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# Expression Analysis of Novel microRNAs in Rice During High Temperature Stress

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**ABSTRACT**
*MicroRNAs (miRNAs) are small non-coding RNAs which play an important role in regulating the genes involved in plant growth and development. Several studies showed that miRNAs are involved in plants response to different kinds of abiotic stresses also. In our previous study, temperature responsive miRNAs were predicted in O.sativa. 27 miRNAs were predicted to be novel in rice using homology search. In continuation to our previous study, expression of 14 novel miRNAs was done in shoot and root of 13 days old seedlings of five different rice cultivars using real time PCR. Expression these miRNAs was analyzed in control and high temperature stress environment. Out of 14 predicted novel miRNAs, two novel miRNAs- miR157a and miR165a showed expression in all five rice cultivars. Interestingly, miR165a showed a differential expression pattern among heat tolerant (N22, IR64 and Rasi) and susceptible (Vandana and Sampada) cultivars suggesting that it might have specific role in high temperature tolerance.*
***Key words:*** *Heat stress, small RNAs, Oryza sativa, N22, genes*

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## INTRODUCTION

MicroRNAs of 18-25 nucleotides play an important role in regulating the expression of genes involved in different biological processes (Khraiwesh et al., 2012). In plants, miRNAs bind to near perfect complementary sites on target mRNAs and thereby direct the mRNAs for cleavage or degradation (Bartel, 2004). Genomic sequence conservation and structural homology across different plant species paved the way to many researchers to predict the miRNAs using computational/bioinformatic approaches. Based on structural homology and sequence conservation, miRNA prediction was done in different plant species like Arabidosis, Rice, Gossypium, Medicago etc. (Rhoades and Bartel 2004; Archak and Nagaraju, 2007; Zhou et al., 2008). Expression of various known and novel miRNAs regulating genes expression has been reported in different plant species (Zhang et al., 2009; Yu et al., 2012; Sun et al., 2014). Several studies in rice reported that miRNAs are involved in abiotic stress responses also. In rice, miRNAs regulation has been reported under drought, salinity, cold and nutrient homeostasis using different approaches (Sunkar and Zhu, 2004; Zhao et al., 2007; Lv et al., 2010; Gao et al., 2011; Agarwal et al., 2015). In Our previous study, temperature responsive miRNAs were predicted in *O.sativa* using homology search by utilizing 154 temperature responsive miRNAs of Arabidopsis (Sailaja et al., 2014). We reported that 55 miRNAs were common in both *O. sativa* and Arabidopsis. Expression analysis of identified miRNAs showed that they were temperature responsive in *O. sativa* also. The study also demonstrated 27 novel rice miRNAs along with their secondary structures which were predicted using homology search. In this study, 14 novel miRNAs were selected to study the expression in shoot and root of 13 days old seedlings of five different rice cultivars. The 14 miRNAs were selected based on minimal free energy index (MFEI) values of novel miRNA prediction. MFEI is a crucial parameter of miRNA prediction which helps in discriminating miRNAs from other non-coding RNAs (Zhang et al., 2006; Dehury et al., 2013; Pan et al., 2015).

## MATERIAL AND METHODS

Seeds of rice (*Oryza sativa*) cultivars N22, Vandana, IR64, Sampada and Rasi were surface sterilized in 0.1% $HgCl_2$ aqueous solution for 3-4 mins followed by 3-4 washes with distilled water. Seeds were then germinated in petridishes at 28°C for three days on moist blotting paper in dark. After three days, germinated seeds were transferred to plastic cups containing yoshida medium (Yoshida et al., 1976). Seedlings were grown in a growth chamber under 13h of light and 11 hrs dark by maintaining diurnal temperature 30°C day/24°C night. Seedlings of five cultivars were grown in two replicates for heat stress treatment. Another two replicates of seedlings with similar conditions were kept as control. Thirteen days old seedlings were subjected to heat stress treatment. Two batches (replicates) of seedlings were maintained in ambient temperature (30°C), while other two batches were exposed to high temperature (42°C) for 6h. Seedlings maintained at ambient temperature were considered as control. Followed by heat stress treatment, samples of all the cultivars were harvested from two biological replicates. Small RNA was isolated from root and shoot tissues of five different cultivars by using mirVana miRNA isolation kit (Ambion, Austin, TX, USA). cDNA synthesis and quantitative PCR (qPCR) conditions were followed as published earlier (Sailaja et al., 2014). Fourteen novel miRNAs and their primer sequences are given in Table1. Two biological replicates and three technical replicates were maintained for expression analysis of miRNAs. The analysis of data was performed as reported previously (Sailaja et al., 2014). The target prediction was done using psRNATarget (http://plantgrn.noble.org/psRNATarget/).

## RESULTS AND DISCUSSION

In order to study the expression with novel miRNAs, qPCR was performed in root and shoot of five genotypes under control and heat stress environment using 14 primers that were derived from 14 novel miRNAs. However, no expression was observed with 12 primers as cT (threshold cycle) value was >35.0 in all the samples. Interestingly, two predicted novel miRNAs- miR157a and miR165a showed expression in all five rice cultivars (Fig.1). Further, miR157a and miR165a showed differential expression patterns in shoot and root while comparing their expression during control and heat stress environment. In comparison to control, heat stress treated shoot and root of IR64 and Sampada showed up-regulation of miR157a while in Vandana, it showed down-regulation in shoot and root by 5.8 fold and 67.2 fold, respectively. In N22 and Rasi, it showed differential expression. N22 showed up-regulation in shoot by 1.49 fold and down-regulation in root by17.0 fold under heat stress. In contrast, Rasi showed down-regulation in shoot (1.4 fold) and up-regulation in root (3.0 fold). Considering the expression pattern of miR157a in five rice genotypes under high temperature stress and the level of heat stress tolerance exhibited by these genotypes shown in our previous study (Sailaja et al., 2015), it is less likely that novel miRNA-miR157a might be contributing heat stress tolerance in rice, though, its expression analysis suggests that it is temperature responsive microRNA. Based on various morphological, physiological, biochemical and yield traits, N22, Rasi and IR64 were suggested as heat tolerant while Vandana and Sampada were heat susceptible (Sailaja et al., 2015). Temperature responsive miRNAs have been reported in various plants (Xin et al., 2010; Jeong et al., 2011; Yu et al., 2012; May et al., 2013).

Expression analysis of miR165a showed down-regulation of this miRNA in root and shoot of N22, IR64 and Rasi under high temperature stress. Our study suggested that all these three genotypes are heat tolerant (Sailaja et al., 2015). The down-regulation of miR165a in root and shoot under high temperature might be causing up-regulation of its target gene. This miRNA showed upregulation in shoot of Vandana and root of Sampada. Both of these genotypes have been characterized as heat susceptible (Sailaja et al., 2015). The expression pattern of miR165a and the high temperature response of these genotypes suggest that it might be associated with heat stress tolerance. Role of miRNAs in heat stress tolerance has been revealed in various reports (Yu et al., 2012; Sailaja et al., 2014; Goswami et al., 2015). The predicted target genes for miR157a and 165a are retrotransposons and RNA binding proteins (Table 2). Retrotransposon activation under stress conditions in plants has been reported (Grandbastien, 1998; Grandbastien et al., 2005). Under elevated temperatures, activation of specific retrotransposons was reported in Arabidopsis (Cavrak et al., 2014). Decreased expression of miR165a suggests the increased expression of corresponding target. RNA binding proteins (predicted target gene of miR165a) play a vital role in posttranscriptional gene regulatory mechanisms like pre-mRNA splicing, capping, mRNA transport, stability, epigenetic regulation, and translation by binding directly or indirectly to other regulatory factors (Floris et al., 2009; Wang et al., 2013). Being a central molecule in regulation of key molecular processes, these RNA binding proteins play an important role in plant stress responses (Kang and Kwak, 2012). Expression analysis of two novel miRNAs (miR157a and 165a) in root and shoot of five rice cultivars showed that homology search is a reliable method for predicting novel miRNAs. This study suggests that miR165a might play an important role during high temperatures stress by increasing the expression of its target transcript RBPs as it showed a distinct expression pattern among susceptible and

tolerant rice cultivars. It will be interesting to further study the role of miR165 and its target gene through transgene expression studies.

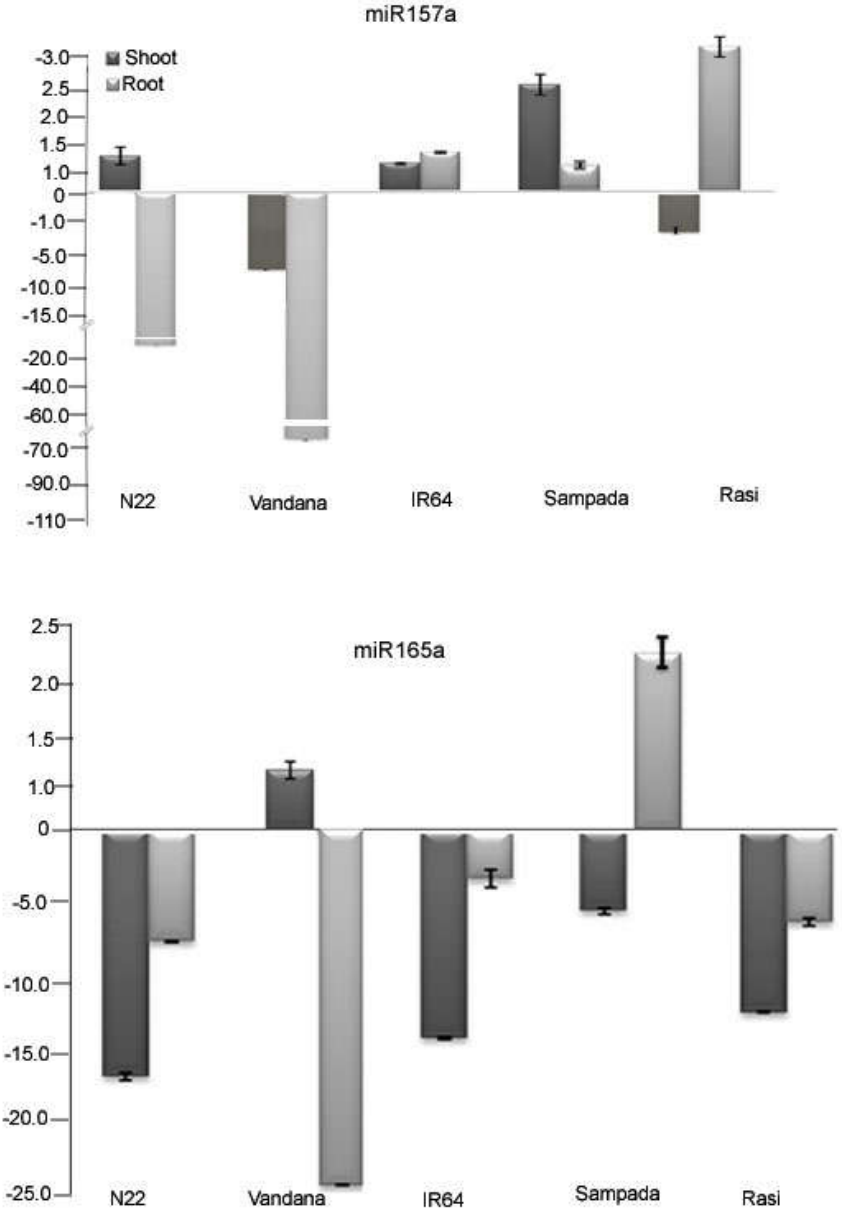
**Table 1.** List of novel miRNAs primers chosen for expression study

| S.No | miRNA | Sequence |
|---|---|---|
| 1 | miR157a | TGTCATAACTCACTGGGGAGCAT |
| 2 | miR165a | TCGGACTCGACAGCCCTTGGG |
| 3 | miR172e | GGACGGCTTGTGGCTATTTGCAT |
| 4 | miR400 | TGTCAAGACCATTCAAAAAGCAA |
| 5 | miR404 | ATTATAGTATAGATTCCGTAAGG |
| 6 | miR823 | TGGCTGGCTCATTCTACACCAA |
| 7 | miR829.1 | GCTGTCTTCCTCAATCCTGGGTT |
| 8 | miR835-5p | TTCTCTCTGTCTCATCTCTATC |
| 9 | miR842 | TCAAATTAAAATTCGGATTCA |
| 10 | miR845a | CTCCTTCTGTTAGCTATTCATT |
| 11 | miR846 | TAGAATATACTCTCCTAGAATTC |
| 12 | miR857 | ATTTCAGTTTGAAAGTGGAT |
| 13 | miR863-3p | TTGATAAAAACATGAATTTATA |
| 14 | miR865-3p | TTTCTCTCCTGATTTATCTTC |

**Table 2: Potential target genes of miR157a and miR165a**

| miRNA | Target Acc. | Expectation (E) | Target Accessibility (UPE) | Target Description | Inhibition |
|---|---|---|---|---|---|
| miR157a | LOC_Os09g31438.1 | 1.0 | 22.658 | OsSPL17 - SBP-box gene family member | Cleavage |
| | LOC_Os08g39890.1 | 1.0 | 22.009 | OsSPL14 - SBP-box gene family member | Cleavage |
| | LOC_Os02g04680.2 | 1.0 | 18.228 | OsSPL3 - SBP-box gene family member | Cleavage |
| | LOC_Os02g04680.1 | 1.0 | 18.228 | OsSPL3 - SBP-box gene family member | Cleavage |
| | LOC_Os08g41940.1 | 1.5 | 19.633 | OsSPL16 - SBP-box gene family member | Cleavage |
| | LOC_Os01g69830.1 | 1.5 | 18.578 | OsSPL2 - SBP-box gene family member | Cleavage |
| | LOC_Os11g30370.1 | 2.0 | 23.318 | OsSPL19 - SBP-box gene family member | Cleavage |
| | LOC_Os09g32944.1 | 2.0 | 12.299 | OsSPL18 - SBP-box gene family member | Cleavage |
| | LOC_Os04g46580.1 | 2.0 | 24.301 | OsSPL7 - SBP-box gene family member | Cleavage |
| | LOC_Os02g07780.1 | 2.0 | 23.318 | OsSPL4 - SBP-box gene family member | Cleavage |
| | LOC_Os02g07780.2 | 2.0 | 12.299 | OsSPL4 - SBP-box gene family member | Cleavage |
| | LOC_Os07g43980.1 | 2.0 | 24.301 | DEAD-box ATP-dependent RNA helicase | Cleavage |
| miR165a | LOC_Os03g01890.1 | 1.5 | 22.598 | START domain containing protein | Cleavage |
| | LOC_Os10g33960.1 | 1.5 | 19.736 | START domain containing protein | Cleavage |
| | LOC_Os12g41860.1 | 1.5 | 20.46 | START domain containing protein | Cleavage |
| | LOC_Os03g43930.1 | 1.5 | 21.231 | START domain containing protein | Cleavage |
| | LOC_Os03g43930.2 | 1.5 | 21.231 | START domain containing protein | Cleavage |
| | LOC_Os10g33960.2 | 1.5 | 19.736 | START domain containing protein | Cleavage |
| | LOC_Os03g01890.2 | 1.5 | 22.598 | START domain containing protein | Cleavage |
| | LOC_Os10g33960.4 | 1.5 | 19.736 | START domain containing protein | Cleavage |
| | LOC_Os10g33960.3 | 1.5 | 19.736 | START domain containing protein | Cleavage |
| | LOC_Os04g23200.1 | 2.5 | 24.586 | expressed protein | Cleavage |
| | LOC_Os01g33740.1 | 2.5 | 19.615 | retrotransposon protein | Cleavage |

Figure.1: Expression of novel miRNAs in five rice cultivars during high temperature stress.

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