**INTRODUCTION**

White tail disease (WTD) was first observed on the giant freshwater prawn, *Macrobrachium rosenbergii*, in 1995 on Guadeloupe Island, French West Indies (Arcier et al., 1999). This disease spread rapidly to many places including Martinique in the French West Indies (Arcier et al., 1999), China (Qian et al., 2003), India (Hameed et al., 2004), Thailand (Yoganandhan et al., 2006), Australia (Owens et al., 2009), Taiwan (Wang et al., 2008) and recently Malaysia (Saedi et al., 2012). This serious global outbreak caused major losses to the giant freshwater prawn industry due to very high mortality rates in the post-larvae within 2–3 days post-infection (Hameed et al., 2004). The virus isolated from prawns showing WTD symptoms was demonstrated to infect healthy prawns (Arcier et al., 1999), suggesting that it is the main causative agent for this disease. Based upon the virus structure, size and genome, it was grouped under the family *Nodaviridae*. This classification is further supported by the fact that the virus is non-enveloped with icosahedral structure and it contains a bipartite linear RNA genome (Bonami et al., 2005; Ravi et al., 2009). The RNA1 segment, which is about 3.12 kb, encodes the RNA-dependent RNA polymerase of the virus while the RNA2 segment encodes the viral capsid protein (NaveenKumar et al., 2013; Pillai et al., 2006). The MrNV capsid protein is a single polypeptide of 371 residues with a calculated molecular mass of about 41.5 kDa (NaveenKumar et al., 2013).

The recombinant MrNV capsid protein is readily produced in *Escherichia coli* and the purified protein assembled into an icosahedral structure resembling the native virus isolated from infected prawns (Goh et al., 2011). Interestingly, the full-length MrNV capsid protein was shown to encapsulate host RNA molecules. This suggests that recombinant DNA technology could provide an alternative means to study capsid–RNA interactions during virus morphogenesis. The first 29 aa of the capsid protein are very rich in positively charged amino acids; therefore this region is postulated to interact with RNA molecules. This postulation is supported by several studies which demonstrated the binding of nucleic acid molecules by arginine- or lysine-rich regions in the capsid proteins of some viruses such as hepatitis B virus (Nassal, 1992), human immunodeficiency virus (Calnan et al., 1994) and alfalfa mosaic virus (Baer et al., 1994). Rao & Grantham (1996) showed that the arginine-rich motif located at positions 11–19 of the capsid protein of brome mosaic virus is crucial for RNA binding. N-terminal deletion of the first 31 residues of the capsid protein of flock house virus (FHV), an alphanodavirus, inhibited RNA-packaging activity (Marshall & Schneemann, 2001), suggesting the
(a) Deletion mutants

(i) MrNVc

(ii) 9ΔMrNVc

(iii) 19ΔMrNVc

(iv) 29ΔMrNVc

(v) 20–29ΔMrNVc

(b) Multiple point mutants

(vi) K20R21R22–K23R24A

(vii) R26R27R29A

(viii) K20R21R22–K23R24R26–R27R29A
importance of the positively charged residues in binding with the viral capsid. However, until now, there has been no information available on the RNA-binding site of the MrNV capsid protein. Therefore, the main objective of this study was to map the RNA-binding site on the MrNV capsid protein. This is the first report to our knowledge describing the RNA-binding region of the MrNV capsid protein.

RESULTS

Deletion and multiple point mutagenesis of MrNV capsid protein

The coding segments of the N-terminal truncated, internal deleted and multiple point mutants were ligated into pTrcHis2 TOPO vectors. All the coding regions in the recombinant plasmids were verified with restriction enzyme analysis and DNA sequencing. The translated products of the N-terminal truncated, internal deletion and multiple point mutants are schematically represented in Fig. 1. The mutants are named after the deleted regions from the N-terminal end and amino acid substitutions.

Expression of the MrNV capsid mutants

All the recombinant plasmids are under the control of the trc promoter and the translated proteins harbour the myc epitope and six histidine (His) residues at their C-terminal ends. Western blot analysis of cell lysates before and after IPTG induction using the anti-His mAb detected the mutated proteins with molecular masses ranging from 40 to 48 kDa, which corresponded well with the calculated mass of the proteins (Fig. 2a). The full-length MrNV capsid protein and mutants were purified with a His-trap HP column. SDS-PAGE revealed that the protein bands decreased gradually in molecular mass, depending on the number of amino acids that have been deleted, and their purities were higher than 90% (Fig. 2b).

MrNV capsid mutants form virus-like particles (VLPs)

All the N-terminal truncated, internal deleted and multiple point mutants were separated by 8–40% sucrose density gradient ultracentrifugation. Fig. 3 shows the sedimentation profiles of all the mutants analysed with the Bradford assay; peaks were observed in fractions 4–9, suggesting the formation of particles which migrated into the sucrose gradient. Transmission electron microscopy (TEM) analysis revealed that all the purified truncated mutants assembled into spherical particles with diameters ranging from 18 to 27 nm (Fig. 4). Dynamic light scattering (DLS) analysis confirmed the formation of VLPs of all the mutants with hydrodynamic diameters ranging from 24.6 to 30.5 nm. The diameters of the VLPs formed by the mutants as analysed by TEM and DLS are summarized in Table 1. The hydrodynamic diameters of the VLPs measured by DLS appeared bigger than the diameters determined with TEM.

Extraction of RNA from the MrNV capsid mutants

RNA was extracted from the freshly purified mutants which formed VLPs. Spectrophotometric analysis at $A_{260}$ revealed that the full-length MrNV capsid protein, and mutants 9ΔMrNVc and 19ΔMrNVc contained about 120–128 ng RNA (μg protein)$^{-1}$, but the RNA content dropped dramatically in mutants 29ΔMrNVc and 20–29ΔMrNVc to about 10–11 ng RNA (μg protein)$^{-1}$ (Table 2). A triple point mutation of arginine residues at positions 26, 27 and 29 to alanines (mutant R26R27R29A) reduced the RNA amount slightly. However, a quintuple point mutation of all the positively charged residues at positions 20–24 to alanines (mutant K20R21R22K23R24A) dramatically decreased the RNA amount to about 28 ng RNA (μg protein)$^{-1}$. A further reduction in RNA content was observed in the octuple point mutant, K20R21R22K23R24R26R27R29A, suggesting that the RNA-binding site is located at residues 20 to 29. Agarose gel electrophoresis of the extracted RNA revealed that the full-length MrNV capsid protein and mutants 9ΔMrNVc, 19ΔMrNVc and R26R27R29A contained significantly higher amounts of RNA than mutant K20R21R22K23R24A. However, the RNA contents of mutants 29ΔMrNVc, 20–29ΔMrNVc and K20R21R22K23R24R26R27R29A were too low to be visualized on the agarose gel (Fig. 5).

In order to study the encapsidation of RNA molecules by the mutated capsid proteins, native agarose gel electrophoresis of the purified VLPs was performed. Fig. 6 shows that RNA molecules migrated together with the VLPs. The migration rates of the RNA molecules (Fig. 6a) were similar to those of VLPs detected in the same gel subsequently stained with Coomassie blue (Fig. 6b), demonstrating that the RNA molecules were encapsidated in the VLPs.
Fig. 2. SDS-PAGE and Western blot analysis of the N-terminal deletion, internal deletion and multiple point mutants of MrNV capsid (MrNVc) protein. (a) Western blot of mutants expressed in *E. coli*. Cell lysates of mutants before (−) and after (+) induction with IPTG were separated on SDS-polyacrylamide gels (12%). The proteins were blotted onto nitrocellulose membranes and detected with anti-His mAb. The full-length MrNV capsid protein and mutants are labelled at the top. (b) The full-length MrNV capsid protein and mutants purified with a His-trap column and analysed on a 12% SDS-polyacrylamide gel stained with Coomassie Brilliant Blue.
DISCUSSION

MrNV capsid protein can be easily and readily expressed in *E. coli*, where it assembles into VLPs resembling the native virus isolated from infected freshwater prawns (Goh et al., 2011). These VLPs were found to package host RNA molecules (Goh et al., 2011). Examination of the first 32 aa of the MrNV capsid protein revealed that this N-terminal region contains 10 positively charged amino acids (Fig. 1), constituting about 31% of the total residues in this region. Most interestingly, 8 out of 10 residues at positions 20–29 in this region are positively charged. Thus, it is hypothesized that the RNA-binding site of MrNV capsid protein is located at the N-terminal end of the capsid protein.

In order to address this hypothesis, three N-terminal deletion mutants (namely 9ΔMrNVc, 19ΔMrNVc and 29ΔMrNVc), one internal deletion mutant (namely 20–29ΔMrNVc) and three multiple point mutants (namely K20R21R22K23R24A, R26R27R29A and K20R21R22K23R24R26R27R29A) were generated and expressed in *E. coli*. The expression of these mutants was confirmed by SDS-PAGE and Western blotting. All these mutated capsid proteins were purified to more than 90% purity by using IMAC.

Mutants 9ΔMrNVc and 19ΔMrNVc, with, respectively, 9 and 19 aa deletions from the N-terminal end, interacted with RNA. This suggests that the first 19 aa of the MrNV capsid protein, containing two positively charged amino acids at positions 3 and 5, do not play a role in RNA–capsid interaction. However, further deletion of 10 aa, as represented by mutant 29ΔMrNVc, affected dramatically the RNA-binding activity. This suggests that residues 20–29, 80% constituted by positively charged amino acids, play an important role in RNA–capsid interaction. This was further supported by an internal deletion mutant, 20–29ΔMrNVc, which possessed the first 19 aa but without the highly positively charged segment. In addition, an octuple point mutant, K20R21R22K23R24R26R27R29A, with all the positively charged residues replaced with alanines contained a very low amount of RNA molecules, as displayed by mutants 29ΔMrNVc and 20–29ΔMrNVc. Therefore, it can be concluded that the highly positively charged segment of residues 20–29 is the binding site for RNA molecules. Marshall & Schneemann (2001) reported that the capsid of FHV contained a different amount of RNA molecules when the first 31 residues of its capsid protein were deleted. This suggests that the RNA-binding site is located at the N-terminal end of the capsid protein.
located at the N-terminal end. Sequence comparison of the first 50 residues of the capsid proteins of 10 nodaviruses is shown in Table S1, available in the online Supplementary Material. Generally, the N-terminal ends of these nodaviruses are very rich in positively charged residues, but the number and distribution of these residues vary. The MrNV capsid protein contains 80% positively charged residues at

![Transmission electron microscopic analysis of MrNV capsid (MrNVc) mutants. Bars, 50 nm.](image)

Table 1. Diameter measurements of MrNV capsid mutants

<table>
<thead>
<tr>
<th>MrNV capsid mutants</th>
<th>Diameter (nm)</th>
<th>TEM (n=100)</th>
<th>DLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MrNVc (full-length)</td>
<td>27 ± 3</td>
<td>30.5 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>9ΔMrNVc</td>
<td>25 ± 3</td>
<td>30.4 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>19ΔMrNVc</td>
<td>25 ± 4</td>
<td>30.4 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>29ΔMrNVc</td>
<td>18 ± 2</td>
<td>28.5 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>20–29ΔMrNVc</td>
<td>18 ± 2</td>
<td>26.1 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>K20R21R22K23R24A</td>
<td>23 ± 4</td>
<td>24.6 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>R26R27R29A</td>
<td>24 ± 4</td>
<td>25.0 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>K20R21R22K23R24R26R27R29A</td>
<td>18 ± 3</td>
<td>28.1 ± 0.0</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. RNA extracted from MrNV capsid mutants

<table>
<thead>
<tr>
<th>MrNV capsid mutants</th>
<th>RNA [ng (μg capsid protein)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>MrNVc (full-length)</td>
<td>127.5 ± 20.3</td>
</tr>
<tr>
<td>9ΔMrNVc</td>
<td>120.0 ± 17.5</td>
</tr>
<tr>
<td>19ΔMrNVc</td>
<td>124.8 ± 18.8</td>
</tr>
<tr>
<td>29ΔMrNVc</td>
<td>11.0 ± 2.0</td>
</tr>
<tr>
<td>20–29ΔMrNVc</td>
<td>10.0 ± 3.5</td>
</tr>
<tr>
<td>K20R21R22K23R24A</td>
<td>27.8 ± 4.2</td>
</tr>
<tr>
<td>R26R27R29A</td>
<td>90.2 ± 10.3</td>
</tr>
<tr>
<td>K20R21R22K23R24R26R27R29A</td>
<td>8.9 ± 3.8</td>
</tr>
</tbody>
</table>
positions 20–29, a distinctive feature that distinguishes it from the other nodaviruses.

All the N-terminal deleted mutants (9ΔMrNVc, 19ΔMrNVc and 29ΔMrNVc) and the internal deleted mutant (20–29ΔMrNVc) with or without the RNA-binding region still assembled into VLPs when observed under TEM and analysed with DLS. This indicates that the RNA-binding site and the capsid assembly domain are located at different positions in the primary structure of the MrNV capsid protein. Two distinct segments of polypeptides which are separately involved in nucleic acid binding and capsid assembly have been reported in several viruses, which include the core protein of the hepatitis B virus (HBcAg), where the first 144 aa are required for capsid assembly, while the C-terminal end (aa 145–183) is extremely basic for the packaging of pre-genomic RNA molecules (Nassal, 1992; Tan et al., 2003). A similar finding was also made in FHV, whereby deletion of the first 31 aa of its capsid protein stopped RNA encapsidation but not virion formation. Further deletion up to 50 aa of FHV capsid protein prevented the virion formation (Dong et al., 1998).

All the truncated and multiple point mutated MrNV capsid proteins assembled into spherical particles resembling the VLPs formed by full-length capsid protein. In general, the hydrodynamic diameters of the VLPs measured by DLS (24.6–30.5 nm) are larger than the diameters determined with TEM (18–27 nm). This could be due to the fact that, in a liquid medium, the charges on the surface of VLPs interact with salts and have an influence on their movements under Brownian motion. As a result, the hydrodynamic diameters measured by DLS are a combination of the size of particles and a hydration layer attached to the particles (Johnson & Gabriel, 1995). Differences in the size of virus particles as measured by DLS and TEM have also been reported for cowpea chlorotic mottle virus (Sikkema et al., 2007), adeno-associated virus serotype 2 (Joo et al., 2011) and turnip yellow mosaic virus (Harding & Johnson, 1985), in which the hydrodynamic diameters are larger than the diameters determined with TEM.

In summary, three N-terminal truncated mutants, one internal deleted mutant and three multiple point mutants were generated. The RNA-binding site of MrNV capsid protein was located at residues 20–29, 80 % constituted by positively charged amino acids. The mutants without the RNA-binding site still assembled into VLPs, suggesting that the capsid assembly domain and the RNA-binding region are located at different positions.

METHODS

Construction of N-terminal deletion, internal deletion and multiple point mutants. Plasmid pTrcHis2-TARNA2 (Goh et al., 2001).
harbouring the coding sequence of MrNV capsid protein, was used as the template in PCRs for the generation of deletion and multiple point mutants (Fig. 1). The primers used in the PCRs are listed in Table S2.

For the construction of the full-length MrNV capsid protein (MrNVc) and the N-terminal deletion mutants (9ΔMrNVc, 19ΔMrNVc and 29ΔMrNVc), PCR was performed in a mixture (50 μl) containing sense and antisense primers (1 μM), plasmid pTrcHis2-TARNA2 (0.5 ng), dNTPs (0.5 mM), 1/6 DreamTaq Buffer containing MgCl2 (2 mM; Thermo Scientific) and DreamTaq polymerase (5 U). The mixtures were incubated at 95 °C for 10 min followed by 30 cycles of 95 °C for 45 s, 53 °C for 45 s and 72 °C for 90 s. The mixtures were then incubated at 72 °C for 30 min.

The coding sequences of the internal deletion mutant (namely 20–29ΔMrNVc) and multiple point mutants (namely K20R21R22K23R24A, R26R27R29A and K20R21R22K23R24R26R27R29A) were generated by using fusion PCR as described by Szewczyk et al. (2006), Heckman & Pease (2007) and Ong et al. (2009). The DNA fragments located upstream and downstream of the deleted or point mutated regions were amplified using two pairs of primers (F1 and R1; F2 and R2; Table S2), in two reaction mixtures (50 μl) containing template plasmid (0.5 ng), primers (1 μM), dNTPs (0.5 mM), 1× DreamTaq Buffer and DreamTaq polymerase (5 U). The PCR profile was as described above. The PCR products were separated on a 1% agarose gel and target bands were excised. The excised DNA bands were purified using a Qiaquick Gel Extraction kit (Qiagen). The purified upstream fragment (20 ng) and the downstream fragment (40 ng) were added into a single reaction mixture (25 μl) containing dNTPs (0.5 μM), 1× DreamTaq Buffer and DreamTaq polymerase (2.5 U). The reaction mixture was incubated at 95 °C for 5 min followed by five cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 120 s, to fuse the upstream and downstream fragments. The second reaction mixture (25 μl), containing primers (F1-20–29ΔMrNVc, R2-20–29ΔMrNVc), dNTPs (0.5 μM), 1× DreamTaq Buffer and DreamTaq polymerase (2.5 U), was added into the first reaction mixture. The total 50 μl reaction mixture was amplified in a 10-cycle PCR at 95 °C for 30 s, 53 °C for 30 s and 72 °C for 120 s.

The PCR products harbouring the coding sequences of the full-length MrNV capsid protein, N-terminal deletion, internal deletion and multiple point mutants were separated on 1% agarose gel, purified and ligated into the pGEM-T vector (Promega). The ligation mixtures were introduced into E. coli TOP 10 cells. The recombinant plasmids were extracted by using the alkaline lysis method (Sambrook et al., 2001) and digested with NcoI and EcoRI. The inserts were purified from 1% agarose gel and ligated into the pTrcHis2 vector which had been digested with the same restriction endonucleases. The ligation mixtures were then introduced into E. coli TOP 10 cells. The recombinant plasmids extracted from the positive transformants were verified with PCR, restriction endonuclease digestion and nucleotide sequencing.
Protein expression and purification. Expression and purification of the mutants were performed as described by Goh et al. (2011). Briefly, E. coli cells harbouring the recombinant plasmids were grown in LB broth (500 ml) containing ampicillin (50 µg ml⁻¹). Expression of the recombinant proteins was induced with IPTG (1 mM). Cells were harvested after 5 h of induction and resuspended in HEPES buffer (25 mM HEPES, 500 mM NaCl, pH 7.4) containing MgCl₂ (4 mM), lysozyme (0.2 mg ml⁻¹), PMSF (2 mM) and DNase I (0.02 mg ml⁻¹). The mixture was incubated at room temperature with continuous rotation for 2 h and sonicated 12 times at 200 Hz for 10 s. The sonicated samples were centrifuged at 12,000 g for 10 min and the supernatant was filtered with syringe filters (0.45 µm; Millipore). The filtered samples were then loaded into a His-Trap HP 1 ml column (GE Healthcare) and the bound proteins were washed with binding buffer A (25 mM HEPES, 500 mM NaCl, 50 mM imidazole, pH 7.4) and binding buffer B (25 mM HEPES, 500 mM NaCl, 200 mM imidazole, pH 7.4). The bound proteins were eluted using elution buffer (25 mM HEPES, 500 mM NaCl, 500 mM imidazole, pH 7.4) and their concentrations were determined by using the Bradford assay (Bradford, 1976). The purified proteins were analysed with SDS-PAGE and Western blotting as described by Goh et al. (2011).

Sucrose density gradient ultracentrifugation. The purified proteins (400 µg) were layered on top of a sucrose gradient [8–40% (w/v)] and centrifuged at 150,000 g for 5 h at 4 °C. Fractions (1 ml) were collected and SDS-PAGE was performed as described by Goh et al. (2011). The concentration of the protein in each fraction was determined by using the Bradford assay.

DLS analysis of purified capsid proteins. The purified proteins were diluted to 100 µg ml⁻¹ using elution buffer and filtered using a syringe filter (0.2 µm). The sizes of the particles were then determined with a DLS machine (DynaPro-801; Protein Solution) and the hydrodynamic radius (R_h) of the particles at 25 °C was determined as described by Tan et al. (2003).

Transmission electron microscopy (TEM). The protein samples were diluted to 10, 25 and 50 µg ml⁻¹ using elution buffer and filtered through a 0.2 µm porosity Pall Acrodisc syringe filter (Pall). The diluted samples (15 µl) were absorbed onto a 200-mesh carbon-coated Formvar copper grid (Agar Scientific) for 5 min and stained with freshly prepared uranyl acetate (2%) for 5 min at room temperature. The stained sample grids were viewed under a transmission electron microscope (Hitachi HT-7700). The particle size was then measured by using the ImageJ software (NIH).

Detection of RNA molecules that associate with mutated MrNV capsid proteins. Freshly purified proteins (100 µg) were extracted with phenol : chloroform : isoamyl alcohol (25:24:1; 300 µl) and centrifuged at 13,000 g, 4 °C for 10 min. The aqueous phase was mixed with chloroform (300 µl), vortexed and centrifuged at 13,000 g, 4 °C for 10 min. The nucleic acids were precipitated with 2-propanol (600 µl) and the pellets were resuspended in nuclease free water (25 µl). Confirmation of RNA molecules was done as described by Goh et al. (2011). The extracted RNA molecules were analysed on 1% agarose gel and quantified with the NanoPhotometer P300 (Implen).

Native agarose gel electrophoresis (NAGE) of MrNV VLPs. NAGE was performed as described by Lee & Tan (2008) and Yoon et al. (2013) with some modifications. Purified VLPs (10 µg) of MrNV capsid proteins were analysed on a native 1.0% (w/v) agarose gel containing the Atlas ClearSight nucleic acid stain (Bioatlas) and electrophoresed in 1 x TBE buffer (200 mM NaOH, 750 mM boric acid, pH 8.3) at 60 V for 2 h at 4 °C. Fluorescence was visualized by UV illumination and protein was then stained with Coomassie blue.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Science, Technology and Innovation of Malaysia (MOSTI) and the Ministry of Higher Education Malaysia (MOHE). Z. H. G. was funded by a National Science Fellowship (NSF) from MOSTI and MyPhD from MOHE. N. A. S. M. was supported by a Graduate Research Fellowship from UPM.

REFERENCES


