Synergistic Regulation of Three *Mx* Genes by NNV Infection and Thermal Stress in Giant Grouper (*Epinephelus lanceolatus*)

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Introduction

The giant grouper (*Epinephelus lanceolatus*) is a key aquaculture species in Taiwan, but Nervous Necrosis Virus (NNV) infection causes up to 100% mortality during the larval stage. As an ectothermic species, the grouper's immunity is highly sensitive to environmental temperature. Therefore, elucidating how viral infection interacts with thermal conditions is essential for developing effective prevention strategies. Myxovirus resistance (Mx) proteins are critical downstream effectors of the Type I interferon (IFN) system. Although our previous findings indicated that ggMx are modulated by NNV, it is still unknown whether the three Mx homologous possess distinct functional roles in inhibiting NNV replication, and the precise regulatory mechanisms of their respective gene promoters remain incompletely understood. This study characterized the transcriptional activation of the three ggMx genes under induced thermal (20, 28, and 36˚C) and viral stressors. Promoter analysis verified that the upstream regions of all three ggMx genes contain conserved interferon-stimulated response elements (ISRE), NF-κB, and heat shock elements (HSE). Co-localization analysis confirmed the physical interaction between Mx proteins and the NNV capsid protein, suggesting that Mx confers protection by directly interfering with viral assembly or trafficking. Overall, these findings clarify the dual-responsiveness of ggMxs to biological and environmental stressors, providing mechanistic insight into NNV resistance and supporting the development of molecular-targeted aquaculture strategies.

Results

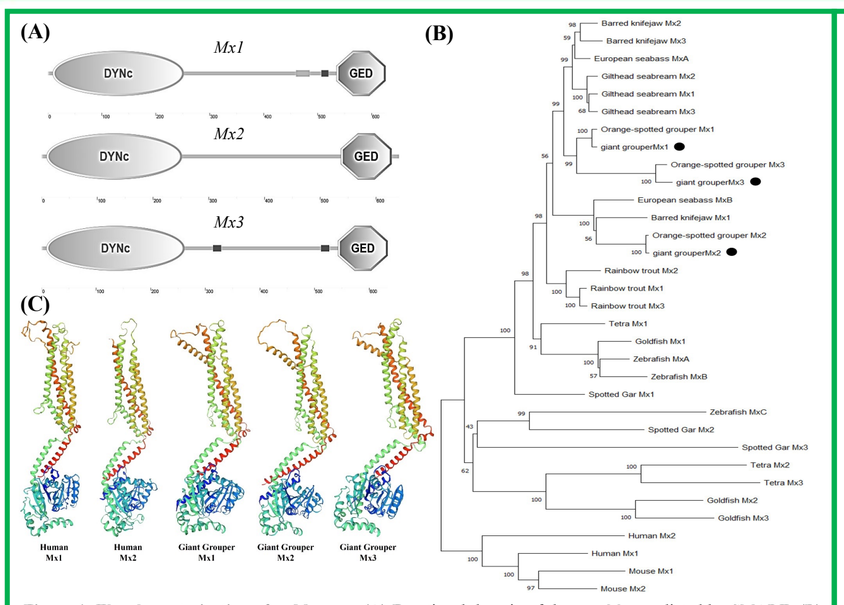


Figure 1. The characterization of *ggMx* gene. (A) Functional domain of three *ggMxs* predicted by SMART. (B) Neighbor-joining analysis of *Mx* homologues among teleost fishes and mammals. (C) Comparison of the predicted 3D protein structures of three ggMx with human Mx. The protein models of three ggMx proteins were predicted by SWISS-MODEL and compared with two human Mx proteins.

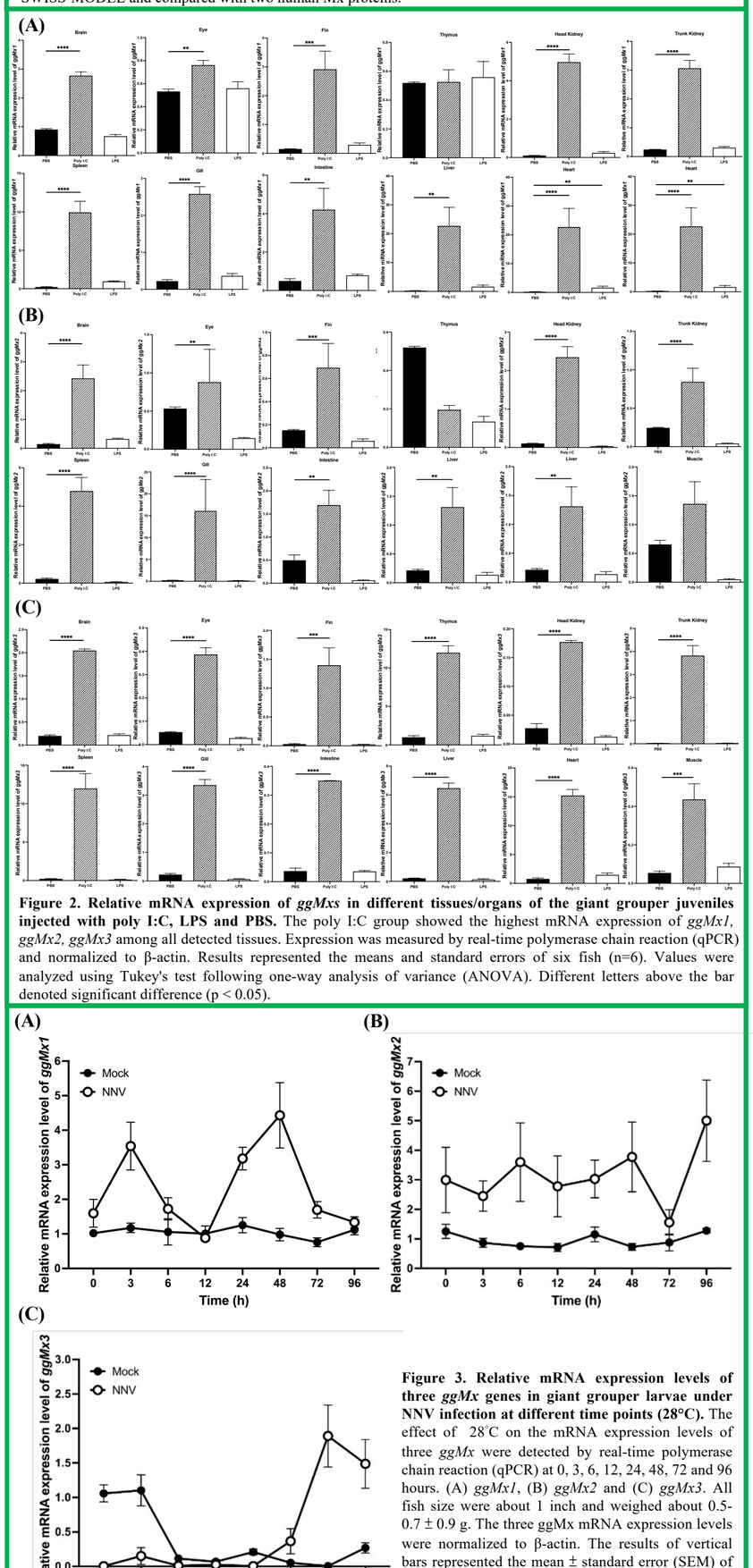


Figure 2. Relative mRNA expression of *ggMxs* in different tissues/organs of the giant grouper juveniles injected with poly I:C, LPS and PBS. The poly I:C group showed the highest mRNA expression of *ggMx1, ggMx2, ggMx3* among all detected tissues. Expression was measured by real-time polymerase chain reaction (qPCR) and normalized to β-actin. Results represented the means and standard errors of six fish (n=6). Values were analyzed using Tukey's test following one-way analysis of variance (ANOVA). Different letters above the bar denoted significant difference ($p < 0.05$).

Figure 3. Relative mRNA expression levels of three *ggMx* genes in giant grouper larvae under NNV infection at different time points (28°C). The effect of 28˚C on the mRNA expression levels of three *ggMx* were detected by real-time polymerase chain reaction (qPCR) at 0, 3, 6, 12, 24, 48, 72 and 96 hours. (A) *ggMx1*, (B) *ggMx2* and (C) *ggMx3*. All fish size were about 1 inch and weighed about 0.5-0.7 ± 0.9 g. The three ggMx mRNA expression levels were normalized to β-actin. The results of vertical bars represented the mean ± standard error (SEM) of six fishes.

Figure 4. Relative mRNA expression of *ggMx1, ggMx2, ggMx3* in the giant grouper juveniles acclimated to 20°C, 28°C, and 36°C. NNV infected giant grouper *ggMx* genes expression time course experiment at various temperatures. The mRNA abundance was measured by real-time polymerase chain reaction (qPCR) and normalized to β-actin. Results represented the means and standard errors of six fish (n=6).

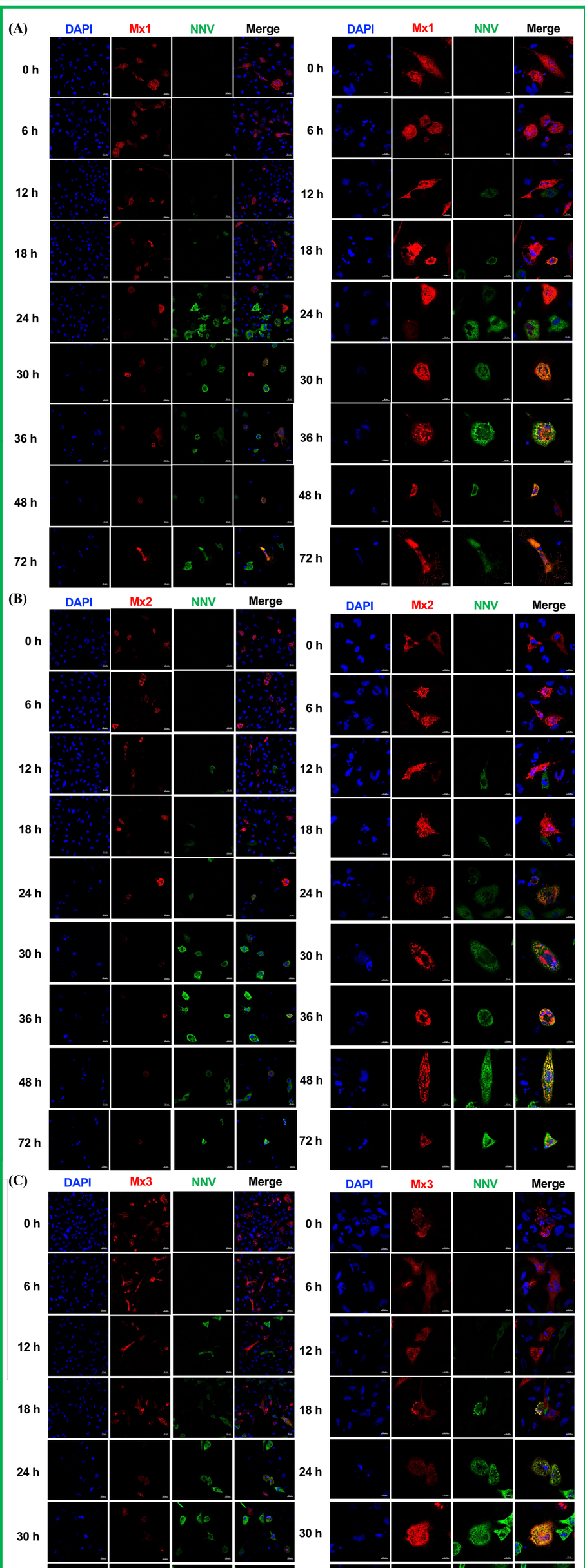


Figure 5. Immunofluorescence staining of the effect of ggMx1,2,3 overexpression in GF-1 cells under NNV infection at different time points (28°C). Immunofluorescence images of ggMx1,2,3 protein (red), NNV CP (green) and nucleus (blue, DAPI) were captured by the LSM 780 confocal microscope. The fluorescent signal intensity of ggMx1,2,3 protein, NNV CP and nucleus were normalized to healthy cells. (A) Immunofluorescence images of ggMx1 protein in GF-1. (B) Immunofluorescence images of ggMx2 protein in GF-1. (C) Immunofluorescence images of ggMx3 protein in GF-1. Images were 40 X magnification with oil objective lens and scale bar was 20 μm.

Figure 6. Dynamics of ggMx1,2,3 overexpression and colocalization in GF-1 cells under NNV infection at different time points (28°C). The value of ggMx1,2,3 protein or NNV / DAPI ratio (%) and colocalization ggMx1,2,3 and NNV (%) were counted immunofluorescence images. Fluorescent puncta were identified based on intensity and counted manually. The results of vertical bars represented the mean ± standard error (SEM) of three microscope slides. (A) The ggMx1 protein overexpression in GF-1. (B) The ggMx2 protein overexpression in GF-1. (C) The ggMx3 protein overexpression in GF-1. Images were 40 X magnification with oil objective lens and scale bar was 20 μm.

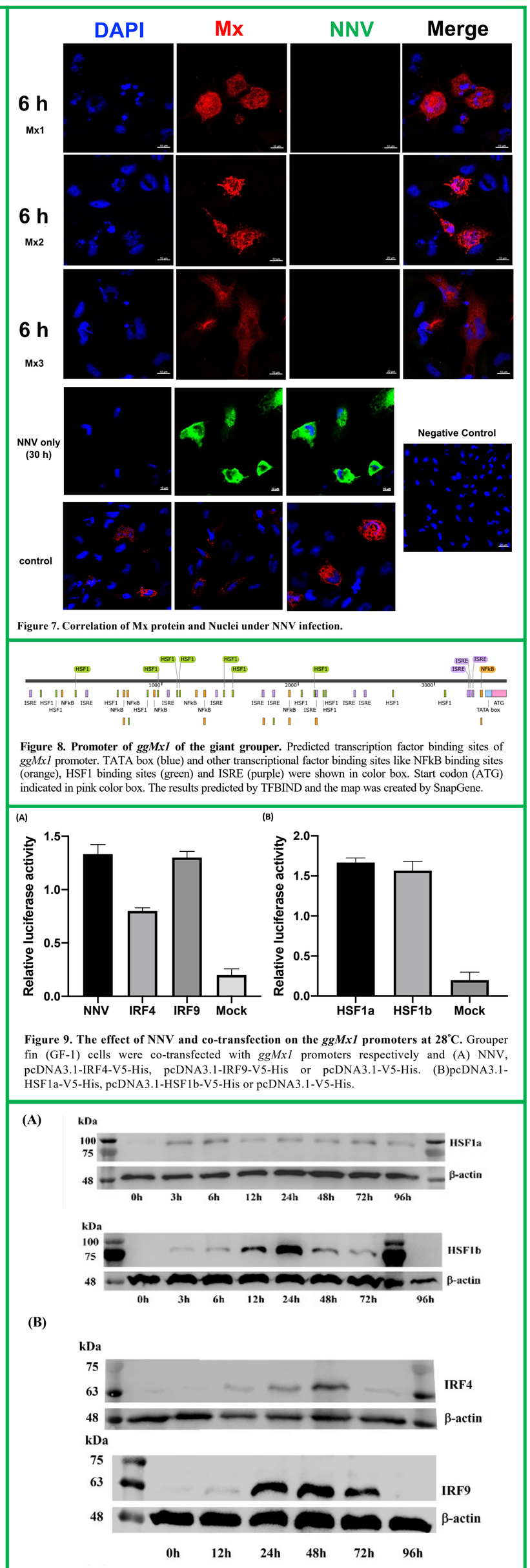


Figure 7. Correlation of Mx protein and Nuclei under NNV infection.

Figure 8. Promoter of *ggMx1* of the giant grouper. Predicted transcription factor binding sites of *ggMx1* promoter. TATA box (blue) and other transcriptional factor binding sites like NFkB binding sites (orange), HSF1 binding sites (green) and ISRE (purple) were shown in color box. Start codon (ATG) indicated in pink color box. The results predicted by TFBIND and the map was created by SnapGene.

Figure 9. The effect of NNV and co-transfection on the *ggMx1* promoters at 28˚C. Grouper fin (GF-1) cells were co-transfected with *ggMx1* promoters respectively and (A) NNV, pcDNA3.1-IRF4-V5-His, pcDNA3.1-IRF9-V5-His or pcDNA3.1-V5-His. (B)pcDNA3.1-HSF1a-V5-His, pcDNA3.1-HSF1b-V5-His or pcDNA3.1-V5-His.

Figure 10. Western blot of overexpression of IRF4, IRF9, HSF1a and HSF1b in IFN pathway. GF-1 cells were co-transfected with three pGL3-ggMxp-Luc respectively with pcDNA3.1-IRF4-V5-His, pcDNA3.1-IRF9-V5-His, pcDNA3.1-HSF1a-V5-His or pcDNA3.1-HSF1b-V5-His. The cell lysates were harvested at different time courses. The samples were run the SDS-PAGE electrophoresis and then the western blotting. GF-1 cells transfected with three *ggMx* promoters respectively were treated with 20°C, 28°C and 36°C. The promoter activities were measured at 0, 3, 6, 12, 24, 48, 72 and 96hr treatment and detected by Dual-Luciferase reporter assay. The *ggMx1p, ggMx2p* and *ggMx3p*. The promoter activities were normalized to pRL-TK.

Conclusion

Figure 11. Pathway of gg*Mx1* in the giant grouper.

Acknowledgement

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