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Identification and assessment of differentially expressed genes involved in growth regulation in *Apostichopus japonicus*

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ABSTRACT. Rapid and efficient growth is a major consideration and challenge for global mariculture. The differential growth rate of the sea cucumber, *Apostichopus japonicus*, has significantly hampered the total production of the industry. In the present study, forward and reverse suppression subtractive hybridization libraries were constructed and sequenced from a fast-growth group and a slow-growth group of the sea cucumber. A total of 142 differentially expressed sequence tags (ESTs) with insertions longer than 150 bp were identified and further analyzed. Fifty-seven of these ESTs (approximately 40%) were functionally annotated for cell structure, energy metabolism, immunity response, and growth factor categories. Six candidate genes, arginine kinase, cytochrome *c* oxidase subunit I, HSP70, β-actin, ferritin, and the ADP-ribosylation factor, were further validated by quantitative PCR. Significant differences were found between the fast- and slow-growth groups ($P < 0.05$) for the expression levels of arginine kinase, cytochrome

c oxidase, HSP70, the ADP-ribosylation factor, and β-actin. However, no significant difference was observed for ferritin. Our results provide promising candidate gene markers for practical size screening, and also further promote marker-assisted selective breeding of this species.

Key words: *Apostichopus japonicus*; Growth rate; qPCR; Suppression subtractive hybridization

INTRODUCTION

Aquaculture of the sea cucumber, *Apostichopus japonicus* (Echinodermata; Holothuroidea), has grown rapidly in recent years and is becoming one of the largest industries in Chinese mariculture. As a rough estimate, the species culture area in China exceeds 1 million acres, with a production value of over 150 million dollars in 2009 (Wang et al., 2009; Liu et al., 2010). To improve the productivity of the industry, the growth phenotype has received considerable attention by researchers, who have determined optimal conditions for growth with respect to temperature (An et al., 2007), nutrition (Yuan et al., 2006; Liu et al., 2009), and salinity (Yuan et al., 2010). However, to the best of our knowledge, the manipulation of growth traits has rarely been investigated in sea cucumber.

The identification and characterization of growth-related genes are the first step in such efforts, and different strategies such as cDNA libraries, homology cloning, and proteomics have been successfully utilized in some previous cases (Wu et al., 2009; Natarajascenivasan et al., 2011; Lenz et al., 2011). Suppression subtractive hybridization (SSH) combines normalization and subtraction in a single procedure, thus allowing the isolation of differentially expressed cDNA that is overexpressed in one population but not in another. In recent years, SSH has been successfully applied as an efficient method to identify differentially expressed genes in oyster (Renault et al., 2011), shrimp (He et al., 2004; Pan et al., 2005; Zhao et al., 2007; James et al., 2010), clam (Prado-Alvarez et al., 2009), and fish (Cui et al., 2011; Li et al., 2011b). However, these studies all focused on the identification of immune-related genes with the challenge of specific pathogens. Indeed, according to literature searches, SSH has rarely been used to analyze growth-related genes.

The goals of the present study were as follows: 1) to construct an SSH library for different growth rates in sea cucumber; 2) to screen and sequence the differentially expressed gene fragments; 3) to validate the expression profiles of certain candidate genes using quantitative PCR (qPCR).

MATERIAL AND METHODS

Experimental animals

Juvenile *A. japonicus* individuals were obtained from Shandong Oriental Ocean Sci-Tech Co., Ltd., in October 2011. Individuals of similar sizes (85 ± 10 g) were selected and cultured at the Ningbo Bowang Aquaculture Company for 5 months. Fifty experimental specimens were randomly selected and cultured in the laboratory for 1 week and then assigned to the following groups, according to their body weight: a fast-growth group (FGG), with a body

weight of 358 ± 35 g, and a slow-growth group (SGG), with a body weight of 158 ± 20 g. The muscles from 10 sea cucumbers from each group were sheared, mixed together, and then ground into a fine powder using liquid nitrogen for the subsequent RNA extraction.

Construction of SSH libraries

The total RNA from the muscle was extracted according to the Trizol protocol (Invitrogen), and cDNA was obtained from 5 μg total RNA using the SMART PCR cDNA Synthesis Kit (Clontech). The SSH assay was then performed with the PCR-Select cDNA Subtraction Kit (Clontech), following manufacturer instructions. Briefly, the cDNA from the FGG was considered as the tester and that from the SGG as the driver, for the forward subtraction; the reverse library was constructed using cDNA from the SGG as the tester and that from the FGG as the driver. The subtracted cDNA pools obtained from the PCR selection protocol were cloned into the pMD-18T vector to generate the SSH libraries in *Escherichia coli* DH5α. White colonies were randomly picked and cultured in Luria-Bertani broth at 37°C for 4-5 h, and were further validated by PCR using M13 forward and reverse primers (Table 1). The positive clones were sequenced by Invitrogen (Shanghai).

Table 1. Primer sequences used in the present study.

Primer	Sequence (5'-3')	Product size (bp)
M13-47	GTCGTGACTGGGAAAACCCTGGCG	
RV-M	GAGCGGATAACAATTTCACACAGG	
PCR primer 1	CTAATACGACTCACTATAGGGC	
Nested PCR primer 1	TCGAGCGGCCGCCCGGGCAGGT	
Nested PCR primer 2R	AGCGTGGTCGCGGCCGAGGT	
18s-F	CGAGTCGTGGGAGATTTTT	198
18s-R	CACTTTGGCTGCTTTGAAC	
FSSH 3-F	TCATGGTAGCTGCTGTGAAGTAGG	234
FSSH 3-R	ACTTGTTCTGATTCTTCGGACACC	
FSSH 30-F	CAGATGGGAGGAGACATGAAGGA	139
FSSH 30-R	TGGATGGGCAAGTCAGAACAAATC	
FSSH 17-F	CTGCTGGATGCTTTCAAGGT	133
FSSH 17-R	CTGTTCCGTCAGGTAGTGGG	
FSSH 28-F	TTG TACGCCAACACTGTTCTT	175
FSSH 28-R	GCCAGACTCGTCGTATTCCT	
FSSH 37-F	AACGAATCTAAAATCATTAGTCAGTG	247
FSSH 37-R	CTACTATTGCTTGGAAAACGAGA	
FSSH 122-F	CCAAGAGAACATTGTCAAGC	220
FSSH 122-R	ATTCGAGTCGAACCTCCG	

Sequencing analysis

The sequences were analyzed using the BLAST algorithm on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast) after removal of the adapter and contaminating vector sequences. The deduced amino acid sequences were analyzed using the Expert Protein Analysis System (http://www.expasy.org/).

Confirmation of differentially expressed sequence tags (ESTs) by qPCR

Sea cucumbers of various sizes, ranging from 105 to 320 g, at the same growth stage

were collected from the indoor ponds of the Pulandian Hatchery in Dalian, China, in April 2012 and were divided into 2 groups for RNA extraction. Muscle tissue was removed and prepared for RNA extraction. First-strand cDNA synthesis was performed based on the Promega M-MLV RT user information (Promega) using total RNA treated with DNase I (Promega) as the template. The cDNA mix was diluted 1:50 for the subsequent experiment. There were 6 replicates for each group.

Six candidate genes, arginine kinase, cytochrome *c* oxidase, HSP70, β-actin, ferritin, and the ADP-ribosylation factor, were selected to validate the efficiency of the SSH library. The gene-specific primers for each differentially expressed gene are shown in Table 1. A pair of 18S RNA primers (Table 1) was used to amplify a 196-bp product, which served as the internal control. The real-time PCR amplification was performed using a Rotor-Gene 6000 real-time PCR detecting system. The reaction was performed in a total volume of 20 μL containing 10 μL 2X SYBR Green Master Mix (Takara), 4 μL diluted cDNA mixture, 1 μL each primer (10 μM), and 4 μL diethylpyrocarbonate-treated water. The thermal profile for the real-time PCR was 94°C for 2 min, followed by 45 cycles of 94°C for 15 s, 56°C for 20 s, and 72°C for 20 s. A dissociation curve analysis of the amplification products was performed at the end of each PCR to confirm that only a single PCR product was amplified and detected. The $2^{-\Delta\Delta CT}$ method was used to analyze the expression level of the differentially expressed genes. The cycle threshold (Ct) for the target amplified genes and the Ct for the internal control were determined for each sample. The difference in the Ct for the target and the internal control, called ΔCT, was calculated to normalize the differences in the amount of template and the efficiency of the RT-PCR. The ΔCT value for the SGG sample was used as the reference sample, henceforth referred to as the calibrator. The ΔCT for each sample was subtracted from the ΔCT of the calibrator, and this difference was called the ΔΔCT. The expression levels of differentially expressed genes were calculated using the $2^{-\Delta\Delta CT}$ method, and the value obtained was denoted the n-fold difference relative to the calibrator. All data are reported in terms of relative mRNA expression as means ± SD. The data were subjected to one-way analysis of variance (ANOVA). P values <0.05 or <0.01 were considered to be significant.

RESULTS

SSH construction and EST sequencing

Two subtracted cDNA libraries (forward and reverse libraries) were constructed in this study. There was an obvious difference between the subtracted and unsubtracted groups based on the results of the agarose gel electrophoresis of the secondary PCR products (Figure 1). The PCR screening revealed that the size of the inserted cDNA fragments ranged from 200 to 900 bp (Figure 2). Eighty-seven and 82 clones were randomly selected for sequencing from the forward and reverse libraries, respectively. A total of 151 sequences were successfully generated, and 142 sequences with insertions longer than 150 bp were used in the sequence homology search. Approximately half of the products did not exhibit sufficient homology with any known genes (Figure 3). More than 40% of the ESTs were identified and functionally annotated in the gene categories for respiratory metabolism (cytochrome *c* oxidase subunit I), membrane structure (β-actin and filamin), energy metabolism (arginine kinase and ADP-ribosylation factor), and growth factor [fibroblast growth factor (FGF) receptor-like 1

precursor, and vascular endothelial growth factor (VEGF) C precursor]. Some of these genes demonstrated more than 1 copy in the library (Tables 2 and 3), namely β-actin, arginine kinase, the ADP-ribosylation factor, and cytochrome *c* oxidase subunit I. Importantly, some candidate genes were detected for the first time, to the best of our knowledge, in sea cucumber, including the VEGF C precursor, the ADP-ribosylation factor, and the FGF receptor-like 1 precursor.

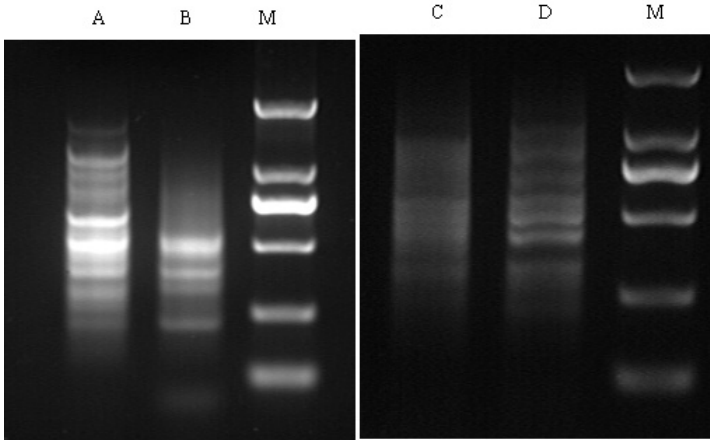

Figure 1. Secondary PCR products of the subtracted library. *Lane A* = unsubtracted sample for the forward library. *Lane B* = subtracted sample for the forward library. *Lane C* = unsubtracted sample for the reverse library. *Lane D* = subtracted sample for the reverse library. *Lane M* = DL2000 DNA marker.

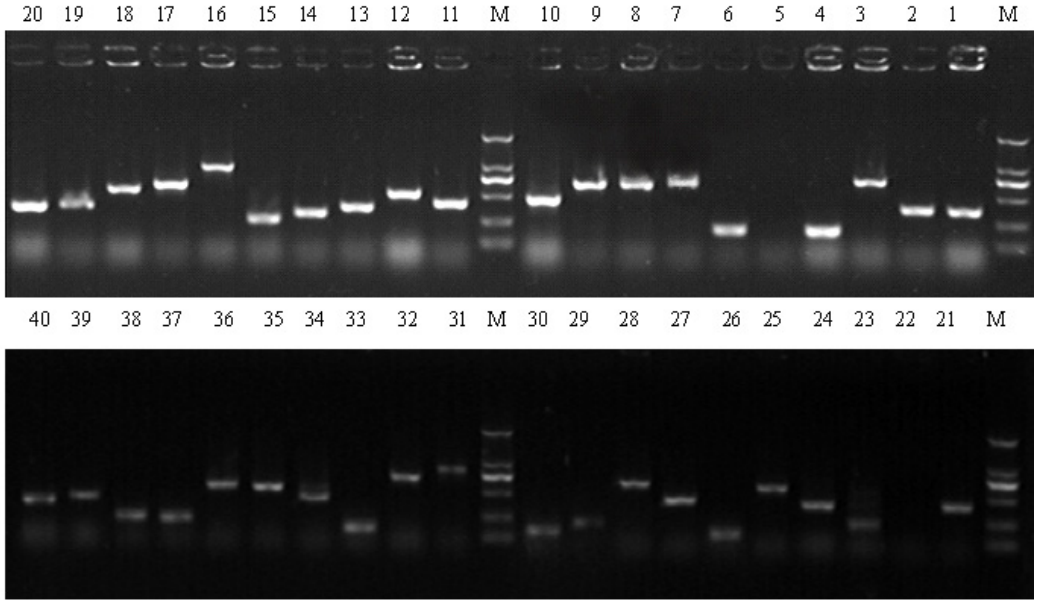

Figure 2. Analysis of the cDNA inserts by PCR amplification using M13 primers. *Lane M* = DL2000 marker. *Lane 1-40* = PCR products from 40 different colonies.

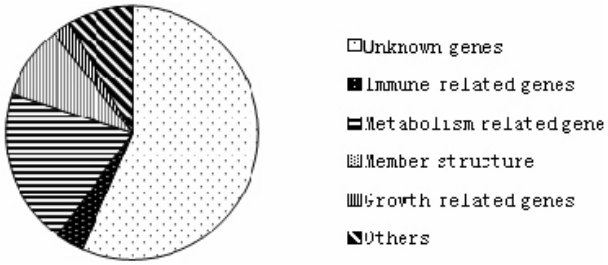

Figure 3. Functional annotation of the sequenced clones from the SSH libraries.

Table 2. Distribution of differentially expressed genes from the forward SSH library.

Clone ID	Identification clones	Accession No.	Species	Identities	Redundancy
FSSH 3	Cytochrome *c* oxidase subunit I	JN836336.1	*Apostichopus japonicus*	99%	3
FSSH 4	Casein kinase 1, delta	XP_002167634.1	*Hydra magnipapillata*	29%	1
FSSH 10	Metalloprotease family M12A	XP_002902411.1	*Phytophthora infestans*	31%	1
FSSH 17	Ferritin mRNA	DQ058411.1	*Apostichopus japonicus*	99%	1
FSSH 18	Filamin	XP_792145.2	*Strongylocentrotus purpuratus*	81%	1
FSSH 20	Hypothetical protein, conserved	CBH18507.1	*Trypanosoma brucei gambiense*	38%	1
FSSH 22	APC family amino acid-polyamine-organocation transporter	YP_001485465.1	*Bacillus pumilus*	45%	1
FSSH 24	Vascular endothelial growth factor C precursor	XP_002159836.1	*Hydra magnipapillata*	64%	1
FSSH 28	β-actin	EU668024.1	*Apostichopus japonicus*	99%	7
FSSH 30	Arginine kinase	AB025275.1	*Apostichopus japonicus*	99%	5
FSSH 37	ADP-ribosylation factor	XP 002260453.1	*Plasmodium knowlesi* strain H	9.3%	2
FSSH 44	Fibroblast growth factor receptor-like 1 precursor	NP_001165523.1	*Strongylocentrotus purpuratus*	59%	1
FSSH 45	CRISPR-associated RAMP protein	YP_003615800.1	*Methanocaldococcus infernus* ME	40%	1
FSSH 47	ABC transporter	XP_001350233.1	*Plasmodium falciparum*	29%	1
FSSH 72	Nbs-lrr resistance protein	XP_003615790.1	*Medicago truncatula*	54%	1
FSSH 81	Myosin-15, putative	XP_002785699.1	*Perkinsus marinus*	35%	1
FSSH 103	Red-type mitochondrial DNA	AB525761.1	*Apostichopus japonicus*	99%	1
FSSH 112	185-kDa silk protein	AAA99803.1	*Chironomus pallidivittatus*	41%	1
FSSH 116	Cell wall-associated hydrolase	CCB82302.1	*Lactobacillus pentosus* MP-10	49%	1
FSSH 122	Heat shock protein 70	EU930813.1	*Apostichopus japonicus*	99%	2

Table 3. Distribution of differentially expressed genes from the reverse SSH library.

Clone ID	Identification clones	Accession No.	Species	Identities	Redundancy
RSSH 2	Exonuclease domain	CBK94522.1	*Eubacterium rectale*	33%	1
RSSH 5	Cadherin-5-like	XP_003417144.1	*Loxodonta africana*	42%	1
RSSH10	Serpentine receptor	NP_504761.2	*Caenorhabditis elegans*	37%	1
RSSH 21	HMG176 isoform D	AEA76318.1	*Mamestra configurata*	40%	1
RSSH 22	Hypothetical protein SPW_4421	ZP_09404117.1	*Streptomyces* sp W007	33%	1
RSSH 25	Very long-chain acyl-CoA synthetase isoform 1	XP_001336957.1	*Danio rerio*	52%	3
RSSH 27	Bifunctional inhibitor/lipid-transfer protein	NP_174848.1	*Arabidopsis thaliana*	35%	1
RSSH 33	Sucrose-6-phosphate hydrolase	ZP_07823322.1	*Streptococcus pseudoporcinus*	36%	1
RSSH 55	NADH dehydrogenase subunit A	ZP_06591397.1	*Streptomyces albus*	38%	2
RSSH 58	CorA-like protein	ZP_08244847.1	*Streptococcus parauberis*	34%	1
RSSH 70	LOC100004228 protein	AAI17651.1	*Danio rerio*	52%	1
RSSH 75	GL27217	XP_002023794.1	*Drosophila persimilis*	65%	1
RSSH 76	CG4168	AAF53442.5	*Drosophila melanogaster*	38%	1
RSSH 91	Fibronectin type-III domain-containing protein 3A	XP_787929.2	*Strongylocentrotus purpuratus*	64%	2
RSSH 106	THAP domain-containing 10-like	XP_002737779.1	*Saccoglossus kowalevskii*	38%	1
RSSH 132	NADH dehydrogenase subunit 5	YP_004347529.1	*Megacopta cribraria*	40%	2
RSSH 143	Novel protein with an immunoglobulin V-set domain	CAX13578.1	*Danio rerio*	28%	1
RSSH147	Filamin isoform A	AAG43431.1	*Drosophila melanogaster*	68%	1

Expression patterns for differentially expressed genes

To confirm the results of the subtracted libraries, 6 ESTs were selected for expression profile analysis using qPCR. Overexpression of 5 genes was observed in the FGG, whereas a downregulation expression profile was only identified for ferritin (Figure 4). ANOVA revealed significant differences for arginine kinase, cytochrome *c* oxidase, HSP70, the ADP-ribosylation factor, and actin between growth groups. However, no significant difference was observed for ferritin.

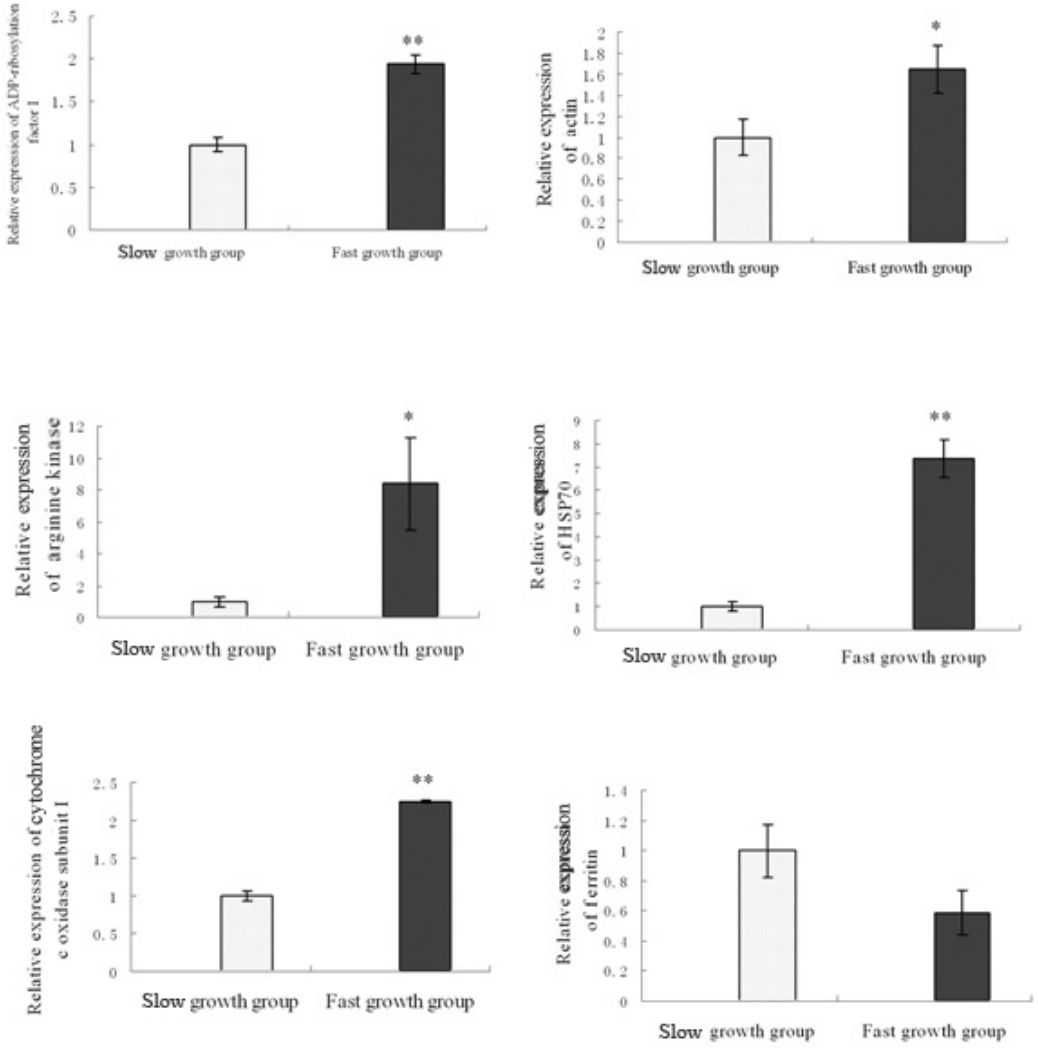

Figure 4. Expression profiles of six selected candidate genes by qPCR using 18S rRNA as the internal control. Each symbol and vertical bar represents the mean ± SD (N = 6). Significant differences from the control are *P < 0.05 and **P < 0.01.

DISCUSSION

As one of the most powerful methods for isolating differentially expressed genes, SSH has been successfully applied for a variety of organisms. In the present study, growth-related genes were investigated and characterized in sea cucumbers exhibiting fast-growth and slow-growth phenotypes. A total of 57 matched ESTs were annotated and divided into 6 categories. Six candidate genes were randomly selected and further evaluated using qPCR, with 5 genes showing significant upregulation compared to the control group (Figure 4).

Unexpectedly, β-actin was the most frequently encountered molecule, with 7 redundancies in our 142 sequencing samples. Significant differences in β-actin expression between the FGG and SGG were also confirmed by a quantitative analysis, implying that it participated in the regulation of sea cucumber growth. This finding was consistent with the fact that β-actin plays important roles in the structure of the cytoskeleton, cell division and motility, and muscle contraction by facilitating the ubiquitination/degradation of the growth hormone receptor (Rico-Bautista et al., 2004; Zhu et al., 2005). In addition to β-actin, 2 growth-related genes validated in other species were also identified in our study: FGF and VEGF. These candidates were not included in subsequent expression analyses due to failure in designing effective primers for qPCR. In higher animals, FGFs have been demonstrated to promote the proliferation, growth, and regeneration of different cells as a powerful mitogen (Baird, 1994). On the other hand, VEGF plays a central role in the maintenance of the human vascular system (Tammela et al., 2005) and promotes the formation of endothelial permeability fibers and cellular elongation in mammals (Ferrara, 1999). Considering that little is known about the function of FGFs or VEGF in regulating *A. japonicus* growth, our future study will address how these molecules might regulate the growth of sea cucumber, focusing on their expression profiles during different developmental stages.

Many studies have revealed connections between growth and energy metabolism (Stelling et al., 2002; Picha et al., 2009; Overturf et al., 2010). To achieve efficient growth, organisms have evolved a finely tuned mechanism to tightly balance energy partitioning and nutrient utilization. Energy metabolism-related enzymes are also considered to be of major importance in regulating growth. Along with the Ras superfamily of regulatory GTPases, the ADP ribosylation factor is part of the Arf family of proteins. These proteins are involved in a diverse spectrum of cellular activities, including regulating cell growth and signal transduction, the organization of the cytoskeleton, the maintenance of organelle structures, and regulating membrane trafficking along the exocytic and endocytic pathways (Sebald et al., 2003; Donaldson and Honda, 2005; D'Souza-Schorey and Chavrier, 2006; Myers and Casanova, 2008). In yeast, the disruption of ARF1 resulted in slow growth and cold sensitivity (Stearns et al., 1990). Another key energy metabolism factor, arginine kinase, belongs to a class of kinases that play roles in the maintenance of ATP levels through the phosphorylation of so-called "phosphagens", which then serve as high-energy sources from which ATP can be rapidly replenished (Pereira et al., 2000).

There has been some evidence that immunity genes are also important components in growth regulation. Ferritin plays a key role in cellular iron metabolism, which includes iron storage and detoxification, processes that have been documented in *A. japonicus* (Li et al., 2011a). Recently, more attention has been paid to the involvement of ferritin in the immunological defense system under external stress (Cullen et al., 2000). Ferritin can protect organ-

isms by sequestering heavy metals (He et al., 2009). Similar to ferritin, HSP70 plays essential roles in protein metabolism under both normal and stressed conditions (Kiang and Tsokos, 1998). The expression of HSP70 was induced by heat and also by the exposure to insecticides and heavy metals, such as cadmium and copper (Yoshimi et al., 2009). Heavy metals could damage the physical performance of sea cucumber and lead to growth retardation, even resulting in death (Sun et al., 2009). The function of molecular chaperones may be to stabilize and protect growth-related proteins, resulting in the ultimate mediation of sea cucumber growth.

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