DDRT-PCR Method for Identifying Differentially Expressed Genes Transcripts in Response to Different High Temperature Treatments of MR253 Rice Seedlings

Enegborienegha Calmday Teke
Department of Biological Sciences, University of Africa, Toru-Orua, Bayelsa State. Nigeria
E-mail:eneteke53@gmail.com

Abstract: *The increasing global temperature is of great concern to everyone, as it affects the cultivation of food crops negatively to meet the increasing demand of a rapid growing world population. Global warming and climate change which are caused by combination of factors including rapid industrialization, vehicular emissions and other anthropogenic activities have led to increase in greenhouse gases (GHGs) that make the climate warmer. This warmer climate poses a severe danger for the cultivation of agricultural crops which are also contending with a lot of similar and other equally detrimental factors present in their environment. The abiotic stress condition which include heat stress, cold stress, drought, salt stress, ultra violet (UV stress) and some toxic chemical materials in the environment negatively affects the growth and yield of agricultural crops. Also, studies have shown that the increasing global temperature will negatively affect the cultivation of rice, leading to poor growth and lower yield. Presented in this paper is a research work that identifies differentially expressed genes transcripts in response to different high temperature treatments of MR253 rice seedlings using DDRT-PCR method. There are a number of proteomic approaches adopted by different Researchers/Authors which provide some insight into the molecular processes of rice response to heat stress which includes; cDNA-AFLP, 2-DE, DNSH and Microarrays. The research work carried out and reported in this paper identifies the changes in gene expression at the mRNA level of MR 253 rice variety seedlings in response to varying intensities of high-temperature stresses at (40°C, 45°C and 50°C) under 1hr with control at 25°C, using Differentially Display Reverse Transcriptase- Polymerase Chain Reaction (DDRT-PCR) technique, in order to show responsive mechanisms to the heat stress. Findings in this research work unravels the suitability of MR 253 rice variety as a parent donor in breeding and also its molecular mechanisms for use under high-temperature conditions in the future.*

Key Words: *Global temperature, Global warming, Climate change, Rice, Heat, Genes, Abiotic, Greenhouse gases, Polymerase.*

1. INTRODUCTION

Rice (*Oryza sativa)* is one the world largest food crop. It provides up to one third of the calories intake of requirement of the global human population mostly for people in Asia, Africa and South America [1]. There are two main distinct type of domesticated rice: (1) *O. sativa;* native to Asia and (2) *O. glaberrima;* native to Africa. However the genus *Oryza* contains 21 other wild varieties that have been domesticated, and crossbreeds developed over time with more other popular varieties over the past century and are been used at various locations globally [2], as shown in Table 1.1.

Table 1.1: Different domesticated species of rice [2]

Section/Species	Accession No.	Locality
O. Sativa	Au73030	China
O. glaberrima	104042	Chad
O. barthii	104140	Cameroon
O. glumaepatula	100968	Brazil, Suriname
O. longistaminata	104977	Kenya
O. meridionalis	103317, 101147	Australia
O. Nivara	106148	Laos
O. rufipogon	105942	China, Thailand
O. punctuate	104071	Cameroon
O. officinalis	105085	Philippines
O. rhizomatis	105448	Sri Lanka

O. minuta	101082	Philippines
O. eichingeri	105160	Uganda
O. alta	105143	Guyana
O. grandiglumis	105669	Brazil
O. latifolia	105141	Costa Rica
O. australiensis	105263	Australia
O. brachyantha	105151	Sierra Leone
O. longiglumis	105148	Indonesia
O. ridleyi	100877	Malaysia
O. schlechteri	82047	Papua New Guinea
O. granulate	106469	China, Vietnam
O. meyeriana	104987	Malaysia

Rice is a monocotyledonous crop, which is normally grown as an annual plant. Rice plant can grow to 1- 1.8 m tall, long, slender leaves 50-100 cm long and 2 -2.5 cm broad. The small wind pollinated flowers are produced in a branched arching to pendulous inflorescence 30-50 cm long. The edible seed is a grain (caryopsis) 5-12 mm long and 2-3 mm thick. Figure 1.1 is an illustration of a typical *O. sativa* plant.

Figure 1.1: Typical rice (*Oryza sativa*) plant. Adapted from [3]

Rice (*oryza sativa L.)* is one of the most import food crops, which supplies the nutritional requirement of about a third of the world population [1], and thus the production of rice is given prime attention by the research community. Studies have shown that the increasing global temperature will negatively affect the cultivation of rice [4], leading to poor growth and lower yield. Thus to mitigate the negative effect posed by this danger for future food security, heat-tolerant rice cultivars with improved yield was developed to withstand the heat stress. Some tolerance acquisition mechanism including ion transport, osmoprotectants, antioxidant defense and signal transduction are means of impacting tolerance to heat stress in rice and other crops [5]. Heat shock proteins (HSPs) play an important part in response heat stress [6].

The MR253 rice (*O. sativa*) was a variety of rice developed in 2010 in Malaysia by researchers in MARDI (Malaysian Agricultural Research and Development Institute). The MR253 rice is a new variety that is resistant to a number of diseases and pest that are prevalent to rice. Some of these diseases include leaf blast, panicle blast, bacterial leaf blight, sheath blight and tungro and also pest such as brown plant hopper. The MR253 rice has early maturity period of 100-104 days, culm height (69.0-74.0 cm), panicle length (23.8 cm), 1000 grain weight (28.5 g) and yield range (5.6-7.0 ts/ha). It also has a 65.8% recovery rate, 80.9% head rice, grain length of 6.9 mm and amylose content of 21.6%.

Local trials shows that the MR253 has the potentials in doing well on marginal soils like peat and high organic soils, hence it is good variety to compensate for limited availability of arable land [7].

But, the increasing global temperature is of great concern to everyone, as it affects the cultivation of food crops negatively to meet the increasing demand of a rapid growing world population. Global warming and climate change which are caused by combination of factors including rapid industrialization, vehicular emissions and other anthropogenic activities have led to increase in greenhouse gases (GHGs) that makes the climate warmer [8]. This warmer climate poses a severe danger for the cultivation of agricultural crops which are also contending with a lot of similar and other equally detrimental factors present in their environment. The abiotic stress condition which include heat stress, cold stress, drought, salt stress, ultra violet (UV stress) and some toxic chemical materials in the environment negatively affects the growth and yield of agricultural crops [9].

The research work reported in this paper identifies the changes in gene expression at the mRNA level of MR 253 rice variety seedlings in response to varying intensities of high-temperature stresses at (40°C, 45°C and 50°C) under 1hr with control at 25°C, using Differentially Display Reverse Transcriptase- Polymerase Chain Reaction (DDRT-PCR) technique, in order to show responsive mechanisms to the heat stress. The DDRT-PCR technique developed by [10] and used in this research work has been used for identification and isolation of differentially expressed genes under various stress conditions from different organisms including plants. Findings in this research work unravels the suitability of MR 253 rice variety as a parent donor in breeding and also its molecular mechanisms for use under high-temperature conditions in the future.

2. LITERATURE REVIEW:

Abiotic stress refers to such excessive factors such as high temperature (heat stress), low temperature (cold stress), low water (drought), high water (flood), high salinity (salt stress), high exposure to ultra violet (UV) radiation among others, emanating from the natural or non-living component of the environment to affect a living organism in a specific environment. Also abiotic stress is been defined as any environmental condition that reduces growth and yield of agricultural crops below their optimum levels. Plants response to abiotic stress is complex and dynamic [11], [12]; they are both elastic (reversible) and plastic (irreversible) depending on the ecology, duration and severity of the stress components. There are situations where such non-living factors which are normally beneficial to the organisms in normal range, will become detrimental when they exceeded their threshold concentration or intensity in the environment, and thus affecting the normal growth and development of such organism in that locality. In [13], it was showed that environmental factors may limit crop productivity as much as 70% in a cropping season.

The various factors responsible for abiotic stress have both positive and negative effects on plant growths depending on the intensity and duration. Abiotic stress in plant reduces growth and development, through inhibition of protein synthesis mechanism [14], [15] and [16]. Also abiotic stress leads to reduced protein folding and generation [15] and therefore the energy metabolic process is affected in severe stress, and the ability to utilize sugar and lipids in photosynthetic process is retarded [9], [17] and [18]. The inabilities of plants to move freely like their animal counterparts, and other motile prokaryotic and eukaryotic organisms make them susceptible to various environmental stresses [12]. These environmental stress which includes high temperature (heat stress), low temperature (cold stress), drought, flood, high salinity (salt stress), toxic chemicals, reactive oxygen species (ROS), and other anthropogenic factors are affecting plants and mainly agricultural crops adversely as reported by [9].

High temperature stress or heat tolerance in a plant is generally defined as the capability of the plant to survive and produce economic yield under high temperature. This however is a special trait that is not possessed by all plants and most rice varieties in particular. Most cultivars of rice grow well in the tropical and sub-tropical climates, where the optimum growth temperature is about (28/22°C); any temperature above 35°C will affect the growth and yield of the rice plant [19]. Rice *(O. sativa*) like other plants can develop different strategies to mitigate the severe effects of high temperature; these mechanisms include short term acclimatization and long term evolutionary adaptation. Some of the major HT stresses tolerance acquisition mechanisms include; ion transporters, osmoprotectants, antioxidant defense, and signal transcriptional factors [5]. Different tissues in plants show variations in development and response on exposure to high temperature. The stress response mechanism is shown by an initial stress signal in ionic and osmotic changes in membrane fluidity, followed by homeostasis and proteins and membranes damage and repair processes [20].

Oxidative stress is one the primary effects of high temperature on plants. Therefore, plants must be protected from HT induced oxidative stress so that they can survive under high temperature. Tolerance to HT stress in most crops has associated with an increased antioxidant capacity [21], [22]. The activities of various antioxidant enzymes are sensitive to temperature and are usually activated under varying temperatures, however their activities usually increase with higher temperatures. [23] observed that catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) showed increased activity at temperatures from ambient to 50°C, while peroxidase (POX) and glutathione reductase (GR) activities decreased at temperature between 20 to 50°C. The importance of various antioxidant enzyme systems in defense against HT stress was demonstrated by [24], in which the activities of the enzymes glutathione S-transferase (GST), APX and CAT were more enhanced in the cultivars that showed better tolerance to HT stress and as

against ROS production. A study comparing the response to HT stress in *O. sativa* and *Z. mays* crops and the antioxidant defense system by [25], shows high level of expression of enzymatic antioxidants like CAT, APX and GR, which are found to be higher in *Z. mays* but no variation in SOD and other non-enzymatic antioxidants like ascorbate (AsA) and glutathione (GSH), at 45/40°C.

An increase in temperature leads to a corresponding increase in expression of antioxidant enzymes until a particular temperature after which a decline occur. The temperature until which increased activities are maintained differs in tolerant and susceptible varieties among different crops [23]. Hence to impact high temperature tolerance in rice (*O. sativa*) the enzyme activities are maintained as the temperature increases. The up-regulation of many genes has been reported to help plants to withstand various stress conditions including high temperature stress, which leads to plants adaptation. Under stress, plants perceive both external and internal signal through various pathways that are used to regulate the various responses for the development of HT tolerance [26]. Exogenous protectants are molecules which possesses the capacity to mitigate the harmful effects of various stress conditions including high temperature when applied on plants. Recently the exogenous treatment of these protectant which includes osmoprotectants, phytohormones, signaling molecules, trace elements among others have shown to reduce the adverse effect on plant growth under HT [27].

Accumulation of various osmolytes such as proline (Pro), glycine betaine (GB) and trehalose (Tre) has shown adaptive mechanism in plants against various abiotic stresses including high temperature stress [28]. Exogenous applications of several phytohormones were found to be effective in ameliorating HT stress in plants. In rice cultivation, the pre-treatment of the seedling with 0.5 mM of salicylic acid (SA) retards high temperature stress (35°C, 48 hr) induces electrolyte osmosis, with reduced Malondialdehyde (MDA) content and superoxide anion radicals production rate, and also increases the content of H_2O_2, proline, soluble sugar, soluble proteins, AsA and GSH production under high temperature stress [29]; these findings shows that SA pretreatment enhances HT tolerance of rice seedling. The cultivation of rice (*O. sativa*) is adversely affected by high temperature stress (Fig. 2.1) like most other plants and cereals in particular; all stages of growth and development of *O. sativa* cultivation, from seedling to maturity are adversely affected [30]. It was reported that a yield decrease of 10% will occur for every 1°C increase in temperature (growing-season minimum) in the dry season [31].

Table 2.1: Effects of HT on rice cultivation. Adapted from [30]

Growth Stage	Threshold Temperature (^{0}C)	Effects and Symptoms	References
Emergence	40	Delay and decrease in emergence	(Yoshida, 1978; Akman, 2009)
Seedling	35	Poor growth rate of seedling	(Yoshida, 1981)
Tillering	32	Reduction in tilling and height	(Yoshida, 1978)
Booting	35	Decreased rate of pollen-grain formation	(Shimazaki et al., 1964)
Anthesis	34	Poor anther dehiscence and sterility	(Jadadish et al., 2007)
Flowering	35	Floret sterility	(Satake & Yoshida, 1978)
Grain formation	34	Reduction of yield	(Morita et al., 2004)
Grain ripening	30	Reduced grain filling rate	(Yoshida, 1981)

3. MATERIALS AND METHOD:

A new rice variety, MR253 *(O. sativa)* seeds obtained from MARDI, Serdang, Malaysia which is blast resistant was used as the planting materials. The seeds were soaked for 24 hours prior to sowing. Twenty seeds of the sample were sown per pot into 12 pots, which were filled with a mixture of black and organic soil with a ratio of 70:30, and adequately watered. The pots were labelled and arranged in a growth chamber according to RCBD (randomized complete block design) with a controlled photo-period of 16 hours and dark-period of 8 hours light cycle, with light supplied with four pieces of 4ft fluorescent bulbs in each layer of the growth chamber. About 25 ml of distilled water was applied to the seedling daily. Temperature reading was taken daily and maintained at an optimum of 25°C. A record of the number and height of germinated seedlings were taken on daily basis. On the 10th day, the seedlings were thinned to one per pot of uniform height plants and liquid fertilizer NPK (15:15:15) was applied. Heat-stress treatment was carried out on the 14-days old seedlings, by exposing them to 40°C, 45°C, and 50°C of temperature for 1 hour in three different preset incubators for a set of two seedlings per treatment, and two seedlings without heat-stress treatment at 25°C was used as control. Each of the treatments and the controls were carried out in two replicates.

3.1 Total RNA Extraction: The total RNA of both the heat-stress treated and control rice seedling samples extracted were carried out in a class II bio-safety cabinet using R&A-BLUE™ Total RNA Extraction kit (iNtRON biotechnology Inc, Korea) according to the manufacturer's protocol. Every sample, both the treated and control whole rice seedlings were harvested and the soil washed away with distilled water thoroughly separately. The cleaned rice seedlings were

ground with ceramic mortar and pistol pre-stored in an ultra-low temperature freezer at -80°C and pre-cooled to -20°C, and 1 ml of R&A BLUE™ extraction reagent was added and homogenized. The mixture was then transferred to 1.5 ml microcentrifuge tube and was separated by centrifugation at 13000 rpm for 10 mins at 4°C. The top homogenate portion was then transferred to a new 1.5 ml microcentrifuge tube and 200 µl of chloroform was added, and stirred properly on vortex for 15-sec to mix and then centrifuged at 13000 rpm for 10 mins at 4°C which separates to three phases (bottom blue organic phase, a whitish middle layer and a top colourless aqueous layer).

The top colourless aqueous phase was then transferred to a new 1.5 ml microcentrifuge tube and 400 µl of isopropanol pre-stored at -20°C for at least 30 mins was added, then, the tube inverted 6-7 times to mix solution properly and then centrifuged at 13000 rpm for 10 mins at 4°C for precipitation of RNA pellets. The supernatant was then discarded carefully without disturbing the pellets at the bottom or side of the tube, and then 1 ml of 75% ethanol was added and mixed properly by inverting the tube 4-5 times and centrifuged at 13000 rpm for 1min at 4°C to wash away and remove any impurities including salts. The supernatant was carefully discarded and the white RNA pellets were dried of any remaining moisture with the aid of thin tissue paper. Finally the RNA pellets were dissolved in 40 µl of RNase free water (ultra-pure water) by passing the solution through pipette tip few times and stored in a -20°C freezer prior to quantification and subsequently in cDNA synthesis.

3.2 Quantification and Qualification of Total RNA: The concentration and purity of the total RNA extracted was determined by using spectrophotometric measurements where the amount of UV-VIS irradiation absorbed by the test materials was applied. Micro-plate reader (EON MICROPLATE READER, BIO TEK'S VERMONT, USA) was used to take reading of Absorbance (A) at A_{260} and A_{280}, where 1.5 µl of each RNA sample was loaded on each plate and RNase free water (ddH_2O) was used as control loaded to the first plate. The reading at 260 nm allows calculation of concentration of nucleic acid in the sample while 280 nm is for protein concentration. The ratio between the readings at (A_{260}/A_{280}) provides an estimation of the purity of the nucleic acid. For pure preparation of RNA product at A_{260}/A_{280} will have a value of approximately 2.0. If there is significant contamination with protein or phenol, the ratio value will be less than 2.0, and accurate quantification become more difficult.

3.3 cDNA Synthesis: The total RNA product was converted to cDNA immediately after extraction and quantification due to its instability. The cDNA was synthesized by using Tetro cDNA synthesis kit (Bioline Inc, UK) according to the manufacturer's protocol. The cDNA mix was prepared on ice; mixed gently with pipette incubated by placing on 45°C heat block for 30 minutes and transferred to 85°C heat block to incubate for 5 minutes for termination. The cDNA product was placed on ice and stored at -20°C freezer for subsequent DDRT-PCR analysis. The summary of reagents used for cDNA synthesis mix is as tabled in Table 3.1.

Table 3.1: Summary of reagents used for cDNA synthesis mix

Reagents	Volume
Total RNA sample	variable1.0 µl
Oligo(dT)	1.0 µl
10 mM dNTP mix	1.0 µl
5x RT buffer	4.0 µl
Ribosafe RNase inhibitor	1.0 µl
Tectro Reverse Transcriptase (200u/µl)	1.0 µl
DEPC- treated water	Top up to 20.0 µl

3.4 Differential-Display Reverse Transcription-PCR (DDRT-PCR): The synthesized cDNA product was amplified by using the differential-display reverse transcription-PCR technique (DDRT-PCR). The cDNA samples were used as a template DNA together with oligo d $(T)_{18}$ primers (reverse) and random primers (forward), Taq polymerase (MyTaq™ Mix PCR Kit- Bioline Inc, UK) and ddH_2O. Six different random primers (Bioneer Inc, Korea) combination were used for the amplification of the genes to ascertain their different level of expression due to the heat-stress treatment, the list of six random primers are shown in Table 3.2. Two actin forward and reverse primers (5-CATGCTATCCCTCGTCTCGACCT -3 and 5- CGCACTTCATGATGGAGTTGTAT -3), from (Bioneer Inc, Korea) specific to the rice actin 1 gene were used as positive control to ascertain the integrity of synthesized cDNA [32]. The reactions were carried out in 10µl total reaction volume as outlined in Table 3.3. The standard protocol of MyTaq Mix was used in this DDRT-PCR amplification. The amplification reactions were done in a PCR thermo-cycler (MJ Research Incorporated Massachusetts, USA) with the following reaction conditions: initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds for denaturation, 40°C for 30 seconds for annealing and 72°C for 1 minute for extension, followed by a final extension step at 72°C for 10 minutes. The PCR products was stored at -20°C and used for subsequently gel electrophoresis analysis.

Table 3.2: List of random primers (Bioneer Inc, Korea)

Random Primer	Sequence ('5-3')
P1	CAGGCCCTTC
P2	AATCGGGCTG
P3	CAGCACCCAC
P4	GACCGCTTGT
P5	AGGTGACCGT
P6	GTCCCGACGA

Table 3.3: Summary of reagents for PCR reaction mix

Substances	Volume per reaction
cDNA samples	0.5 µl
Forward primer (random primer)	0.5 µl
Reverse primer(oligo dT primer)	0.5 µl
My taq mix, 2x	5.0 µl
Water (ddH2O)	Top up to 10.0 µl

3.5 Gel Electrophoresis and Visualization of the DDRT-PCR Products: The DDRT-PCR products were run on 1.5% (w/v) agarose gel to determine the gene transcripts by comparing them with a standard 100bp DNA ladder (1_{st} BASE Axil Scientific, Singapore). The 1.5% agarose gel was prepared by dissolving 1.5 g of agarose into 100 ml of 1xTAE buffer solution (40mM Tris, 20mM acetic acid, and 1mM EDTA). Then 5 µl of SYBR$^{(R)}$ Safe nucleic acid staining dye was added to the heated agarose/buffer mixture and mixed properly before pouring into the gel casting box to solidify for approximately 30 mins. The gel was then placed into electrophoresis tank, and the tank filled with 1xTAE buffer as the running buffer, which would transmit the electric current. Then 7 µl of each PCR product samples was mixed with 3 µl of DNA loading dye and the mixture was loaded into the wells from second well, while the first well was loaded with 5 µl of 100bp DNA ladder (1_{st} BASE Axil Scientific, Singapore). The setup was run for 1 hour and 30 minutes at 80V. The cDNA gel bands were then visualized under UV trans-illuminator and photographed with GelDoc (AlphaImager Mini, Proteinsimple, USA) and also captured with a compact canon camera lens 20.1megapixel.

3.6 Determination of High Temperature Induced Bands on Gel: Based on the comparative intensity of the cDNA bands on the gel pictures, transcripts expressed as a result of high temperature were determined and grouped as up-regulated or induced fragments and down-regulated or suppressed fragment. The comparison was visually carried out in respect between the heat treated and the control samples. Determination of the band sizes was done manually by comparison of samples bands with that of the standard molecular ladder used (1_{st} BASE 100bp DNA ladder), which has eleven bands ranging from 100bp to 1500bp to get the various approximate values of the bands molecular weights.

4. RESULTS AND DISCUSSION:

The spectrometer reading of RNA values of the heat treated and control samples as obtained from the micro-plate reader were used for the determination of quantity of water added to achieve the desire concentration for the cDNA synthesis according to the protocol of the manufacturer of the kit as shown in Table 4.1.

4.1 Comparative Identification of Transcripts Induced Under High Temperature Stress: The identification and comparative analysis of expressed transcript of different plant cells types of the same plant under heat stress would give an insight into the response mechanism of the induced stress. The DDRT-PCR method used in the research work shows the various up-regulated and down-regulated cDNA bands, indicating the presence or absence of bands, thus indicating the quantitative and qualitative differences as also reported in similar work on wheat by [33].

Table 4.1: RNA concentration for cDNA synthesis data for 20µl per reaction

RNA Samples	RNA concentration (ng/µl)	RNA concentration (µg/µl)	5/RNA (µg)	H_2O added (20-8-RNA)µl
T_1R_1	2277.98	2.277	2.196	9.8
T_1R_2	1379.98	1.379	3.626	8.4
T_2R_1	1333.22	1.333	3.751	8.2
T_2R_2	2324.54	2.324	2.151	9.8
T_3R_1	2066.07	2.066	2.420	9.6
T_3R_2	1568.59	1.568	3.188	8.8
C_1	1521.33	1.521	3.287	8.7
C_2	2925.51	2.925	1.709	10.3

Note: T_1R_1 and T_1R_2 for 40°C; T2R1 and T_2R_2 for 45°C; T3R1 and T_3R_2 for 50°C; C_1 and C_2 for control samples at 25°C. This research work, in an attempt to identify transcripts of MR253 rice that are induced under heat stress by DDRT-PCR used two primers (Act1:5-CATGCTATCCCTCGTCTCGACCT-3 and 5-CGCACTTCATGATGGAGTTGTAT-3) specific to rice actin 1 gene as positive control, which resulted in the amplification of fragments for both heat treated and control samples. The result of the positive control primers (Fig.4.1) is an indication that both the heat treated and control samples used in the DDRT-PCR is of high quality.

Figure 4.1: Agarose gel image of positive control bands of the two primers that are specific to actin 1 gene.

Lane M: 100bp ladder (1_{st} base), lane 1 and 2: heat treated samples at 40°C, lane 3 and 4: heat treated samples at 45°C, lane 5 and 6: heat treated samples at 50°C, lane 7 and 8: control samples.

The results of six different random primers tested independently with the anchored oligo d $(T)_{18}$ primer on the heat treated and control samples are shown as follows: P1 and P2 (Fig 4.2), P3 and P4 (Fig 4.3), P5 and P6 (Fig 4.4).

Figure 4.2: Agarose gel image of DDRT-PCR profile of heat treated and control samples using random primers P1 and P2, with anchored oligo d $(T)_{18}$ primer.

Lane M: 100bp ladder (1_{st} base), lane 1 and 2: heat treated sample at 40°C, lane 3 and 4: heat treated samples at 45°C, lane 5 and 6: heat treated samples at 50°C, lane 7 and 8: control samples. Up-regulated fragments are indicated by red arrows, while down-regulated fragments are indicated by green arrows.

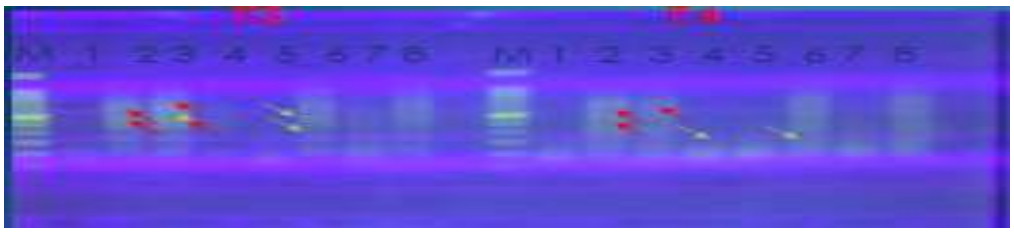

Figure 4.3: Agarose gel image of DDRT-PCR profile of heat treated and control sample using random primers P3 and P4 with anchored oligo d$(T)_{18}$ primer.

Lane M: 100bp ladder (1_{st} base), lane 1 and 2: heat treated sample at 40°C, lane 3 and 4: heat treated samples at 45°C, lane 5 and 6: heat treated samples at 50°C, lane 7 and 8: control samples. Up-regulated fragments are indicated by red arrows, while down-regulated fragments are indicated by green arrows.

Figure 4.4: Agarose gel image of DDRT-PCR profile of heat treated and control sample using random primers P5 and P6 with anchored oligo d $(T)_{18}$ primer.

Lane M: 100bp ladder (1_{st} base), lane 1 and 2: heat treated sample at 40°C, lane 3 and 4: heat treated samples at 45°C, lane 5 and 6: heat treated samples at 50°C, lane 7 and 8: control samples. Up-regulated fragments are indicated by red arrows, while down-regulated fragments are indicated by green arrows.

The agarose electrophoresis gel images of the six primers tested independently with the anchored oligo $d(T)_{18}$ indicates that P1 and P2 (Fig.4.2), P3 and P4 (Fig. 4.3) and P5 and P6 (Fig. 4.4) all show amplified cDNA and different number of up-regulated and down-regulated gene transcripts as shown in Table 4.2.

Table 4.2: Molecular weights estimate of up-regulated and down-regulated fragments of heat treated seedlings compared to the control samples.

Primers	Up-regulated fragments			Down-regulated fragments		
	40°C	45°C	50°C	40°C	45°C	50°C
P1	450bp, 650bp	650bp	550bp			650bp
P2	250bp, 310bp, 650bp		450bp, 650bp			480bp
P3	450bp, 650bp	550bp, 650bp				450bp, 650bp
P4	350bp, 650bp	660bp			450bp	250bp
P5		480bp, 720bp	650bp	650bp		480bp
P6	480bp, 720bp					
Subtotal	11	6	4	1	1	6
Total	21			8		

The DDRT-PCR reaction shows series of faint and diffused cDNA bands and the results. This reaction was earlier discovered by Alves-Costa and Wasko (2015) when conducting a similar work on the brain tissues of *L. macrocephalus* using random primers in DDRT-PCR technique. However many fragments of differentially expressed cDNA band were observed, but the more visible and intense fragments were reported here by comparing the heat treated and control samples. The various intensities of heat exposure on the seedlings did not show a regular pattern of up-regulation of the heat treated samples, but faint bands observed on the control samples might be as result of differences in concentration and the presence of impurities. Based on the comparative intensity of cDNA bands observed on the gel pictures, 21 gene transcripts were induced (up-regulated), while 8 gene transcripts were suppressed (down-regulated) as a result of the high temperature treatment, when compared with the control sample. The result implies that the biological processes of the rice seedlings are affected by the increased temperatures of 40°C, 45°C and 50°C.

5. CONCLUSION:

This research work has identified some cDNA fragments which are differentially displayed in MR253 rice seedlings exposed to varying intensities of heat stress using the DDRT-PCR technique. The agarose gel images result shows that more genes fragments observed at the lower temperature treatment; a total of 11 bands were up-regulated at 40°C, but only 6 bands and 4 bands were up-regulated at 45°C and 50°C respectively. Also 8 bands were observed to be down-regulated at 50°C; this result indicates that at higher temperatures, most of the defense apparatus that were induced to withstand the heat stress are greatly damaged or suppressed. Thus it can be said that these identified fragments are mainly due to the high temperature stress response by the seedlings as the control samples do not show corresponding fragments. The use of random primers is often associated with non-reproducible results as observed in this research work on MR253 rice with varying intensities of high temperature exposure; this can be optimized by using longer sequence specific primers. However the cDNA fragments detected among the samples will be useful for the characterization of specific primers for the identification of high temperature stress responsive markers rice in the future.

Though the actin 1 forward and reverse primers used in research work gives visible bands among both the heat treated and control sample, but the bands appears below range of the DNA ladder (1_{st} base 100bp DNA ladder) used in the research work. Thus, for future work, a different DNA ladder with wider range of bandwidth should be used in order to achieve an approximate value of the actin products before proceeding to use the random primers. Also future work on this work should be carried out with more plant samples, which should be three replicates per treatment for reliable comparison. In subsequent works, twenty random primers of longer length should be used to ascertain their reproducibility and reliability and that which is followed by tailored made specific primers. Finally, since the fragments molecular weights that are observed in this research work were based on manual approximation, an electronic estimation

should be done to achieve more accurate results. Gene sequencing should be done on the discovered fragment to know the exact sequences of the various discovered bands.

ACKNOWLEDGEMENT:

My special thanks go to the Head of Bioscience Department, Malaysia University of Science and Technology; Dr Tang Sui Yan and my supervisor, Dr Tan Fui Ching for their guidance. Also, I thank all the Biotechnology laboratory workers and my fellow post graduates students, more especially, Mr Titus Innocent.

REFERENCES :

1. Khush, G. S. (2001). Green revolution: the way forward. *Nature Reviews Genetics*, 2(10), 815-822.
2. Vaughan, D. A., Morishima, H., and Kadowaki, K. (2003). Diversity in the Oryza genus. *Current opinion in plant biology*, 6(2), 139-146.
3. Cao, Y., Zhang, Q., Chen, Y., Zhao, H., Lang, Y., Yu, C., and Yang, J. (2013). Identification of differential expression genes in leaves of rice (Oryza sativa L.) in response to heat stress by cDNA-AFLP analysis. *BioMed research international*.
4. Shah, F., Huang, J., Cui, K., Nie, L., Shah, T., Chen, C., and Wang, K. (2011). Impact of high-temperature stress on rice plant and its traits related to tolerance. *The Journal of Agricultural Science*, 149(05), 545-556.
5. Wang, W., Vinocur, B., Shoseyov, O., and Altman, A. (2004). Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends in plant science*, 9(5), 244-252.
6. Baniwal, S. K., Bharti, K., Chan, K. Y., Fauth, M., Ganguli, A., Kotak, S. ... and Von Koskull-DÖring, P. (2004). Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. *Journal of biosciences*, 29(4), 471-487.
7. Khaing, E. E., Ahmad, Z. A. M., MuiYun, W., and Ismail, M. R. (2014). Effects of Silicon, Copper and Zinc Applications on Sheath Blight Disease Severity on Rice. *World Journal of Agricultural Research*, 2(6), 309-314.
8. Intergovernmental Panel on Climate Change. (2014). Climate Change 2013: The physical science basis: Working group I contribution to the fifth assessment report of the Intergovernmental Panel on Climate Change. Cambridge University Press.
9. Cramer, G. R., Ergül, A., Grimplet, J., Tillett, R. L., Tattersall, E. A., Bohlman, M. C., ... and Cushman, J. C. (2007). Water and salinity stress in grapevines: early and late changes in transcript and metabolite profiles. *Functional & integrative genomics*, 7(2), 111-134.
10. Liang, P., and Pardee, A. B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science*, 257(5072), 967-971.
11. Skirycz, A., and Inzé, D. (2010). More from less: plant growth under limited water. *Current Opinion in Biotechnology*, 21(2), 197-203.
12. Cramer, G. R. (2010). Abiotic stress and plant responses from the whole vine to the genes. *Australian Journal of Grape and Wine Research*, 16(s1), 86-93.
13. Chaves, M. M., Maroco, J. P., and Pereira, J. S. (2003). Understanding plant responses to drought—from genes to the whole plant. *Functional plant biology*, 30(3), 239-264.
14. Vincent, D., Ergül, A., Bohlman, M. C., Tattersall, E. A., Tillett, R. L., Wheatley, M. D. ... and Cramer, G. R. (2007). Proteomic analysis reveals differences between *Vitis vinifera L.* cv. Chardonnay and cv. *Cabernet Sauvignon* and their responses to water deficit and salinity. *Journal of experimental botany*, 58(7), 1873-1892.
15. Liu, J. X., and Howell, S. H. (2010). Endoplasmic reticulum protein quality control and its relationship to environmental stress responses in plants. *The Plant Cell*, 22(9), 2930-2942.
16. Kumar, S., Kaur, R., Kaur, N., Bhandhari, K., Kaushal, N., Gupta, K., ... and Nayyar, H. (2011). Heat-stress induced inhibition in growth and chlorosis in mungbean (*Phaseolus aureus Roxb.*) is partly mitigated by ascorbic acid application and is related to reduction in oxidative stress. *Acta Physiologiae Plantarum*, 33(6), 2091-2101.
17. Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., ... and Harter, K. (2007). The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *The Plant Journal*, 50(2), 347-363.
18. Pinheiro, C., and Chaves, M. M. (2011). Photosynthesis and drought: can we make metabolic connections from available data? *Journal of experimental botany*, 62(3), 869-882.
19. Baker, J.T., Allen, L.H., Jr. and Boote, K.J. (1995). Potential climate change effects on rice: carbon dioxide and temperature. pp. 31-47. In: Climate Change and Agriculture: Analysis of Potential International Impacts. American Society of Agronomy Special Publication No. 59, American Society of Agronomy, Madison, 382 pp.

20. Vinocur, B., and Altman, A. (2005). Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Current opinion in biotechnology*, 16(2), 123-132.
21. Almeselmani, M., Deshmukh, P. S., Sairam, R. K., Kushwaha, S. R., and Singh, T. P. (2006). Protective role of antioxidant enzymes under high temperature stress. *Plant Science*, 171(3), 382-388.
22. Ara, N., Nakkanong, K., Lv, W., Yang, J., Hu, Z., and Zhang, M. (2013). Antioxidant enzymatic activities and gene expression associated with heat tolerance in the stems and roots of two cucurbit species ("*Cucurbita maxima"* and *"Cucurbita moschata*") and their interspecific inbred line "Maxchata*".International journal of molecular sciences*, 14(12), 24008-24028.
23. Chakraborty, U., and Pradhan, D. (2011). High temperature-induced oxidative stress in *Lens culinaris*, role of antioxidants and amelioration of stress by chemical pre-treatments. *Journal of Plant Interactions*, 6(1), 43-52.
24. Almeselmani, M., Deshmukh, P., and Sairam, R. (2009). High temperature stress tolerance in wheat genotypes: role of antioxidant defense enzymes. *Acta Agronomica Hungaric*a, 57(1), 1-14.
25. Kumar, S., Gupta, D., and Nayyar, H. (2012). Comparative response of maize and rice genotypes to heat stress: status of oxidative stress and antioxidants. *Actaphysiologiae plantarum*, 34(1), 75-86.
26. Kaur, N., and Gupta, A. K. (2005). Signal transduction pathways under abiotic stresses in *plants. Curr Sci*, 88(11), 1771-1780.
27. Hasanuzzaman, M., Hossain, M. A., and Fujita, M. (2011). Selenium-induced up-regulation of the antioxidant defense and methylglyoxal detoxification system reduces salinity-induced damage in rapeseed seedlings. *Biological trace element research*, 143(3), 1704-1721.
28. Sakamoto, A., and Murata, N. (2002). The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. *Plant, cell & environment*, 25(2), 163-171.
29. Zhang, X., Li, J., Liu, A., Zou, J., Zhou, X., Xiang, J. ... and Chen, X. (2012). Expression profile in rice panicle: insights into heat response mechanism at reproductive stage. *PLOS ONE*, 7 (11).
30. Shah, F., Huang, J., Cui, K., Nie, L., Shah, T., Chen, C., and Wang, K. (2011). Impact of high-temperature stress on rice plant and its traits related to tolerance. *The Journal of Agricultural Science*, 149(05), 545-556.
31. Peng, S., Huang, J., Sheehy, J. E., Laza, R. C., Visperas, R. M., Zhong, X., ... and Cassman, K. G. (2004). Rice yields decline with higher night temperature from global warming. *Proceedings of the National Academy of Sciences of the United States of America*, 101(27), 9971-9975.
32. Xv, J., Wei, X., Li, X., Chen, L., Feng, D., and Zhu, Z. (2001). Isolation of rice EPSP synthase cDNA and its sequence analysis and copy number determination. *Acta Botanica Sinica*, 44(2), 188-192.
33. Luo, K., Zhang, G., Wang, C., Ouellet, T., Wu, J., Zhu, Q., and Zhao, H. (2014). Candidate genes expressed in tolerant common wheat with resistant to English grain aphid. *Journal of Economic Entomology*, 107(5), 1977-1984.