The Journal of Microbiology, December 2008, p. 709-719
DOI 10.1007/s12275-008-0224-x

Vol. 46, No. 6

Molecular Characterization of ORFs 2 to 7 of Korean Porcine Reproductive and Respiratory Syndrome Virus (CA) and Its Protein Expression by Recombinant Baculoviruses

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(Received September 11, 2008 / Accepted September 30, 2008)

To determine the characteristics of the Korean porcine reproductive and respiratory syndrome virus (PRRSV), CA, which was isolated from the serum of an infected pig in 2006, we investigated the nucleotide sequence and expression of the structural ORFs (ORFs 2 to 7) using the bApGOZA system. We found that the structural ORFs 2 to 7 of CA consisted of 3188 nucleotides that were the same as those formed from VR-2332. Comparison of the CA with the other strains revealed nucleotide sequence identity ranging from 89.8 to 99.5%. To better understand the genetic relationships between other strains, phylogenetic analyses were performed. The CA strain was closely related to the other North American genotype strains but formed a distinct branch with high bootstrap support. Additionally, expression levels of the PRRSV proteins in insect cells were strong or partially weak. The results of this study have implications for both the taxonomy of PRRSV and vaccine development.

***Keywords*:** PRRSV, CA strain, phylogenetic analysis, baculovirus, bApGOZA system

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important diseases affecting the swine industry worldwide, characterized by reproductive failure in late term gestation in sows and respiratory disease in pigs of all ages. The causative agent, the PRRS virus (PRRSV), is a member of the family Arteriviridae and belongs to the order Nidovirals, along with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and the simian hemorrhagic fever virus (SHFV) (Cavanagh, 1997; Snijder and Meulenberg, 1998). PRRSV is an enveloped virus containing a 15 kb positive strand RNA genome (Meulenberg *et al*., 1997) with a poly (A) tail at its 3'-end. The viral genome contains nine open reading frames (ORFs). The first two ORFs (1a and 1b) comprise the 5'-terminal two-thirds of the viral genome and encode the RNA-dependent RNA polymerase. The ORFs 2∼7 are postulated to encode for structural proteins. Proteins encoded by ORFs 2∼4 are designated GP2, GP3, and GP4 and, postulated to be associated with the viral membrane (Mardassi *et al*., 1995; Van Nieuwstadt *et al*., 1996). The major structural proteins are 15 kDa nucleocapsid (N), 18∼19 kDa matrix (M), and 25 kDa envelope (GP5) proteins that are encoded by ORFs 7, 6, and 5, respectively (Meulenberg *et al*., 1995; Murtaugh *et al*., 1995). Despite the strong immunogenicity of the N protein, it has not been associated with protection. The M protein may be involved in virus assembly and budding and contains three highly hydrophobic regions in its N-terminal half, which are assumed to represent potential membrane-spanning domains, with only 10∼18 amino acids being exposed on the virion surface (Mardassi *et al*., 1995; Mardassi *et al*., 1996; Plagemann, 1996). GP5 is the most important glycoprotein of PRRSV involved in the generation of PRRSV-neutralizing antibodies and protective immunity (Bastos *et al*., 2004; Jiang *et al*., 2006).

PRRSVs can be divided into European (type I) and North American (type II) genotypes, which are represented by the Lelystad and VR-2332 strains, respectively. The two genotypes share less than 60% nucleotide identity (Nelsen *et al*., 1999) and are distinguished not only genetically but also antigenically (Meng, 2000). Genetic variation among PRRSVs within each genotype also has been reported, and viruses tend to be clustered by geographical origin (Forsberg *et al*., 2002; Larochelle *et al*., 2003; Mateu *et al*., 2003; Thanawongnuwech *et al*., 2004). However, the genetics of the Korean PRRSVs are not yet well characterized. Kang *et al*. (2004) determined the first complete genomic sequence

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from the Korean PRRSV isolate PL97-1 and then compared it with the sequences of 11 other PRRSV isolates (3 each from the US and, China, 1 each from Canada and the UK, and 3 vaccine strains). They found that PL97-1 was most closely related to the North American genotype VR-2332, a VR-2332-derived vaccine strain, and the Chinese genotype BJ-4, but they did not further characterize the Korean PRRSV genes. Recently, Cha *et al*. (2006) and Yoon *et al*. (2008) determined the complete ORF5 and ORF7 sequences of Korean PRRSV isolates and compared them with previously published sequences, respectively. Previous phylogenetic analyses have mainly focused on partial ORF5 sequences (Meng *et al*., 1995a; Mateu *et al*., 2003), which appears to be the most variable protein when the American and European isolates are compared and which shows the highest degree of genetic diversity within a single genotype. Other studies have also investigated the PRRSV genetic relationships by comparing the ORF7 nucleotide sequences (Forsberg *et al*., 2002; Stadejek *et al*., 2002) or other proteins (Meng *et al*., 1995b; Oleksiewicz *et al*., 2000).

The baculovirus expression vector system is a helper-independent system that has found extensive use for the expression of heterologous genes. *Autographa californica* nucleopolyhedrovirus (AcNPV) is the prototype baculovirus strain and the virus most commonly used in expression vector systems. The target gene is cloned into an appropriate transfer vector such that it is flanked by viral DNA sequences to allow recombination. Insect cells are then co-transfected with viral DNA and the modified transfer vector. Double recombination between these DNAs results in the transfer of the target gene to the viral genome. bApGOZA system is AcNPV-based recombinant baculovirus (Je *et al*., 2001) and combines recombination with a deleted viral genome lacking part of the essential ORF1629 (Kitts and Possee, 1993) and recombination with a viral genome maintained in *E. coli* (Luckow *et al*., 1993). Therefore, the bApGOZA system facilitates the rapid and efficient generation of recombinant virus.

The aims of this study were (1) to determine the nucleotide sequences of the structural ORFs (ORFs 2 to 7) of CA, the PRRSV recently isolated in Korea; (2) to assess the phylogenetic relationships between CA and those from other countries; and (3) to characterize the proteins encoded by ORFs 2 to 7.

Materials and Methods

Virus and cells

The CA strain (KCTC 11131BP) was isolated from the sera of field pigs at the acute stage of PRRSV infection in 2006. The virus was passaged twice on sub-confluent monolayers of MARC-145 cells in minimum essential medium supplemented with 10% fetal bovine serum (Gibco BRL, UK), nonessential amino acids, sodium pyruvate, and antibiotics in 5% CO_2 at 37°C. The virus was collected when 70% of the cells showed cytopathology. The supernatant was stored

Table 1. Primers used for amplification and sequencing of PRRS viral genome

Primer	Sequence[a]	Fragment position
ORF2F	5'-GGAATTCGCCACCATGAAATGGGGTCCATGCAAA-3'	12073-12843
ORF2R	5'-AACTGCAGTCACCGTGAGTTCGAAAGAAAAA-3'	
ORF3F	5'-GGAATTCGCCACCATGGTTAATAGCTGTACATTC-3'	12696-13460
ORF3R	5'-AACTGCAGCTATCGCCGTACGGCACTGAG-3'	
ORF4F	5'-GGAATTCGCCACCATGGCTTCGTCCCTTCTTTT-3'	13241-13777
ORF4R	5'-AACTGCAGTCAAATTGCCAACAGAATGGCAA-3'	
ORF5F	5'-GGAATTCGCCACCATGTTGGAGAAATGCTTGACC-3'	13788-14390
ORF5R	5'-AACTGCAGCTAAGGACGACCCCATTGTTCC-3'	
ORF6F	5'-GGAATTCGCCACCATGGGGTCGTCCTTAGATGACT-3'	14375-14899
ORF6R	5'-AACTGCAGTTATTTGGCATATTTGACAAGGTT-3'	
ORF7F	5'-GGAATTCGCCACCATGCCAAATAACAACGGCAA-3'	14889-15260
ORF7R	5'-AACTGCAGTCATGCTGAGGGTGATGCTGTG-3'	
ORF1-2F	5'-CAAACATACCTGGGGGTTTGAAT-3'	11871-12373
ORF1-2R	5'-GGTGCCATAACATCCCCAAAGG-3'	
ORF4-5F	5'-GGGATTTAAGGTGGTATTTGGCA-3'	13576-14086
ORF4-5R	5'-CCGGCGGTAGACACAGTGACC-3'	
ORF5-6F	5'-GGCGCTACTCATGTACCAGATA-3'	14188-14673
ORF5-6R	5'-CGGCATCTGGAGGTGATGAATTTC-3'	
ORF6-7F	5'-GGCCCCTGCCCACCACGTTG-3'	14704-15195
ORF6-7R	5'-CCACAGTGTAACTTATCCTCCC-3'	
ORF7-eF	5'-GCGACTGAAGATGATGTCAGAC-3'	15060-15411
ORF7-eR	5'-AATTTCGGCCGCATGGTTCTCG-3'	

[a] Restriction sites are underlined

Table 2. PRRSV isolates used in this study

Virus strain	Country of isolation	GenBank accession no.
PA8	Canada, 1995	AF176348
CH-1a	China, 1996	AY032626
HB-1(sh)/2002	China, 2002	AY150312
BJ-4	China, 2000	AF331831
Em2007	China	EU262603
EDRD-1	Japan, 1992	AB288356
PL97-1	Korea, 1997	AY585241
PL97-1/LP1	Korea	AY612613
Lelystad	Netherlands, 1991	M96262
01CB1	Thailand	DQ864705
01NP1.2	Thailand	DQ056373
VR-2332	USA, 1989	U87392
16244B	USA, 1997	AF046869
NVSL 97-7985	USA, 1997	AF325691
P129	USA, 1995	AF494042
MN184C	USA	EF488739
RespPRRS MLV	Vaccine strain	AF066183
SP	Vaccine strain (Prime Pac)	AF184212
CA	Korea, 2006	This study, FJ194950

at -80°C as the viral stock. *Spodoptera frugiperda* (Sf) 21 cells were cultured at 27°C in Sf-900 II SFM media (Gibco BRL, UK) following previously published procedures (O'Reilly *et al*., 1992).

RT-PCR

Viral RNA was extracted from infected cell cultures with Viral Gene-spin™ Kit (iNtRON Biotechnology, Korea) as recommended by the manufacturer. The viral RNA was used as a template for cDNA synthesis using RNA LA PCR Kit (TaKaRa, Japan). Primers were based on the consensus sequence of the fully sequenced PRRSV RNA genomes available from GenBank. PCR amplification was performed using AccuPower PCR Premix (Bioneer Co., Korea). Primers used for PCR amplification are listed in Table 1.

Cloning and nucleotide sequencing of PRRSV ORFs 2 to 7

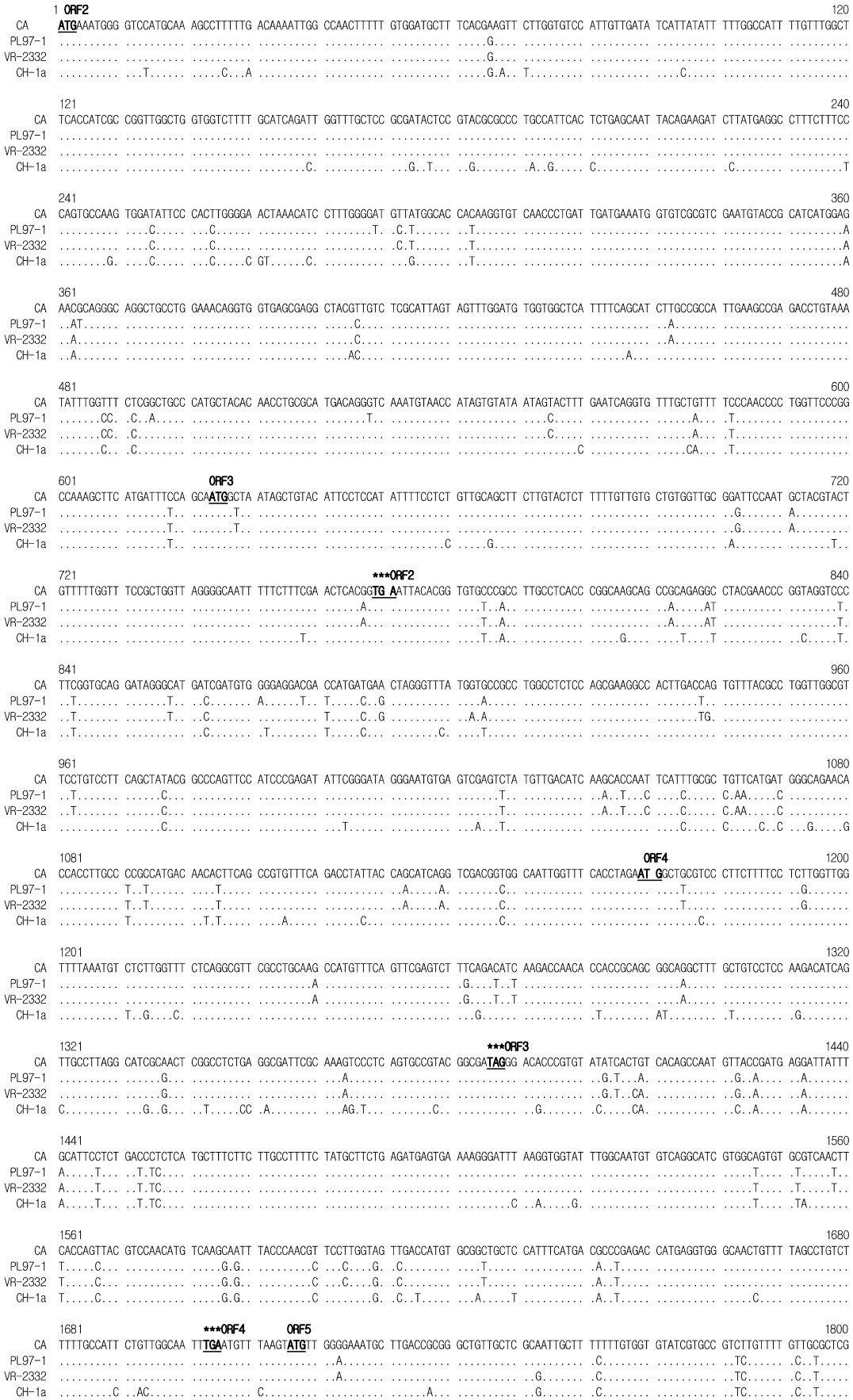
The purified RT-PCR products were cloned into the T&A cloning vector (RBC Bioscience, Taiwan), and the plasmids were named as pTA-ORF2, -ORF3, -ORF4, -ORF5, -ORF6, and -ORF7, respectively. The positive clones were first screened by agarose gel electrophoresis of the plasmids isolated with a LaboPass Mini-prep Kit (CosmoGenetech, Korea), followed by restriction endonuclease digestion and/or nucleic acid sequencing. Restriction endonucleases were purchased from commercial sources (TaKaRa, Japan), and used according to the supplier's instructions. Nucleic acid sequencing of the clones and/or PCR fragments was performed by SolGent Co. (Daejeon, Korea) using ABI 3730XL Capillary DNA Sequencer. This nucleotide sequence has been submitted to the GenBank database under accession number FJ194950. Antigenic determinants were predicted using a computer program, PROTEAN (DNASTAR, USA), which takes into account hydrophilicity, surface probability, chain flexibility, hydropathy, and secondary structure.

Multiple alignments and phylogenetic analyses

The GenBank accession numbers of the fully sequenced PRRSV strains used in the sequence alignments and phylogenetic analyses are detailed in Table 2. Multiple sequence alignments were performed using the CLUSTAL X program (Thompson *et al.*, 1997). Percentage sequence divergences between aligned nucleotide sequences were calculated using CLUSTAL X. The phylogenetic unrooted and rooted trees were reconstructed on aligned nucleotide sequences by using the neighbor-joining method (Saitou and Nei, 1987). The Lelystad virus (M96262) was used as the outgroup in all analyses. Constructed neighbor-joining trees were subjected to bootstrap analysis using 1000 replicates (Felsenstein, 1985) to assess confidence values of virus groupings, and a distance matrix was obtained from bootstrapped datasets by the Kimura method (Kimura, 1980). All trees were drawn using TreeView software (Page, 1996).

Construction of recombinant transfer vectors and viruses

The plasmids (pTA-ORFs 2 to 7) were digested with *Eco*RI and *Pst*I, and then cloned into the transfer vector pBacPAK9 (Clontech, USA), to get the recombinant transfer plasmid pBPRSV-ORFs 2 to 7. Sf21 cells were co-transfected with a


1 ORF2 120
CA ATGAAATGGG GTCCATGCAA AGCCTTTTTG ACAAAATTGG CCAACTTTTT GTGGATGCTT TCACGAAGTT CTTGGTGTCC ATTGTTGATA TCATTATATT TTTGGCCATT TTGTTTGGCT
PL97-1 ....G.... ....
VR-2332 ....G.... ....
CH-1a ....T..... .....C...A ....G.A.. T..... ....C..... ....
121 240
CA TCACCATCGC CGGTTGGCTG GTGGTCTTTT GCATCAGATT GGTTTGCTCC GCGATACTCC GTACGCGCCC TGCCATTCAC TCTGAGCAAT TACAGAAGAT CTTATGAGGC CTTTCTTTCC
PL97-1 ....
VR-2332 ....
CH-1a ....C. ....G..T... ..G..... .A..G..... C..... ..... .C..... .....T
241 360
CA CAGTGCCAAG TGGATATTCC CACTTGGGGA ACTAAACATC CTTTGGGGAT GTTATGGCAC CACAAGGTGT CAACCCTGAT TGATGAAATG GTGTCGCGTC GAATGTACCG CATCATGGAG
PL97-1 ....C..... ...C..... ....T. .C.T..... ..T..... ....A
VR-2332 ....C..... ...C..... ....C.T..... ..T..... ....A
CH-1a .....G. ....C..... ...C.....C GT......C. ....G..... ..T..... ....A
361 480
CA AACGCAGGGC AGGCTGCCTG GAAACAGGTG GTGAGCGAGG CTACGTTGTC TCGCATTAGT AGTTTGGATG TGGTGGCTCA TTTTCAGCAT CTTGCCGCCA TTGAAGCCGA GACCTGTAAA
PL97-1 ..AT..... ....C.... ....A..... ....
VR-2332 ..A..... ....C.... ....A..... ....
CH-1a ..A..... ....AC.... ....A... ....
481 600
CA TATTTGGTTT CTCGGCTGCC CATGCTACAC AACCTGCGCA TGACAGGGTC AAATGTAACC ATAGTGTATA ATAGTACTTT GAATCAGGTG TTTGCTGTTT TCCCAACCCC TGGTTCCCGG
PL97-1 .....CC. .C..A..... ....T.. ....C..... ....A... .T..... .....
VR-2332 .....CC. .C..... ....C..... ....A... .T..... .....
CH-1a .....C.. .C..... ....C ....CA... .T..... .....
601 ORF3 720
CA CCAAAGCTTC ATGATTTCCA GCAATGGCTA ATAGCTGTAC ATTCCTCCAT ATTTTCCTCT GTTGCAGCTT CTTGTACTCT TTTTGTTGTG CTGTGGTTGC GGATTCCAAT GCTACGTACT
PL97-1 ....T.. .....T.. ....G..... A.....
VR-2332 ....T.. .....T.. ....G..... A.....
CH-1a ....T.. ....C .....G.... .... .A..... .....T..
721 ***ORF2 840
CA GTTTTTGGTT TCCGCTGGTT AGGGGCAATT TTTCTTTCGA ACTCACGGTG AATTACACGG TGTGCCCGCC TTGCCTCACC CGGCAAGCAG CCGCAGAGGC CTACGAACCC GGTAGGTCCC
PL97-1 ....A... ....T..A.. ....A....AT ....T.
VR-2332 ....A... ....T..A.. ....A....AT ....T.
CH-1a ....T.. ....T..A.. ....G.... ....T....T ....C.....T.
841 960
CA TTCGGTGCAG GATAGGGCAT GATCGATGTG GGGAGGACGA CCATGATGAA CTAGGGTTTA TGGTGCCGCC TGGCCTCTCC AGCGAAGGCC ACTTGACCAG TGTTTACGCC TGGTTGGCGT
PL97-1 ..T..... .....T.. ..C..... A.....T.. T.....C..G ....A..... ....T.. ....
VR-2332 ..T..... .....T.. ..C..... ..... T.....C..G ....A.A..... ....TG. ....
CH-1a ..T..... ....C..... .T..... T.....C... .....C. ....T..... ....
961 1080
CA TCCTGTCCTT CAGCTATACG GCCCAGTTCC ATCCCGAGAT ATTCGGGATA GGGAATGTGA GTCGAGTCTA TGTTGACATC AAGCACCAAT TCATTTGCGC TGTTCATGAT GGGCAGAACA
PL97-1 ..T..... ......C... ....T.. ....A..T...C ....C..... C.AA.....C .....
VR-2332 ..T..... ......C... ....T.. ....A..T...C ....C..... C.AA.....C .....
CH-1a ....C... ....T..... ....A...T.. ....C..... C.....C..C ...G.....G
1081 ORF4 1200
CA CCACCTTGCC CCGCCATGAC AACACTTCAG CCGTGTTTCA GACCTATTAC CAGCATCAGG TCGACGGTGG CAATTGGTTT CACCTAGAAT GGCTGCGTCC CTTCTTTTCC TCTTGGTTGG
PL97-1 ..... T..T..... ....T..... ....A.....A. .....C.. ....T..... ....G.....
VR-2332 ..... T..T..... ....T..... ....A.....A. .....C.. ....T..... ....G.....
CH-1a ..... T..... ..T.T..... ....A..... ......C... ....C.. ....C.. ....
1201 1320
CA TTTTAAATGT CTCTTGGTTT CTCAGGCGTT CGCCTGCAAG CCATGTTTCA GTTCGAGTCT TTCAGACATC AAGACCAACA CCACCGCAGC GGCAGGCTTT GCTGTCCTCC AAGACATCAG
PL97-1 ....A .... .G....T..T ....A..... ....
VR-2332 ....A .... .G....T..T ....A..... ....
CH-1a ..... T..G....C. ....G..... ..... .T..... AT..... .T..... .G.....
1321 ***ORF3 1440
CA TTGCCTTAGG CATCGCAACT CGGCCTCTGA GGCGATTCGC AAAGTCCCTC AGTGCCGTAC GGCGATAGGG ACACCCGTGT ATATCACTGT CACAGCCAAT GTTACCGATG AGGATTATTT
PL97-1 ....G... ....A..... ....G.T...A. ....G..A.... ..A.....
VR-2332 ....G... ....A..... ....G.T..CA. ....G..A.... ..A.....
CH-1a C..... ...G..G... ..T.....CC .A..... ...AG.T... .....C.. ....G..... .C.....CA. ....C..A.... ..A.....
1441 1560
CA GCATTCCTCT GACCCTCTCA TGCTTTCTTC TTGCCTTTTC TATGCTTCTG AGATGAGTGA AAAGGGATTT AAGGTGGTAT TTGGCAATGT GTCAGGCATC GTGGCAGTGT GCGTCAACTT
PL97-1 A.....T... ..T.TC.... ....T.... .T.....T..
VR-2332 A.....T... ..T.TC.... ....T.... .T.....T..
CH-1a A.....T... ..T.TC.... ....C ..A.....G. ....T.... .TA.....
1561 1680
CA CACCAGTTAC GTCCAACATG TCAAGCAATT TACCCAACGT TCCTTGGTAG TTGACCATGT GCGGCTGCTC CATTTCATGA CGCCCGAGAC CATGAGGTGG GCAACTGTTT TAGCCTGTCT
PL97-1 T.....C... ....G.G.. ....C ...C....G. .C..... ....T..... ..... .A..T..... ....
VR-2332 T.....C... ....G.G.. ....C ...C....G. .C..... ....T..... ..... .A..T..... ....
CH-1a T.....C... ....G.G.. ....C ....G. .C..T..... ...A.....T ..... .A..T..... ....C.... .....
1681 ***ORF4 ORF5 1800
CA TTTTGCCATT CTGTTGGCAA TTTGAATGTT TAAGTATGTT GGGGAAATGC TTGACCGCGG GCTGTTGCTC GCAATTGCTT TTTTTGTGGT GTATCGTGCC GTCTTGTTTT GTTGCGCTCG
PL97-1 ....A..... .... .C..... ....TC..... .C..T.....
VR-2332 ....A..... ....G..... .C..... ....TC..... .C..T.....
CH-1a .....C ..AC..... ..... C..... ....A... ....G..... .C..... ....TC..... .C..T.....

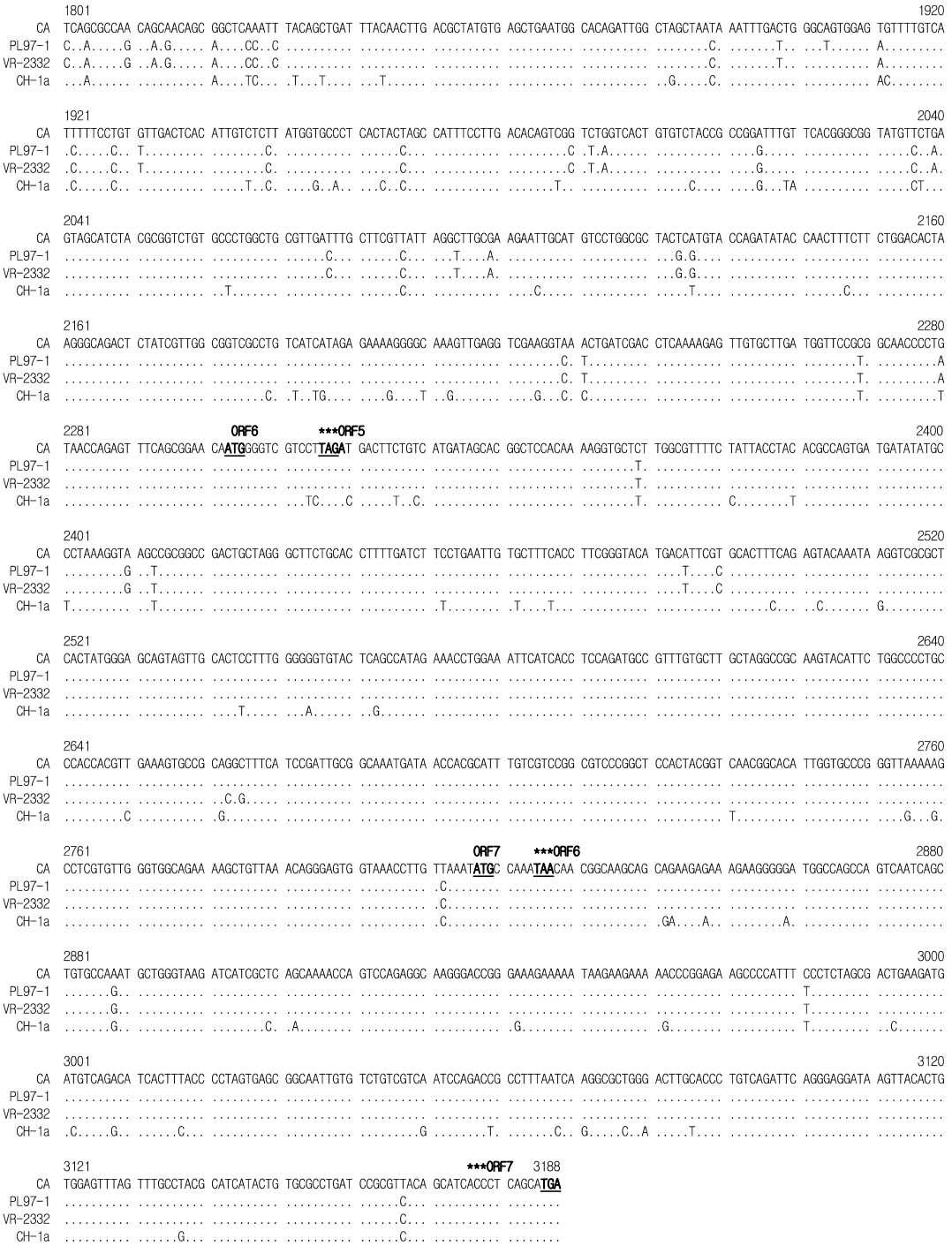


Fig. 1. Nucleotide sequence comparison of the structural ORFs 2 to 7 of CA strain sequenced in this study with other known PRRSV isolates. The start and stop codons of each ORF are shown in bold and underlined. The stop codon is indicated with asterisks.

mixture of purified transfer vector each containing a PRRSV gene, bApGOZA DNA (Je *et al*., 2001), and Cellfectin (Invitrogen, USA), according to the manufacturer's instruction. Briefly, 100 ng of bApGOZA DNA and 500 ng of transfer vector DNA were mixed in a polystrene tube. Fifty microliters of Cellfectin was gently mixed with the DNA solution, and the mixture was incubated at room temperature for 1 h. The Cellfectin-DNA complexes were added dropwise to the medium covering the cells while the dish was gently swirled. After incubating at 27°C for 4 h, media were removed, and the cells were washed twice with fresh medium. Five days after adding the Cellfectin-DNA complexes to the cells, the medium containing viruses released by the transfected cells was transferred to a sterile container

Table 3. Percent nucleotide (NT) and deduced amino acid (AA) identities of the structural ORFs between CV and PL97-1, VR-2332, and CH-1a

ORF	Protein product	PL97-1		VR-2332		CH-1a	
		NT	AA	NT	AA	NT	AA
ORF2	GP2	96.8	96.9	97.3	97.7	94.6	95.3
ORF3	GP3	94.0	94.1	94.0	94.1	92.7	93.7
ORF4	GP4	93.7	94.4	93.3	94.4	89.8	92.1
ORF5	GP5	93.5	90.5	93.4	90.5	91.2	91.0
ORF6	M	99.0	99.4	98.5	98.9	95.4	98.3
ORF7	N	99.5	100	99.2	100	94.1	99.2

and stored at 4°C. A standard plaque assay procedure (O'Reilly *et al*., 1992) was used to obtain viral plaques from dilutions of the media harvested from the co-transfections. Individual plaques were examined under a microscope and scored for polyhedra production.

Western blot

Sf21 cells were infected with AcNPV (wild type) or each recombinant virus in a 60-mm diameter dish (2×10^6 cells) at a multiplicity of infection (MOI) of 5 PFU per cell. After incubation at 27°C, cells were harvested at 3 days post-inoculation (p.i.). For SDS-PAGE (Laemmli, 1970) of cell lysates, AcNPV- or recombinant virus-infected cells were washed twice with PBS and mixed with protein sample buffer and boiled. The total cellular lysates were subjected to 12% SDS-PAGE. For Coomassie stains, gels were washed with deionized water and stained with BioSafe Coomassie. For Western blot analysis, 12% SDS-PAGE was performed as described above. Proteins of cellular lysates were blotted on-to a nitrocellulose membrane (Pall Corp., USA). After transfer, the membrane was incubated for 60 min with gentle agitation in 5% (w/v) non-fat dry milk in TBST buffer (20 mM Tris-HCl; pH 7.5, 150 mM NaCl, 0.05% Tween 20). The blot was incubated with PRRSV-specific antiserum (Choongang Vaccine Lab., Korea) in TBST for 1 h and washed. Subsequently, the membrane was incubated with rabbit anti-pig IgG horseradish peroxidase conjugate for 30 min at room temperature. After repeated washing, the immunoreactive bands were visualized with the ECL Western Blotting Detection System (Elpis Biotech, Korea).

Results and Discussion

Comparison of the structural ORFs sequences

To characterize CA, the Korean PRRSV strain that was isolated from the serum of an infected pig in 2006, we determined the nucleotide sequence of its structural ORFs (ORFs 2 to 7) as described in 'Materials and Methods' in detail. Primers for each ORF based on the consensus sequence of the other PRRSV RNA genomes are available from GenBank. Therefore, to confirm that the nucleotide sequence of the primer region was corrected, we performed additional PCR amplification using ORF1-2, ORF4-5, ORF 5-6, ORF6-7, and ORF7-e primer (Table 1). The N-terminal region of ORF2 and the C-terminal region of ORF3, and the N-terminal region of ORF3 and the C-terminal region of ORF4 were overlapped by 148 bp and 220 bp, respectively, these regions were excluded from analysis. The sequence was aligned with that of the other fully sequenced PRRSV genomes. As shown in Fig. 1, the length of CA structural ORFs is 3188 bp long, which was the same length as PL97-1 (Korean isolate), VR-2332 (American isolate), and CH-1a (Chinese isolate). No additions or deletions were observed in the genomic sequence of CA. As previously reported, each ORF partially overlapped. A detailed sequence identity among CA, PL97-1, VR-2332, and CH-1a for each ORF is summarized in Table 3. The identities between CA and other PRRSV strains ranged from 89.8 to 99.5% for the nucleotide sequences and from 90.5 to 100% for the amino acid sequences. The CA strain was most closely related to PL97-1, the Korean isolate, but it was not perfectly identical. Also, it is distantly related to the European genotype (data not shown).

Phylogenetic analysis of the structural ORFs sequences

To establish the genetic relationships of the sequenced CA strains, phylogenetic analyses were performed on the structural ORFs. A phylogenetic tree reveals that there are two distinct phylogenetic groups based on their geographical origin (Fig. 2A and B). The European genotype consists of the Dutch Lelystad strain and the 01CB1 (Thailander) strain, while the North American genotype contains the other 16 PRRSV isolates, which are from Canada, China, Japan, Korea, Thailand, and the USA. Two separate clusters with high bootstrap support were defined in the North American genotype. In the first cluster, the six isolates from Canada (PA8), VR-2332-derived vaccine strain (RespPRRS MLV), China (BJ4), Thailand (01NP1.2), and Korea (PL97-1 and PL97-1/LP1) were closely related to the VR-2332, but each formed a single minor branch. The American 16244B strain was grouped in the first cluster but formed a single minor branch. The CA strain isolated in this study also grouped in the first cluster but formed a single minor branch. As we expected, the CA strain belongs to the North American genotype. In the second cluster, three Chinese isolates CH-1a, HB-1(sh)/2002 and Em2007 were most closely related to the American P129 strain. The American NVSL 97-7985 strain was also grouped in the second cluster but formed a single minor branch. The SP vaccine strain, EDRD-1, and MN184C belonged to the North American genotype but formed a distinct branch. Subsequently, we performed phylogenetic analyses based on the ORF5 and ORF7 using the

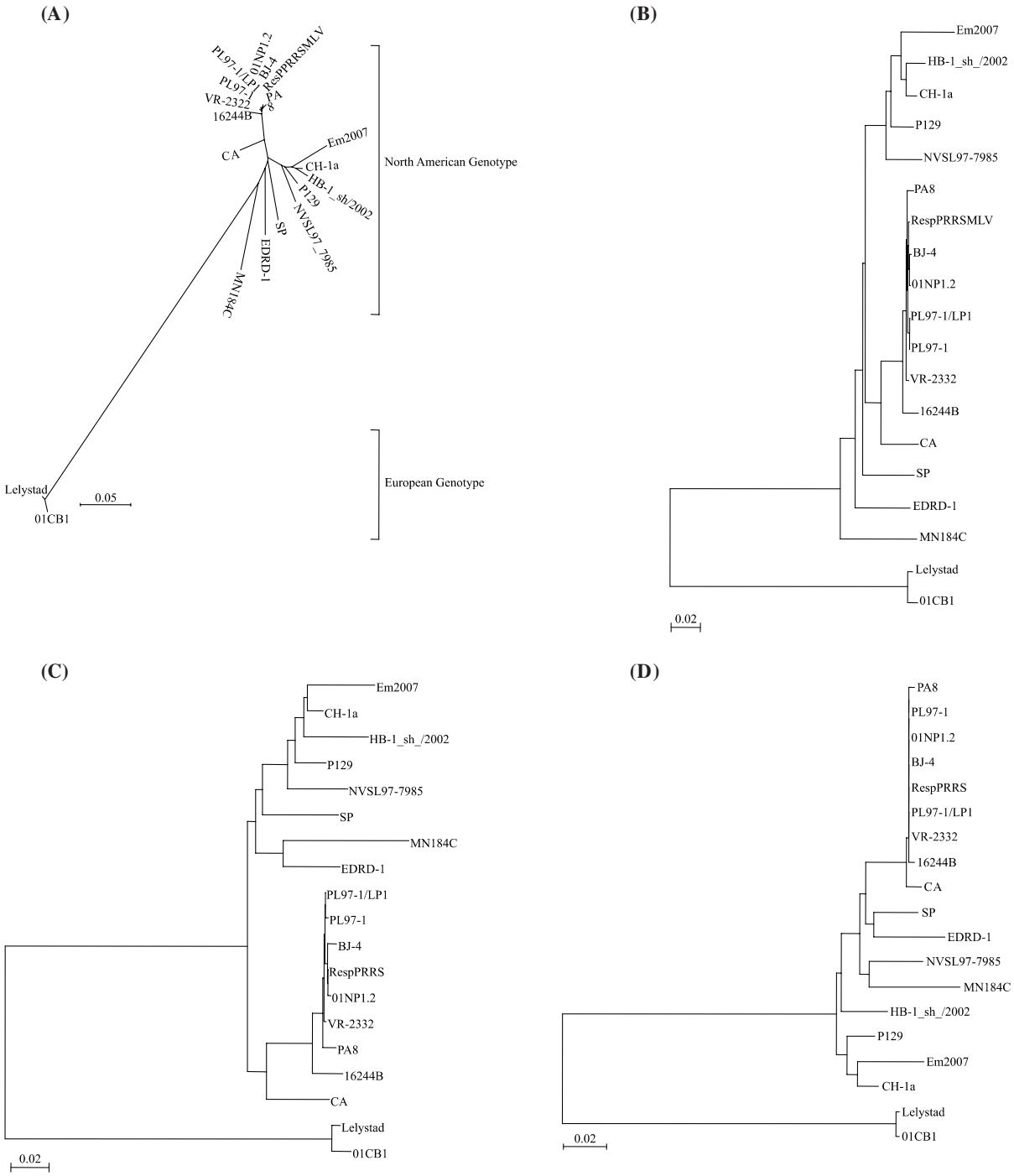


Fig. 2. Phylogenetic trees based on the nucleotide sequence of the structural ORFs 2 to 7 (A and B), ORF5 (C), and ORF7 (D) of CA strain. The tree was constructed by the neighbor-joining method based on genetic distances calculated by Kimura's two-parameter method. Lelystad virus was used as the outgroup. The reliability of the tree was assessed by bootstrap analysis with 1000 replications. Scale bars at the bottom of each tree represent the number of nucleotide substitutions per site. The references for the sequences of PRRSV isolates used in the phylogenetic analyses are cited in Table 2.

18 fully sequenced strains. ORF5, which encodes for the major envelope protein of the virus, has been the target gene for molecular epidemiology of PRRSVs by sequence analysis because ORF5 has shown the highest genetic variability among PRRSVs (Key *et al*., 2001). Kang *et al.* (2004) noted that the phylogenetic tree derived from ORF7 most closely resembles the tree derived from the full-length genomes of PRRSVs. The phylogenetic trees based on the ORF5 and 7 genes revealed overall similar tree topologies to the whole structural ORFs-based phylogenetic tree, but

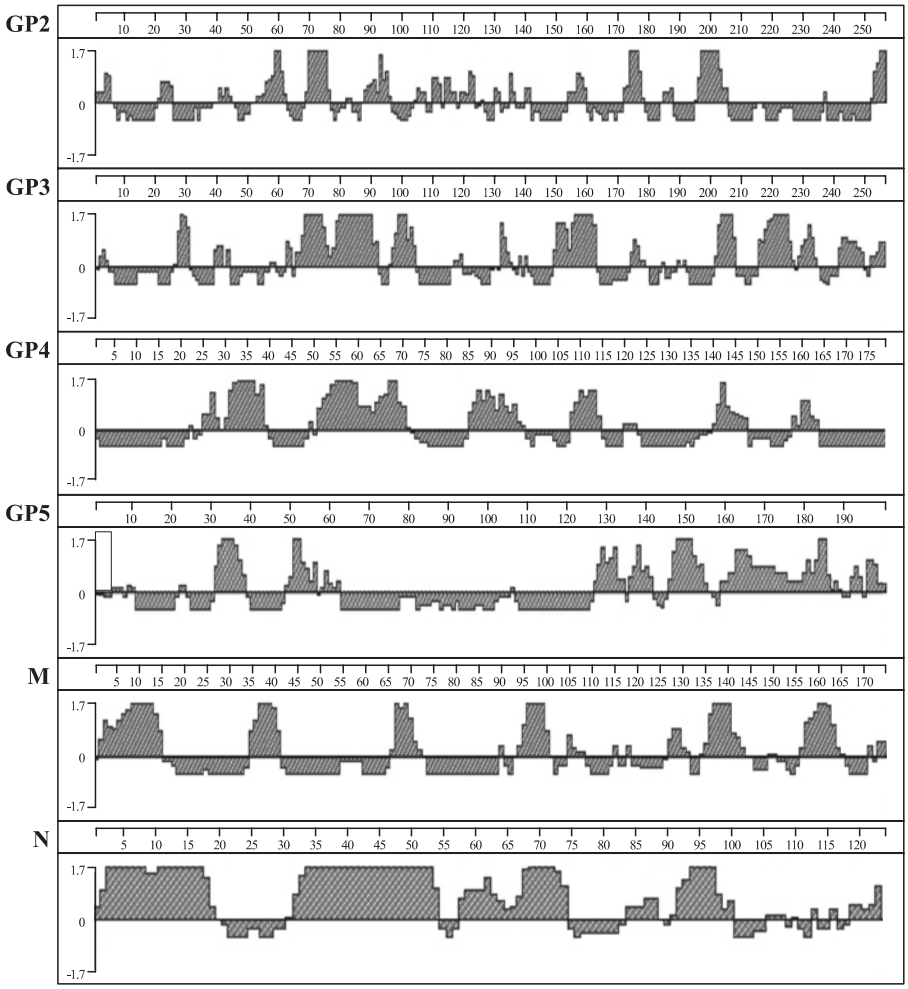


Fig. 3. Antigenicity plot for the structural proteins of CA strain. The Jameson-Wolf antigenicity plot was generated using a computer program, PROTEAN (DNASTAR, USA). *x*-axis values are amino acid residues for each protein; the *y*-axis represents the antigenic index. A high score indicates high antigenicity (Jameson and Wolf, 1988).

with minor differences. In the ORF5 tree, the CA strain was related to members of the first cluster of the North American genotype, but it forms a distinct branch (Fig. 2C). In the ORF7 tree, the CA strain was related to the members of the first cluster of the North American genotype, but it forms a single minor branch (Fig. 2D). The ORF7 gene-based analysis also showed high genetic variation exists not only between two genotypes but also within each genotype. Although the functional significance of this genetic variation remains to be determined, antigenic differences between two genotypes on a serological basis have been reported (Katz *et al*., 1995; Sorensen *et al*., 1998).

Predicted antigenic differences

Figure 3 shows the antigenicity plots of the structural proteins (GP2, GP3, GP4, GP5, M, and N) of the CA strain. As previously reported, the N protein was highly antigenic, which therefore makes it a suitable candidate for detection with virus-specific antibodies and diagnosis of the disease (Seuberlich *et al*., 2002; Inoue *et al*., 2007). However, the most important glycoprotein of PRRSV involved in the generation of PRRSV-neutralizing antibodies and protective immunity is the GP5 protein (Bastos *et al*., 2004; Jiang *et al*., 2006). As shown in Fig. 3, GP5 protein is triple-membrane spanning, and it likely functions as a receptor-binding protein (Mardassi *et al*., 1995; Meng *et al*., 1995a; Murtaugh *et al*., 1995). According to several reports, the GP3, GP4, and M proteins are also highly antigenic and may be involved in viral neutralization along with the GP5 protein (Meng *et al*., 1995b; Meulenberg *et al*., 1997; Hedges *et al*., 1999).

Expression of PRRSV proteins

To generate the recombinant baculoviruses, Sf21 cells were co-transfected with a mixture of bApGOZA and pBPRSV-ORFs 2 to 7 as described in Methods. The new baculoviruses were called rApRSV-ORF2, rApRSV-ORF3, rApRSV-ORF4, rApRSV-ORF5, rApRSV-ORF6, and rApRSV-ORF7 (Fig. 4A). They contained the respective ORF gene inserted in the genome as demonstrated by PCR (Fig. 4B). Sf21

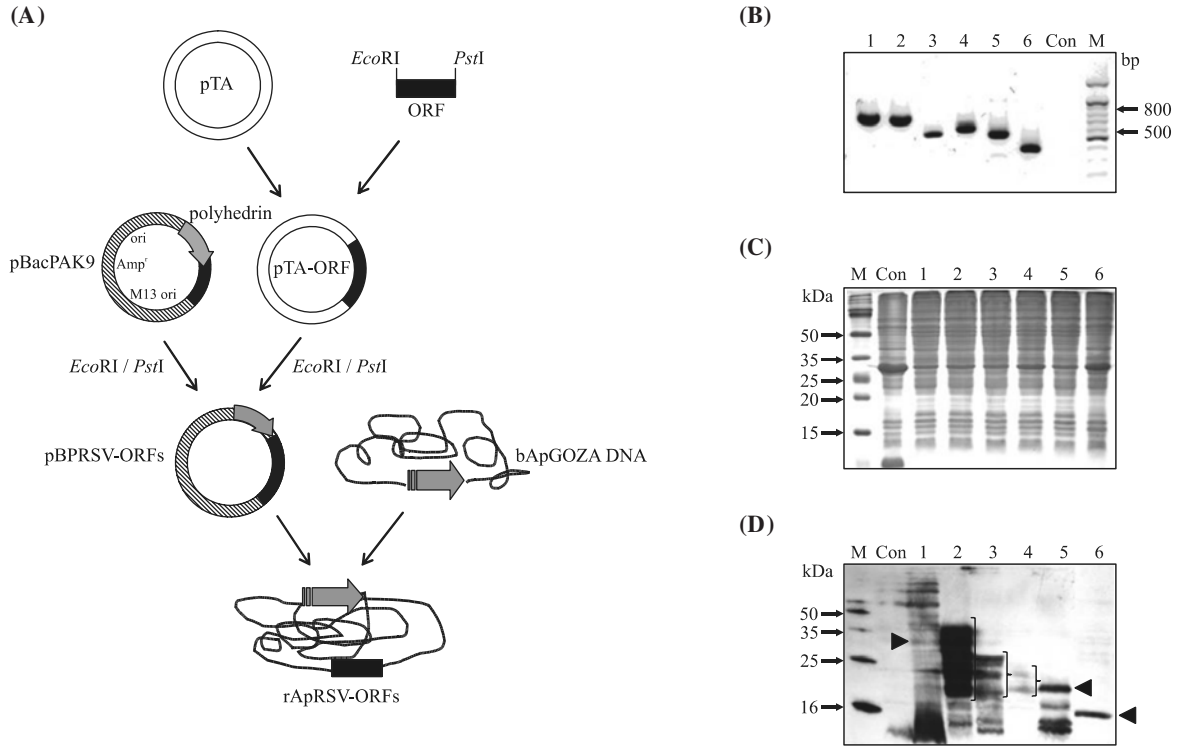


Fig. 4. Expression of CA structural proteins in insect cells. Construction (A) and PCR identification (B) of recombinant viruses SDS-PAGE (C) and Western blotting (D) of recombinant proteins. Sf21 cells were infected at an MOI of 5 PFU per cell with the recombinant viruses. Cells were harvested at 3 days post-infection. Proteins were separated on a 12% SDS-PAGE, transferred to nitrocellulose membranes for immunoblot analysis and reacted with porcine anti-PRRSV antibodies (1:100). ORF, each gene of ORFs 2 to 7; M, DNA (B) and protein (C and D) size marker; Con, wild type AcNPV; lane 1, rApRSV-ORF2; 2, rApRSV-ORF3; 3, rApRSV- ORF4; 4, rApRSV-ORF5; 5, rApRSV-ORF6; 6, rApRSV-ORF7. The recombinant proteins are indicated with arrowheads and black brackets.

cells were infected at a MOI of 5 PFU per cell with each recombinant baculovirus and incubated at 27°C until harvesting of the cultures. Expression of the PRRSV proteins was analyzed by 12% SDS-PAGE and Western blotting. Using Coomassie blue staining, detection was difficult except for the ORF7 proteins (Fig. 4C). To confirm the presence of PRRSV proteins, the proteins were transferred to nitrocellulose and analyzed by immunoblotting (Fig. 4D). As shown in Fig. 4D, expression levels of the GP2 and GP5 proteins were not high. This result could be explained by the high hydrophobicity of these proteins, which contain several transmembrane domains. For GP3, several bands were also observed. The major band showed a MW of 30 kDa, the size of the non-glycosylated form. The size and distribution of the other lower bands may correspond to degraded forms of the ORF3. For GP4, three major bands were observed of MW: 19 kDa, 22 kDa, and 25 kDa, that would correspond to nonglycosylated and glycosylated and different glycosylated forms (Plana Duran *et al*., 1997; Dea *et al*., 2000), respectively. A possible explanation for the various patterns of PRRSV proteins expression might be due to incomplete glycosylation in insect cells. The M (19 kDa) and N (14 kDa) proteins provided a clear positive reaction with anti-PRRSV pig antisera. A negative control consisting of Sf21 cells infected with wild-type AcNPV did not show any reactivity against the PRRSV-specific pig antiserum, as expected. All the expressed PRRSV proteins were insoluble, except ORF7, which was partially soluble. They remained associated with the cell membranes, confirming the hydrophobicity and membrane-associated nature of these proteins.

In summary, this study provides the nucleotide sequences of the CA structural ORFs and phylogenetic tree analyzed to date. Also, each of the structural proteins of CA strain was expressed using a recombinant baculovirus expression system. Our major goal in the very near future is the development of new, safe, effective, and economic vaccines against this pathogen using an insect bioreactor. Additionally, further studies for the characterization of expressed protein are needed.

Acknowledgements

This work was supported by a grant (Project No. 200704010 34002) from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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