3. RESULTS

3.1. Identification of Pm-syntenin binding protein

To identify haemocyte proteins that bind Pm-syntenin, we performed a yeast two-hybrid screen with the full length of Pm-syntenin as bait. The Pm-syntenin encodes a 322 amino acid protein containing two PDZ domains in tandem. These two domains span amino acids 135 to 218 (PDZ1) and 219 to 299 (PDZ2), respectively (Table 1). The screening of the cDNA library obtained from WSSV infected shrimps led to the isolation of seven independent clones including an in-frame α_2M cDNA. The transformants were re-streaked onto selective media containing 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside. Fig. 1A demonstrates the blue colony due to the cleavage of substrate by α-galactosidase, the enzyme that is expressed when the *MEL1* promoter was activated. Sequence analysis showed that clone SA1 consists of 546 bp sequences inserted into the pGADT7 vector. The analysis of amino acid sequence of SA1 revealed it to be identical to the carboxyl-terminal of α_2M. The sequence of the 546 bp was

used to design a set of primers for 5' RACE and the known sequences were extended to 861 bp (SA2) which spans 287 amino acids of shrimp α_2M -like protein (GenBank accession number **AY826818**). These 287 residues of SA2 also bind to full-length Pm-syntenin. The amino acid sequence of SA2 was compared with the sequence of previously known α_2M. SA2 shows a similarity to the C-terminal of the α_2Ms of the kuruma shrimp, *Marsupenaeus japonicus* (78% identity, 87% similarity, Genbank accession number **AB108542**), horseshoe crab, *Limulus polyphemus* (32% identity, 51% similarity, Genbank accession number **T18544**), honey bee *Apis mellifera* (33% identity, 56% similarity, Genbank accession number **XM392454**) and soft tick *Ornithodoros moubata* (34%identity, 51% similarity, Genbank accession number **AF538967**). The expression of full-length Pm-syntenin or SA1 or SA2 alone did not proceed on SD medium and did not activate the *lac-Z* reporter gene of AH109 yeast cells, whereas the expression of both molecules together did so (Fig. 1B). These results reveal that SA1, SA2 is a novel syntenin binding protein in the yeast two-hybrid assay and the binding is located at the C-terminal of an α_2M-like protein.

3.2. The N-Terminus of Pm-syntenin contributes to SA1 binding

Pm-syntenin can be classified as a member of the PDZ containing proteins. It is similar to syntenin-1 that has been found to use PDZ domains to bind to various kinds of molecules by recognizing the ligands of short C-terminal binding motifs (Lin et al., 1999; Torres et al., 1998; Grootjans et al., 2000; Fernandez-Larrea et al., 1999; Koroll et al., 2001; Geijsen et al., 2001, Hirbec et al., 2002). Apart from the PDZ motif, it was found that the N-terminal sequence of syntenin is necessary for its interaction with the C-terminal of eIF5A (Li et al., 2004). To further define which domain of Pm-syntenin is required for the interaction with α_2M, the AD-α_2MSA1 was cotransformed with individual plasmids that contained the complete sequence of Pm-syntenin (BD-syntenin), ie the 131 amino acids of the N-terminal (BD-NS), PDZ1-PDZ2 (BD-PDZ1,2; amino acids 117-322) and PDZ1 (BD-PDZ1; amino acids 117-219). Table 1

shows the results of the growth of yeast cells with a blue colony (+) in the selective medium when the interaction of the two proteins occurred and no growth of yeast cells (-) when there is no interaction of the two proteins tested. Yeast cotransformed with AD-α_2MSA1 and BD-NS can grow on SD medium producing a blue colony, and also gave a strong β-galactosidase reporter expression (Fig. 1B), but no interaction was observed with any of the PDZ domains (Table 1, No.5-6). These data suggest that Pm-syntenin binds α_2M through its N-terminal 131 amino acids *in vivo*, in the yeast two-hybrid system.

3.3. GST pull-down assays

To confirm the interaction between syntenin and α_2M, we carried out a GST pull-down experiment. GST-α_2M was coupled to Glutathione Sepharose 4B beads and then incubated in the presence of the 6xHis-syntenin. Anti-His Tag antibody and Anti -GST antibody were used to visualize the specific protein in a western blot analysis. As shown in Fig. 2, Glutathione beads effectively pulled down both GST and GST-α_2M, although pull-down efficiency was higher with GST than with GST-α_2M as is shown in lane 3 and 4 of Fig. 2, respectively. When the same membrane was probed with anti-His-Tag antibody, 6xHis-Pm-syntenin was only present in the pull-down product of GST-α_2M (lane 2), being absent in the GST pull-down product (lane 1). These data suggest that GST-α_2M, not GST alone, was capable of co-precipitating Pm-syntenin. In the same GST pull-down system, there was no association present between GST-α_2M and the PDZ1 or PDZ1,2 domain of Pm-syntenin.

In order to further validate the presence of a specific interaction between α_2M and syntenin, we performed a reverse pull-down assay where *in vitro* translated and radio-labeled α_2M was incubated with either GST-syntenin or GST alone. As is shown in Fig. 3, the amounts of α_2M added to the reaction mixtures were equal (lanes 1 and 2, input). In addition, GST-syntenin and GST proteins were equally pulled down by glutathione beads (lane 1 and 2, Coomassie). In this system, GST-syntenin, not GST alone, was capable of co-precipitating α_2M

(lanes1 and 2, Pull-down). Taken together with the data shown in Fig. 2 and 3 and Table 1, it is now clear that Pm-syntenin specifically binds the C-terminus receptor binding domain of α_2M, through its N-terminus portion.

3.4. Gene expression of α_2M in haemocytes of shrimp

To address whether α_2M functions as an immune effector in *P. monodon*, we performed RT-PCR analyses and a semi-quantitative assay on RNA extracts from normal and WSSV-injected shrimps using specific primers designed from the SA1 sequences. RT-PCR of β-actin was used as an internal control in the semi-quantitative analysis and to insure that the RT-PCR for each sample contained the same amount of total RNA and that the extracted RNA was intact. The α_2M was observed in five examples each of normal shrimp and WSSV-infected shrimp as shown in Fig. 4. The level of α_2M expression increased when the shrimp was infected with WSSV.