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# Screening of molecular markers associated with heat stress in rice (*Oryza sativa*)

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**ABSTRACT**

By the end of the 21st Century predicted temperature raise is 2–4°C poses a challenge to rice production so, there is an urge to produce new heat resistant varieties which can withstand in such climate. The present research was carried out to screen molecular markers from MR 253 Malaysian rice variety of 14 days old seedling stage. To investigate molecular markers in high temperature environments, DDRT-PCR analysis was performed by comparing non-treated samples with samples treated under 40 and 45$^0$C of heat stress for 10 min, 30 min, 1 hr, 3 hr and 6 hr. Out of 20 random primers, total 11 primers were designated as heat responsive marker in which 6 primers showed 25 bands at 40 $^0$C and 11 primers indicated 30 bands at 45 $^0$C of heat stress. Results of analysis revealed that these random primers could be considered as a molecular marker and used as a good indicator to assess heat stress effect. Thus, these markers can be considered in genetic resource and breeding programme.

## INTRODUCTION

With the prospective growth of world's population toward 10 billion by 2050, the demand of rice is growing faster than for other crops. In the future, there will be many challenges for achieving higher productivity and good yield (IPCC 2007). Due to global warming heat stress is one of the most serious threat to crop yield loss (Boyer, 1982; Teixeira *et al*., 2013; Tebaldi *et al*., 2006). Heat stress affect rice plant at almost all stages of growth especially at booting, flowering, grain-filling seriously affects spikelet fertility, increased probability of male sterility and grain quality (Jagadish *et al*., 2008; Matsui *et al*., 1997a, 1997b, 2001; Maruyama *et al*., Prasad *et al*., 2006; 2013; Satake & Yoshida, 1978; Weerakoon *et al*., 2008; Horie *et al*. 1996). The growth responses of rice to high temperature are still poorly understood (Nagai & Makino 2009). Enormous quantity of rice is being destroyed each year due to environmental stresses. There is an urgent need to produce stress-resistant varieties, which can complete the future demand of food. Many studies has been conducted on molecular studies of rice, in which DNA based molecular markers have proven to be powerful tools in the assessment of genetic relationships within and among the species of rice.

Liang & Pardee developed a new PCR-based technique called Differential Display (DDRT-PCR) in 1992. This technique focused on detecting differentially expressed genes at mRNA level among differentially treated sample at same time. DDRT-PCR is easy and most sensitive technique as like normal PCR. This technique is recently used in many studies for studying differentially expressed gene by various stresses. In this approach, random primers used in DDRT-PCR to identify specified molecular markers to examine the response of rice



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seedling to heat treatment. This molecular marker technology gives a novel tool for DNA fingerprinting and hybridization. These molecular markers, in combination with linkage maps and genomics, can be used to alter and improve plant traits on the basis of genotypic assays.

## MATERIALS AND METHODS

***Plant material, Growth conditions of rice seedlings:***

The seeds of rice variety MR 253 (*Oryza sativa*) were obtained from MARDI (Malaysian Agricultural Research and Development Institute, Serdang, Malaysia). MR 253 is a new Malaysian rice variety, which is resistant to blast. The rice seeds were soaked overnight in RO water before planted in a mixture of 30% of organic and 70% black soil in pots. In each pot 20 seeds were sowed and germination rate ontained was 35-40%. All seedlings were maintained for 14 days and were arranged according to standard RCBD design (Randomized Complete Block Design) in growth chamber, at 25°C for 16/8 hours light and dark condition (Hakim M. *et al*., 2010; Lee D. G. *et al*., 2011). 14 days after sowing, the seedlings were subjected to heat stress at 40 and $45^0$C (10 min, 30 min, 1 hour, 3 hours and 6 hours) were given to plants for further molecular analysis. Whole seedlings were harvested after treatment and proceed for total RNA extraction. The entire experiment was performed in replicates.

***Preparation of RNA extraction and cDNA synthesis:***

Total RNA was isolated from whole plant seedlings for all harvested plants using R&A Blue Total RNA Extraction Kit (iNtRON biotechnology Inc., Korea) according to the protocol provided by manufacturer. 5ug of total RNA was used for cDNA synthesis using oligo d(T) primers. cDNA synthesis were done by using Tetro cDNA Synthesis Kit (Bioline Inc, UK) according to manufacturer's instruction. First strand cDNAs were stored at -$20^o$C before being used in DDRT-PCR reactions.

***DDRT – PCR amplification:***

DDRT–PCR reaction was carried out in 50 ul reaction volume with containing 0.8 ul of cDNA, 0.5 ul random, 0.5 ul oligo d $(T)_{18}$ primers, 23.2 ul $H_2O$ and 25 ul Taq master mix (MyTaq Mix PCR Kit - Bioline Inc, UK). The amplification reactions were carried out in a PCR thermo-cycler (MJ Research Incorporated) with the following conditions: 35 cycles of 95ºC for 5 minute, 40ºC for 30 seconds and 72ºC for 1 minute. The 20 random primers used in the reactions are listed in Table 1. The primers (Act1: 5'-CATGCTATCCCTCGTCTCGACCT-3' and 5'- CGCACTTCATGATGGAGTTGTAT-3') specific to the rice actin 1 gene were used as a positive control to check the integrity and quality of newly synthesis cDNA (Xu Jun-Wang *et al*., 2002)

***Agarose gel electrophoresis and visualization of amplified products:***

PCR products were further checked by agarose gel electrophoresis (1.5% of agarose with TAE buffer using Red Safe$^{TM}$ nucleic acid staining dye, for 1 hour and 30 minutes at 80V). The gel was visualized under UV transilluminator and documented using a compact canon camera lens 12.1 megapixel.

***Data analysis:***

***Molecular weight estimation:***

Molecular weight was calculated using the AlphaView Software version 3.4, AlphaImager MINI instrument, based on a comparison of amplified products with the known size of DNA fragments of 100bp DNA ladder (which consist of 12 bands from 100 to 1500bp).

***Analysis of agarose gel electrophoresis products and molecular markers selection:***

Total numbers of amplified bands were scored manually. Only discrete fragments with medium to high intensity were taken into consideration by comparing between the treated and control samples based on the presence or absence of bands.

## RESULTS AND DISCUSSION

The results of present study indicated considerable molecular markers of heat responses at two different high temperatures. Among 20 primers used in this study, results of 11 primers were taken into consideration based on intensity and quality of amplified bands. Each marker was identified by the presence and absence of band. The selected primers produced total 55 distinct bands responsive to heat treatment.

***Molecular screening of rice seedlings to 40 °C heat stress under varying duration:***

Under heat treatment of 40 °C at 5 five different time intervals (10 min, 30 min, 1 hour, 3 hours and 6 hours) multiple bands showed in control samples in all 20 primers. Whereas, treated seedlings produced a total of 25 discrete bands (6 in treatment 1, 5 in treatment 2, 5 in treatment 3, 6 in treatment 4 and 3 in treatment 5) in heat treatment. These 25 bands, were produced in 6 primers and they are: P3, P9, P12 and P13 in treatment 1, P4, P9, P12 and P13 in treatment 2, P3, P4, P12 and P13 in treatment 3, P3, P4, P9, P12, P13 and P20 in treatment 4 and P4, P9 and P13 in treatment 5 (Table 1). These 25 bands were however found to be absent in control samples (Fig. 1).

***Molecular screening of rice seedlings to 45 °C heat stress under varying duration:***

Different duration of heat treatment of 45 °C (10 min, 30 min, 1 hour, 3 hours and 6 hours), the control samples produced multiple bands for all 20 primers whereas treated seedlings produced a total of 30 discrete bands (7 in treatment 1, 4 in treatment 2, 11 in treatment 3, 6 in treatment 4 and 2 in treatment 5). These 30 bands were produced by 11 different primers and they are: P4, P6, P9, P12 and P13 in treatment 1, P4, P10, P12 and P17 in treatment 2, P1, P2, P3, P4, P9, P10, P12, P13 and P20 in treatment 3, P4, P9, P12, P13 and P20 in treatment 4 and P13 and P20 in treatment 5 (Table 1). These 30 bands were however found to be absent in the control samples (Fig. 2).

**Table 1:** Amplified products from rice seedlings subjected to heat stress.

| Primer | Sequence (5' - 3') | Heat (40°C) - bands in MW | | | | | Heat (45°C) - bands in MW | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | T1 (10min) | T2 (30min) | T3 (1hr) | T4 (3 hr) | T5 (6 hr) | T1 (10min) | T2 (30min) | T3 (1 hr) | T4 (3 hr) | T5 (6 hr) |
| P1 | CAGGCCCTTC | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1/200 | 0 | 1 |
| P2 | AATCGGGCTG | 0 | 0 | 2 | 1 | 1 | 2 | 1 | 1/200 | 1 | 1 |
| P3 | CAGCACCCAC | 2/ 900, 1000 | 3 | 1/200 | 1/1000 | 0 | 0 | 0 | 1/200 | 1 | 1 |
| P4 | GACCGCTTGT | 2 | 1/400 | 1/400 | 1/400 | 1/400 | 1/400 | 1/100 | 1/200 | 1/400 | 0 |
| P5 | AGGTGACCGT | 2 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| P6 | GTCCCGACGA | 3 | 1 | 3 | 0 | 1 | 2/200,250 | 1 | 1 | 0 | 1 |
| P7 | GACGGATCAG | 2 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| P8 | GTGTGCCCCA | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| P9 | GGGAATTCGC | 1/ 200 | 1/350 | 1 | 1/200 | 1/200 | 1/250 | 1 | 1/300 | 1/300 | 2 |
| P10 | GGCTGCAGAA | 3 | 2 | 0 | 1 | 0 | 0 | 1/300 | 2/300, 400 | 0 | 0 |
| P11 | GAAACGGGTG | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 2 | 2 | 2 |
| P12 | CAGCACCCAC | 2/ 900,1000 | 2/ 900,1000 | 2/ 900,1000 | 1/1000 | 0 | 2/900, 1000 | 1/900 | 2/900. 1000 | 2/900, 1000 | 1 |
| P13 | GATGACCGCC | 1/500 | 1/500 | 1/500 | 1/500 | 1/500 | 1/500 | 0 | 1/500 | 1/500 | 1/500 |
| P14 | ACCCGGTCAC | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| P15 | TCGGCGGTTC | 3 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| P16 | GTCTCCGCAA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| P17 | CCGCCCAAAC | 1 | 0 | 0 | 0 | 0 | 1 | 1/700 | 0 | 0 | 1 |
| P18 | CACCTTTCCC | 2 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 |
| P19 | AGCGAGCAAG | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| P20 | GAACACTGGG | 1 | 1 | 0 | 1/400 | 0 | 0 | 0 | 1/300 | 1/300 | 1/300 |
| Total bands | | 6 | 5 | 5 | 6 | 3 | 7 | 4 | 11 | 6 | 2 |

T1 – treatment 1, T2 – treatment 2, T3 – Treatment 3, T4 – treatment 4 and T5 – treatment 5.

*Common: bands also appeared in the control samples

*Specific/MW: bands present exclusively in treated samples / molecular weight in base pair.

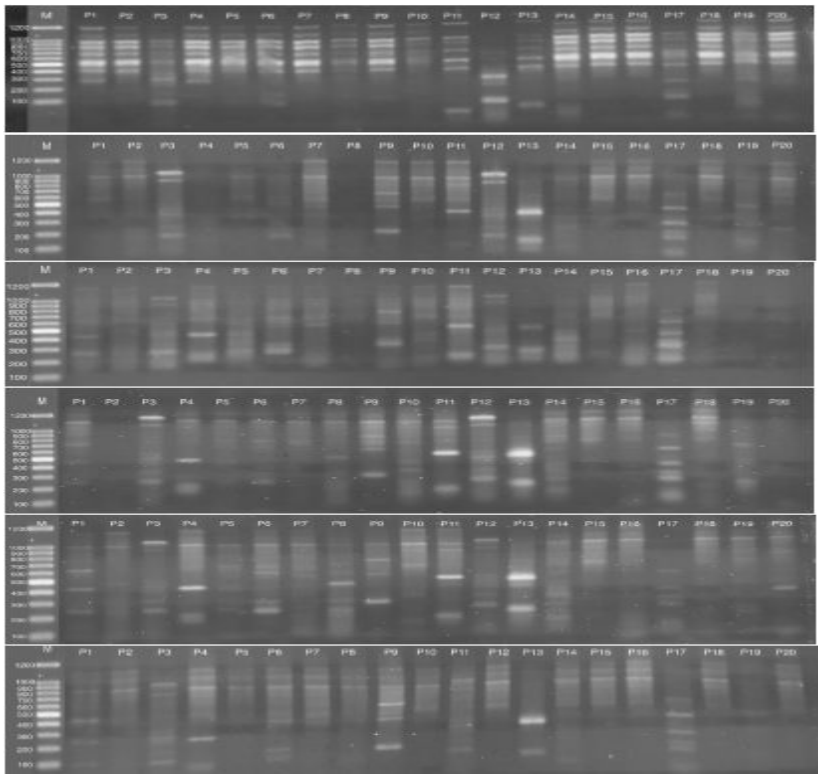


**Fig. 1:** Comparison of Agarose gel electrophoresis result between Control and heat-treated at 40 °C on plant seedling- 1(10 mins), 2 (30 mins), 3 (60 mins), 4 (3 hours) and 5 (6 hours). M- 100 bp Ladder Marker (First base Company) and lane 1 to 20 is represents for random primers 1-20.

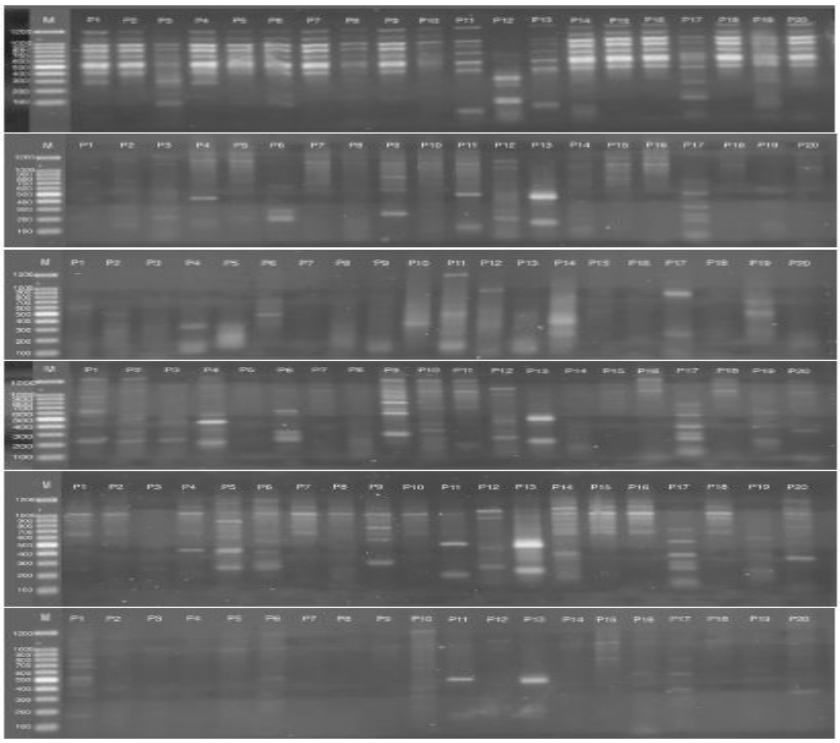


**Fig. 2:** Comparison of Agarose gel electrophoresis result between Control and heat-treated at 45 °C on plant seedling- 1(10 mins), 2 (30 mins), 3 (60 mins), 4 (3 hours) and 5 (6 hours). M- 100 bp Ladder Marker (First base Company) and lane 1 to 20 is represents for random primers 1-20.

***Specificity of molecular markers for the detection of stress response in rice seedlings:***

At both temperatures of 40 and 45 °C heat treated samples produced 55 distinct bands, which were amplified from 11 out of 20 random primers used in the analysis. Primers P1, P2, P6, P10 and P17 formed bands only at 45 °C of heat stress. Other 6 primers (P3, P4, P9, P12, P13 and P20) showed exclusive bands in both temperatures in maximum number of time intervals accept few durations such as P3, P4, P9, P12, P13 and P20. These primers are suitable for detecting heat stress in rice plant seedlings.

***Conclusion:***

Molecular marker has made enormous advance in recent year in both genomics and molecular mapping of the genes. They have proven to be a very useful tool for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted. These markers have generated a vast amount of information, which has helped to generate numerous databases to preserve and popularize it.

In the present study, it has been proposed that the random primers can be used as molecular markers to assess the response of rice seedlings to heat stress. But further studies are still required for the development of heat-resistant rice to cope with the challenges of climate change. Thus, this kind of study will be a very useful tool for marker-assisted selection in rice.

The present discovered molecular markers for heat responsive in rice cultivars will be acceleration of breeding program for development of new hybrids varieties having more tolerance to heat. In conclusion, our goal was to find DNA based molecular markers linked to heat tolerance in order to use them in marker-assisted breeding programs.

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