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Identification of immune-related genes in adductor muscle tissue of the Japanese scallop *Mizuhopecten yessoensis* by expressed sequence tags analysis

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ABSTRACT

The Japanese scallop *Mizuhopecten yessoensis* is economically important shellfish in Asia. In this study, a normalized cDNA library of the scallop's adductor muscle of *M. yessoensis* was constructed. A total of 3061 unigene were identified. BlastX homology analysis indicated that 1522 of these unigenes were homologues of known genes. Interestingly, based on sequence similarities 49 immune-related genes were identified in the cDNA library of adductor muscle. These unigenes related to immunity were classified into 5 broad groups according to their predicted functions: immune recognition, stress response genes, proteases and protease inhibitors, cell signal transduction factors and other genes. The numerous immune genes discovered in this study may provide us with important information on how to efficiently modulate immune and stress responses to diseases and environmental pressure in mollusks.

Key word: Expressed sequence tag; Immune; Japanese scallop; *Mizuhopecten yessoensis*

The Japanese scallop, *Mizuhopecten yessoensis*, widely inhabits the cold coasts of the northern islands of Japan, the northern part of the Korean Peninsula, Sakhalin and the Kuril Islands. As commercially important marine product, high demand has driven the growth of a scallop aquaculture industry in Asian countries including Korea, Japan, and China. Scallop adductor muscle is the main edible part, which is rich in nutrition. The adductor muscle is in attention of scientists as it offers an excellent model for understanding muscle physiology. In this study, a normalized cDNA library of the scallop's adductor muscle of *M. yessoensis* was constructed. A total of 5334 ESTs were sequenced, from which 3061 unigenes were identified. Blastx homology analysis indicated that 1522 of these unigenes were homologues of known genes. Interestingly, 49 immune-related genes were identified in the cDNA library based on sequence similarities.

Immune-related genes play an important role in the growth of cultured animals because they can prevent certain diseases. Traditionally, immune genes were studied by gene clone and protein expression characterized, and finally its function was determined (Nilsen *et al.* 1999). To prevent infectious diseases, genes related to immunity will be helpful for early elimination of pathogen of contaminated individuals. Expressed sequence tags (ESTs) analysis is one of the most effective ways for gene discovery and genome mapping in many aquaculture species (Song *et al.* 2006, Wang *et al.* 2009).

Immune genes are usually expressed in immune organs. However many immune genes were also expressed in other organs, like body wall, adductor muscle, abdominal muscle and mammary gland. Skeletal muscle cells can actively participate in local immune reactions (Hohlfeld and Engel 1994). Furthermore, myoblasts can secrete or express various cytokines, chemokines and cell adhesion molecules, as well as receptors of the innate immune system (Figarella-Branger *et al.* 2003). In recent years, there were some studies about immune-related genes in shellfishes, such as *Chlamys farreri* (Cong *et al.* 2009, Wang *et al.* 2009), *Mytilus edulis* (Charlet *et al.* 1996), *Ruditapes philippinarum* (Kang *et al.* 2006), *Ruditapes decussatus* (Prado-Alvarez *et al.* 2009), *Pinctada fucata* (Zhang *et al.* 2009). However, the reports about immune-related genes in adductor muscle are few. Our work was originally focused on identifying growth-related genes of the scallop's adductor muscle. However in our study many immune-related genes were found. These immune-related genes found in muscle-specific normalized cDNA library could be used as markers for selective breeding programs and disease resistance of cultured *M. yessoensis*.

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MATERIALS AND METHODS

Three healthy male individuals of *M. yessoensis* (18-months-old) were sampled from Zhangzi Island in Dalian, China. They were cultured on ropes suspended from rafts in natural cultured sea area. The adductor muscles were removed and immediately placed in liquid nitrogen for total RNAs extraction and cDNA library construction. A cDNA library from *M. yessoensis* adductor muscle was constructed and large-scale ESTs were sequenced to study functions of genes of *M. yessoensis* (Meng *et al.* 2010).

Partial nucleotide sequences of the 5' -end of the cDNAs inserts were determined by single-pass sequencing using M13 primer on DNA Analysis System. All of the ESTs were assembled into unigenes using PHRED/PHRAP/CONSED software (http://www.phrap.org). To infer putative functions of these unigenes, the Blastx programs were used to perform similarity searches of the unigenes against the GenBank nonredundant protein database at National Center for Biotechnology Information (NCBI) with a cut-off E value $\leq 10^{-5}$.

RESULTS AND DISCUSSION

A total of 5334 single clones were selected from the library for high-throughput DNA sequencing, of which 4595 high quality ESTs were recovered. These ESTs have been submitted to GenBank (GenBank Accession No. GT067737–GT067746; GT086406-GT087795; GT565072-GT566465; GT568893-GT570693). In total, 3061 unigenes with an average length of 544 bases (ranging from 266 to 2364 bases) consisting of 654 contigs and 2407 singletons were assembled.

Of the 3061 uniquegenes, 1522 uniquegenes were annotated based on sequence similarity searches of GenBank nonredundant protein database. Surprisingly, 49 uniquegenes related to immunity were found (Table 1). These unigenes related to immunity were classified into 5 broad groups according to their predicted functions: immune recognition, stress response genes, proteases and protease inhibitors, cell signal transduction factors and other genes. These immune genes found in the adductor muscle cDNA library can offer information of immune functions of *M. yessoensis*.

Among the genes related to immune recognition, a peptidoglycan recognition protein S1 precursor was found in our cDNA library indicating that there are peptidoglycan recognition proteins (PGRPs) in the body of *M. yessoensis* (Acc. No. GT087277). PGRPs are a family of pattern-recognition receptors binding to bacterial peptidoglycan which are highly conserved from insects to humans (Kang *et al.* 1998). PGRP cDNA sequences have been cloned from adductor muscle in bay scallop (*Argopecten irradians*) and Zhikong scallop (*Chlamys farreri*). Galectins play important roles in animal innate immune responses. Galectin found in our study (Acc. No. GT565257) was also found in some other shellfishes. Autophagy is an important form of programmed cell death that regulates the growth and development of cells. Autophagy is related to the pathogenesis of several immune-mediated diseases (Hussey *et al.* 2009). The EST of autophagy protein gene was obtained from the cDNA library indicating that *M. yessoensis* has autophagic functions to defend against intracellular pathogens (Acc. No. GT087102).

Among the genes related to stress response genes, ferritins are ubiquitous and conserved iron storage proteins of most living organisms and have been indirectly linked to innate immune response (Zandman-Goddard and Shoenfeld 2007). In our study, two ferritin related genes were found (Acc. No. GT569879; GT565349). In shellfishes, ferritin has been identified in *C. farreri* (Wang *et al.* 2009), *Argopecten irradians irradians* (Song *et al.* 2006), *C. gigas* (Gueguen *et al.* 2003) and *Sinonovacula constricta* (Feng *et al.* 2009). Heat shock proteins (HSPs) are well known for their role in host-defense against diseases and stresses (Robert 2003). Large number HSPs were found in this study indicating that they may be involved in a wide range of physiological processes in *M. yessoensis* (Acc. No. GT086752; GT569807; GT086783; GT569528; GT086564; GT565812; GT565708). Proteasomes are implicated in stress response by removing damaged, denatured, or misfolded proteins and involved in metabolic adaptation by degrading transcriptional regulators (Jiang *et al.* 2009). Five kinds of proteasomes-related unigenes were found in this study (Acc. No. GT566010 and GT570079; GT087675 and GT565089; GT56921 and GT569561; GT570676; GT086487).

Among the genes related to proteases and protease inhibitors, one EST related to cathepsin B was found in our study (Acc. No. GT569872). Cathepsins are considered as intracellular proteases capable of mediating caspase-independent cell death and can also act in concert with caspases in apoptotic cell death (Vasiljeva *et al.* 2007). Through past investigations, cathepsin has been also reported in several shellfishes. Some other proteases and protease inhibitors were also found, such as matrix metalloproteinase (Acc. No. GT568984), glutathione peroxidase (Acc. No. GT087671), adenosine deaminase (Acc. No. GT086999) and glutathione-S-transferases (Acc. No. GT570300; GT086811; GT086873; GT086626; GT566170; GT569283).

Among the genes related to cell signal transduction factors, integrin-associated protein belongs to immunoglobulin superfamily of membrane proteins. One EST matching to integrin in this study indicates that it has essential functions during the cell signal transduction of immune reaction process (Acc. No. GT087254). Tumor necrosis factor receptor-associated factor 6 (TRAF6) represents a key molecule in innate and antigen-specific immune responses against viral infection (Konno *et al.* 2009). TRAF6 found in our study can offer clues for the existence of Toll signaling pathway and TNF-a or TNF-a-like molecules in *M. yessoensis* (Acc. No. GT087069).

Among the other genes, astacin is a kind of natural

Table 1. Unique genes (contigs and singletons) related to immunity from *M.yessoensis* muscle tissue

Category	GenBank Accession No.	Homology ID	E-value	Annotation
Immune recognition	GT565257	gb|ABG75998.1|	1.00E–33	galectin
	GT087102	emb|CAJ31284.1|	2.00E-06	autophagy protein 5
	GT087585	gb|AAT97080.1|	1.00E-48	B cell receptor associated protein-like protein
	GT570593	ref|NP_291079.2|	7.00E-21	B-cell CLL/lymphoma 3
	GT568951	gb|ACH92125.1|	2.00E-37	B-cell translocation gene 1
	GT566115	gb|EEB18255.1|	2.00E-46	immunodeficiency virus type I enhancer binding protein
	GT565942	ref|XP_001893973.1|	1.00E-10	Immunoglobulin I-set domain containing protein
	GT087277	gb|AAY53765.1|	1.00E-116	peptidoglycan recognition protein S1 precursor
Proteases and	GT086999	gb|ACC69106.1|	5.00E-23	adenosine deaminase-like protein
protease inhibitors	GT569849	gb|ACO52475.1|	3.00E-32	RNA transcript variant b adenosine deaminase
	GT569872	emb|CAH04630.1|	3.00E-46	cathepsin B
	GT087671	ref|NP_001007282.2|	6.00E-56	glutathione peroxidase 1
	GT570300	gb|ACO08456.1|	2.00E-23	Glutathione S-transferase kappa 1
	GT086811	emb|CAE11863.1|	7.00E-22	glutathione S-transferase sigma class protein
	GT086873	gb|ABO26600.1|	2.00E-49	omega class glutathione-s-transferase 1
	GT086626	gb|ABO26601.1|	5.00E-38	omega class glutathione-s-transferase 2
	GT566170	ref|XP_001193599.1|	5.00E-07	microsomal glutathione S-transferase 3-like
	GT569283	gb|ABO26603.1|	5.00E-31	sigma class glutathione-s-transferase 2
	GT568984	gb|AAY85550.1|	1.00E-132	matrix metalloproteinase
	GT569364	gb|ACR15167.1|	3.00E-11	cysteine protease inhibitor
Stress proteins	GT569472	gb|AAR11779.1|	4.00E-47	cyclophilin A
	GT569879	gb|AAV66906.1|	5.00E–75	ferritin CFC
	GT565349	gb|AAQ12076.1|	1.00E-47	ferritin-like protein
	GT086752	ref|NP_001012934.1|	3.00E-77	heat shock 60kDa protein 1
	GT569807	ref|NP_001095497.1|	7.00E-19	heat shock 70kD protein 12B
	GT086783	dbj|BAA11036.1|	3.00E-59	heat shock protein 105 kDa beta
	GT569528	gb|EEC06453.1|	1.00E-21	heat shock protein 20.6
	GT086564	gb|AAS17724.1|	2.00E-48	heat shock protein 70
	GT565812	gb|ABS50431.1|	4.00E-75	heat shock protein 90
	GT565708	ref|XP_514486.2|	1.00E-22	heat shock 70kD protein 12B
	GT565980	dbj|BAE78580.1|	4.00E-79	manganese-superoxide dismutase
	GT566302	gb|ABD58974.1|	1.00E-65	superoxide dismutase
	GT566010	ref|NP_001135241.1|	9.00E-42	26S proteasome non-ATPase regulatory subunit 12
	GT570079	gb|EEC04613.1|	5.00E-35	26S proteasome non-ATPase regulatory subunit
	GT087675	ref|XP_001925937.1|	2.00E-65	proteasome 26S subunit, non-ATPase, 1
	GT565089	gb|EDL38769.1|	5.00E-67	proteasome 26S subunit, non-ATPase, 4
	GT569211	ref|NP_001132918.1|	1.00E-115	proteasome subunit, alpha type, 6
	GT569561	gb|EDL26822.1|	9.00E-73	proteasome subunit, beta type 7
	GT086487	gb|ABO26645.1|	6.00E-54	proteasome alpha type 2
	GT570676	gb|ABO26681.1|	4.00E-64	proteasome subunit N3
Signal transduction	GT087254	gb|EDK99884.1|	1.00E-46	integrin alpha FG-GAP repeat containing 2
	GT087069	gb|ABC73694.1|	1.00E-114	TRAF6
	GT569461	gb|ABI79459.1|	8.00E-29	LPS-induced TNF-alpha factor
Other genes	GT569536	ref|XP_001845663.1|	5.00E-33	15 kDa selenoprotein
	GT569296	gb|ABI93178.1|	2.00E-11	selenoprotein M
	GT086930	ref|NP_840072.3|	6.00E-14	selenoprotein W, 1
	GT569492	gb|AAX56337.1|	2.00E-14	astacin-like protein
	GT570636	gb|AAV73827.1|	8.00E-26	thioredoxin
	GT568936	gb|ACQ72915.1|	1.00E-24	bactericidal permeability increasing protein

carotenoids. With highly active anti-oxidant properties astacin can enhance immunity through increasing resistance to viral, bacterial, fungal and parasitic organisms (Cai *et al.* 2003). Thioredoxin plays an important role as a costimulatory molecule involved in immune processes (Schenk *et al.* 1996). Anti-microbial peptides are important components of the innate immune systems of most living organisms. The bactericidal permeability-increasing protein is a highly conserved host-defence molecule produced and stored by myeloid cells (Elsbach 1994). These immune-related genes encourage further studies on possible immune functions of *M. yessoensis* as found in other species.

The mining of EST databases for immune-related genes holds great potential for addressing questions in host-defense mechanisms and for the disease control in aquaculture species. Invertebrates are believed to lack adaptive immunity. They rely exclusively on their innate immune response to efficiently defend themselves against a variety of pathogens. However, several adaptive immune-related genes have been recently discovered in invertebrates (Loker *et al.* 2004), such as the fibrinogen-related proteins of mollusks and the immunoglobulin-type variable region containing chitin-binding proteins of amphioxus. Some immune-related genes were previously thought to be specific to vertebrate immunity but are found in various invertebrates. One example is complement component 3 which was first identified as an EST from lipopolysaccharide activated coelomocytes of the purple sea urchin, *Strongylocentrotus purpuratus* (Smith *et al.* 1996).

Although adductor muscle is not an immune organ, there were immune genes found in adductor muscle tissues (Roberts and Goetz 2003). We focused on the identification of immune-related genes which are concern in cultured bivalves. The biological function and importance of these genes in *M. yessoensis* have yet to be confirmed, but they provide candidate genes for further characterization. The numerous immune genes discovered in this study may provide us with important information on how to efficiently modulate immune and stress responses to diseases and environmental pressure in mollusks.

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