

## Expression Analysis of Conserve MicroRNAs in Rice Under Abiotic Stress Condition



### Biotechnology

**KEYWORDS :** miRNA; targeted Transcription factor; abiotic stress; stress related cis elements; *Oryza sativa*

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### ABSTRACT

*MicroRNAs (miRNAs) are a class of endogenous small regulatory RNA molecules (20–24 nucleotides) that play important role in various aspects in plant development and come out as gene expression regulators at post-transcriptional levels in plants. Plant microRNAs indulge in miscellaneous biological processes together with growth and stress responses. Salt and drought stresses are one of the most serious abiotic stresses of crop plant world-wide. Although the expression of miRNAs under abiotic stress is not well understood and our efforts are just beginning to be explored it. We analyzed the promoters of 11 conserve miRNAs families (miR156, miR159, miR160, miR164, miR166, miR167, miR169, miR171, miR172, miR393, miR396) that contained all the stress-related cis-elements. Among these miRNAs we found only two miRNAs- miRNA396 and miRNA169 showing up regulation in salt and drought stress respectively.*

### INTRODUCTION

Plants are sessile in nature that's why they have developed several mechanisms to cope up with environmental stresses. Due to global warming, earth temperature increases gradually, this causes the conversion of previously wet region to more arid and the deposition of salt into low lying grass and farm lands. Drought and salt stress are two wide ranges of abiotic stresses which causes severe damage of crop growth and productivity. Although various research unit has been dedicated towards abiotic stress inducing genes but the mechanism of these stress inducing gene expression remain largely unknown. MicroRNAs are approximate 21 nucleotides short sequences of endogenous highly conserve class of non coding RNAs that regulate gene expression at post transcriptional level. MicroRNAs have enormous role in plant development including metabolic process and organ development (Palatnik et al., 2003). Not only in developmental process but also have been shown to play an important role in environmental abiotic stress such as drought (Zao et al., 2007) salinity, heavy metals and cold (Zhou et al., 2008). MicroRNA398 was first stress reported miRNA regulated by oxidative stresses. During sulphate and inorganic phosphate starvation responses miRNA395 and miRNA399 were identified respectively (Fujii et al., 2005).

Various technique and approaches have been developed (Biochemical, Molecular and Bioinformatics) for miRNA analysis and detection. In which Stem loop RT-PCR is fast sensitive and specific miRNA expression profiling and suitable for quantification of miRNA expression (Varkonyi-Gassic et al., 2007). The gene responsive to salt and drought stresses are poorly understood regarding regulatory mechanism. Being as a gene regulator, miRNA play an important role in plant tolerance to abiotic stresses (Zhang et al., 2005). Rice is widely used cereal staple crop all over the world so it is necessary to understand the metabolic process to look up its yield, nutrition quality and stress management in near future because of tremendous increase in population. Perhaps majority of biological characteristic is governed by miRNAs. On that basis we can assume high significance to make the rice miRNA as a better tool to increase the rice yields. The aim of this study was to analysis of expression of conserve miRNAs under salt and drought stress which target to transcription factors in rice.

### Materials and Methods

#### Seed sterilization and stress treatment

Rice seeds obtained from (Indian Agricultural Research Institute, New Delhi, India) were washed with mild detergent solution and rinsed with distilled water. Seeds of *Oryza sativa* var. Pusa Basmati-1 were sterilized and grown under control condition at 28

°C in day or 25 °C in night with 12hrs light/12hrs dark photoperiods. After 14 days of germination. Uniform-sized seedlings were subjected to 200 mM of NaCl for different time interval maximum up to 72 hrs at 28 °C. For drought treatment, seedlings were carefully transferred from their pots onto dry paper in the growth room and drought stress was applied for various periods. The samples were frozen in liquid nitrogen and stored at -80 °C for further analysis. Total RNA was isolated from 80-100 mg shoot tissue of rice using RNeasy plant mini kit from QIAGEN according to the manufacturer's instructions. The RNA quality was checked by agarose gel electrophoresis and nanodrop.

#### Primer design for conserve miRNA

Conserve miRNA strongly participate in plant development because they regulate different transcription factor gene. We found these targets in Indica rice with the help of miRU target prediction algorithm. Plant miRNAs recognize their target mRNAs by near-perfect base pairing; computational sequence similarity search can be used to identify potential targets. The conserve rice miRNA sequences were downloaded from Sanger institute miRBase sequence Database (Release12.0) (Griffiths-Jones et al., 2008). The stem loop RT primers were designed according to Chen et al., (2005). On the basis of single nucleotide differences of each miRNA family members, introduce degenerated nucleotides to amplify miRNA. Primer sequence data of selected miRNAs are presented in Table 3 and 4. The specificity of stem-loop RT primers to individual miRNA is conferred by a six nucleotide extension at the 3' end; this extension is a reverse complement of the last 6 nucleotides at the 3' end of the miRNA (Fig 2). Forward primers are specific to the miRNA sequence but exclude the last six nucleotides at the 3' end of the miRNA. A 5' extension of 5–7 nucleotides is added to each forward primer to increase the melting temperature; these sequences were chosen randomly and are relatively GC-rich.

#### cis-acting element analysis of conserve miRNA promoter

Micro RNA (miRNA) originated from long primary transcript (pri-miRNA) and finally it makes a shape of miRNA when it processed from precursors miRNA (pre-miRNA). Stress gene of miRNA has been analyzed by the presence of cis acting elements extensively (Zhang et al., 2005). The sequences of promoter region were downloaded from PMRD and the cis-element was predicted via PLACE. In the promoter region we searched ABRE (ABA responsive element: ACGTG), DRE (Dehydration responsive element: RYCGAC), ERD (Early responsive to dehydration element: ACGT), LTRE (Low temperature responsive element: CCGAC), MYB (CTAACA, CNGTTR), MYC (CANNTG). Both MYB and MYC are found in promoters of dehydration responsive gene and salt induced cis-element.

### Isolation of RNA and cDNA preparation

Total RNA was isolated from 80-100 mg leaf tissue of rice using RNeasy plant mini kit from QIAGEN. Reverse transcription reaction carried out in 10 $\mu$ l reaction mixture contain RNA, 50nM stem loop RT primer, 50 unit reverse transcriptase, 0.25 mM dNTPs, 1X reverse transcriptase buffer and 4 unit RNAase inhibitor. For reverse transcriptase incubation start at 16 °C for 30 minutes followed by pulse RT of 60 cycles for 30 second at 30 °C, 30 second at 42 °C and 1 second at 50 °C. For reaction termination incubate at 85 °C for 5 minutes to snatch the reverse transcriptase activity.

### Stem-loop RT (Reverse transcriptase) PCR

Stem loop RT and PCR primers were designed according to Chen et al., (2005). Total RNA was treated with DNase-I (Promega) to remove residue of genomic DNA. Four hundred nanograms RNA (DNase treated) were used to generate first strand cDNA (Varkonyi-Gassics et al., 2007). We prepare 19 micro lit PCR reaction mixture contain 15.4 $\mu$ l nuclease free water, 2  $\mu$ l 10X PCR buffer, 0.4 $\mu$ l 10Mm dNTPs mix, 0.4 $\mu$ l forward primer(10 $\mu$ M),0.4 $\mu$ l reverse primer(10 $\mu$ M) and 0.4 $\mu$ l Phusion Taq (Finyzyme). After the preparation of master mix we separately added 1 $\mu$ l RT product. RT product act as template for this PCR, in our designed experiment we used here different RT product from control to salt stress at different time interval. Reaction mix placed on thermal cycler pre heated block (94 °C) and incubation period was 94 °C for 2 min followed by 20-40cycles of 94 °C for 15s and 60 °C for 1 min (Erika et al., 2007).PCR product visualized by gel electrophoresis on 4% agarose gel in 1X TAE (Fig 5a and 5b) . We wanted aim to check the expression of conserve miRNA in rice under salt and drought stress at different time interval in this context separately we amplified each conserve miRNA under 200mM salt stress at different time interval with the help of Stem-loop RT-PCR (End point PCR).The most important parameters in this stem loop RT PCR was re folding of stem loop in to its stable structure when primer subjected to high temperature. During the reaction if stem loop folded incompletely due to this increase the chances of non specific cDNA may occurs. Due to the same we faced the problem of non specific PCR amplification. We prepared the step wise dilution of total RNA of rice shoots for establishment of sensitivity of stem loop RT PCR. In semiquantitative manner we performed the amplification at fixed cycle (20-30 cycles). We detected the amplification of tested miRNA from 400ng total RNA. In the selected cycle there were no amplification in minus-RT except weak band of primer dimmer. Though we got non specific PCR amplification above 30 PCR cycle. Three biological replicates were run for each miRNA for each treatment and then results were analyzed.

### Result and discussion

Twenty miRNAs are highly conserved in three sequenced plant genome *Arabidopsis*, *Oryza sativa* and *Populus trichocarpa* (Jones Rhoades et al., 2006). Our aim was to study the expression of those conserve miRNA which targeted to transcription factor (Table 1 and 2). Tentatively we choose eleven miRNA which target to transcription factor. Conserve plant miRNA regulate homologous target at identical target sites in every species in which they exist and these target can be simply predicted (Rhoades et al., 2002). We designed the primers (Table 3 and 4) according to Chen et al., (2005). Mature miRNA are conserve in different plant species (Fig 1). The members of miRNA in same families differ from each other by only one to three nucleotides or identical (Fig 1). We used stem loop RT-PCR for expression of miRNA because due to cross hybridization might be occurred in microarray as well as in northern blots.

### Expression of known miRNAs altered by salinity and drought stress in rice (PB 1-Pusa Basmati 1)

In our designed experiment stem loop RT-PCR approach was used to detect mature miRNA species in rice seedling shoot.

Expression of conserve miRNA which targeted to transcription factor was detected using two step process in the first the stem loop RT primer was hybridized to a miRNA and then reversely transcribed in the pulsed RT reaction in the second step the RT product was amplified according to experiment and generally quantified by semiquantitative manner (Fig 5 and 6). For Semi quantitative study of miRNA first we ensured the equal concentration of total RNA from different plant samples (stress or without stress) at different time interval up to 72 hrs under salt and drought stress condition. We determined the cycling parameter as well as MgCl<sub>2</sub> concentration along with actin control and the condition were chosen such that none of the stem loop RT product reached at Plateau stage at the end of amplification. Expression of selected mature miRNA altered during salt (200mM NaCl) and drought stress in a time dependent manner.

During stem loop RT-PCR we observed that transcript of all miRNA accumulate at 18 hrs maximally and we did not found any amplification beyond that. These 11 miRNA get induced (Up/down) in both the stress. In our data we did not saw the amplification of all miRNA (Eleven conserve miRNA) up to 72hrs although we found amplification only up to 18hrs. We hypothesized that may be they are required at low level at this stage or their expression is inadequate on order of plant development, which make it difficult to identified the changes under stress conditions. We found miRNA169 exhibiting good expression pattern in comparison to others and showing up regulation during drought stress condition. We know that the most of the miRNAs are involved in the regulation of crucial development process. Plant in stress condition always has shown abnormal developmental phenotype. It is evident that most of the miRNA targeted genes participate in development as well as stress regulation. So we speculate that these miRNA which targeted to transcription factors might be co regulated by both environmental factors and developmental cue.

### Analysis of cis-element exist in the promoters of miRNA

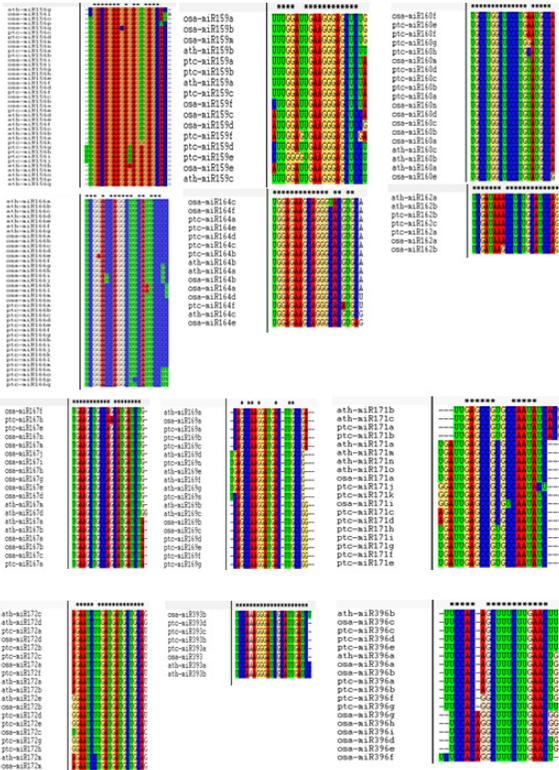
MicroRNA originated from long primary transcript (Pri-miRNA) and finally it makes a shape of miRNA processed from precursor's miRNA (Pre-miRNA). Stress gene of miRNA has been analyzed by the presence of cis acting elements extensively (Zhang et al., 2005).The sequences of promoter region were downloaded from PMRD and the cis-element was predicted via PLACE. On promoter we search ABRE (ABA responsive element: ACGTG), DRE (Dehydration responsive element: RYCGAC), ERD (Early responsive to dehydration element: ACGT), LTRE (Low temperature responsive element: CCGAC), MYB (CTAACA, CNGTTR), MYC (CANNTG)-Both are found in promoters of dehydration responsive gene and salt induced cis-element. We got all type of abiotic stress related cis elements on the promoters of selected miRNAs (Fig 4).

### Conclusion

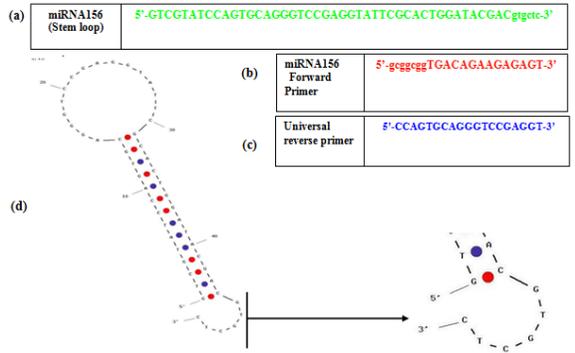
Computational method identified miRNA that are conserve between *Arabidopsis* and rice. On this evidence we chose 11 miRNA which target to those transcription factors which have great importance in plant development. It is evident that most of the miRNA (gene regulators) expression altered during the abiotic stresses (Liu et al., 2008). In this report we investigated the changes in miRNA expression level under salt stress (200mM NaCl) and drought stress in rice variety: Pusa Basmati 1. We exhibited it with the semiquantitative Stem loop RT-PCR to test whether the stress influence the expression of miRNA. Stem loop RT-PCR is most reliable sensitive and affordable methods because in microarray, miRNA belonging to same family have similar expression pattern due to one or two nucleotides difference in each family members. That's why they could not be differentiated in microarray analysis because of cross hybridization. Using Stem Loop RT-PCR we analyzed 11 different conserve miRNA (which target to transcription factor) in two week

old rice seedling shoot exposed under salt and drought conditions. We failed to detect the expression of miRNA up to 72hrs . We hypothesized that may be they are required at low level at this stage or their expression is inadequate on order of plant development, which make it difficult to identify the changes under stress conditions. In addition most of the miRNA involve in the regulation of crucial development process. Plant in stress condition always has shown abnormal developmental phenotype. We found two miRNAs miRNA396 and miRNA169 showing up regulation in salt and drought stress respectively. So we speculate that these miRNA which targeted to transcription factors might be co regulated by both environmental factors and developmental cue. This introductory data indicate that miRNA might play an important role in abiotic stresses in plant and help to with stand to survive against stresses. Due to potential uncertainty in this experiment it is not reasonable to just estimate the exact effects of these miRNA in plants. This data help to understand the relation of miRNA with abiotic stresses, initial point for future studies and sustained efforts are needed to confirm the function of miRNAs in stress responses and adaptation.

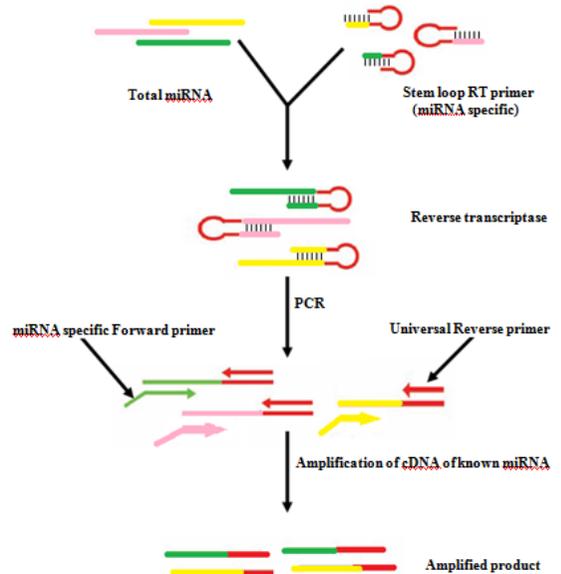
**Acknowledgments** Authors acknowledge financial support from UGC in the form of Major Research Project. One of the author MKW is also thankful to UGC for BSR fellowship.



**Fig1: Conserved miRNAs alignment showing in Arabidopsis, Populus and Oryza sativa.**



**Fig 2: Diagram showing the primer design for stem-loop RT-PCR for detection of microRNAs. (a) The stem-loop RT primers have a universal backbone and provide a miRNA-specific extension. The specificity of a stem-loop RT primer to an individual miRNA is conferred by a six-nucleotide extension at the 3'-end which is a reverse complement of the last six nucleotides at the 3'-end of the miRNA. The backbone sequence can form a stem-loop structure because of the complementarity between the nucleotides in the 5'- and 3'-end. (b) Forward primers are specific to the miRNA sequence but exclude the six nucleotides at the 3'-end of the miRNA. (c) Universal reverse primer. (d) Showing the loop structure of stem loop primer.**



**Fig 3: Schematic showing stem loop RT-PCR mechanism. In this method the stem primer binds to the 3' portion of miRNA and then initiate the reverse transcription, after the reverse transcription RT product amplify with the help of miRNA specific forward primer and universal reverse primer.**

miRNA family	Targeted Transcription factor family	Conservation status			References
		Arabidopsis	Oryza	Populus	
miR156	SBP	√	√	√	Reinhart et al., 2002
miR159	MYB	√	√	√	Reinhart et al., 2002
miR160	ARF	√	√	√	Reinhart et al., 2002
miR164	NAC	√	√	√	Reinhart et al., 2002
miR166	HD-ZIPIII	√	√	√	Reinhart et al., 2002

miR167	ARF	√	√	√	Reinhart et al., 2002
miR169	HAP2	√	√	√	Reinhart et al., 2002
miR171	SCL	√	√	√	Reinhart et al., 2002
miR172	AP2	√	√	√	Park et al., 2002
miR393	bZIP	√	√	√	Jones Rhoades and Bartel, 2004.
miR396	GRF	√	√	√	Jones Rhoades and Bartel, 2004.

**Table1: miRNA and their predicted target.** The miRNA (previously known) in listed table is conserve (*Arabidopsis*, *Oryza* and *Populus*) and targeted to transcription factors.

miRNA	Predicted function	Target Gene Accession No
miR-NA156	Squamosa promoter-binding protein SPL2, SPL9, SPL10	SBP(OsIBCD032899)
miR 159	Plant growth, anther development and flowering time. (Palatnik <i>et al.</i> ,2004)	MYB(OSIBCD018450)
miR-NA164	Apical meristem establishment, embryonic development and meristem formation. (Aida <i>et al.</i> ,2004,Mallory <i>etal.</i> ,2004)	NAC(OSIBCD014570)
miR160	Plant growth, root and shoot and vascular development. Apical dormence	ARF(OSIBCD014998,021834)
miRNA 166	Homeobox-leucine zipper transcription factor (HB-14); homeo-domain-leucine zipper protein Revoluta (REV)	HD-ZIPIII (LOC_Os12g41860.1 )
MiRNA 167	Plant growth root and shoot	ARF (LOC_Os04g57610)
miR-NA169	CCAAT-binding transcription factor	HAP2 (LOC_Os03g07880.1 )
miRNA 171	Scarecrow-like transcription factor 6 (SCL6)	SCL(LOC_Os06g01620.1 )
miR-NA172	Plant flowering time and floral morphology. (Aukerman and Sakai,2003)	AP2(OSIBCD015814,012428)
miR-NA393	bHLH transcription factors	bZIP(OsIBCD028791)
miR-NA396	GRF factors	GRF(OsIBCD011542)

**Table 2: miRNA targeted transcription factor and its accessions no in Pusa Basmati 1.** We targeted these conserve miRNA in rice (Pusa Basmati 1) with the help miR U target finders. These conserve miRNA strongly participate in plant development because they regulate different transcription factor gene.

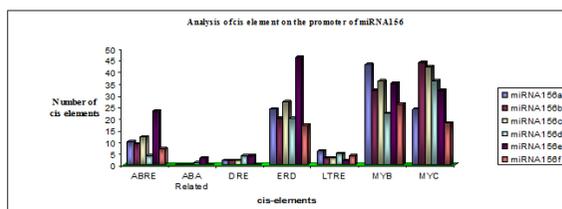
**Table3: Stem loop primers for rice conserve miRNA.**

MicroRNAs	Forward primer for stem loop RT PCR
miRNA156	5'-gcggcggTGACAGAAGAGAGT-3'
miRNA159	5'-gcggcggATTGGATTGAAGGGA-3'
miRNA160	5'-cgccTGCCTGGCTCCCTGT-3'
miRNA162a	5'-gcggcggTCGATAAACCTCTGC-3'
miRNA164	5'-cacgTGGAGAAGCAGGGCA-3'
miRNA166	5'-gcgcgTCGGACCAGGCTTCA-3'
miRNA167	5'-gggcgTGAAGCTGCCAGCAT-3'
miRNA169a,b,c	5'-gcggcgCAGCCAAGGATGACT-3'
miRNA169d-p	5'-gcggcgcTAGCCAAGGATGAAT-3'
miRNA171	5'-gccgTGATTGAGCCGTGCC-3'
miRNA172	5'-ggcgcAGAATCTTGATGATG-3'
miRNA393	5'-gcggcgTCCAAAGGGATCGCA-3'
miRNA396	5'-gcggcggTTCCACAGCTTTCTT-3'
Universal reverse primer	5'-CCAGTGCAGGGTCCGAGGT-3'

MicroRNAs	Stem loop primers
miRNA156	5'-GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGACTGGATACGACgtgctc-3'
miRNA159a,b,f	5'GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGAC(t/c)agagc3'
miRNA159c,d,e	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGAC(t/c)ggagc-3'
miRNA160	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGAC(t/c)ggcat-3'
miRNA164	5'-GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGACTGGATACGACcgcagc-3'
miRNA166	5'-GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGACTGGATACGACctggat-3'
miRNA167	5'-GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGACTGGATACGACggggaa-3'
miRNA169	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGAC(t/c)(c/a)ggca-3'
miRNA171	5'-GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGACTGGATACGACgatatt-3'
miRNA172	5'-GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGACTGGATACGACatgcag-3'
miRNA393	5'-GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGACTGGATACGACagatcaa-3'
miRNA396	5'-GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGACTGGATACGACgagttc-3'

**Table4: Forwards and universal reverse primer**

**Fig 4: Exhibiting abiotic stress related cis acting elements on the promoter of different miRNAs**



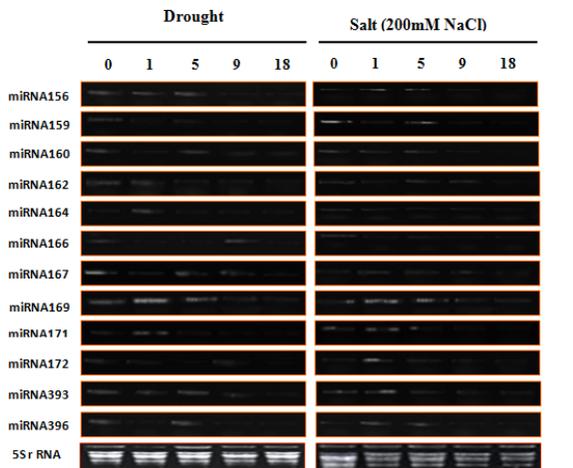
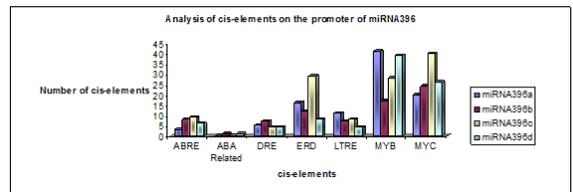
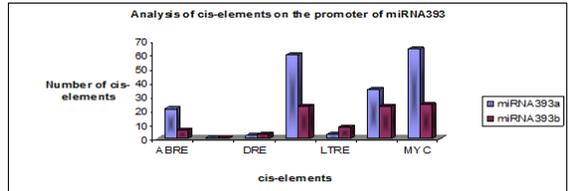
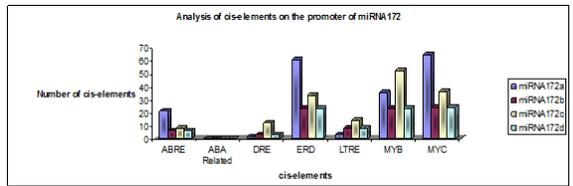
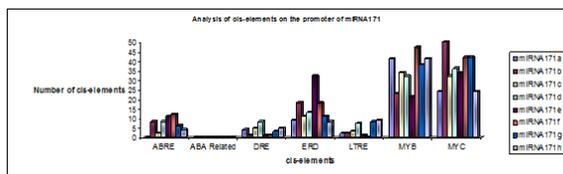
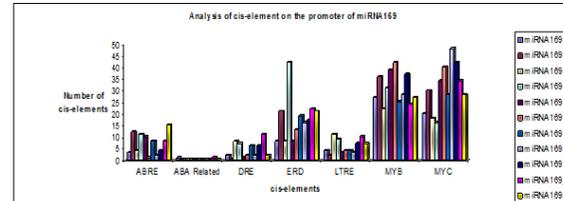
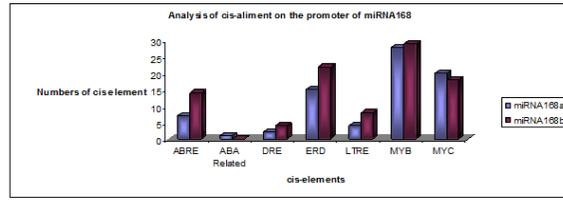
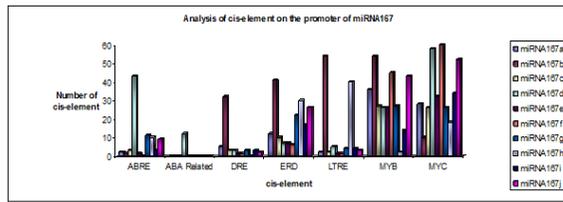
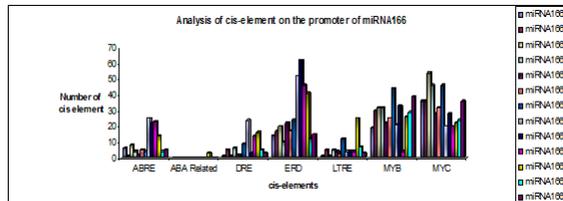
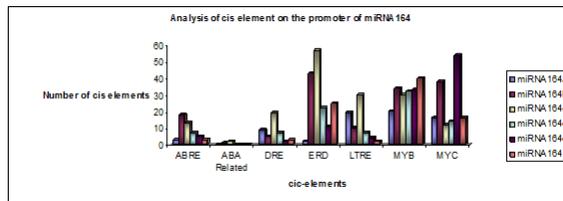
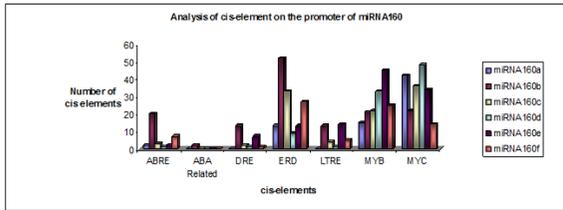
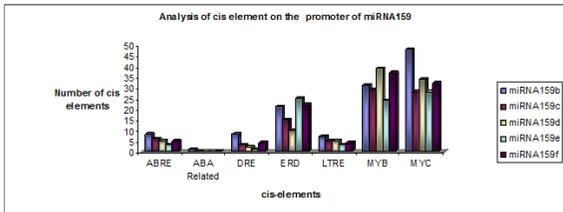


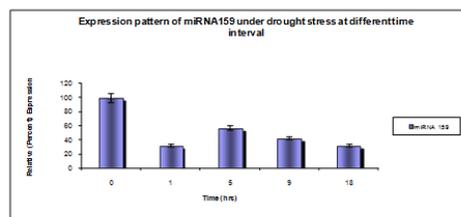
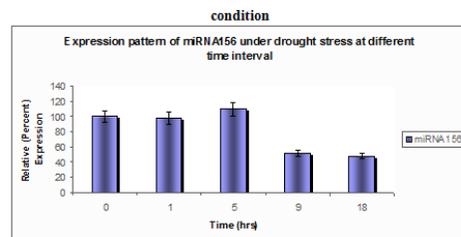
Fig 6: Expression level of selected miRNAs by Stem-loop RT-PCR under drought and salt (200mM NaCl) stress at different time course.

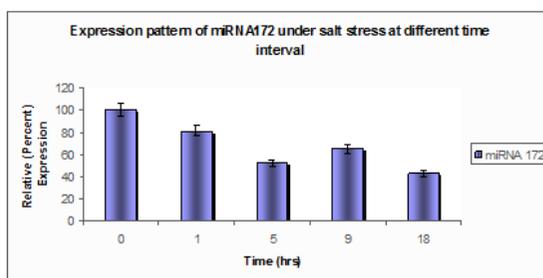
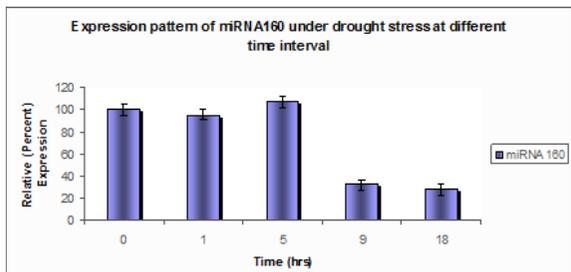
The sensitivity of the stem-loop RT-PCR

Fig 5a: Stem loop RT-PCR analysis of selected miRNA in rice seedling shoots at different cycling parameters.

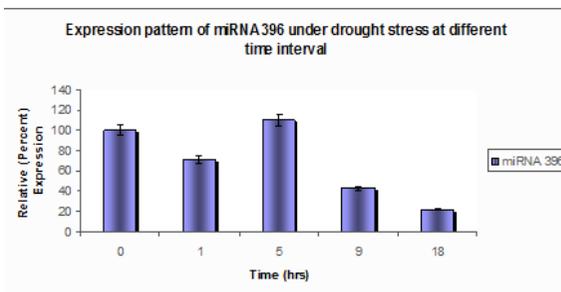
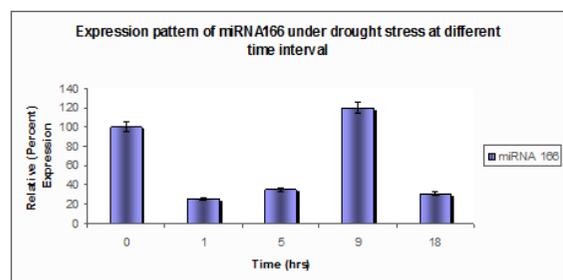
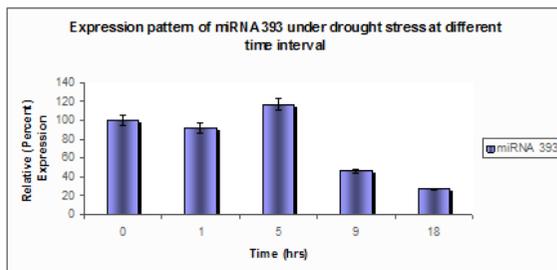
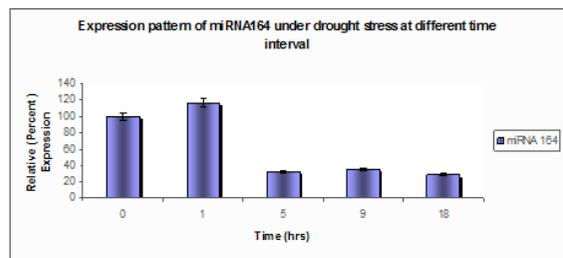
Fig 5b: Stem-loop RT-PCR analyses of miRNAs. The amounts of RNA ng/micro lit used for reverse transcription reactions are indicated on the top.

Expression pattern of conserve miRNA in rice via stem loop RT-PCR under drought stress

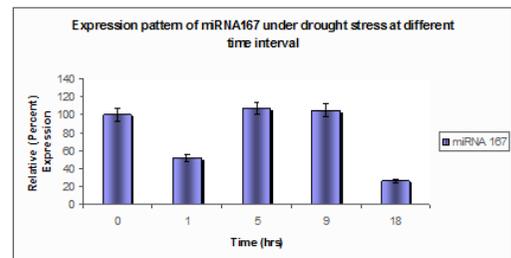




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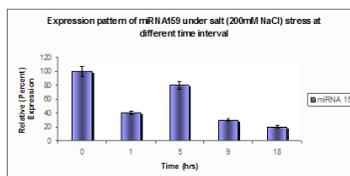
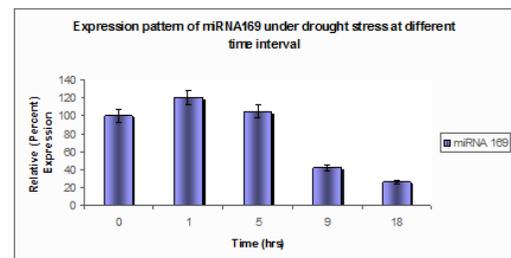


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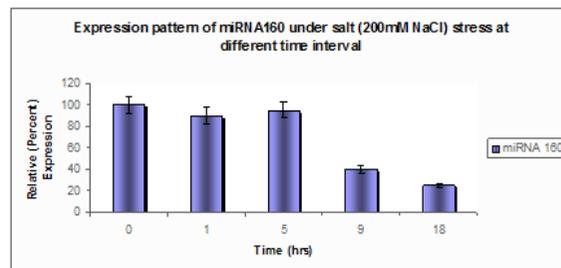
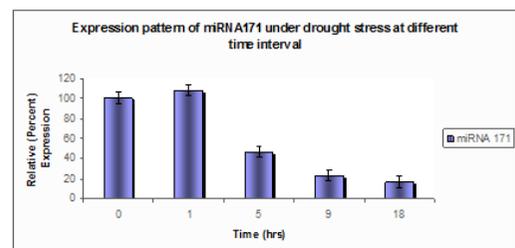


**Fig 7a:** Relative percent expression of various conserved miRNA in rice seedlings shoot under drought stress at different time course in rice (Pusa basmati 1). Three biological replicates were run for each mature miRNA. This graph was plotted on the basis of band intensity obtained by semi-quantitative stem loop RT-PCR. The band intensity for each sample was normalized. The normalized value for healthy tissue was taken as 100 while the value for other stress samples was given with respect to healthy control. Actin is used as an internal loading control.

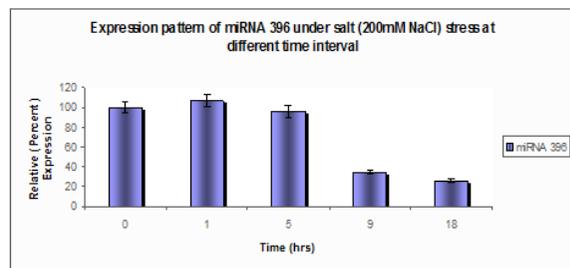
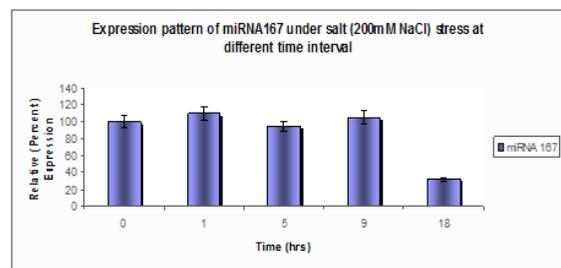
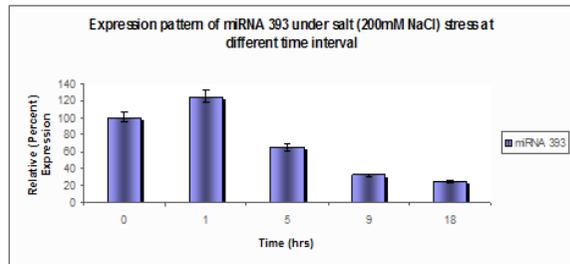
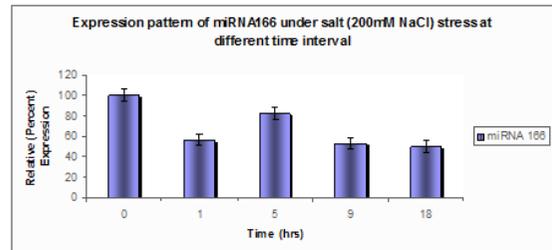
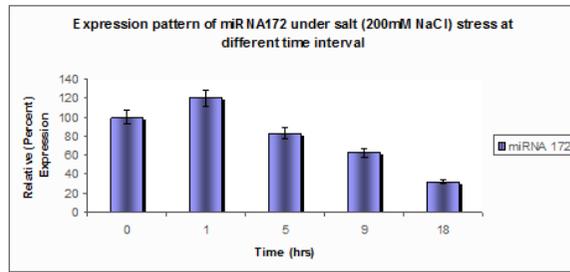
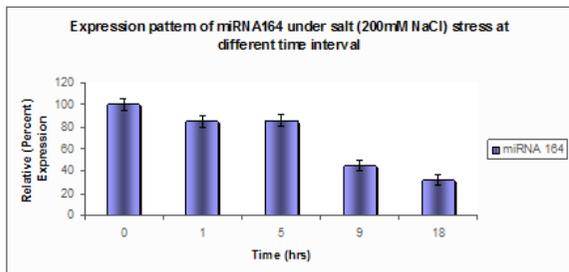
**Expression pattern of conserved miRNA in rice via stem loop endpoint RT-PCR under salt stress (200mM, NaCl) condition**



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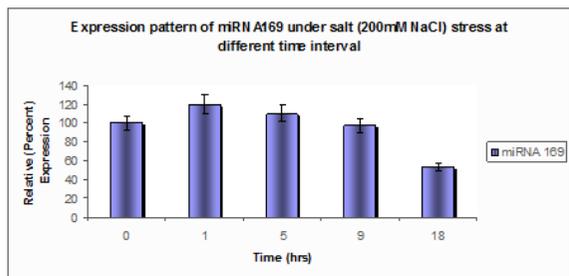


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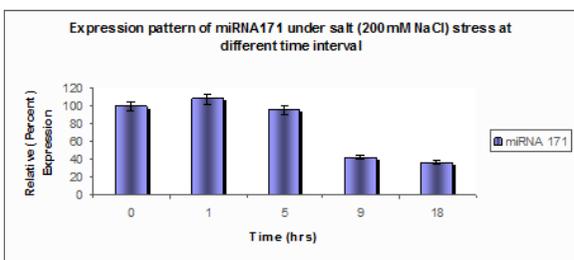


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**Fig 7b: Relative percent expression of selected conserved miRNA in rice seedlings under salt stress (200mM, NaCl) at different time course in rice (Pusa basmati 1). Three biological replicates were run for each mature miRNA. This graph was plotted on the basis of band intensity obtained by semi-quantitative stem loop RT PCR. The band intensity for each sample was normalized. The normalized value for healthy tissue was taken as 100 while the value for other stress samples was given with respect to healthy control. Actin is used as an internal loading control.**



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