ABSTRACT

The NS3 protein of *Rice hoja blanca tenuivirus* has previously been demonstrated that it is a viral suppressor of RNA silencing which binds to the small interfering RNA molecule as a dimer. Structure determination serves as the main tool to study the suppressing mechanism of the RNA silencing of this protein. However, the full length NS3 protein resisted all crystallization attempts for structure determination. To investigate the functional domain of the NS3 protein, proteolytic enzyme digestion was performed on the full length NS3 protein to identify the stable and rigid region. Truncated NS3 protein with amino acid of 108 residues (21-128) has resisted the enzyme digestion which is believed to be the functional core of the NS3 protein. Electrophoretic mobility shift assays and *Agrobacterium* infiltration have further testified that this truncated fragment is involved in the siRNA binding and RNA silencing-suppressing activity. Infiltration with the NS3 truncated construct has resulted in complete reactivation of the GFP expression in the GFP silencing transgenic plant. The truncated NS3 protein was observed to have better siRNA binding affinity and greater suppression activity as compared to the full length NS3 protein which suggests that it might be the functional core of the NS3 protein.

INTRODUCTION

RNA silencing is an evolutionarily conserved mechanism that targets and degrades aberrant endogenous and exogenous RNA molecules (Hemmes, 2007; Sontheimer, 2005). The process includes the endonucleolytic cleavage of longer double-strand RNA molecules into short interfering RNA (siRNA) or miRNA species which will then be incorporated into the RNA-induced silencing complex (RISC) and trigger sequence specific degradation of the RNA targets. (Schnettler, 2008).

NS3 is a viral suppressor protein encoded by the *Rice hoja Blanca tenuivirus* which is a negative strand RNA virus that will infect both plants and their arthropod vectors (Ramirez, 1992; Ramirez, 1993). NS3 protein has high affinity for 21 nt double strand siRNAs and it can bind to siRNA as a dimer to suppress the RNA silencing mechanism (Hemmes, 2007). In vivo, it has been reported NS3 also interfere with the miRNA regulated RNA silencing pathway and induce developmental abnormalities in plants (Hemmes, 2007).

To establish a more detailed model for the molecular basis of NS3 of the RNA silencing suppressor, we would like to investigate the functional domain of the NS3 which is responsible for the siRNA binding and RNAi suppressing activity in vitro. To gain a better insight of the NS3 functional domain, RNA silencing suppression and siRNA binding capability were tested respectively with *Agrobacterium tumefaciens* co-Infiltration assay and electrophoretic mobility shift assay. However, this NS3 fragment protein with the functional domain resisted all crystallization efforts due to precipitation of the protein at high concentration and this has prevented structure determination.

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METHODS AND MATERIALS

Proteolytic enzymes (trypsin and chymotrypsin) were used to digest the NS3 full length protein. The digested fragments were analyzed by Mass Spectrometry. From the location of the proteolytic cleavage site and the secondary structure prediction analyses, the chosen fragments were cloned into the pET28a vector with a SUMO tag at the N-terminal. The recombinant plasmids were then transformed into the Escherichia coli (BL21/DE3 strain) cell.

The cells were expressed and grown for overnight. The bacterial cells were harvested and ruptured by high pressure homogenizer and centrifuged. The supernatant was collected and purified using Ni²⁺ affinity column. After elution, protease Ulp 1 enzyme was then added to the sample to cut off the SUMO tag. NS3 truncated protein was further purified by gel filtration column to remove undesired proteins. SUMO tag was removed by passing through the Ni²⁺ affinity column. The flow through which contained the purified truncated protein was concentrated.

Electrophoretic mobility shift assays were used to test the binding affinity of the purified truncated protein NS3 and the full length NS3 to the siRNA. To examine the RNA silencing suppression activity, both full length NS3 and truncated NS3 were subjected to Agrobacterium tumefaciens co-infiltration assays. The GFP fluorescence expression in Nicotina benthamiana leaves was monitored.

RESULTS

Mass spectrometry analysis showed that sample treated with both trypsin and chymotrypsin produced the same fragment of protein with amino acid of 21 to 124 residues. From the secondary structure prediction, the C-terminal beta strands presents from the amino acids 121 to 126. To avoid breaking a predicted secondary structural element, residue 128 was chosen as the C-terminus. A 108-residues of NS3 protein construct consisting of residue 21 (the primary cleavage site of trypsin and chemotrypsin) to residue 128 was cloned and expressed in the E.coli.

The truncated NS3 protein fusion with the N-terminal SUMO tag approximately 27 KDa was successfully purified by Ni²⁺ affinity column chromatography. Presence of the sum-tag might affect the functional region of the NS3 protein; therefore, the SUMO tag was cleaved by SUMO protease enzyme Ulp 1. The mixture of sumo tag and truncate protein NS3 was separated by passing through the the Ni²⁺ affinity column. The pure truncated NS3 protein was concentrated until 4 mg/ml. Further concentrating would cause precipitation of the NS3 truncated protein.

Electrophoresis Mobility Shift Assay result showed that the truncated NS3 was able to bind to the dsRNA which indicated that the siRNA binding domain lies in the truncated region. Truncated NS3 protein showed siRNA binding even at low concentration (3 mg/ml) which showed that it has high affinity for 21nt siRNA. Increase in the volume of the protein has increased the amount of the siRNA-protein complexes formed. Consistent to the previous studies, EMSA results showed that the full length NS3 protein (5 mg/ml) bound to the 21-nt double strand siRNA. However, it was observed that there are some smear regions around the siRNA-protein complexes. This is caused by the dissociation of the siRNA-protein complex during electrophoresis, indicating that the binding to the siRNA is not very efficient.

In vitro Agrobacterium co-infiltration assay was used to examine the RNA silencing suppressing activity of the truncated NS3 protein. After 3 days postinoculation, both NS3 full
length protein and NS3 truncated protein showed a complete reversal of the GFP silencing and became GFP fluorescent in infected tissue. This result showed that truncated NS3 (21-128) has retained the suppressing RNA silencing ability. NS3 truncated protein was observed to have greater RNA silencing suppression activity than the NS3 full length protein. The suppression of the NS3 protein is markedly less than that of the suppression of the truncated protein as indicated by the colour of the spot. This strongly suggests that the RNA silencing domain might lies within the truncated NS3 protein with the amino acid residues of 21 to 128.

**DISCUSSION**

The full length NS3 protein was initially subjected to crystallization, however, all attempts failed due to the protein highly unstable and precipitated easily. To solve the problem, a more stable region of the NS3 protein was determined through proteolytic enzyme digestion method as a rigid and stable region will resist enzyme digestion. The protein domain is usually a compact structure that is independently folded. The folding of the structure protects the inner amino acid residue from the digestion by making it inaccessible to the enzyme. Therefore, the region of the protein is highly stable which normally carries a function.

SUMO tag was chosen to clone with the NS3 truncated protein in the pET expression system as it produces high levels of soluble protein in bacteria. In addition, SUMO tag contains 6X Histidine which can bind specifically to the Ni$^{2+}$ ligand of the chromatography matrix which facilitates the purification process. One drawback of using the SUMO tag is that it involves extra purification step to remove the SUMO moiety and this might cause protein lost during the purification processes. Precipitation occurred when the truncated protein was concentrated to 5 mg/ml. All attempts to crystallization were not successfully due to low protein concentration. It was observed that this truncated protein has even lower solubility than the full length NS3 protein.

Truncated NS3 protein consists of the siRNA binding domain where it can readily bind to siRNA even at low protein concentration (3 mg/ml). It formed a single band of protein-siRNA complexes on the film without any smearing regions. This indicates that the protein bound firmly to the siRNA without dissociation, suggesting the high binding efficiency of the truncated protein NS3. Whereas in the case of the full length NS3 protein, results showed that it bound relatively weak as compared to the truncated NS3 protein. Smearing regions can be clearly observed and this was due to the dissociation of the protein-siRNA complex during electrophoresis. A possible explanation is that the full length NS3 protein might contain some inhibition region which is responsible for the inhibition mechanism of the siRNA binding under certain circumstances. Removal of some portions of the NS3 protein has improved the binding efficiency as the inhibition regions might have been removed in the truncated protein.

Upon infiltration with the NS3 truncated gene, transgenic plants silencing the GFP transgene showed a strong recovery of GFP fluorescence, suggesting that RNA silencing is suppressed in the infected plant cells. No GFP fluorescence was observed when the transgenic plants were infiltrated with the 3SS-GFP construct alone. When the 35S-GFP gene co-injected with the NS3 gene constructs, it is able to restore transient expression of GFP in the infiltrated regions. This is because the NS3 protein will bind to the dsRNA formed to suppress the silencing activity. None of the cases showed suppression of silencing beyond the inoculation region which indicates that the suppressor protein was unable to induce a mobile silencing suppressing signal to the other part of the transgenic plant. NS3 truncated gene showed greater RNA silencing suppression activity as compared to the NS3 full length protein. This has further testified that the truncated NS3 sequence could be the functional core of the
protein which consists of the siRNA binding domain that responsible for the RNA silencing suppression activity.

NS3 protein resisted all crystallization attempts due to its high instability and low solubility which causes precipitation. More extensive research needs to be carried out in the future to improve the solubility of the NS3 protein. MBP tag can be used as the fusion partner as it can enhance the solubility of the protein. The solubility of the truncated protein might also improved by adding an appropriate buffer. In future studies, truncated fragment of the NS3 can be tested in vivo to investigate whether it will interfere with the miRNA regulated RNA silencing pathway which subsequently regulates the gene expression of the plants.

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REFERENCES


