Eur J Plant Pathol (2011) 131:9–14
DOI 10.1007/s10658-011-9797-z

Molecular and biological characterization of *Chrysanthemum stem necrosis virus* isolates from distinct regions in Japan

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Accepted: 18 April 2011 / Published online: 8 May 2011

Abstract Three isolates of *Chrysanthemum stem necrosis virus* (CSNV) were obtained from chrysanthemum plants in distinct regions of Japan in 2006 and 2007. All the original host plants showed severe necrotic symptoms on the leaves and stems. Amino acid sequence data of the nucleocapsid protein genes of the three isolates (CbCh07A, TcCh07A, and GnCh07S) showed high identities with those of two other CSNV isolates, HiCh06A L1 from Japan and Chry1 from Brazil. Furthermore, for the first time the complete nucleotide sequence of the S RNA was determined for CSNV (isolate HiCh06A). In phylogenetic analysis based on the non-structural protein genes from the genus *Tospovirus*, HiCh06A L1 was placed in the same genetic group as *Tomato spotted wilt virus* (TSWV) and *Impatiens necrotic spot virus*. Host range examination for isolates HiCh06A L1 and CbCh07A showed that green pepper (cv. 'Kyoyutaka', 'Saitamawase', 'Tosakatsura', 'L3 sarara' and 'L3 miogi') and tomato (cv. 'Sekaiichitomato') were systemically susceptible hosts, whereas TSWV-resistant *Solanaceae* species, *Capsicum chinense*, *Lycopersicon peruvianum* and a TSWV-resistant cultivar of green pepper (cv. TSR miogi), were resistant.

Keywords Tospovirus · Thrips · N gene · NSs gene

Tospoviruses, which belong to the family *Bunyaviridae*, cause economically serious damage in many agricultural crops. The viruses consist of eight assigned and six tentative species (Nichol et al. 2005), and are transmitted by thrips species in a circulative-propagative manner. Tospovirus species have a tripartite single-stranded RNA genome which contains negative sense L RNA, ambisense M RNA and S RNA (German et al. 1992).

The tospovirus *Chrysanthemum stem necrosis virus* (CSNV) was first reported in Brazil as a pathogen which induces stem necrosis, necrotic spots and rings on the leaves of tomato and chrysanthemum (*Dendranthema grandiflorum*) (Duarte et al. 1993; Duarte et al. 1995; Nagata et al. 1995; Nagata et al. 1998). Subsequently, occurrences of the CSNV disease were reported in the

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Netherlands (Verhoeven et al. 1996), the United Kingdom (Mumford et al. 2003), Slovenia (Ravnikar et al. 2003), and Japan (Matsuura et al. 2007). Currently, CSNV is considered as a worldwide-emerging virus. Nagata et al. (2007) first reported the complete nucleotide sequence of the M RNA of CSNV, although neither the entire L RNA nor S RNA sequences have been reported. *Frankliniella occidentalis* and *F. schultzei* have been identified as vector species of CSNV (Nagata and De Ávila 2000).

Chrysanthemum is one of the most economically important cut flowers in Japan. In 2006, severe necrotic symptoms were observed on the leaves and stems of chrysanthemum in greenhouses in Hiroshima Prefecture (Pref.) in the west of Japan. Subsequently, Matsuura et al. (2007) identified the causal agent as an isolate of CSNV named as HiCh06A L1. In only a few years, CSNV infections were found in open fields of three distinct regions in Japan. Single lesion isolates of CSNV were obtained from the three regions, CbCh07A from Chiba Pref., TcCh07A from Tochigi Pref., and GnCh07S from Gunma Pref.

The aim of this study was accumulation of molecular and biological information of CSNV, which could be helpful to understand infection strategies of the virus and to trace virus spread around the globe. Initially, nucleotide sequences of the nucleocapsid protein (N) gene of the three CSNV isolates were determined to analyze their genetic relations. We then sequenced the entire CSNV S RNA derived from HiCh06A L1. Furthermore, the host range of CSNV was studied after mechanical inoculation of HiCh06A L1 and CbCh07.

Initial molecular characterization of the three isolates was carried out on the open reading frame (ORF) of the N gene. The CSNV isolates were maintained in an air-conditioned greenhouse at 25°C/20°C (day/night) through mechanical inoculation onto *Datura stramonium*. Total RNA was extracted from *D. stramonium* systemically infected with each of CbCh07A, TcCh07A, and GnCh07S using an ISOGEN kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Oligonucleotide primers, CSNV-N5 (5′-GAGCGACTGCGGAATACTCT-3′) and CSNV-N3 (5′-GACACACTTTAAATCTTTAACA CACC-3′), were designed to amplify the N gene ORF on the basis of the nucleotide information from isolate Chry1 (GenBank Acc. No. AF067068; Bezerra et al. 1999). Reverse transcription (RT)-PCR using One-Step RT-PCR Kit (Invitrogen) and the N gene-specific primers was performed by RT at 53°C for 30 min before inactivation of reverse transcriptase at 94°C for 2 min, and by subsequent thermocycling reactions consisting of the first step for 40 cycles (94°C for 15 s, 53°C for 30 s, and 68°C for 1 min) followed by the second step (68°C for 5 min). The N gene-targeting RT-PCR successfully amplified an approximately 950-bp fragment which covers the entire ORF of the N genes of the three CSNV isolates. No amplification was observed for plants infected by *Tomato spotted wilt virus* (TSWV) and *Impatiens necrotic spot virus* (INSV) (data not shown). The amplified DNA fragments were cloned into pCR®2.1-TOPO® vector of TOPO TA Cloning® Kit (Invitrogen). Sequence comparison and pair-wise alignment were performed utilizing Geneious Pro (Biomatters, Auckland, New Zealand).

By complete sequencing, the N genes of the CSNV isolates were each shown to be 780 nucleotides long. The amino acid sequence data of the N genes showed pair-wise identities between 97.7% and 98.5% with that of CSNV Chry1 from Brazil (Acc. No. AF067068) and 98.1% to 99.2% with HiCh06A L1 (Acc. No. AB438998) also from Japan. By contrast, they showed the second highest pair-wise identities between 78.4% and 79.9% with that of *Zucchini lethal chlorosis virus* (Acc. No. AF067069). The high identities of the five CSNV isolates, therefore, suggested a common origin. Furthermore, the N genes of all the three isolates contained the conserved regions 1, 2, and 3 which are observed commonly in tospoviruses (Okuda and Hanada 2001). The sequences of the N genes have been submitted to the DDBJ data bases (Accession No. AB600870 for CbCh07A, AB600871 for GnCh07S, and AB600872 for TcCh07A).

The ambisense S RNA of TSWV encodes a non-structural protein (NSs) gene in the sense and the nucleocapsid protein designated as the N gene in the minus sense (De Haan et al. 1990). The NSs protein has been identified as an RNA silencing suppressor (RSS) which acts during plant infection (Takeda et al. 2002; Bucher et al. 2003). For better taxonomic characterization of CSNV, the complete nucleotide sequence of S RNA of HiCh06A L1 was determined using 5′/3′ RACE Kit, 2nd Generation (Roche) and oligonucleotide primers designed for

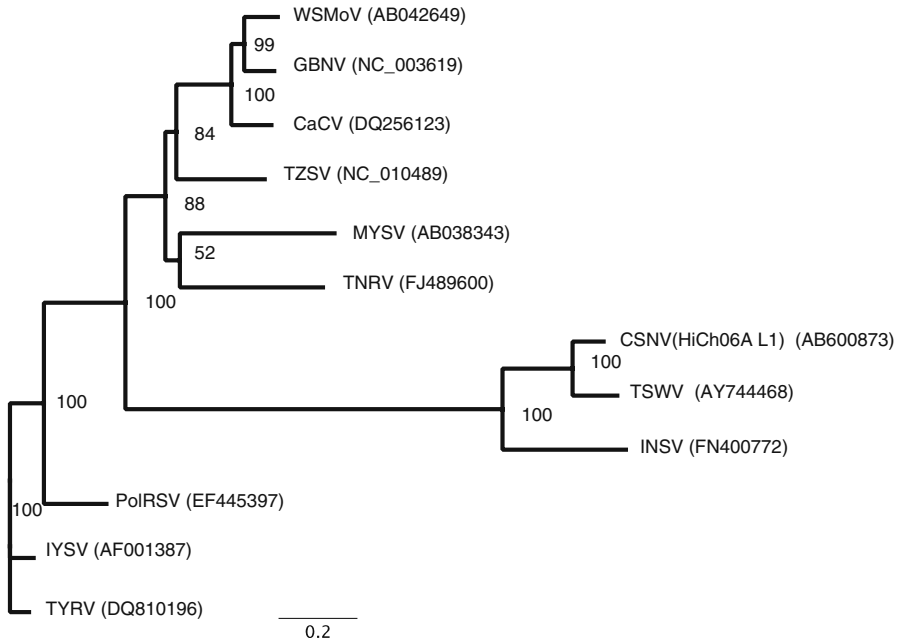

Fig. 1 Phylogenetic analysis of CSNV (HiCh06A L1) and members of the genus *Tospovirus* based on the alignment of the amino acid sequence of the NSs gene. Bootstrap values are indicated on the nodes. The bar represents a *p*-distance of 0.2. Sequences included in this analysis are as follows (with virus acronyms in parentheses): *Groundnut bud necrosis virus* (GBNV), *Watermelon silver mottle virus* (WSMoV), *Capsicum chlorosis virus* (CaCV), *Tomato zonate spot virus* (TZSV), *Melon yellow spot virus* (MYSV), *Tomato necrotic ringspot virus* (TNRV), *Tomato spotted wilt virus* (TSWV-CA1), *Impatiens necrotic spot virus* (INSV), *Poligonum ringspot tospovirus* (PolRSV), *Iris yellow spot virus* (IYSV), *Tomato yellow ring virus* (TYRV). GenBank accession numbers of the sequences used in this phylogenetic analysis are shown in parentheses after virus names

amplification of 5′ and 3′ cDNA ends. Based on the determined sequence information in the process of 5′/3′ RACE, S RNA-specific oligonucleotide primers were newly synthesized to extend the S RNA sequence by sequence-walking. The nucleotide sequence data revealed that the S RNA of CSNV was 2,940 nucleotides, and that the NSs gene contained 1,392 nucleotides (464 amino acids). The nucleotide and the deduced amino acid sequence of the NSs genes showed 73% and 77% identities to those of TSWV (Acc. No. NC_002051). The 5′- and 3 ′-untranslated regions (UTR) of S RNA are 79 and 152 nucleotides long, respectively. Moreover, the 3′-UTR of S RNA showed 98% sequence similarity with the corresponding regions of Chry1. The intergenic region between the NSs gene and the N gene of S RNA is 532 nucleotides in length. A phylogenetic tree based on the neighbour-joining method using Clustal W (version 1.8) (Thompson et al. 1997) was executed under the control of the Geneious Pro program. All calculation parameters were set as default values. In phylogenetic analysis, performed on the NSs genes from the genus *Tospovirus*, the NSs gene of HiCh06A L1 clustered in the same genetic group as TSWV and INSV (Fig. 1). Interestingly, the clustering of CSNV with TSWV and INSV was similar to that based on the N genes of tospoviruses (Hassani-Mehraban et al. 2010). The phylogenetic inference also was supported by the maximum likelihood method using PHYML (Guindon and Gascuel 2003) (data not shown). The complete nucleotide sequence of the S RNA of HiCh06A L1 has been submitted to the DDBJ database (Accession No. AB600873).

To investigate an experimental host range of the CSNV isolates found in Japan, HiCh06A L1 and TcCh07A were mechanically inoculated onto plant species listed in Table 1 using extracts from leaf tissues of systemically infected *D. stramonium*. The extracts were prepared in 10 mM sodium

Table 1 Host range experimentally determined for CSNV

Host		HiCh06A L1				TcCh07A			
		Local		Systemic		Local		Systemic	
Brassicaceae	*Brassica rapa var. glabra*	…	−	…	−	…	−	…	−
Chenopodiaceae	*Spinacia oleracea L.*	CS[a)]	+[b)]	…	−	CS	+	…	−
Asteraceae	*Achillea fillipendulina*	…	−	…	−	…	−	…	−
	Calendula officinalis	…	−	…	−	…	−	…	−
	Carthamus tinctorius	…	−	…	−	…	−	…	−
	Centaurea suaveolens	…	−	…	−	…	−	…	−
	Coleostephus myconis	…	−	…	−	…	−	…	−
	Coreopsis grandiflora ST. NO. 307986	…	−	…	−	…	−	…	−
	Dendranthema grandiflorum	…	−	…	−	…	−	…	−
	Dorotheanthus bellidiformis	…	−	…	−	…	−	…	−
	Felicia heterophylla	…	−	…	−	…	−	…	−
	Gazania splendens cv. Tarentmix	…	−	…	−	…	−	…	−
	Gazania splendens cv. F1 Daybreak red stripe	…	−	…	−	…	−	…	−
	Helianthus annus cv. Pinotiogold	NS (1/4)/…(3/4) [c)]	+/−	…	−	…	−	…	−
	Helianthus annus cv. F1 sunrich fresh orange	…	−	…	−	…	−	…	−
	Helichrysum bracteatum	…	−	…	−	…	−	…	−
	Leucanthemum paludosum	…	−	…	−	…	−	…	−
	Melampodium paludosum	…	−	…	−	…	−	…	−
	Senecio cruenta	…	−	…	−	…	−	…	−
	Tagetes tenuifolia	…	−	…	−	…	−	…	−
	Tanacetum parthenium	…	−	…	−	…	−	…	−
	Zinnia elegans cv. F1 swizzle cherry and ivory	NS (1/3)/…(2/3)	+/−	…	−	NS (1/3)/…(2/3)	+/−	…	−
Cucurbitaceae	*Lagenaria var.gporda*	…	−	…	−	…	−	…	−
Fabacaea	*Vigna unguiculata*	CS	+	…	−	CS	+	…	−
Solanaceae	*Datura stramonium*	LA M NS NR	+	M VC	+	LA M NS NR	+	M VC	+
	Capsicum annuum cv. TSR miogi	NS	+	…	−	NS	+	…	−
	Capsicum annuum cv. Kyoyutaka	NS NR	+	Cr VC	+	NS NR	+	Cr VC	+
	Capsicum annuum cv. Saitamawase	LA Cr CS NS	+	Cr CS	+	LA Cr CS NS	+	Cr CS	+
	Capsicum annuum cv. Tosakatsura	Cr CS NS	+	Cr	+	Cr CS NS	+	Cr	+
	Capsicum annuum cv. L3 sarara	Cr CS NS	+	Cr	+	Cr CS NS	+	Cr	+

	Cr CS NS	+	Cr CS NS	+	Cr CS NS	+	Cr CS NS	+
Capsicum annuum cv. L3 miogi	LA NS	+	…	–	LA NS	+	…	–
Capsicum chinense PI152225	LA NS	+	…	–	LA NS	+	…	–
Capsicum chinense PI159236	LA NS	+	…	–	LA NS	+	…	–
Lycopersicon peruvianum LA111 76L	LA NS	+	…	–	LA NS	+	…	–
Lycopersicon peruvianum PI126944	LA NS	+	…	–	LA NS	+	…	–
Nicotiana benthamiana	CS	+	VC M	+	CS	+	VC M	+
Petunia hybrida	NS[d]	+	…	–	NS	+	…	–
Solanum lycopersicum cv. Sekaiichitomato	CS NS	+	Cr CS NS	+	CS NS	+	Cr CS NS	+
Solanum melongena L.	CS	+	…	–	CS	+	…	–

a) Symptoms *Cr* crinkle; *CS* chlorotic spot; *LA* leaf abscission; *M* mosaic; *NR* necrotic rings; *NS* necrotic spots; *VC* veinal clearing; …: no symptoms
b) RT-PCR + : positive, –: negative c) Number of plants displaying symptoms over number of inoculated plants d) Matsuura et al. (2007)
Four replicates were tested for each host plant except for *Helianthus annus* cv. Pinotiogold and *Zinnia elegans* cv. F1 swizzle cherry and ivory

phosphate buffer, pH 7.0, containing 10 mM sodium diethyldithiocarbamate. The susceptibility of the plants was evaluated by monitoring the symptoms on both inoculated and upper leaves at 2 weeks post inoculation. The plants were grown in an air-conditioned greenhouse at 25°C/20°C (day/night). Local and systemic infections were checked by the N gene specific RT-PCR described above. *Nicotiana benthamiana*, and *Solanum lycopersicon* were systemically infected by both CSNV isolates. Although *Capsicum annuum* cultivars 'Kyoyutaka', 'Saitama-wase', 'Tosakatsura', 'L3 Sarara', and 'L3 Miogi' were infected systemically, TSWV-resistant cultivar 'TSR miogi' was not and only produced local lesions. Similarly as reported by Bezerra et al. (1999), CSNV infections were confined to necrotic local lesions on the inoculated leaves of *Capsicum chinense* PI159236. Furthermore, other TSWV-resistant sources, *C. chinense* PI152225 (Boiteux 1995), *Lycopersicon peruvianum* LA11176L, and *L. peruvianum* PI126944 (Rosello et al. 1999) only displayed necrotic local lesions. No latent infections were detected by RT-PCR in plant parts without symptoms. The reactions of both CSNV isolates were nearly identical and are not inconsistent with those reported by Bezerra et al. (1999). The results suggest that the resistance reported for TSWV also applied for CSNV.

To our knowledge, this study is the first report of the complete nucleotide sequence of S RNA of CSNV. In conclusion, the genetic information will be helpful to analyze the host plant-CSNV interactions, because the NSs gene coded in S RNA is thought to be an RNA silencing suppressor which is indispensable for extensive infection of the virus in host plants. Also, the application of the resistant cultivars or sources on the basis of the results obtained from our and other research groups will contribute to development of optimal control strategies for CSNV.

Acknowledgement Authors would like to thank Mr. Shinichiro Okazaki, Oita Prefectural Agriculture, Forestry and Fisheries Research Center, for providing *F. occidentalis* and for the helpful suggestions with regard to the transmission tests, and Mr. Osamu Nunomura, Japan Horticultural Production and Research Institute, Division of extension for production, for providing 'TSR miogi'. Germplasm of *Capsicum chinense* and *Lycopersicon peruvianum* was provided by National Institute of Agrobiological Sciences, Japan.

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