Egyptian Journal of Aquatic Research (2016) 42, 313–318

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Egyptian Journal of Aquatic Research

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FULL LENGTH ARTICLE

Differential expression analysis in Egyptian redbelly tilapia (*Tilapia Zillii*) exposed to cold stress

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Received 27 March 2016; revised 27 May 2016; accepted 29 June 2016
Available online 30 August 2016

KEYWORDS

Tilapia zillii;
F-DD;
Cold stress;
Fish adaptation;
Molecular ecology

Abstract Fish can adapt to a wide range of temperature changes, however, the molecular mechanisms that underlie these adaptations are not fully understood. In this study, fluorescently labeled differential display (FDD) technique was used to detect mRNAs that are differentially expressed in hepatic tissues of *Tilapia zillii* fish subjected to a temperature reduction regime that combined both accelerated and stationary cold stresses. The two arbitrary primers (DD1 and DD2) with the Oligo $d(T)_{23}$-C generated a total of 18 and 37 unknown messenger RNA (UMR) bands, respectively. Both DD1 & DD2 arbitrary primers with oligo $d(T)_{23}$-C scored four stress-related unidentified fragments after refining. Using the two arbitrary primers with the Oligo $d(T)_{23}$-G, a total of 371 and 372 UmRs were yielded, respectively. Both arbitrary primers scored nine stress-related unidentified fragments after refining. All UmR were tested for its correlation to time and temperature factors scaled for the current experiment, only four were found to be correlated for both factors. Thus, *T. zillii* responded to temperature reduction by adjusting the expression of a set number of genes that may be required for their adaptation and tolerance to low environmental temperature.

Introduction

Tilapia fishes became an economically important fish used for world aquaculture production (Charo-Karisa et al., 2005). Tilapias come in the second place after carps for the worldwide production (FAO, 2005-2013). Generally, tilapia are warm water fish, they require thermal environment of 25–28 °C that is optimum for their reproduction and feeding (Wohlfarth and Hulata, 1983). They cannot survive temperatures less than 10–12 °C for more than few days (Chervinski, 1982). Consequently, cold tolerance is an economically important trait in tilapia, as severe mortalities occur during the winter in temperate climate countries (Tave et al., 1990; Sarig, 1993 and Starling et al., 1995).

Generally, two types of mechanisms are used by different fish species to tolerate low temperatures. The first mechanism was found in the teleost fish inhabiting the polar regions, is

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Peer review under responsibility of National Institute of Oceanography and Fisheries.

http://dx.doi.org/10.1016/j.ejar.2016.06.005
1687-4285

achieved by producing anti-water-crystallization proteins known as antifreeze proteins (AFPs) that protect cellular contents from being frozen in subzero temperatures (Yang et al., 2013). The other mechanism was found in the fishes inhabiting temperate regions. This mechanism mainly acts by keeping or maintaining the fluidity of cell membranes and this can be achieved by increasing the proportion of unsaturated fatty acids to saturated fatty acids. Such increase was directly proportional to the increasing of desaturase enzymes (e.g. Delta 9 destaurase) (Michaud and Denlinger, 2006; Kayukawa et al., 2007).

Wohlfarth and Hulata (1983) and Cnaani et al. (2000), studied the cold tolerance of some tilapia species and their hybrids. They found that the genetic variation in cold tolerance seems to have a large dominance component. However, in Nile tilapia, *Oreochromis niloticus*, Tave et al. (1989, 1990) and Behrends et al. (1990) suggested that cold tolerance is a quantitative trait, controlled by additive genes.

Salah (2014) used the cooling degree-days (CDD) parameter to measure the ability of fish species; *O. niloticus*, *Sarotherodon galilaeus* and *Tilapia zillii* to tolerate the low temperatures. They tested the association between the simple sequence repeat (SSR) locus UNH 118 provided by Cnaani et al. (2003) and the CDD score of each species. Numerically the *T. zillii* was shown to be the most tolerant species down to 6.5 °C (CDD = 143). Nevertheless, the results of the SSR application revealed that *O. niloticus* was the only correlated species to the UNH118 variation. This might support the fact that cold tolerance in Nile tilapia is a quantitative trait. While in *T. zillii*, there was no significant correlation between the alleles found and the CDD values detected despite being numerically the most cold-tolerant species of the tested species. Therefore, the cold tolerance of *T. zillii* might have a dominant component as reported by Wohlfarth and Hulata (1983) and Cnaani et al. (2000). In addition, *T. zillii* may tolerate low temperatures quantitatively by two ways either by a Quantitative Trait Loci (QTL) for cold tolerance different from that present in *O. niloticus* or by enhancing the gene expression for a set of genes that indirectly enables the fish to tolerate low temperatures.

Fishes, as poikilothermic animals, undergo rapid changes with different temperatures and are able to tolerate the gradual temperature changes that are common in natural systems as long as they fall within the range of non-lethal temperatures. This can be achieved by developing a variety of behavioral, cellular, and physiological responses (Crawshaw and Hammel, 1974; Cossins et al., 1977, 2006). Physiological responses to low temperatures in fish were frequently investigated on molecular levels using differential display techniques (a.k.a. Differential display (DD), Fluorescent Differential Display (F-DD), Microarrays or Micro-chip technology) (e.g. Ju et al., 2002; Jason and Somero, 2004; Bradley and Somero, 2009; and Zerai et al., 2010; Niea et al., 2015).

Differential display technique (Liang and Pardee, 1992) was developed to identify and clone differentially expressed genes. The principle of the DD technique is the reverse transcription of mRNA with oligo-dT primers anchored to the poly (A) tail, followed by a PCR reaction using both the anchor primer and a second short primer with an arbitrary sequence. This technique has been also used to identify differences in gene expression after exposure to xenobiotics in aquatic species (Denslow et al., 2001; Larkin et al., 2002; Maples and Bain, 2004; Roling et al., 2004), and other Ionic liquids (ILs) such as 1-hexadecyl-3-methylimidazolium chloride ([C16mim]Cl) (Nan et al., 2016). In addition, DD technique has been also applied for detecting changes in gene expression associated with cold temperature stresses (Podrabsky and Somero, 2004.). The goal of this work was to detect messenger RNAs that are differentially expressed in hepatic tissue of *T. zillii* subjected to a regime of controlled temperature reduction, and to record all cases of up and down regulated genes that are incorporated in the process of cold tolerance during winter.

Materials and methods

Fish stocks

T. zillii stock was obtained from the earthen ponds of El-Quanater station for fish research, National Institute of Oceanography and Fisheries (NIOF). Average weights and lengths of the experimental fish samples were 60 ± 20 g and 13 ± 3 cm, respectively.

Experiment description

A specialized incubator was established containing 90 L glass pond (50 × 60 × 70 cm) with aeration pumps filled with de-chlorinated water. Water temperature in the glass pond was monitored by a sensor adjusted through the control unit located outside the incubator. The tested fish were stocked in external tanks for five days for primary laboratory adaptation.

Initially, water temperature was gradually reduced during one week to 20 °C, at which the fish were held for other four days. Water temperature was then reduced at a rate of 2 °C/2 h until it reached 10 °C. One fish sample was collected every two hours with each temperature reduction; the last two samples were taken at 10 °C/24 h and 10 °C/48 h. Fish feeding stops exactly before the beginning of the experiment and, no diet was allowed during the experiment.

RNA extraction

Liver tissues were removed from fish samples collected at 18 °C, 14 °C, 10 °C, 10 °C/24 h and 10 °C/48 h with a control sample taken at 27 °C. Small pieces from liver tissues were sampled, homogenized by homogenizer, and total RNA was extracted from the homogenized samples using RNeasy Mini Kit (Qiagen) Cat. 74104, according to the manufacturer's instructions. During RNA extraction, Dietylpyrocarbonate (DEPEC) treated water was used, all the glassware used were sterilized at 250 °C overnight. The purified total RNA was dissolved in TE buffer, quantified using Nano-drop spectrophotometer device and stored at −80 °C.

Differential display of mRNA

Complementary DNA (cDNA) was generated in reverse transcriptase (RT) reactions using High Capacity RNA-to-cDNA™ kit (Cat. 4387406, Invitrogen™) as described by the manufacturer. PCRs were carried out using Qiagen Taq-PCR Master Mix Kit (Cat. 201443) as described by the manufacturer. Reaction mixes were submitted to 40 cycles of

PCR as follows: 94 °C for 15 s, 40 °C for 2 min and 72 °C for 30 s followed by an elongation step at 72 °C for 5 min. Each step was tested by 1.5% agarose gel electrophoresis stained with ethidium bromide Eth Br solution. PCRs were carried out using DD1 (5′-AAGCTTTGGTCAG-3′) and DD2 (5′-AAGCTTGATTGCC-3′) as forward primers with Oligo d $(T)_{23}$-C and Oligo d$(T)_{23}$-G as reverse primers. 2 µl of the 1st PCR product (obtained using DD1 & DD2 primers with Oligo d$(T)_{23}$-C (FAM-labeled)) was mixed with 2 µl of the 2nd PCR product (obtained using DD1& DD2 primers with Oligo d $(T)_{23}$-G (NED/CY3-labeled), the volume was then completed to 10 µl by distilled water. The PCR products were then sent to Macrogen services for the fragment analysis. The fragment analysis provided a way for separating the PCR product fragments and detecting Fluorescence intensity. Trace files obtained from the fragment analysis service were loaded separately into a new project file generated by Peakscanner™ for analysis.

Statistical analysis

The relation between the experimental factors (Time and Temperature) was plotted in graphical method for regression test. Peak heights of the genes that showed changes in their expression profile were tested and correlated with each factor by Student *T*-test and correlation coefficients, respectively using calculation option embedded in Microsoft Excel 2013. To compare between differential bands, all peaks were standardized by subtracting each value from the sample mean and dividing the result by the sample standard deviation, known as z-score transformation.

Results

Differential display scored bands

Using the two arbitrary primers (DD1 and DD2) with the Oligo d$(T)_{23}$-C, a total of 18 and 37 unknown messenger RNA (UMR) bands were yielded, respectively. Both DD1 & DD2 arbitrary primers scored four significantly expressed fragments after refining. These four genes were expressed in all the samples stated and showed changes in their expression profiles in each of DD1 & DD2 primers respectively (Table A.1).

Using the two arbitrary primers (DD1 and DD2) with the Oligo d$(T)_{23}$-G, a total of 371 and 372 unknown messenger RNA (UMR) bands were yielded, respectively. Both DD1 & DD2 arbitrary primers scored nine significantly expressed fragments after refining.

The nine scored fragments by primer DD1-G were categorized as: three genes expressed in all the samples and showed changes in their expression profile (52, 54, and 84 bp); one down-regulated gene (87 bp); four genes expressed only in the stationary phase (58, 227, 354 and 360 bp); one genes expressed only during the accelerated phase and degraded during the initiation of the stationary phase (175 bp).

Concerning the fragments scored by primer DD2-G, nine significantly expressed fragments after refining were categorized as: four genes expressed in all the sampled states and showed changes in their expression profile (51, 58, 77 and 84 bp); two down-regulated genes (93 and 572 bp); one up-regulated genes (106 bp); four genes expressed only in the stationary phase; one gene expressed only during the accelerated phase and degraded before the stationary phase (96 bp); while another gene expressed during the accelerated phase and degraded in a slower rate during the initiation of the stationary phase (69 bp) (Table A.1).

Student *T*-test showed to be significant in all comparisons, which indicates that there are relevant differences between the expression peaks for the tested fragments with both experimental factors (Time and Temperature). Correlation tests showed different strengths and directions depending on the tested factor. Correlation test to time showed that: UmR-9 (r-value = 0.96), UmR-11(r-value = 0.80) and UmR-13 (r-value = 0.71) genes showed strong positive correlation ($0.64 < r\text{-value} < 0.96$). UmR-1 ($r$-value = 0.41), UmR-2 ($r$-value = 0.34), UmR-12 ($r$-value = 0.37), UmR-14 ($r$-value = 0.58) and UmR-15 (r-value = 0.53) genes showed moderate positive correlation ($0.32 < r\text{-value} < 0.64$). UmR-4 gene showed a high negative correlation (r-value = −0.72). UmR-3 (r-value = 0.13), UmR-5 (r-value = 0.06), UmR-7 (r-value = 0.00), UmR-10 (r-value = 0.19) genes showed weak positive correlation (zero < r-value < 0.32), while, UmR-8 (r-value = −0.29) and UmR-6 (r-value = −0.05) gene showed weak negative correlation.

Correlation test to temperature showed that: UmR-4 (r-value = 0.72) and UmR-8 (r-value = 0.76) genes showed strong positive correlation. UmR-1 (r-value = 0.25), UmR-2

Table A.1 Band statistics scored using DD1& DD2 primers with the Oligo d$(T)_{23}$-C and d$(T)_{23}$-G using fragment analysis method. Mw = Molecular weight, NB = Number of bands, UpReg = Up-regulated genes, FDeg = Fast RNA-degradation after shock, SDeg = Slow RNA-degradation after shock, DReg = Down-regulated genes, UpSt = Up-regulated genes in the stationary phase, CEP = Changes in expression patterns, TSB = Total significant bands and TB = Total screened bands.

Categories	DD1-C		DD2-C		DD1-C		DD1-C	
	NB	Mw (bp)	NB	Mw (bp)	NB	Mw (bp)	NB	Mw (bp)
UpReg	0	–	0	–	0	–	1	106
FDeg	0	–	0	–	0	–	1	96
SDeg	0	–	0	–	1	175	1	69
Dreg	0	–	0	–	1	87	2	93, 572
UpSt	0	–	0	–	4	58, 227, 354, 360	0	–
CEP	4	53, 55, 93, 162	4	53, 55, 93, 162	3	52, 54, 84	4	51, 58, 77, 84
TSB	4	53–162	4	53–162	9	52–360	9	51–572
TB	18	53–227	37	53–162	371	51–631	372	51–638

Table B.1 Correlation test values (r) for peak values of the unknown messenger RNA (UmR) that showed changes in their expression profile, to the experimental factors (time and temperature). Only the positive moderate to strong correlated genes to time, and negative moderate to strong correlated genes to temperature, are marked with *.

	Oligo $d(T)_{23}$	C				G			
DD1	Temperature (°C)/Time (h)	UmR-1	UmR-2	UmR-3	UmR-4	UmR-5	UmR-6	UmR-7	
	MW (bp)	53	55	93	162	52	54	84	
	24 °C/0 h	1274	3434	10322	1970	225	71	3340	
	18 °C/2 h	1116	2165	7984	1880	252	85	2804	
	14 °C/6 h	353	2023	19460	875	102	84	4374	
	10 °C/10 h	914	2268	7299	1582	209	65	2422	
	10 °C/34 h	327	963	13774	1049	73	90	2255	
	10 °C/58 h	1648	3791	12213	724	239	73	3471	
	r(Time)	0.41*	0.34*	0.13	−0.72*	0.06	−0.05	0.00	
	r(Temp.)	0.25	0.34*	−0.17	0.73*	0.37*	−0.06	0.26	
DD2	Temperature (°C)/Time (h)	UmR-8	UmR-9	UmR-10	UmR-11	UmR-12	UmR-13	UmR-14	UmR-15
	MW (bp)	53	55	93	162	51	58	77	84
	24 °C/0 h	792	1548	5943	1251	304	750	427	4101
	18 °C/2 h	804	1800	8503	1324	291	725	385	3768
	14 °C/6 h	396	1846	19398	934	114	336	164	102
	10 °C/10 h	893	1546	6574	1646	169	336	271	2108
	10 °C/34 h	800	2125	7114	1461	233	571	358	3417
	10 °C/58 h	1266	4478	12200	2003	313	1151	510	5090
	r(Time)	0.76*	0.96*	0.19	0.80*	0.37*	0.71*	0.58*	0.53*
	r(Temp.)	−0.30*	−0.45*	−0.26*	−0.53*	0.37*	0.09	0.16	0.15

(r-value = 0.34), UmR-5 (r-value = 0.37), UmR-7 (r-value = 0.26) and UmR-12 (r-value = 0.37) genes showed moderate positive correlation ($0.25 < r\text{-value} < 0.50$). UmR-11 gene showed strong negative correlation (r-value = −0.53). UmR-13 (r-value = 0.09), UmR-14 (r-value = 0.16) and UmR-15 (r-value = 0.15) genes showed weak positive correlation. UmR-9 (r-value = −0.45) and UmR-10 (r-value = −0.26) genes showed moderate negative correlation, while UmR-3 (r-value = −0.17) and UmR-6 (r-value = −0.06) genes showed weak negative correlation (Table B.1).

By plotting the detected changes in the expression-profile of the 15 UMRs screened by DD1 and DD2 primers showed different curve responses to time and temperature, in which UMR-8, UMR-9 and UMR-11 were statistically fitter to both factors.

Discussion

In this study, the molecular responses for low temperature acclimation of *T. zillii* was examined using differential display technique. According to Salah (2014), *T. zillii* showed the highest survival rate to the lowering of the aquarium temperature in comparison with *O. niloticus*, and *S. galilaeus* of the river Nile. *T. zillii* samples survived down to 6.5 °C while fish samples were still active and healthy. For that, it was used as a model of study among the three species to investigate the Tilapia physiological potentials to survive the low temperatures during winter season in Egypt.

Zerai et al. (2010) studied the effect of the low temperature on Nile Tilapia using Reverse transcription PCR (RT-PCR) technique, in which they designed two separate experiments based on the rate of decreasing the temperature, the first named acute for having high rate of lowering the aquarium temperature, and vice versa for the chronic experiment. Ju et al. (2002) designed a different type of experiment in which they concentrated on the pre-experimental acclamation prior to acute shock exposure to the low temperature. In the current study, we followed the acute cold stress until reaching 10 °C (accelerated phase) then this cold stress was extended for another 48 h (stationary phase) with sampling every 24 h in order to find newly expressed patterns that can explain the process of tolerance that Tilapia follows for the fluctuation of temperature during winter season and to cover the cold stress.

By such, two factors were used: the first is the time (t) in a linear upstream starting from zero to 48 h, and the second is the temperature (T), in a downstream curve from 20 °C down to 10 °C and stationed on 10 °C for two days. Studying the correlation of the obtained expression pattern to the experimental factors would reveal the genetic expression patterns that correspond to the coup of the stressing conditions. The analysis of the data of the expression patterns differentially displayed can be categorized as follows:

(a) Up-regulated genes: (1) Up-regulated genes in all samples acclimated to lower temperatures (from 18 °C to 10 °C and the stationary phase). (2) Up-regulated genes only appeared in the stationary phase. Ju et al. (2002) reported three groups of up-regulated gene by the analysis of brain tissues of channel catfish using microarray technology upon cold acclimation, that were: (i) genes coding for transcription factors and gene products that are functioning in signal transduction pathways such as zinc-finger proteins, calmodulin kinase inhibitor, interferon regulatory factor 3, and inorganic pyrophosphatase; (ii) gene products that are involved in lipid metabolism such as TB2 and acyl CoA binding protein; and (iii) Gene products that participated in the translational machinery such as ribosomal proteins. However

Zerai et al. (2010) found that the expression pattern for cold tolerance genes differs from one tissue to another, especially for the genes involved in lipid metabolism such as delta 9 desaturase. (2) Up-regulated genes only appeared in the stationary phase.

(b) Genes that were transiently induced during the accelerated cold stress phase, but after the stationary phase began, their RNAs showed complete degradation. This resembles the behavior of heat shock protein 70 (Hsp70) that was rapidly induced to a high level after the temperature shift. It was highly induced during the temperature decrease period, and high-level expression lasted for less than 2 h and decreased to its normal level of expression after 2 h at 12 °C (Ju et al., 2002).

(c) Genes that were transiently induced during the accelerated cold stress phase, but their expression extended to the 10 °C for 24 h then completely disappeared in 10 °C at 48 h. Ju et al. (2002) specifies a category of genes that their expression increased rapidly when exposed to a temperature of 12 °C. However, the over expression lasted for 6 weeks after incubation at the low temperature. The recorded genes found to encode chaperone, transcriptional factor, proteins involved in cell signal transduction and the stress-inducible phospho-protein I.

(d) Down-regulated gene that are expressed only in the control sample, but absent in all other samples. Those might be ribosomal protein genes, indicating reduced metabolic activities after extended incubation at the low temperature (Ju et al., 2002).

(e) Genes with changes in their expression profiles. However, the expression of these genes was variable in respect to temperature and time. Correlation testes between the expression pattern and the experimental factors showed different levels of positive and negative correlations, in which they grouped as follows: (1) it was found that, UmR8, UmR9, and UmR11 were the fragments that were positively correlated with time and negatively correlated with temperature factors. The attitude of expression pattern for these fragments increased by lowering temperature (cold increase) as time passed. These might be the genes expressed due to cold acclimation as a mean of cold tolerance. (2) On the other hand, the expression of some genes was found to be positively correlated only with the time factor but not affected by the stressing conditions (temperature reduction), and this appeared in fragments, UmR13, UmR14 & UmR15. These genes might be expressed due to the adaptation to the experimental conditions in the laboratory. (3) The expression of another group of genes revealed a case of cold hardening. This was shown by fragments that their expression was negatively correlated with temperature factor only, in which the attitude of expression pattern increased by lowering temperature from 18 °C to 14 °C (accelerated cold stress phase). However by increasing the stress conditions (lowering the temperature below the optimum temperature) the expression decreased, until the temperature fixed at 10 °C (stationary phase), then it increased again. This case may be fit along with the fragment UmR10. (4) UmR4 fragment expression decreased with increasing the cold stress but have a negative correlation with the time factor that shows a drastic decrease in the gene expression under the stressing conditions by time; however, it did not reach to be down-regulated. Genes related to such category are reduced through the experimental duration due to the stressing conditions. (5) UmR3, UmR6 and UmR7 fragments expression were neither directly correlated with temperature nor with time; while UmR1, UmR2 and UmR12 fragments showed positive moderate correlation for both factors. Generally, both types can be discarded as adaptive-related expressed genes to the experimental conditions.

It is important to mention the vital role of desaturase enzymes in the process of cold tolerance in all warm water fish. Δ9D is known to be associated with cold in many fish species (Tiku et al., 1996; Trueman et al., 2000; Gracey et al., 2004; Cossins et al., 2006) including tilapia. They are involved in membrane lipid metabolism and act to increase the proportion of the unsaturated fatty acids to the saturated fatty acids to sustain membrane fluidity during cold stress. Among these desaturases, Delta-9-Desaturase (Δ9D) was frequently studied as a marker for fish adaptation to low temperatures. Zerai et al. (2010) studied the expression of Δ9D gene in Nile tilapia exposed to separate experiments of acute and chronic cold stress. They found that the expression of Δ9D gene was not significantly affected during the acute cold stress. Nevertheless, the production of Δ9D in chronic cold exposure increases significantly up to 16-fold. In our experiment, the expression pattern that is similar to Δ9D gene was not apparently found. This may be attributed to that the duration of the chronic cold stress phase as it was more extended for about 12 weeks rather than 48 h as in our case. However, Tiku et al. (1996) were able to detect an increase in the transcription of the Δ9D gene in carp liver from 8- to 0-fold following 48–60 h exposure to 0 °C that strongly suggest that the DD primers used in the current study were not complementary to the sequence of Δ9D gene.

In conclusion, *T. zillii* was able to adapt to low temperature by adjusting the expression of some genes that may be required for their tolerance to low temperature. These genes could be grouped into five categories: (i) up-regulated genes; (ii) down-regulated; (iii) transiently enhanced during the accelerated cold stress phase but their expression did not extend during the stationary phase; (iv) transiently enhanced during the accelerated cold stress phase but their expression extended during the stationary phase; (v) some genes appeared only due to the extended exposure to low temperatures; and (vi) group of genes undergo changes in their expression profile and their expression was variable with respect to temperature and time. All these molecular modifications enabled the fish to adapt and survive healthy during exposure to cold temperatures. The current study recommends the usage of next-generation sequencing (RAD-NGS) (Hohenlohe et al., 2012) for sequencing the produced UMRs to exactly identify the genes that are incorporated in the process of cold tolerance in *T. zillii* fish.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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