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Nucleic Acid Binding Activity of Pns6 Encoded by Genome Segment 6 of Rice Ragged Stunt Oryzavirus

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Abstract The ORF of genome segment 6 (S6) of rice ragged stunt oryzavirus (RRSV) Philippines isolate was cloned and sequenced based on the S6 sequence of the Thailand isolate. Pns6, the 71 kD product of S6 expressed in *E. coli,* was demonstrated to be a viral non-structural protein of RRSV by Western blotting. The gel mobility shift assays showed that Pns6 had nucleic acid binding activity. Pns6 could interact with single- and double-stranded forms of DNA and RNA, showing a preference for single-stranded nucleic acid and a slight preference for RRSV ssRNA over the rice ssRNA, as demonstrated by both competition and displacement assays. The binding of Pns6 to nucleic acids is strong and sequence non-specific. By using five truncated derivatives of Pns6, it was found that the basic region from amino acid 201 to 273 of Pns6 was the unique nucleic acid binding domain. Subcellular fractionation of leaf tissues of RRSV-infected rice plants and subsequent Western blotting had shown that Pns6 accumulated predominately in the cytoplasmic membrane fraction. The possible role of RRSV Pns6 in virus replication and assembly is discussed.

Key words rice ragged stunt oryzavirus (RRSV); nucleic acid binding; non-structural protein; S6

Rice ragged stunt disease, caused by rice ragged stunt oryzavirus (RRSV), was first discovered in 1976–1977 in Indonesia and Philippines [1]. Subsequently the disease was found in most rice-growing countries in south-eastern and far-eastern Asia [2] and may inflict heavy loss on the crop.

RRSV is the type species of the genus *Oryzavirus* in the family *Reoviridae*. The virus particle is icosahedral with a diameter of about 65–70 nm and the genome consists of 10 double stranded RNA (dsRNA) segments with molecular weights ranging from 0.76×10^6 to 2.46×10^6 Daltons [3]. All 10 genomic segments possess identical conserved nucleotide sequences, with GAUAAA at 5′-terminus and GUGC at 3′-terminus [4].

The complete nucleotide sequences of all genomic segments, S1-S10, of RRSV Thailand isolate have been determined and could be obtained from GenBank. The sequence of S9 and S10 of Philippines and India isolates have also been determined by several laboratories [5–12]. The virion is composed of 6 major, highly immunoreactive structural proteins with estimated M_r of 31, 39, 43, 70, 90 and 120 kD, respectively, and several minor structural proteins [13]. Three proteins with M_r of 33, 63 and 88 kD have already been identified by *in vitro* translation of RRSV genomic segments and immunoprecipitation, and were assigned as nonstructural [14]. More recently, it has been shown that the expression products of S7 and S10 with M_r of 68 and 33 kD are non-structural proteins [6]. S5 encodes a 91 kD structural protein [5]. S8 encodes a 67 kD structural protein that is endowed with self-aggregation and self-cleavage abilities. The 43 kD protein, one of the cleavage product, may take part in virus assembly and another product, the 26 kD protein may act as a self-cleavage proteinase [9,15]. Among the viral proteins mentioned above, the function of the P9 protein, the product of S9, is well established. P9 is a 39 kD protein that contributes largely as a viral spike protein and plays an important role

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in virus transmission by the insect vector [16,17].

It is imperative that functions of all other viral proteins, either structural or non-structural, are elucidated in order to gain insights into the molecular biology of the virus and the control of the disease. In the present study, RRSV S6 gene was expressed in *E. coli* and the product of S6 purified from the soluble fraction of the cell lysate. We demonstrate that the S6 product is a 71 kD nonstructural protein with a nucleic acid binding activity. The protein interacts both with single- and double-strand forms of RNA and DNA in a sequence-independent manner and it is preferential for single-stranded nucleic acids. The nucleic acid binding domain is located in the basic region of Pns6, from amino acid 201 to 273. The Pns6 protein may play an important role in virus replication and assembly.

Materials and Methods

Rice plants and virus

Rice strain Aijiao Nante, which is susceptible to RRSV infection, was used for virus propagation and assays of virus infection. The Philippines isolate of RRSV was kindly provided by Dr. H. Koganezawa of the International Rice Research Institute (IRRI) in the Philippines and maintained at the Zhejiang Academy of Agricultural Science in Hangzhou, China.

Extraction of total RNA from rice plant

Total RNA was extracted from fresh healthy rice plants with an RNeasy plant mini kit (Qiagen).

Subcellular fractionation of leaf tissues of RRSV-infected rice and detection of Pns6

Subcellular fractionation of leaf tissues of RRSV-infected and healthy rice plants was as described [18], with slight modification. Leaf tissues were pulverized after freezing in liquid nitrogen and lysed by grinding in a mortar with two volumes of ice-cold grinding buffer (100 mM Tris-HCl, pH 8, 10 mM EDTA and 5 mM DTT). The tissues were again triturated in a glass homogenizer and the homogenates were centrifuged at 1000 *g* for 10 min. The crude cell wall pellet was washed, heated in boiling water, and then lyophilized and stored for use. The supernatant was further fractionated into pellet (P30) and supernatant (S30) at 30,000 *g*. These two fractions were heated and then lyophilized. All three lyophilized preparations, i.e., crude cell wall, S30 and P30, were dissolved in PBS and the protein concentrations were adjusted by pre-electrophoresis before the proteins were separated by 12% SDS-PAGE and then transferred to nitrocellulose. Pns6 protein was detected using a rabbit antibody raised against Pns6 and then with sheep anti-rabbit serum. Immunoreactive bands were visualized with ECL reagents according to the supplier's protocols (Amersham International).

Preparations of double- and single-stranded DNA (dsDNA and ssDNA)

dsDNA used for binding assays was generated by plasmid digestion. DNA fragments were obtained by single digestion with *Eco*RI or by double digestion of plasmid pTXB1-S6 with *Nde*I and *Eco*RI.

Preparation of single-stranded DNA from phage M13 vector (M13-AIA) containing an inhibitor gene was performed as described [19].

Preparation of single-stranded RNA (ssRNA) by *in vitro* runoff transcription

Generation of ssRNA probes by *in vitro* runoff transcription was performed basically as described [20]. Plasmid pUC119-S9 was digested with *Bam*HI and *Eco*RI to generate the full-length RRSV S9 segment, which was then inserted into corresponding cloning sites of pSPT18 (Boehringer Mannheim). Purified template DNA was linearized by digesting pSPT18-S9 with *Eco*RI for trans-cription of plus-strand-ssRNA or with *Bam*HI for minus-strand-ssRNA. One μg of linear DNA template was mixed with RNA polymerase buffer containing 40 mM Tris-HCl, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, pH 7.9, 0.5 mM ATP, CTP, GTP and UTP, 100 μg/ml BSA, 2–10 U/μl RNase inhibitor-HPR I (TaKaRa Biotechnology Co., Ltd.) and 1 U/μl of SP6 RNA polymerase (New England Biolabs) for preparation of plus-strand-ssRNA, or 1 U/μl T7 RNA polymerase for minus-strand-ssRNA. For labeling ssRNA, 0.2 mM [α-^{32}P]UTP plus 0.1 mM unlabeled UTP was used. The reaction mixture was incubated for 60 min at 40 °C for SP6 polymerase or at 37 °C for T7 polymerase. The DNA template was then removed by treatment with RNase-free DNase I (TaKaRa Biotechnology Co., Ltd.) after the transcription reaction was completed. The *in vitro* transcription products were detected either by agarose gel electrophoresis, or SDS-PAGE, followed by autoradiography.

Construction and sequencing of the plasmid pTXB1-S6

The dsRNA of RRSV, as the template, was extracted from infected rice as described [21]. The ORF encoding

Pns6 was amplified by RT-PCR, primed by GGGAATTCCATATGCAGCTCTTCATAGTCAAAC with an *Nde*I site at 5′-terminus and by CCGGAATTCATCAAGCTCCTTACATTCAG with an *Eco*RI site at 3′-terminus. Both primers were designed according to the S6-ORF sequence of RRSV (Thailand isolate) (GenBank accession No. AF020337). The amplified S6-ORF segment was inserted into the expression plasmid pTXB1 (New England Biolabs) between *Nde*I and *Eco*RI sites. The S6-ORF is located upstream of the Mxe intein/chitin binding domain (Mxe intein/CBD). Pns6 could be self-cleaved by addition of DTT and released from chitin beads. The recombinant pTXB1-S6 clones were sequenced with forward and reverse sequencing primers of pTXB1 and two internal primers in the forward direction followed by aligning analyses against the corresponding region of RRSV S6.

Expression and purification of Pns6

pTXB1-S6 was transferred to *E. coli* DE3 (BL21). For large-scale expression of Pns6, the recombinant cells were incubated in four liters of Luria-Bertani medium at 37 °C for about 2.5 h until the cell density reached A_{600}=1.0–1.2. After induction with 0.3 mM IPTG, the cells were shifted to 15 °C and incubated for 4 h for optimal protein expression. The cells were pelleted by low speed centrifugation, washed with the column buffer (20 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 8.0), resuspended in 200 ml of column buffer containing 2 mg/L leupeptin and lysed by sonication. The lysate was clarified by centrifugation at 12,000 *g* for 30 min. After addition of Tween-20 to the supernatant to a final concentration of 0.2%, 20 ml of chitin beads slurry balanced with column buffer were added to the mixture with gentle stirring overnight. Chitin beads were precipitated by allowing the slurry to stand for about 10 min and then the supernatant was removed. The chitin beads slurry were washed three times with 100 ml of washing buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, pH 8.0) containing 0.2% Tween-20 and three more times with 100 ml washing buffer only. Self-cleavage was induced by the addition of 50 mM DTT to the slurry and incubation for 72 h at 4 °C. After cleavage, the slurry was subjected to chromatography and the cleaved Pns6 without the CBD fusion part in the eluate was collected. The protein solution with DTT was dialyzed against column buffer at 4 °C overnight and purified protein was stored at –20 °C prior to use.

Cloning and expression of truncated mutants of Pns6

For cloning and expression of different forms of Pns6, truncated at the N-terminus or C-terminus, the plasmids were constructed using PCR products of S6 ORF fragment amplified with the following primers:

P+ (sense, 1, *Nde*I): 5′-GGGAATTCCATATGCAGCTCTTCATAGTCAAAC-3′

Pt1 (antisense, 1620, *Eco*RI): 5′-GGAATTCGAGGCAATCTATTTTATCATA-3′

Pt2 (antisense, 819, *Eco*RI): 5′-GGAATTCTTTATCTTTTGTCCGACCC-3′

Pt3 (antisense, 600, *Eco*RI): 5′-GGAATTCACTTGCCGAACCAACTTCAC-3′

Pt4 (sense, 601, *Nde*I): 5′-GGGAATTCCATATGAAACGTAGGCAAAGATTGG-3′

Pt5 (sense, 820, *Nde*I): 5′-GGGAATTCCATATGGCCCAAGAGGTTAGAGAG-3′

P– (antisense, 1782, *Eco*RI): 5′-CCGGAATTCATCAAGCTCCTTACATTCAG-3′

The truncated forms of T1, T2, T3, T4 and T5 were obtained by PCR amplification with primers P+/Pt1, P+/Pt2, P+/Pt3, Pt4/P– and Pt5/P– respectively. These truncated mutants were inserted into the expression plasmid PTXB1 (New England Biolabs) between *Nde*I and *Eco*RI sites. All plasmid constructs were then checked by sequencing. Positive clones were transferred into *E. coil* strain DE3(BL21) competent cells. The protein expression and purification were carried out in a similar way as in the case of Pns6, only with a minor change of the protein expression induction temperature (22 °C instead of 15 °C).

Western blotting

Equal amounts of RRSV Pns6 and P8 protein, already known as a viral structural protein [9,15] in SDS-polyacrylamide gels were blotted onto nitrocellulose membranes at 40 V for 2 h at 0 °C in transfer buffer [25 mM Tris, 192 mM glycine, 20% methanol (*V*/*V*)]. The membrane was probed with rabbit anti-RRSV IgG at a dilution of 1:50, and the secondary antibody (goat anti-rabbit) at a dilution of 1:5000. Both polyclonal antibodies were prepared in our laboratory. The immuno-reacted protein bands were visualized with diaminobenzidine.

All truncated forms of Pns6 were confirmed by Western blot analysis using a rabbit antiserum against Pns6, prepared in our lab too, as the primary antibody at a dilution of 1:100 and the secondary antibody (goat anti-rabbit) at a dilution of 1:5000. The results were visualized with ECL reagents.

Agarose gel mobility shift assays

Different amounts of purified Pns6 and other proteins

served as controls were dissolved in column buffer and incubated with different amounts of RRSV dsRNA and ssRNA, dsDNA or ssDNA for 45 min at 37 °C. For reactions with ssRNA, RNase inhibitor was supplemented as in runoff transcription. The samples were subjected to electrophoresis in a 0.8% agarose-TAE gel (agarose-Tris-acetate-EDTA gel) [20], and stained with 1 μg/ml ethidium bromide for 30 min.

Competition assays

Competition assays were performed basically as described [22]. Different amounts of unlabeled rice total ssRNA, RRSV dsRNA or certain amount of ssDNA (indicated in figure legends) as competitors were mixed with labeled RRSV ssRNA in DEPC-treated water containing 2 to 10 U/μl HPR I before adding Pns6 and incubation for 15 min at 37 °C. Samples were then subjected to electrophoresis in 5% polyacrylamide-TBE non-denaturing gels at 190 V for 30 min and visualized by exposing gels to X-ray film (Kodak).

Displacement assays with ssRNA and ssDNA

ssRNA or ssDNA mixed with purified Pns6 was first incubated at 37 °C for 30 min. Then ssRNA of or ssDNA displacers were added to the reaction mixture at 37 °C for 30 min. Samples were analyzed as described for agarose gel mobility shift assays.

Characterizations of nucleic acid binding properties of Pns6 by heating and salt treatments

Pns6 (5 μg) was first heated in water at either 60 °C or 80 °C for 5 min prior to incubation with 0.5 μg ssRNA and 40 U RNase inhibitor. For salt treatments, the binding of Pns6 with ssRNA was performed in binding buffer containing different concentrations of NaCl (0 mM, 250 mM, 500 mM and 750 mM). All mixtures were incubated at 37 °C for 45 min and then subjected to electrophoresis in a 0.8% agarose-TAE gel.

Results

Comparison of S6 ORF sequences of Philippines and Thailand isolates of RRSV

The RRSV-S6 has a single ORF region. The coding region of S6 of the Philippines isolate (GenBank accession No. AF527634) is composed of 1782 bp, 6 bp longer than that of the Thailand isolate (GenBank accession No. AF020337), and encodes a polypeptide of 594 amino acids. Alignment of the ORFs of the Philippines and Thailand isolates showed that there were four single-nucleotide insertions and one two-nucleotide insertion in the ORF of the Philippines isolate between nucleotides 1550 to 1593, which led to variations in the sequence of 17 amino acids. Three amino acid substitutions are caused by three among seven separate single-point nucleotide substitutions. All the polymorphic sites in the Philippines isolate were concentrated in the 3′ terminal region of the S6 sequence and were confirmed by repeated sequencing of the RT-PCR products.

Expression of Pns6 in *E. coli* and detection with Western blot

The fusion protein Pns6-Mxe intein/CBD was expressed and accumulated in induced *E. coli* cells as shown by Western blotting with anti-CBD serum s. Purified Pns6, free of Mxe intein/CBD after self-cleavage in presence of DTT, appeared as a single major band in SDS-PAGE (Fig. 1). The estimated molecular weight of Pns6 is 71 kD, slightly larger than 67 kD predicted from the amino acid sequence. Western blot analysis showed that Pns6 was not detectable using anti-RRSV serum, whereas the viral structural protein P8, was detected (Fig. 1). Thus we concluded that Pns6 is a non-structural protein of RRSV.

Subcellular localization of Pns6 in RRSV-infected leaf tissues

Subcellular fractionation and subsequent Western

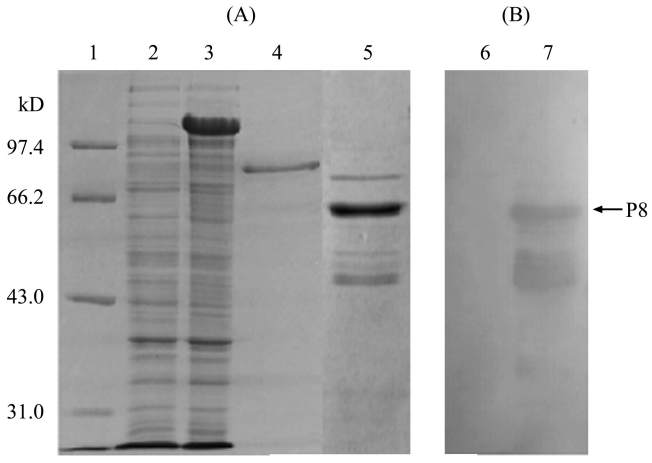

Fig. 1 Expression and purification of RRSV Pns6 and Western blotting with anti-RRSV serum

(A) SDS-PAGE of expressed and purified Pns6. 1, molecular weight standards; 2, crude extract from uninduced cells; 3, crude extract from induced cells; 4, purified Pns6; 5, purified P8. (B) Western blotting with anti-RRSV serum. 6, purified Pns6; 7, purified P8.

blotting showed that Pns6 was detected only in the P30 fraction, i.e., the cytoplasmic membrane fraction of infected rice leaves. As shown in Fig. 2, no Pns6-immunoreactive bands could be detected either in the nuclei and cell wall fraction of infected leaves or in the soluble and cell wall fractions of healthy rice leaves.

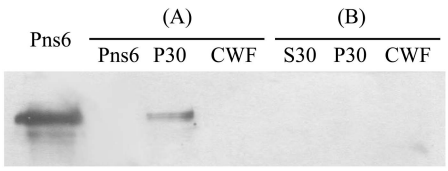

Fig. 2 Western blot analysis of Pns6 in subcellular fractionation of leaf tissues of RRSV-infected (A) and health (B) rice plants

Nucleic acid binding activity of RRSV Pns6

Binding properties of Pns6 with dsDNA, ssDNA, dsRNA and ssRNA were investigated by gel mobility shift assays. The retardation for RRSV dsRNAs intensified gradually with increasing amounts of Pns6 [Fig. 3(C)]. It could be seen that Pns6 had shown an higher affinity of binding with larger segments of dsRNA or dsDNA [Fig. 3 (C,D)]. The retardation profiles of single-nucleic acids after

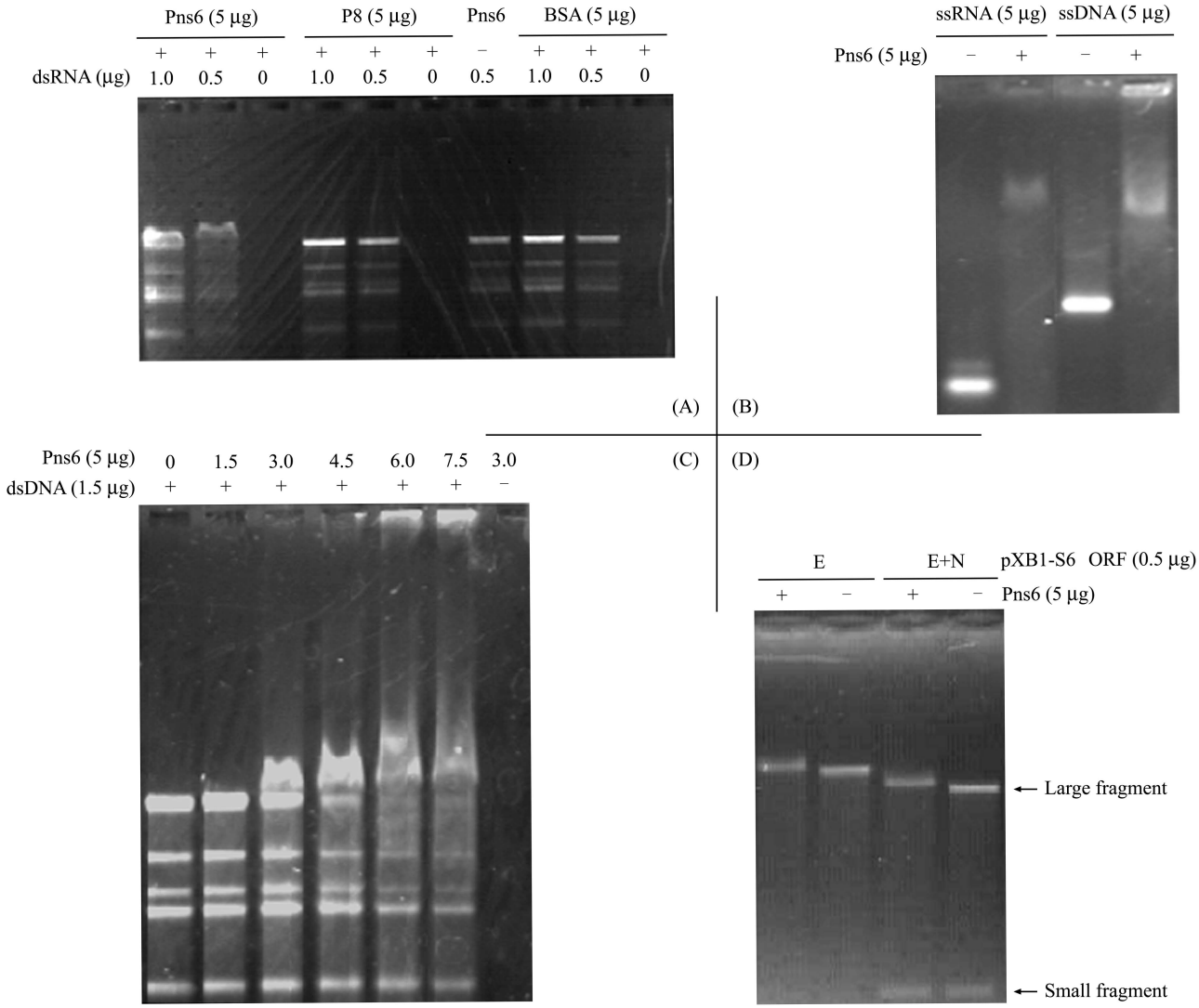

Fig. 3 Binding of Pns6 with different kinds of nucleic acids in gel mobility shift assay

(A) Pns6 with different amounts of dsRNAs of RRSV. Both P8 and BSA were set as the control. (B) Pns6 with ssRNA and ssDNA. (C) Different amounts of Pns6 with dsRNAs, showing the preference of Pns6 to the larger dsRNA fragment. (D) Pns6 with plasmid dsDNA fragments showing the preference of Pns6 to larger dsDNA fragments.

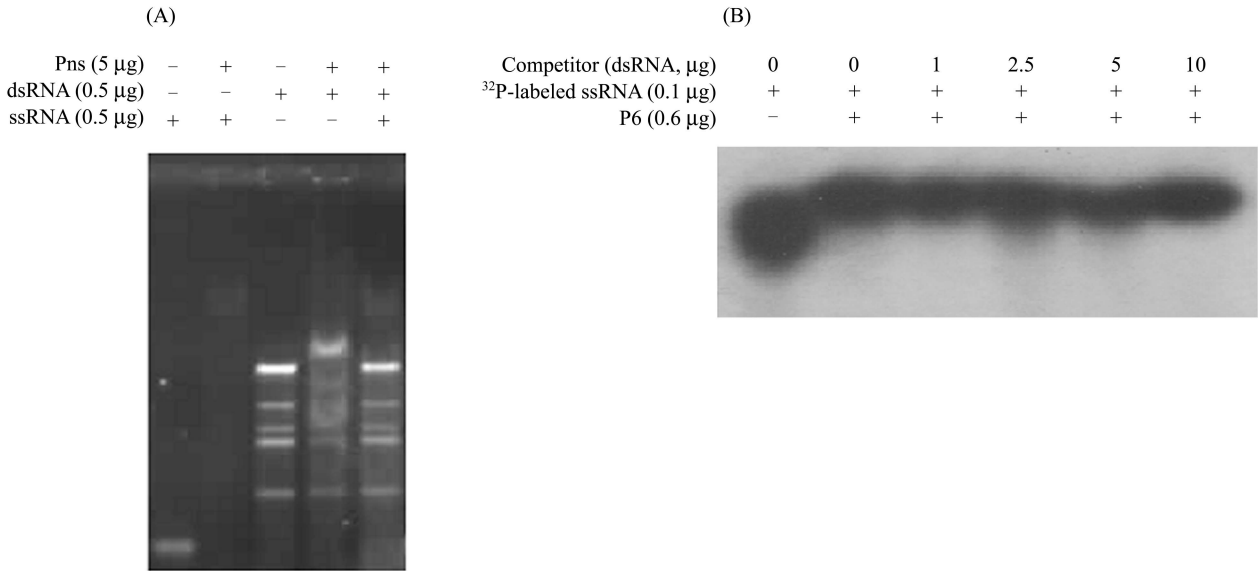

Fig. 4 Displacement assays of Pns6-bound dsRNA by ssRNA (A), and competition assays of dsRNA with ssRNA for Pns6 binding (B)

binding with Pns6 were quite different from that of dsRNA and the contrasts were much sharper [Fig. 3(B)].

Displacement and competition assays with single- and double-stranded nucleic acids

The displacement assays with either plus or minus S9 ssRNA and RRSV dsRNAs showed that after equal amounts of ssRNA were added to the prebinding mixture of Pns6 and dsRNA, the shifted bands of dsRNA returned to their original position, whereas the ssRNA band was shifted [Fig. 4(A)]. On the other hand, when different amounts of unlabeled dsRNA were added to the P^{32}-labeled ssRNA solution and then the mixtures were incubated with Pns6, the competition assays showed that the profile of the shifted ssRNA band did not change with increasing amount of dsRNA. These results indicated that Pns6 showed a preferential binding with the single-stranded RNA [Fig. 4(B)].

Competition and displacement assays of Pns6 with ssRNA and ssDNA

The preliminary binding experiments showed that when the amount of added Pns6 was 6 times more than the nucleic acid, the binding could be saturated. The competition and displacement assays with ssRNA and ssDNA showed that neither ssRNA nor ssDNA was competitive to, or be displaced by each other, meaning that Pns6 had a similar binding affinity to both ssRNA and ssDNA (Fig. 5).

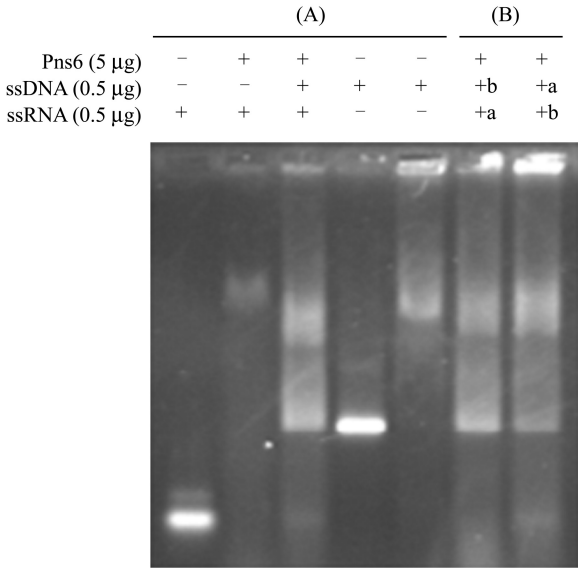

Fig. 5 Competition (A) and displacement (B) assays between ssRNA and ssDNA for binding to Pns6

a, first added; b, second added.

Competition assays of Pns6 with RRSV ssRNA and total RNA of rice plant

The competition assays with *in vitro* transcripted ssRNA and the total rice RNA had demonstrated that the rice ssRNA could be competitive to both of labeled probes of plus- or minus-ssRNA transcripted from RRSV S9 in the

binding mixture with Pns6 at levels of saturation of the labeled ssRNA, but there maybe still some slight preference of RRSV RNAs over rice RNA, because when the added rice RNA was 2.5 time more than ssRNA, the rice RNA is unable to competite the RRSV ssRNA (Fig. 6).

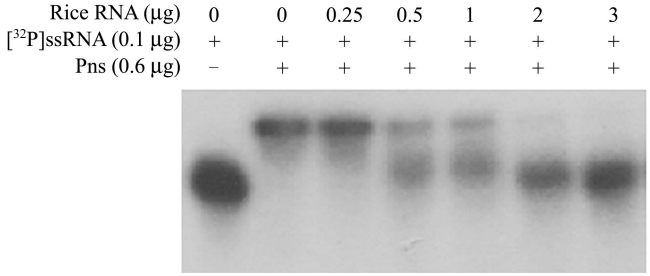

Fig. 6 Competition assays of total rice RNA with ^{32}P-labeled RRSV S9 ssRNA for binding to Pns6

Stability of Pns6:ssRNA complex

The stability of Pns6:ssRNA complex with respect to salt concentration was often used as a criterion to evaluate the strength of a protein:RNA association. As shown in Fig. 7(A), Pns6 did bind ssRNA to its maximal extent from 0 and up to 250 mM NaCl. In 500 mM NaCl, Pns6 still retained about 80% of its binding capacity which decreased drastically in 750 mM NaCl solution. The 50% binding of Pns6 was reached around 600 mM NaCl [Fig. 7(A)].

The heating of Pns6 at 60 °C and 80 °C for 5 min did not affect its RNA-binding activity [Fig. 7(B)], showing thus the activity was quite stable at the temperature tested.

Binding of truncated mutants of Pns6 to nucleic acids

Three C-terminal truncated (T1, T2 and T3) and two N-terminal truncated (T4 and T5) mutants of Pns6 were expressed in *E. coli* [Fig. 8(A)]. The molecular mass of these proteins estimated by migration in SDS-PAGE were consistent with those deduced from their amino acid sequences [Fig. 8(B)]. By Western blot with anti-serum against Pns6 it is confirmed that the five mutants were all derivatives of Pns6 (date not shown). The mobility shift assays showed that T1, T2 and T4 mutants retained the ssRNA binding activity, but both T3 and T5 did not [Fig. 8(C)]. The results indicated that the basic region (i.e., from aa 201 to aa 273) of Pns6 was the unique nucleic acid binding domain [Fig. 8(A)].

Discussion

One of the hurdles in the research on biological function of viral proteins is the procurement of fairly large amount of purified protein. In the present study we have shown that the product of RRSV-S6 could be expressed in *E. coli* as a fusion protein with Mxe intein/CBD and easily purified. We have presented here the evidences that Pns6 is a non-structural protein with a nucleic acid binding activity and other features and properties of this protein. The deletion mutations have pinpointed the basic region from amino acids 201 to 273 of Pns6 as the unique nucleic acid binding domain. Besides, this is located in the N-terminal fragment of Pns6 molecular, and is conservative in both Thailand and Philippines isolates of RRSV.

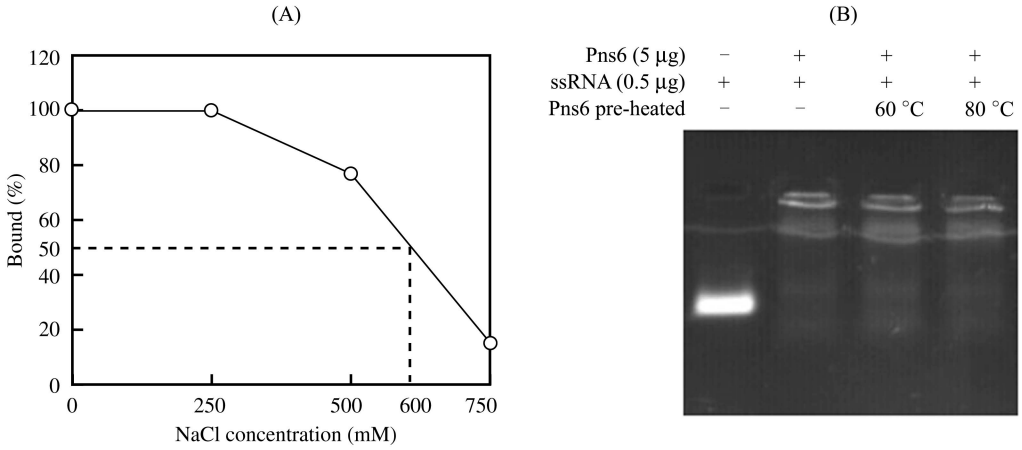

Fig. 7 Stability of Pns6:ssRNA complex at different salt concentrations (A), and effect of heating on RNA binding activity of Pns6 (B)

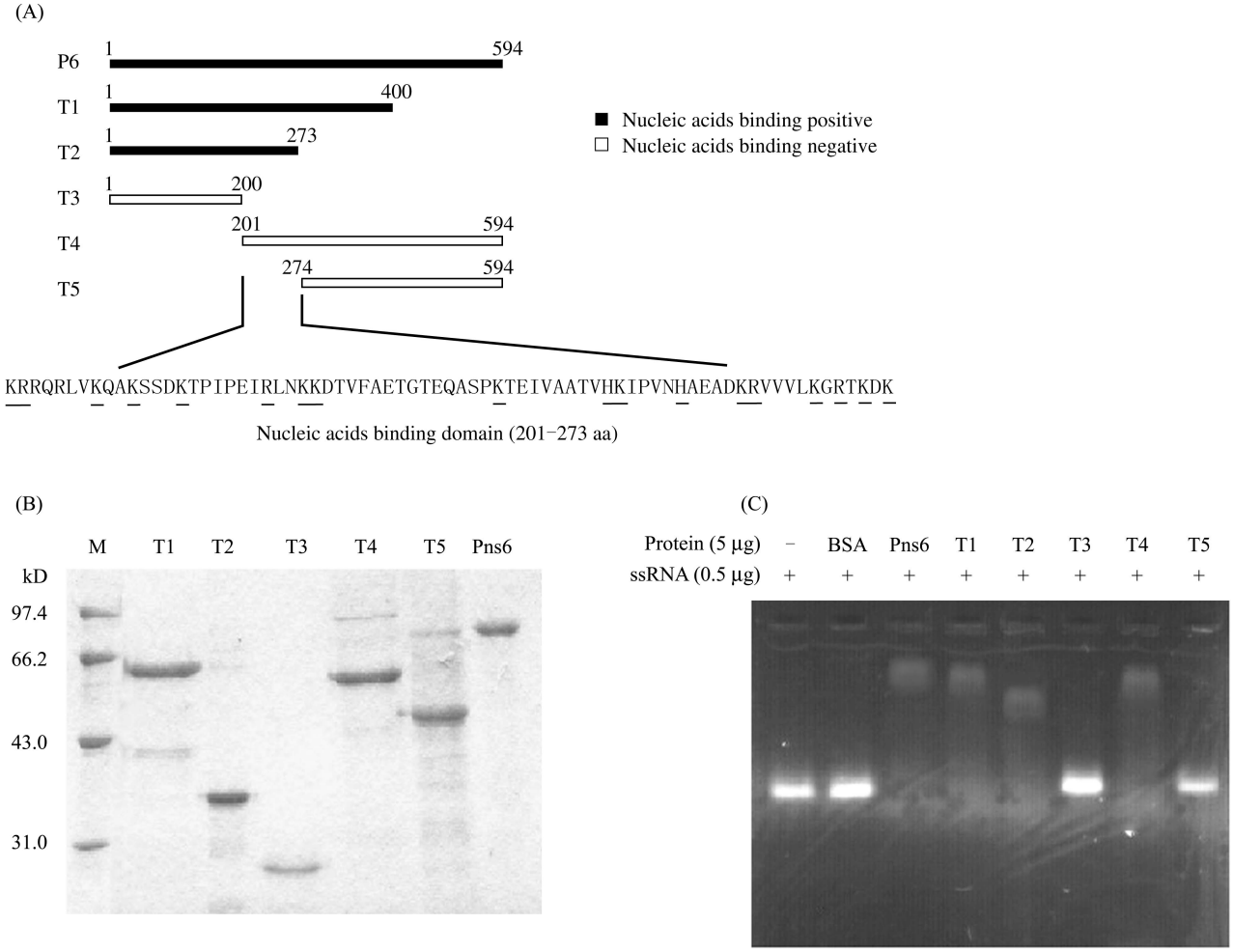

Fig. 8 Schematic diagrams of Pns6 and its 5 truncated mutants (A), 12% SDS-PAGE of purified truncated mutants of Pns6 (B), and gel mobility shift assays of truncated mutants of Pns6 with ssRNA (C)

The numbers refer to amino acids. Underlines indicate the basic amino acids. In (C), Pns6 was used as a positive and BSA a negative control.

Although more in-depth studies are needed to illustrate the function of Pns6, it would be insightful to discuss our present results with reference to numerous and diversified studies on the nucleic acid binding activity of viral non-structural protein.

The biological functions of viral nucleic acid binding proteins are well documented in two groups. In the first group the movement proteins (MPs) of plant viruses, including the well-known P30 MP of TMV are involved [18,23]. Like RRSV-Pns6, the movement proteins display similar sequence non-specific and co-operative nucleic acid binding activity. P30 MP of TMV requires a higher than 0.8 M NaCl to dissociate the protein:RNA complex. The preferential binding to single stranded nucleic acids have also been demonstrated in P30 MP and many MPs of other plant viruses, such as CMV, CaMV, red clover necrotic mosaic virus (RCNMV) and carnation mottle virus [24–27]. To date no specific MP has yet been identified for any viruses in the *Reoviridae*. Many aspects of nucleic acid binding activity of Pns6 are similar to those of MPs. Nevertheless, there are differences between Pns6 of RRSV and MPs of other plant viruses. Most MPs are associated with cell wall and membrane fractions from leaf tissue of transgenic plants expressing the MP gene and from virus-infected plants as well [18,27], whereas in RRSV, like CMV 3a protein [24] Pns6 is associated with the cytoplasmic membrane fraction (P30), suggesting it could probably be a candidate of MP, Although this conclusion does not exclude the possibilities of other functions of membrane associated Pns6. In the second group are the viral regulatory proteins. One characteristic of these proteins is that a zinc-finger motif with other

basic regions is present and involved in nucleic acid binding activity. The structure-depending feature is similar to that of a group of transcription factor proteins. S11, a nonstructural protein of rice dwarf phytoreovirus, contains a putative zinc finger and five flanking basic regions at the C-terminus. The Pns11 of RDV interacts with single-and double-stranded forms of DNA and RNA in a sequence-nonspecific manner [28]. However, no significant sequence similarity between Pns11 and Pns6 of RRSV has been found and, what is more, the biological function of Pns11 still has to be elucidated. There are more examples of plant virus proteins that contain putative zinc-finger motifs and basic regions and could probably function as a regulatory protein such as the capsid protein of tobacco streak virus and alfalfa mosaic virus, and the 12 kD protein of potato virus M [29,30]. The comparisons on structural characterizations between nucleic acid binding proteins of these plant viruses and Pns6 of RRSV fail to justify Pns6 as a candidate of regulatory proteins.

A number of studies on animal and plant viruses indicated that the virus replication complexes are closely associated with cellular membranes [31–35].

In one of our previous studies we have reported that Pns6 together with RRSV nucleic acids can activate and accelerate the *in vitro* self-aggregation of a viral structural protein P8, the product of RRSV-S8 [15]. However, we did not find, in our GST-pull down experiments, any interactions between P8 and P9, a major spike protein of RRSV (data not shown). We have assumed therefore that P8 may function as an inner coat protein and participate in the preformation or formation of the inner capsid [15]. Since RRSV has an active RNA-dependent RNA polymerase [36], it could synthesize the complementary minus strands from the already packaged positive strand genomic segments inside the polymerase particle, as described by Bamford and Wickner [37]. The Pns6 bound with nucleic acid may function as a helper factor in the assortment of nucleic acids in packaging and in the virus assembly, in forming viral inner capsid. However, we can not exclude the multifunction of the viral nucleic acid binding proteins, like RRSV Pns6. A nonstructural protein of avian or mammalian reoviruses, δNS, functions in a sequence-nonspecific manner by binding with ssRNA. It plays a direct role in packaging and replicating the reovirus genome by forming a nucleus-protein complex. In addition, δNS may play other roles in life cycle of the virus, such as stabilizing the viral mRNAs prior translation or packing, localizing mRNA to appropriate intracellular compartment, etc. [20]. Currently studies on biological significance of nucleic acid binding of Pns6 in life cycle of RRSV are underway in our laboratory.

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