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Identification, Characterization, and Expression of a PRQFVamide-Related Peptide in Cephalopod *Sepiella japonica*

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Neuropeptides, as neurotransmitters and neuromodulators, have a variety of physiological functions in the mollusk. Here, a *PRQFVamide-related peptide* gene was cloned from cuttlefish *Sepiella japonica* (designated as *SjPRQFVRP*, GenBank Accession No: OK999997). The full length of *SjPRQFVRP* is 1748 bp, including an open reading frame (ORF) of 738 bp encoding 245 amino acids. The putative precursor protein comprises one signal peptide and four different mature pentapeptides: fourteen copies of PMEFLamide, three copies of RMEFLamide, one copy of AMEFLamide and GMEFLamide. Multiple alignments showed *Sj*PRQFVRP shared 71% identity with that of *Octopus vulgaris* and supported the phylogenetic analysis. The spatio-temporal expression pattern showed that *SjPRQFVRP* mRNA was widely expressed among the 13 tissues and primarily abundantly expressed in the brain and optic lobe during the whole development stage. *In situ* hybridization data indicated that *SjPRQFVRP* was detected in the vertical lobe, subvertical lobe, anterior basal lobe, anterior pedal lobe, and optic lobes of the brain. Subcellular localization analysis revealed that the *Sj*PRQFVRP protein was localized in the cytoplasm of HEK293 cells. Collectively, the results will provide a foundation for further exploring the mechanism of *SjPRQFVRP* function in cephalopods.

Keywords: neuropeptide, *Sepiella japonica*, cuttlefish, cephalopod, PRQFVamide-related peptide

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INTRODUCTION

Neuropeptides are signaling molecular produced and released primarily by neurons and engaged in diverse physiological processes (Wang et al., 2015). A large number of bioactive neuropeptides have been found in vertebrates with multiple functions in the regulation of learning and memory, reproduction, energy homeostasis, cardiovascular activity, and stress response (Corbière et al., 2019). Since the first neuropeptide eledoisin was sequenced from octopus *Eledone moschata* (Erspamer and Anastasi, 1962), neuropeptides have been extensively studied in multiple invertebrates, including mollusks (Price and Greenberg, 1977; Pulst et al., 1988), nematodes (Davenport et al., 1988; Keating et al., 1995), and arthropods (Marder et al., 1986; Nambu et al., 1988). These neuropeptides have also been linked to various physiological and behavioral processes such as learning and memory (Beets et al., 2012), metabolism (Kaufmann and Brown, 2008; Wang et al., 2013), longevity (Waterson et al., 2014), and reproduction (Garrison et al., 2012).

Diverse neuropeptides have been identified in cephalopods. It was reported that they play a vital role in regulating feeding (Zhang and Tublitz, 2013), reproduction (Di Cristo et al., 2005), muscle contraction (Go et al., 2011), memory (Bardou et al., 2010), chromatophore activity (Zhang et al., 2012), and heart activities (Springer et al., 2004). FMRFamide (Phe-Met-Arg-Phe-NH_2) and FMRFamide-related peptides (FaRPs) were identified in *Loligo pealei* (Burbach et al., 2013), *Sepia officinalis* (Chrachri, 2020), and *Sepia pharaonis* (Zhu et al., 2020); GnRH (Gonadotropin-releasing hormone) was reported in *S. officinalis* (Di Cristo et al., 2009), *Sepia lycidas* (Murata et al., 2021) and *S. pharaonis* (Song et al., 2021); APGWamide (Ala-Pro-Gly-Trp-NH_2) was reported in *Octopus vulgaris* (Di Cristo et al., 2005), and *Idiosepius pygmaeus* (Sirinupong et al., 2011); Small cardioactive peptide (sCAP) was identified in *Octopus minor* (Iwakoshi et al., 2000), *O. vulgaris* (Kanda and Minakata, 2006), and *Sepiella japonica* (Li et al., 2019).

The -FVamide neuropeptides are purified from *Aplysia* with -FVamide in their C-terminus. These neuropeptides contain AMRPs (Fujisawa et al., 1999), enterins (Furukawa et al., 2001), and PRQFVamide (Furukawa et al., 2003). PRQFVamide was firstly identified from the central nervous system (CNS) and gut of *Aplysia californica* (Furukawa et al., 2003). Through immunohistochemistry, the PRQFVamide immunopositive signals were observed in the gut, especially where related to the vasculature (Furukawa et al., 2003). Moreover, synthetic PRQFVamide not only could inhibit contractions of the gut and vasculature but also could reduce the excitability of B4/5 and B31/32 neurons of the buccal feeding circuit (Furukawa et al., 2003). Later, the same research group discovered that activation of PRQFVamide-containing neurons could lead to the organized rhythmic output of the feeding central pattern generator (CPG) (Dembrow et al., 2003). It is poorly studied when we search for the *PRQFVamide* gene or protein from the National Center for Biotechnology Information (NCBI). Only one or two sequences from *Deroceras reticulatum*, *A. californica*, *O. vulgaris*, *Mizuhopecten yessoensis*, and *Lingula anatina* can be found. And another sequence from *Charonia tritonis* was only found in Uniprot. PRQFVamide was only reported in two cephalopods, *S. officinalis* and *O. vulgaris*. In *S. officinalis*, the incomplete *PRQFVamide* preprohormones showed very large precursors (Zatylny Gaudin et al., 2016). Moreover, researchers obtained five mature products from the hemolymph of the cuttlefish, and one of them was the pentapeptide PMEFLamide (Zatylny Gaudin et al., 2016). A study on *O. vulgaris* indicated that the *PRQFVamide* was expressed in the gastric ganglion. The gene expression level was suppressed with the higher levels of the octopuses with "high" *Aggregata* parasite load, while those with "low" parasite load showed an increased expression (Baldascino et al., 2017). However, the underlined function and regulatory mechanisms of the PRQFVamide in cephalopod are poorly understood.

The common Chinese cuttlefish *S. japonica* used to be one of the four traditional major marine fishery species in the East China Sea (Wu et al., 2010b). Because of great economic importance and ecological value, *S. japonica* is a promising aquaculture species. However, due to overexploitation and the deterioration of environmental conditions, the resources of *S. japonica* have been severely damaged since the mid-1970s. Great efforts have been poured on it to recover its ecological resources. Although artificial breeding and some achievements have been made in China, precocious puberty and disease arose in cuttlefish during artificial breeding (Wu et al., 2010a; Lü et al., 2019). Thus, exploring the potential physiological functions of PRQFVamide-related peptide in *S. japonica* (termed as *SjPRQFVRP*) is urgently needed and our work has important scientific and practical significance.

In this study, the main objectives aim to (1) clone the full-length cDNA of *SjPRQFVRP*; (2) detect the spatio-temporal expression during the whole developmental stages with quantitative Real-time PCR (qRT-PCR); (3) investigate the tissues distribution profiles by *in situ* hybridization (ISH); (4) observe the subcellular localization in HEK293 cells. The results will inform us the follow up studies based on different approaches that the gene might be involved in various physiological functions in *S. japonica*.

MATERIALS AND METHODS

Sample Collection

Healthy *S. japonica* was collected from a local aquaculture base (29°53′ N, 122°18′ E, Xixuan Island, Zhejiang Province, China). Cuttlefishes were cultured in aerated seawater at approximately 25°C on a 14/10 h light/dark cycle and fed with shrimp twice daily. On the basis of the growth time and gonad appearance, cuttlefishes were divided into six different developmental stages (stage I–VI) as previously reported (Jiang et al., 2007; Luo et al., 2014). Three cuttlefishes were used for the tissue distribution analysis at each developmental stage: stage I-II (17.3 ± 4.7 g), stage III (52.7 ± 5.1 g), stage IV (60.8 ± 4.3 g), stage V (70.0 ± 9.4 g), stage VI (99.1 ± 6.4 g). After anesthetized on ice for 1 min, experimental cuttlefishes were dissected. The brain, optic lobe, heart, liver, intestine, stomach, pancreas, gill, muscle, skin, ovary, nidamental gland, and accessory nidamental gland were removed separately and put in RNAstore (CoWin Biosciences, Jiangsu, China) at −80°C. The brain of stage I-II was fixed in 4% paraformaldehyde (PFA) overnight at 4°C. All experiments were conducted according to the protocols and guidelines approved by the Ethics Committee of Zhejiang Ocean University and the Academy of Experimental Animal Center of Zhejiang Ocean University.

RNA Extraction and cDNA Synthesis

To isolate total RNA from tissues, Trizol reagent (Takara, Kyodo, Japan) was used as described in previous studies (Li et al., 2019). Then the quality, purity, and integrity of RNAs were assessed by UV spectroscopy (A260/A280) on Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, United states) and agarose gel electrophoresis. The mRNA was reverse transcribed into first-strand cDNA using PrimeScript^{TM} RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio Inc., Kyodo, Japan). The first-strand cDNA was used as the template.

Full-Length cDNA Amplification

To amplify the *SjPRQFVRP* sequence, PRQFVRP-F/R primers (**Table 1**) were designed in accordance with the unigene annotated in *S. japonica* transcription (Lü et al., 2016). The PCR amplification reaction was conducted with 2 × Easy Taq Mix (Sangon Biotech, Shanghai, China). The target band was gel-purified and ligated to pMD19-T Vector (Takara Bio Inc., Kyodo, Japan). The insert was sent to the company for sequence determination (Sangon Biotech, Shanghai, China).

According to the *SjPRQFVRP* sequence, specific primers (**Table 1**) for 5′- and 3′-RACE were designed. RACE reactions were conducted following the instructions of SMARTer^{TM} RACE cDNA Amplification Kit (Clontech, Fitchburg, MA, United states) as a previous study (Zheng et al., 2016). UPM (10 × Universal Primer A Mix, **Table 1**) and 5′-PRQFVRP-RACE (**Table 1**) were used to perform the 5′-RACE of the *SjPRQFVRP*. The final reaction volume of 50 μl contained 41.5 μl Master Mix, 2.5 μl cDNA of the brain, 1.0 μl 5′-PRQFVRP-RACE, and 5 μl UPM. The PCR reaction program was 25 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 2 min. The next steps were the same as conventional PCR. The annealing temperature was adjusted according to the primer temperature. The 3′-RACE of the *SjPRQFVRP* used 3′-PRQFVRP-RACE (**Table 1**) and UPM. The PCR reaction was similar to that mentioned above for 5′-RACE. All the products were purified using DNA gel extraction kit (Sangon Biotech, Shanghai, China) and ligated into the pMD19-T vector for sequence determination. All primers used in the present study are listed in **Table 1**.

Bioinformatics Analysis of *Sj*PRQFVRP

To get the target sequence, three conserved nucleotide sequences (XM 029790420.1, NM 001204600.1, KY659273.1) (**Supplementary Table 1**) were acquired from the NCBI database[1] and were aligned with the Blast (Blastx and Blastn). Once gaining the full length of *SjPRQFVRP*, the open reading frame (ORF) of the *SjPRQFVRP* gene was predicted in NCBI. Online software NetPhos 3.1 Server[2] was used to predict the phosphorylation sites. The polyadenylation signal, cleavage sites, amidation site, and mature peptides were marked as mentioned in previous studies (Veenstra, 2000; Proudfoot, 2011; Zatylny Gaudin et al., 2016). Online software Expasy-ProtParam[3] was used to translate it into amino acid sequence and predict the molecular weight (MW) and theoretical isoelectric point (*pI*) of the protein. The signal peptide was predicted by SignalP 5.0.[4] Multiple alignments were performed through software DNAMAN (Lynnon biosoft, San Ramon, CA, United states). The phylogenic tree was established by the maximum likelihood method (MEGA-X) and the bootstrap test was assessed from 1,000 replications to assure reliability.

Quantitative Expression Analysis of *SjPRQFVRP*

qRT-PCR was performed with primers PRQFVRP-RT-F/R (**Table 1**) as previously described (Li et al., 2018) on a CFX Connect Real-time PCR amplifier (Bio-Rad, Richmond,

[1]https://www.ncbi.nlm.nih.gov/

[2]https://services.healthtech.dtu.dk/service.php?NetPhos-3.1

[3]http://web.expasy.org/protparam/

[4]https://services.healthtech.dtu.dk/service.php?SignalP-5.0

TABLE 1 | Primers used in this study.

Primer	Sequence (5′–3′)	Position	Application
PRQFVRP-F	ATTCTGAAGTTTCCCATT	342–359	Core sequence cloning
PRQFVRP-R	CGTTGTCTATTTGCTCCTA	1503–1521	
10 × Universal Primer A Mix	Long,CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT; Short, CTAATACGACTCACTATAGGGC	/	RACE
5′-PRQFVRP-RACE	CTGCGAGAGGTTTCAGGACTGTGTCTTTCA	549–578	5′-RACE
5′-PRQFVRP-N1	AGAAGTGCCACCACATACATTG	444–465	
5′-PRQFVRP-N2	AAAGAAGAGAAAGTCGCCCGT	316–336	
3′-PRQFVRP-RACE	TCGCGCAACCCTTTACAACTCAACCTTCT	1332–1360	3′-RACE
3′-PRQFVRP-N1	AACAACCACTACCAAACACCA	1378–1398	
3′-PRQFVRP-N2	ATTAGGAGCAAATAGACAAC	1501–1520	
PRQFVRP-RT-F	CTTTAATGCAGCAGAACCCG	511–530	qRT-PCR
PRQFVRP-RT-R	GTCACCATCCTCAGTACCAGAC	644–661	
β-*actin*-F	GCCAGTTGCTCGTTACAG	/	qRT-PCR
β-*actin*-R	GCCAACAATAGATGGGAAT	/	
GAPDH-F	TGGTTCCTTGGCTTTTGCT	/	qRT-PCR
GAPDH-R	GGTGGTGGTGCGGGTAGT	/	
PRQFVRP-ISH-F	ACACAGTCCTGAAACCTCTCGC	555–566	ISH
PRQFVRP-ISH-R	ATTCCATGGGTCTCTTCCCTAA	1010–1031	
PRQFVRP-*Xho*I-F	CCGCTCGAGATGAGGATCTATTGGCCAAT	428–447	Subcellular localization
PRQFVRP-*Hin*dIII-R	CCCAAGCTTACCTAAAAATTCCATTGGGC	1164–1173	

The underline indicates restriction endonuclease recognition sequence.

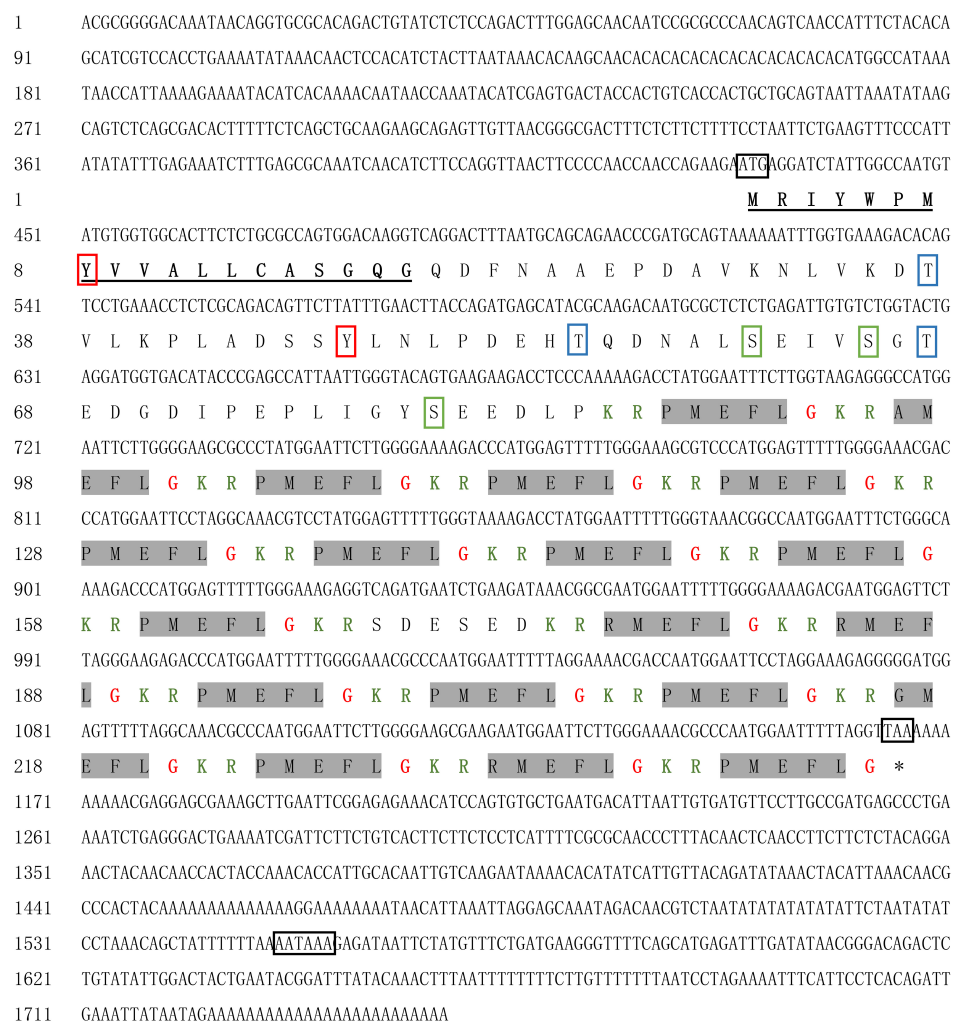

FIGURE 1 | Nucleotide sequence and deduced amino acid sequence of *SjPRQFVRP*. The initiation codon (ATG), stop codon (TAA), and polyadenylation signal (AATAAA) are boxed in black. The deduced signal peptide is underlined in black. The putative mature peptides are shaded in gray. The cleavage sites are identified in green color, and glycine used for C-terminus amidation is marked in red. The predicted phosphorylation sites are shown in colorful frames (Tyr site in red, Thr site in blue, and Ser site in green).

VA, United states) with TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Kyodo, Japan). *GAPDH* (Huo et al., 2018) and β-*actin* (JN564496.1) were taken as double internal controls. The reactions were performed with three individual repeats and three independent experiments in each tissue. PCR specificity was assessed in the terms of a melting curve.

The *SjPRQFVRP* mRNA levels were analyzed using the threshold and Ct (threshold cycle) values. The mRNA expression level detected in the muscle tissue was used as the reference value according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All data were normalized with the mRNA expression level in the muscle and presented as mean ± standard deviation (SD) (n = 3). The Least Significant Difference (LSD) multiple comparison test was performed by SPSS 21. Differences were considered significant at $p < 0.05$.

In situ Hybridization

The specific primers (PRQFVRP-ISH-F/R, **Table 1**) were designed for ISH. The PCR was carried out with brain cDNA as a template. The gel-purified PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, WI, United states) and sequenced. The sense and anti-sense probes were synthesized by *in vitro* transcription with the DIG RNA Labeling kit (Roche Diagnostics, Mannheim, Germany) as previously described (Li et al., 2019).

The fixed brain at stage I-II was washed with 1 × PBST (0.01 M PBS, 1% Tween-20) and dehydrated with gradient methanol (25, 50, 75, and 100%). The dehydrated tissue was embedded into the paraffin and sectioned with an ultra-microtome at a thickness of 7 μm.

In situ hybridization procedure was described as a previous study (Li et al., 2019). Briefly, on the first day, sections were

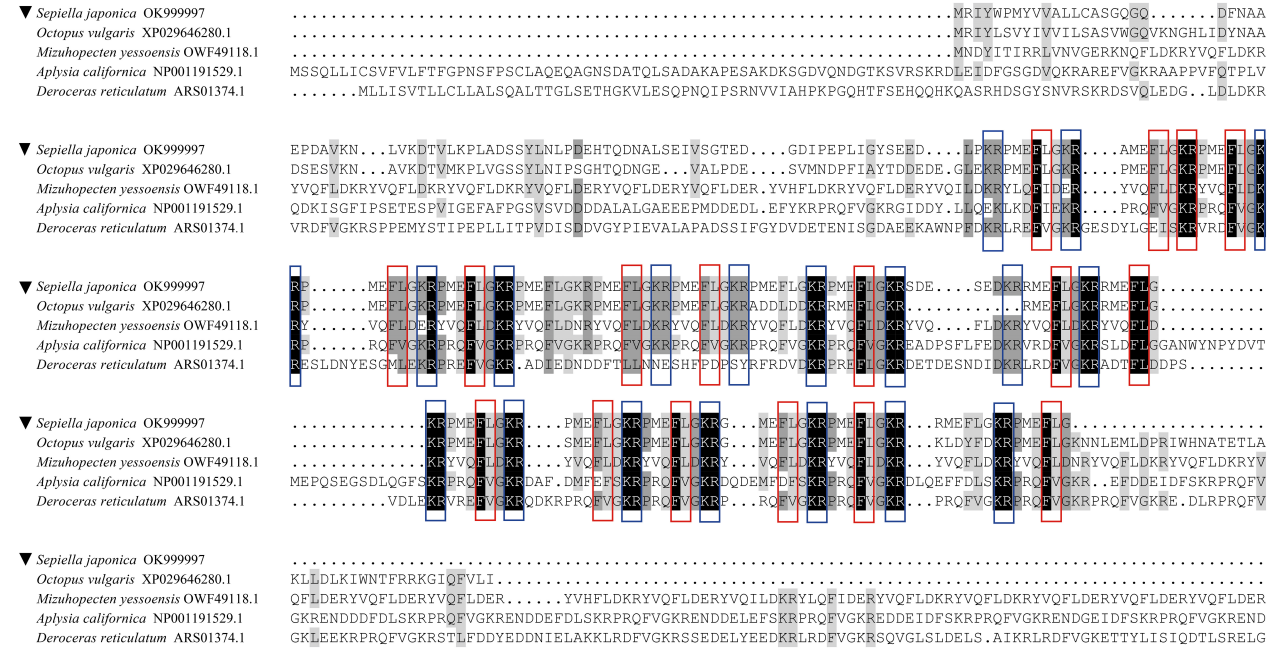

FIGURE 2 | Multiple alignments of the *Sj*PRQFVRP with PRQFVamide of other species. The *Sj*PRQFVRP is noted by ▼. The GenBank accession numbers of the sequences are as follows: *Sepiella japonica* (OK999997), *Octopus vulgaris* (XP 029646280.1), *Mizuhopecten yessoensis* (OWF49118.1), *Aplysia californica* (NP 001191529.1), and *Deroceras reticulatum* (ARS01374.1). Identical residues in all sequences are showed with black boxes. Conservative substitutions are showed with gray boxes. The red boxes are used to indicate the conservative position in the predicted mature peptide (FX). The blue boxes are used to indicate the conservative cleavage sites (KR/R).

dewaxed in xylene and rehydrated in gradient alcohol. Then, the slices were digested with Proteinase K (1 μg/ml) for 8 min at 37°C. Later, the slices were transferred to Tris/Glycine buffer to stop the reaction and fixed in 4% PFA. Pre-hybridization was conducted to reduce non-specific hybridization for 2 h at 42°C. Then slices were incubated at 55°C for 14–16 h with sense or anti-sense RNA probes (3 μg/ml). On the second day, the slices were washed at 37°C with gradient saline sodium citrate (SSC). Following the wash, sections were blocked for 2 h at room temperature (RT) with blocking buffer (2% goat serum, 2 mg/ml BSA, 0.01 M PBS). Next, slides were incubated in anti-DIG-AP Fab fragments (1:1000, Roche Diagnostics, Mannheim, Germany) overnight at 4°C. On the third day, sections were stained with NBT/BCIP (Roche Diagnostics, Mannheim, Germany). Signals were checked every 30 min. Then stained slides were mounted in glycerol and observed with a microscope (Nikon, Tokyo, Japan).

Subcellular Localization of *Sj*PRQFVRP Precursor Protein

To construct recombinant plasmids *Sj*PRQFVRP-EGFP, PCR was used to amplify the ORF of *SjPRQFVRP* with specific primers (**Table 1**) containing restriction sites *Xho*I and *Hin*dIII. Then, the PCR products were ligated into pEGFP-N1 and sequenced.

Human embryonic kidney 293 (HEK293) cells were cultured at 37°C with 5% CO_2 for 24 h before transfection. The medium was discarded and 2 ml Opti-MEM (Gibco, Waltham, MA, United states) was added to the plate. The recombinant plasmids *Sj*PRQFVRP-EGFP and Lipo6000^{TM} Transfection Reagent (Beyotime Biotechnology, Shanghai, China) were diluted with Opti-MEM for 5 min at RT, respectively. The mixture of two diluents was transfected into HEK293 cells and the cells were incubated for 6 h at 37°C. Then, the medium was removed and the cells were cultured with a fresh medium for 24 h. After fixation with 4% PFA overnight, the cytomembrane and nucleus were stained with DiI (7 μM) (Beyotime Biotechnology, Shanghai, China) and DAPI (5 μg/ml) (Beyotime Biotechnology, Shanghai, China), respectively. The stained cells were visualized under TCS SP5II laser scanning confocal microscope (Leica, Wetzlar, Germany).

RESULTS

Characteristics of Full-Length *SjPRQFVRP* cDNA

The full-length cDNA of *SjPRQFVRP* is 1748 bp, including 428 bp untranslated region (UTR) in the 5′-terminus, 582 bp of UTR in the 3′-terminus, and a 738 bp ORF encoding 245 amino acids (aa) (**Figure 1**). The putative precursor protein had a signal peptide of 19 aa and four different pentapeptides: 14 copies of PMEFLamide, three copies of RMEFLamide, one copy of AMEFLamide and GMEFLamide. The sequence MEFL was followed by a glycine which is an amidation

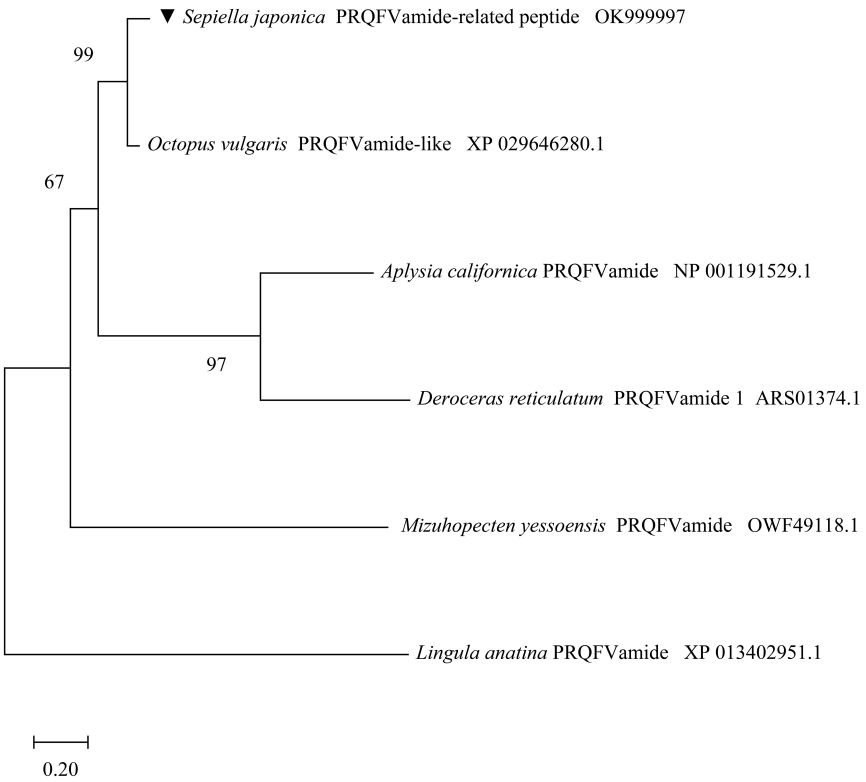

FIGURE 3 | Phylogenetic tree of *Sj*PRQFVRP with five other PRQFVamide using the maximum likelihood method. The *Sj*PRQFVRP is noted by ▼. All protein sequences are obtained from GenBank of the NCBI, and the GenBank accession numbers are as follows: *Sepiella japonica* (OK999997), *Octopus vulgaris* (XP 029646280.1), *Mizuhopecten yessoensis* (OWF49118.1), *Aplysia californica* (NP 001191529.1), *Deroceras reticulatum* (ARS01374.1), and *Lingula anatina* (XP 013402951.1). The PRQFVamide of *L. anatina* serves as the outgroup. The topological stability of the tree is achieved by running 1,000 bootstrap replications. Bootstrap values (%) are indicated by numbers at the nodes.

site. The predicted MW was 28.6 kDa and the theoretical *pI* was 9.47.

Homology and Phylogenetic Analysis

The amino acid sequences of PRQFVamide downloaded from the NCBI database from the other four representative species and the *Sj*PRQFVRP were used for alignment analysis. The results indicated that *Sj*PRQFVRP shared 71% identity to the PRQFVamide-like of *O. vulgaris* (XP 029646280.1), and 47, 46, 45% identity to those of *M. yessoensis* (OWF49118.1), *A. californica* (NP 001191529.1), and *D. reticulatum* (ARS01374.1), respectively. The multiple alignments results showed that *Sj*PRQFVRP had some conserved amino acids with other PRQFVamide, such as cleavage sites (KR/R) and partial mature peptide amino acids (FL/FV) (**Figure 2**).

To determine the evolutionary status of the *Sj*PRQFVRP, a phylogenetic tree was generated from *Sj*PRQFVRP and the other five PRQFVamide sequences by the maximum likelihood method (MEGA-X) (**Figure 3**). Phylogenetics reveals that the PRQFVamide from five mollusks were clustered. Moreover, a close relationship between *Sj*PRQFVRP and PRQFVamide-like of *O. vulgaris* was observed at a high confidence value (99%), suggesting they were close relatives. The PRQFVamide of brachiopod *L. anatina* (XP 013402951.1), as an outgroup, was distantly related to those of the mollusks.

Quantitative Expression Analysis of *SjPRQFVRP*

As shown in **Figure 4**, the mRNA expression level of *SjPRQFVRP* at different development stages from females was detected. The transcripts of the *SjPRQFVRP* in the muscle were taken as the reference.

At stage I-II (**Figure 4A**), the expression of *SjPRQFVRP* in the intestine was approximately 10 times that of the muscle, followed in the liver, which was about 8 times higher ($p < 0.05$). At stage III (**Figure 4B**), the expression level of *SjPRQFVRP* in the brain and optic lobe was significantly higher than that of other tissues, with the levels were approximately 125 times and 60 times that of the muscle, respectively ($p < 0.05$). At stage IV (**Figure 4C**), the expression pattern is very similar to that of stage III, with the brain and optic lobe showing very high levels ($p < 0.05$). The difference with a slightly increasing level in the nidamental gland and accessory nidamental gland has been detected. At stage V (**Figure 4D**), the optic lobe had higher expression level than that of other tissues and was around 450 times that of the muscle ($p < 0.05$), followed by the brain, liver, and nidamental gland.

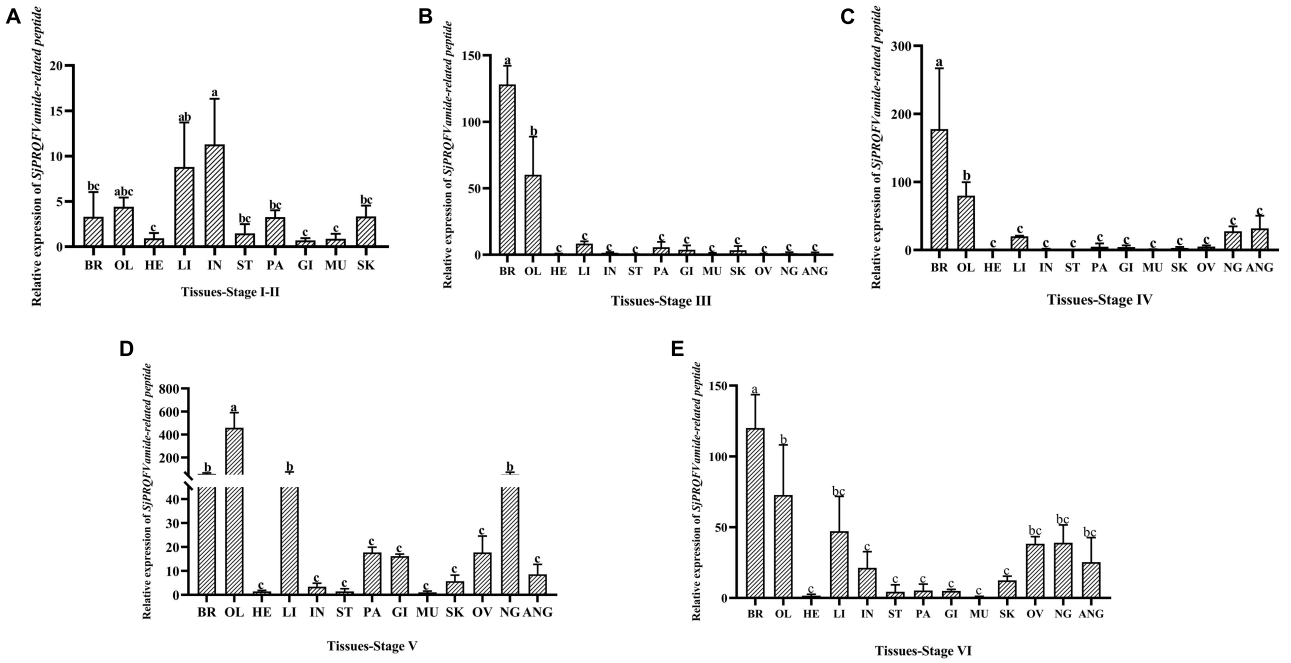

FIGURE 4 | Expression analysis of *SjPRQFVRP* from diverse tissues at different development stages of female cuttlefish. **(A)** Stage I-II; **(B)** stage III; **(C)** stage IV; **(D)** stage V; **(E)** stage VI. In each case, the transcripts of the muscle were served as the reference value. Vertical bars represent the mean ± SD (n = 3). The analyses were conducted in tissues including the brain (BR), optic lobe (OL), heart (HE), liver (LI), intestine (IN), stomach (ST), pancreas (PA), gill (GI), muscle (MU), skin (SK), ovary (OV), nidamental gland (NG), and accessory nidamental gland (ANG). *GAPDH* and β-*actin* served as reference genes. Different letters on the bars indicate statistically significant differences ($p < 0.05$).

At stage VI (**Figure 4E**), the relative expression in the brain was significantly higher than other tissues and was around 120 times that of the muscle ($p < 0.05$).

To better elucidate our data, the stage-point expression levels of *SjPRQFVRP* in one single tissue were re-quantified in **Figure 5**. As shown in **Figure 5A**, for the brain tissue, the expression level of *SjPRQFVRP* showed a steady increase from stage I-II to IV and reached the peak at stage IV ($p < 0.05$), then the expression level decreased at stage V and recovered to the level of stage III at stage VI. In the optic lobe (**Figure 5B**) and pancreas (**Figure 5E**), the transcripts of *SjPRQFVRP* at stage V were significantly higher than that of other stages ($p < 0.05$). In the liver (**Figure 5C**), it showed an increasing trend, and the peak was at stage V and VI ($p < 0.05$). In the ovary (**Figure 5F**), from stage III to VI, the expression showed a continuous increase. In the nidamental gland (**Figure 5G**), from stage III to stage V, the expression increased until stage V reached the peak ($p < 0.05$). In the accessory nidamental gland (**Figure 5H**), the highest expression of *SjPRQFVRP* appeared at stage IV ($p < 0.05$).

Distribution of *SjPRQFVRP* mRNA in the Brain

The *SjPRQFVRP* mRNA distribution in the cuttlefish brain at stage I-II was determined by ISH. As shown in **Figure 6**, no signal was detected with the sense probe (**Figures 6A,C**). In contrast, specific staining was detected in various regions of the cuttlefish brain with the anti-sense probe (**Figures 6B,D**). In the supraesophageal mass (**Figure 6B**), positive signals were intensively appeared in cells of the anterior basal lobes (abl) and followed in the vertical lobe (vl) and subvertical lobe (svl). In the suboesophageal mass, the positive signals were primarily observed in the anterior pedal lobe (apl) (**Figure 6B**). In other words, the positive signals were mainly distributed in the peripheral cells of the functional lobes of supraesophageal and suboesophageal masses. In the optic lobe (ol) (**Figure 6D**), the staining in the central medulla (med) was the most extensive and intense. The edge of the medulla cells was stained as weakly as the inner granulosa cells (inn. gr. cel) and outer granulosa cells (out. gr. cel). Moreover, the esophagus (**Figure 6B**) was also observed some weak signals at stage I-II.

Subcellular Localization of *Sj*PRQFVRP

Figure 7 showed the localization of *Sj*PRQFVRP protein in the HEK293 cells. Strong and clear green signals were detected in the cytoplasm of the transfected cells (**Figure 7A**) but not in the membrane and nucleus (**Figures 7B,C**), indicating that *Sj*PRQFVRP was localized in the cytoplasm of the cells (**Figure 7D**).

DISCUSSION

In this study, *Sj*PRQFVRP contained four pentapeptides: 14 copies of PMEFLamide, three copies of RMEFLamide, one

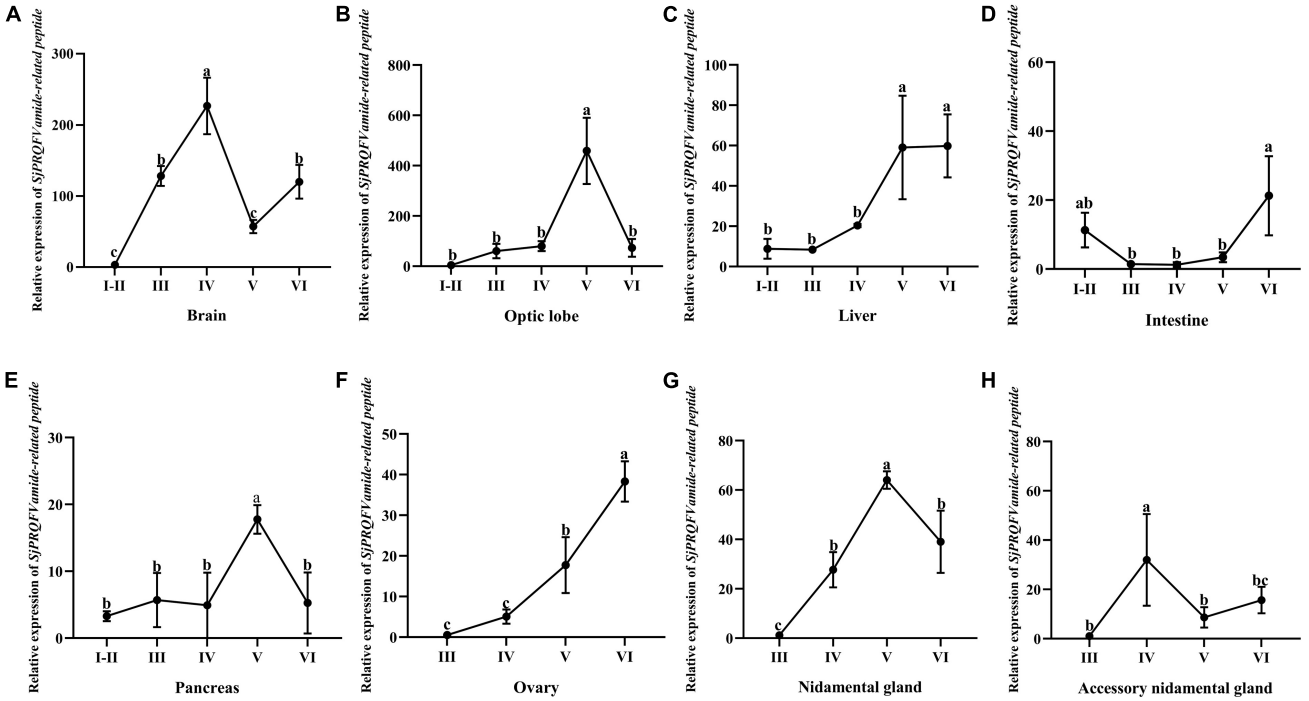

FIGURE 5 | The expression level of *SjPRQFVRP* of females in different tissues at different stages. Vertical bars represent the mean ± SD ($n = 3$). *GAPDH* and β-*actin* served as reference genes. Different letters indicate statistically significant differences ($p < 0.05$). **(A)** Expression trend of *SjPRQFVRP* in the brain. **(B)** Expression trend of *SjPRQFVRP* in the optic lobe. **(C)** Expression trend of *SjPRQFVRP* in the liver. **(D)** Expression trend of *SjPRQFVRP* in the intestine. **(E)** Expression trend of *SjPRQFVRP* in the pancreas. **(F)** Expression trend of *SjPRQFVRP* in the ovary. **(G)** Expression trend of *SjPRQFVRP* in the nidamental gland. **(H)** Expression trend of *SjPRQFVRP* in the accessory nidamental gland.

copy of AMEFLamide and GMEFLamide. In *A. californica*, PRQFVamide precursor protein contained PRQFVamide and other four kinds of pentapeptides: AREFVamide, VRDFVamide, VREFVamide, and IREFVamide (Furukawa et al., 2003). These five predicted pentapeptides were amidated and shared three immobile amino acids: the second amino acid is an arginine, the fourth is a phenylalanine, and the fifth is a valine (Furukawa et al., 2003). Compared with the PRQFVamide of *A. californica*, the predicted mature peptides of *Sj*PRQFVRP did not contain PRQFVamide or the other four peptides. But similar to the PRQFVamide of *A. californica*, all four pentapeptides of *Sj*PRQFVRP were amidated and shared four invariant amino acids (-MEFLamide). In addition, they had a similar structure feature at C-terminus: FXamide (X = hydrophobic amino acid). In *S. officinalis*, there were two sequences of incomplete *PRQFVamide* precursor with two different signal peptides and two termination codons at the C-terminus (Zatylny Gaudin et al., 2016). Moreover, five mature products were recovered from hemolymph in *S. officinalis*, including PMEFLamide (Zatylny Gaudin et al., 2016). However, in the predicted peptides of *Sj*PRQFVRP, we did not find the other four mature peptides like the ones recovered in the hemolymph of *S. officinalis*. One possible explanation is that there are other transcripts of PRQFVamide in the cuttlefish, and this probably result from alternative splicing (Zatylny Gaudin et al., 2016).

Some interesting discoveries have been made by this study through the qRT-PCR data. 1) The *SjPRQFVRP* expression level was increased in the ovary from stage III to VI. 2) The expression in the nidamental gland was also increased from stage III to V but reduced at stage VI. 3) The expression in the liver increased and reached the peak at stage V and stage VI. The synthesis and release of neuropeptides were found to be a general feature of most cells of the nervous system (Kiss, 2011). However, researchers found that neuropeptides are also produced and released by non-neuronal cells such as glands or endocrine cells (Pirger et al., 2008; Kiss, 2011). Therefore, the expression of *SjPRQFVRP* in liver and nidamental gland might be related to its potential function. Nidamental gland is a typical secretory gland. It is mainly used to form a third layer of egg membrane to protect the fertilized eggs (Wang et al., 2021). Liver is an important organ for metabolism and innervated by a large number of afferent and efferent nerves (sympathetic and parasympathetic) in vertebrate (Yi et al., 2010). Otherwise, liver is also one of the synthesis organs of vitellogenin, which is transported to the ovary to be accumulated and stored in the form of vitellin (Tian et al., 2014). However, the function of the liver in cephalopods is still unclear. Given these interesting discoveries, more efforts are needed to further study in *S. japonica*.

A previous study reported that the cephalopod brain was divided into the supraesophageal mass, suboesophageal mass,

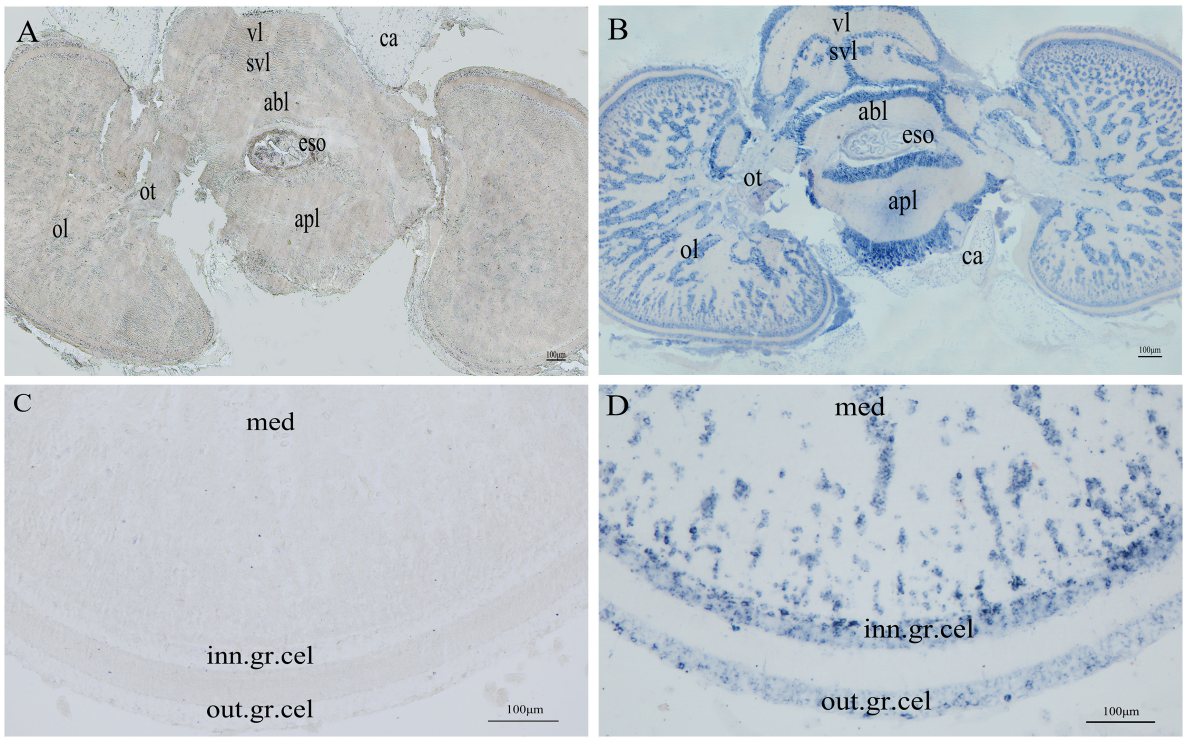

FIGURE 6 | Distribution of *SjPRQFVRP* mRNA in the brain. **(A)** The brain of stage I-II was stained with sense *SjPRQFVRP* probe; **(B)** The brain of stage I-II was stained with anti-sense *SjPRQFVRP* probe; **(C)** The optic lobe was stained with the sense *SjPRQFVRP* probe; **(D)** The optic lobe was stained with the anti-sense *SjPRQFVRP* probe. Vl, vertical lobe; svl, subvertical lobe; abl, anterior basal lobe; eso, esophagus; apl, anterior pedal lobe; ca, cartilage; ot, optic tract; ol, optic lobe; med, medulla; out. gr. cel, outer granulosa cell; inn. gr. cel, inner granulosa cell.

and optic lobes (Yu et al., 2011). Various nerve lobes have been found in cephalopod brain with different functions. The vertical lobe stores memory information and accurately identifies the opposite position (Young, 1960). The subvertical lobe receives nerve impulses from the vertical lobe and transmits them to the optic lobe (Hochner et al., 2006). The reproductive nerve lobes are mainly the subpeduncle lobe and optic gland (Messenger, 1967; Froesch, 1974). The anterior and posterior basal lobes control the direction of steering movement and tentacle movement and color change by chromatophores (Shigeno and Yamamoto, 2002). The anterior and posterior pedal lobes also regulate tentacle movements, while the brachial lobe coordinates the movements of the arms (Shigeno and Yamamoto, 2002). It is reported that various neuropeptides distribute in different parts of the cephalopod brain, where is likely to be related to their function. In *S. japonica*, *SjLFRFamide* mRNA was highly expressed in the brain of both male and female cuttlefish. It was detected in several different functional lobes, mainly in the subpeduncle lobe, subvertical lobe, and all regions of suboesophageal mass. These findings suggested that *SjLFRFamide* might function in regulating reproduction and feeding (Cao et al., 2016). The distribution pattern of *SjFMRFamide* mRNA was similar to that of the *SjLFRFamide* in the *S. japonica* brain (Li et al., 2018). In *S. officinalis*, the *SOFaRP2* gene was detected in the posterior chromatophore, anterior chromatophore, lateral basal, and optic lobes (Zhang et al., 2012; Zhang and Tublitz, 2013). These observations supported the idea that *SOFaRP2* might be related to regulate chromatophore activity, feeding behavior, learning and memory. *FMRFamide* transcripts were also detected in the brain and expressed at the early life of *S. pharaonis* (Zhu et al., 2020). Additionally, strongly positive signals of *GnRH* were observed in the different lobes of the supraesophageal, suboesophageal mass, and the medulla of the optic lobe in *S. pharaonis*, suggesting that *GnRH* might have a role in regulating feeding and reproduction (Song et al., 2021). In this manuscript, the brain at stage I-II was selected to preliminarily explore its potential functionally relevant localization in cuttlefish at early developmental stages. The *SjPRQFVRP* was distributed in several functional lobes of the brain at stage I-II, including the vertical lobe, subvertical lobe, anterior basal lobe, anterior pedal lobe, and optic lobes. The distribution of *SjPRQFVRP* in the brain is similar to the distribution of neuropeptides mentioned above. However, to further explore the role of *SjPRQFVRP* during the growth and development of cuttlefish, more experiments are needed.

Moreover, in *Aplysia*, researchers described the distribution of *PRQFVamide* on the buccal and cerebral ganglion and found that the gene might have effect on some systems as a modulator, especially the feeding system (Dembrow et al., 2003;

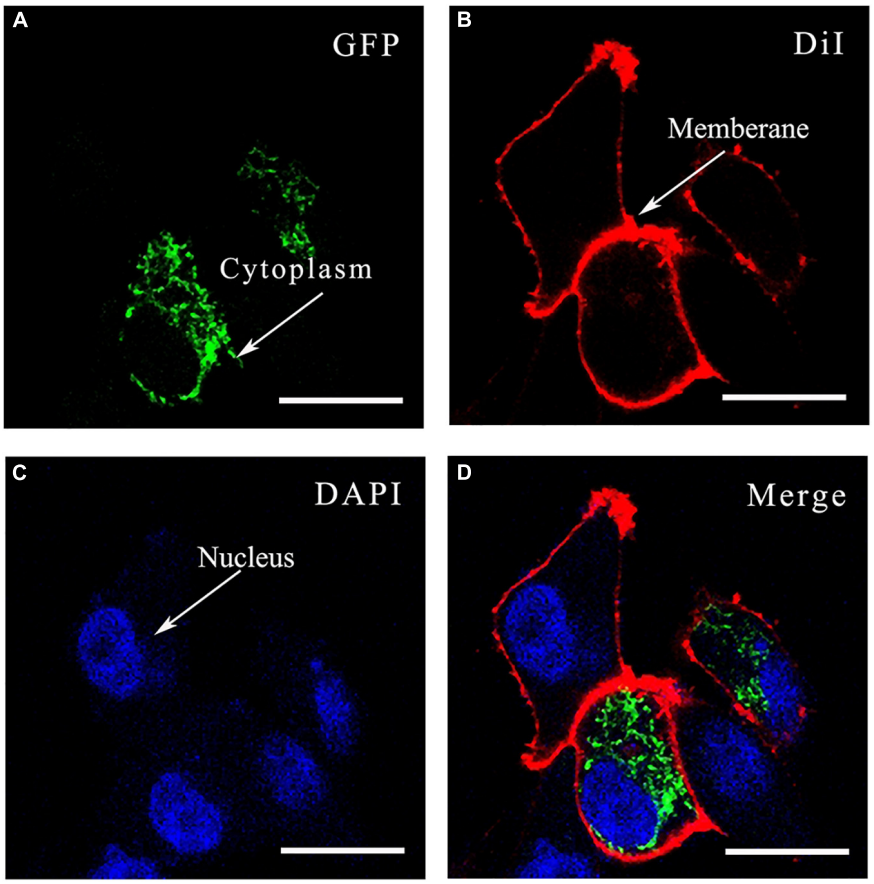

FIGURE 7 | The localization of *Sj*PRQFVRP-EGFP fusion protein in HEK293 cells. **(A)** GFP: *Sj*PRQFVRP-EGFP fusion protein was stained green; **(B)** DiI: cytomembranes of cells were stained red with DiI; **(C)** DAPI: nucleus of cells was stained blue with DAPI; **(D)** Merge: *Sj*PRQFVRP protein was mapped in the cytoplasm of HEK293 cells. Scale bars = 20 μm.

Furukawa et al., 2003). Whether *SjPRQFVRP* plays a part in the feeding system like that of *Aplysia* still needs to be confirmed by further experiments.

The subcellular localization suggests that *Sj*PRQFVRP was in the cytoplasm of HEK293 cells. At present, the PRQFVamide receptor has not been found yet. In a future experiment, we can start with the PRQFVamide receptor and conduct a functional exploration of *Sj*PRQFVRP in *S. japonica*.

CONCLUSION

In conclusion, our study described the full-length sequence of *SjPRQFVRP* cDNA in *S. japonica*. qRT-PCR analysis and ISH of the brain indicated the dominant expression of *SjPRQFVRP* throughout different developmental stages in *S. japonica*. In brief, the results informed us the follow up studies based on different approaches that the gene might be involved in various physiological functions in *S. japonica*. This study could also be of the theoretical basis for the restoration of cuttlefish germplasm resources.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, OK999997.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of Zhejiang Ocean University and the Academy of Experimental Animal Center of Zhejiang Ocean University.

AUTHOR CONTRIBUTIONS

C-FC and L-BZ conceived and designed the study, reviewed, revised, and edited the manuscript, and contributed the reagents, materials, and analytical tools. J-YQ performed the investigation, analyzed the data, and led the writing-original draft. All authors contributed critically to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.805209/full#supplementary-material

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