Identification and Allelic Variants Associated With Cold Tolerance of PmPIAS in Pinctada fucata martensii

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The protein inhibitor of activated STAT (PIAS) functions in diverse aspects, including immune response, cell apoptosis, cell differentiation, and proliferation. In the present study, the PIAS in the pearl oyster Pinctada fucata martensii was characterized. The sequence features of PmPIAS were similar to that of other PIAS sequences with PIAS typical domains, including SAP, Pro-Ile-Asn-Ile-Thr (PINIT), RLD domain, AD, and S/T-rich region. Homologous analysis showed that PmPIAS protein sequence showed the conserved primary structure compared with other species. Ribbon representation of PIAS protein sequences also showed a conserved structure among species, and the PINIT domain and RLD domain showed the conserved structure compared with the sequence of Homo sapiens. The expression pattern of PmPIAS in different tissues showed significant high expression in the gonad. PmPIAS also exhibited a significantly higher expression in the 1 and 2 days after cold tolerance stress (17°C) and showed its potential in the cold tolerance. The SNP analysis of the exon region of PmPIAS obtained 18 SNPs, and among them, 11 SNPs showed significance among different genotypes and alleles between cold tolerance selection line and base stock, which showed their potential in the breeding for cold tolerance traits.

Keywords: Pinctada fucata martensii, PIAS, cold tolerance, SNPs, expression pattern

INTRODUCTION

In aquaculture, changes in temperature influence the growth, development, reproductive ability, and survival of organisms (Viergutz et al., 2012; Aagesen and Hase, 2014). The fluctuating temperature could compromise and alter the immune function and cause bacteria to proliferate and accumulate in tissues, leading to diseases and stress (Yu et al., 2009). Pearl oysters as warm-water shellfish species were cultured in natural seas and exposed in diverse temperature ranges and their corresponding effects (Adzigbli et al., 2020b). Pinctada fucata martensii, as the main marine pearl called "South China sea pearls" production in China (Yang et al., 2019; Zhang et al., 2021), hold a low enduring capacity to extreme temperatures and just hold an optimal range of 23–28°C (Deng et al., 2010). During pearl culture, the juvenile of pearl oyster is transferred to a culturing site in the sea and thus exposed to various environmental perturbations that affect its survival and growth in the natural sea (Hao et al., 2019; Adzigbli et al., 2020b). The sensitivity of pearl oysters to temperature change has limited their culture area, which in turn affects the pearling industry. Therefore, understanding the response mechanism of the pearl oyster to environmental stress and exploring the genetic mechanism of cold tolerance in pearl oyster are essential for the breeding production.
Recently, multiple researches were performed to explore the mechanism of temperature stress such as *Mytilus galloprovincialis*, *Mytilus trossulus*, and *Crassostrea gigas*, and plenty of genes were identified especially genes like HSPs or HSP-related genes (Ioannou et al., 2009; Fields et al., 2012; Zhu et al., 2016). Through the analysis of high-throughput data, cellular senescence, homeostatic flexibility, inhibition of apoptosis, lysosome protein-encoding genes, and immune-related genes participated in the process of temperature tolerance (Ibarz et al., 2010; Zhu et al., 2016; Wang et al., 2018). Liu et al. (2018) showed that stearoyl-CoA desaturase showed a significantly higher expression in the low temperature tolerance, which showed the potential function of monounsaturated fatty acid in the response of temperature (Liu et al., 2018). Although many genes and related metabolic pathway have been reported to be involved in temperature stress, small genetic markers that are important for breeding were screened.

Pearl oysters rely on their innate immunity in response to diverse stress including cold tolerance and diseases (Adzigbli et al., 2020a). The protein inhibitor of activated signal transducer and activator of transcription (PIAS) is a key small ubiquitin-related modifier protein (SUMO) E3 ligase, which participates not only in cytokines but also in various immune signaling pathways (Shuai and Liu, 2005) and may be involved in diverse immune response to stimuli. In addition, extreme temperatures have been known to influence apoptosis, with PIAS protein performing this function in various organisms. PIAS proteins could modulate the function and localization of many proteins, including many components of two important pathways, the NF-κB and JAK-STAT signaling pathway (Schmidt and Müller, 2002; Shuai and Liu, 2003). Furthermore, diverse functions have been ascribed to PIAS in transvertebrates and invertebrates including immune response, cell apoptosis, cell differentiation, and proliferation (Duval et al., 2003; Myllymäki and Rämet, 2014). Duck PIAS2 could interact with duck interferon regulatory factor 7 and inhibits IFN-β promoter activation induced by the duck IRF7 (Zu et al., 2020). Amphioxus PIAS inhibits NF-κB by binding with upstream signaling adaptor TICAM-like and MyD88 (Wang et al., 2017). Although most of the PIAS research has focused on model species like Drosophila and mammals, some information is available for aquatic organisms like shrimp *Litopenaeus vannamei* (Zhang et al., 2019).

### MATERIALS AND METHODS

#### Experimental Samples

In the present study, *P. f. martensii* was sampled and acquired from Xuwen, Zhanjiang, Guangdong Province, China.
Allelic Variants of PmPIAS (20°250′ N, 109°570′ E). Six individuals about 1.5 years old with shell length ranging from 5 to 6 cm were utilized for tissue expression pattern including adductor muscle (A), gill (GI), hepatopancreas (HE), mantle (M), foot (F), and gonad (GO). For the expression analysis of PmPIAS in cold tolerance, a total of 150 individuals about 1.5 years old with shell length ranging from 5 to 6 cm were collected and cultured. The water temperature was set at 17°C as cold tolerance and 22°C as control group according to the previous research of Liu et al. (2018) and Wang et al. (2018). The salinity was 30%. During the experiment, the pearl oysters were cultured with the same amounts of single-cell algae. Eight individuals from both groups were randomly obtained at 6 h, 1, 2, 3, 5, 10, and 15 days. GI tissues were collected for further analysis.

Gene Cloning and Sequence Analysis of PmPIAS

A partial sequence of the PIAS gene used for cloning was acquired from P. f. martensii genome data (Du et al., 2017). RACE technology was utilized for the full length of the PmPIAS gene. In order to obtain the template for the nested PCR, the total RNA was extracted with Trizol reagent (Invitrogen). SMART™ RACE cDNA amplification kit was used to prepare 5’ and 3’ RACE templates that were amplified via nested PCR to acquire the full-length sequence of PmPIAS with primers in Table 1. The PCR product was detected using 1% agarose electrophoresis. The gene fragments with appropriate length were sequenced, jointed with DNAMAN software, and then analyzed with BLAST1. Open reading frame (ORF) was obtained with ORF finder tool2. PIAS protein sequence analysis from different species was performed by Clustal omega3, SMART4, PHYRE25, and Chimera 1.13.1.

PmPIAS Expression Pattern in Tissues, Development, and Cold Tolerance

Real-time quantitative PCR (qRT-PCR) analysis was performed with Thermo Scientific DyNaMo Flash SYBR Green qPCR Kit (Thermo Scientific) in Applied Biosystems 7500/7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, United States) to identify the expression pattern of PmPIAS. Expression analysis of different tissues was performed. The qRT-PCR program was 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 30 s according to the manufacturer’s instructions (Lei et al., 2017). Different stages of development from transcriptomes were collected and analyzed for the expression profiles of

1 https://blast.ncbi.nlm.nih.gov/Blast.cgi
2 https://www.ncbi.nlm.nih.gov/orffinder/
3 https://www.ebi.ac.uk/Tools/msa/clustalo/
4 http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1
5 http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index

FIGURE 2 | Multiple-sequence alignment and structural organization of PmPIAS aa sequences. Dark blue background indicates conserved aa, pink background indicates aa with strong similarity, light blue indicates aa with weak similarity, and the numbers on the right show the position of sequence alignment. The frame showed the conserved motif (LXXLL). The accession numbers for sequences used in this alignment and structure analysis are as follows: Crassostrea virginica (XP_022331287.1), Mizuhopecten yessoensis (XP_021340266.1), Octopus vulgaris (XP_029634642.1), and Lingula anatina (XP_013385044.1).
**Allelic Variants of PmPIAS**

Expression pattern of eight individuals from different time points after cold tolerance was performed. Primers used in the present experiment are shown in Table 1.

### SNP Screening of PmPIAS

The pearl oysters utilized for the SNP screening were sampled from the third line selected for resistance to cold tolerance. In September 2013, pearl oysters were sampled from a base stock farm in Leizhou of Zhanjiang, Guangdong. The oysters were transported to Nan’ao Island of Shantou, Guangdong, with a temperature of 2–3°C lower than that in Leizhou of Zhanjiang, Guangdong, and cultured in the natural sea. The pearl oyster stayed over winter at Nan’ao Island of Shantou, and the endured individuals were utilized to culture the first line in April 2014. The procedures for larval, juvenile, and adult rearing were explained by Deng et al. (2010). In November 2015 and April 2017, low-temperature resistant selection lines F2 and F3 were cultivated in accordance with the same route. In November 2018, adductor muscles of samples were obtained from the low-temperature resistant selection line (R) and the base stock (W) (shell length ranging from 5 to 6 cm). DNAs from W and R were extracted for SNP identification in the exon region of PmPIAS with the method of resequencing (Lei et al., 2019; Yang et al., 2020), and the data were deposited in European Variation Archive, accession number PRJEB43188.

### Statistical Analysis

Popgene32 software was used to calculate the number of alleles, the expected heterozygosity (He), the allele frequency, the effective number of alleles (Ne), and the observed heterozygosity (Ho). PIC software was used to calculate the polymorphism information content of the SNP loci (Yang et al., 2020). Statistical differences in SNPs between the R and W were obtained using chi-square test on SPSS 16.0 software (Chicago, IL). The expression of PmPIAS at tissues and different time points after cold tolerance was calculated using the $2^{-\Delta Ct}$ method with $\beta$-actin gene as the reference gene and then analyzed by SPSS 16.0. $P < 0.05$ was considered statistically significant.

### RESULTS

#### Cloning and Sequence Analysis of PmPIAS

The full length of PmPIAS was 2,313 bp with 5’UTR of 14 bp and 3’UTR of 337 bp. The ORF of PmPIAS was 1,962 bp, encoding 653 amino acids (Figure 1). The deduced aa sequence of PmPIAS featured a theoretical molecular weight of 70.53 kDa. Domain analysis showed that PmPIAS had typical PIAS domains, including SAP, PINIT, RLD domain, AD, and S/T-rich region. The complete sequence of PmPIAS was deposited in GenBank under the accession number MW326754.

#### Structural and Homologous Analysis

Homologous analysis was performed with PIAS sequence from Crassostrea virginica (XP_022331287.1), Mizuhopecten yessoensis (XP_021340266.1), Octopus vulgaris (XP_029634642.1), and Lingula anatina (XP_013385044.1) to determine the homology of PmPIAS with other species. The results showed that PmPIAS had the highest identity with C. virginica (75.75%) (Figure 2). The PmPIAS hold the identities of 60.67, 64.38, and 61.43%, compared with M. yessoensis, O. vulgaris, and L. anatina, respectively. The predicted structural organization of PIAS was performed among C. virginica (XP_022331287.1), M. yessoensis (XP_021340266.1), O. vulgaris (XP_029634642.1), and L. anatina (XP_013385044.1). PmPIAS protein sequence showed the conserved primary structure compared with other species (Figure 3).

#### PIAS Advanced Structure Analysis of Different Species

The PIAS protein sequences from P. f. martensii, C. virginica (XP_022331287.1), M. yessoensis (XP_021340266.1), O. vulgaris (XP_029634642.1), L. anatina (XP_013385044.1), and H. sapiens (AAI11061.1) were submitted to Phyre server for homology modeling. Ribbon representation of these protein sequences

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**FIGURE 3** | The structural organization analysis of PmPIAS. The frame of yellow, light blue, green, dark blue, and red represents SAP, PINIT, RLD domain, AD, and S/T-rich region, respectively. Accession numbers for sequences used in this alignment and structure analysis are as follows: Crassostrea virginica (XP_022331287.1), Mizuhopecten yessoensis (XP_021340266.1), Octopus vulgaris (XP_029634642.1), and Lingula anatina (XP_013385044.1).
FIGURE 4 | Ribbon representation of PIAS protein sequences from different species. (A–F) Show the protein sequences from *P. f. martensi*, *Crassostrea virginica* (XP_022331287.1), *Homo sapiens* (AAI11061.1), *Lingula anatina* (XP_013385044.1), *Mizuhopecten yessoensis* (XP_021340266.1), and *Octopus vulgaris* (XP_029634642.1), respectively. The blue and green represented the PINIT and RLD domains, respectively.

FIGURE 5 | Expression pattern of *PmPIAS* in tissues and cold tolerance. (A) Presents the expression pattern of *PmPIAS* in tissues. M, mantle; F, foot; GI, gill; GO, gonad; A, abductor muscle; HE, hepatopancreas. (B) Shows the expression pattern of *PmPIAS* under cold tolerance stress (17°C). Columns with different letters are significantly different (*P* < 0.05).
showed similar structure. Furthermore, the PINIT domain and RLD domain showed the conserved structure compared with the sequence of Homo sapiens (Figure 4).

**Expression Analysis of PmPIAS in Tissues, Development, and Cold Tolerance**

qPCR analysis was conducted to ascertain the PmPIAS expression in the various tissues examined (Figure 5A). The result showed that PmPIAS hold the significant high expression in GO compared with other tissues. Analysis of the developmental transcriptome of *P. f. martensii* indicated that PmPIAS was widely expressed in the development stages and showed high expression in trophophore, followed by fertilized egg and juveniles (Supplementary Figure S1). The temporal expression of PmPIAS under cold tolerance stress was examined via qPCR. The most significant high expressions among the different time points were observed at 1 and 2 days (Figure 5B).

**Genetic Polymorphism Analysis of SNPs From PmPIAS Exon Region**

Eighteen SNP loci were identified in the exon region of *PmPIAS*. The polymorphism information of these SNP loci was analyzed, and the results showed that the PIC ranged from 0.1038 to 0.3749. Among them, nine SNPs with low polymorphism (PIC < 0.25) and nine SNPs with moderate polymorphism (0.25 < PIC < 0.5) were found. Ho ranged from 0.1167 to 0.6000. The range of He is 0.1108–0.3749. HWE analysis indicated that 18 SNP did not deviated from HWE (P > 0.05) (Table 2).

| SNP name | Position | Ho      | He      | PIC      | HWE     |
|----------|----------|---------|---------|----------|---------|
| SNP1     | 4,900,234 (C > T) | 0.4500  | 0.4167  | 0.3278  | 0.5306  |
| SNP2     | 4,900,243 (C > T) | 0.2167  | 0.2688  | 0.2310  | 0.1242  |
| SNP3     | 4,900,273 (C > T) | 0.2167  | 0.1948  | 0.1745  | 0.3677  |
| SNP4     | 4,900,303 (T > G) | 0.5333  | 0.4952  | 0.3705  | 0.5478  |
| SNP5     | 4,901,462 (G > A) | 0.1167  | 0.1399  | 0.1291  | 0.1739  |
| SNP6     | 4,901,547 (G > A) | 0.4500  | 0.4973  | 0.3716  | 0.4571  |
| SNP7     | 4,902,338 (A > G) | 0.1500  | 0.1399  | 0.1291  | 0.5553  |
| SNP8     | 4,902,368 (C > T) | 0.1667  | 0.1815  | 0.1638  | 0.5097  |
| SNP9     | 4,903,720 (G > A) | 0.4167  | 0.5041  | 0.3749  | 0.1756  |
| SNP10    | 4,903,771 (C > A) | 0.1833  | 0.1679  | 0.1527  | 0.4578  |
| SNP11    | 4,904,485 (T > C) | 0.1167  | 0.1108  | 0.1038  | 0.6581  |
| SNP12    | 4,904,527 (T > C) | 0.2667  | 0.1331  | 0.2044  | 0.2503  |
| SNP13    | 4,904,822 (C > T) | 0.3333  | 0.3227  | 0.2688  | 0.7947  |
| SNP14    | 4,904,900 (C > T) | 0.5167  | 0.4637  | 0.3541  | 0.3718  |
| SNP15    | 4,904,903 (A > G) | 0.1167  | 0.1108  | 0.1038  | 0.6581  |
| SNP16    | 4,910,826 (C > T) | 0.5667  | 0.4768  | 0.3610  | 0.1403  |
| SNP17    | 4,910,841 (T > G) | 0.6000  | 0.4840  | 0.3648  | 0.0611  |
| SNP18    | 4,910,873 (G > A) | 0.5667  | 0.4768  | 0.3610  | 0.1403  |

**SNP Analysis of Genotype and Allele From PmPIAS Between R and W**

A total of 18 SNP sites were detected in the exon region of PmPIAS, 11 of which significantly differed between W and R (P < 0.05). Among them, the frequencies of genotype TT of g.4900234 was 70% in the R, and 26.7% in the W; the genotype CT of g.4900243 in the two lines were 40 and 3.3%, respectively. The frequencies of genotype GG of g.4903720 in the W and R were 50 and 6.67%, respectively. The frequencies of genotype CT of g.4904822 in the W and R were 56.7 and 10%, respectively (Table 3). These results indicated the potential of the 11 SNPs in cold tolerance selection program.

**DISCUSSION**

As a member of the negative regulators of the JAK/STAT signaling pathway, PIAS participates in the regulation of immune responses, which showed its potential in response to cold tolerance. However, in the bivalve, limited reports have been researched about PIAS proteins. In the present study, a PIAS gene from *P. f. martensii* was cloned and SNPs in the exon region of *PmPIAS* were obtained to explore the genetic information for breeding.

Researches have reported that the PIAS protein family exhibit high similarity in conserved domains including the conserved RLD, PINIT motif, C-terminal Ser/Thr amino acids enriched region (S/T), AD, and LXXLL signature motif in the SAP domain (Duval et al., 2003; Suzuki et al., 2009). Among them, SAP domain aids the function of the PIAS gene, which is associated with sequence- or structure-specific DNA binding (Aravind and Koonin, 2000). The LXXLL signature motif was found to mediate the interactions between nuclear receptors and their coregulators (Glass and Rosenfeld, 2000). RLD domain is required for the SUMO-E3-ligase activity of PIAS proteins and may be involved in the interaction with other proteins (Kotaja et al., 2002). Amphioxus PIAS inhibited NF-κB activation by co-localizing and binding with TRAF6, and the interaction relied on the N-terminal SAP and PINIT domains of PIAS (Fu et al., 2020). PIASy binds to MafA through the SAP domain and negatively regulates the insulin gene promoter through a novel SIM1-dependent mechanism (Onishi and Kataoka, 2019). In the present study, the protein sequence feature of PmPIAS also contained all conserved domains and motifs, which were consistent with PIAS proteins from other species like *Crassostrea virginica, Mizuhopecten yessoensis, Octopus vulgaris, Scylla paramamosain,* and *Lingula anatina* (Shuai, 2006; Huang et al., 2015). Advanced structure analysis of PIAS proteins from *H. sapiens* and bivalve also showed the conserved structure of PINIT and RLD domains in the PmPIAS. Therefore, PmPIAS may be a member of the PIAS protein family.

PIAS protein participates in regulating various immune signaling pathways and immune response to stimuli (Shuai and Liu, 2005). In *L. vannamei*, LvPIAS exhibited an immune response function after bacteria and virus stimulation with a significant expression pattern within 48 h post-stimulation and inhibited the transcriptional activity of LvSTAT, which
indicated that there was a feedback loop between LvSTAT and LvPIAS (Zhang et al., 2019). PIAS of Scylla paramamosain was involved in the pathogen-resistant activities of mud scab (Huang et al., 2015). Expression pattern of PmPIAS in the different tissues and developmental stages indicated that this gene may play an important role in the life process of P. f. martensii. Xenopus PIAS plays important roles in mesodermal induction and patterning during early frog development (Burn et al., 2011). After cold tolerance, PmPIAS expressed significantly highly at the early time points to respond to the stress, which indicated its important function in the cold tolerance stress response. In the Arabidopsis, the ectopic expression of IZ1 (a SIZ/PIAS-Type SUMO E3 Ligase) could also improve the cold tolerance (Li et al., 2020). Researches have reported that, after low temperature tolerance, Pm-SCD, Pm-HK, and PmHSP70 genes showed a significantly higher expression at different time points compared with the control group, which means the pearl oysters utilized different biological processes at different times to respond to the low temperature tolerance (Liu et al., 2018, 2020). Temperature tolerance has been reported to induce the immune system, and transcriptome analysis of low water temperature stress showed that several immune-related genes and pathways were presented in response to exposure to low temperatures in pearl oysters (Wang et al., 2018). Immune-related genes were also upregulated after cold exposure of Drosophila melanogaster and suggested that immunomodulation plays an important role in response to cold stress (Zhang et al., 2011; Tusong et al., 2017).

In order to explore the potential of PmPIAS in the breeding program for the cold tolerance selection, exon region SNPs of PmPIAS were identified. Various studies have reported that particular environmental stresses increase SNPs. Different cold tolerances for Nile tilapia strains from Ghana, Egypt, and Ivory Coast suggest the influence of geographic location and natural selection on cold tolerance in tilapia (Sifa et al., 2002). The further the geographic location from the equator, the more cold-tolerant the strain of Nile tilapia. The two sequenced natural populations of bay scallop Argopecten irradians presented a substantial difference in T allele frequency implying that the SNP all-53308-760 T/C may have been subjected to natural selection for temperature adaptation, and the higher frequency of T allele in the southern subspecies is the consequence of local adaptation (Du et al., 2014). In the present study, 11 SNP sites were found in the exon region of PmPIAS, and they demonstrated significant differences between the R and W in genotype and allele. Accumulating evidence suggested that genetic polymorphisms in the coding regions could affect protein activity or effect of translation (Gottler et al., 2008; Frydenberg et al., 2010). The SNPs detected in the present study may provide a potential site for the cold tolerance selection in the future.

**CONCLUSION**

The full-length characterization of PmPIAS showed its conserved primary and advanced structures in the protein sequence. Expression analysis demonstrated the wide distribution of PmPIAS in pearl oyster and showed a significant increase after cold tolerance. Eighteen SNPs were identified in the exon region of PmPIAS, and the SNPs detected in the present study may provide a potential site for the cold tolerance selection in the future.
region of \textit{PmPIAS}, and 11 SNPs showed the potential in the cold tolerance breeding of pearl oysters. This study provided a potential molecular marker for the selective breeding of cold tolerance of \textit{P. f. martensii}.

DATA AVAILABILITY STATEMENT

The sequence information of \textit{PmPIAS} presented in the study are deposited in the GenBank repository, accession number MW326754. The SNP data are deposited in the European Variation Archive, accession number PRJEB43188.

AUTHOR CONTRIBUTIONS

RH and QW: conceptualization. ZL: formal analysis and writing—original draft preparation. LA and QC: resources. RH, QW, YL, and YD: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.634838/full#supplementary-material
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