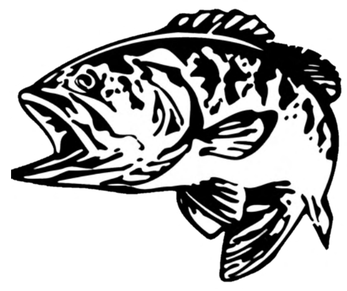
Protein kinase R (PKR) an antiviral protein through the eif2α in the giant grouper (*Epinephelus lanceolatus*) for virus resistance

Tzong-Yueh Chen[1,2,3,4] *, Sih-Shien Lee[1]

1. Department of Biotechnology and Bioindustry Sciences, National Cheng Kung University, Tainan, Taiwan
2. University Center for Bioscience and Biotechnology, National Cheng Kung University, Tainan, Taiwan
3. Translational Center for Marine Biotechnology, National Cheng Kung University, Tainan, Taiwan
4. Agriculture Biotechnology Research Center, National Cheng Kung University, Tainan, Taiwan



Abstract

The giant grouper (Epinephelus lanceolatus) is an important aquaculture species in Taiwan's aquaculture industry. However, its vulnerability to Nervous Necrosis Virus (NNV) during early stages hampers fry survival. Investigating the immune response, this study focused on Protein Kinase R (PKR), a vital antiviral protein pathway in fish and mammals. Cloning and sequencing the full-length PKR gene (1566 bp, encoding a 522-amino-acid peptide), followed by bioinformatics and phylogenetic analysis, elucidated its characteristics. Real-time PCR revealed highest expression of PKR in juvenile grouper fins, influenced by environmental temperature. Experiments with poly I:C and LPS injections showed elevated PKR expression in response to dsRNA. Fluctuations in PKR expression correlated with NNV infection severity in juvenile organs and behavior. Overexpression and siRNA inhibition experiments demonstrated role of PKR in suppressing viral replication via eif2a phosphorylation. These findings unveil significance in grouper immunity, especially against NNV, shedding light on potential strategies for disease management.

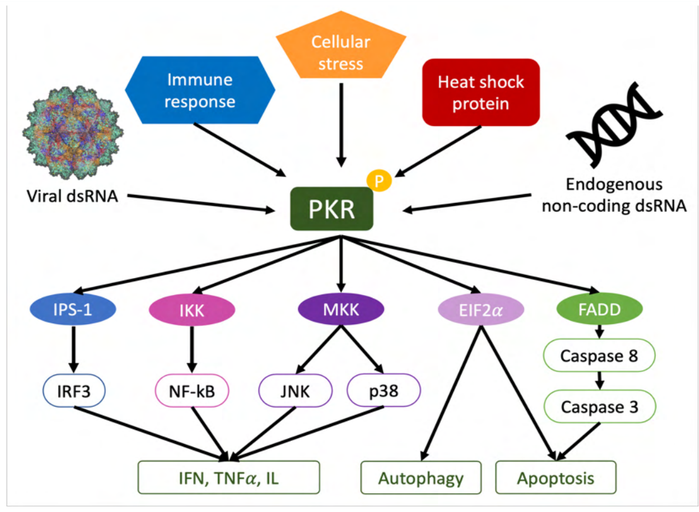


Fig.1 Protein Kinase R (PKR) signal transduction pathway

Results

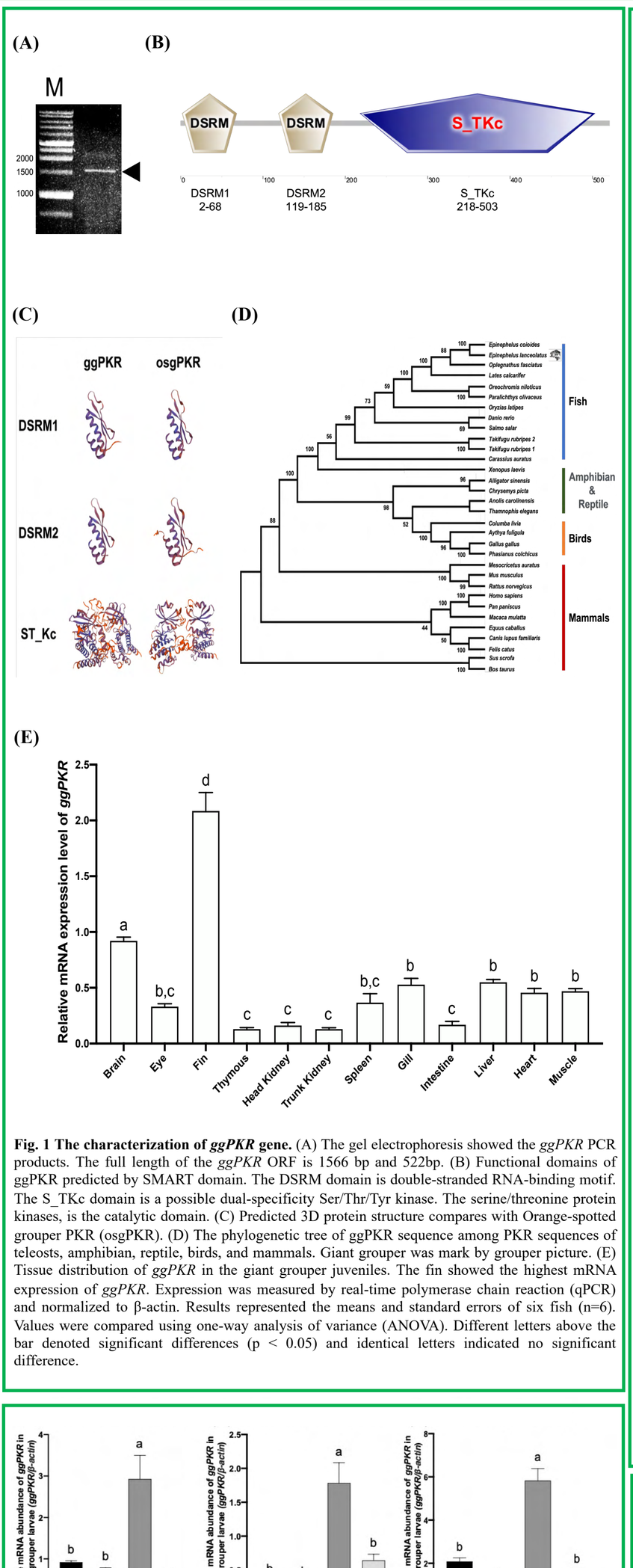


Fig. 1 The characterization of *ggPKR* gene. (A) The gel electrophoresis showed the *ggPKR* PCR products. The full length of the *ggPKR* ORF is 1566 bp and 522bp. (B) Functional domains of ggPKR predicted by SMART domain. The DSRM domain is double-stranded RNA-binding motif. The S_TKc domain is a possible dual-specificity Ser/Thr/Tyr kinase. The serine/threonine protein kinases, is the catalytic domain. (C) Predicted 3D protein structure compares with Orange-spotted grouper PKR (osgPKR). (D) The phylogenetic tree of ggPKR sequence among PKR sequences of teleosts, amphibian, reptile, birds, and mammals. Giant grouper was mark by grouper picture. (E) Tissue distribution of *ggPKR* in the giant grouper juveniles. The fin showed the highest mRNA expression of *ggPKR*. 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 compared using one-way analysis of variance (ANOVA). Different letters above the bar denoted significant differences ($p < 0.05$) and identical letters indicated no significant difference.

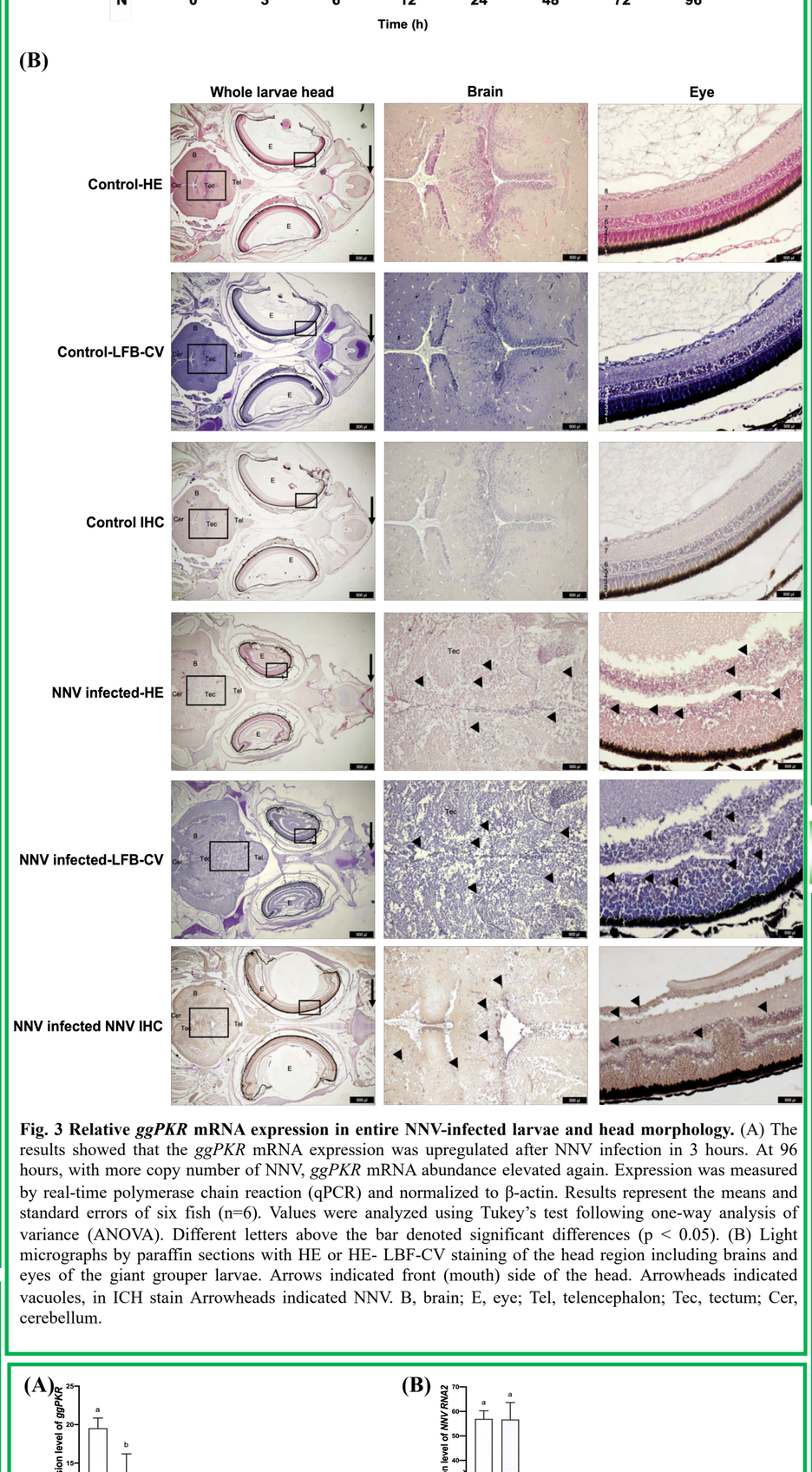


Fig. 3 Relative *ggPKR* mRNA expression in entire NNV-infected larvae and head morphology. (A) The results showed that the *ggPKR* mRNA expression was upregulated after NNV infection in 3 hours. At 96 hours, with more copy number of NNV, *ggPKR* mRNA abundance elevated again. Expression was measured by real-time polymerase chain reaction (qPCR) and normalized to β-actin. Results represent 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 differences ($p < 0.05$). (B) Light micrographs by paraffin sections with HE or HE- LBF-CV staining of the head region including brains and eyes of the giant grouper larvae. Arrows indicated front (mouth) side of the head. Arrowheads indicated vacuoles, in ICH stain Arrowheads indicated NNV. B, brain; E, eye; Tel, telencephalon; Tec, tectum; Cer, cerebellum.

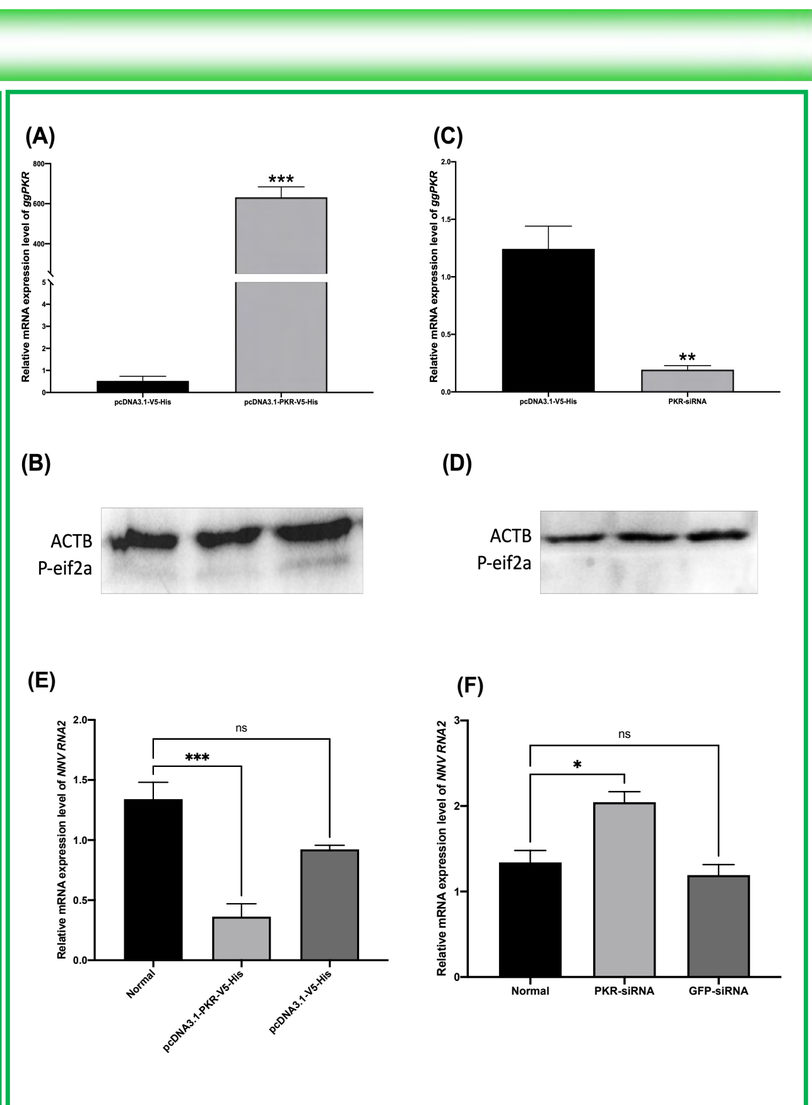


Fig. 6 ggPKR gain of function (A, B) and loss of function (C, D). (A) Relative mRNA expression of ggPKR gain of function. (B) Western blot analysis of cell gain of function of p-eif2a and ACTB as control. (C) Relative mRNA expression of ggPKR loss of function. (D) Western blot analysis of cell loss of function of p-eif2a and ACTB as control. Results represented the means and standard errors of three dish of cell (n=3). (E, F) Relative mRNA expression of NNV RNA2. Relative mRNA expression of NNV RNA2 in the cell with gain of function PKR significantly lower than other two groups. The results shows that PKR can inhibited the replication of NNV virus. Results represented the means and standard errors of three dish of cell (n=3). Values were analyzed using Tukey's test following one-way analysis of variance (ANOVA). Different letters above the bar denoted significant difference ($p < 0.05$).

Fig. 2 Relative mRNA expression of *ggPKR* 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 *ggPKR* among all detected tissues. The LPS showed the highest mRNA expression of *ggPKR* in livers. 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 ($p < 0.05$).

Fig. 4 Tissue distribution of *ggPKR* in the giant grouper juveniles infected with NNV. (A) Relative expression of *ggPKR* and (B) relative expression of *NNV RNA2*. (C) Light micrographs by paraffin sections with *ggPKR in situ* or NNV IHC staining of brains and eyes of the giant grouper juveniles. The brain and eye tissues showed more mRNA expression of *ggPKR* and more NNV. 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 differences ($p < 0.05$).

Fig. 5 ggPKR and dsRNA interaction. (A) Confocal laser scanning micrograph shows Co-localization of ggPKR and dsRNA. PKR (green), dsRNA (red), Merge (orange) of PKR and dsRNA. The orange color in the merged images indicates colocalization between overexpression ggPKR proteins and dsRNA; white arrow indicates the cells contain PKR and dsRNA. The red color shows only dsRNA; white arrowheads indicate the cell only shows dsRNA. (B) RNA-Binding Protein Immunoprecipitation with His-tag antibody detected NNV dsRNA with R-T PCR.

Summary

- **The molecular cloning of *ggPKR* revealed that it is an anti-viral protein.**
- **The tissue distribution of *ggPKR* after immunostimulant experiment** revealed that Poly I:C-injection group showed the highest expression of *ggPKR* than the other treatment groups.
- **The *ggPKR* gene expression induced after NNV infection.** With time course treatment, *ggPKR* expression level been induced. The NNV symptom been proved by the paraffin section.
- **The NNV RNA expression, the higher the *ggPKR* expression.**
- **NNV replication been stop by ggPKR with eif2α phosphorylation pathway.** ggPKR and NNV dsRNA interaction and phosphorylation of eif2α. With phosphorylation of eif2α. Relative NNV RNA2 expression in PKR gain of function were less than PKR loss of function.
- **According to the findings, *ggPKR* gene response to viral infection, and its interaction with NNV dsRNA. More over, PKR inhibited NNV replication by phosphorylated eif2α.**

Acknowledgement

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