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Complete Genome Sequence of the RNAs 3 and 4 Segments of *Rice stripe virus* Isolates in Korea and their Phylogenetic Relationships with Japan and China Isolates

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The complete genome sequences of RNA3 and RNA4 of the 13 different *Rice stripe virus* (RSV) isolates were determined and characterized in this study to address the possible causes of the recent re-emergence of RSV that affected many rice fields in Korea. The genome size of each RNA segment varied among isolates and significant differences were observed in the intergenic region. There was up to 4% average divergence in the RNA4 nucleotide sequence among 13 Korean isolates and only 1.4% in the RNA3. Phylogenetic relationships among different Korean isolates revealed that there were at least 2 types of RNA3 and 4 distinct types of RNA4 genomes present in Korea. However, Korean isolates with one type of RNA3 predominate over the other while the occurrences of the RSV Korean isolates with the 4 types of RNA4 genome were not correlated to specific geographical areas. Results further indicate that RNA4 had diverged more than RNA3 and these differences in accumulation of mutations in the individual RNA segments indicate that genetic reassortment were likely to contribute to the genetic divergence in the 13 Korean isolates. All of the Korean-RNA3 sequences except for one isolate grouped with Chinese isolates (JY and Z). In contrast, the RNA 4 sequences segregated together with either Chinese (JY and Z) and Japanese (M and T) isolates but genetic relationships of Korean isolates- RNAs 3 and 4 segments to Chinese-Y isolate were low. Altogether, these results suggest that the occurrence of mixtures of RNAs 3 and 4 genotypes in the natural population of RSV may have contributed to the sudden outbreak in Korea.

Keywords : phylogenetic relationship, rice, *Rice stripe virus*, *Tenuivirus*

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Rice stripe virus (RSV) is one of the important diseases of rice (*Oryza sativa* L.) in temperate countries such as Japan, China, Taiwan, the USSR and Korea. The causal agent virus had filamentous morphology and transmitted actively by small brown planthopper (BPH, *Laodelphax striatelleus*) in a persistent manner to rice plants. RSV-infected rice plants showed chlorotic to yellowish white stripes on the leaves and stunting of the plants (Hibino, 1996; Toriyama et al., 2000).

RSV is the type species of the genus *Tenuivirus*. Other members in the *Tenuivirus* genus include *Maize stripe virus* (MStV), *Rice hoja blanca virus* (RHBV), and *Rice grassy stunt virus* (RGSV). RSV consists of 4 single-stranded (ss) RNA segments named RNAs 1-4 designated according to the size of the genome where RNA1 is the largest (*ca* 8.9 Kb). The total genome size of RSV is *ca.* 19 Kb and encodes seven proteins. Except for RNA1, RNAs 2, 3 and 4 are ambisense wherein open reading frames (ORFs) are present at 5' proximal ends of both viral (v) and viral complementary (vc) senses. The ORFs are separated by an intergenic region (IGR) which has A-U rich sequences and a stable secondary structure capable of forming a hairpin loop, which is required for transcriptional termination of the ambisense genes (Hamamatsu et al., 1993). *Tenuivirus* belongs tentatively to the family *Bunyaviridae* and is likely to be distantly related to the genus *Phlebovirus*, an animal virus group based on their weak sequence similarities in the genes for RNA-dependent RNA polymerase (RdRp), glycoprotein and coat protein genome encoded by vcRNA1, vcRNA2, and vcRNA3, respectively (Kakutani et al., 1990; Falk and Tsai, 1998).

In Korea, the first outbreak of RSV disease was recorded in 1965 which had affected 40% of the rice hills (Toriyama, 2000) and recently re-emerged as a major rice pathogen in 2007 and 2008, caused a total of 84% affected paddy fields in the entire country (HS Choi and KH Kim, unpublished). Concurrently, concerted efforts are under-

way by all concerned authorities to find a solution to this malady.

The genome sequences of Korean RSV isolates have not been determined yet. Therefore, in order to determine the possible causes underlying the recent epidemics, it is essential to obtain information on the genome sequences of the isolates of RSV from Korea. We prioritized segments RNAs 3 and 4 of the 13 Korean isolates for sequence determination. RNA 3 has 2.1 Kb and encodes 35 kDa-nucleocapsid protein encoded by vcRNA3 (Kakutani et al., 1991; Zhu et al., 1991). RNA 4 consists of 2.5 Kb and a 21 kDa-major disease-specific protein encoded by vRNA4 (Kakutani et al., 1990; Zhu et al., 1992). In addition, recent vital roles of the RNAs 3 and 4 nonstructural (NS) proteins were reported. vRNA3 encoded the 23.9 kDa-NS3 which acts as a post-transcriptional gene silencing suppressor (Hemmes et al., 2007) and 32 kDa-NSvc 4 encoded by vcRNA4 is a movement protein (Xiong et al., 2008). In this study, we report the complete nucleotide sequences of RNAs 3 and 4 of 13 RSV isolates and investigated their phylogenetic relationships to those reported in China and Japan.

Table 1. Sources of Korean-*Rice stripe virus* field isolates and the accessions numbers for RNA 3 and 4 of Korean, Japanese and Chinese isolates used in this study

Source (Location, Province)[a]	RSV Isolate Code	Accession No.	Reference
Buan1, Jeonbuk	RNA3 BA1-JB	FJ602675	This study
	RNA4 BA1-JB	FJ602688	This study
Buan2, Jeonbuk	RNA3 BA2-JB	FJ602676	This study
	RNA4 BA2-JB	FJ602689	This study
Gunsan, Jeonbuk	RNA3GS-JB	FJ602677	This study
	RNA4GS-JB	FJ602690	This study
Iksan, Jeonbuk	RNA3 IS-JB	FJ602678	This study
	RNA4 IS-JB	FJ602691	This study
Gochang, Jeonbuk	RNA3 GC-JB	FJ602679	This study
	RNA4 GC-JB	FJ602692	This study
Muan, Jeonnam	RNA3 MA-JN	FJ602680	This study
	RNA4 MA-JN	FJ602693	This study
Jindo, Jeonnam	RNA3 JD-JN	FJ602681	This study
	RNA4 JD-JN	FJ602694	This study
Yeonggwang, Jeonnam	RNA3 YG-JN	FJ602687	This study
	RNA4 YG-JN	FJ602700	This study
Shinan, Jeonnam	RNA3 SA-JN	FJ602685	This study
	RNA4 SA-JN	FJ602698	This study
Wando, Jeonnam	RNA3 WD-JN	FJ602686	This study
	RNA4 WD-JN	FJ602699	This study
Chongyang, Chungnam	RNA3 CY-CN	FJ602682	This study
	RNA4 CY-CN	FJ602695	This study
Seochon, Chungnam	RNA3 SC-CN	FJ602683	This study
	RNA4 SC-CN	FJ602696	This study
Andeok, Jeju-do	RNA3 AD-JJ	FJ602684	This study
	RNA4 AD-JJ	FJ602697	This study
Japan-M	RNA3	D01094	Kakutani et al., 1991
	RNA4	D01039	Kakutani et al., 1990
Japan-T	RNA3	NC_003776	Zhu et al., 1991
	RNA4	NC003753	Toriyama et al., 1994
China-JY	RNA3	EF141329	Genbank, unpublished
	RNA4	EF141330	Genbank, unpublished
China-Y	RNA3	Y11095.1	Qu et al., 1997
	RNA4	Y11096	Qu et al., 1997
China-Z	RNA3	DQ333944	Zhang et al., 2007
	RNA4	DQ333945	Zhang et al., 2007

[a]All Korean isolates were collected in 2007 except for SA-JN and WD-JN that were collected in 2008.

Material and Methods

RSV isolates and total RNA extraction. Infected rice plants showing typical RSV symptoms were collected from 4 provinces in Korea including Chungnam (CN), Jeonbuk (JB), Jeonnam (JN) and Jeju (JJ) in 2007 and 2008. RSV infection was confirmed in each collected sample by RT PCR (Lee et al., 2004). Approximately 100 mg of powdered leaf tissues were used to extract total RNAs using TRI Reagent (Molecular Research Center, Inc.) or RNAeasy kit (Qiagen) following manufacture's protocols and kept at −80°C. The isolate nomenclature and geographical origin of the 13 Korean isolates were shown in Table 1 and Fig. 1, respectively.

cDNA synthesis, cloning and sequencing. Complete sequences of RNAs 3 and 4 were obtained using assembly of overlapping amplified fragments (0.9 to 1.5 kb) using primers in Table 2. Oligonucleotide primers of each fragment were designed based on sequences conserved among RNAs 3 and 4 of Japan-T, China-JY and China-Z isolates available in the GenBank (Table 1). Approximately, 50 μg of RNA was denatured by heating for 1 min at 100°C and immediately chilled on ice water for 3 min. cDNA was

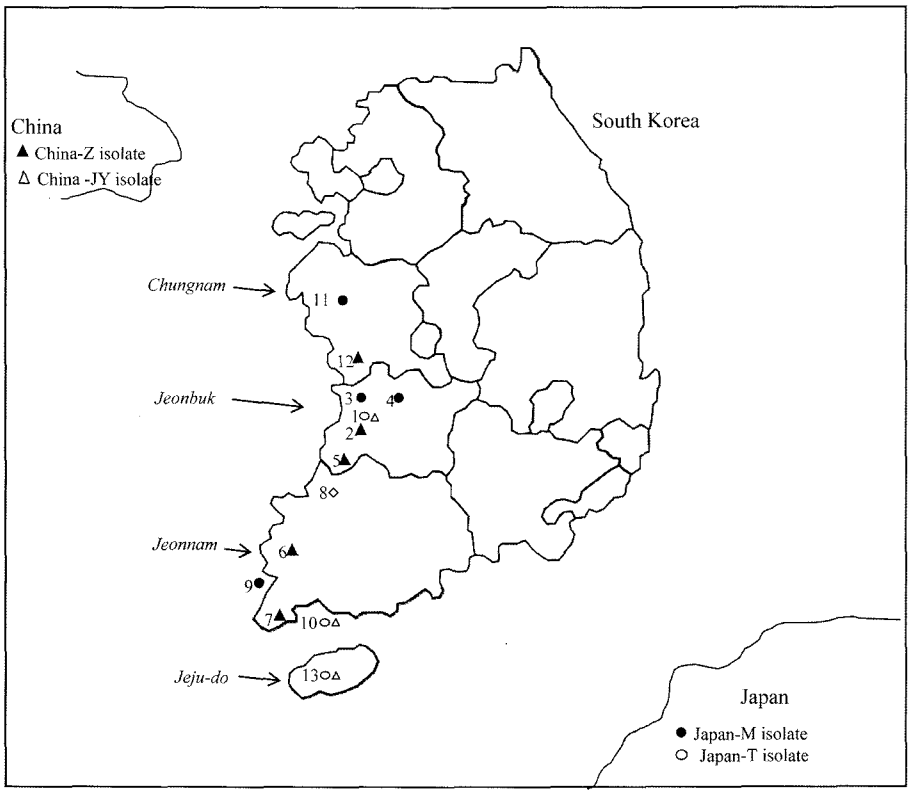

Fig. 1. Map of Korea showing the location of RSV isolates carrying different RNA4 genotypes in Korea. Closed circles and triangles resembled with Japan-M isolate and China-JY isolates; Open circles and triangles resembled with Japan-T isolate and China-Z isolates; Diamond resembled with unknown isolate. Numbers correspond to the Korean isolates in Table 3.

Table 2. Oligonucleotides primers used for amplifying RSV-RNAs 3 and 4 genome

RNA segment	Primer Name	Oligonucleotides	Sense	Nucleotide location	PCR product
RNA3	RS3-23	ACACAAAGTCCTGGGTAAAATAG	+	1-23	
	RS3-24	AYTTTGTCTGTGTGGTTYTGGGC	−	1283-1305	~1.3 kb
	RS3-25.1	CGAAGATGTCTCGGATGTTTTTT	+	935-958	
	RS3-26	ACACAAAGTCWGGGTAATAAAAT	−	2486-2508	~1.5 kb
RNA4	RS4-27	ACACAAAGTCCAGGGCA	+	1-17	
	RS4-28	TCGGTGTGGTGAAGCAG	−	1307-1323	~1.3 kb
	RS4-29	TGYTAGCATACTCYGGA	+	1219-1235	
	RS4-30	ACACAMAGTCAKGGYAT	−	2145-2161	~0.9 kb

(A) RNA3

	BA1 -JB	BA2 -JB	GC- JB	GS- JB	IS- JB	CY- CN	SC- CN	JD- JN	MA- JN	SA- JN	WD- JN	YG- JN	AD- JJ	Japan -M	Japan -T	China -JY	China -Z	China -Y
BA1-JB		98.2	98.4	98.3	98.5	99.0	97.4	98.8	97.9	98.5	98.8	98.6	98.7	96.6	96.6	98.4	98.9	95.6
BA2-JB	1.5		98.0	97.9	98.3	98.3	97.3	98.7	97.5	98.1	98.2	98.1	98.3	96.4	96.6	98.1	98.5	95.2
GC-JB	1.3	1.7		98.1	98.4	98.4	97.7	98.7	97.6	98.3	98.4	99.3	98.5	96.6	95.7	98.2	98.6	95.1
GS-JB	1.4	2.0	1.6		98.0	98.4	97.1	98.3	97.4	98.1	98.2	98.2	98.3	96.2	95.9	98.1	98.5	95.0
IS-JB	1.1	1.4	1.5	1.8		98.5	97.4	98.8	97.8	98.4	98.4	98.7	98.6	96.5	96.1	98.3	98.5	95.4
CY-CN	0.8	1.5	1.3	1.3	1.1		97.3	98.7	97.8	98.6	98.8	98.6	98.7	96.5	96.1	98.3	98.9	95.5
SC-CN	2.1	2.5	2.3	2.6	2.2	2.2		97.7	97.3	97.5	97.3	97.5	97.6	96.7	95.8	97.6	97.6	94.7
JD-JN	1.2	1.3	1.3	1.8	1.1	1.3	2.2		97.9	98.3	98.6	98.7	98.7	96.7	96.4	98.3	98.7	95.8
MA-JN	2.0	2.3	2.3	2.5	2.0	2.0	2.5	2.2		97.8	97.9	97.8	97.9	96.5	95.9	97.9	97.8	94.9
SA-JN	1.6	2.0	1.8	2.1	1.8	1.5	2.6	1.8	2.5		98.6	98.5	98.6	96.2	96.0	98.1	98.3	95.4
WD-JN	1.1	1.8	1.6	1.7	1.5	1.1	2.5	1.6	2.3	1.5		98.7	98.8	96.5	96.2	98.2	98.8	95.6
YG-JN	1.1	1.5	0.6	1.5	1.2	1.0	2.2	1.2	2.1	1.5	1.3		98.8	96.7	95.8	98.3	98.8	95.5
AD-JJ	1.2	1.7	1.5	1.8	1.5	1.2	2.5	1.5	2.2	1.4	1.3	1.2		96.5	96.3	98.3	98.7	95.5
Japan-M	3.4	3.6	3.5	3.8	3.5	3.4	3.5	3.5	3.7	3.7	3.5	3.3	3.4		98.1	96.4	96.3	94.6
Japan-T	3.4	3.7	3.6	4.0	3.5	3.5	3.5	3.6	3.9	3.9	3.7	3.4	3.6	1.9		96.4	96.2	93.8
China-JY	1.7	1.9	1.7	2.0	1.7	1.6	2.3	1.8	2.1	2.1	1.8	1.5	1.8	3.5	3.5		98.2	95.2
China-Z	1.1	1.6	1.3	1.4	1.4	1.1	2.4	1.2	2.3	1.7	1.3	1.0	1.4	3.7	3.8	1.8		95.5
China-Y	4.3	4.7	4.7	5.0	4.4	4.3	5.3	4.4	5.2	4.7	4.5	4.3	4.7	5.6	6.0	5.0	4.7	

(B) RNA4

	BA1 -JB	BA2- JB	GC- JB	GS- JB	IS-JB	CY- CN	SC- CN	JD-JN	MA- JN	SA- JN	WD- JN	YG- JN	AD- JJ	Japan -M	Japan -T	China- JY	China -Z	China -Y
BA1-JB		96.1	96.1	95.9	95.1	95.2	96.1	95.9	95.9	95.3	96.8	95.8	97.2	95.6	96.7	97.1	95.5	91.6
BA2-JB	3.9		98.4	95.5	95.3	94.9	95.7	96.0	96.5	95.4	96.2	96.0	96.2	95.7	96.0	96.3	95.6	91.7
GC-JB	3.9	1.6		95.3	95.0	94.8	95.7	95.8	96.5	95.2	96.0	95.7	96.1	95.5	96.0	96.0	95.6	91.6
GS-JB	4.0	4.5	4.6		97.7	97.3	95.4	98.3	95.4	97.8	96.0	95.9	96.3	98.1	95.7	95.7	95.4	91.7
IS-JB	4.7	4.7	4.9	2.3		97.3	94.8	98.5	95.1	97.8	95.5	95.3	95.7	98.0	95.2	95.2	95.1	91.2
CY-CN	4.8	5.2	5.2	2.7	2.7		94.6	98.0	94.9	97.4	95.3	95.1	95.7	97.6	95.0	95.3	94.8	91.2
SC-CN	4.0	4.4	4.4	4.8	5.2	5.6		95.5	95.9	95.0	95.6	95.5	96.0	95.3	95.7	95.6	96.8	91.5
JD-JN	4.0	4.1	4.1	1.7	1.5	2.0	4.6		95.7	98.5	96.2	96.0	96.4	98.6	96.0	96.2	95.5	92.1
MA-JN	3.9	3.5	3.5	4.4	4.7	5.0	4.1	4.1		95.0	96.1	95.7	96.2	95.3	95.9	95.9	95.8	91.7
SA-JN	4.5	4.6	4.6	2.2	2.1	2.7	5.0	1.6	4.6		95.6	95.6	95.9	98.2	95.4	95.5	95.1	91.5
WD-JN	3.1	3.8	4.0	3.8	4.5	4.8	4.5	3.8	3.7	4.4		96.0	98.4	95.7	96.8	96.8	96.0	91.6
YG-JN	4.1	4.1	4.2	4.0	4.5	4.9	4.6	4.0	4.1	4.2	4.0		96.2	95.8	95.7	95.9	95.3	91.8
AD-JJ	2.8	3.9	4.0	3.7	4.3	4.5	4.2	3.7	3.7	4.1	4.2	4.0		96.0	97.0	97.1	96.3	91.7
Japan-M	4.4	4.4	4.5	2.0	1.9	2.4	4.9	1.4	4.5	1.9	4.3	4.2	4.1		95.6	95.6	95.2	91.9
Japan-T	3.3	4.1	4.1	4.3	4.8	5.1	4.5	4.1	4.0	4.7	3.2	4.4	3.0	4..3		96.8	95.4	91.6
China-JY	2.9	3.8	4.0	4.3	4.8	4.9	4.5	3.9	4.1	4.5	3.3	4.1	2.9	4.4	3.2		95.3	91.8
China-Z	4.5	4.6	4.5	4.7	4.9	5.3	3.3	4.6	4.2	4.9	4.1	4.7	3.7	4.9	4.7	4.8		91.4
China-Y	8.5	8.5	8.5	8.6	8.9	9.1	8.7	8.2	8.3	8.8	8.6	8.2	8.6	8.3	8.5	8.2	8.8	

Fig. 2. Nucleotide sequence similarity matrix index among Korean, Japanese and Chinese isolates based on RNA3 (A) and RNA4 (B) sequences. Values in upper and lower diagonal shaded frames indicate percent nucleotide sequence identity and divergence, respectively.

synthesized using M-MLV reverse transcriptase (Promega) and incubated at 42°C for 1 hr. A total of 50 μl cDNA reaction volume was added to Ex Taq (Takara) polymerase chain reaction mixture and was amplified in a thermal cycler (My Cycler™, Bio-Rad) for 35 cycles at 53°C annealing temperature. The 5' and 3' terminal regions were confirmed following the 5'/3' RACE (Miranda et al., 2000). The 3' end of cDNA was tailed with oligo(G) using dGTP and terminal deoxynucleotidyl transferase (Takara). G-tailed cDNA was amplified by *Taq* DNA polymerase (Type Ex, Takara) using d(C)12 or d(C)12ACACA, in which ACACA is conserved in all 5' termini of *tenuivirus* RNA, and an internal primer used for cDNA synthesis. For cloning and sequencing individual PCR fragments were extracted from 0.8% agarose gel and purified using Qiaquick gel extraction kit (Qiagen) and directly cloned into pGEM-T Easy vector (Promega). All clones (at least 2 independent clones) or gel extracted PCR products were submitted to National Instrumentation Center for Environmental Management, Seoul National University, Korea, for sequencing using vector sequence primer, M13 in both orientations and PCR primers, respectively. Sequences obtained were initially subjected to the BLAST search program for confirmation of related RSV sequences.

Sequence alignments and phylogenetic analyses. The determined sequences of the Korean isolates derived from at least 2 independent clones and or gel extracted PCR products were initially analyzed using BioEdit sequence alignment editor, version 7.0.9 (Hall, 1999). The ClustalW method (Thompson et al., 1994) of MegAlign from the DNAStar 5.01 (1993-2001) package was used to calculate distance matrices based on the full-length sequences of RNA3 and RNA4. The phylogenetic relationships of the sequences were determined by the neighboring-joining (NJ) algorithm of PHYLIP Version 3.5 (Falsenstein, 1993). Trees constructed from these matrices by the NJ method (Saito and Nei, 1987) with a boostrap value for each internal node using 1000 random replications. In addition, RNA3 and RNA4 sequences were subjected to Recombination Detection Program (RDP) v.327 software (http://darwin.uvigo.es/rdp/rdp.html) for possible recombination among different RSV isolates. The accession numbers of each Korean-RSV isolates including the two RSV isolates M and T from Japan and three isolates JY, Y and Z from China are listed in Table 1 that were used to establish genetic relationships with the 13 Korean isolates determined in this study.

Results

RNAs 3 and 4 sequences analyses of the 13 Korean RSV isolates.

The average percent divergence in the RNA3 nucleotide sequences of the 13 Korean isolates was 1.4% (Fig. 2A) and 4.0% in RNA4 (Fig. 2B). The numbers of nucleotides (nts) of the 5' and 3' untranslated regions (UTR) and ORFs 1 and 2 in RNAs 3 and 4 were exactly the same among all 13 Korean isolates and those of RSV RNAs 3 and 4 genomes previously reported (Kakutani et al., 1990, 1991; Qu et al., 1997; Toriyama et al., 1994; Zhang 2007; Zhu, et al., 1991). The 5' UTR, ORF1, ORF2 and 3' UTR of RNA3 consisted of 65, 636 (212 aa), 969 (323 aa), and 92 nts, respectively; whereas they were 54, 537 (179 aa), 861 (287 aa), and 51 nts, respectively for RNA4. However, nucleotide changes by substitutions were observed in the ORF regions of both RNAs 3 and 4, although the rate of deduced amino acid changes (aa) was relatively low i.e., 0.3% (1 out of 323 aa) for RNA3-ORF2 and 3.0% (7 out of 212 aa) for RNA3-ORF1 and also for ORFs 1 and 2 of RNA4 (6 and 10 out of 179 and 287 aa, respectively). The nts of 5' UTR of RNAs 3 and 4 were completely identical, but 1 or 2 nts changes were found at the downstream of 3' UTR among some Korean isolates (data not shown). The total nucleotide numbers of RNAs3 and RNA4 genomes (Table 3) varied among the Korean RSV isolates and those reported previously (Kakutani et al., 1990, 1991; Qu et al., 1997; Toriyama et al., 1994; Zhang 2007; Zhu et al., 1991). Significant nts differences due to multiple nt deletions and insertions were observed at the IGR among the Korean,

Table 3. Total nucleotide numbers of the intergenic region and complete genome of RNAs 3 and 4 of *Rice stripe virus* isolates in Korea, Japan, and China

Isolate		RNA3		RNA4	
No.	Code	IGR	Total	IGR	Total
1	BA1-JB	732	2494	651	2155
2	BA2-JB	724	2485	654	2157
3	GS-JB	720	2482	638	2141
4	IS-JB	721	2482	637	2140
5	GC-JB	728	2490	652	2155
6	MA-JN	724	2485	651	2154
7	JD-JN	710	2472	636	2139
8	YG-JN	729	2491	654	2157
9	WD-JN	719	2481	653	2156
10	SA-JN	710	2472	637	2140
11	CY-CN	722	2484	636	2135
12	SC-CN	733	2495	655	2158
13	AD-JJ	716	2478	654	2157
14	Japan-M	713	2475	634	2137
15	Japan-T	742	2504	654	2157
16	China-JY	714	2476	655	2157
17	China-Y	749	2511	732	2235
18	China-Z	714	2476	653	2156

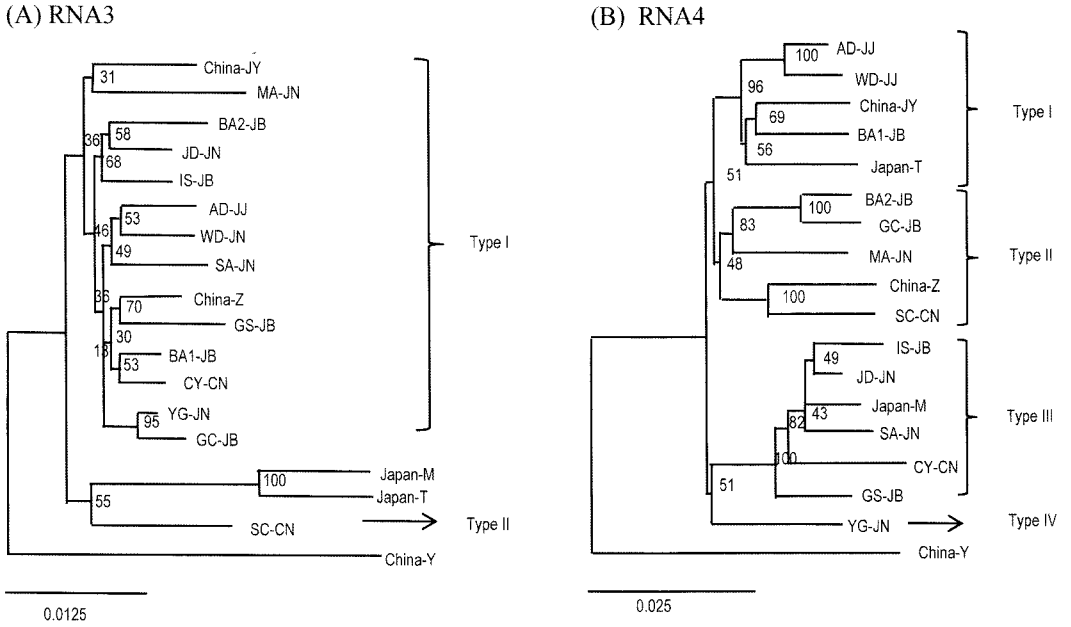

Fig. 3. Phylogenetic trees constructed based on complete genomic sequences of RNA3 (A) and RNA4 (B) of the 13 Korean, 2 Japanese and 3 Chinese isolates. Trees constructed from these matrices by the NJ method with a boostrap value for each internal node using 1,000 random replications.

Japanese and Chinese isolates. Apparently, the differences in the sizes of RNAs 3 and 4 were in the IGR (Table 3) and average percent nucleotide variation was higher in RNA4 (7.8%) than RNA3 (3.2%). Interestingly, in the RNA4 sequences of the 13 Korean isolates examined in this study, 5 isolates showed a consecutive of 19 nts deletions at the upstream of IGR, which was also observed in the Japan-M isolate (Qu et al., 1997; Zhu et al., 1992). The 5 isolates were not restricted in one particular province but occurred in the three provinces including Jeonbuk, Jeonnam and Chungnam. The RNA4 of Chinese-Y isolate which had an insertion of 84 nts was longest among those of the isolates examined (Table 3).

Phylogenetic relationships among the Korean, Japanese and Chinese RSV isolates. The genetic relationships among 13 Korean, 2 Japanese and 3 Chinese RSV isolates based on the complete nucleotide sequences of RSV RNA3 and RNA4 are shown in Fig. 3 (panels A and B). The Korean isolates grouped into 2 major clades based on the RNA3 sequences, indicating that there were 2 types of RNA3 genome present in the natural RSV population (Fig. 3A). The proportions of isolates belonging to each type, however, were not even. It appeared that 12 out of 13 isolates grouped together and their nucleotide sequences shared 97-99% homology with each other (Fig. 2A). In addition, Chinese isolates JY and Z included in this group suggesting that most Korean isolates were nearly identical having similar ancestors with those of Chinese isolates -JY and -Z based on the RNA3 sequences. The SC-CN isolate, however, seemed to have another distinct type of RNA3 genome with 95-97% nucleotide sequence identity with the rest Korean isolates and appeared to have an ancestor different from that of the first group. The 2 Japanese isolates M and T with 95-96% sequence similarity formed a separate group and appeared to originate from another ancestor (Fig. 2A). On the other hand, the RNA4 genome of the 13 Korean isolates separated into 4 major clades, indicating that 4 distinct types of RNA4 genome were present in Korea (Fig. 3B). The proportions of isolates in each group were nearly equal, indicating that RNA4 diverged more than RNA3 and this was supported by the percent nucleotide divergence (ave. 1.4% RNA3 and 4.0% RNA4 nucleotide sequences) obtained from the matrix similarity index analysis (Fig. 2). The first group consisted of 3 Korean isolates from BA1-JB, AD-JJ, WD-JN had 95-98% sequence similarity with those of China-JY and Japan-T isolates (Fig. 2B). The second group consisted of 4 Korean isolates from BA2-JB, GC, JB, MA-JN, SC-CN and shared 95-98% sequence homology with China-Z isolates. The third group includes 5 Korean isolates from IS-JN, JD-JN, CY-CN, GS-JB, SA-JN formed another group with 95-98% sequence similarity with Japan-M isolate. These 5 Korean and Japan-M isolates were actually those that showed the 19 nts deletion at the IGR. Apparently, YG-JN isolate had 95-96% sequence homology among other isolates formed another branch and appeared to have another type of RNA4 genome with unknown

sequence origin. Meanwhile the Chinese isolate-Y appeared to be very distinct with relatively lower nucleotide sequence identity with RNA3 (93-95%) and RNA4 (91-92%) from those of the rest of the RSV isolates analyzed in this study, indicating that its ancestor is far different from those for other groups (Fig. 2B).

Occurrence of different RSV RNA3 and RNA4 genotypes in Korea. The 13 Korean isolates analyzed in this study showed different types of RNAs 3 and 4 sequences. The occurrence of RNA3-type I genotype was present in all provinces analyzed in this study and the other type of RNA3 genome was only observed in SC-CN (Fig. 3A). On the other hand, the occurrences of all the 4 types of RNA4 genome were almost present in each province as vividly seen in the map (Fig. 1). Interestingly, the 4 types of RNA 4 were all present in the province of Jeonnam (JN). In addition, even the two isolates BA1-JN and BA2-JN collected in one single location showed different types of RNA4 indicating that the 4 types of RNA4 genome were not correlated to specific geographical areas.

Discussion

In this study, the complete nucleotide sequences of RSV RNA segments 3 and 4 of the 13 Korean isolates were determined to examine a possible association between the recent RSV outbreak in Korea and its genetic variations. The complete sequences of RNAs 3 and 4 of the 13 Korean RSV isolates showed significant nucleotide sequence divergence (ave. 1.4% RNA3 and 4.0% RNA4, Fig. 1a and b) which resulted in different types of RNA3 and RNA4 genomes (Fig. 3). In addition, the overall relationships between isolates showed different topology of the phylogenetic trees constructed which revealed 2 and 4 distinct types of RNAs 3 and 4 sequences, respectively (Fig. 3). However, based on RNA3 sequences, the majority of the isolates (12 out of 13) grouped into one type (type 1) which has the same ancestor with those of China- JY and -Z isolates. The other type consisted of only one isolate and may represent another type that was significantly different from the type 1, and was seemingly derived from other unknown ancestor (Fig. 3A). Interestingly, the 13 Korean isolates were divided into 4 distinct groups based on the RNA4 sequences and each group derived from different ancestors. Type 1 shared similarity with those of China-JY and Japan-T isolates. Type II from China-JY and Type III from Japan-M isolate and one isolate (YG-JN) branched out represents Type IV. The Type III consisted of isolates with 19nt deletions and apparently these 19 nts deletion was also seen in one of the two samples collected in 2008. Moreover, these different types of RNA4 genome were not confined in one locality but also occurred in different provinces (Fig. 1) indicating that the occurrence of the 4 types of RNA4 genome were not correlated to specific geographical areas. All Korean isolates, however, were distantly related with Chinese Y isolate based on the RNA 3 and 4 segment sequences.

In this study, we have demonstrated that there were near identities in RNA3 and a significant divergence in RNA4 in the 13 geographical RSV isolates in Korea. The differences in the divergence of RNAs 3 and 4 genome segments are not known but selective pressures may occur in either or both the insect and plant hosts. The RNA 3 codes for a capsid protein on vcRNA which is expressed in both plants an insect vectors whereas RNA4 codes for noncapsid protein also referred as disease specific protein which is detected in abundance in host plants but not detectable in viruliferous insects (Falk et al., 1987; Liang et al., 2005; Lijun et al., 2003). Recently, these segments have identified roles for their nostructural protein encoded on vRNA3 and vcRNA4 which are responsible for gene silencing suppressor (Hemmes et al., 2007) and movement protein (Xiong et al., 2008), respectively.

The generation of different RSV-RNA 3 and 4 genotypes among the Korean isolates could be explained by genetic recombination (Jegger et al., 2006; Kakutani et al., 1990). Another possibility is natural genetic reassortment which is often reported for plant (Gu et al., 2007; Miranda et al., 2000) and animal RNA viruses (Henderson et al., 2005; Rodriguez et al., 1998) with segmented genomes. We have performed RDP and results of our analyses showed that possible recombination was only detected between isolates SC-CN and China-Z isolates in RNA4 genome (data not shown) but the rest of the isolates in both RNA's 3 and 4 genome were negative of possible recombination indicating that the general source of genetic variations is most likely due to genetic reassortment. Previous report, however, on the comparison of RSV-RNA 3 and 4 nucleotide sequences of Japan-M and -T and China-Y isolates showed no obvious patterns of differences, indicating no proof of genetic reassortment (Qu et al., 1997). On another hand, other members of tenuiviruses have been reported with multiple deletion/insertions in the intergenic region on nucleotide level in both RNAs 3 and 4. For instance, the two isolates of RHBV (de Miranda et al., 1997) and RGSV (Miranda et al., 2000) where RNAs 3 and 4 are equivalent to RNAs 5 and 6 also indicated that RNA4 (or RNA6 in RGSV) is more diverged than RNA3 (or RNA5 in RGSV) suggesting a possibility of reassortment. However, the involvement of genetic reassortment in the formation of natural populations of RSV in Korea would be more evident once the complete sequences of RSV genome and chronological RSV sequences become available.

Although, there are other several factors may have led to the re-emergence of RSV disease in Korea. These factors may include changes in the insect population, the virus virulence, the environment favoring the disease occurrence and even change in the cultivation practices causing breakdown of resistant cultivars. The proportion of BPH carrying RSV investigated in 4 provinces of Korea during 2007-2008 ranged from 1-22% (Kim et al., unpublished). This relatively high percent of active transmitter of BPH population in Korea could be one of the causes of rapid spread of RSV infection in the entire country. Apparently, during these periods, high incidences of RSV were also observed in China (Wang et al., 2008). Geographical proximity of Korea, Japan and China possibly explains why the RSV isolates in these countries shared high genome sequence homology. The nucleotide sequences of the natural population of RSV revealed in this study demonstrated that different genotypes of RNAs 3 and 4 are present in Korea. However, the biological significance of the difference in RNA3 and 4 genomes is not yet determined. Identification and isolation of RSV-infected plants that carries these different types of RNAs 3 and 4 genome segments may facilitate a better understanding of the molecular mechanism underlying the pathogenesis of RSV. Such information also might be useful for characterization of evolution and epidemiological patterns of RSV to develop control strategies of RSV particularly in Korea.

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