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Identification, classification and expression analysis of the Ras superfamily genes in the Pacific white shrimp, *Litopenaeus vannamei*

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The Ras superfamily of small guanosine triphosphatases (GTPases) are a large group of small GTP-binding proteins, which play crucial roles in basic cellular processes in all eukaryotes. In this study, by analyzing the gene structure, temporal and spatial expression patterns, a total of 108 Ras superfamily genes were identified in the genome of the Pacific white shrimp *Litopenaeus vannamei*. We found these genes included not only the classical Ras GTPase superfamily members, but also some unconventional and novel Ras GTPase proteins, which have unknown functions and unique expression patterns. All Ras superfamily genes of *L. vannamei* were highly conserved within the core G domain and closely related in phylogeny, but they might have two different evolutionary origins. In addition, different Ras GTPase genes exhibited distinct expression patterns in different tissues, development/molting stages and WSSV infection samples of *L. vannamei*, suggesting that they may have a high functional specialization, and play important roles in regulating the biological processes of cell differentiation, growth and development, immune response, etc. This study provides important clues for the structure, classification, evolution and function of Ras superfamily in shrimp.

KEYWORDS

Ras superfamily, GTPase proteins, *Litopenaeus vannamei*, gene structure and function, gene expression, immunity, growth

1 Introduction

The Ras superfamily, also known as small guanosine triphosphatases (GTPases), or small G proteins, is a group of monomeric protein family with GTPase hydrolytic activity. They are low molecular weight (20–30 kDa) and similar to the α-subunit of G-proteins and regulate many biological processes as molecular switches, alternating between an active GTP-bound state and an inactive GDP-bound state (Phillips et al., 2008). Ras was the first members of the

superfamily to be discovered, and then Ran, Rho, Rab, Arf and other families (Marcus and Mattos, 2020). They control different signal transduction pathways in cells, including proliferation, differentiation, morphology, polarity, adhesion, migration, survival, apoptosis, etc. (Goitre et al., 2014).

Ras superfamily proteins are universal components of signaling pathways in eukaryotic organisms, including vertebrates, invertebrates, yeasts and plants (Cetkovic et al., 2007). In human, using a somewhat broader definition of sequence similarity reveals an extended superfamily of more than 170 Ras superfamily members (Colicelli, 2004). *Drosophila melanogaster* has 68 members: 13 Ras proteins, 7 Rhos, 30 Rabs, 17 Arfs, and 1 Ran (Rojas et al., 2012). The budding yeast *Saccharomyces cerevisiae* contains 29 members: 1 Sar and 1 SRβ, 6 Arfs, 10 Rabs, 6 Rhos, 4 Ras, and 2 Rans (Garcia-Ranea and Valencia, 1998). Studies have shown that the proteins of each family appeared very early in the evolution of eukaryotes, and then expanded to varying degrees in various species (Colicelli, 2004; Jiang and Ramachandran, 2006).

At present, there are limited studies on Ras GTPases in crustaceans, which mainly focused on their function in immunity of shrimp. In Kuruma shrimp *Marsupenaeus japonicus*, Ras, Ran and Rab genes have been found, they all play important roles in resistance to virus (Ménasché et al., 2000). In Chinese shrimp *Fenneropenaeus chinensis*, the expression of Rap gene was up-regulated in both *Vibrio harveyi* and white spot syndrome virus (WSSV) infection (Ren et al., 2012). Another study showed that after WSSV infection, the expression of *FcRas* was significantly up-regulated in muscle of *F. chinensis*, while it was significantly down-regulated in hepatopancreas (Li et al., 2020). These results suggest that different Ras superfamily members may participate in the process of anti-bacterial and viral immunity in different ways in shrimp.

Ras GTPases participate in various biological processes, and many studies have confirmed that they play an important role in growth (Sato et al., 2008; Geng et al., 2016; Liu et al., 2021). In the Pacific white shrimp *Litopenaeus vannamei*, the most economically valuable aquaculture shrimp in the world, through genome wide association study (GWAS) analysis, we found several genomic markers related to body weight and body length were mapped to *Rap-2a*, which is a member of the Ras superfamily (Yu et al., 2019). Rap-2a, as part of several signaling cascades, may regulate cytoskeletal rearrangement, cell migration, and cell diffusion (Taira et al., 2004). Through RT-qPCR analysis, *Rap-2a* was found to have high expression in lymphatic organ, hepatopancrea, intestine and stomach, it was negatively regulated by NF-κB and contributed to growth (Yu et al., 2019; Wang et al., 2022). Additionally, another shrimp growth trait candidate gene, *MMD2*, was identified by our group (Wang et al., 2020); and studies showed that MMD2 can enhance the retention and activation of Ras protein in Golgi complex, and subsequently lead to the enhancement of ERK (extracellular signal-regulated kinase) signal (Huang et al., 2012). These studies suggest that the Ras superfamily may play key roles in shrimp growth.

In this study, through the analyses of genome and transcriptome data, we found that *L. vannamei* has a large number of Ras superfamily genes. In this study, we identified 108 Ras superfamily genes, and analyzed their gene structure, classification and expression patterns. These studies have provided an important basis for further explore the structure, evolution and function of the Ras superfamily in shrimp.

2 Materials and methods

2.1 Experimental animals

The experimental shrimp were cultured in the laboratory of the Institute of Oceanology, Chinese Academy of Sciences (Qingdao, Shandong, China), at a temperature of 25 ± 1°C, salinity of 30%, and pH of 7.5 ± 0.1, the photoperiod was maintained at 12L:12D. The aquaculture seawater was filtered, sterilized, and continuously oxygenated. All shrimp were fed three times per day at 9:00 a.m., 2:00 p.m., and 7:00 p.m. with equal weights of commercial food pellets (Dale Feed Company, Yantai, China). The average weight of the shrimp was 4.0 ± 0.8g (7.5 ± 0.5cm). The animal study was reviewed and approved by the ethics committee of the Institute of Oceanology, Chinese Academy of Sciences. We declare that all animal experiments in this study were conducted in accordance with the guidelines of UK Animals Act, 1986 and EU Directive 2010/63/EU. In these experiments, no any endangered or protected species were used.

2.2 Identification of Ras superfamily gene members

We collected all genes annotated as Ras superfamily or small G protein genes from the *L. vannamei* genome database (http://www.shrimpbase.net). At the same time, we screened all Ras superfamily genes in the previous RNA-Seq data from different developmental stages, molting stages, different adult tissues, and WSSV infection of the shrimp (Wei et al., 2014; Gao et al., 2015; Wang et al., 2019; Zhang et al., 2019). After comparing all the obtained sequences, eliminating redundant sequences, merging overlapping fragments and connecting broken genes, all non-redundant candidate sequences were initially identified and compared by blastx (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and SMART (https://smart.embl.de/) to confirm the gene members of Ras superfamily.

2.3 Phylogenetic analysis of Ras superfamily of *L. vannamei*

In order to determine evolutionary relationships of the Ras superfamily members in *L. vannamei*, all identified Ras GTPase homologous sequences of this species and a number of representative sequences of other animals, fungi, protists and plants were aligned by ClustalW in MEGA X (www.megasoftware.net/), and phylogenetic trees were constructed using the maximum likelihood (ML) method of MEGA X with 1000 bootstrap repeats (Whelan and Goldman, 2001; Kumar et al., 2018). The phylogenetic trees were then visualized using iTOL (Letunic and Bork, 2007).

2.4 Gene structure and conservative motif

In order to illustrate the gene structure of the Ras GTPases of *L. vannamei*, the gene location, gene length, open reading frame (ORF), exon number and deduced amino acid number of all the obtained genes were analyzed in detail. ExPASy (http://web.expasy.org/protparam/) was used for protein molecular weight, isoelectric point analysis. Using the gene structure display server (GSDS) program (Hu et al., 2015), the coding sequence (CDS) of each gene was compared with the genome sequence of *L. vannamei*, and the intron and exon arrangement diagram of each gene was obtained. The gene structure of Ras GTPase proteins was drawn by TBools software (Chen et al., 2020), and the conservative domains analysis was carried out according to the amino acid sequences. In order to better analyze the conserved motifs in Ras superfamily, a conserved motif map using the Multiple EM for Motif Elicitation (MEME, https://meme-suite.org/meme/tools/meme) program was constructed to provide more detailed evidence for clarifying the structural characteristics between different categories of Ras superfamily numbers.

2.5 Gene expression analyses

According to the previous RNA-Seq data at different developmental stages, molting stages, different adult tissues and WSSV infection of *L. vannamei* (Wei et al., 2014; Gao et al., 2015; Zhang et al., 2019), the RPKM (Reads per kilo base per million mapped reads) value of each unigene or transcript were obtained. The expression heatmaps of Ras GTPase genes in different transcriptomes were constructed and a part of representative genes were verified and analyzed using RT-qPCR subsequently.

2.6 RNA isolation and cDNA synthesis

The healthy WSSV-free shrimp *L. vannamei* (9 ± 1g) were collected from laboratory culture tanks for WSSV infection study, each shrimp was injected into 1000 copies of live WSSV particles suspended in 10μl sterile phosphate-buffered saline (PBS) *in vivo* WSSV challenge group, the control group shrimps were injected into the same volume of PBS. A total of 15 individuals were randomly assigned in each group and equally divided into three parallel subgroups as biological replicates. At 6 hpi, same size shrimp were picked, sacrificed and dissected. A total of 3 tissues, hemocyte, lymphoid (Oka) organ, hepatopancreas were sampled and frozen in liquid nitrogen and stored at -80 °C for total RNA extraction.

About 4.0 ± 0.8g (7.5 ± 0.5cm) untreated shrimp were picked, sacrificed and dissected. A total of 12 tissues, hemocyte, muscle, intestines, stomach, lymphoid organ, gill, hepatopancreas, eye stalk, brain, ventral nerve, epidermis and heart, were sampled and frozen in liquid nitrogen and stored at -80 °C for total RNA extraction. According to the manufacturer's instructions, RNAiso Plusreagent (TaKaRa, Japan) was used to isolate total RNA from these tissues. Then, the quality and the concentration of RNA were detected using 1% agarose gel electrophoresis and Nanodrop 2000 (Thermo Fisher Scientific, United States). The first-strand cDNA was synthesized with the PrimeScript First Strand cDNA Synthesis Kit (TaKaRa, Japan) using 1.5 μg RNA as template. The specific cDNA synthesis were as follows: the first step was to remove genomic DNA, 5×genomic DNA eraser buffer was added to the template at 42°C for 5 min, and then moving on to step 2 immediately, at 37°C for 1 h and 85°C for 5 s. Finally, the cDNA was stored at -80°C until use.

2.7 Real-time quantitative PCR

The 18S rRNA gene was selected as the internal reference gene. Primer3Plus (http://www.primer3plus) was used to design thirteen pairs of primers of the selected Ras superfamily genes. All primer sequences used in this study are shown in Supplementary Table S1. Then, an Eppendorf Mastercycler ep realplex (Eppendorf, Hamburg, Germany) was used to perform the RT-qPCR. The SuperReal PreMix Plus (SYBR Green) (TIANGEN, Beijing, China), template, primers, and DEPC-treated water were mixed in a certain proportion (Supplementary Table S2); each sample includes four technical replicates. After qPCR, melting temperature (Tm) analysis showed a single peak and a single PCR band was identified, indicating that both primers were suitable for further experiments. The qPCR steps were as follows: 94°C for 2 min, 40 cycles of 94°C for 20 s, 62°C or 55°C for 20 s (the annealing temperature of rtRas superfamily genes-F/R and rt18S-F/R were 62 and 55°C, respectively), and 72°C for 20 s. Eventually, relative expression levels were analyzed by the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001).

2.8 Statistical analyses

The different groups were subjected to one-way ANOVA tests using SPSS (https://www.ibm.com/cn-zh/analytics/spss-statistics-software) (version 20).

3 Results

3.1 Identification and classification of the Ras superfamily genes

In this study, a total of 108 Ras superfamily genes of *L. vannamei* were screened and identified from genome and transcriptome data. The identified genes were classified and analyzed according to gene homology, conserved domain, gene annotation and evolutionary relationship. Using the SMART tool, the domains of 50 sequences were identified as the classical domains of the Ras superfamily: Ras, Rab, Arf (Sar), Rho respectively, and 49 sequences contained a small-GTPase domain, which have similar GTP binding conserved motifs to the classical Ras superfamily domains (Table 1), all them shared a set of conserved G box GDP/GTP-binding motif elements (Figure 1). Among the small-GTPase domain containing sequences, five have large molecular weight, they have not only a small-GTPase domain, but also other domains, such as BTB, FF, EFh, and RPT1 (Table 1). The remaining 9 sequences do not have the classical Ras superfamily domains or the small-GTPase domain, but they all have GTPase-

TABLE 1 Basic information of the Ras superfamily members in *L. vannamei* genome.

Proposed name	Gene_id	Genome position	Position	Exon	Length (aa)	Domain	pI	Predicted MW (KD)	NCBI_id
LvRas1	LVAN10661	LVANscaffold_1774	616354-616965(-)	1	203	RAS	9.25	22.5	ROT75450.1
LvRas2	LVAN00103	LVANscaffold_77	13355-24063(-)	4	193	RAS	6.21	21.9	XP_027206906.1
LvRas3	LVAN06862	LVANscaffold_1264	1151303-1155602(-)	3	173	RAS	5.13	19.5	XP_027233334.1
LvRas4	LVAN12623	LVANscaffold_2024	396324-398769(+)	3	186	RAS	6.37	20.1	XP_027216805.1
LvRas5	LVAN24533	LVANscaffold_4064	359946-366495(-)	5	182	RAS	5.67	20.5	XP_027235111.1
LvRas6	LVAN21358	LVANscaffold_3190	91647-99055(-)	6	185	RAS	5.96	21.2	ROT64798.1
LvRas7	LVAN23009	LVANscaffold_3481	297108-307390(+)	NA	345	RAS	9.74	39.0	ROT63158.1
LvRas8	LVAN07607	LVANscaffold_1371	296593-307664(+)	3	110	pfamRas	10.87	12.5	XP_027209547.1
LvRas9	LVAN21643	LVANscaffold_3244	538288-547990(-)	6	263	SmallGTPase	8.69	29.1	ROT64508.1
LvRas10	LVAN12750	LVANscaffold_2031	142221-145529(-)	2	123	SmallGTPase	9.65	14.5	QBA57436.1
LvRas11	LVAN22364	LVANscaffold_3378	325461-325907(+)	1	148	SmallGTPase	5.28	17.3	ROT63798.1
LvRas12	LVAN00405	LVANscaffold_207	728705-729996(+)	3	250	SmallGTPase	8.10	27.5	XP_027217457.1
LvRas13	LVAN25101	LVANscaffold_4396	28904-29844(-)	3	231	SmallGTPase	7.64	25.9	XP_027236068.1
LvRas14	LVAN01975	LVANscaffold_591	25670-28817(+)	NA	302	SmallGTPase	10.9	31.8	ROT84101.1
LvRas15	LVAN01445	LVANscaffold_514	389157-389615(-)	1	152	SmallGTPase	6.64	17.0	ROT84676.1
LvRas16	LVAN07913	LVANscaffold_1417	189305-202140(-)	NA	593	SmallGTPase	10.03	64.7	ROT78217.1
LvRas17	LVAN20599	LVANscaffold_3071	80071-83245(-)	3	190	SmallGTPase	9.34	21.4	XP_027228024.1
LvRas18	LVAN07484	LVANscaffold_1351	39073-51286(+)	NA	345	SmallGTPase	8.91	37.3	ROT78630.1
LvRas19	LVAN07483	LVANscaffold_1351	28496-34926(-)	NA	412	SmallGTPase	10.29	45.8	ROT78629.1
LvRas20	LVAN18345	LVANscaffold_2759	299702-311847(+)	5	157	SmallGTPase	4.82	17.2	ROT67801.1
LvRas21	LVAN09982	LVANscaffold_1688	287125-292722(+)	3	100	SmallGTPase	9.44	11.2	ROT76149.1
LvRas22	LVAN24642	LVANscaffold_4124	510279-523240(+)	7	405	RAS	9.51	43.8	ROT61531.1
LvRas23	LVAN02724	LVANscaffold_694	49272-57249(+)	3	178	SmallGTPase	7.69	20.3	ROT83387.1
LvRas24	LVAN15227	LVANscaffold_2371	387067-396008(+)	3	196	SmallGTPase	9.25	22.0	ROT70918.1
LvRab1	LVAN22146	LVANscaffold_3340	1162103-1162573(+)	2	81	pfamRas	4.49	9.2	KAG7176781.1
LvRab2	LVAN22145	LVANscaffold_3340	1130781-1131492(+)	NA	82	SmallGTPase	4.48	9.2	ROT64023.1
LvRab3	LVAN21354	LVANscaffold_3190	10914-11931(-)	3	133	SmallGTPase	6.29	14.6	ROT64794.1
LvRab4	LVAN21137	LVANscaffold_3156	515327-518808(-)	2	105	SmallGTPase	4.82	12.1	ROT65026.1
LvRab5	LVAN07945	LVANscaffold_1421	131603-134012(+)	3	139	SmallGTPase	8.44	15.5	ROT78183.1
LvRab6	LVAN08022	LVANscaffold_1431	386949-389496(-)	4	142	SmallGTPase	9.24	16.3	ROT78100.1
LvRab7	LVAN15167	LVANscaffold_2365	1060966-1065462(+)	4	201	SmallGTPase	5.69	22.2	XP_027220508.1
LvRab8	LVAN11009	LVANscaffold_1811	15772-16133(+)	2	78	SmallGTPase	7.95	8.9	ROT75118.1
LvRab9	LVAN21138	LVANscaffold_3156	519598-523474(-)	2	66	SmallGTPase	9.59	7.1	ROT65027.1
LvRab10	LVAN05619	LVANscaffold_1114	1435106-1455690(+)	5	249	SmallGTPase	9.21	27.6	XP_027215650.1
LvRab11	LVAN06487	LVANscaffold_1213	337968-340688(-)	3	114	SmallGTPase	8.66	13.0	ROT79637.1
LvRab12	LVAN03293	LVANscaffold_773	452339-455136(+)	3	206	SmallGTPase	5.21	23.1	XP_027206950.1
LvRab13	LVAN18392	LVANscaffold_2765	222508-228145(+)	5	224	SmallGTPase	6.97	24.5	XP_027224884.1
LvRab14	LVAN17383	LVANscaffold_2637	892303-910421(+)	6	194	RAB	5.05	22.1	ROT68780.1
LvRab15	LVAN17826	LVANscaffold_2691	268423-311239(-)	NA	384	RAB	6.67	42.1	ROT68336.1

(Continued)

TABLE 1 Continued

Proposed name	Gene_id	Genome position	Position	Exon	Length (aa)	Domain	pI	Predicted MW (KD)	NCBI_id
LvRab16	LVAN01202	LVANscaffold_474	391941-401150(+)	6	217	RAB	5.79	24.7	XP_027237208.1
LvRab17	LVAN23564	LVANscaffold_3614	106113-109647(+)	NA	219	RAB	6.12	25.1	XP_027233441.1
LvRab18	LVAN06823	LVANscaffold_1261	385184-391030(+)	6	206	RAB	5.27	22.8	XP_027232796.1
LvRab19	LVAN19404	LVANscaffold_2894	247281-268433(-)	6	204	RAB	8.28	23.0	XP_027226368.1
LvRab20	LVAN13572	LVANscaffold_2136	305166-308307(-)	4	206	RAB	7.8	22.5	XP_027218190.1
LvRab21	LVAN17219	LVANscaffold_2622	284408-287130(+)	4	363	RAB	9.04	40.7	ROT68938.1
LvRab22	LVAN21341	LVANscaffold_3181	573342-580823(+)	5	189	RAB	6.62	20.8	ROT64825.1
LvRab23	LVAN19840	LVANscaffold_2961	234638-240111(+)	6	212	RAB	5.81	23.3	XP_027226949.1
LvRab24	LVAN08561	LVANscaffold_1512	344592-359247(-)	5	234	RAB	5.39	27.2	XP_027210828.1
LvRab25	LVAN23562	LVANscaffold_3604	336239-338604(-)	4	262	RAB	8.83	29.4	ROT62609.1
LvRab26	LVAN02399	LVANscaffold_643	203945-213216(-)	5	197	RAB	6.18	22.6	ROT83704.1
LvRab27	LVAN20526	LVANscaffold_3065	313188-320729(-)	5	179	RAB	5.24	19.9	ROT65648.1
LvRab28	LVAN23177	LVANscaffold_3512	57445-60789(-)	5	183	RAB	9.32	20.1	ROT62985.1
LvRab29	LVAN23755	LVANscaffold_3698	81369-130338(-)	8	211	RAB	5.61	23.7	XP_027233704.1
LvRab30	LVAN17622	LVANscaffold_2661	481957-494370(+)	7	381	RAB	5.67	42.3	ROT68514.1
LvRab31	LVAN25515	LVANscaffold_4650	206670-210018(+)	3	167	RAB	5.56	18.3	ROT60664.1
LvRab32	LVAN24208	LVANscaffold_3904	466888-469532(+)	5	233	RAB	7.13	26.6	XP_027234523.1
LvRab33	LVAN06435	LVANscaffold_1204	472272-477666(+)	6	315	RAB	9.91	36.0	ROT79683.1
LvRab34	LVAN16310	LVANscaffold_2515	307052-319306(+)	NA	619	PfamRAS	4.58	66.8	ROT69842.1
LvRab35	LVAN19904	LVANscaffold_2967	1290009-1299116(-)	6	208	SmallGTPase	5.6	24.1	XP_027227050.1
LvRho1	LVAN13930	LVANscaffold_2195	90058-90238(+)	1	60	SmallGTPase	4.41	6.8	XP_022196088.1
LvRho2	LVAN14373	LVANscaffold_2254	134051-138120(-)	4	183	Rho	8.64	20.2	XP_027219355.1
LvRho3	LVAN12953	LVANscaffold_2058	134518-135800(-)	3	192	Rho	6.00	21.6	XP_027217324.1
LvRho4	LVAN20900	LVANscaffold_3111	404322-409551(+)	NA	335	Rho	8.4	37.9	ROT65265.1
LvRho5	LVAN09834	LVANscaffold_1676	687493-691897(+)	4	191	Rho	6.16	21.4	XP_027212701.1
LvRho6	LVAN04453	LVANscaffold_939	26591-30653(-)	3	135	Rho	8.93	15.0	ROT81660.1
LvRho7	LVAN19782	LVANscaffold_2947	161137-195305(-)	NA	875	SmallGTPase, EFh, Pfam_EF_assoc_2, RPT1	6.07	99.0	ROT66379.1
LvRho8	LVAN22095	LVANscaffold_3334	511668-520107(-)	14	763	SmallGTPase, BTB	8.7	87.5	XP_027230767.1
LvRho9	LVAN22094	LVANscaffold_3334	491137-499763(-)	15	733	SmallGTPase, BTB	8.63	84.1	ROT64065.1
LvRho10	LVAN21019	LVANscaffold_3135	598754-667877(-)	NA	1312	SmallGTPase, FF	5.42	148.1	ROT65153.1
LvArf1	LVAN23222	LVANscaffold_3522	439556-440887(+)	NA	141	SmallGTPase	10.27	15.6	ROT62941.1
LvArf2	LVAN07698	LVANscaffold_1383	60984-74708(-)	NA	476	SmallGTPase	7.62	52.2	XP_027209663.1
LvArf3	LVAN20888	LVANscaffold_3109	392595-396458(+)	3	175	SmallGTPase	8.96	20.1	XP_027228467.1
LvArf4	LVAN22103	LVANscaffold_3334	726975-728151(-)	5	249	SmallGTPase	7.86	28.0	ROT64074.1
LvArf5	LVAN13735	LVANscaffold_2161	981884-984055(+)	4	390	SmallGTPase	9.65	43.1	ROT72413.1
LvArf6	LVAN02781	LVANscaffold_700	574778-595351(-)	4	185	SmallGTPase	7.63	20.9	ROT83323.1
LvArf7	LVAN06381	LVANscaffold_1197	212280-220139(-)	5	202	ARF	5.15	22.7	XP_027226427.1
LvArf8	LVAN25495	LVANscaffold_4639	182954-184883(-)	3	191	ARF	7.89	21.7	XP_027236976.1

(Continued)

TABLE 1 Continued

Proposed name	Gene_id	Genome position	Position	Exon	Length (aa)	Domain	pI	Predicted MW (KD)	NCBI_id
LvArf9	LVAN07222	LVANscaffold_1316	619788-624265(+)	4	182	ARF	9.48	20.5	XP_027238771.1
LvArf10	LVAN21095	LVANscaffold_3150	128335-137020(+)	4	186	ARF	7.64	21.2	XP_027228934.1
LvArf11	LVAN02780	LVANscaffold_700	507639-508457(-)	2	257	ARF	9.68	28.2	XP_027239458.1
LvArf12	LVAN14933	LVANscaffold_2332	332905-338395(-)	5	249	ARF	9.84	28.2	XP_027220166.1
LvArf13	LVAN04965	LVANscaffold_1012	28249-30524(-)	NA	295	ARF	9.68	32.8	ROT81146.1
LvArf14	LVAN22101	LVANscaffold_3334	713171-715382(+)	NA	359	ARF	9.71	39.7	ROT64072.1
LvArf15	LVAN22102	LVANscaffold_3334	721812-724398(-)	NA	349	ARF	9.9	37.3	ROT64073.1
LvArf16	LVAN05779	LVANscaffold_1129	397116-421186(+)	4	180	ARF	6.61	20.5	ROT80345.1
LvArf17	LVAN01006	LVANscaffold_447	452224-459676(+)	5	188	ARF	5.56	21.5	XP_027236265.1
LvArf18	LVAN00936	LVANscaffold_413	460488-464478(+)	4	159	ARF	6.08	18.0	ROT85182.1
LvArf19	LVAN10426	LVANscaffold_1745	96079-98495(+)	5	207	SAR	7.8	23.5	ROT63200.1
LvArf20	LVAN22995	LVANscaffold_3478	971535-973975(+)	5	207	SAR	7.8	23.5	ROT63200.1
LvRan	LVAN25465	LVANscaffold_4616	23557-26360(-)	4	261	SmallGTPase	8.76	29.7	ROT60705.1
LvRbj	LVAN22098	LVANscaffold_3334	618311-624575(+)	6	273	SmallGTPase, DNAJ	8.63	30.3	XP_027230770.1
LvRGK1	LVAN00207	LVANscaffold_157	9522-19442(+)	NA	269	SmallGTPase	9.62	29.7	ROT85896.1
LvRGK2	LVAN15533	LVANscaffold_2408	915535-917586(-)	2	170	SmallGTPase	9.82	19.3	ROT70608.1
LvRGK3	LVAN13377	LVANscaffold_2115	313476-324918(+)	8	310	SmallGTPase	6.16	34.1	ROT72725.1
LvGPN1	LVAN02036	LVANscaffold_597	208499-213319(+)	6	283	GPN-loop GTPase 3-like	4.33	32.3	XP_027238371.1
LvGPN2	LVAN10675	LVANscaffold_1774	818255-823119(+)	6	316	GPN-loop GTPase 2-like	4.72	35.3	XP_027213967.1
LvGPN3	LVAN14725	LVANscaffold_2302	140684-144789(-)	NA	402	GPN-loop GTPase 1-like	4.68	46.0	XP_027219880.1
LvREM	LVAN10561	LVANscaffold_1758	117850-120070(-)	5	228	SmallGTPase	7.65	25.6	XP_027213765.1
LvIFT1	LVAN15495	LVANscaffold_2403	622328-630954(+)	6	189	SmallGTPase	7.72	21.5	XP_027220942.1
LvIFT2	LVAN16277	LVANscaffold_2510	134322-139941(+)	4	186	PfamROC	5.37	20.7	XP_027222074.1
LvRGBP1	LVAN23245	LVANscaffold_3526	817681-821300(+)	8	300	PfamROC	6.52	35.0	XP_027232892.1
LvRGBP2	LVAN10418	LVANscaffold_1743	12487-28108(+)	8	403	PfamSRPRB	4.63	45.9	ROT75709.1
LvOBG1	LVAN13973	LVANscaffold_2198	107630-120662(-)	7	346	PfamGTP1_OBG, PfamMMR_HSR1	8.38	38.6	ROT72156.1
LvOBG2	LVAN09529	LVANscaffold_1637	267545-277265(-)	9	437	PfamGTP1_OBG, PfamMMR_HSR1	8.12	48.0	ROT76592.1
Lvseptin	LVAN02721	LVANscaffold_693	43543-54935(+)	NA	308	Pfam_septin	8.18	34.1	ROT83391.1
LvEFCAB4B	LVAN23704	LVANscaffold_3675	563504-574292(-)	NA	466	SmallGTPase	5.79	49.6	ROT62468.1
LvSRP	LVAN12281	LVANscaffold_1980	125372-130524(+)	5	238	SmallGTPase	8.54	26.5	ROT73853.1
LvGPo	LVAN23290	LVANscaffold_3533	210346-225082(-)	5	249	SmallGTPase	4.87	28.8	XP_027232961.1

related domains, and all the residues are perfectly conserved with Ras GTPases, mainly G1 and G3 motif (Figure 2A, corresponding to motif symbol 1 and 2).

From the perspective of gene location, these Ras superfamily genes were distributed in 98 scaffolds, Ras, Rho, Rab and Arf families each had 1-2 two-gene clusters, and Arfs also had a three-gene cluster (*LvArf4*, *LvArf14*, and *LvArf15*) (Table 1).

In order to distinguish these Ras superfamily members, we compared identity as assayed by smartblast and blastp, and found that most of them were classical Ras GTPases, such as Ras, Rab, Arf, Rho, Ran, named LvRas1-24, LvRab1-35, LvArf1-20, LvRho1-10, LvRan. A small number (18) are unconventional or newly discovered members of the Ras superfamily, named LvRbj, LvRGK1-3, LvGPN1-3, LvREM,

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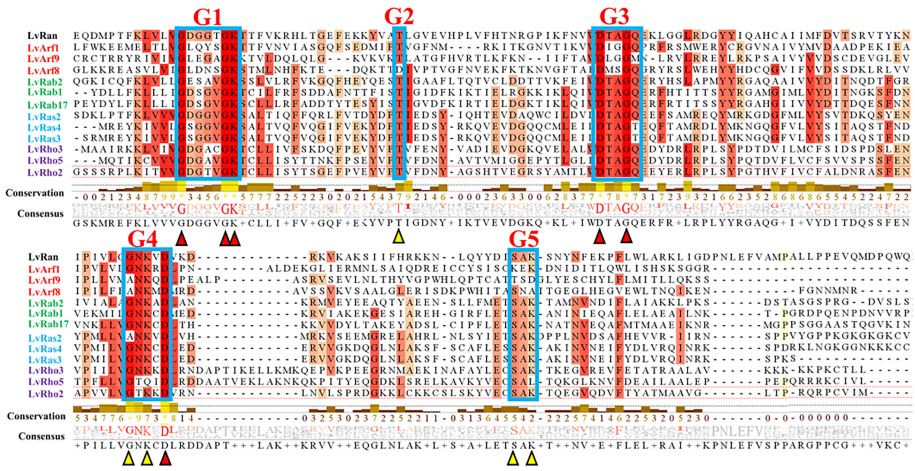

FIGURE 1
Multiple sequence alignment of some classical Ras superfamily protein sequences of *L. vannamei*. Red indicates complete agreement and orange indicates 75% ~ 99% homology. Conserved residues: G1 (GXXXXGKS/T), G2 (T), G3 (DXXGQ/H/T), G4 (T/NKXD) and G5 (C/SAK/L/T) (X stands for any amino acid). Red and yellow triangles represent major sites of conserved motifs.

LvIFT1-2, LvRGBP1-2, LvOBG1-2, Lvseptin, LvEFCAB4B, LvSRP, LvGPo (Table 1).

3.2 Phylogenetic analysis of the Ras superfamily numbers

In order to clarify the phylogenetic relationship among Ras superfamily members of *L. vannamei*, we constructed phylogenetic trees using Ras superfamily genes from *L. vannamei* and those identified from other species (Table 1 and Supplementary Table S3). Phylogenetic analysis showed that the Ras GTPase proteins were clustered in the clades of each subfamily, although they come from different species (Figure 2A). The result suggested that the Ras superfamily members of *L. vannamei* could be divided into two large monophyletic groups. The Ras family and Rho family could be clustered into a clade, the number of Rab family is the largest (35 members) and form a clade with Ran family, then clustered with Ras

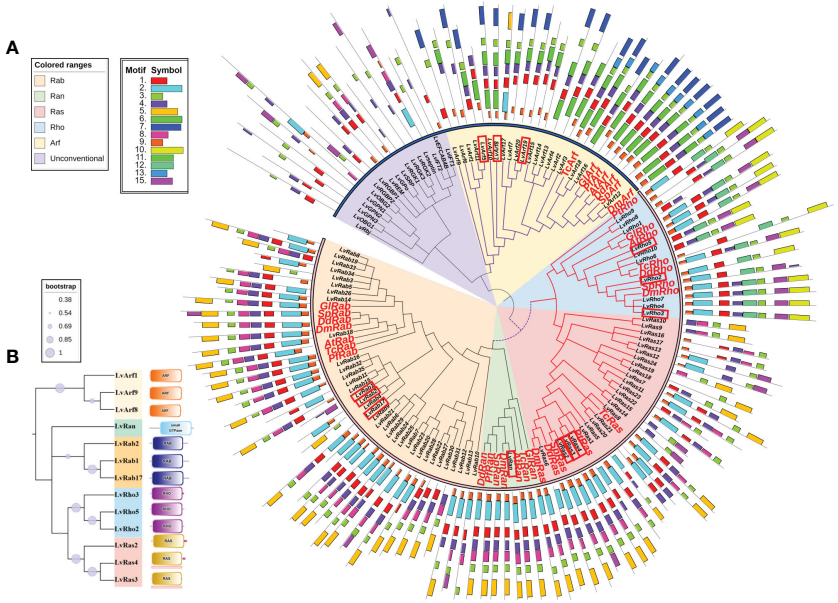

FIGURE 2
(A) Phylogenetic tree of Ras superfamily genes of *L. vannamei* and other species. The accession numbers of amino acid sequences used in phylogenetic trees were shown in Table 1 and Supplementary Table S3. The red box marks the most typical GTPases with high expression. The orange shadow represents Rab family; The green shadow represents Ran family; The red shadow represents Ras family; The blue shadow represents Rho family; The yellow shadow represents Arf family. The purple shadow represents Unconventional Ras superfamily. The phylogenetic tree periphery represents motifs of different families of *L. vannamei* and other species, the colored boxes indicate the conserved motifs. **(B)** Domains of classical Ras GTPases of different families of *L. vannamei*. The tree on the left illustrates the phylogenetic relationships of various families of Ras GTPases. On the right are the different domains of each family.

family and Rho family into a larger clade. Arf family members were clustered together and far away from other four families, other unconventional Ras superfamily numbers were clustered together, and then clustered with the Arf family clade formed another large clade (Figure 2A).

3.3 Conserved motifs and gene structure of the Ras superfamily numbers

According to the analyses of gene structure and conserved motifs, most Ras superfamily genes share a set of conserved G box GDP/GTP-binding motif elements: GXXXXGKS/T, T, DXXGQ/TE, NXXD and SXK (X stands for any amino acid), a few genes contain only G1 box, G3 box, and G4 box (Figures 1, 2B). However, different Ras GTPases have many specific motifs, resulting in different domains (Figure 2), and the differences of their amino acid sequences mainly occur in the amino terminal and carboxyl terminal, which are considered as major protein modification sites (Figure 1).

3.4 Gene expression patterns of the Ras superfamily of *L. vannamei*

In order to clarify the expression patterns of Ras superfamily genes of *L. vannamei*, we summarized four different transcriptional profiles of 108 Ras GTPase genes into heatmaps of different tissues, development stages, molting stages and WSSV infection status (Figures 3–6; Supplementary Figures S1-6).

In different tissues of adult shrimp, most members of Ras superfamily showed low expression, a few genes were highly expressed in their respective families, such as *LvRab18*, *LvRab20*, *LvRho3*, *LvRho5*, *LvRas4*, *LvRas10*, *LvArf1*, *LvArf10*, and *LvRan*. (Figure 3). *LvRan* had the highest expression level in all the examined tissues, and its expression level is more than 3-5 times that of other members (Figure 3; Supplementary Figures S1, S2A). Other highly expressed Ras superfamily genes usually presented in some specific tissues: *LvRas4* was highly expressed in hemocyte and intestines; *LvRab18* was highly expressed in muscle; *LvArf1* was highly expressed in brain, thoracic ganglion, ventral nerve and intestines; and *LvRho5* was highly expressed in antenna, hepatopancreas and intestines. Some unconventional Ras superfamily members were highly expressed in testis and ovary tissues, such as *LvGPN1, LvGPN3, LvIFT2 and LvOBG1*.

At early development, most members of Ras superfamily were expressed at a higher level. *LvRas1, LvRas2*, *LvRas5* and *LvRas10* are the main expressed Ras family genes, in addition to *LvRas10* at gastrula (gast) and limb bud embryo (Lbe) stages, the expression of Ras genes was not very obvious in early development as a whole. The Rab family showed two expression patterns, some genes were highly expressed mainly during the zygote to gast stages, while others were highly expressed after limb bud embryo stages. Arf12, Rho1 and Rho5 were the most expressed genes in their respective families, and their expression trends remained relatively stable during different stages (Figure 4; Supplementary Figure S3). Ran was the gene with the highest expression of all Ras superfamily numbers in the early developmental stages of *L. vannamei*, but with little fluctuations (Supplementary Figure S4A). Interestingly, most of the unconventional Ras superfamily genes had high expression levels at early development stages. *LvSRP* and *LvGPo* were highly expressed at the whole stages, showing that play an important role in early development. *LvREM*, *LvGPN2*, *LvIFT2*, and *LvRGBP1* had similar expression patterns, they were all highly expressed after gastrula stage (gast). In contrast, LvRbj and LvGPN1 were highly expressed before gastrula stage (gast) (Supplementary Figures S4B-H).

In different molting stages of *L. vannamei*, most members of Ras superfamily showed lower expression (Figure 5; Supplementary Figure S5), and only a few genes were highly expressed in respective different families, such as *LvRab18*, *LvRho1*, *LvArf12*, *LvRan*, *LvRas5*. Similarly, a few unconventional members were highly expressed at different molting stages, such as *LvRGBP1*, *LvSRP* and *LvGPo* (Supplementary Figures S6B-H).

The hemocyte, hepatopancreas, lymphoid (Oka) organs are the three main immune related tissues. The expression level of many Ras superfamily genes was up-regulated in different organs after WSSV infection. In lymphoid organs, 19 Rab genes, 8 Rho genes, 11 Ras

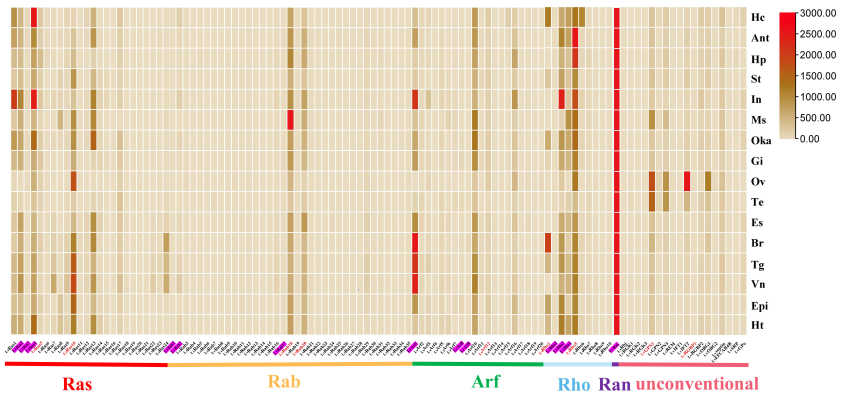

FIGURE 3
Ras superfamily gene expression profiles in different tissues of *L. vannamei*. Adult tissues: Hc, hemocyte; Ant, antenna; Ms, muscle; In, intestines; Ov, ovary; St, stomach; Oka, lymphoid organ; Gi, gill; Hp, hepatopancreas; Te, testis; Es, eye stalk; Br, brain; Tg, thoracic ganglion; Vn, ventral nerve; Epi, epidermis; and Ht, heart. Pink shading represents members of the classical Ras superfamily in each classification, red fonts represent members of the Ras superfamily with validated expression levels in each classification.

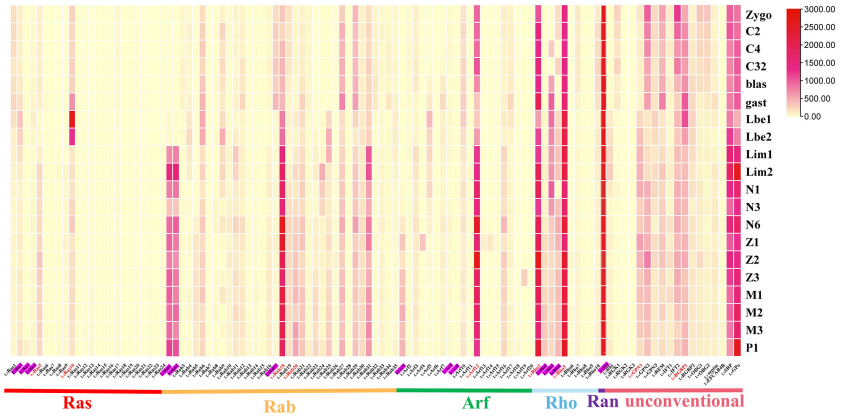

FIGURE 4
Ras superfamily gene expression profiles at different early developmental stages of *L. vannamei*. Early development stages: zygote (zygo), 2 cells (C2), 4 cells (C4), 32 cells (C32), blastula (blast), gastrula (gast), limb bud embryo I (Lbe1), limb bud embryo II (Lbe2), larva in membrane I (Lim1), larva in membrane II (Lim2), nauplius I (N1), nauplius III (N3), nauplius VI (N6), zoea I (Z1), zoea II (Z2), zoea III (Z3), mysis I (M1), mysis II (M2), mysis III (M3), and post larvae 1 (P1). Pink shading represents members of the classical Ras superfamily in each classification, red fonts represent members of the Ras superfamily with validated expression levels in each classification.

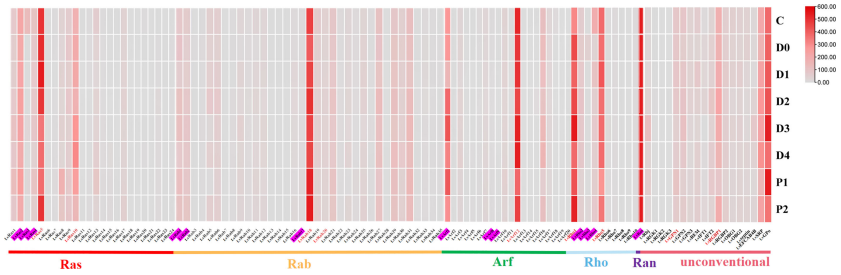

FIGURE 5
Ras superfamily gene expression profiles at different molting stages of *L. vannamei*. Molting stages: intermolting phase (C), premolting phase (D0, D1, D2, D3, and D4), and postmolting phase (P1 and P2). Pink shading represents members of the classical Ras superfamily in each classification, red fonts represent members of the Ras superfamily with validated expression levels in each classification.

genes, 10 Arf genes, *LvRGBP2*, *LvSRP*, *LvEFCAB4B* and *LvGPo* were significantly up-regulated. In hepatopancreas, 11 Rab genes, 5 Rho genes, 4 Ras genes, 10 Arf genes, *LvIFT1* and *LvGPN2* genes were significantly up-regulated. In hematocyte, 6 Rab genes, 1 Rho gene, 7 Ras genes and 8 Arf genes were up-regulated, most unconventional Ras superfamily genes were also significantly up-regulated (except for *LvRGKs*, *LvSRP* and *LvRGBP2*) (Figure 6; Supplementary Table S2).

The types of up-regulated Ras superfamily genes were different in different tissues, and it is possible that these genes play different roles in the immune process. For example, LvRan was up-regulated in hemocyte and hepatopancreas, but down-regulated in lymphoid organs after WSSV infection. In lymphoid organs, most of the Ras superfamily genes were up-regulated and a few genes were down-regulated, while was opposite in hepatopancreas. Among them, some

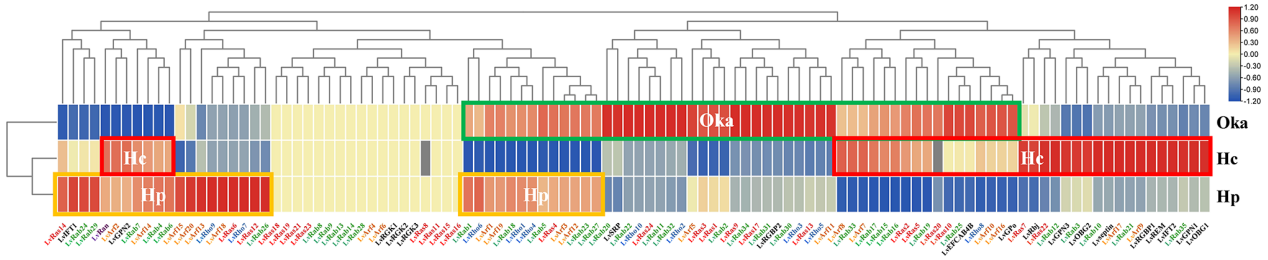

FIGURE 6
Changes of Ras superfamily gene expressions after WSSV infection of *L. vannamei*. The orange line (Hp) shows high expression in hepatopancreas tissue, the red line (Hc) shows high expression in hemocyte, the green line (Oka) shows high expression in lymphoid organ. Sterile phosphate-buffered saline (PBS) as control group, the infection lasts for 6 hours.

Ras and Arf genes and *LvRGK* showed no changes in their expression levels in the three organs after WSSV infection, indicating that these genes are not affected by immune response.

3.5 Expression verification of some Ras superfamily genes

In order to verify the accuracy of the expression level of different tissues, the expression of twelve Ras superfamily genes were verified by qRT-PCR in twelve different adult tissues (Figure 7). *LvRas5* and *LvRas10* were highly expressed in hepatopancreas and ventral nerve respectively, and *LvRas6* was highly expressed in ventral nerve, lymphoid organ and brain. *LvRab18*, *LvRab20*, *LvRho1* and *LvRho5* were relatively expressed in most tissues except eyestalk, epidermis and muscle, among the expression tissues, these genes were mainly highly expressed in ventral nerve, brain, lymphoid organ and hepatopancreas, which is similar to the expressed pattern of verified Ras genes above. *LvArf1* and *LvArf12* were expressed in most tissues, and the three tissues with the highest expression were brain, ventral nerve and gill. *LvRan* was highly expressed in ventral nerve, and is about 2-4 times higher than other tissues. In the unconventional Ras superfamily members, *LvGPN1* and *LvRGBP1* had similar expression patterns with the classical Ras superfamily members, which were mainly highly expressed in hepatopancreas, brain, heart and other tissues. In sum, the most expression levels of these genes were consistent with respective RNA-Seq results, and some differences might be caused by individual differences. We found that almost all verified members of Ras superfamily were expressed in almost all detected tissues, and showed the lowest expression level in eyestalk, epidermis and muscle tissues. It is possible that Ras GTPases play a weak role in these tissues.

4 Discussion

The Ras superfamily is a large gene family, which generally contains fewer members in invertebrates and more in vertebrates, such as 68 in *D. melanogaster* and 46 in *C. elegans*, correspondingly, 170 in human and 137 in *Xenopus tropicalis* (Rojas et al., 2012). In metazoans, although the total number of Ras superfamily varies in different species, the Ras superfamily has traditionally been divided into five major branches: Ras, Rho, Rab, Ran, and Arf/Sar. Among them, the Rab family has the most members, and the Ran family has

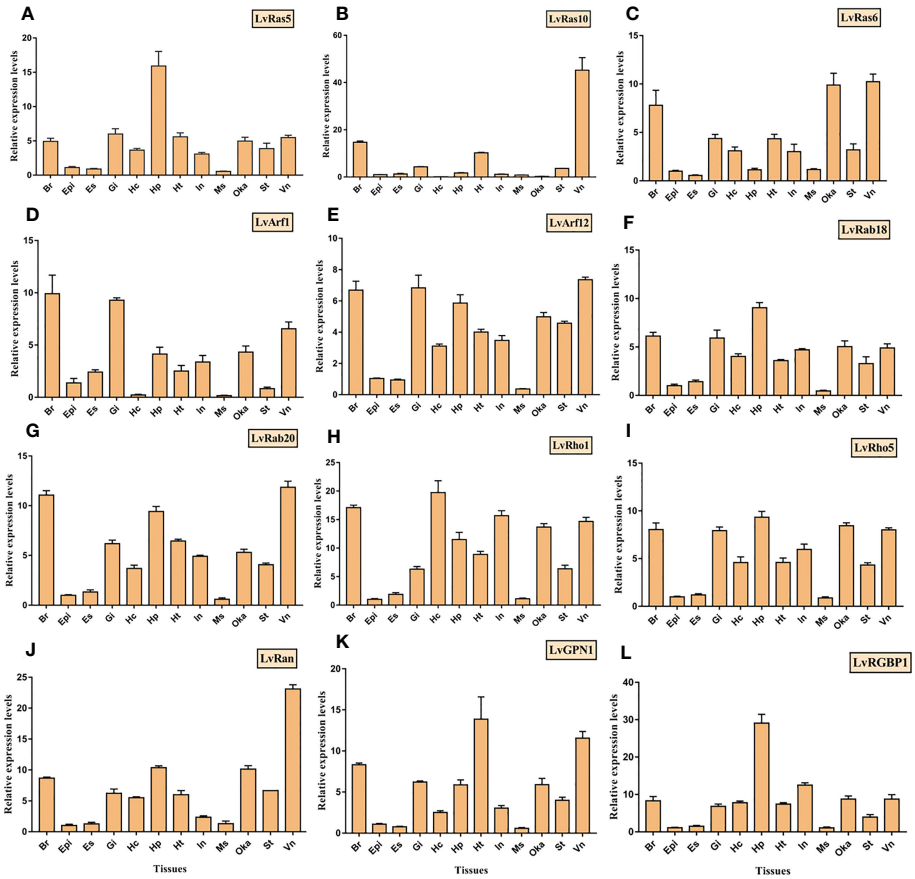

FIGURE 7
Tissue distributions of 12 Ras superfamily genes were detected by RT-qPCR, and **(A–L)** was LvRas5, LvRas10, LvRas6, LvArf1, LvArf12, LvRab18, LvRab20, LvRho1, LvRho5, LvRan, LvGPN1, LvRGBP1, respectively. Adult tissues: Hc, hemocyte; Ant, antenna; Ms, muscle; In, intestines; Ov, ovary; St, stomach; Oka, lymphoid organ; Gi, gill; Hp, hepatopancreas; Te, testis; Es, eye stalk; Br, brain; Tg, thoracic ganglion; Vn, ventral nerve; Epi, epidermis; and Ht, heart." . Please note that "LvRas5, LvRas10, LvRas6, LvArf1, LvArf12, LvRab18, LvRab20, LvRho1, LvRho5, LvRan, LvGPN1, LvRGBP1.

the least members (usually one). In this study, shrimp obviously has an expanded Ras superfamily (108 members), in which, Contains five classic Ras families and with the most Rabs and the least Rans, implying a high degree of conservation of the structure and pattern of this superfamily.

From the perspective of gene structure, most these Ras GTPase have a close genetic relationship and very similar binding GTP related motifs, indicating that these genes have the same ancestor, and the gene structure differentiation originated from different functional requirements in the process of evolution. Phylogenetic analysis showed that among these different families, Ras and Rho families are closely related, clustered on the same branch and have high homology; Rab family cluster with Ran, then combine with Ras and Rho into a larger branch. However, Arf family is a relatively separate branch, and the unconventional Ras superfamily numbers are clustered into larger branch with the Arf family. The result indicated that the members of Ras superfamily might come from two different origins, which is consistent with previous studies: Ras, Rho, Rab and Ran were probably derived from Cyanobacteria or proteobacteria, or the common ancestor of both; and Arf was probably derived from Methanogenus (Dong et al., 2007).

Comprehensive snapshots of the patterns of gene expression can provide a path toward a global and dynamic understanding of gene functions and their roles in particular biological processes or events. In this study, the expression analysis of Ras superfamily genes showed that the expression of different Ras GTPases was different under different conditions (different tissues, development/molting stages and WSSV infection). In general, from the expression pattern, it can be concluded that the functions of the Ras superfamily of shrimp are similar to other animals, which are very diverse and complex (Table 2). Only a few members of each family are highly expressed, suggesting that these genes are critical in the corresponding biological processes, while other members may correspond to other specific conditions, allowing for more fine-grained regulation. During early development, unconventional Ras family members are generally expressed more actively than the classical Ras superfamily members, which suggested that the unconventional members play a vital role in early developmental stages. In addition, after WSSV infection, almost all of Ras superfamily genes are up-regulated in different tissue, which at least suggests that these genes play an important role in development and pathogenesis.

4.1 The Ras family

Ras family is the most typical and common class of Ras superfamily. In *L. vannamei*, multiple Ras family members such as *Ras*, *Rap*, *Ral*, *Rheb*, *RHEs* and *RIT* were found. Gene expression analysis showed that these Ras genes were expressed in different tissues, indicating their respective functions. Most members of the Ras family in mammals are called oncogenes and well-studied. Ras proteins receive signals from cell surface receptors, these signals are transmitted among proteins through different pathways, and finally affect a variety of biological functions, such as development, proliferation, differentiation, and survival (Goitre et al., 2014). If the *Ras* gene was mutated, these signal pathways will be destroyed, which will lead to a variety of tumors and cancers (Bos, 1989; Goodsell, 1999; Murugan et al., 2019). In addition, some Ras family genes are considered to be involved in animal growth and reproduction. For example, the *Ras* gene is related to the size of catfish head (Geng et al., 2016), it is involved in the regulation of insulin pathway during oocyte vitellogenesis in female oysters (Jouaux et al., 2012). During vitellogenesis of the marine flounder *Solea senegalensis*, the expression of *Ras* homologous gene was up-regulated (Tingaud-Sequeira et al., 2009). In *L. vannamei*, Ras family genes also were highly expressed in ovary, such as *LvRas4* and *LvRas10*. In previous study, we conducted growth candidate gene association analysis in two independent populations of *L. vannamei*, the results showed that the SNP of Ras related protein gene *Rap-2a* (*LvRas1*) was significantly correlated with growth traits (Yu et al., 2019). The above research proves that Ras family may play an important role in growth and reproduction of shrimp.

TABLE 2 The possible function of Ras superfamily in *L. vannamei*.

Groups	Gene number	Possible functions
Ras family	24	growth and reproduction, nervous system development and signal transduction
Rab family	35	vesicle morphology, vesicle transport and immune regulation
Rho family	10	development, cytoskeleton dynamics and immunity
Ran family	1	the gonad development and antiviral immunity
Arf family	20	nervous system development
RJL family	1	immune and neural regulation
RGK family	3	regulates calcium and insulin signaling pathways
GPN family	3	immune regulation
IFT family	2	cilia assembly and maintenance
OBG family	2	unknown
RGBP family	2	unknown
Others	5	unknown

There are also other classic Ras family genes in *L. vannamei*, such as *M-Ras* and Ras related protein *R-Ras2*. *M-Ras* alone constitutes a small Ras subfamily in mammals, in *L. vannamei*, *M-Ras* gene was named as *LvRas2*, and showed high relative expression under most conditions, indicating that *M-Ras* plays an important role in shrimp. Classical Ras binds to Raf and activates ERK pathway (Endo, 2020), while *M-Ras* mediated cell transformation related to the weak activation of RAF/MEK/ERK pathway, other downstream effectors are also involved in the pathway, which can induce neuronal differentiation (Castro et al., 2012). In mice, both classical *Ras* and *M-Ras* are highly expressed in the central nervous system (Sun et al., 2006). In addition, neuronal differentiation of p12 cells in rats also requires the induction of classical *Ras* and *M-Ras* (Sassone-Corsi et al., 1989). *R-Ras2* has also been shown to be essential for correct axonal myelination and accurate neurotransmission in mouse (Gutierrez-Erlandsson et al., 2013; Sanz-Rodriguez et al., 2018). In *L. vannamei*, both *M-Ras* (*LvRas2*) and *R-Ras2* (*LvRas6*) gene were highly expressed in the thoracic ganglion, which further showed that Ras family genes may play an important role in nervous system development and signal transduction.

At present, there are few studies on Ras family of *L. vannamei*, but as these typical Ras superfamily proteins may play an important role in metabolism, growth, reproduction and neural development, the related mechanisms need to be further studied.

4.2 The Rab family

Rab is a kind of regulatory small molecule GTPase protein, found on eukaryotic cell and organelle membrane (Seabra et al., 2002), and it also is a group with the largest number of genes of the Ras superfamily in *L. vannamei*. For example, there are 57 Rab genes in *Arabidopsis*, 30 in *D. melanogaster*, and 61 in *Mus musculus* (Rojas et al., 2012). The main members of Rab family include *Rab1*, *Rab3A* and *Rab5c*.

Rab, called *Ypt* in yeast, plays an important role in vesicle transport (Takai et al., 2001). *Rab8* and *Rab5* in human have the same function as *YPT* in yeast, and they share high sequence similarity (Molendijk et al., 2004). It has also been reported that Rab protein plays a role in vesicle transport in plants. For example, *Rab1*, *Rab2*, *Rab4*, *Rab5* and *Rab6* in *Arabidopsis* have been proven to play an important role in regulating the morphology of endoplasmic reticulum, Golgi apparatus and plasma membrane vesicles (Stenmark and Olkkonen, 2001). In the squid *Loligo pealei*, it was verified that *Myo5a* and *Rab3A* directly bind and interact with synaptic vesicles (SVs) and participate in the transport of neuronal vesicles (Wöllert et al., 2011). In *L. vannamei*, studies showed that *Rab* might be related to the resistance mechanism of shrimp induced by environmental stress (Wang et al., 2015). In addition, *Rab* gene also plays an important role in shrimp immunity, *Rab* like protein had been proved to interact with immune related membrane proteins to regulate the phagocytosis of shrimp hemolymph cells against WSSV, and *Rab27* mutation leads to immune deficiency (Wu et al., 2008; Han et al., 2011; Chen et al., 2021). Similarly, *Rab* gene *(Rab6a)* in *M. japonicus* was up-regulated during WSSV infection (Wu and Zhang, 2007). In this study of WSSV-infected *L. vannamei*, several Rab family genes were significantly up-regulated in immune organs such as Oka organ, hepatopancreas and hemocyte. Therefore, Rab family may affect vesicle morphology, vesicle transport and immune regulation in shrimp.

4.3 The Rho family

In 1985, Madaule found a Ras superfamily member Rho (RAS homolog) in the marine gastropod molluscs *Aplysia* (Kawasaki et al., 2004). The study confirmed that Ras has highly sequence homology with Rho, with 35% consistency in amino acid sequence. Moreover, they have the same C-terminal required for membrane attachment. In *L. vannamei*, Rho family were found in the same clade of the phylogenetic tree with Ras family. These results suggest that Rho family is closely related to Ras family. In vertebrates, the Rho family has undergone considerable expansion and differentiated into more than 10 subfamilies, including *Rho subtypes (A, B, C, D, G, E)*, *Rac subtype*s (1, 2, 3), *Cdc42*, *Rnd* (1, 2, 3), *TCL*, *Rho H/TTF*, *Chp*, *Wrch-1*, *Rif*, *Rho BTB1*, *Rho BTB2*, *Miro-1 and Miro-2* (Burridge and Wennerberg, 2004; Wennerberg and Der, 2004). Five Rho subfamilies, including *Rhol*, *Cdc42*, *Rac2*, *MIG-2*, *Rho BTB1*, were identified in *L. vannamei.*

As a representative and well-studied member of Rho family, *Cdc42* plays a significant role in a variety of cellular processes that are dependent on the actin cytoskeleton, such as cytokinesis, cell migration, phagocytosis, morphogenesis, axon myelination, intracellular trafficking, and tumor occurrence (Etienne-Manneville and Hall, 2002; Sahai and Marshall, 2002; Moon and Zheng, 2003). *Rac* is another member of the Rho family with more research. In mammals, *Rac* is mainly involved in promoting the malignant proliferation and migration of tumor cells (Chan et al., 2007). Compared with normal striated muscle tissue, the expression of *Rac1* and *Cdc42* was significantly high ($P < 0.05$) in rhabdomyosarcoma (RMS) tissue (Li et al., 2021). In addition, Rac also plays a role in immunity, the expression of *Rac2* gene in the large yellow croaker *Pseudosciaena crocea* was significantly up-regulated after challenge by *Vibrio parahaemolyticus* (Liu et al., 2017). Injection of *Vibrio alginolyticus* into *L. vannamei* induced up-regulated expression of *LvRac1* (*LvRho1*) in hepatopancreas, then after knocking down *LvRac1* and stimulating *V. alginolyticus*, the mortality of *L. vannamei* was significantly increased relative to that of the control group (Cha et al., 2015). Rho1 is another important Rho GTPase protein, and previous research has shown a role for this in immunity, cytoskeleton dynamics and embryonic development. For example, *Rho1* affected the development of eggs by regulating the hormone level of the braconid wasp *Microplitis mediator* (Magie and Parkhurst, 2005). *Rho1* also regulated the rearrangement of cytoskeleton, and then affected the cellular immunity of the cotton bollworm *Helicoverpa armigera* (Li et al., 2010). In the purple sea urchin *Strongylocentrotus purpuratus*, Rho could affect *SpROCK* expression by the Rho dependent signal pathway, which is essential for early embryonic development (Aguirre-Armenta et al., 2011). In this study, we suggest Rho family may play an important role in development, cytoskeleton dynamics and immunity in *L. vannamei.*

4.4 The Ran family

Although the Ran is small family in number, it plays an important role in many species. In *H. armigera*, it was reported that Ran participated in the 20-hydroxyecdysone (20E) signal transduction pathway by regulating the location of ecdysone receptor-B1 (EcR-B1) (He et al., 2010). In the brown planthopper *Nilaparvata lugens*, *NlRan* knockdown significantly delayed development and affected reproduction (Liao et al., 2019). Only one Ran gene exists in *L. vannamei*, and its expression is higher than that of other Ras superfamily genes. It was previously reported that the highest expression of *Ran* is the black tiger shrimp, *Penaeus monodon is* in the ovary (Zhou et al., 2012). In the expression profile of testis maturation stages of scallop, *Ran* expression increased dramatically during meiosis and spermatogenesis (Hino et al., 2012). In addition, there is a similar situation in mammals. *Ran* had a high level of expression from the late pachytene spermatocytes to early round spermatocytes in mice (López-Casas et al., 2003). Moreover, the cellular localization of *Ran* also changed during spermatogenesis (Kierszenbaum et al., 2002). On the other hand, *Ran* may also involve in the immune process. In the Kuruma shrimp *M. japonicus*, *Ran* played a vital role in antiviral immunity (Han and Zhang, 2007). Another study found that *Ran* interacts with myosin in *M. japonicus*, which can regulate blood cell phagocytosis. RNAi knockdown led to a significant increase in virus copy number in *M. japonicus*, and overexpression of *Ran* resulted in a significant decrease in virus copy number (Liu et al., 2009). When IL-4 and lysophosphatidylcholine were respectively injected into shrimp, the results indicated that the two molecules could enhance the Ran GTPase activity and improve hemocytic phagocytosis against WSSV (Zhao et al., 2011). In our study, *LvRan* was significantly up-regulated in hemocytes and hepatopancreas, but down-regulated in Oka after WSSV infection. The above research suggests that Ran may participate in the 20E signaling pathway, regulate the gonad development and antiviral immunity, and then affect the growth and reproduction in shrimp.

4.5 The Arf family

The Arf family is mainly divided into three subfamilies: Arf, Arf like proteins (ARLs) and Sar1. Relevant studies have confirmed that Arf and the components that promote Arf function played an important role in mediating the transport of endoplasmic reticulum to the Golgi (Balch et al., 1992; Dong et al., 2010). For example, *Arf1* regulated vesicle formation, Golgi assembly and promoting vesicle division, and *Arf6* promoted membrane invagination on the cell surface during endocytosis (D'Souza-Schorey and Chavrier, 2006). Arf and Rab have similar functions in vesicle formation and transportation, indicating that the connection between GTPase-mediated signaling pathways requires different Ras superfamily proteins to fulfill a common task by a cooperation (Mitin et al., 2005). In *D. melanogaster*, *Arf6* was only involved in spermatogenesis (Lambaerts et al., 2009), it was not required for early development in mice (Doherty and McMahon, 2009). In *L. vannamei*, *Arf4* was almost not expressed in the early development stages, however, it had a higher expression in adults. There is almost no research on Arf in crustaceans, we found most Arf genes highly expressed in brain and ventral nerve of *L. vannamei*, so it is speculated that Arf should have a certain effect on the nervous system in shrimp.

4.6 The unconventional Ras GTPase families in *L. vannamei*

The researches of the five classical Ras GTPase families have been very in-depth. Furthermore, many genes have regions predicted as or similar to the small GTPase domain, but they're unconventional, for example, RJL family, RGK family, GPN family, IFT family, OBG family and Septin family. They have the same motif as classical Ras superfamily members, and the prediction region acts as a signal converter or molecular switch, called GTP-binding protein and has GTPase activity. Unlike the other members of the classical Ras GTPase, most these non-classical members showed extremely low levels of expression in adult tissues of *L. vannamei*, but most of them showed high levels of expression during early development and WSSV infection, this indicates that their functions are relatively specific and may be related to early development and immunity.

4.6.1 RJL family

RJL is a new member of Ras superfamily reported in recent years (Gao et al., 2019). Besides the GTP binding domain, members of this family also contain an additional DnaJ domain, so they are also named the DnaJ family. The family is divided into two subfamilies: Rjl and Rbj. Different from other Ras superfamily members, The RJL family lacks membrane targeting signal and their hydrolysis ability of GTP is impaired. The RJL family exists in many protozoa and deuterostome metazoans, but is obviously missing in some intermediate phyla, indicating an interesting possibility of horizontal gene transfer (HGT) between lower and higher eukaryotes (Nepomuceno-Silva et al., 2004). In human gastrointestinal cancers, Rbj was dysregulated and could promote tumor progression, the activation of MEK and ERK by Rbj indicated that RJL family might have a role in MEK/ERK signaling pathway (Gao et al., 2019; Chen et al., 2021). No any RJL family member has been reported in shrimp so far. In this study, the Rjl gene with GTP binding domain and DnaJ domain was found in *L. vannamei*, and called *LvRbj*. This gene was little highly expressed in the limb bud embryo (Lbe) and Lim stages, while in the adult, it only had low expression in eyestalk and blood cells, and only significantly highly expressed in hemolymph after WSSV interference, so *LvRbj* might involve in immune and neural regulation of shrimp.

4.6.2 RGK family

In this study, we identified three unconventional Ras superfamily genes of *L. vannamei*, *LvRGK1-3*. The RGK family includes Rad, Rem, Rem2 and Gem/Kir, which are called "distant cousins" of the typical G proteins and constitute the first unconventional Ras subfamily with a novel effector binding mechanism distinct from that of other Ras GTPases (Miranda et al., 2021). Rad (Ras associated with diabetes) is mainly in skeletal muscle and cardiac muscle, and its expression increase by an average of 8.6 times in muscle of type II diabetes. It is speculated that Rad may be an inhibitor of Ras, interfering with the function of

normal Ras, Rab or Rap (Reynet and Kahn, 1993). In addition, Rad can be phosphorylated by PKA, but it does not affect GTP binding and GTPase activity like classical Ras superfamily members, indicating it may have a specific GAP-like activity regulation mechanism (Zhu et al., 1995). Rem is the first Ras-related GTP-binding protein whose mRNA levels are regulated by repression after stimulation (Finlin and Andres, 1997). In human, RGK GTPase family genes bind directly to Ca^{2+} channel β-subunits (CaVβ), serve as regulators of Ca^{2+} channel activity (Finlin et al., 2003). In conclusion, RGK family is mainly highly expressed in skeletal muscle, cardiac muscle and other tissues, and plays a major role in the calcium and insulin signaling pathway. In *L. vannamei*, the overall expression level of RGK family was low, but they were higher expressed in skeletal muscle and cardiac muscle than most other tissues.

4.6.3 GPN family

GPN-loop GTPase (GPN) is a member of P-loop NTPase, which has a GTP binding domain similar to the classical Ras superfamily (Forget et al., 2010). However, this family is rare and there are few relevant studies. In the yeast *S. cerevisiae*, the deletion of *Gpn1* or its homologous genes *Gpn2* and *Gpn3* was fatal (Giaever et al., 2002), Gpn1, Gpn2, and Gpn3 were all essential proteins for cell growth, and deletion of each one resulted in cell death, suggesting that these GPN-like GTPases may be necessary for survival and their functions are not redundant (Liu et al., 2020). Three GPN subfamily genes were found in *L. vannamei*: *LvGPN1*, *LvGPN2* and *LvGPN3*, their expression patterns were similar, mainly in gonads and muscles, and significantly increased in hemocyte after WSSV infection, but the specific role is unknown.

4.6.4 IFT family

The intraflagellar transport (IFT) family contains at least 20 different proteins and can be resolved into two smaller subunits, complexes A and B (Cole et al., 1998), complexe A contains 6 protein subunits (IFT43, 121, 122, 139, 140, 144), and complexe B contains 14 protein subunits (IFT20, 22, 25, 27, 46, 52, 54, 57, 70, 72, 74, 80, 81, 88, 172) (Fan et al., 2010). Only IFT-B complexes were found in *L. vannamei*: IFT22 and IFT27. As core subunits of IFT-B complexes, IFT22 and IFT27 have significant sequence homology with members of Ras superfamily. *IFT27* was predicted to be Rab-like GTPase and proved to binding to GTP (Qin et al., 2007). IFT complex has a function similar to small GTPase, but its GTPase activity is very low, due to the lack of conserved catalytic Gln sites, so some GTP-activating proteins (GAPs) are needed to exchange between GTP and GDP (Bhogaraju et al., 2011). Therefore, the IFT complex, as an important structure in cilia assembly and maintenance, may be required for ciliogenesis by ferrying ciliary components using IFT complexes as cargo adaptors and may be necessary for normal life activities.

4.6.5 OBG family

OBG like-GTPase is a subfamily of P-loop GTPase, which was originally found downstream of *Spo0b* in the Gram-positive bacteria *Bacillus subtilis* (Trach and Hoch, 1989). Although these proteins contain GTP binding domains and conserved from bacteria to human, their sequence homology with other GTP-binding proteins is low (Kukimoto-Niino et al., 2004), so they are divided into a new group: OBG family, which contains three domains: OBG folding, G domain and OBG c-terminal region (OCT). In this study, an OBG-like GTPases protein of *L. vannamei* was identified, which has three domains, GTP1_OBG (OBG folding), FeOB_N, MMR_HSR1, among them MMR_HSR1 interacts with 50S ribosome and is necessary for binding adenine and guanine nucleotides to have complete activity. At present, research on OBG-like GTPases mainly focused on bacteria, yeast and plant chloroplasts (Chigri et al., 2009; Lin et al., 2018), and there are few reports in animals.

4.6.6 Septin family

Septin, a unique polymeric Ras superfamily protein with GTPase activity, was found in *L. vannamei*. Septin was described mainly as a spatial regulator of protein localization and interaction in the budding yeast, it is the key to their asymmetric cell shape and division (Spiliotis and McMurray, 2020). In human, *septin2* is a cancer promoting gene, its overexpression can promote the proliferation of gastric cancer cells and inhibit apoptosis (Li et al., 2018). *Septin2* gene was highly expressed in liver cancer tissues and corresponding adjacent tissues (Xu et al., 2019). Septin is classified as a member of the Ras superfamily because it has the same GTP binding motif as most Ras GTPases, and it is closely related to cell proliferation and oncogenesis. In crustaceans, the function of Septin is unclear.

5 Conclusion

In this study, based on genome and transcriptome data, we have conducted comprehensive analyses of gene structure, protein domain, and expression patterns of the Ras superfamily members in the economically important shrimp, *L. vannamei*. The results showed that the Ras superfamily is relatively complete in shrimp, a total of 108 Ras superfamily genes were identified. We found that shrimp contained not only all classical Ras superfamily members, but also some unconventional and novel Ras superfamily genes, these genes shared common conserved domain and motifs. From a phylogenetic point of view, Ras superfamily of *L. vannamei* are divided into two clades, They had different expression patterns and might have diversified functions in development, growth and immune response. These works provide important clues for future research on the function of Ras superfamily genes in crustaceans, which is of great significance for understanding growth development and immunity mechanism and promoting genetic breeding of shrimp.

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 in the article/Supplementary Material.

Author contributions

XJZ, SS, SY, and FL conceived and designed the experiments. SS and XJZ performed the experiments and data analyses. SS wrote the manuscript and prepared all the figures. XJZ, JY, and XXZ reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1063857/full#supplementary-material

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