2. Materials and Methods

2.1. Plasmid construction

The DNA sequence encoding the open reading frame (ORF) of Pm-syntenin was amplified by PCR from a plasmid obtained from previous work (Bangrak et al., 2002): a forward primer flanked by *Eco*RI site (5′ CGAATTCATGGGTCTGTACCCGTC 3′) and

reverse primer flanked by the *Bam*HI site (5'GGGATCCTTACAGGTCGGGAATG 3'). The amplified product was subcloned in frame into the pGBKT7 (CLONTECH), a bait vector that encodes the Gal4 DNA-binding domain and the plasmid was named as BD-syntenin. The other three bait plasmids were constructed as follow; BD-NS was constructed by isolating the *Eco*RI-*Xho*I fragment of the N-terminal (amino acid 1- 131) of Pm-syntenin and subcloned into pGBKT7; a plasmid containing PDZ1 and PDZ2 domains (BD-PDZ1,2) was obtained by using PCR with a forward primer 5' CCGAATTCATGGTGGCTCCCATCTC 3' and a reverse primer 5'CGGGATCCTTACAGGTCGGGAATG 3' and the PCR product was subcloned into *Eco*RI-*Bam*HI of pGBKT7; a plasmid of PDZ1(BD-PDZ1) was constructed by ligating the PCR product that was amplified by a forward primer 5' CCGAATTCATGGTGGCTCCCA TCTC 3' and a reverse primer 5'CTTCTCGAGTTATGGCCTGTCTCTAATGGC3' into the *Eco*RI-*Sal*I site of a pGBKT7 vector. All plasmid constructs were verified by sequencing.

2.2. Library construction

Total RNA was isolated from the haemocyte of WSSV infected shrimps using a Trizol reagent (GIBCO BRL). Poly(A)$^+$RNA was purified and cDNA carrying a *Eco*RI - *Xho*I restriction site was constructed by using the Zap Express cDNA synthesis kit (Stratagene). After site fractionation, cDNA of 800-1500 bp were pooled and ligated into the pGADT7 prey vector previously digested with the *Eco*RI and *Xho*I and transformed into bacteria. The library has a titer of 1.5×10^5. Plasmid DNA was prepared using the QAIprep Spin Miniprep Kit (QAIGEN).

2.3. Yeast two- hybrid screening

Yeast two-hybrid screening was carried out with the MATCHMAKER Gal4 Two-Hybrid System 3 (CLONTECH). The bait BD-syntenin was used to screen 10^5 independent recombinant clones of haemocyte cDNA library in *Saccharomyces cerevisiae* strain AH109. Positive clones were selected for growth on a synthetic dropout (SD) plate lacking adenine,

histidine, leucine and tryptophan (SD/-ade/-his/-leu/-trp) containing 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside. Plasmid DNA was isolated from those clones that activated all four yeast reporter genes [*ADE2, HIS3, MEL1* (encode α-galactosidase) and *lacZ* (encode β-galactosidase)] and transformed into *E. coli* Top10F' to recover the plasmid for sequencing. Gene database searches were performed through the National Center for Biotechnology Information using the Blast X. To confirm the screening result, recovered plasmids (containing both BD-syntenin and AD-syntenin binding proteins) were retransformed into a host strain and plated on SD/-ade/-his/-leu/-trp. The transformants were tested for β-galactosidase activity by filter lift assay according to the manufacturer's instruction.

2.4. Expression and purification of recombinant Pm-syntenin

A *Bam*HI - *Pst*I fragment containing the entire coding region of Pm-syntenin was obtained from previous work (Bangrak et al., 2002). These recombinant plasmids as well as insertless pQE40 were transformed into *E. coli* M15 and the proteins were also prepared according to Bangrak et al (2002).

2.5. Expression and Purification of GST-fusion protein

A GST fusion protein encompassing 181 amino acids residues of the C-terminal region of alpha 2-macroglobulin (α_2M) was cloned into the pGEX-4T-1 vector (Amersham Biosciences) at *Eco*RI and *Xho*I sites. The protein was expressed in *E.coli* BL21 (Amersham Biosciences). Protein production was accomplished by standard methods of bacterial growth in the presence of ampicillin, followed by induction with 0.5 mM IPTG. The GST fusion protein was purified by using Glutathione Sepharose 4B resin (Amersham Biosciences) and was analyzed for purity by 12.5% SDS-PAGE and kept at -80°C until ready for use.

The same method was also used to prepare GST-syntenin for the next experiment.

2.6. *In vitro* binding assays (*In vitro* pull-down assays)

For pull-down experiments, there are two methods. First, the interaction between 6xHis-syntenin and GST-α_2M was examined by incubating purified GST- α_2M fusion protein (1 μg) with a Glutathione Sepharose 4B resin (50 μl of 50% bed slurry) for 1 hour. Purified recombinant 6xHis -syntenin protein was added, and incubation was continued for another 2 hours at room temperature. After incubation, the beads were washed ten times with phosphate-buffered saline, pH7. The fusion protein was eluted by the addition of buffer containing reduced glutathione. The proteins were resolved by 12.5 % SDS-PAGE and transferred onto nitrocellulose membrane. Blots were incubated with Anti-His Tag antibody (His-Probe horseradish peroxidase conjugated, Pierce; diluted 1:20000) and the blots were visualized using ECL detection reagent (Pierce). To confirm the presence of GST-fusion protein, the blots were tested with Anti-GST (Amescham Biosciences; 1:2000) and conjugated goat anti-mouse IgG-alkaline phosphatase (Pierce; diluted 1: 20000). Lumi-Phos was used as a substrate for detecting chemiluminescence.

In the second method, the full length cDNA of Pm-syntenin was cloned into pGEX-4T-1 (Amersham Biosciences) and the recombinant protein was prepared according to the above protocol. A DNA template of pGADT7-α_2M that already contains the T7 RNA polymerase promoter was used in an *in vitro* transcription/translation reaction by the TNT quick-coupled transcription/translation system (Promega). The α_2M protein was radiolabeled with ^{35}S-methionine (MP Biomedical Inc.). The *in vitro* binding reaction was generated by adding ^{35}S-α_2M and GST-syntenin into buffer A, containing 50 mM HEPES, pH 7.5, 70 mM KCL, 0.5 mM ATP, 5 mM $MgSO_4$,1 mM dithiothreitol (DTT), 0.05% nondidet P-40, 2 μg/ml BSA, 0.5 mM PMSF, and protease inhibitor mixture (Roche Molecular Biochemicals) and allowed to form complexes at 4 °C for 90 min. The Glutathione 4B bead was then added and incubated for 90 min. The complex was washed with buffer A four times and twice with buffer B (buffer A

supplemented with 0.1% Nondidet P-40). Binding proteins were eluted into SDS-loading buffer (50 mM Tris-HCL, pH 6.8, 100mM DTT, 2% SDS, 0.1 % bromophenol blue, and 10% glycerol), boiled for 5 minutes and loaded on to 12.5 % SDS-PAGE gels. The result was visualized by fluorography and imaged with a phosphoimager system (Bio-Rad).

2.7. Analysis of expression of Pm-α_2M transcript

To determine the expression of the Pm-α_2M in shrimp, semi-quantitative RT-PCR analysis was performed according to the previous work (Bangrak et al., 2002). The primer sequences of α_2M are 5'CGAGATCTACCATTAATGAGGATAAC 3' (sense primer) and 5'CTAAGCTTTCAATCGCACCCTTCGA 3' (anti-sense primer). β-Actin primers were used to amplify a PCR product of 500 bp in the RT-PCR experiments as an internal control of gene expression. The identity of the PCR products was confirmed by sequencing.