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Unraveling complex viral infections in cassava (*Manihot esculenta* Crantz) from Colombia

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ABSTRACT

In the Americas, different disease symptoms have been reported in cassava including leaf mosaics, vein clearings, mottles, ring spots, leaf distortions and undeveloped and deformed storage roots. Some viruses have been identified and associated with these symptoms while others have been reported in symptomless plants or latent infections. We observed that reoviruses associated with severe root symptoms (RS) of Cassava Frogskin Disease (CFSD) are not associated with leaf symptoms (LS) observed in the cassava indicator plant 'Secundina'. Neither were these LS associated with the previously characterized *Cassava common mosaic virus*, *Cassava virus X*, *Cassava vein mosaic virus* or phytoplasma, suggesting the presence of additional pathogens. In order to explain LS observed in cassava we used a combination of biological, serological and molecular tests. Here, we report three newly described viruses belonging to the families *Secoviridae*, *Alphaflexiviridae* and *Luteoviridae* found in cassava plants showing severe RS associated with CFSD. All tested plants were infected by a mix of viruses that induced distinct LS in 'Secundina'. Out of the three newly described viruses, a member of family *Secoviridae* could experimentally induce LS in single infection. Our results confirm the common occurrence of complex viral infections in cassava field-collected since the 1980s.

1. Introduction

Cassava (*Manihot esculenta* Crantz) is native to the southern border of the Amazon basin where it has been eaten for centuries by indigenous people (Olsen and Schaal, 1999). It was then spread and cultivated in Latin America, West India, Africa and Asia increasing worldwide production to about 250 million tons per year (FAOSTAT, 2011). Colombia is the third largest producer of cassava in Latin America and has increased its production by ~30% over the last 4 years (FAOSTAT, 2011). Cassava is an important source of calories for human consumption and has played an important role in subsistence agriculture and food security because it requires few inputs and tolerates dry weather conditions (FAO, 2013). However, cassava is vegetatively propagated and thus infections tend to accumulate over different crop cycles with a cumulative negative effect on the quality of cassava planting material and plant yield potential (Calvert et al., 2012).

Virus infections have a devastating effect on cassava yield in Africa (Legg et al., 2006; Mbanzibwa et al., 2011), and there is evidence for the occurrence of several viral diseases affecting cassava in the Americas which cause significant yield losses (Calvert et al., 2012). Cassava Vein Mosaic Disease (CVMD) and Cassava Common Mosaic Disease (CCMD) were the first two cassava diseases reported in South America (Costa, 1940; Silva et al., 1963). Affected plants show characteristic leaf symptoms (LS) that have been associated with *Cassava vein mosaic virus* (CsVMV; Family *Caulimoviridae*, Genus *Cavemovirus*) and *Cassava common mosaic virus* (CsCMV; Family *Alphaflexiviridae*, Genus *Potexvirus*), respectively. CVMD has been reported only in Brazil (Calvert et al., 1995) and little research has been published on the epidemiology and control of its associated virus. CCMD has been reported in Brazil, Colombia, Paraguay, Africa and Asia (Silberrschmid, 1938; Costa and Kitajima, 1972; Chen et al., 1981; Aiton et al., 1988), and although the disease has been considered a minor problem in the Americas, prolonged cold periods can cause severe LS and high yield losses (Costa and Kitajima, 1972). Purification of CsCMV (Fig. 1A) and genome sequencing allowed its classification as a member of genus *Potexvirus* (Kitajima et al., 1965; Calvert et al., 1996). ELISA tests using a polyclonal antiserum against a Brazilian isolate showed no serological relation between CsCMV and *Cassava virus X* (CsVX), another putative *Potexvirus* member associated with symptomless infections in cassava (Fig. 1B) (Harrison et al., 1986). Distinct strains of CsCMV have different serological and biological properties (Elliot and Zettler, 1987; Marys and Izaguirre-Mayoral, 1995) compared to

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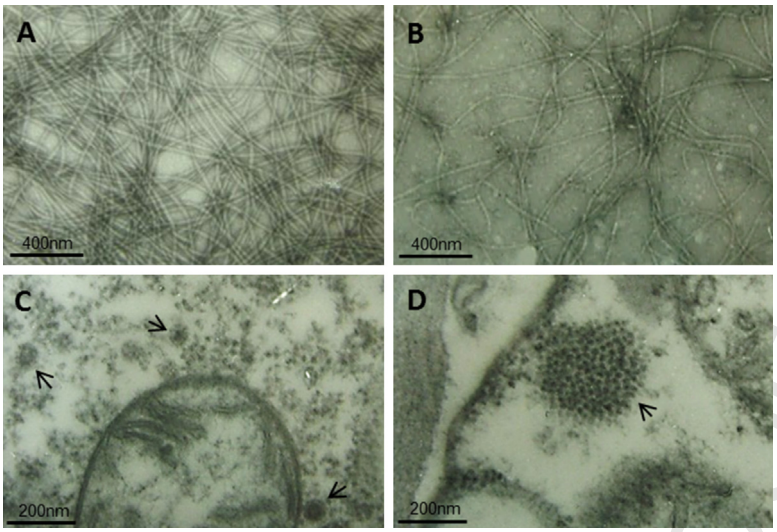

Fig. 1. Photographs of viral particles or virus-like particles derived from cassava plants infected with CsCMV and CsVX or affected by CFSD, CCMD and 'Cassava Caribbean mosaic disease', observed by transmission electron microscopy. (A) CsCMV purified particles from a cassava plant displaying CCMD symptoms (image from 1990), (B) CsVX purified particles (image from 1990) from plants affected by CFSD, (C) Leaf section of a parenchyma cell derived from a cassava plant affected by 'Cassava Caribbean mosaic disease' and CFSD (FSD80) showing spherical-like particles of around 70 nm in diameter, (D) or spherical-like particles of around 25 nm (image from 1990). Size bars are shown for each picture.

the Brazilian isolate used as a reference (Nolt et al., 1991) suggesting greater variability among potexviruses in cassava. Indeed a putative potexvirus named *Cassava Colombian symptomless virus* (CsCSLV) was reported in cassava (Lennon et al., 1986) but not associated with LS. To our knowledge there is no original sample, antisera or sequence information available to identify this virus.

In 1971 in the Department of Cauca, Colombia, a disease that affected cassava storage roots caused yield losses up to 89% (Pineda et al., 1983). Due to the type of root symptoms (RS) observed, the disease was named Cassava Frogskin Disease (CFSD) (Fig. 2E–H). Mild symptoms include the enlargement of the corky layer to form raised lip-shaped fissures in the roots (Fig. 2E), but the disease is symptomless in leaves of most cassava landraces. Severely affected roots present constriction zones and failure of the storage root to accumulate starch (Fig. 2F–H). CFSD has been associated with diverse RS reported in Brazil, Venezuela, Costa Rica, Panama and Peru, and it is the major constraint to cassava production in Latin America (Chaparro-Martinez and Trujillo-Pinto, 2001; Calvert and Thresh, 2002; Calvert et al., 2012). Different pathogens have been associated with CFSD; initial studies aiming to identify the causal agent of the disease detected particles similar to those of members of genus *Closterovirus* and mycoplasma-like structures (Pineda et al., 1980). In 1981, a disease similar to CFSD but with additional severe LS was described in the Caribbean coastal region of Colombia (Department of Magdalena) affecting the local cassava landrace 'Secundina' (COL2063). The associated LS were characterized by bright yellow mosaics (Fig. 2C) and the disease was named 'Caribbean mosaic' (Pineda et al., 1982). Later studies performed by Nolt et al. (1992) suggested that 'Caribbean mosaic' and CFSD were the same disease, and the mosaic symptoms induced in 'Secundina' upon grafting were then recommended for indexing of CFSD (Nolt et al., 1992). More detailed microscopy analysis of plants displaying typical LS of 'Caribbean mosaic' revealed the presence of elongated (*not shown*) as well as spherical virus-like particles (Fig. 1C and D) suggesting mixed virus infections. In 1982, CsVX was first isolated from a symptomless cassava plant (Angel et al., 1987) and then detected by ELISA in cassava plants affected by CFSD (Nolt et al., 1992). Thus, over the years, different viruses have been detected in plants showing RS associated with CFSD. More recently, the analysis of double-stranded RNA (dsRNAs) and total DNA from cassava roots allowed the identification and partial characterization of *Cassava frogskin associated virus* (CsFSaV; Family *Reoviridae*, Genus unassigned) (Calvert et al., 2008) and a phytoplasma (Alvarez et al., 2009), both detected in plants displaying RS of CFSD.

Additional evidence of mixed infections in cassava comes from recent studies: (1) the work by Calvert et al. (2008) first reported CsFSaV association to CFSD, but also indicated the presence of a second type of isometric particles in CFSD-affected plants. (2) A recent report on a phytoplasma associated with CFSD where it is shown that in single-infection, the phytoplasma does not induce LS or RS on cassava indicator plants (Alvarez et al., 2009). (3) Preliminary observations during virus indexing showing that plants infected with CsFSaV induce different kinds of LS in the indicator plant 'Secundina', suggesting the presence of additional uncharacterized viruses or viral strains. Based on these observations we have used serological, biological and molecular methods to identify additional viruses in cassava plants showing varying degrees of LS. We thus unraveled and confirmed suspected disease-associated mixed virus infections in cassava and reported the presence of three novel viruses belonging to the families *Alphaflexiviridae*, *Secoviridae* and *Luteoviridae* in plants collected since the 1980s in different regions of Colombia. The results indicate that mixed virus infections in cassava are more common and widespread than previously known and that they are associated with a variety of LS in 'Secundina'.

2. Materials and methods

2.1. Virus isolates and plant material

Plants from the *in vitro* cassava germplasm collection maintained at the International Center of Tropical Agriculture (CIAT), Colombia (Table 1, upper half) were propagated in a humid chamber for 3 weeks before being transferred to a greenhouse at CIAT as indicated below. Stem cuttings of different cassava varieties, displaying CFSD root symptoms, were collected between 1980 and 2012 from various regions of Colombia including farms and experimental fields (Table 1, lower half). Plants were grown and maintained at $28 \pm 5\,^{\circ}C$ and 70–80% relative humidity in a quarantine insect-proof greenhouse at CIAT, in 25 cm × 25 cm plastic pots with a sterile substrate mix consisting of 2 parts soil and 1 part sand.

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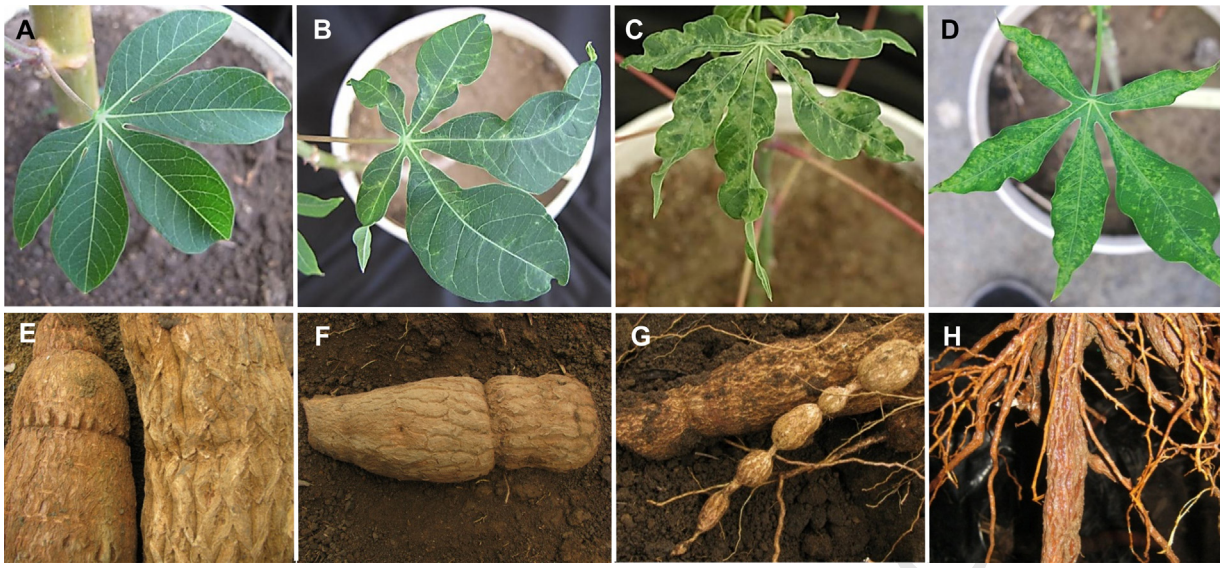

Fig. 2. Variety of virus-like disease symptoms on cassava leaves and roots observed under greenhouse conditions at CIAT. (A) healthy 'Secundina' (COL2063), (B) 'Secundina' leaf expressing mild mosaic and leaf distortion grafted on sample FSD5, (C) 'Secundina' leaf with severe chlorosis and deformation after grafting on BIP2-284 rootstock, (D) leaf showing mottle and ringspot symptoms (CsCMV). The root symptoms range from very mild to very severe, (E) CFSD-root symptoms showing two different severity of lip-shaped fissures, (F) root with constriction zones and lip-shaped fissures, (G) root with multiple constrictions, (E–G) correspond to samples of plant BIP2-284 and (H) severely affected root of cassava variety 'Brasilera' (COL2737) with no accumulation of starch.

Plants from the cassava variety CM5460-10 that were collected in Valle del Cauca, Colombia in 1998 showing severe RS and LS, were used as positive controls for all characterized viruses. Virus-free cassava plants of the landrace 'Secundina' (COL2063) were obtained *in vitro* from the germplasm bank maintained at CIAT. A lyophilized leaf sample from a cassava plant infected with *Cassava green mottle virus* (CsGMV; Family *Secoviridae*, Genus *Nepovirus*) was provided by Dr. Bryan D. Harrison in 1987 and used as inoculum for transmission to *Nicotiana benthamiana* Domin. and *N. tabacum* L. Cassava plants infected with Colombian isolates of CsCMV and CsVX were maintained under the same greenhouse conditions as described above and used as positive controls in ELISA tests. Total DNA of a CsVMV-infected cassava plant (kindly provided by Dr. Eduardo Andrade-EMBRAPA) was used as positive control in PCR tests. Tomato plants infected with *Tomato torrado virus* (ToTV; Family *Secoviridae*, Genus *Torradovirus*) were provided by Florentino Vivas (Syngenta-Colombia) and used as positive controls for RT-PCR detection and biological assays.

2.2. Total RNA isolation and deep sequencing

Total RNA was isolated from 200 to 400 mg of cassava leaf tissue by using the CTAB method and following standard protocols. Total RNA was incubated with 1 U of DNase (Invitrogen) for 1 h at 37 °C followed by precipitation with Lithium Chloride (LiCl). High molecular weight RNA quality was visualized by agarose gel electrophoresis and GelRed staining. The concentration and purity was measured by calculating the absorbance at 260 nm/280 nm using a nanodrop1000 (Thermo Scientific). Low molecular weight RNA was obtained by LiCl precipitation and analyzed by electrophoresis on 4% agarose gel. For deep sequencing (DS), 21–24 nucleotide (nt) RNA including small interfering RNAs (siRNAs) were isolated from two selected common cultivars CM5460-10 and CMC40-4 grown and collected in Cauca and Valle del Cauca regions, respectively. A third sample was from cultivar FSD29-a collected in the Cauca region but introduced there from the Amazonas (Table 1, lower half). All these samples showed RS associated with CFSD and they induced clear LS upon grafting on 'Secundina'. Each sample (barcoded separately) consisted of a mix of siRNA obtained from 2 to 3 plants from the same cultivar. Lyophilized RNA was sent to Fasteris (www.fasteris.com; Switzerland) for DS using the Illumina platform (GSIII). Velvet was used for *de novo* sequence assembly of contigs (Kreuze et al., 2009), and viral sequences were identified by comparison to the GenBank virus sequence database using BLASTX and BLASTN. Based on contigs sequence information we designed specific primers and confirmed the presence of virus sequences in the original plants and then in a collection of *in vitro* and field-collected samples.

2.3. Detection by RT-PCR, cloning, sequencing and phylogenetic analysis

Cassava plants were virus-indexed using RT-PCR. Between 2 and 4 μg of RNA and 200–400 ng of random primers were denatured at 65 °C for 5 min, followed by one minute on ice. First strand cDNA was synthesized at 37 °C for 1.5 h after addition of 1× first stranded buffer, 10 mM DTT, 40 U RNaseOUT, 0.5 mM dNTPs and 200 U M-MLV reverse transcriptase (Invitrogen) to a final volume of 20 μl. CsFSaV was detected as previously described (Calvert et al., 2008). To fill gaps between siRNA contigs and to standardize a detection method for the three novel viruses, primers were designed based on alignments with related viral genomes used as references. The following primer pairs were used to index cassava plants shown in Table 2: for 'Cassava new alphaflexivirus' (CsNAV): CsNAV-F4 (5′-TTC AAC CAT CAC TTT ACA TCA GAC-3′) and CsNAV-R5 (5′-AAC T TG ACC ACG TGT AAC TTC AGC-3′); for 'Cassava polero-like virus' (CsPLV): CsPLV-F2 (5′-TTG CAT TCA AAG ATC AGT TCT CTC-3′) and CsPLV-R3 (5′-TGG TTG ACA GCT GTT TCA GAG G-3′); and for 'Cassava Torrado-like virus' (CsTLV) RNA segment 1: CsTLV-1F (5′-GAC TCA ATG AAG GAG GAG GAT AGA-3′) and CsTLV-1R (5′-ACC AGA GCT TGT CCT AAT AGC AAC-3′) and RNA segment 2: CsTLV-2F (5′-GAT CGC TGA GAG TTT ATG TGC TTA-3′) and CsTLV-2R (5′-ACA GGT GTT CTG AGT TAC ACC AAA-3′). Specific primers were used to amplify a region of the RNA1 of members of the genus *Torradovirus* as described by Verbeek et al. (2012). RT-PCR was performed on an Eppendorf thermocycler, and amplifications were obtained by incubating the reaction first to a denaturation step at 94 °C for 5 min, followed by 35 cycles of 1 min denaturation at 94 °C,

Table 1
List of plants infecetd by CsFSaV analyzed in this work. Samples were vegetatively propagated (stem cuttings) and maintained in an insect-proof screenhouse. Indicator plants of cultivar 'Secundina' were propagated in vitro and used for graftindexing as described in Section 2. Mosaic symptoms in 'Secundina' were observed and recorded 2–4 weeks after graft-inoculation. N/A = not apply; N.D. = not determined due to death of plants.

	Isolate	Host cultivar	Collection data	Root FSD symptoms	Secundina graft
In vitro	BRA1299	BRA1299	Valle del Cauca, Colombia	N/A	(−)
	BRA383	BRA383	Brazil, 1982	N/A	(−)
	BRA504	BRA504	Minas Gerais, Brazil. 1982	N/A	Mosaic
	CM2772-3	CM2772-3	Valle del Cauca, Colombia.	N/A	(−)
	CM3306-4	CM3306-4	Valle del Cauca, Colombia.	N/A	(−)
	CM523-7	CM523-7	Valle del Cauca, Colombia.	N/A	(−)
	CM4574-7	CMC4574-7	Valle del Cauca, Colombia.	N/A	(−)
	COL1185	COL1185	Santander, Colombia. 1970	N/A	(−)
	COL1287	COL1287	Boyaca, Colombia. 1970	N/A	(−)
	COL1505	COL1505	Venezuela, 1970	N/A	(−)
	COL2215	COL2215	Magdalena, Colombia. 1982	N/A	(−)
	COL911B	COL911B	Bolivar, Colombia. 1984	N/A	(−)
	CR29	CR29	Limon, Costa Rica. 1984	N/A	(−)
	CR158*	CR158	Costa rica. 1961	N/A	ND
	CR169*	CR169	Panama. 1977	N/A	ND
	ECU43	ECU43	Manabi, Ecuador. 1970	N/A	(−)
	ECU72	ECU72	Zamora, Ecuador. 1970	N/A	(−)
	HMC1	HMC1	Valle del Cauca, Colombia.	N/A	(−)
	PAN51	PAN51	Ocu, Panamá. 1970	N/A	(−)
	PAR36	PAR36	Amambay, Paraguay. 1983	N/A	(−)
	SG700-3	SG700-3	Valle del Cauca, Colombia.	N/A	(−)
	VEN24	VEN24	Venezuela, 1971	N/A	(−)
	VEN77	VEN77	Venezuela, 1971	N/A	(−)
Field collected	AMZ16	ND	Amazonas, Colombia. 1990	(+)	Mosaic
	AMZ9-a	ND	Amazonas, Colombia. 1990	(+)	Mosaic
	AMZ9-a-P2	ND	Amazonas, Colombia. 1990	(+)	Mosaic
	AMZ9-b	ND	Amazonas, Colombia. 1990	(+)	Mosaic
	AMZ9-b-P2	ND	Amazonas, Colombia. 1990	(+)	Mosaic
	Cauca1B	COL2215	Cauca, Colombia.2005	(+)	Mosaic
	CMC40-4	CMC40	Valle del Cauca, Colombia. 2012	(+)	Mosaic
	CMC40-5	CMC40	Valle del Cauca, Colombia. 2012	(−)	ND
	CM5460-10	CM5460-10	Valle del Cauca, Colombia. 1998	(+)	Mosaic
	COL2737-1	COL2737	Yopal, Colombia. 2013	(−)	ND
	COL2737-3	COL2737	Yopal, Colombia. 2013	(+)	ND
	COL911B-b	COL911B	Bolivar, Colombia. 1984	(ND)	ND
	FSD23-a	ND	Cauca, Colombia. 1984	(+)	Mosaic
	FSD23-b	ND	Cauca, Colombia. 1984	(+)	Mosaic
	FSD29-a	COL1684	Cauca, Colombia. 1990	(+)	Mosaic
	FSD29-b	COL1684	Cauca, Colombia. 1990	(+)	Mosaic
	FSD5	COL2063	Magdalena, Colombia. 1980	(+)	Mosaic
	FSD80	COL2063	Magdalena, Colombia 1980	(+)	Mosaic
	FSD86	COL2063	Magdalena, Colombia 1980	(+)	Mosaic
	HEL4-a	ND	Amazonas, Colombia. 1992	(+)	Mosaic
	HEL4-b	ND	Amazonas, Colombia. 1992	(+)	Mosaic
	Llano1A	COL2177	Meta, Colombia. 2005	(+)	Mosaic
	RegTolimaA	COL2063	Tolima, Colombia 1992	(+)	Mosaic
	SM909A	SM909-25	Valle del Cauca, Colombia. 2001	(+)	Mosaic
	VEN-Costa	COL2215	Sucre, Colombia 2005	(+)	Mosaic

Table 2
Selected cassava field-collected plants used for virus indexing. These plants were originally collected because they showed different levels of severity of RS associated to CFSD. Plants were grown under green house conditions at CIAT and evaluated for leaf symptoms after their first propagation. All these plants induced LS in 'Secundina' grafting tests. NR: not registered in the year when they were collected.

No.	Isolate	Host Cultivar	Collection data	Host LS
1	AMZ16	ND	Amazonas, Colombia. 1990	–
2	AMZ9	ND	Amazonas, Colombia. 1990	NR
3	HEL4	ND	Amazonas, Colombia. 1992	NR
4	FSD23	ND	Cauca, Colombia. 1984	NR
5	FSD29/Sec	COL2063/Graft	Valle del Cauca, Colombia. 2011	+
6	SM3375-113	SM3375-113	Valle del Cauca, Colombia. 2010	NR
7	CMC40	COL1468	Valle del Cauca, Colombia. 2012	–
8	BIPD-289-25	BIPD-289-25	Cauca, Colombia. 2008	–
9	SM909A	SM909-25	Valle del Cauca, Colombia. 2001	+
10	CM4574-7	CM4574-7	Cauca, Colombia. 2010	NR
11	RegTolimaA	COL2063	Tolima, Colombia 1992	–
12	Catumare	ND	Valle del Cauca, Colombia. 2003	–
13	Nataima31	CG48931	Valle del Cauca, Colombia. 2010	NR
14	FSD80	COL2063	Magdalena, Colombia 1980	+
15	FSD86	COL2063	Magdalena, Colombia 1980	+
16	CM5460-10	CM5460-10	Valle del Cauca, Colombia. 1998	+

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45 s annealing at 55 °C and 1.3 min extension at 72 °C. Detection of CsTLV was performed with an annealing at 48 °C and 1 min extension. RT-PCR products were cloned and virus identity confirmed by sequencing (Macrogen). For detection of CsVMV we used primers SST-FW: 5′-TGA GCA GGT ACA ATT CAT CTG ATA CTG A-3′ and SST-RE: 5′-CTT CGT ATT CTG GCA GTA TAG G-3′ and total DNA extraction as described in Calvert et al. (1995). Phylogenetic trees were produced by neighbor-joining with 1000 bootstrap replications using MEGA4 (Kumar et al., 2008) based on the complete amino acid (aa) sequences of RdRp sequences of novel cassava viruses and members of the families *Luteoviridae*, *Reoviridae*, *Alphaflexiviridae* and *Secoviridae* (Fig. 3). All virus sequence identities and similarities were calculated by using BLAST. Phytoplasma detection of selected samples was done as described in Alvarez et al. (2009).

2.4. Sap-inoculation and graft-inoculation of indicator plants

N. benthamiana and *N. tabacum* plants were tested for their response to CsGMV and CsTLV through sap-inoculation. Two plants at 3–5 leaf stage were selected for mechanical inoculation with each virus. Leaf tissue from plants infected with each virus was ground separately in sterile water (inoculum) and used to inoculate indicator plants using a cotton wool pad. For graft inoculation three 4-week old 'Secundina' plants were used as rootstocks per inoculum and stem-cuttings from 4-week old test plants (Tables 1 and 2) were used as inoculum. The grafting junction was protected with Parafilm and plants were kept for 4 weeks for symptoms to develop. Plants inoculated with water alone or grafts from virus-free 'Secundina' plants were used as controls. Virus transmission and systemic infection of grafts was confirmed by RT-PCR and ELISA 4 weeks after inoculation.

2.5. ELISA detection of CsCMV and CsVX

To determine the presence of CsCMV and CsVX, we used double antibody sandwich ELISA (DAS-ELISA) as described by Clark and Adams (1977) and Nolt et al. (1991). Briefly, purified immunoglobulin were bound to the surface of a microtiter plate and were incubated at 37 °C for 4 h. Wells were washed with PBS-Tween and incubated for 6 h with plant extracts diluted 1/10. After washing with PBS-Tween, conjugated-gamma globulins were added and incubated for another 4 h. p-Nitrophenylphosphate substrate was incubated at room temperature. Readings were performed after 60 min by measuring the absorbance at 450 nm. *In vitro* propagated plants that tested negative for CsCMV, CsVX, CsVMV and the viruses newly presented in this work, were used as negative controls.

3. Results

3.1. Identification of novel viruses from CFSD-affected plants

BLAST analysis of siRNA contigs obtained identified sequences of the previous associated reovirus CsFSaV (Calvert et al., 2008) and sequences from viruses belonging to the families *Luteoviridae, Alphaflexiviridae* and *Secoviridae*. We used this sequence information to design PCR primers in order to screen cassava *in vitro*-grown plants (infected with CsFSaV) and field-collected plants for the presence of the different viruses detected by DS (Table 1). Most *in vitro* plants were single infected with the reovirus CsFSaV, and interestingly they did not induce LS after grafting to 'Secundina' (Table 1, upper half). These results confirmed that CsFSaV does not cause LS in single infections. Some of these *in vitro* plants were positive for the newly described viruses in single infections (*not shown*), but in grafting experiments only CsTLV induced LS in single infections (Fig. 4A). On the other hand when we screened field-collected samples we detected the presence of CsFSaV in mixed infections

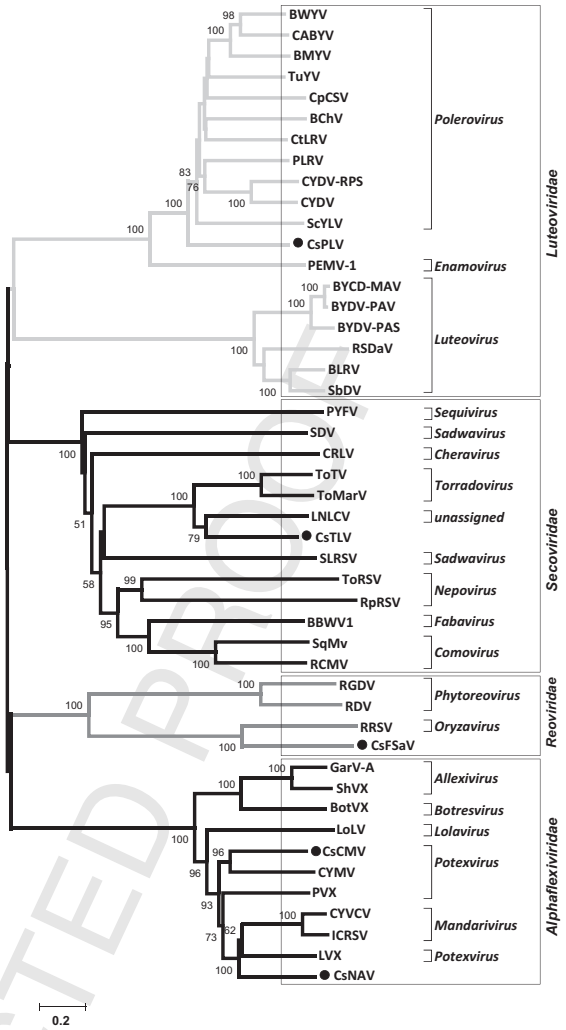

Fig. 3. Phylogenetic relationship of novel cassava viruses. Tree was obtained using aa sequences from the replicase genes aligned using CLUSTALW (Jeanmougin et al., 1998). Genera are indicated with brackets and virus names in abbreviations. Previous reported cassava viruses as well as novel viruses from the Americas are indicated by a black circle. Tree was produced with MEGA5 (Kumar et al., 2008). Numbers of branches indicate percentage of bootstrap support out of 1000 bootstraps replications and the scale bar indicates amino acid substitutions per site. Sequences used for the *Luteoviridae*: BYCD-MAV, *Barley yellow dwarf virus-MAV* (NC_003680.1); BYDV-PAS, *Barley yellow dwarf virus-PAS* (NC_002160.2); BYDV-PAV, *Barley yellow dwarf virus-PAV* (NC_004750.1); BLRV, *Bean leafroll virus* (NC_003369.1); BChV, *Beet chlorosis virus* (NC_002766.1); BMYV, *Beet mild yellowing virus* (NC_003491.1); BWYV, *Beet western yellows virus* (NC_004756.1); CtRLV, *Carrot red leaf virus* (NC_006265.1); CYDV-RPS, *Cereal yellow dwarf virus-RPS* (NC_004751.1); CpCSV, *Chickpea chlorotic stunt virus* (NC_008249.1); CABYV, *Cucurbit aphid-borne yellows virus* (NC_003688.1); PEMV-1, *Pea enation mosaic virus-1* (NC_003629.1); PLRV, *Potato leafroll virus* (NC_001747.1); RSDaV, *Rose spring dwarf-associated virus* (NC_010806.1); SbDV, *Soybean dwarf virus* (NC_003056.1); ScYLV, Sugarcane yellow leaf *virus* (NC_000874.1) and TuYV, *Turnip yellows virus* (NC_003743.1). *Reoviridae*: RRSV, *Rice ragged stunt virus* (NC_003771.1); RDV, *Rice dwarf virus* (RDU73201.1); RGDV, *Rice gall dwarf virus* (AB254451.1). *Alphaflexiviridae*: BotVX, *Botrytis virus X* (NC_005132.1); CsCMV, *Cassava common mosaic virus* (NC_001658.1); CYVCV, Citrus yellow vein clearing virus (JX040635); CYMV, *Clover yellow mosaic virus* (NC_001753.1); GarV-A, *Garlic virus A* (NC_003375.1); ICRSV, *Indian citrus ringspot virus* (NC_003093.1); LVX, *Lily virus X* (NC_007192.1); LoLV, *Lolium latent virus* (NC_010434.1); PVX, *Potato virus X* (NC_011620.1) and ShVX, *Shallot virus X* (NC_003795.1). Secoviridae: BBWV1, *Broad bean wilt virus* (NC_005289.1); CRLV, *Cherry rasp leaf virus* (NC_006271.1); LNLCV, Lettuce necrotic leaf curl virus (KC855267); MCDV, *Maize chlorotic dwarf virus* (NC_003626.1); PYFV, *Parsnip yellow fleck virus* (NC_003628.1); RpRSV, *Raspberry ringspot virus* (NC_005266.1); RCMV, *Red clover mottle virus* (NC_003741.1); SDV, *Satsuma dwarf virus* (NC_003785.2); SqMV, *Squash mosaic virus* (NC_003799.1); SLRSV, *Strawberry latent ringspot virus* (NC_006964.1); ToMarV, *Tomato marchitez virus* (NC_010987.1); ToRSV, *Tomato ringspot virus* (NC_003840.1) and ToTV, *Tomato torrado virus* (NC_009013.1).

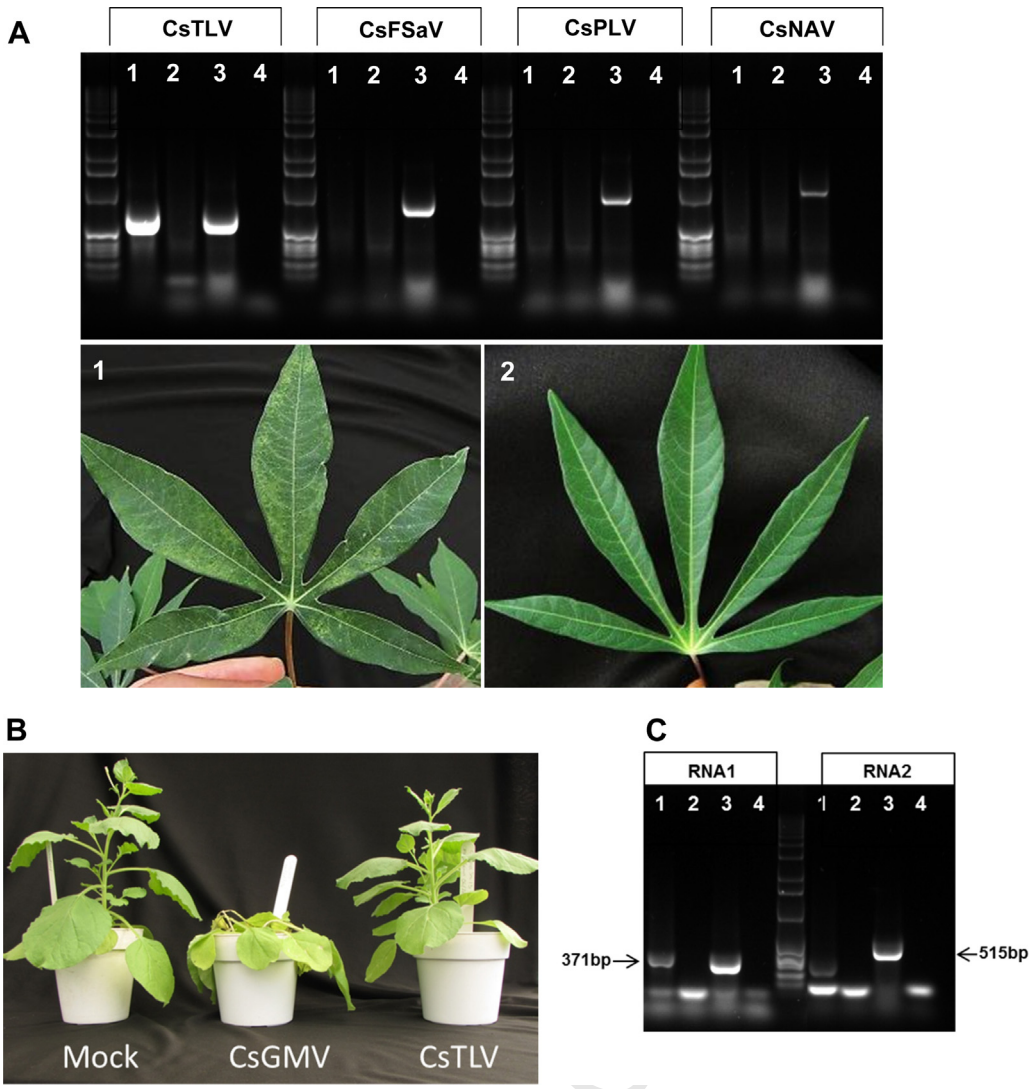

Fig. 4. CsTLV induces leaf symptoms in 'Secundina' and is distinct from CsGMV. (A) RT-PCRs using specific primers to detect CsTLV, CsFSaV, CsPLV and CsNAV from 'Secundina' plants displaying mild leaf symptoms. 1: COL2063/Secundina-13; 2: COL2063/Secundina-4 (negative control); 3: CM5460-10 (positive control); 4: reaction control. 1 kb marker is used. COL2061/Secundina-13 is single infected with CsTLV. (B) Sap-inoculation of *N. benthamiana* plants with CsGMV and CsTLV leaf extracts. (C) RT-PCR using a generic pair of primers for the genus *Torradovirus* (Verbeek et al., 2012). The primer pair for RNA1 detected ToTV (3) and CsTLV (1) while RNA2 primer pair detected only ToTV (3). No PCR product was obtained in CsGMV sample (2) or the PCR reaction control (4). Mock: plants inoculated with sterile water.

with CsTLV, CsPLV and CsNAV and in all tested cases, LS in the indicator 'Secundina' correlated with the detection of mixed infections (Table 1, lower half and Fig. 5). Only few field-collected samples were positive for phytoplasma (*not shown*), confirming previous results indicating that infection by phytoplasma is not sufficient to induce LS or RS in cassava indicator plants (Alvarez et al., 2009). The results show that grafting in 'Secundina' should be complemented with RT-PCR for virus indexing in cassava. Thus by using a combination of biological studies, molecular analyses and DS we detected and identified novel viruses and mixed infections in cassava.

3.2. A member of the genus Polerovirus infecting cassava plants

BLASTX identified contigs with ~62% aa sequence similarity and coverage of ~65% (*e*-value 0.0) to *Chickpea chlorotic stunt virus* (AAY90038) a member of the family *Luteoviridae* (Genus *Polerovirus*) To validate this finding and to complete gaps between contigs we performed RT-PCR to produce overlapping sequence fragments and complete a genomic region of 2990 nt (Genbank nr. KC505249). Although the assembled sequence does not encompass the P0 region (used for genus demarcation), by using the aa sequence of the P1–P2 read-through gene of members of the family *Luteoviridae* we conducted phylogenetic analysis confirming that this cassava virus is evolutionarily closer to members of the genus *Polerovirus* (Fig. 3). We named this virus 'Cassava polero-like virus' (CsPLV) and if confirmed it would be the first virus from this family reported to infect cassava plants. CsPLV sequences were detected in samples collected in the south/central and northern regions of Colombia (Fig. 5A) and they were graft-transmissible to 'Secundina' (Fig. 6).

3.3. Identification of a novel cassava Alphaflexivirid member serologically unrelated to CsCMV or CsVX

Previous reports have suggested the presence of different members of the *Alphaflexiviridae* family infecting cassava, including CsCMV, CsVX, CsCSLV and 'Cassava Caribbean mosaic virus' (Genus *Potexvirus*) (Silva et al., 1963; Kitajima et al., 1965; Lennon et al., 1986). Based on DS data, four overlapping RT-PCR fragments produced a genomic region of 5419 nt (Genbank nr. KC505252) and

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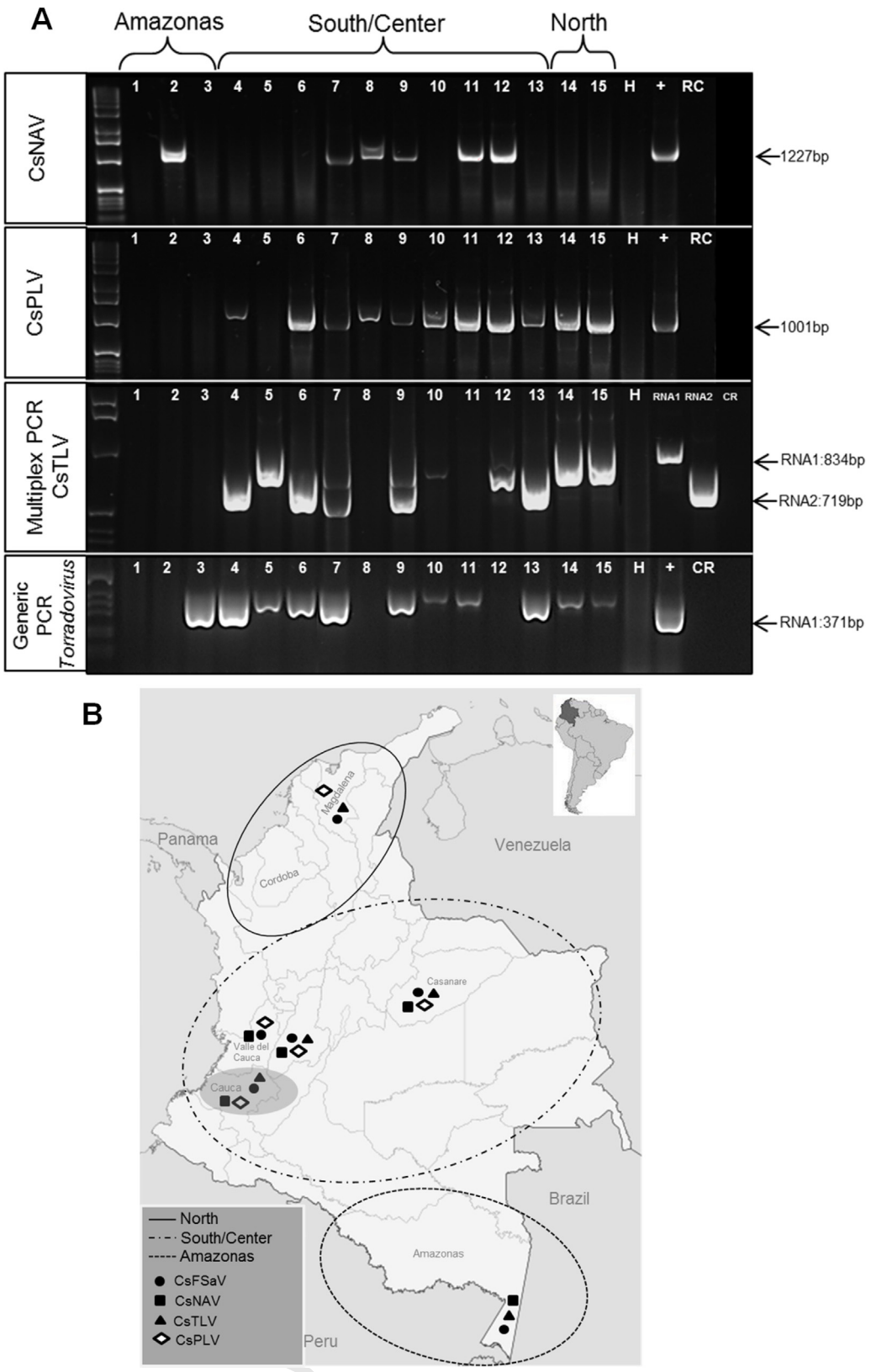

Fig. 5. Occurrence of mixed viral infections in CSFD-affected cassava plants from three regions of Colombia. (A) RT-PCRs of field collected plants displaying CFSD-root symptoms (see details in Table 2) from: Amazonas (1: AMZ9, 2: AMZ16 and 3: HEL4), south/central (4: FSD23, 5: FSD29/Sec, 6: SM3375-113, 7: CMC40, 8: BIPD-289-25, 9: SM909-25, 10: CM4574-7, 11: Regional Tolima, 12: Catumare and 13: Nataima31) and northern Colombia (14: FSD80, 15: FSD86). H: healthy (COL2063), (+): CM5460-10 and RC: reaction control. (B) Map of Colombia showing the geographic distribution of viruses in each sampled region. Circles represent the three different regions where samples were collected. We confirmed the virus identity of each PCR band by sequencing.

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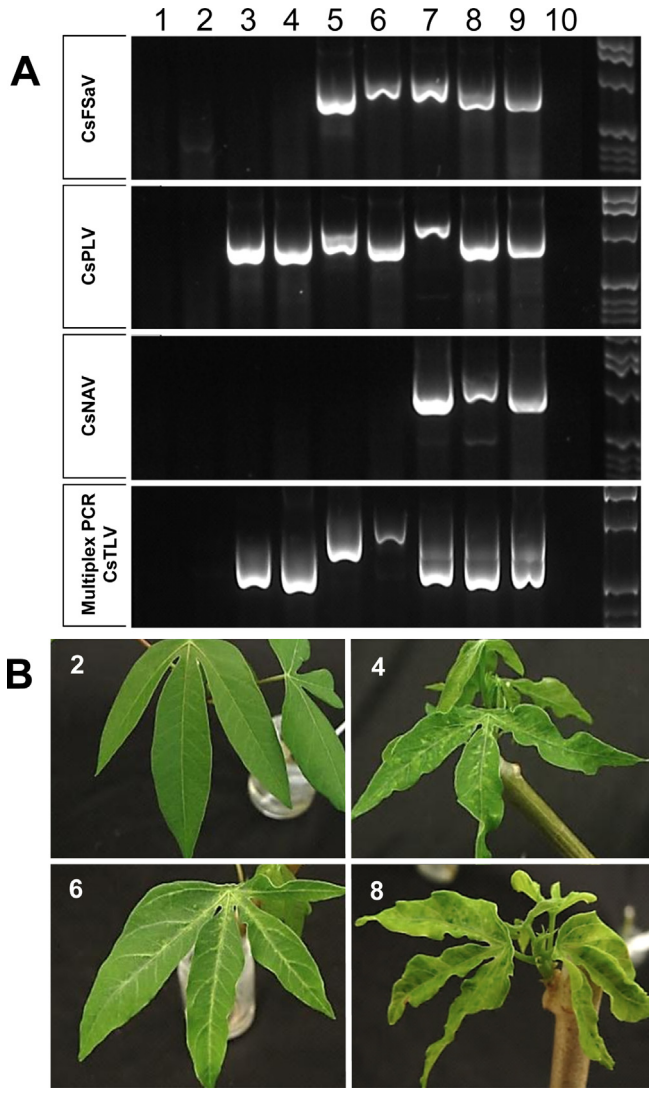

Fig. 6. A variety of LS on 'Secundina' plants graft-infected with differential mixed viral infections. (A) RT-PCR of the rootstocks (1: COL2063; 3: SM3375-113; 5: FSD86; 7:SM909-25) and the grafted plants (2: COL2063/COL2063; 4:SM3375-113/COL2063; 6: FSD86/COL2063; 8: SM909-25/COL2063) from three plants collected in the field with CFSD-root symptoms. Sample (9) corresponds to the positive control (CM5460-10) and (10) to the PCR reaction control. (B) Different LS on grafted 'Secundina' plants (2: COL2063/COL2063; 4:SM3375-113/COL2063; 6: FSD86/COL2063; 8: SM909-25/COL2063). Sample 8, infected by four viruses displayed severe LS as compared to samples infected by 2 or 3 viruses. Multiplex PCR detection of CsTLV was carried out using the primer pairs designed in this work.

four open reading frames (ORFs) were detected in an isolate from the landrace CM5460-10 (Table 2). BLASTX searches with the predicted ORF1 nt sequence showed ~51% aa sequence similarity to the replication protein of *Lily virus X* (YP263303) (Family *Alphaflexiviridae*, Genus *Potexvirus*) with a coverage of 98% (*e*-value 0.0), while ORF4 shows a ~46% aa sequence similarity to the core protein of *Pepino mosaic virus* (Genbank nr. ACI01032) (Family *Alphaflexiviridae*, Genus *Potexvirus*) with a coverage of ~89% (*e*-value 7×10^{-57}). ORF2 and OFR3 corresponded to TGB1 and TGB2 found in members of the genus *Potexvirus*. The characteristic TGB3 of the triple gene block of members of the *Alphaflexiviridae* was missing in this isolate. Phylogenetic analysis from aligned aa sequences of the replication protein of different *Alphaflexiviridae* family members clustered the previously undescribed virus within the genus *Potexvirus* and close to *Lily virus X* which also lacks an ATG for the TGB3 gene (Chen et al., 2005) (Fig. 3). Interestingly, CM5460-10 was also positive for CsVX which was mechanically transmitted to *N. benthamiana*. However primers designed for different genomic regions of CsNAV did not amplify a PCR product in *N. benthamiana* samples positive to CsVX. This indicates that landrace CM5460-10 is double infected by CsNAV and CsVX and that CsNAV did not infect *N. benthamiana* by mechanical inoculation. In addition antisera against CsCMV or CsVX did not react with other samples positive for CsNAV by RT-PCR. This indicates that CsNAV is distinct from the potexviruses CsVX and CsCMV but until its genome characterization is completed we will refer to this virus as 'Cassava new alphaflexivirus' (CsNAV).

3.4. A novel virus species related to the genus Torradovirus infecting cassava

Other virus particles reported in cassava are the isometric members of the family *Secoviridae*, CsGMV and *Cassava American latent virus* (CsALV; Genus *Nepovirus*), which have been previously characterized but not at sequence level (Lennon et al., 1987; Walter et al., 1989). We have preliminarily completed a region of 1980 nt of the RNA1 segment (GenBank: KC_505250) and 2992 nt of the RNA2 segment (GenBank: KC_505251) of a member of the *Secoviridae* family. Both sequences show no significant nt similarity to known virus species by BLASTN. However, BLASTX analysis showed a sequence similarity of CsTLV to members of the genus *Torradovirus*. RNA1 translated sequence has 34% aa similarity to *Tomato torrado virus* (ToTV) (ACB47566.1) with a 99% coverage (*e*-value of 4×10^{-102}), whereas RNA2 sequence has 32% aa similarity to *Tomato chocolate virus* (ToChV) (AC_U01026) with a 99% coverage (*e*-value of 5×10^{-157}). Phylogenetic analysis using the aa sequence of the RdRp of other members of the family *Secoviridae* and partial genome organization analysis supported BLAST results and place CsTLV in the genus *Torradovirus* (Fig. 3). We provisionally named this new virus Cassava torrado-like virus (CsTLV) but due to low sequence similarity to other members of the genus, different host and different associated symptoms, CsTLV could be considered a new species in the genus *Torradovirus*. We used several indicator plants to compare inocula of CsTLV and CsGMV, the latter a putative *Secoviridae* reported from the Solomon Islands in 1987. CsTLV inoculum was obtained from the cassava cultivar COL2063, in apparent single infection (Fig. 4A). Symptoms developed on sap-inoculated *N. benthamiana* and *N. tabacum* with CsGMV were as previously described, including wilting and systemic necrosis (Lennon et al., 1987). *N. benthamiana* and *N. tabacum* inoculated with CsTLV extracts did not develop visible symptoms (Fig. 4B). CsTLV was not detected in inoculated plants by RT-PCR tests, suggesting CsTLV could not be mechanically transmitted by sap inoculation to *Nicotiana* spp. Verbeek et al. (2012) recently reported PCR primers for the generic detection of torradoviruses. Using these primers (targeting the RNA1) we detected CsTLV in cassava plants from all collected regions and in some samples that were negative using our primers (samples 3 and 11 in Fig. 5A). Similar experiments could not be performed with CsALV due to the lack of inoculum, however unlike CsALV (Walter et al., 1989), CsTLV caused LS in 'Secundina' in the absence of the other viruses as determined in two independent assays (Fig. 4A).

3.5. Detection of mixed infections in cassava and differential symptom expression

Cassava stems have been collected in the Amazonas, the south/central and the northern regions of Colombia since 1980 from plants showing symptoms of CFSD. Those plants (Table 2) were evaluated by RT-PCR for the presence of CsPLV, CsNAV and CsTLV. We selected 15 plants collected in different regions of Colombia to check for the presence of different viruses. Of these, 11 plants, except for the plants collected in the Amazonas region,

Table 3
List of viruses reported infecting cassava in the Americas.

Name	Classification	Reference	Genbank	Antiserum
Cassava common mosaic virus (CsCMV)	*Alphaflexiviridae/Potexvirus*	Silva et al. (1963), Kitajima et al. (1965)	NC_001658	A
Cassava vein mosaic virus (CSVMV)	*Caulimoviridae/Cavemovirus*	Costa (1940)	NC_001648	NA
Cassava symptomless virus (CsSLV)	*Rhabdoviridae/Nucleorhabdovirus*	Kitajima and Costa (1979)	NA	NA
Cassava virus X (CsVX)	*Alphaflexiviridae/Potexvirus*	Lennon et al. (1986)	NA	A
Cassava Caribbean mosaic virus	*Alphaflexiviridae/Potexviru*[a]	Lennon et al. (1986)	NA	NA
Cassava Colombian symptomless virus	*Alphaflexiviridae/Potexvirus*	Lennon et al. (1986)	NA	NA
Cassava American latent virus	*Secoviridae/Nepovirus*[a]	Walter et al. (1989)	NA	NA
Cassava frogskin-associated virus (CsFSaV)	*Reoviridae/Oryzavirus*	Calvert et al. (2008), Carvajal-Yepes et al. (2014)	DQ_139870	NA
Cassava polero-like virus (CsPLV)	*Luteoviridae/Polerovirus*	This work	KC_505249	NA
Cassava torrado-like virus (CsTLV)	*Secoviridae/Torradovirus*	This work	KC_505250, KC_505151	NA
Cassava new alphaflexivirus (CsNAV)	*Alphaflexiviridae/Potexvirus*	This work	KC_505252	NA

A: available; NA: not available.
[a] Tentative member.

were positive for CsPLV (Fig. 5A). CsNAV was detected in 6 plants and was not found in the samples from the northern region of Colombia (Fig. 5A). CsTLV was detected in material collected in all regions, but using a multiplex PCR to detect RNA1 and RNA2 segments (replicase and capsid protein regions, respectively) we detected both RNAs only in samples from the south/central regions of Colombia, while in samples originating from the north and some from the south/central regions only RNA1 could be detected using these primers (Fig. 5A). These results suggest that there is sequence variability in CsTLV isolates. Expression of LS with differential mixed infections was studied using selected field-collected samples as inoculum to graft-infect the cassava cultivar 'Secundina' and then compare LS in the same cassava genotype (Fig. 6). Stem cuttings of the plants with double, triple or quadruple infections were side-grafted on 'Secundina' and transmission of viruses and symptoms was confirmed by RT-PCR and visual inspection, respectively (Fig. 6A). Plants started to develop a variety of LS such as chlorotic spots, mosaics, vein chlorosis and yellowing, leaf deformation and mottling 3–4 weeks after inoculation. The most severely affected leaves were those of plants infected by at least 3 to 4 viruses (Fig. 6B). These samples were negative for CsCMV, CsVX and CsVMV as tested by ELISA and RT-PCR except for sample CM5460-10 which was positive to CsVX by ELISA.

4. Discussion

Cassava virus studies in the Americas have focused on the properties of isolated viruses and have given little attention to virus interactions in mixed infections in terms of variety of symptoms, disease severity and effect on yield (Lennon et al., 1987; Aiton et al., 1988; Walter et al., 1989; Calvert et al., 1995). Some of these viruses have been associated with distinct LS (*e.g.*, CsCMV, CsVMV) and at least one of them to severe RS (CsFSaV). The potexviruses CsCSLV, CsALV, 'Cassava symptomless virus' (CsSLV) and CsVX (Table 3) are not associated with LS or RS (Kitajima and Costa, 1979; Lennon et al., 1986; Walter et al., 1989). However, the result of their mixed infections with heterologous viruses has not yet been investigated. It is important to investigate the effect of mixed infections because vegetatively propagated crops tend to accumulate pathogens over successive growing seasons as exemplified by sweetpotato (Clark et al., 2012). Furthermore, the number of reports on mixed virus infections has increased recently showing that they are commonly found in nature (Rentería-Canett et al., 2011; Syller, 2012). In this work we showed that mixed virus infections are also commonly found in cassava and most importantly that they are associated with distinct LS and RS.

Viruses co-infecting the same host could interact in unexpected manners including synergisms and antagonisms (Untiveros et al., 2007; Rentería-Canett et al., 2011), and understanding these interactions is of crucial significance for predicting viral pathogenesis and virus evolution and to develop efficient and stable control strategies (Read and Taylor, 2001). Most importantly, synergistic interactions can have a significant effect on the outcome of a disease with devastating results. This is because virus synergistic interactions can affect host range, enhanced cell-to-cell and long distance movement, and result in better transmissibility by insect vectors, and enhanced viral concentrations or symptoms by one or several of the co-infecting viruses (Latham and Wilson, 2008). Cassava mosaic disease in Africa and Sweet potato virus disease occurring worldwide are examples of naturally occurring synergistic virus diseases (Pita et al., 2001; MuKasa et al., 2006; Untiveros et al., 2007).

As part of the extensive effort over the last 30 years to identify the causal agent of CFSD, several viruses and other pathogens have been reported (Table 3 and Fig. 1) (Lennon et al., 1986; Calvert et al., 2008). It is noteworthy that the RS that farmers and pathologists conventionally associate to CFSD are not well defined and that they vary with the age of the plant and the affected cassava landrace (Fig. 2E–H). Of all reported viruses so far, only reoviruses have been found consistently associated with RS in CFSD-affected plants (Calvert et al., 2008); however, reoviruses are not associated with LS in single infections in the indicator plant 'Secundina' (Table 1). We therefore evaluated a collection of cassava plants field-collected since the 1980s from different regions of Colombia and affected by CFSD (Table 2) (Nolt et al., 1991, 1992; Calvert et al., 2008). Next we used available antisera, biological assays, DS and RT-PCR of these samples to check for previously characterized cassava viruses and for the presence of suspected additional viruses (Fig. 3). To validate the identity of the viral sequences obtained by DS and test infectivity we designed specific primers and transmission studies to indicator plants (Fig. 6A-B). Although we cannot rule out the presence of additional viruses that may have escaped detection by DS, electron microscopy examination, molecular tests and biological assays showed no evidence of additional virus particles or disease symptoms in indicator plants (*not shown*).

Viruses in the family *Luteoviridae* have spherical particles; they are phloem-limited and transmitted by aphids (King et al., 2012). Although virus transmission in cassava has focused on whiteflies, it is interesting to point out that during whitefly-transmission studies Angel et al. (1989) noted the presence of aphids in proportions ranging from 1 to 50% in relation to whiteflies and other insects in several cassava fields of Colombia. Therefore it is likely that poleroviruses are transmitted to cassava. Further studies are needed to confirm the identity of this virus, identify its insect vector(s) and assess virus-vector-plant relationships.

The alphaflexivird CsNAV lacks a TGB3 gene; a gene with a role in virus movement through the plasmodesmata (Tilsner et al., 2013), and although the related *Lily virus X* lacks a TGB3 ATG codon

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(Chen et al., 2005), further characterization of additional CsNAV isolates needs to be done to fully determine its genome organization. Sequencing of CsVX will also be needed to confirm that CsNAV and CsVX are distinct potexviruses as the serological and RT-PCR tests suggest.

CsTLV would be the third virus in the family *Secoviridae* reported to infect cassava together with CsGMV from the Solomon Islands and CsALV reported in Guyana and Brazil (Lennon et al., 1987; Walter et al., 1989). The variability in the detection of CsTLV RNA1 and RNA2 (Fig. 5B) suggests the presence of different strains, mixed infections and possibly reassortment, therefore additional isolates need to be sequence-characterized in order to exclude false negatives during virus indexing. It is interesting that CsTLV could cause symptoms in single-infection in 'Secundina' and that it belongs to a genus (*Torradovirus*) whose members can be transmitted by whiteflies. In a previous work at least one species of whiteflies (*Aleurotrachelus socialis* Bondar) has been associated with transmission of LS from CFSD-affected plants to 'Secundina' (Angel et al., 1987, 1989); therefore it will be interesting to investigate potential whitefly transmission of CsTLV and its associated LS (Fig. 4A) and/or RS.

Mixed infections in cassava were not completely unexpected. When we reviewed electron microscopy and biological data collected over the years at CIAT we could identify different viral-like particles in symptomatic cassava plants (Fig. 1). This was important because previous studies performed by Nolt et al. (1992) suggested that the agent graft-transmitted from plants infected with CFSD and 'Caribbean mosaic' was the same. Nevertheless, microscopy studies performed in the 1980s showed the presence of elongated particles (*data not shown*) and two kinds of spherical virus-like particles (~25 nm and ~70 nm) (Fig. 1C-D) in plants affected by CFSD and 'Caribbean mosaic'. Virus particles of members of the families *Secoviridae* and *Luteoviridae* range from 25 to 30 nm, while the *Reoviridae* have a particle size of about 60–80 nm in diameter (King et al., 2012). Spherical particles observed in the 1980s could be related to CsPLV and/or CsTLV in co-infection with CsFSaV, thus the viruses here described may have been infecting cassava crops for a long time contributing to yield reductions that at the time were associated with apparent single virus infections (Calvert et al., 2012). It is also possible that a correct identification of the causal agent of CFSD has been masked by mixed infections mainly because LS in 'Secundina' were used as a diagnostic tool for the disease. This also would explain why over 30 years of research different pathogens have been associated with CFSD.

Our results with differential viral infections in 'Secundina' revealed a variety of LS (Fig. 6B). Interestingly these were similar to those observed in association with previously characterized viruses which have not been detected in these samples. For example CsCMV causes dark and light green patches delimited by veins (Costa and Kitajima, 1972) (Fig. 1D). CsVMV is associated with vein chlorosis that coalesces to form ringspots and in severe cases, leaf deformation (Calvert and Thresh, 2002). And although CsVX is a putative potexvirus that does not causes symptoms in most cassava landraces, it also has been reported in plants affected by CFSD (Walter et al., 1989). A more precise description of LS and RS associated with specific virus(es) and their correct characterization and detection will contribute to improve virus indexing, breeding for resistance, control of specific insect vectors, disease transmission and a better understanding of the epidemiology of the disease(s). Based on the results presented here, we hypothesize that differential mixed virus infections could also explain the diversity of RS observed in CFSD-affected plants (Fig. 2). As a priority we are studying the role of these viruses and their interactions in the development of roots symptoms in CFSD, a disease that in the light of these new results should be better defined.

Acknowledgments

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