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Characterization of a novel envelope protein WSV010 of shrimp white spot syndrome virus and its interaction with a major viral structural protein VP24

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Abstract

White spot syndrome virus is one of the most serious viral pathogens causing huge mortality in shrimp farming. Here we report characterization of WSV010, a novel structural protein identified by our recent shotgun proteomics study. Its ORF contains 294 nucleotides encoding 97 amino acids. Transcription analysis using RT-PCR showed that *wsv010* is a late gene. Localization analyses by Western blot and immunoelectron microscopy demonstrated that WSV010 is a viral envelope protein. Furthermore, the pull-down assay revealed that WSV010 could interact with VP24, which is a major envelope protein. Since WSV010 lacks a transmembrane domain, these results suggest that WSV010 may anchor to the envelope through interaction with VP24. Previous studies indicated that VP24 could also interact with VP28 and VP26. Therefore, we propose that VP24 may act as a linker protein to associate these envelope proteins together to form a complex, which may play an important role in viral morphogenesis and viral infection.

Keywords: White spot syndrome virus; WSV010; Envelope protein; VP24

Introduction

White spot syndrome virus (WSSV) is the most major serious shrimp pathogen, which can cause up to 100% mortality of shrimp within 7 to 10 days. Thus it is a cause of huge economic losses to the shrimp farming industry (Wang et al., 2000). Moreover, it can also infect a large variety of other marine to freshwater crustacean species, including freshwater crabs and crayfish. WSSV is an enveloped, double-stranded DNA virus (Wang et al., 1995). Its isolates from China, Taiwan and Thailand have been sequenced by three groups respectively (Chen et al., 2002; van Hulten et al., 2001; Yang et al., 2001). The first proteomic study of WSSV by using one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry or electrospray ionization tandem mass spectrometry (ESI-MS/MS) utilizing a quadrupole time-of-flight (Q-TOF) mass spectrometer has led to identification of 18 structural proteins (Huang et al., 2002b). Additional seven structural proteins were identified by two-dimensional gel electrophoresis and mass spectrometry (Zhang et al., 2004). Twenty new WSSV structural proteins were identified by one-dimensional SDS–PAGE and the on-line liquid chromatography (LC)-ESI Q-TOF mass spectrometer (Tsai et al., 2004). Most recently, our group has applied shotgun proteomics approach by using offline coupling LC system with MALDI-TOF/TOF MS/MS to investigate the WSSV proteome (Li et al., submitted). WSV010 is one of the novel viral structural proteins that have been identified by this approach.

Here we report characterization of WSV010 by transcription analysis and localization study. Transcription analysis using RT-PCR showed that *wsv010* is a late gene. WSV010 was identified as an envelope protein by Western blot analysis and

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the immunoelectron microscopy (IEM) technique. In an effort to further understand the function of this protein, a pull-down assay was performed to study interactions of WSV010 with the virus and host proteins. We found that WSV010 could interact with a major WSSV envelope protein VP24.

Results

Temporal analysis of wsv010 gene transcription

The transcription analysis of *wsv010* was carried out by RT-PCR. RNAs were extracted from shrimp tissues before infection (0 h) and at 2, 4, 6, 8, 12, 24 and 72 h after the WSSV challenge (Fig. 1). The *wsv010* gene specific transcript was first detected at 24 hpi (hours post infection). A major structure gene *vp28* detected from 12 hpi was used as a positive control and shrimp *β-actin* gene as a loading control. This result indicated that *wsv010* is a late gene.

Expression of WSV010 and antibody preparation

wsv010 was cloned into the modified pET vector with N-terminal Avi-tag and C-terminal 6× His tag. The recombinant protein was overexpressed in the *E. coli* strain BL21 Star (DE3) (Fig. 2A). The Avi-tag enables the recombinant protein to be biotinylated directly in *E. coli*. The expressed fusion protein was purified using the Ni-NTA resin under native condition. The identity of the WSV010 fusion protein was confirmed by mass spectrometry using MS/MS sequencing (Table 1). A rabbit polyclonal antibody against WSV010 was successfully raised using the purified recombinant protein. The specificity of the antibody was tested with the recombinant WSV010 (Fig. 2B). The optimal dilution of the polyclonal antibody was 1:1000 and it could be used to detect the purified recombinant protein in amounts as low as 0.2 μg.

Localization of WSV010 by Western blot analysis

Purified virions, envelope proteins and nucleocapsid proteins of WSSV were separated by SDS–PAGE for Western blot analysis with the polyclonal anti-WSV010 antibody. Western blot results showed that WSV010 polyclonal antibody recognized the protein specifically in the whole virion and the viral

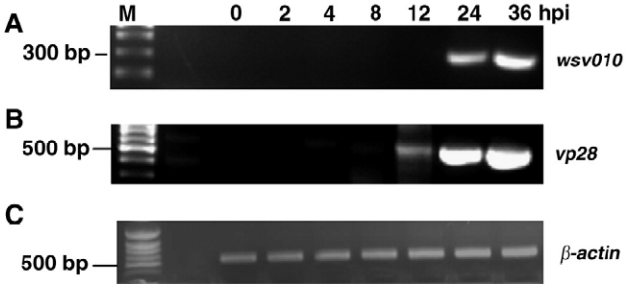

Fig. 1. Temporal analysis of *wsv010* gene transcription. RT-PCR was conducted using (A) *wsv010* specific primers, (B) *vp28* specific primers, and (C) Shrimp *β-actin* specific primers, respectively.

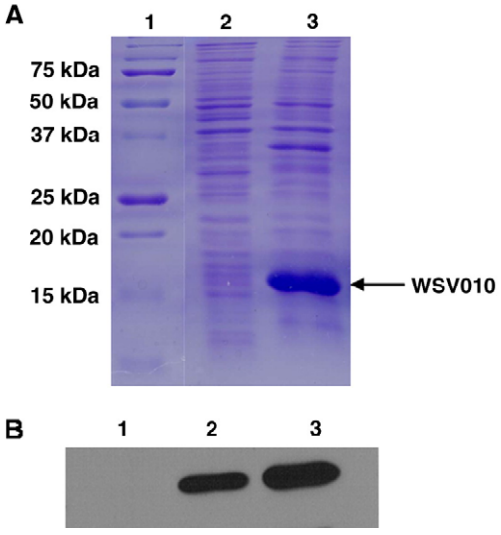

Fig. 2. Expression and detection of WSV010 expressed in *E. coli*. (A) Expression of WSV010 in *E. coli*. Lanes 1, the Precision Plus Prestained Dual Color marker (Biorad); lane 2, uninduced bacterial lysate of WSV010; and lane 3, induced bacterial lysate of WSV010. (B) Detection of WSV010 with its polyclonal antibody. The amounts of the recombinant WSV010 used from lane 1 to lane 3 are 0.02 μg, 0.2 μg and 1 μg, respectively.

envelope fraction, but not in the nucleocapsid fraction (Fig. 3A). It is noted that WSV010 exists as a dimer or a complex in virus but as a monomer in the recombinant protein. One possibility is that WSV010 could associate with itself to form a dimer or the protein with similar molecular weight to form a complex *in vivo*. The failure of the recombinant protein to form a dimer by itself may be due to the existence of the N- and C-terminal tags. Envelope protein VP28 was used as a control (Fig. 3B). These results proved that WSV010 is associated with the viral envelope and not with the nucleocapsid.

Localization of WSV010 by IEM

IEM technique was further applied to confirm the localization of WSV010 on the envelope. Both 0.1% Tween 20 treated WSSV virions and the nucleocapsid fraction were spotted onto nickel grids separately to carry out immunogold labeling experiments. The gold particles were located on the surface of WSSV virions using WSV010 polyclonal antibody as the primary antibody (Fig. 4A). However, no gold particles could be found on the nucleocapsids (Fig. 4B). When pre-immune rabbit serum was used as the primary antibody in the control experiment, no gold particles could be detected on WSSV virions (Fig. 4C). These results demonstrated that WSV010 should be an envelope protein, which is consistent with the Western blot result.

Pull down of VP24 by WSV010

The biotinylated recombinant WSV010 protein was used as a bait to pull down proteins from epithelial tissue of the healthy and the infected crayfish, respectively. Eluted proteins were resolved by SDS–PAGE followed by the silver staining. A

Table 1
Measured and calculated molecular masses of tryptic peptides which match WSV010 of shrimp white spot syndrome virus

Start	End	Peptide	Observed	Mr(expt)	Mr(calc)	Delta
10	26	SAITLVLQSPEFVNDVK	1860.02	1859.01	1859.00	0.00
10	41	SAITLVLQSPEFVNDVKQEASQVVEGLIPSIR	3466.95	3465.94	3465.87	0.07
27	41	QEASQVVEGLIPSIR	1625.89	1624.89	1624.88	0.01

Protein sequence of WSV010 (matched peptides are highlighted in bold).
1 MDILEDIYK**S** **AITLVLQSPE** **FVNDVKQEAS** **QVVEGLIPSI** **R**EAVFRRLLE
51 EERKKHEDEV GDVEDKRQAV IDKANTMITT MAAEYLESVD ILEEFGF

unique band was found in the pull-down fraction of WSV010 with the infected epithelial tissue proteins (Fig. 5A), but not in the bait control (WSV010 only) and the negative controls (epithelial tissue proteins only). This band was identified as VP24 of WSSV by mass spectrometry analysis (Table 2). The interaction of WSV010 with VP24 was further confirmed by Western blot using the anti-VP24 polyclonal antibody (Fig. 5B). No such band was detected in the wash fraction and negative controls.

Discussion

To date, a total of 59 structural proteins have been identified by gel-based proteomic studies (Huang et al., 2002b; Tsai et al., 2004; Xie et al., 2006; Zhang et al., 2004) and our shotgun proteomic study (Li et al., submitted). WSV010 was identified as a novel structural protein firstly by the shotgun proteomic approach. The failure to identify WSV010 using gel-based proteomic studies is probably due to its low abundance in WSSV virions. Homology search of WSV010 against GenBank using BLAST shows no significant similarity to any other known proteins in the database.

In this paper, we describe that transcription analysis with RT-PCR showed that *wsv010* is a late gene, which could be detected only at 24 hpi onwards. WSV010 was identified as an envelope protein by Western blot analysis and further confirmed by IEM observation. These results are consistent with the hypothesis that the capsid proteins should appear early to form the nucleocapsid and that envelope proteins appear at a later stage to form the envelope surrounding the nucleocapsid (Tsai et al., 2004).

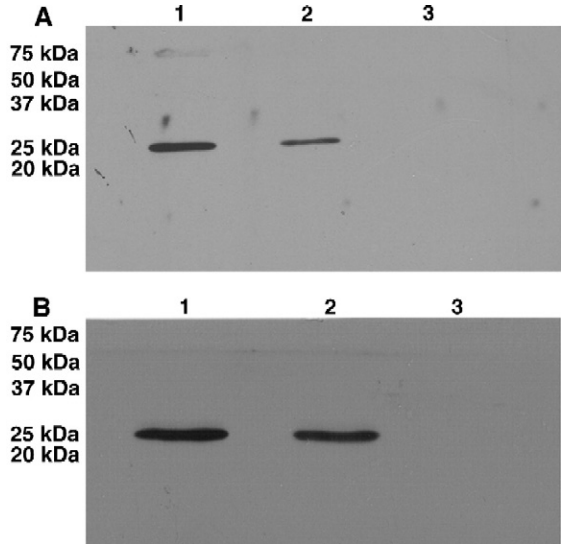

Fig. 3. Western blot analysis of WSV010 localization in WSSV. Lane 1, total viral proteins; lane 2, envelope proteins; lane 3, nucleocapsid proteins. (A) Rabbit anti-WSV010 antibody was used as the primary antibody. (B) Mouse anti-VP28 antibody was used as the primary antibody. The molecular weight marker used was the Precision Plus Prestained Dual Color marker (Biorad).

Among viral structural proteins, envelope proteins play vital roles, such as binding with receptors or penetrating into host cells by membrane fusion (Alicia and Helenius, 2004). Although many envelope proteins have transmembrane domains, WSV010 lacks a predicted transmembrane domain. Three known WSSV proteins, VP31, VP39 and VP281, are also envelope proteins without transmembrane domains (Huang et al., 2002a; Li et al., 2005; Zhu et al., 2006). It has been reported that envelope proteins without transmembrane domain can anchor on the membrane by interacting with other proteins containing transmembrane domains (Boyle and Compton, 1998). Moreover, protein–protein interactions among structural proteins are not unusual for enveloped viruses, which are believed to be essential for virion morphogenesis (Chiu and Chang, 2002; Szajner et al., 2003; Vittone et al., 2005). With this purpose, we performed the pull-down assay to try to identify proteins capable of interacting with WSV010. As we expected, we successfully detected the interaction of WSV010 with VP24, a major envelope protein of WSSV. This result suggests that association of WSV010 with VP24 either directly or indirectly may allow WSV010 to anchor to the envelope. The previous study indicated that VP24 could also interact with important structural proteins VP28 and VP26 to form a complex that plays a role in virus infection (Xie and Yang, 2006). Thus, we postulate that VP24 may play a role as a linker protein to link up the structural proteins VP28, VP26, WSV010, and probably other envelope proteins, to form a complex on the viral envelope. The possibility of existence of this complex will be tested in future study. Since both VP28 and VP26 are important for virus infection, it will be interesting to determine if WSV010 is also critical for virus entry. The characterization of the interaction of WSV010 with VP24 will enrich our understanding of viral morphogenesis and viral infection.

Materials and methods

Temporal analysis of wsv010 gene transcription

Infected *Penaeus monodon* shrimps were sampled at different time points and the total RNA was extracted from

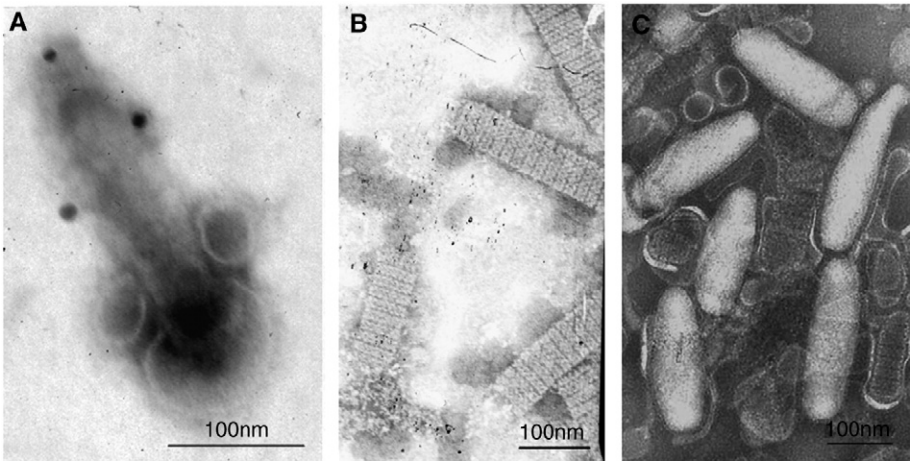

Fig. 4. Localization of WSV010 in WSSV by IEM. (A) 0.1% Tween 20 treated virus particle with the immunogold-labeled anti-WSV010 antibody. (B) WSSV nucleocapsid detected with the anti-WSV010 antibody. (C) Virus particles detected with the immunogold-labeled preserum.

the gills by TRIzol extraction (Invitrogen, Carlsbad, CA). Five micrograms of total RNA isolates was reverse-transcribed into cDNA using a SuperScript III First Strand Synthesis System (Invitrogen), and further PCR amplified with the sense primer 5′-ATGGACATTTTGGAAGACATC-3′ and the anti-sense primer 5′-AAACCCAAACTCTTCTAAAATATC-3′. WSSV late gene *vp28* was used as a positive control and the shrimp *β-actin* gene was used as a loading control. The PCR cycling parameters were as follows: 2 min at 95 °C; 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, 32 cycles, 15 min at 72 °C.

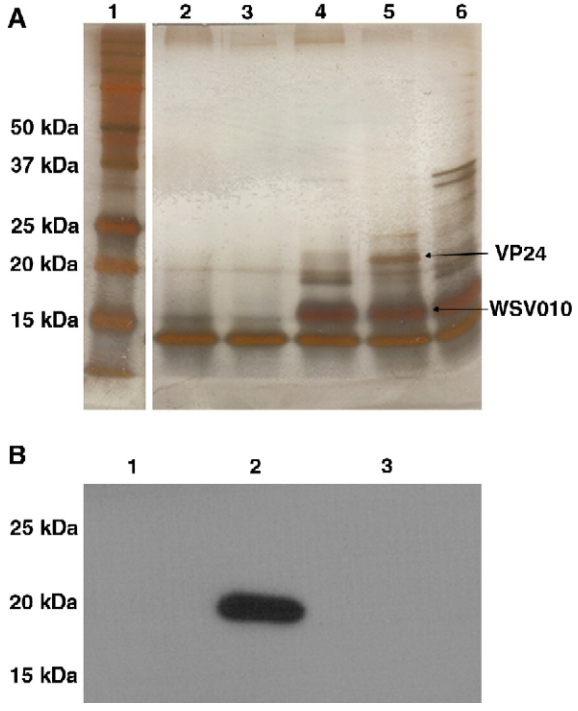

Fig. 5. Pull down of VP24 by WSV010. (A) Elution fractions of WSV010 pull-down assay. Lane 1, molecular mass marker; lane 2, the negative control 1 (infected epithelial tissue proteins only); lane 3, the negative control 2 (healthy epithelial tissue proteins only); lane 4, WSV010 with healthy epithelial tissue proteins; lane 5, WSV010 interacting with infected epithelial tissue proteins; lane 6, the bait control sample (WSV010 only). (B) Western blot analysis with the anti-VP24 polyclonal antibody. Lane 1, the wash fraction of WSV010 interacting with infected epithelial tissue proteins; lane 2, the elution fraction of WSV010 interacting with infected epithelial tissue proteins; and lane 3, the elution fraction of the negative control (infected epithelial tissue proteins only). The molecular weight marker used was the Precision Plus Prestained Dual Color marker (Biorad).

Proliferation and isolation of the WSSV virions

The origin of the virus used in this study is from WSSV-infected *Penaeus chinensis* (China isolate). Its genome accession number is AF332093. Virus inoculums were collected from hemolymph of infected red claw crayfish *Cherax quadricarinatus* (Wu et al., 2002). The extracted hemolymph was diluted 1:4 with phosphate-buffered saline (PBS) and stored at −80 °C as the stock. Before injection into the experimental crayfish, the stock hemolymph was diluted 10 times in PBS and centrifuged at 1000×*g* for 10 min and then followed by filtration of the supernatant with a 0.45-μm filter. Each healthy crayfish was injected with 0.3–0.4 ml of the virus inocula intramuscularly between the second and third abdominal segments. Hemolymph was collected from moribund crayfish during 4th to 6th day after infection. After centrifugation at 2000×*g* for 10 min, the supernatant was layered on top of a 30 to 60% (w/v) stepwise sucrose gradient and ultracentrifuged at 53,000×*g* (Beckman XL-90, Rotor SW28) for 1 h at 4 °C. The virus band was collected and then mixed with TN buffer (20 mM Tris–HCl and 400 mM NaCl, pH 7.4) and repelleted at 53,000×*g* for 1 h at 4 °C. The white pellet was washed with TN buffer at 12,000×*g* for 0.5 h at 4 °C for three times and then resuspended in TN buffer. The purified virus samples were subjected to negative staining with 2% phosphotungstic acid and examined under the transmission electron microscope JEOL JEM 2010F.

Separation of virus envelope and nucleocapsid proteins

The purified virions were treated with lysis buffer (20 mM Tris–HCl, 1% Triton X-100, 0.5 M NaCl, pH 7.4) for 1 h at 4 °C and aliquoted into two equal volumes. One half was kept as total

Table 2
Measured and calculated molecular masses of tryptic peptides which match VP24 of shrimp white spot syndrome virus

Start	End	Peptide	Observed	Mr(expt)	Mr(calc)	Delta
38	58	DKDAYPVESEIINLTINGVAR	2317.23	2316.22	2316.20	0.02
59	72	GNHFNFVNGTLQTR	1604.82	1603.81	1603.79	0.03
132	149	DITVDSVSLSPGLNATGR	1802.02	1801.02	1800.92	0.10

Protein sequence of VP24 (matched peptides are highlighted in bold).
1 MHMWGVYAAI LAGLTLILVV ISIVVTNIEL NKKLDKK**DKD AYPVESEIIN**
51 LTINGVARGN HFNFVNGTLQ TRNYGKVYVA GQGTSDSELV KRKGDIILTS
101 LLGDGDHTLN VNKAXSKELE LYARVYNNTK R**DITVDSVSL SPGLNATGR**E
151 FSANKFVLYF KPTVLKKNRI NTLVFGATFD EDIDDTNRHY LLSMRFSPGN
201 DLFKVGEK

virus proteins, and the other half was subjected to centrifugation at 12,000×*g* for 0.5 h at 4 °C. The supernatant was kept on ice as the envelope fraction. Then the pellet fraction was washed with the lysis buffer and centrifuged. Finally, the pellets were resuspended with an equal volume of lysis buffer as the envelope fraction.

Expression of WSV010 and antibody preparation

The full-length gene of *wsv010* was PCR amplified and inserted into the modified pET vector containing N-terminal Avi-tag and C-terminal 6× His tag (unpublished data). The construct was transformed into an *E. coli* strain BL21 Star (DE3) (Invitrogen) after sequencing. The recombinant WSV010 protein was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and cultured at 37 °C for 3 h. The protein was purified using Ni-NTA resin under native condition. The corresponding band was sent for MALDI-TOF/TOF mass spectrometry analysis (Protein and Proteomics Centre, National University of Singapore). The purified protein was used to raise rabbit polyclonal antibody by Bam Biotech Co., LTD (Xiamen, Fujian, PR China).

Western blot analysis of WSV010 localization

Proteins from virions, envelope and nucleocapsid fractions were resolved on a 15% SDS–PAGE gel and Western blot analysis was performed. After transfer, the nitrocellular membrane was blocked with 5% nonfat milk in TBST buffer (20 mM Tris base, 137 mM NaCl, 0.1% Tween 20, pH 7.6) for 1 h at room temperature. Then the membrane was incubated with anti-WSV010 antibody for 1 h at room temperature (1:1000 dilutions). After washing with TBST for 3 times, anti-rabbit horseradish peroxidase-conjugated secondary antibody was added and incubated for 1 h at room temperature (1:5000 dilution, GE Healthcare, Uppsala, Sweden). After washing with TBST buffer, supersignal west pico chemiluminescent substrate (Pierce, Rockford, IL) was used to identify the localization of WSV010 band. Mouse anti-VP28 antibody was used as a control.

Localization of WSV010 by immunoelectron microscopy

Purified virus was collected as described above. To verify the localization of WSV010 on virus particles, the virus was treated with 0.1% Tween 20 to partially separate the envelope from the nucleocapsid. Then the treated virus and the nucleocapsid fraction were spotted onto carbon-coated nickel grids for 5 min. The samples were blocked with 5% BSA in 0.2 M phosphate buffer (pH 7.3) for 0.5 h at room temperature. Rabbit anti-WSV010 antibody was used as the primary antibody for 2 h incubation at room temperature. Pre-immune rabbit serum was used as a negative control. After washing with the phosphate buffer for 3 times, goat anti-rabbit IgG conjugated with 15-nm gold beads (Electron Microscopy Sciences, Hatfield, PA) was used as the secondary antibody and the grids were incubated for 2 h at room temperature. After washing with the buffer, the samples were stained with 2% solution of phosphotungstic acid and examined under the transmission electron microscope Philips CM 10.

Pull-down assay of WSV010

Prey proteins for pull-down assay were extracted from epithelial tissues of healthy and infected crayfish. The infected crayfish epithelial tissue contains proteins from the host as well as WSSV. Firstly, the prey proteins were prepared by manual homogenization in liquid nitrogen and then dissolved in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40). After centrifugation at 10,000×*g* for 15 min, the supernatant was saved and stored at −80 °C until use. Since the recombinant WSV010 could be biotinylated *in vivo* when expressed in *E. coli*, the pull-down assays were performed according to the protocol of Profound Pull-down Biotinylated Protein:Protein Interaction Kit (Pierce Biotechnology, IL, USA). Briefly, excessive recombinant protein of WSV010 was immobilized on streptavidin–agarose beads in a column at 4 °C for 1 h. After blocking excess streptavidin group with biotin, the prey proteins were added to interact with the bait protein at 4 °C overnight. After wash with the lysis buffer, unbound proteins and eluted (bound) protein fractions were analyzed by electrophoresis on 15% SDS–PAGE gels and stained with silver nitrate. Silver-stained bands were excised and identified by mass spectrometry. The eluted proteins were also transferred to nitrocellulose membrane for Western blot analysis (Huynh et al., 2004).

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