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Biological and Molecular Characterization of a Korean Isolate of *Orthotospovirus chrysanthinecrocaulis* (Formerly Chrysanthemum Stem Necrosis Virus) Isolated from *Chrysanthemum morifolium*

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Biological and molecular characterization of a Korean isolate of *Orthotospovirus chrysanthinecrocaulis* (formerly known as chrysanthemum stem necrosis virus, CSNV) isolated from *Chrysanthemum morifolium* was determined using host range and sequence analysis in this study. Twenty-three species of indicator plants inoculated mechanically CSNV-Kr was investigated for determination of host range. CSNV-Kr induced various local and systemic symptoms in the inoculated plant species. CSNV-Kr could not infect three plant species and induced symptomless in systemic leaves in *Nicotiana tabacum* cultivars, though the plant samples reacted positively with the antiserum to CSNV by double-antibody sandwich–enzyme-linked immunosorbent assay. The complete genome sequence of CSNV-Kr was determined. The L RNA of CSNV-Kr consists of 8,959 nucleotides (nt) and encodes a putative RNA-dependent RNA polymerase. The M RNA of CSNV-Kr consists of 4,835 nt and encodes the movement protein (NSm) and the glycoprotein precursor (Gn/Gc protein). The S RNA of CNSV-Kr consists of 2,836 nt and encodes NSs protein and N protein. The Gn/Gc and N sequence of CSNV-Kr were compared with those of previously published CSNV isolates originating from different countries at nucleotide and amino acid levels. The Gn/GC sequence of CSNV-Kr shared 98.8–99.5% identity with CSNV isolated from other countries and the N sequence of CSNV-Kr shared 98.8–99.6% identity. No particular region of variability could be found in either grouping of viruses. All of the CSNV isolates did not show any relationship according to geographical origins and isolation hosts, suggesting no distinct segregation of the CSNV isolates.

Keywords: CSNV, Host range, Phylogenetic tree, Sequence, Symptom

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Introduction

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is one of the most important ornamental cut flower crops in the world. Chrysanthemum cultivars are grown for cult flowers, as potted flowering plants, or garden plants (Anderson, 2006; Yoon et al., 2020b). Cultivation area of chrysanthemum is 309.1 ha in South Korea (Ministry of Agriculture, Food and Rural Affairs, 2020) and chrysanthemum can be divided into two types (called standard-type and spray-type) depending on flower size. To date, 13 viruses and two viroids have been reported to infect chrysanthemum cultivars (Trolinger et al., 2018; Yoon et al., 2020b). *Orthotospovirus chrysanthinecrocaulis* is a member of the genus *Orthotospovirus* in the family *Tospoviridae* which includes 26 species and two tentative species (Koonin et al., 2019). Formerly chrysanthe-

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mum stem necrosis orthotospovirus (CSNV) was renamed to *O. chrysanthinecrocaulis* to binomials as required by the recently amended the International Code of Virus Classification and Nomenclature (ICVCN) (Adkins et al., 2022).

CSNV was first reported on chrysanthemum (*Dendranthema grandiflorum*) in Brazil (Duarte et al., 1995). Later, chrysanthemum plants were naturally infected with CSNV in the Netherlands (Verhoeven et al., 1996), the United Kingdom (Mumford et al., 2003), Slovenia (Boben et al., 2007; Ravnikar et al., 2003), Japan (Matsuura et al., 2007), Iran (Jafarpour et al., 2010), Belgium (De Jonghe et al., 2013), South Korea (Yoon et al., 2017), and Italy (EPPO Datasheet, 2020). CSNV was also detected from tomato (*Solanum lycopersicum*) plants in Brazil (Nagata et al., 1998) and in Japan (Kuwabara and Sakai 2008), from aster (*Callistephus chinensis*) and Russell prairie gentian (*Eustoma grandiflorum*) plants in Japan (Momonoi et al., 2011), and from Russell prairie gentian plants in Brazil (Duarte et al., 2014).

At least, two thrips species (the family *Thripidae*) have been reported to be associated with CSNV transmission (Nagata and de Ávila, 2000; Okuda et al., 2013), and the transmission of CSNV occur in a persistent and propagative manner similar to other orthotospoviruses (Maris et al., 2004; Nagata and De Ávila, 2000; Okuda et al., 2013). In particular, western flower thrips (*Fankliniella occidentalis* Pergande) and common blossom thrips (*F. schultzei* Trybom) but not *F. intosa*, *T. tabaci,* or *T. palmi* are reportedly the most efficient vectors (Chung et al., 2006; Nagata and De Ávila, 2000; Ogada et al., 2016; Okuda et al., 2013).

The genome of CSNV consists of three single-stranded RNAs. The negative-sense large RNA (L RNA) segment encodes the RNA-dependent RNA polymerase (RdRp) required for viral RNA replication and mRNA transcription (Adkins et al., 1995; de Haan et al., 1991). The ambi-sense M RNA segment encodes a cell-to-cell movement protein (NSm) and a glycoprotein precursor of Gn and Gc (Kormelink et al., 1992). The glycoproteins (Gn and Gc) are required for particle maturation and are present as spikes on the surface of the virus envelope membrane (Kikkert et al., 1999; Ribeiro et al., 2008). Gn and Gc also play a major role as determinants for thrips vector transmission (Sin et al., 2005). The ambi-sense S RNA segment encodes a nonstructural protein (NSs) that acts as an RNA-silencing suppressor against the plant innate immunity system (Bucher et al., 2003; Schnettler et al., 2010; Takeda et al., 2002). Also, The S RNA segment encodes a nucleocapsid protein (N) required for the formation of rinonucleoprotein complexes (Guo et al., 2017; Komoda et al., 2017; Li et al., 2015) and viral intracellular movement (Feng et al., 2013; Ribeiro et al., 2013). So far, only a limited number of complete genomic sequences of CSNV isolates are available in the NCBI database, all of which are from isolates originating in Japan (Dullemans et al., 2015; Takeshita et al., 2011), Slovenia (Pecman et al., 2017), or South Korea (in this study). The aim of this study was to report genomic and biological information of a Korean isolate of CSNV to understand spread of CSNV isolates worldwide, host range by mechanical inoculation and genetic relationship between the Korean isolate and other isolates.

Materials and Methods

Virus source and host range test. A Korean isolate of CSNV (CSNV-Kr) was identified from chrysanthemum (*Chrysanthemum morifolium* cv. Jinba) in 2013 (Yoon et al., 2017). A leaf of a chrysanthemum plant infected with CSNV-Kr was ground in 10 mM potassium phosphate buffer (pH 7.0), which used as a inoculum. Then, six *Chenopodium quinoa* plants were mechanically inoculated with the inoculum of CSNV-Kr. After two successive transfers from a single local legion in the leaf of C. quinoa, CSNV-Kr was obtained from leaves of systemically infected *Nicotiana benthamiana* plants. The inoculum of CSNV-Kr was prepared from systemically infected *N. benthamiana* for all experiments in this study. The host range of CSNV-Kr was determined by mechanical inoculation onto several plant species using extracts from systemically infected *N. benthamiana* plants in 10 mM potassium phosphate buffer (pH 7.0). The symptoms were checked until 28 days post-inoculation. The inoculated plant species were checked for infection of CSNV-Kr by double-antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA) using an antiserum specific to CSNV (DSMZ, Braunschweig, Germany). Briefly, 0.2 g of each leaf sample was homogenized with 800 µl of extraction buffer solution (50 mM sodium phosphate containing 20 mM sodium sulfite) in a 2-ml sterile microcentrifuge tube. The homogenate was centrifuged at 14,000 rpm for 2 min, and the lysate was used for DAS-ELISA. Each sample was processed in duplicate. Each DAS-ELISA plate contained two CSNV-infected, two healthy, and two buffer controls. Plates were read with an automated plate reader (Titertek, Huntsville, AL, USA) at 405

nm. A sample was considered positive if the optical density (OD_{405}) was greater than three times the mean of the healthy controls (Yoon et al., 2011, 2020b).

RNA extraction and high-throughput sequencing analysis. Total RNA was extracted from leaf samples using an RNeasy Plant Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Briefly, 0.1 g of leaf tissue of *N. benthamiana* was ground with lysis buffer from the kit in a 1.5-ml microcentrifuge tube using a bead beater. Subsequently, a contaminated DNA in the eluted total RNA solution was removed from the samples by on-column DNase digestion with the RNase-Free DNase Set (Qiagen) according to the manufacturer's protocol. RNA was eluted from the columns with 50 µl nuclease-free water, and the concentration was measured using NanoDrop a Quant-iT RiboGreen RNA assay kit according to the manufacturer's instructions (Qiagen). We generated the RNA-seq libraries using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Each library was paired-end (100 bp×2) sequenced by Illumina's HiSeq 2000 system (Macrogen, Daejeon, Korea). All raw sequence data were *de novo* assembled by the Trinity program with default parameters as described previously (Jo et al., 2020). The obtained contigs were subjected to BLASTX search with E-value $1e^{-10}$ as a cutoff against the plant viral database derived from the NCBI. The 5′/3′-terminal sequence of CSNV-Kr was amplified using SMARTer RACE 5′/3′ kit (TaKaRa Bio., Shiga, Japan) according to the manufacturer's instructions. The 5′-terminal sequence was amplified by reverse transcription polymerase chain reaction (RT-PCR) using a generic primer (J13) for the sequence ends of tospoviruses (Cortez et al., 2001) and an internal specific primer. The cDNA synthesis was conducted using M-MLV reverse transcriptase according to the manufacturer's instructions (ThermoScientific, Waltham, MA, USA). PCR was carried out using Platinum *Taq* DNA Polymerase High Fidelity (ThermoScientific) under the thermal cycling conditions as follows: pre-denaturation at 94℃ for 2 min, 35 cycles of 94℃ for 30 sec, 55-60℃ for 30 sec, and 72℃ for 1 min, and a final extension of 72℃ for 10 min. The amplicons that were obtained were sequenced directly by the Sanger method. The assembled complete genome sequences of CSNV-Kr have been deposited to GenBank (NCBI accession nos. LC126116, LC126117, and LC126118).

Phylogenetic tree analysis. Analysis of the nucleotide (nt) and deduced amino acid (aa) sequences were done using BLAST search and DNASTAR Lasergene Genomics Suite software (ThermoScientific). All CSNV-associated contigs were selected using the BLASTX results. Of the CSNV-associated contigs, we selected viral contigs with sizes greater than 1,000 bp to identify CSNV sequences that covered all open reading frames (ORFs) using the NCBI ORF-finder. For phylogenetic analysis of CSNV, partial or complete RNA sequences for reported CSNV isolates were obtained from NCBI (Table 1). Nt sequences were aligned using CLUSTAL W implemented in MEGA X followed by manual modification (Kumar et al., 2018). For comparison, the 12 CSNV N gene sequences available from the GenBank database were added to our data set. The sequences from the database that were redundant, or smaller than the size of full-length CSNV N gene or the size of full-length CSNV Gn-GC gene, were omitted. Aligned nt sequences or deduced aa sequences were used to construct phylogenetic trees using the MEGA X software package (Kumar et al., 2018). Full-length sequences of CSNV RNAs L, M, and S segments were aligned and manually adjusted using CLUSTAL W (Kumar et al., 2018). Phylogenetic trees were constructed based on the neighbor-joining (NJ) method and Maximum likelihood method. Bootstrap resampling (1,000 replications) was used to measure the reliability of individual nodes in each phylogenetic tree.

Results and Discussion

The virus isolate CSNV-Kr was isolated from symptomatic chrysanthemum plants in Changwon, South Korea and showed typical pathological properties similar to previously reported CSNV isolates (Yoon et al., 2017). CSNV-Kr was propagated in *N. benthamiana* plants after two single local transfers on *Chenopodium quinoa* as described previously (Yoon et al., 2017). To determine host range of CSNV-Kr, 23 species of indicator plants were inoculated mechanically with CSNV-Kr. Host range and symptoms of CSNV-Kr were summarized in Table 2. Symptomatic plants reacted positively with an antiserum to CSNV by DAS-ELISA. Some cultivars of *N. tabacum* showed symptomless in systemic leaves, but the plant samples reacted positively with the antiserum to CSNV by DAS-ELISA (Table 1). Although the difference of induction time and severity of symptoms was observed in a few indicator plant species, symptomatology of CSNV-

Table 1. List of CSNV isolates for multiple alignment and phylogenetic tree analysis in this study

Isolate name	Isolation host	Country	Segment	Accession no.	Length (nt)
CSNV-Kr	*Chrysanthemum* x *morifolium* 'Jinba'	Korea	L	LC126116	8,959
			M	LC126117	4,835
			S	LC126118	2,936
PD4412741	*Chrysanthemum* x *morifolium*	Japan	L	NC_027718	8,955
			M	NC_027720	4,830
			S	NC_027719	2,947
TcCh07A	*Chrysanthemum*	Japan	L	KF493773	8,960
			M	KF493772	4,828
			S	KF493771	2,936
HiCH06A L1	*Chrysanthemum*	Japan	S	AB438998	940
HiCh06A	*Chrysanthemum*	Japan	S	AB600873	2,940
CbCh07A	*Chrysanthemum*	Japan	S	AB600870	783
GnCh07S	*Chrysanthemum*	Japan	S	AB600871	783
Ca-03	*Aster*	Japan	S	AF067068	1,053
Eu-03	Russell prairie gentian	Japan	S	AB597291	940
China	*Chrysanthemum*	China	S	JQ764839	845
ChrysLO_FVR_ILVO2012	*Chrysanthemum*	Belgium	S	KC525102	937
NIB-V38	*Chrysanthemum*	Slovenia	L	MF093683	8,959
			M	MF093684	4,829
			S	MF093685	2,948
DSMZ PV-0529	*Chrysanthemum*	Germany	L	MW051791	8,915
			M	MW051792	4,830
			S	MW051793	2,950
Chry-1	*Chrysanthemum*	Brazil	M	AB274026	4,088

CSNV, chrysanthemum stem necrosis virus.

Kr is similar to other previously reported CSNV isolates from chrysanthemum (Bezerra et al., 1999; Matsuura et al., 2007; Momonoi et al., 2011). It is noteworthy that CSNV-Kr induced systemically chlorotic spots and mosaic symptoms in *Capsicum annuum* and chlorotic lesions, mosaic and top necrosis in *Solanum lycopersicum* (Table 2). It is possible that CSNV is transmitted by thrips to chili peppers and tomatoes, so the virus is likely to become one of potential threat in chili pepper and tomato farms. In addition, CSNV-Kr induced necrotic local lesions on the inoculated leaves of *C. amaranticolor* and *C. quinoa,* so these plant species could be useful for single isolation of CSMV isolates. These results suggest that CSNV-Kr has pathological properties similar to those of other previously reported CSNV isolates.

Symptoms in plant species are the effects of plant viruses on growth and development of plants, indicating a disturbance in the normal courses of physiological processes or genetic disorders (Bos, 1976; Hull, 2014). Development of systemic symptoms in plants depends on titer of viruses, mode of transmission, infection timing, crop cultivars, age and growth conditions of plants, watering, soil conditions including types of nutrients and environment including temperature and relative humidity, so called plant-virus-environment interactions (Hull, 2014). The symptom expression can

Table 2. Reaction of indicator plant species inoculated mechanically with CSNV-Kr

Plant species	CSNV-Kr	
	Symptoms	ELISA
Capsicum annuum	NR/CS, M[a]	+/+[b]
Chenopodium amaranticola	CS, NS/–	+/–
C. quinoa	CS, NS/–	+/–
Cucumis sativus	NS/–	+/–
Cucurbita pepo	–/–	–/–
Datura stramonium	CS, NS/CS, M	+/+
Glycine max	–/–	–/–
Gomphrena globosa	NS, NR/–	+/–
Impatiens balsamiana	CS, NS/–	–/–
Lactuca sativa	N/–	+/–
Nicotiana benthamiana	CS, NR/PD, W	+/+
N. clevelandii	NR/PD	+/+
N. glutinosa	NR/–	+/–
N. occidentalis	NR/TN	+/+
N. rustica	NR/–	+/–
N. tabacum cv. Burley 21	NR/–	+/+
N. tabacum cv. Ky57	NR/–	+/+
N. tabacum cv. Samsun NN	NR/–	+/+
N. tabacum cv. White Burley	NR/–	+/+
N. tabacum cv. Xanthi-nc	NR/–	+/+
Physalis floridana	NR/–	+/+
Petunia hybrida	NR, NS/–	+/–
Phaseolus vulgaris	–/–	–/–
Solanum lycopersicum	CL, NS/CL, M, TN	+/+
Tetragonia tetragonioides	CS/–	+/–
Vigna unguiculata	CL, NR/–	+/–
Zennia elagans	CL, NR/–	+/+

CSNV, chrysanthemum stem necrosis virus.

[a]Inoculated leaves/upper leaves. Symptoms were assessed by observations in the inoculated and upper leaves until 28 days after inoculation. The symptoms were briefly indicated as follows: CL, chlorotic lesions; CS, chlorotic spot; M, mosaic; N, necrosis; NR, necrotic rings; NS, necrotic spots; PD, plant death; TN, top necrosis; W, wilt; –, no symptoms.

[b]Double-antibody sandwich–enzyme-linked immunosorbent assay (ELISA) was performed from sap extracted from indicator plant species 14 days after inoculation as described by Yoon et al. (2020a) Plus symbol (+) indicates the optical density (OD_{405}) was greater than three times the mean of the healthy controls (Yoon et al., 2011, 2020a). And negative symbol (–) indicates the optical density (OD_{405}) was similar to that of the mean of the healthy controls.

be indicated by the final results of the successful infection or by the process of changing the appearance of the diseased organs, particularly on chloroplast and mitochondria. In field conditions, many crops infected with plant viruses sometimes show distinct symptoms, allowing identification of the causal virus by naked eyes. However, it is impossible to identify a causal virus by observation of symptoms in crops in many farms, thus it is required to identify accurately a causal virus using immunological, genetic, or biological methods. Owing to their broad host range, multiple vector species, and appearance of new species, tospoviruses have become distributed worldwide and cause serious economic losses in many agricultural crops and ornamental plants (Komoda et al., 2017). The virus is a major threat to chrysanthemum production in greenhouses and crop fields worldwide (Chung et al., 2006; Yoon et al., 2017). Host range, particularly on host response to CSNV infection did not show any significant pathological changes of CSV isolates in the world, though a few plant species showed different responses according to challenging CSNV isolates. Cultivars and greenhouse conditions are likely to affect expression of local responses or systemic symptoms while CSNV infection steps.

To further molecularly characterize CSNV-Kr total RNA extracted from one *N. benthamiana* plant systemically infected with CSNV-Kr was subjected to high-throughput sequencing, 5′/3′-RACE, and the Sanger sequencing. In order to eliminate non-viral sequences, the obtained virus-associated contigs were again subjected to BLASTX search against the NCBI non-redundant protein database. As a result, we obtained only virus-associated contigs from each library. The large RNA (L RNA) segment of CSNV-Kr consists of 8,959 nt and contains a 5′-untranslated region (UTR) of 292 nt and a 3′-UTR of 34 nt, and one ORF of 8,634 nt in an antisense arrangement coding for the putative RdRp (L protein) of 2,877 aa with a predicted molecular mass of 331.13 kDa. Analysis of the L protein of CSNV-Kr revealed the presence of all conserved PDDEXK nuclease-like and Bunyavirus RdRp motifs (Dullemans et al., 2015). The M RNA segment of CSNV-Kr consists of 4,835 nt and contains a 5′-UTR of 101 nt, a 3′-UTR of 83 nt, and two ambi-sense ORFs of 912 and 3,408 nt. The M RNA segment encodes for NSm and the precursor of the Gn/Gc proteins. The ORFS of CSNV-Kr are separated by an intergenic region (IGR) of 331 nt which contains A and U-rich sequences for formation of a stable hairpin structure, similar to other orthotospoviruses. The S RNA segment of CSNV-Kr

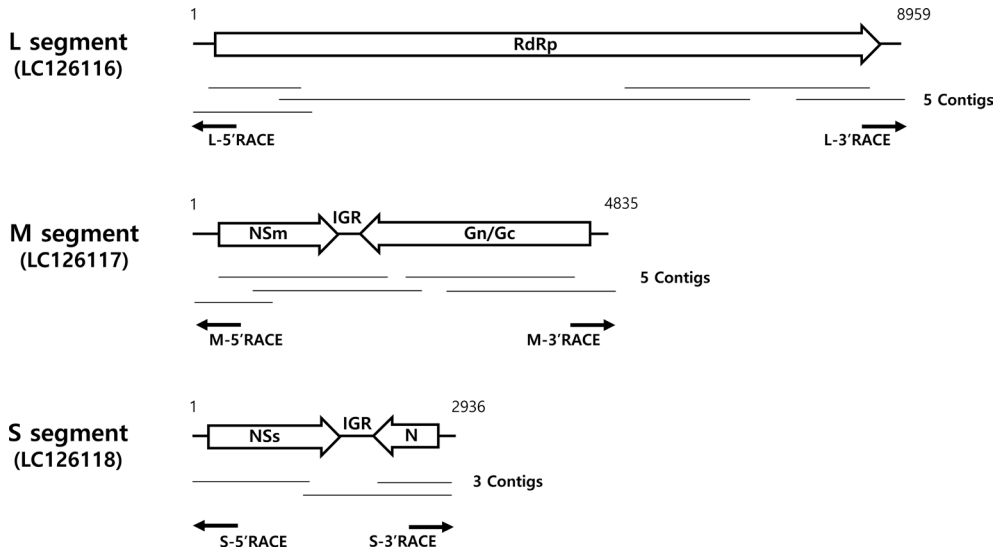

Fig. 1. Schematic representation of tripartite RNA genome of chrysanthemum stem necrosis virus (CSNV) Kr isolate. CSNV-associated contigs obtained from the analyzed high-throughput sequencing and 5′/3′-rapid amplification of cDNA ends were represented in each L, M, and S segments.

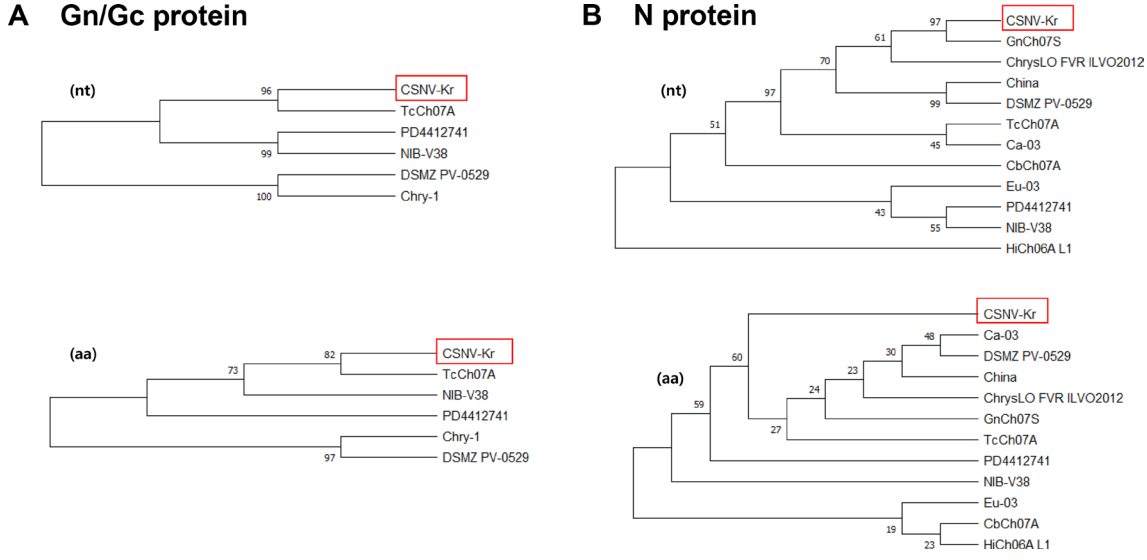

Fig. 2. Phylogenetic tree of chrysanthemum stem necrosis virus (CSNV) isolate Kr and other previously reported CSNV isolates based on alignment of nucleotide and amino acid sequences of Gn/Gc (A) and nucleotide and amino acid sequences of N (B) using the neighbor-joining method in MEGA X package. Numbers among the lines indicate the frequency of the cluster after bootstrap analysis (1,000 replicates). The source of N amino acid sequences and Gn/GC amino acid sequences of CSNV isolates are indicated as GenBank accession numbers at the right side, respectively. CSNV-Kr was indicated with red rectangles. Bootstrap values out of 1,000 replicates are indicated at the nodes.

of 2,936 nt consists of a 5′-UTR of 79 nt a 3′-UTR of 152 nt and two ambi-sense ORFs of 1,404 nt and 783 nt. The ORFs encode for the NSs and N proteins, and the ORFs are separated by an IGR of 529 nt (Fig. 1).

Multiple alignment analysis showed that L RNA sequence of CSNV-Kr showed 96.8–98.9% identity with those of other published CSNV isolates at nt level. The RdRp encoded in L RNA of CSNV-Kr showed 98.8–99.3% identity with those of other published CSNV isolates at aa level. The Gn/Gc protein of CSNV-Kr showed 98.8–99.5% aa identity with those of other published CSNV isolates. Phylogenetic tree analysis deduced from the Gn/Gc alignment showed the

Gn/Gc protein of CSNV-Kr had the closest relationship with CSNV-TcCho7A (Japanese isolate, accession no. KF493772) (Fig. 2A). The N protein of CSNV-Kr showed 98.8–99.6% identity with those of other published CSNV isolates at aa level. Phylogenetic tree analysis deduced from the N alignment showed the N protein of CSNV-Kr was classified into one cluster and had the closest relationship with CSNV-DSMZ PV-0529 (NCBI accession no. MW051793) (Fig. 2B). By pairwise alignment analysis, there was no distinct variable region among the encoded N proteins of 16 CSNV isolates between the Korean isolate and other previously reported isolates. Due to high sequence identity, the NJ trees constructed from the six Gn/Gc sequences and the 12 N sequences revealed no distinct segregation of the CSNV isolates, belonged to one monophyletic cluster. All of the CSNV isolates did not show any relationship according to geographical origins and isolation hosts, as expected by matrix of sequence homology.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

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