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Differential gene expression analysis implicates wild *Magallana bilineata* (Röding, 1798) to be in an enhanced immunological status than laboratory-maintained oysters

Esha Arshad, MA Pradeep, CS Subin and KK Vijayan

Abstract
Oysters are filter-feeding bivalves thriving in estuaries and inter-tidal zones. As sessile invertebrates, oysters have evolved a well-developed stress tolerance mechanism to tolerate various environmental stressors such as altered hydrological parameters, microbial pathogens, and anthropogenic influences. The focus of this study was to identify the key genes which enable the Indian oyster (*Magallana bilineata*) to overcome the turbulent conditions in their environment. Suppression subtractive hybridization (SSH) technique was used to generate the differentially expressed genes in wild *Magallana bilineata* in comparison to laboratory-maintained ones. The method revealed twenty functionally relevant genes with roles in immunity, stress, cellular processes, cytoskeleton, and lipid metabolism. The diverse set of genes obtained from the SSH library are known to have direct and indirect roles in oyster immunity, suggesting that the wild oysters are more immune stimulated and active than laboratory-maintained ones. The expression of ten potentially upregulated genes was analysed using quantitative real-time PCR.

Keywords: *Magallana bilineata*, SSH, stress, immunity, real-time PCR

1. Introduction
The marine environment constitutes the most mercurial of all habitats owing to its seasonal climatic fluctuations and increasingly, due to anthropogenic threats. These changes vastly influence the aquatic ecosystems, especially, coastal and estuarine habitats and cause significant physiological stress on the animals [1, 2]. These animals in such a challenging environment may flourish or perish, depending on its capability to acclimatize to natural variations and anthropogenic factors [3]. Learning the mechanisms by which marine organisms attune to the local disturbances is pivotal to gain a better perspective of their physiological tolerance limits as well as the plasticity of the ecosystem.
Molluscs such as oysters are key ecosystem engineers and play vital roles in the effective functioning of marine, freshwater and terrestrial ecosystems [4]. They are model organisms to study the effect of climatic change on marine ecosystems. Oysters, in their natural environment, are exposed to a range of environmental stressors which they withstand to thrive. Stressors like altered abiotic hydrological parameters including temperature, salinity, and pH occur regularly in their habitat [5, 6]. The oysters are vulnerable to a motley of microbes in their waters which they accumulate due to their filter feeding nature and are also periodically exposed to air during daily and seasonal cycles [7]. In addition to all this, several anthropogenic factors like water and sediment pollution can have adverse effects on the animal. Increased pollution also increases the susceptibility of the oyster to aquatic epizootics [8].
The resilient nature of these immobile animals can be attributed to their phenotypic plasticity and modification of gene expression to maintain cellular homeostasis [9]. Cues regarding the complex mechanisms by which the oysters get its sturdiness have been opened up by the recent whole-genome sequencing of the Pacific oyster. Researchers were able to confirm the role and interplay of a multitude of genes that assist in environmental adaptation [10, 4]. With the help of various genomic tools, it is possible to identify the specific set of genes which can regulate the essential physiological processes required for the animals to cope with the changing environment.

Numerous studies on the impact of environmental parameters on the transcriptome of oysters have been conducted and have identified genes or suites of genes involved in functional pathways that help combat stress [11, 3, 12, 13, 14, 15]. Most of these studies have focussed on the effect of single stress on the animal *in vitro* and described genes associated with that stress. This situation is unlike their natural environment, where multiple stressors act simultaneously leading to a complex stress landscape producing a different physiological response [3]. Meta-analysis studies have tried to identify the critical molecular systems that are common to a broad range of stressors, by pooling microarray and suppression subtractive hybridization (SSH) analysis data of oysters exposed to environmental parameters [16]. However, very few works have been conducted to analyse the gene expression of the bivalves in response to the combined effects of all the physicochemical conditions in the environment.

Magallana bilineata, (previously known in India and the Indian sub-continent as *Crassostrea madrasensis* (Preston, 1916) or the Indian backwater oyster), is among the most common and widely distributed oysters found along the Indian subcontinent. In India, it is distributed along the coastal regions of the Arabian Sea and the Bay of Bengal occupying the estuaries, bays, intertidal zones and backwaters [17]. These oysters are euryhaline and are widely cultivated in the southern states of India as a food source owing to their high nutritional value [18] and as a raw material for many small-scale industries. The Kodungallur-Azhikode estuary (part of the Cochin backwater system, Kerala, India), which constitutes our study area as the "wild environment" is a microtidal estuary which drains into the Arabian Sea. The estuary shows remarkable variation in environmental parameters during seasonal fluctuations, and recently, the Trophic Index TRIX analysis of the estuary showed apparent eutrophication as a consequence of severe anthropogenic influences [19, 20]. The Cochin backwaters constitute the largest wetland ecosystem in the southwest coast of India and is also a heavily polluted estuary owing to continuous discharge of industry effluents and untreated domestic waste [21].

In this work presented here, an effort was made to learn the transcriptomic profile of the Indian oyster, *M. bilineata*, in its natural environment as compared to those maintained in laboratory conditions. It will enable us to have a fair understanding of the common genes expressed by the organism to tide over the sub-optimal conditions often present in the wild. We performed a suppression subtractive hybridization to find out the up-regulated genes in the digestive tissues of the oysters from natural habitat (wild conditions), against the oysters maintained in controlled laboratory conditions. The SSH analysis revealed an array of genes associated with many physiological processes such as lipid metabolism, cellular functions, immunity and, energetic metabolism, expressed by the oysters of the Kodungallur-Azhikode estuary as part of their daily belligerence to survival. The various number of immune-related genes obtained from the SSH library indicates that wild *M. bilineata* are more immunologically stimulated and active than laboratory-maintained ones. Oysters are known to encode for a vast repertoire of immune genes and several gene families related to immune response are highly enriched. These sturdy animals also possess numerous functionally divergent immune genes which play multiple roles including biotic and abiotic stress management [22]; and thereby supporting its sustenance in the wild environment.

2. Materials and Methods

2.1 Oyster Collection & Treatment

Adult oysters were collected from Sathar Island region of Kodungallur-Azhikode estuary (10°11′26.34″N and 76°11′28.88″E), Ernakulam, India. Twelve wild oysters were immediately shucked, and digestive tissues were transferred to RNAlater stabilisation solution (Ambion life technologies, USA) for preservation until the time of the experiment. Another set of oysters were transported to wet laboratory (CMFRI hatchery) and maintained in controlled conditions. The animals were cleaned to remove grit and epiphytes and then acclimatized for seven days in aerated tanks with filtered water at 25-28ºC and salinity of 22 ppt. Microalgae *Isochrysis* and *Nanochloropsis* were fed to the oysters daily, except on the day before the experiment. At the end of the acclimatization period, 12 control oysters were sacrificed and digestive tissues transferred to RNAlater stabilisation solution.

2.2 Environmental Analysis

In order to document the critical environmental parameters that influenced the wild (test) oysters, water samples were simultaneously collected from the oyster sampling site and analysed. Water from oyster tanks was used to analyse the 'control' environmental conditions. Water temperature and salinity were determined at the site using a precision thermometer and `salinometer respectively. pH and turbidity were measured using pH meter Cyberscan PC5500 (Eutech Instruments, Singapore) and a digital turbidity meter, Micro1000 IR (HF Scientific, USA) respectively. Samples for dissolved oxygen analysis were fixed at the site and measured using modified Winkler's method. The chlorophyll -a contents, dissolved inorganic nutrients *viz* ammonia-nitrogen (NH4–N), nitrate-nitrogen (NO3–N), nitrite-nitrogen (NO2–N), inorganic phosphate-phosphorus (PO4–P) were collected by standard methods and computed using spectrophotometric methods [23, 24].

2.3 RNA Extraction

Total RNA from digestive tissue of all sets of samples *viz*. wild, control, were isolated using TRIzol reagent (Ambion life technologies, USA) as per manufacturer's protocol. For both wild and control samples, an equal weight of tissue from 5 oysters was mixed and used for RNA isolation. Poly A^+ RNA from all pools of total RNA was isolated using Oligotex mRNA mini kit (Qiagen, USA), also following manufacturer's instructions. The integrity of total RNA as well as purified mRNA was checked using a spectrophotometer, Biophotometer plus (Eppendorf, Germany), at 260 nm and further in 1.5% agarose gel electrophoresis.

The digestive glands of bivalves are critical organs involved in functions ranging from carrying out basic processes such as endo-cellular digestion to essential roles such as detoxification of many organic and inorganic toxic substances [12, 25]. Due to their roles in accumulation and detoxification of xenobiotics, the digestive glands are sites of multiple oxidative reactions and hence, involved in strong free radical generation. Furthermore, changes in temperature and food availability also induce oxyradical generation in the digestive organs, thereby causing an increase in antioxidative defence [26]. Bivalves exposed to hypoxia, heavy metal contamination, and other environmental pollutants, showed organ-specific overexpression of HSP 70, Metallothionines, and enzymes such as catalase and superoxide dismutase in digestive glands

[26, 12, 13]. Thus, the digestive glands of oysters are of significance in determining the oysters' stress response to long-term, chronic exposure to stressors in the environment [27].

2.4 Suppression Subtractive Hybridization (SSH)

The SSH assay was performed by adhering to the PCR select cDNA subtraction kit procedure (Clonetech, USA), utilizing two µg of wild and control Poly A+ RNA as starting material. Hybridization and subtraction procedures were performed in the forward direction to obtain the up-regulated genes in the wild (tester) sample, in comparison with the control (driver) sample. The differentially expressed PCR products obtained after subtraction were cloned onto pJET1.2 plasmid using CloneJET PCR cloning kit (Thermo Fisher Scientific, USA) and transformed to TOP10 competent cells. A total of 150 random clones from the SSH library were screened, and positive clones were grown in LB liquid medium with Ampicillin ($100mgL^{-1}$) for plasmid isolation using GeneJet plasmid miniprep kit (Thermo Fisher Scientific, USA). The sequences generated from the plasmids were checked for sequencing errors, removed of vector and adaptor sequences and contig aligned with the help of Seqman (Lasergene) assembly program. After that, the sequences were checked for homology to known sequences in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). EST's with significant match values (E values < 1×10^{-5}) were assigned putative functions using QuickGO (www.ebi.ac.uk/QuickGO). The EST sequences were submitted to the GenBank database (GenBank accession no's from MH887438 to MH887466).

2.5 Real-Time PCR Analysis

Real-time PCR was used to investigate the expression of 10 candidate genes identified by SSH. The selected genes were: (i) Mitochondrial ATP Synthase Lipid Binding Protein (ii) Pancreatic triacylglycerol lipase-like (iii) Astacin like protein (iv) Ferritin (v) Calreticulin (vi) ATP dependent RNA polymerase (vii) Poly ADP ribose polymerase (viii) Apoptotic chromatin condensation inducer in the nucleus (ix) Rhamnose binding lectin and (x) Snaclec B6 like Lectin. 18S ribosomal gene was used as the PCR internal control to normalize the gene expression by real-time PCR.

1 µg of total RNA from digestive tissues of wild and control samples were DNAse treated to remove genomic DNA contamination and then reverse transcribed using iScript cDNA synthesis kit (Biorad, USA). The cDNA generated was diluted and used as a template in a 20 µl reaction mixture with 5 µl iQ SYBR green supermix (Biorad, USA), sterile water and each specific primer pairs. The PCR reaction conditions included 10 minutes of DNA denaturation at 94ºC, followed by 40 amplification cycles of 94ºC – 10 seconds and 60ºC – 30 seconds each. A final melting curve analysis programme from 99^0C to 55ºC decreasing by 0.5º C every 10 seconds was carried out to ensure that a single specific product was detected. All real-time PCR experiments were conducted with biological triplicates in ROCHE (Switzerland) light Cycler 96 system. The list of primers used in real-time PCR are given in table 1.

Real-time expression data are presented in this study, as the change in relative expression, normalized to the reference gene (ribosomal 18S), using the method explained by Pfaffl [28]. The relative expression is determined using the equation:

Expression Ratio = $\{(E_{target})^{\triangle Cttarget\ (Control-Sample)}\}/\{(E_{ref})^{\triangle Ctref\ (Control-Sample)}\}$;

where E_{target} and E_{ref} are the PCR efficiency of the target and reference gene respectively, and Ct is the crossing threshold.

Table 1: List of primers used for real-time PCR

	Gene	Primer Sequence
1.	ATP synthase lipid binding protein, mitochondrial like-F	CCACATGCGTAGTGAAAGCC
2.	ATP synthase lipid binding protein, mitochondrial like-R	CCAGCTGCCAAAAGGAAACA
3.	Pancreatic Triacyl glycerol lipase like- F	CGAGGTGATCCTAGTGACGC
4.	Pancreatic Triacyl glycerol lipase like-R	CACGAAATCGGCGTCACATC
5.	Astacin like-F	CTGCTTCAGCGGGTATTTCC
6.	Astacin like-R	GGATGGGGCATGGCTATTGA
7.	Ferritin- F	ACGCCTTGACTCCTAAAGCC
8.	Ferritin- R	CCTCCGTTACCGATTCCCAA
9.	Calreticulin-F	AGCCACCCCAGATTGACAAC
10.	Calreticulin- R	AGTCTGGGGTGTACTCTGGG
11.	ATP dependent RNA helicase-F	GAGGAGCATCGGTTCAACAAGA
12.	ATP dependent RNA helicase-R	TCCAGGTCAGTCCTCAGCTG
13.	Poly ADP ribose polymerase-F	CTGTGCCTCTCCCCAGTGTA
14.	Poly ADP ribose polymerase-R	AGGATCCAGAAGGCACTCCA
15.	Apoptotic chromatin condensation inducer in the nucleus-F	AGAAGAACAGGCAGCAGCTC
16.	Apoptotic chromatin condensation inducer in the nucleus-R	CACGCCATGGACTTCTCTCC
17.	Rhamnose Binding Lectin-F	ACCTGTTCGAAGTCATGGCAC
18.	Rhamnose Binding Lectin-R	GACTCTACAGAAGCGTCTACCAC
19.	Snaclec B6 Like lectin-F	GATCTGGGCCTCCAATGGAC
20.	Snaclec B6 Like lectin-R	GTAGGCCTCGGAGTGACAAC

2.6 Ethical Statement

All experimental protocols were approved and regularly assessed by the Institute Research Council (IRC) of ICAR-Central Marine Fisheries Research Institute. The Priority Setting, Monitoring, and Evaluation (PME) cell of ICAR-Central Marine Fisheries Research Institute have approved the manuscript for submission considering set criteria, including ethical aspects

3. Results

3.1 Environmental Analysis

Table 2 gives the values of environmental parameters for the wild environment and laboratory conditions considered in this study. The oysters and circumambient water samples were collected from the Sathar Island region of Kodungallur-Azhikode estuary during May (2016), which is a cusp period of the Pre-monsoon season. Water temperature at the site during sampling was found to be on the warmer side, at 31ºC and salinity at 15 ppt. In comparison, the temperature and salinity of the laboratory oyster tanks were maintained to be at 28ºC and salinity at 22 ppt, congenial to the oysters [29,30]. The dissolved oxygen levels and the inorganic nutrient contents of the sampling site were sub-optimal; but typical of any productive, tropical estuarine system. The inorganic

nutrient levels in the laboratory tanks were below the detectable levels.

Table 2: Environmental parameter variables observed at the time of oyster sampling.

Environmental Parameter	Sampling Site	Laboratory
Temperature (°C)	31	28
pH	7.44	7.42
Salinity (ppt)	15	22
Turbidity (NTU)	6.60	0.05
DO (mg/ml)	4.46	5.82
Chlorophyll-a (mg/m^3)	8.0	--
NH_4^+ (µmoles/L)	2.3	--
NO_2-N (µmoles/L)	0.695	--
NO_3-N (µmoles/L)	1.2	--
PO_4-P (µmoles/L)	0.5	--

3.2 Identification of Differentially Expressed Genes in Wild M. bilineata

The main objective of this study is to identify the differentially expressed genes in wild *M. bilineata* in contrast to the laboratory-maintained ones. A total of 150 positive clones from the SSH library were sequenced. The sequences obtained were contig aligned and evaluated using NCBI BLAST query search. The BLAST analysis results revealed the expression of twenty functionally important genes, three uncharacterized proteins and six ribosomal proteins from 25 contigs and 14 singletons. The genes identified from SSH can be clustered into the following functional categories: (i) Lipid Metabolism (ii) Stress/ Immunity (iii) Cell structure (iv) Cellular functions/ Replication/ Transcription/ Translation and (v) energetic metabolism (figure 1). Table 3 enlists the genes obtained from the SSH library.

Table 3: cDNA Clones Identified from SSH Library.

Putative gene/Category	e-Value	Homolog Species	Accession Number
Lipid Metabolism			
Pancreatic Lipase related protein1	2e-43	*Crassostrea gigas*	MH887449
Ganglioside GM2 activator	3e-33	*Crassostrea gigas*	MH887446
PREDICTED: Fatty acid-2-Hydroxylase	2e-44	*Crassostrea gigas*	MH887456
Stress/Immunity			
Carnosine Synthase like protein	1e-78	*Crassostrea gigas*	MH887442
L-Rhamnose binding Lectin CSL3	1e-10	*Crassostrea gigas*	MH887451
Calreticulin	1e-88	*Crassostrea gigas*	MH887441
Snaclec B6 like protein	3e-30	*Crassostrea gigas*	MH887452
Ferritin	8e-10	*Crassostrea ariakensis*	MH887445
Tumor necrosis Factor 14	2e-10	*Crassostrea hongkongensis*	MH887455
Cell Structure			
Tenascin-X	2e-30	*Crassostrea gigas*	MH887454
Collagen α-(VI) Chain	3e-45	*Crassostrea gigas*	MH887444
Energetic Metabolism			
ATP synthase lipid binding protein, mitochondrial like	1e-75	*Crassostrea gigas*	MH887447
Cellular Functions/Replication/Transcription/Translation			
Astacin Like protein	1e-98	*Crassostrea gigas*	MH887439
ATP dependent RNA helicase DDX24	3e-43	*Crassostrea gigas*	MH887440
Putative Helicase with Zinc Finger like domain	6e-11	*Crassostrea gigas*	MH887457
Poly ADP ribose polymerase 14	4e-64	*Crassostrea gigas*	MH887450
Cysteine and histidine domain-containing protein 1	7e-25	*Crassostrea gigas*	MH887443
Apoptotic Chromatin condensation inducer in the nucleus	1e-10	*Crassostrea gigas*	MH887438
PREDICTED: Splicing factor 3B subunit 6 like protein	5e-20	*Crassostrea gigas*	MH887453
Nascent polypeptide-associated complex subunit alpha-like	2e-44	*Crassostrea gigas*	MH887448
Unknown functions			
PREDICTED Uncharacterized protein LOC105337218	1e-71	*Crassostrea gigas*	MH887459
Uncharacterized LOC105317444	8e-21	*Crassostrea gigas*	MH887460
PREDICTED Uncharacterized protein LOC105319519	3e-80	*Crassostrea gigas*	MH887458
Ribosomal Proteins			
60S Ribosomal Protein L11	2e-08	*Crassostrea gigas*	MH887463
60S Ribosomal Protein L19	2e-82	*Crassostrea gigas*	MH887466
40S Ribosomal Protein S15 like	1e-33	*Crassostrea gigas*	MH887462
PREDICTED 60S ribosomal protein L22-like	1e-11	*Crassostrea virginica*	MH887464
60S ribosomal protein L40	3e-45	*Crassostrea hongkongensis*	MH887465
40S ribosomal S7	3e-71	*Crassostrea gigas*	MH887461

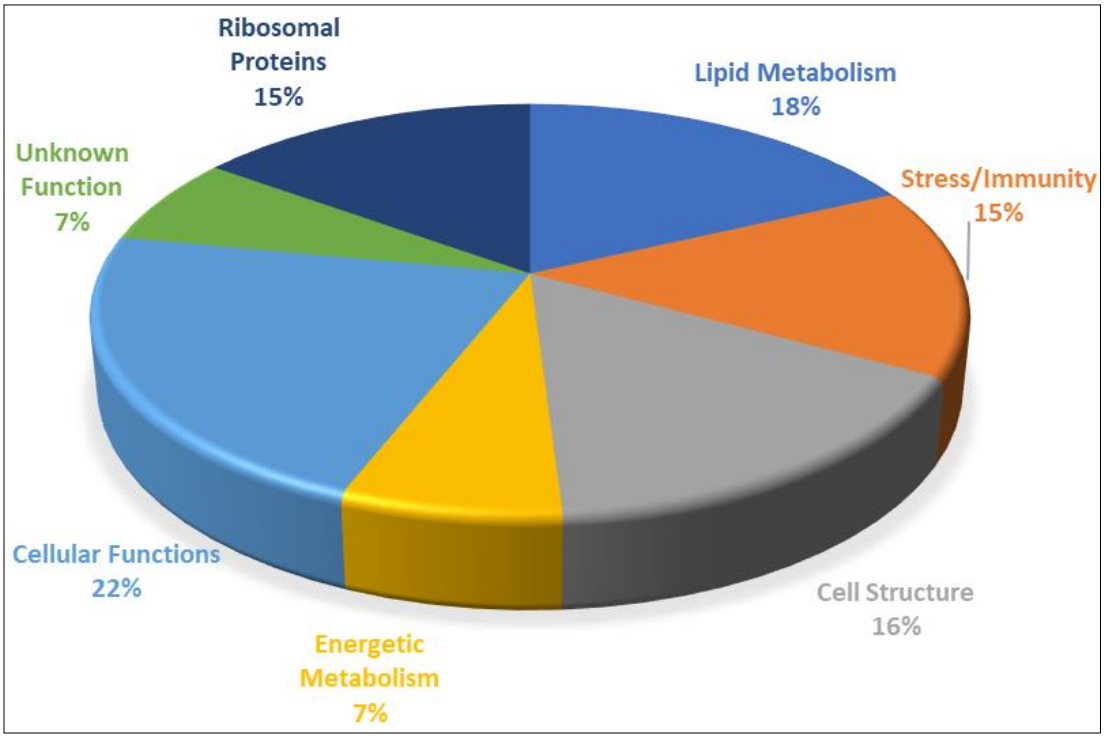

Fig 1: Functional classification of genes identified from SSH library

3.3 Real-Time Expression of Upregulated Genes.

Real-time PCR analysis was utilized to validate the SSH assay by assessing the relative expression of ten target genes obtained from the SSH library. The expression of the selected genes was studied and compared in wild and control samples of *M. bilineata*. Expression patterns of the different cDNA transcripts in various samples are shown in figure 2. mRNA transcripts of all the genes used for expression analysis were found to be higher in the wild (test) than the laboratory control.

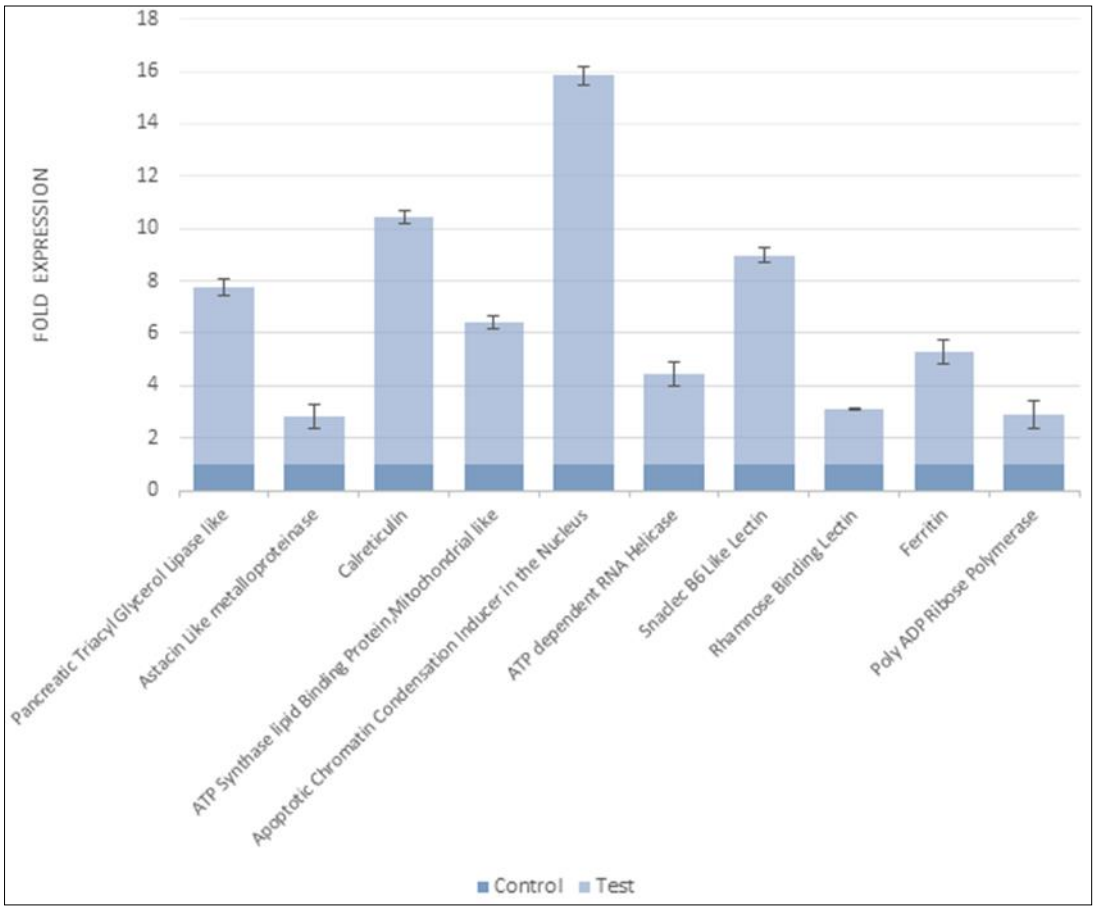

Fig 2: Differential expression of selected genes in wild oysters relative to control oysters using qRT-PCR. The expression is presented relative to the housekeeping gene 18S. The additional amount of relative gene expression in test compared to control is represented by light blue bars.

Eight among the transcripts *viz* Pancreatic triacylglycerol lipase-like, Calreticulin, Mitochondrial ATP synthase lipid

binding protein, Apoptotic chromatin condensation inducer in the nucleus, Snaclec B6 like lectin, ATP dependent RNA helicase, Rhamnose Binding Lectin, and Ferritin, were found to be significantly over-expressed in the wild oysters. The fold expression changes ranged from 13.846±0.35 for Apoptotic chromatin condensation inducer in the nucleus to 2.11±0.06 for Rhamnose Binding Lectin.

4. Discussion

Oysters are sturdy animals capable of overcoming unfavourable environmental conditions to successfully colonize the estuaries, creeks, bays, lagoons and intertidal areas. The highly polymorphic genome of the oyster combined with the complex interplay of a diverse set of genes is responsible for the oyster's adaptation to sessile life [5, 10, 31, 4, 32]. Numerous studies have tried to elicit the gene expression of oysters in response to a particular stress [11, 13, 14, 33, 15, 34] but very few have tried to learn the concerted gene expression of the animals in response to the slew of stressors existing in its natural environment. This work aims at acquiring a better understanding of the variety of transcriptomic reactions by which the oysters in its wild environment succeed over the turbulent conditions.

Using suppression subtractive hybridization, we were able to generate a clone library containing genes potentially up-regulated in the wild conditions alone. 150 clones processed from this library yielded 20 functionally essential genes, all of which showed high homologies with the genes of other *Crassostrea/ Magallana* species. The genes identified encoded for proteins involved in functions such as Immunity, lipid metabolism, energetic metabolism, extracellular matrix, and cellular functions. This agrees with a meta-analysis report conducted on several recent studies involving the gene expression of oysters in response to environmental stress [16]. They have described the most common genes altered due to stress as belonging to the mitochondrial electron transport chain, translation and protein processing, subcellular stress responses and cytoskeleton. It is also worth mentioning that all the genes identified through this study are first reports of the genes in the Indian oyster, *M. bilineata*.

Heightened cellular energy demand is amongst the primary responses of animals to environmental stress. This SSH library we generated showed the up-regulation of a nuclear gene mitochondrial like ATP synthase lipid binding protein, which is a mitochondrial protein likely involved in energy production through ATP hydrolysis. Increased energy production fuels the adaptive cellular processes enabling it to overcome stress. However, this can also have a detrimental effect on the cells as overproduction of energy increases the amount of cytotoxic reactive oxygen species which in turn can affect the cytoskeletal and mitochondrial integrity. It is worth noting that in our work, 16% of the genes identified from the SSH library are involved in extracellular matrix maintenance. The main genes in this category include Tenascin-X, and Collagen-α-6 chain, both of which are components of the extracellular matrix participating in maintaining the cytoskeletal integrity.

Molluscs and other invertebrates are equipped with an advanced innate immunity to fight against the high microbial load present in the marine environment. The wild *M. bilineata* showed the up-regulation of two lectins, namely Rhamnose binding lectin and Snaclec b6 like lectin. Lectins have non-self-recognition properties and are hence thought to be involved in agglutination or opsonization during phagocytosis [5, 35, 36]. The SSH analysis revealed the expression of Ferritin, which is also a gene involved in immunity by regulating iron concentration to destroy microbial agents and protecting cells from oxidative damage [37, 38]. The elevated expression of the described genes in the wild samples was expected, due to the endless variety of pathogens and parasites in the marine waters which are accumulated by the filter feeding oysters.

The vast majority of the proteins differentially expressed in the SSH clone library are those involved in crucial cellular processes and among that is the gene ATP dependent RNA helicase DDX24, which is an RNA helicase molecule functioning in ribosome assembly, translation initiation, nuclear and mitochondrial RNA splicing. Also identified were several small but critical molecules acting as transcriptional activators, chaperons, and spliceosome assembly components; namely Helicase with Zinc finger domain, Cysteine and histidine-containing domain protein 1, and splicing factor 3b subunit-like protein. Cysteine and histidine-containing domain protein 1(CHORD1) has implicated functions as co-chaperone of Heat shock protein 90 and hence suspected to have roles in stress response and immunity [39]. Calreticulin, an endoplasmic reticulin chaperon molecule involved in maintaining cellular homeostasis, host immunity [40] and known to be activated by a variety of chemical and biological stresses [41], was also found to be up-regulated in wild oysters.

Effective stress response in an ancient but adept organism such as the oysters requires the synergistic activity of many genes. In molluscs, apoptosis constitutes an essential mode of immune response that is prompted by various stimuli that include exposure to environmental pollutants like pesticides, insecticides, heavy metals, toxic insults, parasites and pathogens [42]. Interestingly in our study, the gene Apoptotic chromatin condensation inducer in the nucleus showed the highest fold expression among the ten genes selected for real-time PCR analysis. This finding hints at the presence of toxic pollutants or other chemicals in the water, at levels requiring a molecular response from the organism. Though we have not documented the status of heavy metals or pesticide pollutant levels in the sampling site, studies conducted on the Cochin backwaters showed high levels of almost all trace elements in the estuary, especially during pre-monsoon season to due to low flushing rates [19]. Heavy metals Cu, Cd, Pb, and Zn were found in alarming rates in both the estuarine waters and in sediments [43]. Reports made decades ago by Nair & Nair. (1986) [44] and Rajendran & Kurian. (1986) [45] states the presence of trace metals in tissues of *M. bilineata* inhabiting the Cochin estuary; however recent studies on the same are nil.

Genes encoding for proteins involved in lipid metabolism is well represented in the SSH library and comprise of Pancreatic triacylglycerol lipase-like, Ganglioside GM2 activator, and fatty acid 2 hydroxylases. Pancreatic triacylglycerol lipases are digestive lipases which hydrolyse triacylglycerides (TAGs) in food, and the real-time expression analysis of the gene shows it to be 6-fold overexpressed in wild oysters than in control. This enhanced expression of lipase in the digestive tissues of the wild *M. bilineata* could stem from the difference in the diet of the tester and driver samples used for SSH. Some studies also suggest the involvement of the "pancreatic triacylglycerol lipase-like" gene in stress response; the gene was found to be overexpressed in situations of hydrocarbon contamination [11], OsHV-1 µVar infection [46] and on exposure to dinoflagellate toxins [47]. Ganglioside GM2 activator-like is also involved in

the metabolism of a special kind of lipids called Ganglions. In oysters, they were found to be up-regulated upon exposure to hydrocarbon contaminants, heavy metals and under hypoxic conditions [11, 8, 48], though the original function of the protein in oysters remain unknown.

An intriguing observation from the study is that there was no differential expression of heat shock proteins or metallothionines in wild oysters. These groups of genes mentioned above are known to be over-expressed in stress conditions especially during temperature shock and heavy metal exposure. The absence of these genes in our SSH Library could in part be because most of the stress studies employ elevated levels of stress parameters, to obtain a molecular and physiological response from the organism. These inflated parameters are often not present in the wild environment and are not ecologically or biologically relevant. A study conducted to identify the transcriptomic response of *C. gigas* to pesticide doses present in normal conditions, using Subtractive hybridization technique, could not identify any heat shock proteins or metallothionines [12].

Oysters have a highly expanded immune system that is enriched for stress and immune responsive genes [4] and forms the basis for the adaptation of oysters to the sessile intertidal life. The findings of this study adequately point out the idea that wild oysters are immunologically more active. Many of the genes differentially expressed in the SSH library are known to have multiple roles in stress management, as described earlier, including biotic and abiotic stress. The complex interplay of these functionally diverged immune genes is what makes the oyster immunity a piece of sophisticated defence machinery and consequently making it more adaptable.

5. Conclusion

Oysters are marine ectotherms having the capability to accommodate to the changing environmental conditions. They are distributed globally and have been the subject of several research works owing to its sturdy nature. Transcriptomic studies to analyse the stress-responsive genes in the Indian oyster, *M. bilineata*, are very few. The current work has been an effort to gain an understanding of the mechanisms by which these estuarine oysters withstand the combined effect of all the stressors present in their habitat. In summary, through this study, we were able to identify several functionally divergent genes with roles in immunity, lipid metabolism, and other cellular processes, triggered as a first step towards the complex process of maintaining normalcy within the cell. The identified genes, all of which have known roles in biotic and abiotic stress management, are being reported for the first time in *M. bilineata*.

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