Proteomic approach: Identification of *Nephotettix virescens* vector protein transmitting the tungro virus in rice

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Abstract. Senoaji W, Rahardjo BT, Tarno H. 2021. Proteomic approach: Identification of *Nephotettix virescens* vector protein transmitting the tungro virus in rice. Biodiversitas 22: 2750-2755. As a vector of tungro virus, *Nephotettix virescens* is one primary pest in rice cultivars. Associated *Rice Tungro Spherical Virus* provides a helper component for *Rice Tungro Bacilliform Virus* acquisition produced the severe symptoms in rice. The vector proteins encoded by the virus become a poorly understood transmission of plant viruses, particularly in semi-persistent mechanisms. It is essential to develop new molecules to interrupt the transmission mechanism of plant viruses by insect vectors. This study aimed to detect the helper proteins in vectors with proteomics. Protein separation was performed using SDS-PAGE, and then was identified by the Liquid Chromatography-Mass Spectrometry (LC-MS/MS) to find the candidate proteins. The result showed that actin was identified in *N. virescens* as responsible protein that related to transmitting tungro viruses into the plant.

Keywords: *Nephotettix virescens*, protein, tungro, SDS-PAGE, MS/MS, actin

INTRODUCTION

*Nephotettix virescens* is one primary pest in rice cultivar because it is the tungro virus vector (Rice tungro virus, RTV) (Khan and Saxena 1985). RTV is an interaction of two different viruses between Rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV), which are transmitted simultaneously or separately by the vector from plants infected with both virus particles (Hibino et al. 1979). The presence of RTSV in the vector body provides a helper component for RTBV acquisition, which is transmitted semi-persistently (Hibino 1996).

The hypothesis that the helper components form a “molecular bridge” can be reciprocal between vector and viruses (Froissart et al. 2002). While studies have shown that the protein encoded by the virus will support the development of new molecules to interrupt the transmission mechanism of plant viruses by vector insects (Fereres and Raccah 2015), non-structural proteins encoded by viral particles mediate virus-vector binding factor. However, the identity and function of helper proteins are also unknown (Ng and Zhou 2015).

A possible approach to detect the presence of helper proteins in *N. virescens* vector is the molecular approach with proteomics. Proteomics can describe an organism's protein identity (Pennington et al. 1997). A tool of tandem mass spectrometry (MS/MS) spectra generated the amino acid sequence and predicted to classify proteins by their similarity to current database entries (Liska and Shevchenko 2003). The sophisticated separation and pre-fractionation techniques have been developed to extend coverage, enhanced dynamic range and sensitivity (Zhang et al. 2014), mass spectrometry-based proteomics being the primary technique for large-scale protein characterization due to the complexity of proteomes. Protein expression on the interaction of vector and plant virus has been a comprehensive study. The helper component protease (HC-pro) of potyvirus in turnip mosaic virus was known to be involved in the formation of polyproteins, transmission, and suppression of antiviral RNA (Guo et al. 2011). Cuticular protein (CPR1) is used by the rice stripe virus to promote movement, replication, and continuity in the hemolymph of *Laodelphax striatellus*, even contributing to persistent transmission (Liu et al. 2015). The role of cuticular structures in the Aphid *Acyrthosiphon pisum* reveals candidate receptors against plant viruses at the stylet's tip (Deshoux et al. 2020). As described previously, protein was identified in several vector insects based on previous studies but till now, protein of *N. virescens* has not been known. So, protein identification is needed to investigate the tungro transmission activity by *N. virescens*.

MATERIALS AND METHODS

*Nephotettix virescens* vectors and tungro transmission test

*Nephotettix virescens* individuals were collected from an endemic area in Pasuruan region, East Java Province, Indonesia, in February 2020. The suspected plant was obtained from the field that indicated tungro symptoms. About 5-10 samples of the suspected plant were taken from a different location. Tungro transmission detected by *Polymerase Chain Reaction* (PCR) (Takahashi et al. 1993). PCR detection was targeted at RTBV gene because it has DNA structure more stable than RTSV. Total DNA
Protein extraction

Protein extraction was based on the symptoms that appeared on the tested plants. Twenty individuals for each group were weighed (ca. 50-100 mg), then extracted using the ReadyPrep™ Protein Extraction Kit (Bio-RAD). The extraction procedure followed the manufacturing protocol.

Separation protein by SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins based on molecular weight (MW) with Laemmli buffer (Laemmli 1970). Samples were separated using SDS-PAGE to obtain major protein. Usually, there are various limitations to 2-dimensional electrophoresis, including the difficulty of separating largest or smallest proteins and acidic or basic proteins (Hattori et al. 2015). The total protein samples were prepared using 12% SDS-PAGE. The sample's buffer solution contained acrylamide, 0.25M Tris-HCl (pH 8.8), 10% SDS, 10% APS, and TEMED. The sample protein concentration was measured using a nanodrop spectrophotometer to determine the concentration ratio to be added to the buffer. RSB buffer ratio with sample was 3:1. The sample and RSB were mixed and then heated at 100°C for 10 minutes. The 10-20 μl of buffered samples were inserted into the electrophoresis well (buffer pH 8.3). The electrophoresis process was run at 100 volts for 60 minutes. The resulting protein bands were stained with Coomassie Brilliant Blue (CBB).
The SDS-PAGE analysis showed the same protein in MW of 49 and 54 kDa, namely, actin-2 or the protein group (member of protein family) actin. It showed that MS has a high level of sensitivity and accuracy. MS was an effort to verify the expected identity.

Target protein in this proteomic study has been identified. Identity obtained has more detailed derivative information on a database. It can be used or developed in subsequent studies, especially the interaction between vectors and tungro viruses. In this study, actin was first identified in the N. virescens, supposed to be involved in the interaction with the tungro virus which showed more severe infectious symptoms (> 30% stunting and yellowing leaves).

**Discussion**

In the SDS-PAGE analysis, both BM estimates showed the same protein. In gel-based proteomic, gel staining is proportional to the total protein content of each gel band. It was not possible to characterize quantitative and qualitative information in more than one protein. It is a problem in most MS/MS identification due to the limited resolution of separation with SDS-PAGE (Campos-trini et al. 2005). However, MS-based proteomics has been used primarily to identify, characterize, and quantify proteins. The accurate results have increased the use and confidence in molecular biology studies (Bensimon et al. 2012).

Identification method in this study uses a bottom-up strategy (Kellie et al. 2010). In the workflow, the protein is cut into smaller peptide fragments using an enzyme (trypsin), and then the peptide is analyzed by MS. Protein identification was based on two substances. First, the observed peptide mass's suitability with the prediction of protein values from the protein sequence database (Peptide-mass fingerprinting, PMF). Second, the peptide sequence's suitability (conclusions from the MS/MS spectrum fragmentation) to the amino acid fragmentation of the protein sequence from the database (database searching). Protein identification can be determined at different levels of precision, depending on the analysis's objectives and success in identifying unique peptides (Szabo and Janaky 2015).
The facts related to actin proteins and actin-binding proteins (ABPs) have been extensively studied. In cells, actin has various functions (multi-functional) from regulating gene expression to maintaining genome integrity. Actin is also called the cytoskeleton or cell skeleton (Mostowy and Shenoy 2015). Actin and ABPs are present in the cytoplasm and nucleus. ABPs regulate actin metabolism in response to various intra and extracellular signals (cell signaling), and where the presence of actin forms as free monomers is called G-actin and as part of microfilaments is called F-actin, both of which function in cell motility and contraction in cell division (Furukawa and Fechheimer, 1997), so circulating actin in the nucleus is a mechanism to maintain cell homeostasis (Davidson and Cadot 2020). There are three actin groups in eukaryotes based on their protein variants (isoforms), namely alpha, beta, and gamma. Alpha-actin occurs in muscle tissue, beta-actin and gamma-actin are often together as components of the cytoskeleton and mediators of internal cell motility (Kelber and Klemke 2011). While actin production is also the key to the process of infection by several pathogenic microorganisms (Kyheroinen and Vartiainen, 2020), it has recently been discovered that actin also plays a role in controlling gene expression and mutations cause some diseases in the alleles of genes that regulate actin production or the proteins associated with them.

The interaction of both actin and ABPs in insect vectors has been studied with several plant viruses. *Myzus persicae* vector is a subtropical climate plant pest. Receptor for activated C kinase (Rack-1), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH3), and actin in the membrane complex binding Beet western yellows virus (BWYV) particles to facilitate transcytosis in the vector body. BWYV is a circular-persistent viral particle, bound by (Rack-1), (GAPDH3), and actin via micropinocytosis and polarized transport across epithelial cells using the cytoskeleton element pathway. However, the actual receptor for BWYV has not been identified (Seddas et al. 2004).

The cytoskeleton is a critical component of the intracellular transport route for the movement of plant viruses. Chen et al. (2015) studied the non-structural protein interaction of the Rice Dwarf Virus (RDV) in its vectors *Nephotettix cincticeps* and *Recilia dorsalis*, which are sucking pests in rice plants. Protein responses revealed that microtubules and actin cytoskeleton in the vector supported the virus's movement across cell surface protrusions. The interaction of virus and insect actin can determine the specific insect vector. RDV and their vector relationship is a persistent transmission mechanism for rice plants (Chen et al. 2011). However, vector proteins with plant viruses that are non-persistent or semi-persistent are still poorly understood or studied.

Transmission of RTBV and RTSV viruses are a semi-persistent mechanism by *N. virescens* vector studied in this study. The transmission test was carried out to provide a vector opportunity to acquisition feeding 2 × 24 hours from infected plants, then transferred to a healthy plant for 2 × 24 hours. The results showed symptoms to transmit both viruses, causing severe symptoms (≥ 30% stunting and yellowing leaves). This treatment has implications for responding to more actin in the vector body during the transmission mechanism than the vector group with mild symptoms, non-symptoms, or vectors in healthy plants.

### Table 2. The results of LC-MS/MS identification with the Mascot algorithm using the Basic local alignment search tool on the UniProt database against the estimated MW of 49 and 54 kDa in the vector group severe symptoms.

| Protein characteristics | 49 kDa<sup>1</sup> | Sample | 54 kDa<sup>1</sup> |
|-------------------------|-------------------|--------|-------------------|
| Accession no. (gi IDs)<sup>2</sup> | A0A125RA11 | A0A125RA11 |  |
| Protein (ID)<sup>3</sup> | Actin 2 | Actin 2 | |
| Species (Taxonomy)<sup>4</sup> | *Nephotettix cincticeps* | *Nephotettix cincticeps* |  |
| Protein domain<sup>5</sup> | actin | actin |  |
| Mowse score<sup>6</sup> | 84 | 414 |  |
| Unique peptides<sup>7</sup> | 2 | 7 |  |
| Sequence coverage (%)<sup>8</sup> | 5 | 23 |  |
| Predicted mass (kDa/IP)<sup>9</sup> | 42.081/5.29 | 42.081/5.29 |  |
| A sequence of matched peptides<sup>10</sup> | AVFPSIVGRPR; GYSFTTTAER | AVFPSIVGRPR; VAPEEHPILLTEAPLNPK; DLTDYLMK; GYSFTTTAER; SYELPDGQVITIGNER; EITALAPSTIK; QYEYDESGPGIVHR |  |

Note: <sup>1</sup>estimation of MW candidate protein on SDS-PAGE; <sup>2</sup>ID number on UniProt database; <sup>3</sup>protein sample identity match to the UniProt database; <sup>4</sup>homologous species; <sup>5</sup>structural and functional unit of protein according to Pfam version 33.1 (http://pfam.xfam.org/); <sup>6</sup>Mascot’s probability score > 54 indicates identity (p < 0.05), a higher score indicates the accuracy of the identity; <sup>7</sup>the number of amino acids in a specific protein sequence found in the sequenced peptide in the MS/MS; <sup>8</sup>The percentage coverage of the sample amino acid sequence to the homologous sequence; <sup>9</sup>Molecular weight and isoelectric point accuracy at a pH indeed; <sup>10</sup>Amino acid sequence match to database ≥ 2 indicates protein identity.
There are two hypotheses for this study. First, upregulated actin occurs in the tissue of the stylet sheath because RTSV and RTBV virus particles interacts intensively with actin. Proteomic analysis of the salivary glands and saliva secreted (watery/gelling saliva) showed that actin and ABPs activity occurs in rice-sucking pests i.e. Nilaparvata lugens and Sogatella furcifera (Huang et al. 2018) but is not expressed on N. cincticeps (Hattori et al. 2015). However, their interactions with plant viruses can be different and specific, especially with the RTBV and RTSV particles being semi-persistent viruses, the actin expressed on the N. virescens vector in this study is homologous to N. cincticeps and exhibited the same function in vector body metabolism. Thus, future studies can be done to the specific interaction mechanism of actin and ABPs proteins against RTBV and RTSV.

Interestingly, three-dimensional (3D) structure of actin expression showed homology to that of N. cincticeps. This information could be a hope regarding the management of pests and diseases in rice plants. 3D structures can be used as a further study with a combination of methods with an in-silico approach (Mishra et al. 2021) to predict and find new molecules as candidate components to control tungro disease from an insect vector perspective.

In conclusion, actin in N. virescens vector interacts with RTSV and RTBV, which causes the tungro symptoms in rice. Actin interactions can be specific with upregulating, although the quantity is not known. Actin could be a protein candidate that be studied further to design biomarkers.

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