana*. Attempts to back-inoculate the virus onto nine different Alstroemeria cultivars failed, as was reported earlier with TSWV [31].

To assess the economic impact of the Alstroemeria isolate in Colombia, the incidence of this virus not only in Alstroemeria but also in other ornamentals as well as vegetables will have to be determined.

To analyse the Alstroemeria isolate serologically and differentiate it from members of established tospovirus species, a DAS-ELISA was performed [4] using antisera directed against TSWV, GRSV, TCSV and INSV nucleocapsid protein. To this end, leaf samples from infected *N. benthamiana* were ground in PBS-Tween 1:20 (w:v) and applied to an ELISA plate pre-coated with IgGs to the respective viruses. Both IgG and AP-conjugate were diluted 1:1,000 from a 1 mg ml^{-1} stock. Thirty minutes after the addition of AP-substrate, absorbance was measured at 405 nm using a FLUOstar OPTIMA (BMG LABTECH GmbH, Offenburg, Germany) microplate reader. The Alstroemeria isolate clearly cross-reacted with antisera to TCSV, GRSV and TSWV, but not with that to INSV (Fig. 2).

To enable taxonomic classification of the Alstroemeria isolate, its N protein gene was cloned and sequenced. To this end, total RNA was extracted from infected *N. benthamiana* plants using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed on 2 µg RNA using AMV reverse transcriptase (Promega, Corp. Madison, WI, USA) in the presence of primers J060 (5′-CAT**GGATCCTGCAG**AGCAATTGTGTCA-3′, containing *Bam*HI and *Pst*I restriction sites [in boldface] and

Fig. 1 Alstroemeria plant showing necrotic streaks on the stems (*left panel*) and leaves (*right panel*) during natural infection by ANSV

Table 1 Host range study of Alstroemeria necrotic streak virus

Plant family and species	Symptoms	
	Local	Systemic
Balsaminaceae		
Impatiens spp.	NL	–
Asteraceae		
Emilia sonchifolia	–	M
Cucurbitaceae		
Cucumis sativus	CL, NL	–
Leguminosae		
Glycine max cv. Sahar	–	–
Phaseolus vulgaris	NL	NL, VN, LD, R
Vicia faba	BS	
Vigna unguiculata	BS	–
Liliaceae		
Alstroemeria sp. cv. Dimension	–	–
Lilium sp. cv. Gironde	–	–
Solanaceae		
Capsicum annuum	CL	VC, Mo, D
Datura stramonium	CL	M, NS
Nicotiana benthamiana	NL	Ru, C,TN
N. glutinosa	NL	NS, TN
N. occidentalis -P1	NL	NL, PN
N. rustica	CL	–
N. tabacum White burley	CCR, CNR	VN
Petunia hybrida	NL	–
Physalis floridana	CL, NL	VC, M, W
Solanum lycopersicum cv. Money maker	CL, NL	NL, NR, GR, R
S. melongena	–	–

BS brown spot, *C* chlorosis, *CCR* concentric chlorotic ring, *CL* chlorotic lesion, *CNR* concentric necrotic ring, *D* dwarfing, *GR* growth reduction, *LD* leaf distortion, *M* mosaic, *Mo* mottling, *NL* necrotic lesion, *NR* necrotic ring, *NS* necrotic spot, *PN* plant necrosis, *R* recovery, *Ru* rugosity, *TN* top necrosis, *VC* veinal chlorosis, *VN* veinal necrosis, *W* wilting, – no symptoms

containing 15 nucleotides identical to the first 15 conserved nts of the 5′-end of the S RNA) and J064 (5′-CTT TGCTTTTCAGCACAGTGCA-3′, complementary to nt 579–601 of the N gene of TSWV, TCSV and GRSV). After polymerase chain reaction (PCR) amplification using the same primers, a DNA fragment of expected size (~700 bp) was amplified and cloned into pGEM-T Easy Vector (Promega Corp., Madison, WI, USA), and its sequence was analysed. Based on the partial N gene sequence obtained, an internal primer, Col-N-down (5′-GT GTTGTTCTGGCTATATACCAGG-3′), was designed and used in combination with a universal hairpin primer (UHP) (5′-CACT**GGATCC**TTTTGTTTTTGTTTTTTG-3′, containing a *Bam*HI restriction site [in boldface]) to obtain the 3′ end of the N gene [7]. In this way, a partial S RNA sequence of 1,240 nucleotides was obtained, containing the entire open reading frame (ORF) of the N gene, flanked at one end by the 5′ untranslated region (UTR) sequence and at the 3′ end with a part of the intergenic region (IGR). The 5′ UTR of the N gene contained at its terminus the highly conserved consensus sequence of eight nucleotides that is found among isolates of all tospovirus species and assumed to play an important role in transcription/replication [19]. The 5′ UTR contained 152 nt and showed the closest similarity (70% identity) to the analogous region of TCSV. The sequenced partial IGR spanned 311 nt and contained stretches highly rich in A and U residues, which is typical for the IGRs of tospoviral S RNA segments. The N ORF consisted of 777 nt, and its deduced protein sequence contained 258 amino acid residues (accession no. GQ478668). Multiple sequence alignment analysis, using Vector NTI (Invitrogen, Landsmeer, The Netherlands), showed that the Alstroemeria isolate was closely related to TCSV (82%), GRSV (81%) and TSWV (80%) (data not shown), in agreement with their serological relationship, and suggested that the Alstroemeria isolate represents a new tospovirus species. Attempts to back-inoculate to Alstroemeria repeatedly failed, like previous attempts to mechanically inoculate TSWV onto Alstroemeria and to back-inoculate IYSV strains ($IYSV_{NL}$ and $IYSV_{BR}$) onto iris and onion [6, 27, 31]. For this reason, the Alstroemeria isolate has been provisionally named Alstroemeria necrotic streak virus (acronym ANSV). Data from a Clustal W alignment [30] of nucleocapsid (N) protein sequences were used as input for phylogenetic analysis using MEGA version 4.0 software [29]. The results showed clustering of ANSV with TCSV, GRSV, and TSWV within the American tospovirus clade and support its origin from the American continent (Fig. 3).

Until now, five different thrips species, i.e. *F. occidentalis*, *F. panamensis*, *F. auripes*, *F. minuta* and *Thrips tabaci*, have been reported in Colombia. The first species has been observed on most flowers of all ornamental plants within greenhouse cultivations, whereas the second species is the most prevalent species outdoors [5]. Considering the fact that *F. occidentalis*, *T. tabaci* and *F. schultzei* are major vectors for viruses of the American tospovirus clade [24], the first two species, which are present in Colombia, were tested as candidate vectors of ANSV. To this end, ten 0–4 h old first instars of both *F. occidentalis* and *T. tabaci* were placed on two ANSV-infected *Datura stramonium* plants for virus acquisition [32]. Then thrips larvae were transferred onto healthy *D. stramonium* plants and kept there for 3 h to transmit the virus, and plants monitored for the next 2 weeks. Leaf samples were collected for total RNA extraction and subsequent RT-PCR analysis using primers J060 and J064. A fragment of about 700 bp was amplified from plants on which *F. occidentalis* had fed, and

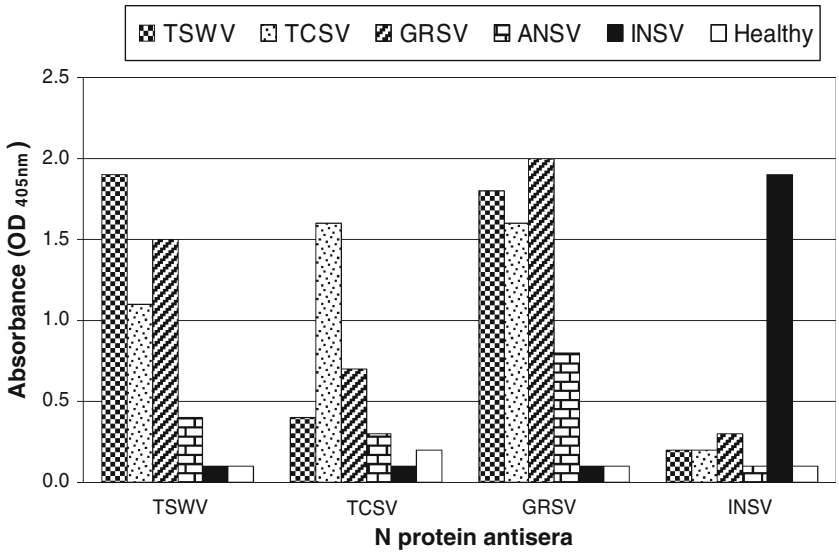

Fig. 2 Serological relationship between alstroemeria necrotic streak virus (ANSV) and four established tospovirus species belonging to the American-continent clade. Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was performed using polyclonal antisera (1:1,000) raised against the N protein of each tospovirus and extracts from infected *N. benthamiana* plants (1:20) as antigen source. Absorbance values were recorded 30 min after addition of substrate

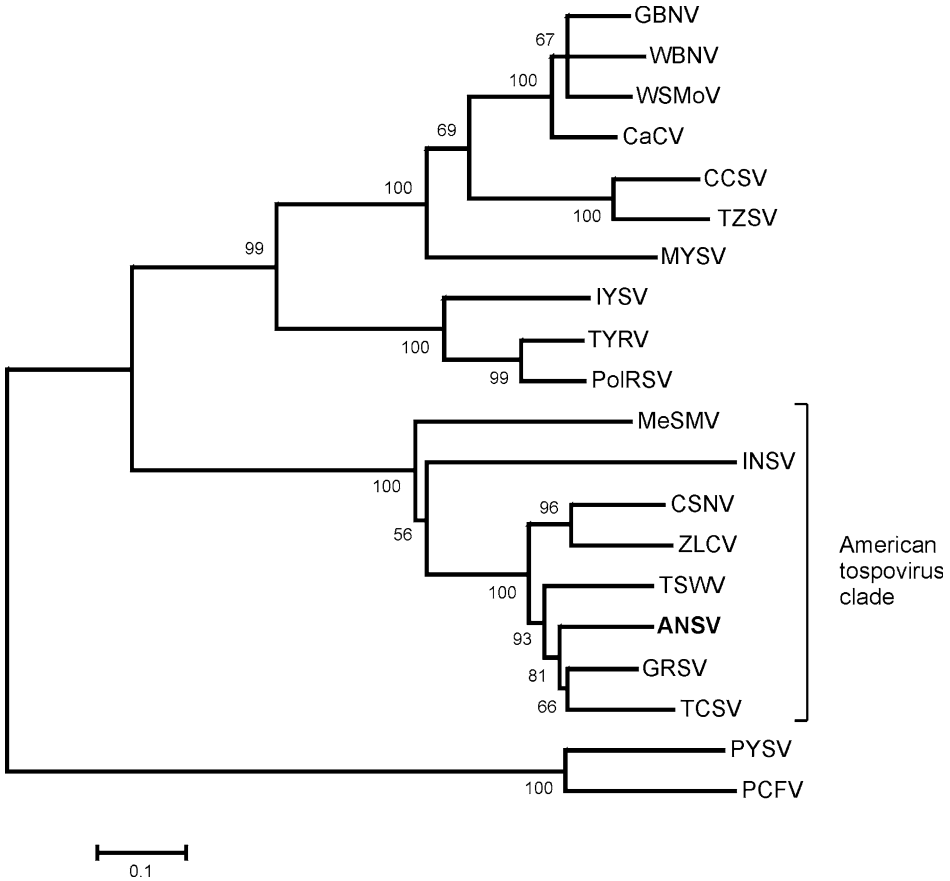

Fig. 3 Phylogenetic relationship of known tospoviruses based on the amino acid sequence of the N protein. The tree was constructed using the neighbour-joining method of MEGA version 4.0. Bootstrap values are shown as percentages derived from 1,000 replicates. Accession numbers (in *parentheses*) of the sequences are from GenBank: alstroemeria necrotic streak virus (ANSV) (GQ478668), capsicum chlorosis virus (CaCV) (AY036058), calla lily chlorotic spot virus (CCSV) (AY867502), chrysanthemum stem necrosis virus (CSNV) (AF067068), groundnut bud necrosis virus (GBNV) (U27809), groundnut ringspot virus (GRSV) (S54327), impatiens necrotic spot virus (INSV) (S40057), iris yellow spot virus (IYSV) (AF001387), melon severe mosaic virus (MeSMV) (EU275149), melon yellow spot virus (MYSV) (AF067151), peanut chlorotic fan-spot virus (PCFV) (AF080526), peanut yellow spot virus (PYSV) (AF013994), Polygonum ringspot virus (PolRSV) (EE445397), tomato chlorotic spot virus (TCSV) (S54325), tomato spotted wilt virus (TSWV) (D00645), tomato yellow ring virus (TYRV) (AY686718), tomato zonate spot virus (TZSV) (EF552433), watermelon bud necrosis virus (WBNV) (AF04567), watermelon silver mottle virus (WSMoV) (Z46419), zucchini lethal chlorosis virus (ZLCV) (AF067069)

this was similar in size as the one amplified from ANSV-infected *N. benthamiana* positive-control plants. On the other hand, no fragment was amplified from plants exposed to *T. tabaci* or healthy *N. benthamiana*, suggesting that *F. occidentalis* is capable of transmitting ANSV (data not shown). Our attempts to thrips-transmit ANSV to Alstroemeria failed. The possibility that ANSV can also be transmitted by other vector species still remains to be investigated.

In Colombia, thrips are usually controlled by insecticides, but their abundance in greenhouses indicates that they have developed resistance against the insecticides used, and this diminishes the efficacy of such pest-management strategies [25]. Alternatively, these thrips species may have a stronger preference for Alstroemeria. As a consequence, the risk of exporting viruliferous thrips-infested plant material has become greater and concomitantly increases the threat of American tospoviruses to crops elsewhere in the world, e.g. of CSNV to Europe [23, 33], especially considering that some of their vectors, like *F. occidentalis*, are already present. The fact that Alstroemeria is vegetatively propagated additionally stresses the global importance of (local) disease management strategies to reduce the incidence of tospovirus infections.

In conclusion, the data presented in this report demonstrate that the Alstroemeria isolate represents a new tospovirus species for which, in light of the symptoms on its natural host, the name Alstroemeria necrotic streak virus (ANSV) is proposed. So far, the virus has not yet been reported either inside or outside of Colombia, possibly due to its (serological) misidentification as an isolate of TSWV, but this can now be tested (by RT-PCR) with the availability of the ANSV N gene sequence.

Acknowledgments The authors would like to acknowledge Könst Alstroemeria BV and Invitrogen Corp. for providing infected ANSV samples and different *Alstroemeria* sp. cultivars and Vector NTI Advance™ 10 license used in this study, respectively.

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