FUNCTIONAL AND EXPRESSIONAL QUANTITATION OF SELECTED microRNAs IN RESPONSE TO LOW TEMPERATURE AND NaCl STRESS CONDITIONS IN LEAF AND ROOT TISSUES OF Arabidopsis thaliana

Thesis submitted to the
University of Agricultural Sciences, Dharwad
in partial fulfillment of the requirements for the
Degree of

MASTER OF SCIENCE (AGRICULTURE)

IN

MOLECULAR BIOLOGY AND BIOTECHNOLOGY

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LIST OF ABBRIVATIONS

Sl. No.	Abbreviations	Description
1	*AGO1*	Argonaute protein
2	*APS*	ATP sulfurylase
3	*ARF*	Auxin Response Factor
4	*DCL*	Dicer-like protein
5	DEPC	Diethyl Pyrocarbonate
6	DIG	Digoxigenin
7	ISH	*In situ* Hybridization
8	*LAC*	Laccase
9	LNA	Locked Nucleic Acid
10	miRNA	microRNA
11	PBS	Phosphate Buffer Saline
12	PFA	Paraformaldehyde
13	*PPR*	Pentatricopeptide repeat
14	RISC	RNA induced silencing complex
15	*SCL*	Scarecrow Like Proteins
16	*SCR*	Scarecrow
17	siRNA	Small interfering RNA
18	tasiRNAs	Trans-acting RNA
19	*UBC24*	Ubiquitin conjugating enzyme

INTRODUCTION

The growth and development of the plants is highly dependent on different environmental conditions such as, temperature, light, availability of water and various soil conditions which strongly affect productivity through worldwide. Plants need to adjust themselves to any changes in these environmental conditions and hence plants have developed different strategies for their better growth and survival. To minimize the cellular damage caused by stress, plants respond by reprogramming gene expression, which results in osmolyte accumulation for osmotic adjustment, up-regulation of antioxidant pathways for reactive oxygen species (ROS) homeostasis, minimizing as well as repairing the damage caused to the DNA, proteins and membranes, and maintaining processes that sustains cellular homeostasis under stress. Modulation of gene expression is a key level of regulation during different stress conditions. Gene regulation by small non-coding RNAs (sncRNAs) is another important mechanism by which plants respond, adjust, survive and ultimately adapt to stressful conditions (Sunkar *et al.,* 2007). In eukaryotes, these small non coding RNAs mainly comprised of microRNAs (miRNAs) and small interfering RNAs (siRNAs). Depending on the organisms, other types of small RNAs have been identified such as transacting siRNA (tasiRNAs) and repeat associated siRNAs (rasi-RNAs) in plants, Piwi interacting RNAs (pi-RNAs) in animal germline (Sunkar, 2010). The miRNAs regulate gene expression by targeting mRNAs for direct cleavage or the inhibition of protein translation. The discovery of the miRNAs as gene regulators has led to the deep understanding of post- transcriptional gene regulation in plants and animals. The miRNAs have emerged as master regulators of plant growth and development. Evidence suggesting that miRNAs play a role in plant stress responses arises from the discovery that miR398 targets genes with known roles in stress tolerance (Sunkar *et al*., 2012).

The microRNAs (miRNAs) were initially identified in *Caenorhabditis elegans* through forward genetic screens of the lin-4 and let-7 mutants (Lee *et al*., 1993). Plant miRNAs were first reported in early 2002. The first miRNAs discovered in plants regulate largely transcription factor genes involved in a variety of plant developmental processes (Llave *et al*., 2002a). In *Arabidopsis thaliana*, miRNAs have been discovered by using direct cloning, bioinformatics analysis and forward genetics approaches. In animals, miRNAs are evolutionarily conserved, many miRNAs, such as miR7 and miR18, are conserved from worm to human (Weber, 2005). The microRNA conservation has also been observed in plants, however, only mature plant miRNAs are conserved (Zhang *et al*., 2005). In animals it has been identified that many miRNAs plays a major roles during developmental timing, cell death, cell proliferation, haematopoiesis and also many miRNAs shown response to different environmental stimuli (Ambros, 2004).

The roles of miRNAs in multiple biological and metabolic processes in plants have been identified through functional analysis of miRNAs. They regulate variety of plant developmental program such as leaf morphogenesis, vascular and root development, shoot formation and floral organ identity. They also play a role in plant responses to different biotic and abiotic stresses. Several stress-regulated miRNAs have been identified and reported in model plants under various biotic and abiotic stress conditions, including nutrient deficiency, drought, salinity, cold, bacterial infection, UV-B radiation and several mechanical stresses (Khraiwesh *et al*., 2011).

The miRNAs regulate gene expression in different tissues and cells temporally and spatially. With the identification of miRNAs several techniques also have been explored for testing and validation of miRNAs in plants*. In situ* hybridization can detect temporal and spatial expression of miRNAs conveniently and directly. *In situ* detection of miRNAs by hybridization with Locked Nucleic Acid (LNA) mediated oligonucleotide probes is more specific and sensitive than that of normal anti-sense RNA probes.

Locked Nucleic Acid (LNA) oligonucleotide probes are special class of bi-cyclic, high affinity RNA analogues with a modified ribose moiety. In LNA probes a 2′ O, 4′ C Methylene bridge is introduced to chemically lock the furanose ring in the sugar- phosphate backbone. These LNA oligonucleotide probes exhibit strong thermal affinity when they hybridized with the complementary RNA molecules and also introduction of LNA residues results in increased stability against endo and 3′-exonucleases (Frieden *et al*., 2003). Because of all these properties LNA mediated probes can be utilized in many biological experiments including microRNA profiling (Mirco *et al.*, 2006), real time expression analysis (Jean *et al.*, 2006), *in situ* detection (Sweetman, 2011) and also in other applications. LNA oligonucleotide can easily be labeled with standard oligonucleotide tags such as DIG, fluorescent dyes, biotin, amino-linkers, etc. Thus, a very high degree of freedom in the design of primers and probes exists for LNA.

Validation of plant miRNAs can be primarily achieved by using two kinds of methods such as, PAGE/Northern hybridization, which detects the existence of miRNA candidates and other method which is widely using for detection and quantification of miRNA is RT-PCR. Quantitative real time polymerase chain reaction (qRT-PCR) is one of the most powerful and sensitive technique available for gene expression analysis, genotyping, target gene validation and measuring RNA interference. The development of novel chemistries and instrumentation platforms has led to wide spread adaptation of real-time RT-PCR as the method of choice for quantify the changes in gene expression. By using real time it is possible to determine the starting concentration of the nucleic acid because it measures PCR amplification as it occurs. Real time PCR contains a fluorescent molecule such as, *TaqMan*® probe or SYBR® Green dye which monitor the progress of the amplification reaction. The fluorescence intensity increases proportionally with each amplification cycle in response to the increased amplicon concentration. Various improvement techniques have been developed such as primer extensions RT-PCR, stem-loop primer RT-PCR and tailed RT-PCR of miRNA. Stem-loop real-time fluorescence quantitative RT-PCR is one of the highly specific and more sensitive methods currently available to test miRNA expression.

Keeping these views in the background, the present study was undertaken with the following objectives:

1. To assess the expression pattern of selected microRNAs in response to salinity stress in leaf tissues of *Arabidopsis thaliana.*
2. To assess the effect of low temperature stress on expression pattern of selected microRNAs in leaf tissue of *Arabidopsis thaliana*.

REVIEW OF LITERATURE

The optimal plant growth and development, plant progression into different phases, also other physiological processes including stress responses is determined by highly coordinated multiple gene regulatory mechanism which involves transcriptional, post transcriptional and post translational regulations. Understanding of post-transcriptional gene regulation in plants and animals has gained momentum after the discovery of miRNAs as gene regulators. The miRNAs have been emerged as master regulators of plant growth and development and ample number of experimental evidences are continue to accumulate to their effect.

2.1 Plant microRNAs

microRNAs are small approximately 21-24 nucleotides, single stranded RNA molecules which regulates target gene expression at post transcriptional level through mRNA cleavage or translational repression. The miRNAs are evolutionarily conserved in animals and many miRNAs, such as miR7 and miR18, are conserved from worm to human (Weber, 2005). It has been considered that animal miRNAs existed at least 420 million years ago, with a common ancestor of metazoans (Pasquinelli *et al.,* 2000). In plants it has been observed that only mature miRNAs are conserved, unlike the conservation of both mature miRNAs and miRNA precursors in animals (Reinhart *et al*., 2002 and Zhang *et al*., 2005). Most of the miRNAs found to be conserved among the angiosperms and which are shown to be involved in transcription factor regulation in developmental control (Axtell and Bowman, 2008).

A total of 338 mature microRNAs have been found in *Arabidopsis thaliana* and deposited in the miRBase (Accessed on May 23rd 2013). A majority of the miRNA families have also been found in other species, such as rice, maize and sorghum. Among the different miRNA families some are highly evolutionarily conserved; there are only a few nucleotide changes among a wide variety of plant species (Zhang *et al*., 2006). Axtell and Bartel (2005) found that the miR159/319 family existed in 10 plant species. The miRNA families 156/157 and 165/166 exist in 9 plant species, ranging all plant lineages. Of these miRNA families, miR156 was found in 36 different plant species (Sunkar and Jagadeeswaran, 2008). It has also been reported that plant pre-miRNAs originated from their target genes by formation of inverted duplications which have been transcribed, but not modified further (Allen *et al*., 2004). The miRNAs may originate anywhere within the plant genome and many genomic regions were found to be sites of miRNAs (Lu *et al*., 2005a). Findings have showed that the origin of miRNA is complex and it involves many different mechanisms including inversion and duplication.

Based on extent of conservation, microRNAs have been classified into highly, moderately, lowly, and non-conserved microRNAs (Jones-Rhoades and Bartel, 2004 and Zhang *et al*., 2005). There are some miRNAs, such as miR163 and miR158 which are considered as non-conserved microRNAs (Jones-Rhoades and Bartel, 2004). The majority of non-conserved microRNAs exhibit low or tissue-specific expression (Zhu *et al*., 2008). It is believed that conserved miRNAs play an important role in conserved gene regulation, such as leaf and flower morphology or signal transduction. Non-conserved miRNAs may play more specific roles in specific plant species, such as the differentiation and elongation of cotton fibers (Mallory *et al*., 2006 and Sunkar *et al*., 2007).

The miRNAs are encoded by endogenous MIR genes, which are transcribed by RNA polymerase II (Pol II) (Lee *et al*., 2004; Xie *et al*., 2005a). The miRNA producing primary transcript is then cleaved by an RNase type III known as Dicer-like protein (DCL) in a successive manner leading to small RNA duplexes with a 2-nt overhang at the 3'-end (Park *et al.,* 2002; Kurihara and Watanabe 2004). Mature miRNAs are produced from pri-miRNAs through sequential processing steps by RNase III-type endonucleases.

The processing of pri-miRNAs to pre-miRNAs by DCL1 is known to be assisted by two other proteins, HYPONASTY LEAVES1 (HYL1) and SERRATE (SE). HYL1 belongs to a family of double-stranded RNA (dsRNA) binding protein and interacts with DCL1. Loss-of-function mutations in the HYL1 gene result in decreased accumulation of miRNAs and concomitant accumulation of pri-miRNAs. SE, a C2H2 zinc finger protein, interacts with DCL1 and HYL1 and plays a role in the processing of pri-miRNAs to pre-miRNAs (Lobbes *et al*. 2006; Yang *et al*., 2006b). After the miRNA/miRNA* duplexes are released from the pre-miRNAs by the DCL1 activities, the duplexes are methylated at the 2′ OH of the 3′-ends by HUA ENHANCER1 (HEN1), a small RNA methyl transferase (Yu *et al.,* 2005; Yang *et al*., 2006c). The methylated miRNA/miRNA* duplexes get selectively incorporated into an ARGONAUTE (AGO)-containing effector complex, known as the RNA-induced silencing complex (RISC) (Xie *et al*., 2010).

After forming the miRNA-RISC assembly, miRNAs direct the RISC to regulate gene expression mRNA cleavage or translational repression mechanisms. The degree of miRNA–mRNA complementarity is a key determinant of the mechanism. Most plant miRNAs have near-perfect or perfect complementarity to their targets and mRNA cleavage is believed to be a predominant mechanism by which miRNAs regulate gene expression in plants. In case of mRNA cleavage mechanism, miRNAs guide the AGO component of RISC to cleave a single Phosphodiester bond of the target mRNA within the miRNA-binding site (Tang *et al*. 2003). The cleaved fragments are then release and degrade, makes the RISC free to recognize and cleave another target mRNA. It has been also reported that plant miRNAs can regulate the gene expression by translation repression. It has also been observed that miR172 can also efficiently guide cleavage of its target transcripts (Schwab *et al*., 2005).

2.2 Role of miRNAs in plant development

Plant growth and developmental processes regulated by miRNAs are quite diverse and enormous. Although miRNAs were identified recently they have become one of the most important gene regulators in both plants and animals. Functional analysis of conserved miRNAs revealed their involvement in multiple biological and metabolic processes in plants. They regulate various aspects of developmental programs including auxin signaling, meristem boundary formation and organ separation, leaf development and polarity, lateral root formation, transition from juvenile-to-adult vegetative phase and from vegetative-to-flowering phase, floral organ identity, and reproduction. They also regulate plant responses to biotic and abiotic stresses (Khraiwesh *et al*., 2011).

Function of miRNAs can be understood by using the plants which are mutants of specific enzymes involved in the biogenesis of miRNAs. Several important enzymes, such as *DCL1*, *AGO1*, *HEN*, *HYL1*, and *HASTY*, have been shown to play very important role in plant miRNA biogenesis and functions. Loss-of-function of any single one of these enzymes caused significantly developmental abnormalities. Loss-of function of the *dcl1* gene show a variety of development abnormalities, including arrested embryos at early developmental stage, altered leaf shape, delayed floral development and caused female sterility (Park *et al*., 2002 and Dugas and Bartel, 2004).

It has also been shown that Loss-of-function of *HASTY* gene blocked miRNA-miRNA* duplex transported from nucleus to cytoplasm and further effected the mature miRNA production, and caused several pleiotropic abnormal phenotypes. Loss-of function of *HASTY* gene have been shown changed leaf and flower morphology, accelerated plant phase change from vegetative growth to reproductive growth (Bollman *et al.,* 2003).

Leaf development and leaf morphology is controlled by miRNAs. In *Arabidopsis thaliana* there are three closely related HD-ZIP transcription factors which include, PHABULOSA (*PHB*), PHAVOLUTA (*PHV*) and REVOLUTA (*REV*). The polarized expression of these transcription factor genes controls the asymmetrical pattern in plant leaves along the adaxial/abaxial (upper/lower) axis (Juarez *et al*., 2004). Dominant mutations in any of these three transcription factor genes (*phb, phv*, and *rev*) results in radialization and adaxialization of leaf and vascular bundles in the stem. Several experiments show that these transcription factors are targets of mir165 and mir166 and their expression is regulated by these two miRNAs (Emery *et al*., 2003).

In addition to miR166 and miR165, miR159/JAW also regulates leaf development by targeting a subset of TCP transcription factor genes (Palatnik *et al*., 2003). Over expression of miRJAW resulted in low levels of all tested TCP mRNAs and caused jaw-D phenotypes, including uneven leaf shape and curvature and over expression of miRJAW-resistant TCP mutants indicated that miRJAW-guided mRNA cleavage was sufficient to restrict TCP function (Palatnik *et al*., 2003).

Flower development and phase change is one of the important biological processes in plant development. In *Arabidopsis* it was demonstrated that an over expression of miR172 cause defects in floral identity including absence of petals and transformation of sepals into carpels and also early flowering (Schwab *et al*., 2005). The miRNA 156 is also involved in phase change and developmental timing by targeting Squamosa promoter binding protein like (*SPL*) transcription factors. Over expression of miRNA 156 resulted in quick initiation of rosette leaves, decreasing the apical dominance and a moderate delay in flowering, suggesting that miRNA 156 affected plant phase change from vegetative growth to reproductive growth (Schwab *et al*., 2005). The scarecrow-like (*SCL*) (GRAS domain) family is a class of plant-specific transcription factors. Three *SCL* genes (*SCL6-II*, *SCL6-III*, and *SCL6-IV*) in *Arabidopsis* and 4 in rice have perfectly complementary sequences with miR171 (Llave *et al*., 2002 and Reinhart *et al*., 2002).

This indicates that miR171 targets these *SCL* genes and plays a significant role in the radial patterning of both roots and shoots and hormone signaling (Kamiya *et al*., 2003).

Cup-shaped cotyledon 1 (*CUC1*) and *CUC2* are two important transcription factors of the NAM/ATAF/CUC (NAC)-domain transcription factor family which is restricted to plants (Riechmann *et al.,* 2000). Loss-of-function of these genes resulted in abnormal floral and shoot development (Hibara *et al.,* 2003). In *Arabidopsis* it has been shown that five members (*CUC1, CUC2, NAM, NAC1*, At5g07680, and At5g61430) of the *NAC* domain gene family have complementary sites with miR164 and they are targets for miR164 (Laufs *et al.,* 2004). The miR164 regulates *NAC* and *CUC2* expression and controls the organ boundaries and root formation (Laufs *et al*., 2004, Guo *et al*., 2005). Loss-of-function miR164 mutants accumulated *NAC* mRNA, resulting in more lateral root formation (Guo *et al*., 2005).

Plant vascular tissues, which originate from procambial cells, provide a pathway for the transport of signaling molecules, water and nutrients. In *Arabidopsis*, differentiation of xylem cells from procambial cells is coupled with the expression level of HD-ZIP-III homeobox genes (*PHB, PHV, REV*, AtHB8 and AtHB15). Studies have shown that HD-ZIP-III mRNAs specifically targeted by miR165 and miR166. miR165 or miR166 over expression causes a drastic reduction in the transcript levels of the HDZIP III genes and results in prominent phenotypes including, alterations of organ polarity, inhibition of vascular development and aberrant differentiation of the inter fascicular fibers (Zhou *et al*., 2007).

Several miRNAs affect signal transduction, especially hormone signaling pathways. Plant hormones, especially auxin which regulates the plant developmental processes and it controls different aspects of plant growth and development influencing Auxin Response Factor (ARF), a plant-specific family of DNA binding proteins (Weijers and Jurgens, 2005). Studies have been confirmed that miR160 and miR167 regulates these ARFs. The miR167 controls the patterns of ARF6 and ARF8 expressions and regulates male and female reproduction, whereas miR160 cleaves ARF10, ARF16 and ARF17 to affect many aspects of plant development (Yang *et al*., 2006). The miR159 mediates GA and ABA signaling by targeting a group of the MYB genes (Achard *et al*., 2004). The induction of miR159 by ABA has been shown to mediate seed germination by directing the degradation of *MYB33* and *MYB101* mRNAs (Reyes and Chua, 2007). Two other miRNAs, miR319 and miR166, have recently found to be downregulated by gibberellin (GA) (Liu *et al*., 2009).

2.3 microRNAs involved in stress response

Being sessile in nature plants are routinely exposing to various biotic and abiotic stresses including drought, salinity, heat, low temperature, heavy metal pollution and also pathogen attack. To overcome from adverse effects caused by variety of stresses plants have evolved with adaptive responses operating at the transcriptional, post-transcriptional, translational, and post-translational levels to cope with these environmental challenges (Sunkar 2012). In plants, a number of miRNAs have been identified as a post transcriptional gene regulator and which play roles in multiple stress responses. Many studies have provided supportive evidence to show that there is a direct link between miRNA regulation and stress response in plants. Upon treatment of diverse stress conditions it has been detected as up and down regulated expression of plant miRNAs. Several stress-related miRNAs were identified based on the sequencing of a library of small RNAs isolated from *Arabidopsis* seedlings exposed to various stresses (Sunkar and Zhu, 2004). In *Arabidopsis NFYA5* transcription factor, promotes drought resistance is found to be regulated by miR169 (Trindade *et al.,* 2010). Liu and colleagues (2008) analyzed 117 miRNAs under salinity, drought, and low-temperature conditions using miRNA chips representing miRNAs identified in *Arabidopsis* and detected seventeen stress inducible miRNAs.

2.3.1 ABA mediated stress responsive miRNAs

ABA is a kind of phytohormone which involves in plant response to various environmental stresses. Studies from Arabidopsis mutant containing a “pleiotropic recessive *Arabidopsis* transposon insertion mutation,” *hyl1* indicated that miRNAs may be involved in ABA-mediated responses (Lu and Fedoroff, 2000). Achard *et al*. (2004), Sunkar and Zhu (2004) independently reported that treating with either ABA or GA regulates miR159 expression. Sunkar and Zhu (2004) also reported the upregulated expression of miR393 and miR402 and down regulation of miR389a upon ABA treatment. Studies from *Arabidopsis* have also been indicated that upregulation of miR160, miR417 and down regulation of miR169 and miR398 in response ABA treatment (Khraiwesh *et al.,* 2011).

2.3.2 microRNA response to drought stress

Drought is one of the major limitations in the agriculture productivity throughout the world. By using high throughput technologies including genome wide gene expression and proteomics, it has been identified that there are number of genes which get alter during drought stress (Yamaguchi-Shinozaki and Shinozaki, 2006). Recently it has been shown that, in response to drought stress several miRNAs expression is also altered (Trindade *et al*., 2009). A study from *Arabidopsis* has been reported that, in response to dehydration stress miR393, miR319 and miR397 get upregulated (Sunkar and Zhu, 2004). Upregulation of miR393, miR160 and miR167 during drought and/or salt stress has been commonly reported in several plant species (Sunkar *et al*., 2012). In *Arabidopsis* miR169 shows down regulation and its target *NFYA5* get induced in response to drought stress. Functional analyses have confirmed that *NFYA5*-overexpressing transgenic lines are drought-tolerant, whereas miR169-overexpressing plants are drought-sensitive (Li *et al*., 2008). It have been also reported that miR169 targets 8 NF transcription factor Y subunit mRNAs in rice (Li *et al*., 2010). Some miRNAs including miR156, miR166, miR171, and miR408 in barley showed differential expression upon dehydration stress (Kantar *et al*., 2010). It was also found that upregulation of miR397a under drought stress in *Arabidopsis* (Ghosh, 2008).

2.3.3 microRNAs in response to salt stress

Salt stress is one of major constraint in agriculture which can reduce crop yields substantially and also threatens survival of crop plants. Several studies indicated that the altered expression of miRNAs in response to salt stress. In *Arabidopsis* miR396, miR168, miR167, miR165, miR319, miR159, miR394, miR156, miR393, miR171, miR158 and miR169 were upregulated in response salt stress (Liu *et al.,* 2008). Studies have been shown that miR396 is a salt responsive plant miRNA which targets growth regulating factor (GRF) transcription factors. In *Arabidopsis* and rice, it has been shown that over expression of miR396 resulted in reduced salt tolerance than that of wild type plants (Chi *et al.,* 2011). Recently it have also been reported that differentiated expression level of miR398, targeting 2 Cu/Zn superoxide dismutases (*CSD1* and *CSD2*), in response to salt stress (Jagadeeswaran *et al.,* 2009). Comparative analysis between two maize (*Zea mays*) lines differing in salt sensitivity (salt-tolerant NC286 and salt-sensitive Huangzao4) indicated differential expression of miRNAs in response to salt stress (Ding *et al.,* 2009). In *P. vulgaris*, it was observed that increased accumulation of miRS1 and miR159.2 in response to NaCl addition (Arenas-Huertero *et al*., 2009).

2.3.4 microRNAs in response to cold and heat stress

Every plant has its own set of temperature requirements for its proper growth and development. Any deviation from their normal required temperature is more harmful to plant cells and leads to high or low temperature stress. To adjust to high or low temperature stress plants have to reprogram their gene expression profiles. Sunkar and Zhu (2004) reported upregulation of miR393, miR397b, miR402 and miR319c expression in *Arabidopsis* in response to cold stress. It is also reported that opposing patterns of miRNA regulation during cold stress (Sunkar *et al*., 2012). Liu *et al*. (2008) found that up and down regulation of miR171 in *Arabidopsis* and rice respectively, in response to cold stress. By using rice specific miRNA microarray chip it was found that differential regulation of 15 miRNA families in response to cold stress (Lv *et al.,* 2010). Studies from *Arabidopsis* reported that increased expression levels of miR165/166, miR169, miR172, miR393, miR396, miR397, miR402, and miR408 in response to cold stress (Zhou *et al*. 2008). In wheat it is also reported that differential expression of miRNAs in response to heat stress. Solexa high-throughput sequencing data showed 32 miRNA families in wheat and among them nine miRNAs including miR156, miR159, miR160, miR166, miR168, miR169, miR393 and miR827 are detected as putatively heat responsive in wheat germline. Mahale. B (2010) in his experiment shown that some miRNAs such as, miR397a, miR161, miR168 and miR171 were upregulated in response to different shoot heat shock regimes in *Arabidopsis*.

2.3.5 miRNAs in response to nutrient homeostasis

Optimal level of nutrients in the soil is also one of the important factors which determines the proper growth and development of the plant. Plants adopted several molecular mechanisms to cope up with nutrient starvation. The microRNA mediated transcriptional regulation of gene expression has been reported for several element deficiencies such as, phosphate (Hsieh *et al*., 2009), sulfate (Rhoades and Bartel, 2004; Rausch *et al.,* 2005) and copper (Yamasaki *et al.,* 2007). Several miRNAs including miR156, miR169, miR395, miR398, miR778, miR827, miR828 and miR2111 were found to be differentially expressed upon phosphate deprivation (Sunkar *et al.,* 2012).

The miR395 has complementary to mRNA of ATP sulfurylase (APS) proteins and plays a role in sulfate metabolism. It was also observed that induced expression of miR395 under low external sulfate concentrations (Rhoades and Bartel, 2004). It was also found that upregulation of miR398 levels in copper deficiency condition results in down regulation of Cu/Zn superoxide dismutase (*CSD1* and *CSD2*) mRNA level (Yamasaki *et al.,* 2007). Recently it has been also found that upregulation of miR397, miR408 and miR857 under low copper conditions (Burkhead *et al.,* 2009).

Various aspects of plant growth and development adversely affected by deprivation in major macronutrient like nitrogen. Various gene regulatory mechanisms, including miRNA mediated gene regulation are operating in plants to adapt to availability of low nitrogen condition. Recently miR393/AFB3 module is implicated that regulation of root system architecture in response to nitrogen availability (Vidal *et al.,* 2010). It was also observed that upregulation of miR393 levels leads to down regulation of its target gene *AFB3*, which controls primary and lateral root growth. In *Arabidopsis* pericycle and root cap miR167a was repressed, where as its target *ARF8* was induced in these tissues in response to nitrogen treatment (Gifford *et al*., 2008).

2.4 *In situ* hybridization

In situ hybridization is one of the most powerful technique which combine both histological as well as cytological approaches with molecular biology techniques in order to detect specific nucleic acid sequence in temporal and spatial manner in samples at the cellular or tissue level.

This technique was first described as a method for localization of nucleic acid hybrids in cytological preparations by Pardue and Gall (1969). Until the early 1980s, radioisotopes were the only labels available for nucleic acid probes and autoradiography was the only means to detect *in situ* hybridized sequences. But many practical inconveniences are imposed by the use of radio activity including, short half life of radio isotopes, safety problem and long exposure periods required by autoradiography. Later a major advance in this method was achieved with the description of *in situ* hybridization using single stranded RNA probes, known as ribo probes (Cox *et al*., 1984). Development of stable nucleic acid labels allowed non radioactive detection through fluorescence or enzymatic reactions demolished problems associated with *in situ* hybridization. *In situ* hybridization can be performed rapidly with multiple colored nucleic acid probes at maximum optical resolution; this made it widespread applicability of this technique in most of the basic as well as applied research.

2.4.1 Non radioactive probes in *in situ* hybridization

Both radioactive and non radioactive probe labels are commonly used for *in situ* hybridization. In spite of the high sensitivity and wide applicability of *in situ* hybridization techniques, their use has been limited to research laboratories. This is mainly due to the problems associated with radioactive probes. But, preparing nucleic acid probes with a stable nonradioactive label removes the major problems associated with the general application of *in situ* hybridization and it also opens new opportunities for combining different labels in one experiment. Availability of many sensitive antibody detection systems made further enhancement in the flexibility of this method. There are several systems available for the non-radioactive labeling of in situ hybridization probes including biotin, digoxigenin, and fluorescein.

Digoxigenin (DIG)-labeled RNA antisense probes are widely used for *in situ* hybridization due to their high sensitivity and specificity. DIG-labeled RNA probes are also stable for more than a year, making them ideal for long-term studies with high consistency and low technical variation. The DIG labeling method is based on a steroid found exclusively in the flowers and leaves of digitalis plants (*Digitalis purpurea, Digitalis orientalis* and *Digitalis lanata*), where it is attached to sugars, to form the glycosides. As the blossoms and the leaves of these plants are the only natural source of digoxigenin, the anti-DIG antibody does not bind to other biological material. The DIG-labeled nucleotides may be incorporated, at a defined density, into nucleic acid probes by DNA polymerases and RNA polymerases. Hybridized DIG-labeled probes may be detected with high affinity anti-digoxigenin (antiDIG) antibodies that are conjugated to alkaline phosphatase, peroxidase, fluorescein, rhodamine, or colloidal gold and these anti-DIG antibodies conjugate determines the detection sensitivity.

2.4.2 Locked Nucleic Acid probes in miRNA detection

In situ detection of RNA by hybridization with complementary probes is a widely used technique for analyzing temporal and spatial expression of RNA.

But, it is not suitable for small RNA molecules like miRNA, siRNA distribution because the shorter sRNA sequences are difficult to detect using an antisense RNA probe, and usually result in a low signal-to-noise ratio, especially for low-abundance miRNAs. So one of most important considerations in the miRNA ISH process is developing and designing special and high affinity binding probes that can easily hybridize with mature miRNAs.

To avoid the sensitivity problem of miRNA ISH technology locked nucleic acid (LNA)-modified oligonucleotide probes were used to enhance the efficiency of hybridization. Locked Nucleic Acid oligonucleotide probes are special class of bi-cyclic, high affinity RNA analogues with a modified ribose moiety, in which the ribose ring is "locked" by a methylene bridge connecting the 2′-O atom with the 4′-C atom. This chemical modification induces a conformational change that brings about enhanced base stacking and phosphate backbone pre-organization (Petersen *et al*., 2002). LNAs possess similar water solubility as that of DNAs or RNAs and also a major structural characteristic of LNA is its close resemblance to natural nucleic acids. Hence, LNA-modified oligonucleotides can be used easily in many biological experimental applications. The introduction of LNA residues resulted in increased stability against endo and exonucleases (Frieden *et al.,* 2003). These properties of LNA – modified oligonucleotides made them as an ideal tool for detecting small RNA molecules.

The usage of locked nucleic acid (LNA)-modified oligonucleotide probes has been shown to significantly improve the sensitivity and specificity of microRNA detection (Valoczi *et al*., 2004). It has been emphasized that the LNA-modified probe shows a strong ability to bind to mature miRNAs rather than pri- or pre-miRNAs, subsequently resulting in detection of high-level mature miRNAs. By using LNA modified oligonucleotide probes. LNA-modifi ed DNA probes have been successfully used to detect miRNA signals in zebra fish and mouse embryos (Wienholds *et al*., 2005). Valoczi *et al*. (2005) detected miR171 accumulation by *in situ* hybridization in *Nicotiana benthamiana* leaves.

By using LNA-modified probers Micro *et al*. (2006) developed novel microarray platform for genome-wide profiling of mature microRNAs (miChip). The spatial distribution of miR172 in leaves, stems, stolons and swollen stolons was examined by using *in situ* hybridization and miR172 was detected in epidermis as well as vascular cells of leaves (Antoine *et al*., 2009). By utilizing miRNA *in situ* hybridization Kidner and Timmermans (2006) elucidated the role of miRNAs in the developmental process of *Arabidopsis* and maize. Expression behavior of the TCP4 transcription factor which is regulated by miR319 during wild type embryogenesis was detected by using ISH technique (Palatnik *et al*., 2003). Expression behavior of miRNAs in *Medicago truntula* root nodule was detected by using in situ hybridization and results showed that miR160, miR169, miR172, miR393 and miR171 expressed abundantly in nodule meristem, miR396 had no expression (Lelandais *et al*., 2009).

2.5 Plant miRNAs and their cognate genes

Regulation of gene expression is one of the essential mechanisms which operate in all the living organism. Since the initial discovery of miRNAs as essential regulators in development of nematode *Caenorhabditis elegans*, thousands of miRNA genes have been identified in animal and plant genomes (Kozomara and Griffiths, 2011). Now miRNAs have emerged as key regulators of gene expression involving diverse biological pathways. The miRNAs negatively regulate gene expression at the post-transcriptional level by binding to the target mRNAs (Bartel, 2004). MiRNAs regulate the gene expression by two different mechanisms including, target mRNA cleavage and translational repression. In plants, it has been shown that most of the miRNAs have significant complementarity to their target genes and regulate their targets by directing mRNA cleavage at single sites in the coding regions (Franco-Zorrilla *et al*., 2007).

Since the first plant miRNAs were cloned in *Arabidopsis thaliana* (Llave *et al*., 2002), many miRNAs and their target genes have been identified and characterized in plants. It has been found that majority of miRNA target genes encode transcriptional factors or other important enzymes which plays important role in plant development as well as responses to various biotic and abiotic stresses (Rhoades *et al.,* 2006). In plants, most miRNAs target multiple targets belonging to the same gene family and also emerging data indicating that miRNAs can selectively regulate the expression of specific target genes under specific conditions (Sunkar *et al.*, 2012). It has been well characterized the involvement of miRNAs in regulation of transcription factors in case of AUXIN RESPONSE FACTOR 10, a target of miR160 (Liu *et al*., 2007). *CUC1* and *CUC2* are two important transcription factors of the NAM/ATAF/CUC (NAC) domain transcription factor family and their expression was controlled by At-miR164 which regulates meristem development and aerial organ boundaries (Laufs *et al*., 2004).

It has been found that in response to drought stress, miR169 is down regulated where as its target *NFYA5* got induced in *Arabidopsis* and functional analysis of the same confirmed that *NFYA5* over expressing transgenic lines are drought tolerant where as miR169 over expressing transgenic lines are drought sensitive (Li *et al*., 2008). Sunkar and Zhu (2004) examined the role of miRNAs in cold stress in *Arabidopsis thaliana* and found that miR397 upregulation upon cold stress. The miR397 was predicted to target laccases, which are involved in lignin synthesis and maintaining cell wall structure and integrity (Sunkar and Zhu, 2004). It have also been reported the accumulation of ATP sulfurylase ASP4 and the sulfate transporter AST68 at low sulfur conditions, which are the targets of miR395 (Allen *et al*., 2005). *SCL6-II, SCL6-III* and *SCL6-IV* are members of GRAS gene family and which are predicted to be targets of miR171 (Llave *et al*., 2002a). Recently, it has been shown that transgenic plants over expressing miR171a exhibit multiple development defects, including reduced shoot branching (Song *et al*., 2011).

2.5.1 The microRNAs in *Arabidopsis thaliana*

The presence of microRNAs in higher plants was first discovered in *Arabidopsis* and later it has been found that some of the miRNAs were conserved in many plant genomes such as, *Oryza sativa*, *Zea mays* and also those of more ancient vascular plant genera such as ferns and non vascular mosses (Axtell and Bartel, 2005).

microRNA399

The miR399 is a microRNA it has been identified in both *Arabidopsis thaliana* and *Oryza sativa* by using computational approaches. The mature sequence of miR399 is excised from the 3' arm of the hairpin. It has been also identified that each plant genome contains multiple copies of miR399. In *Arabidopsis thaliana* there are six miRNA precursors have been identified and all give rise to an almost identical mature miR339 sequence. Studies have been reported that microRNA399 (miR399) controls inorganic phosphate (Pi) homeostasis by regulating the expression of UBC24 encoding a ubiquitin-conjugating E2 enzyme in *Arabidopsis thaliana* (Chiou *et al*., 2006). Quantitative RT-PCR and RNA gel blot data revealed that upregulation of miR399 in response to Pi deficiency (Lin *et al*., 2008).

microRNA168

The miR168 is one of the miRNA which control its own expression and also the other miRNAs by targeting specific proteins involved in the posttranscriptional gene silencing pathway. In particular, miR168 regulates the function of all miRNAs by targeting AGO1 expression, therefore modulating its actual levels and consequently RISC activity (Xie *et al*., 2003). MiR168 is the one of the most commonly detected stress inducible miRNA. Existence of homologs of miR168 has been identified in various plant species including dicots such as tobacco, poplar and Arabidopsis and also in monocots like maize and rice (Li *et al*., 2012). In the *Arabidopsis* genome two miR168 paralogs (miR168a and miR168b) has been reported and only miR168a transcript has been isolated and shown to be involved in AGO1 post-transcriptional gene silencing in *Arabidopsis* (Gazzani *et al*., 2009). Recent studies using microarray followed by reverse transcription (RT)-PCR analyses revealed increased accumulation of miR168 transcripts under high salinity, drought, and cold conditions in *Arabidopsis* (Liu *et al*., 2008). Li *et al*. (2012) adopted northern blot technique to measure the abundance of mature miR168 under various abiotic stress conditions including, drought, 300 mM NaCl and cold stress and identified increased levels of mature miR168 under all the treatments in two weeks old wild type Arabidopsis seedlings (Li *et al*., 2012).

microRNA395

The miR395 is a microRNA which has been identified in both *Arabidopsis thaliana* and *Oryza sativa* by using computational approaches. Studies have been reported that miR395 targets mRNAs coding for ATP sulfurylases encoded by APS genes (*APS1* and *APS4*). Significant upregulation of miR395 expression have been identified during sulfate limitation (Liang *et al*., 2010). RT-PCR analyses in *Arabidopsis* revealed that decreased levels of *APS1* and *APS4* transcripts in response to sulfate deficiency (Liang and Yu, 2010). Recently, deep sequencing results in *Arabidopsis* showed that repressed expression of miR395 in response nitrogen starvation (Liang *et al.,* 2012).

microRNA161

The miR161 is one of the nonconserved miRNA which was found in *Arabidopsis thaliana* and it is represented by single gene rather than multigene families (Rhoades and Bartel, 2004).

It has been reported that miR161 was found on the chromosome number 1 in *Arabidopsis thaliana* (Reinhart *et al*., 2002). Studies have been indicated that miR161 targets several mRNAs coding for pentatricopeptide repeat proteins (PPRs) (Rhoades and Bartel, 2004). Recently microarray analyses on *Arabidopsis thaliana* infected with the Oil-seed Rape Mosaic Virus (ORMV) revealed that upregulation of miR161 upon ORMV infection and RT-PCR as well as northern blot analysis confirmed the microarray analyses results (Hajdarpašić and Ruggenthaler, 2012).

microRNA397

The miR397 is a short RNA molecule and which is predicted to be target mRNAs coding for laccases. A bioinformatics analysis of the *Arabidopsis* genome and target validation analysis have been suggested that miR397 family targets *LAC2, LAC4* and *LAC17* genes (Rhoades and Bartel, 2004). The increased levels of miR397 has been identified in Arabidopsis in response to cold stress (Sunkar and Zhu 2004; Zhou *et al*., 2008). Quantitative real time PCR analysis revealed that the expression of *LAC2*, *LAC4* and *LAC17* were negatively correlated with the expression pattern of miR397 in response to copper limitation (Abdel-Ghany and Pilon, 2008).

microRNA171

The miR171 is a well conserved miRNA family known to regulate members of the SCARECROW-LIKE (*SCL*) transcription factor family. In *Arabidopsis*, there are three MIR171 genes (a, b and c) have been identified and predicted to regulate three SCL6 genes (*SCLII, III, IV*) (Engstrom *et al.,* 2011). Studies have been reported that miR171 acts mainly by down-regulating SCL6 genes to control a wide range of developmental processes during shoot development (Wang *et al*., 2010). Liu *et al.* (2008) reported that miR171 shown to be induced by drought, cold and salt stress in *Arabidopsis* seedlings (Liu *et al*., 2008).

2.5.2 The miRNA target genes

Laccases (*LAC2* and *LAC17*)

Laccases or p-diphenol: dioxygen oxidoreductases are multi copper containing glycoproteins and found to be widely distributed in higher plants including both monocots and dicots. Studies have been identified that laccases catalyzes oxidation of various phenolic, inorganic and/or aromatic amine substrates through simultaneous reduction of molecular oxygen to water (Reinhammar and Malmstroem 1981). It has been identified that *Arabidopsis thaliana* contains 17 annotated laccase genes with four sub groups (McCaig *et al*., 2005). Different functions of laccases in plants have been proposed including, lignin synthesis (Ranocha *et al*., 2002), wound healing (Dean and Eriksson, 1994), iron acquisition (Hoopes and Dean, 2004), response to stress (Liang *et al*., 2006), and maintenance of cell wall structure and integrity (Ranocha *et al*., 2002). Studies have been revealed that, in *Arabidopsis thaliana*, rice and poplar laccase genes were targeted by miR408 (Rhoades *et al*., 2006).

Argonaute proteins (AGO)

Argonaute (AGO) proteins are catalytic component of the RNA induced silencing complex (RISC) that uses base pairing to silence complementary mRNA at the posttranscriptional level (Carthew and Sontheimer, 2009). AGO proteins bind to short interfering RNAs (siRNAs) and microRNAs (miRNAs) through a conserved PAZ domain. In *Arabidopsis thaliana*, 10 different AGO proteins have been identified (Vaucheret, 2008). The most studied Arabidopsis AGO protein is *AGO1*, which is a core component of the RNA-induced silencing complex, which associates with miRNAs and inhibits target genes by mRNA cleavage and/or translational repression (Vaucheret *et al*., 2004; Vaucheret, 2008; Voinnet, 2009). A recent report showed that a decrease in *AGO1* confers hypersensitivity to ABA, whereas an increase in the level of *AGO1* leads to ABA hyposensitivity (Earley *et al*., 2010).

ATP sulfurylase genes

ATP sulfurylase is the first enzyme in the sulfate assimilation pathway, which provides reduced sulfate for the synthesis of cysteine, methionine and glutathione. In *Arabidopsis thaliana* four different APS genes (*APS1, APS2, APS3* and *APS4*) have been identified and which encodes ATP sulfurylase isoforms. From the previous studies it has been predicted that *ASP1, ASP3* and *ASP4* proteins function in plastid whereas *ASP2* functions in cytosol (Rotte and Leustek, 2000).

Recently it has been identified that miR395 regulates the accumulation of sulfate by targeting ASP genes (Kawashima *et al*., 2009). Quantitative RT-PCR data showed that decreased level of *APS1* transcripts in both shoot and root tissues in response to sulfate limitation (Liang *et al*., 2010).

Ubiquitin conjugating enzyme (UBC24)

These are enzymes which perform the second step in the ubiquitylation reaction which targets a protein for degradation via the proteasome. It has been identified that ubiquitylation plays important functions in many aspects of plant growth and development, including phytohormone and light signalling, embryogenesis, organogenesis, leaf senescence and plant defence (Dreher and Callis, 2007). In *Arabidopsis thaliana*, 37 proteins with a UBC domain and active-site cysteine has been predicted (Kraft *et al*., 2005). Among the 37 proteins, *UBC24* has been described for its involvement in phosphate signalling (Bari *et al*., 2006). Recently it has been identified that *UBC 24* is targeted by miR399 and the expression of *UBC24* down regulated whereas the level of miR399 is increased in response low phosphate conditions (Chiou *et al*., 2006).

Pentatricopeptide repeat (PPR)

The pentatricopeptide repeat (PPR) is a helical repeat motif found in an exceptionally large family of RNA–binding proteins that functions in mitochondrial and chloroplast gene expression. PPR proteins harbor between 2 and 30 repeats and typically bind single-stranded RNA in a sequence-specific fashion (Barkan *et al*., 2012). It has been identified that PPR proteins localize primarily to mitochondria and chloroplasts where they influence various aspects of RNA metabolism (Linneweber and Small, 2008). Studies have been reported that miR161 targets several mRNAs coding for PPRs (Rhoades *et al*., 2002).

SCARECROW (SCR)

SCARECROW (SCR) is a member of the plant-specific GRAS gene family encoding putative transcription factors, and plays a significant role in the radial patterning of both roots and shoots and hormone signalling (Helariutta *et al.,* 2000; Kamiya *et al*., 2003). It has been reported that three members of the *Arabidopsis* SCR-like putative transcription factors (SCL), *SCL6-II*, *SCL6-III* and *SCL6-IV* possess perfectly complementary target sites for miR171 (Llave *et al*., 2002a; Reinhart *et al*., 2002). Four SCL genes in *Oryza sativa* have also been shown to contain the miR171 target site (Reinhart *et al*., 2002). It has previously been demonstrated that *SCL6-III* and *SCL6-IV* are regulated by miR171-mediated cleavage (Llave *et al*., 2002b). In *Arabidopsis*, Northern and qRT-PCR expression analysis showed that increased levels of miR171 target *SCL6-III* in miR171_3B decoy plants (Ivashuta *et al*., 2011).

2.6 Expression analysis of microRNAs

The miRNA and target mRNA expression level measurement presents valuable information about the miRNAs functions. Understanding about the miRNA mediated gene regulation is highly dependent on availability of various strategies and different methodological approaches for accurate detection of miRNA expression levels in specific tissues. Till today several methodologies have been developed for rapid and specific detection in expression of miRNAs and target mRNAs including, direct cloning, real time quantitative PCR, high-throughput deep-sequencing, and hybridization-based methods such as northern blotting, in situ hybridization and miRNA-microarray methods (Eldem *et al*., 2013). It is also important to have efficient and reliable methods for detection of miRNAs and target mRNAs in specific cells and tissues. Less abundant miRNAs routinely escape from detection with standard technologies such as, cloning, northern blotting and micro array methods. Sensitive qRT-PCR techniques are available for sensitive and specific detection of miRNAs.

Real-time PCR (qPCR) is one of the most powerful and sensitive gene analysis techniques available. It is used for a broad range of applications including quantitative gene expression analysis, genotyping, copy number, drug target validation, biomarker discovery, pathogen detection, and measuring RNA interference. Real-time PCR measures PCR amplification as it occurs, so that it is possible to determine the starting concentration of nucleic acid. The more sensitive and reproducible method of real time QPCR measures the fluorescence at each cycle as the amplification progresses. Every real-time PCR contains a fluorescent reporter molecule such as *TaqMan*® probe or SYBR® Green dye to monitor the accumulation of PCR product. The fluorescence intensity increases proportionally with each amplification cycle in response to the increased amplicon concentration.

Basically there are two quantification methods are available to measure the level of gene expression which includes absolute quantification and relative quantification and each method is suitable for different application.

Absolute quantification is one of the most direct and precise approach for analyzing quantitative data and it requires a standard curve that is prepared from a dilution series of control template (plasmid containing a cloned gene of interest (GOI), genomic DNA, cDNA, synthetic oligos, in vitro transcripts, or total RNA) of known concentration. In this method the standard curve is generated by plotting the log of the initial template copy number against the Ct generated for each dilution. Comparison of the Ct values of the unknown samples to the standard curve allows the quantification of unknown sample.

Relative quantification or comparative quantification measures the relative change in mRNA expression levels. It determines the changes in steady state mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA. Relative quantification does not require standards with known concentrations and this is based on the expression levels of a target gene versus a reference gene. The reference gene has to be chosen in such a way that its expression does not change under the experimental conditions or between different tissues. There are various mathematical models are available for calculation of expression of a target gene in relation to an adequate reference gene (Pfaffl *et al*., 2004). Two types of relative quantification models are available to determine the relative expression:

(1) Without efficiency correction

$R = 2^{-(\Delta Ct\ sample - \Delta Ct\ control)}$

$R = 2^{-\Delta\Delta Ct}$

(2) With kinetic PCR correction efficiency

$$Ratio = \frac{(E\ target)^{\Delta Ct\ (sample\ -\ \Delta Ct\ contol)}}{(E\ ref)^{\Delta Ct\ (sample\ -\ \Delta Ct\ contol)}} \div \frac{(E\ ref)^{Ct\ control}}{(E\ ref)^{Ct\ control}}$$

For calculation of gene expression most of the methods assumed the amplification efficiency of the reaction as ideal or 1; means the PCR product concentration doubles during every cycle within the exponential phase of the reaction (Gibson *et al*., 1996). The PCR amplification efficiency bears the biggest impact on amplification kinetics and the accuracy of the quantification results are influenced by the amplification efficiency. Many PCR reactions do not have ideal amplification efficiencies, and calculations without an appropriate correction factor may overestimate starting concentration (Liu and Saint, 2002).

LinRegPCR program have been used for the analysis of real time RT-PCR Data. This program imports non-baseline corrected data and performs a baseline correction on each sample separately, determines a window-of-linearity and then uses linear regression analysis to determine the PCR efficiency per sample from the slope of the regression line. Linear regression analysis can be used to calculate the intercept and the slope log (No) and log (Eff.) respectively, from the straight line that fits best to the included data points.

2.7 The real time quantification of plant miRNAs by stem loop RT-PCR

For better understanding about miRNA mediated gene regulation it is necessary to have efficient and reliable detection and quantification method for miRNA expression analysis. Currently a number of miRNA quantification/ profiling methods are available including miRNA microarrays (Nelson *et al*., 2004), SYBR- based miRNA RT-qPCR assays (Shi and Chiang, 2005) and invader assay (Allawi *et al.,* 2004). The miRNA represent a relatively abundant class of transcripts and their expression levels vary greatly among different cells and tissues. Less abundant miRNAs routinely escape detection with technologies such as cloning, northern hybridization and microarray analysis (Chen *et al*., 2005). The qRT-PCR has been extensively used in measurement of gene expression patterns of miRNAs with its high level of sensitivity, specificity, accuracy and practical use (Bin *et al*., 2011). In general, there are two common RT-PCR methods are available for measurement of miRNA expression levels including, SYBR Green based miRNA RT-qPCR assays and Stem-loop RT based *TaqMan*, which are differentiated from each other in terms of chemical reaction (Eldem *et al*., 2013).

Stem loop RT-PCR method is one of the novel method developed for miRNA quantification which detect and quantify mature miRNAs in a fast, specific, accurate and reliable manner (Chen *et al*., 2005). In this method first, a miRNA specific stem loop RT primer is hybridized to the miRNA and then reverse transcribed. Next, the RT product is amplified and monitored in real time using a miRNA-specific forward primer and the universal reverse primer. Stem-loop RT-based *TaqMan* method uses a target-specific fluorogenic probe that enables the rapid detection and quantification of desired miRNAs (Mestdagh *et al*., 2008). *TaqMan* miRNA assays are specific for mature miRNAs and discriminate among related miRNAs that differ by as little as one nucleotide and also not affected by genomic DNA contamination. Chen *et al*. (2005) developed an unusual and mature miRNA-specific stem-loop RT primer that can easily hybridize in a sequence-specific manner with its target mature miRNA. It was also reported that the stem loop RT primers used in the reverse-transcription step are considerably better when compared with conventional ones (Eldem *et al*., 2013).

By using stem loop RT-PCR four different plant miRNAs such as (miR156a, miR159a, miR166a and miR167a) expression levels have been quantified (Wu *et al.,* 2007). Feng *et al*. (2009) used SYBR Green I chemistry in the stem-loop real-time RT-PCR assay to quantify the expression changes of certain tomato miRNAs (miR159, miR162, miR164, miR165/166, miR167, miR168 and miR171) upon CMV and TAV infections. It has been also identified that altered accumulation level of selected miRNAs and mRNA targets in tomato seedlings, indicating that the miRNA pathway was interrupted by *Cucumber mosaic virus* and *Tomato aspermy virus infection* (Feng *et al*., 2009). Stem-loop real-time fluorescence quantitative PCR was used successfully to detect miRNA398 in *A. thaliana* and poplar under the ABA and salt stress (Jia *et al*., 2009). It has been showed that miRNA398 was increasing firstly and then began to decline at 48h, then reached the lowest in poplar and miR398 was unchanged under salt stress in *Arabidopsis thaliana*, while opposite under ABA stress in poplar.

2.8 The miRNA target gene expression analysis

Gene specific experimental validation of individual miRNA: mRNA interactions are important for functional analysis of miRNAs. For a true target of a specific miRNA, the modulation of miRNA concentration should correspond to a predictable change in the amount of protein encoded by the target mRNA (Ding *et al*., 2012). The downstream effects of differential miRNAs have been identified at the mRNA level by qRT-PCR. In *P. euphratica* 15 miRNA and target pairs were validated by Real-time qPCR upon salt stress and it have been identified that only 14 miRNA and target pairs have consistent expression changes comparing with high throughput sequencing results (Li *et al*., 2013).

For validation of regulatory roles of miRNAs in response to phytohormone signaling Liu *et al*. (2009) analyzed the expression profiling of corresponding targets of phytohormone-regulated microRNAs in rice. It has been shown that the expression levels of five *in vivo* validated targets, AP2 (the target of miR166), GaMyB (the target of miR159), HD-ZIP (the target of miR172), PINHEAD (the target of miR168) and HIRA (the target of miR167) were just contrary to the expression levels of their corresponding miRNAs (Liu *et al*., 2009). It has been also observed that when miRNAs levels were down-regulated the levels of their corresponding targets were up-regulated. In rice by using real time PCR transcript abundance was determined for SPB and AP2 which are targeted by miR156 and miR172 respectively and based on real time data it have been identified that the moderate abundance of SBP genes in rice seedlings (Li *et al*., 2010).

MATERIALS AND METHODS

The current study on 'Functional analysis of selected miRNAs in response to abiotic stress in *Arabidopsis thaliana'* was undertaken for expression profiling of selected miRNAs under NaCl (300 mM) and low temperature (4 ±1 °C) stress treatments in leaf and root tissues using LNA mediated *in situ* hybridization and stem loop RT-PCR based approaches. The material used and methods employed are presented below.

3.1 Growing of *Arabidopsis thaliana* plants

The *Arabidopsis thaliana* seeds used in this study were of Columbia ecotype (*Col-0*) and obtained from University of Missouri, Columbia, USA. Due to their high ABA and low GA_3 concentration The *Arabidopsis thaliana* seeds possess a dormancy period. Chilling treatment was adopted to break the dormancy processed by the *Arabidopsis thaliana* seeds. The seeds were sown in the media mixture containing perlite and vermiculite in the ratio of 2:1 in plastic pots. After sowing, the pots were placed in cold room maintained at 4 °C for four days. On the 5^{th} day of sowing, pots were transferred to culture room maintained at 25 ±2 °C, a light intensity of 1000 lux with 16/8 hr light-dark duration. Recommended nutrients were provided using all plant food tablets (Rantanshi Agro-Hortitech, Mumbai). The plants were regularly irrigated in every 4 days of interval.

To meet both technical and biological replication *Arabidopsis thaliana* seedlings were maintained in three different batches wherein each batch contained required number of plants. In order to ensure availability of plants for various experiments during the course of investigation the plants were grown in three independent attempts. Thirty days old *Arabidopsis thaliana* plants were used for all the experiments. To ensure proper growth and development of *Arabidopsis thaliana* plants one seedling per pot was maintained.

3.2 Induction of low temperature stress to *Arabidopsis thaliana* plants

Initially well grown 30 days old healthy *Arabidopsis thaliana* plants along with the pots were used for induction of low temperature stress. The *Arabidopsis thaliana* plants were exposed to low temperature (4±1 °C) for increasing durations of 1 hr, 2 hr and 3 hr exposure separately. The required level of low temperature for specific duration was maintained by using incubator (Brnenska Medicinska Technika, Germany). Leaf tissues were harvested from each treatment and immediately used for fixation. Well watered plants grown under ambient culture room conditions were used as controls. Plants were grown separately with three replicates of each treatment.

3.3 Shoot low temperature stress treatment to harvest ambient root tissues of *Arabidopsis thaliana*

The low temperature stress treatment to shoot system was achieved to get an idea of its effect on expression pattern of miRNAs in root systems which are at normal ambient temperature. To maintain unaltered root zone temperature over an extended period a module comprising two thermocol boxes (inner small and outer big) and warmed ice packs were used. The outer box accommodated the inner small box leaving about 8cm space on all the sides where the warmed ice packs were placed. The warmed ice packs helped in neutralizing low temperature effect and double layered thermocol box helped in maintaining constant temperature in root zone. The inner small thermocol box accommodated the pot carrying the *Arabidopsis thaliana* plants. The top portion of the pot was covered with the thermocol layer: a slit in the thermocol layer was made through which the plant stood erect. The whole set up was tested for its ability to maintain the ambient root temperature when the shoot portion was exposed to low temperature stress in an incubator (Brnenska Medicinska Technika, Germany) with the help of Thermo/Hygro/Clock probe (Equinox). This system was used harvest the root tissues from each treatment and immediately used for fixation.

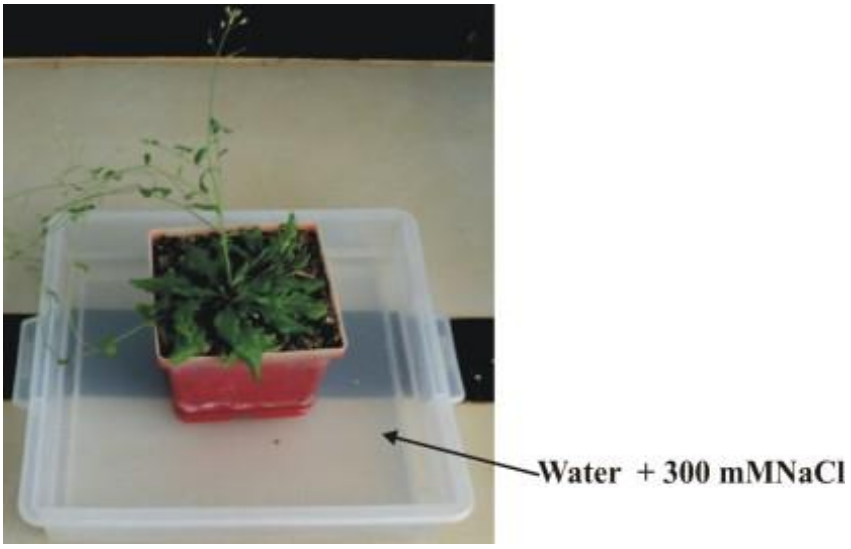
3.4 Induction of salinity stress to *Arabidopsis thaliana* plants

The well grown 30 days old healthy plants were used for induction of salinity stress. Pots carrying *Arabidopsis thaliana* plants were subjected to 300mM concentration NaCl treatment and plants were exposed for 3 hr, 6 hr, 12 hr and 24 hr duration separately to study the effect of salinity stress with increasing duration of exposure on the expression pattern of selected miRNAs. Plants were grown separately with 3 replicates of each treatment. Normal watered plants grown under ambient culture room conditions were used as controls. All the NaCl stressed and different exposure periods leaf and root tissues were harvested and immediately used for fixation.

Well grown healthy Arabidopsis thaliana plant

Thermal insulating model used to study the shoot low temperature stress

On expression pattern of miRNAs in root tissues



Induction of salinity stress to well grown healthy Arabidopsis plant

Plate 1: Materials used in functional assessment of selected miRNAs in Response to low temperature and NaCl treatments

3.5 *In situ* hybridization in *Arabidopsis thaliana*

In situ hybridization is one of the most powerful technique which combine both histological as well as cytological approaches with molecular biology techniques in order to detect specific nucleic acid sequence in temporal and spatial manner in samples at the cellular or tissue level. At each step sufficient care was taken to ensure RNase free environment in order to retain the miRNA in the tissues. All the details related to composition of various reagents and solutions used in the course of *in situ* hybridization are given in the appendix (I-V)

3.5.1 Preparation of RNase free water

Diethyl pyrocarbonate (DEPC) (Sigma Aldrich, USA) was used to treat the millipure water for preparation of all the solutions required for *in situ* hybridization. The DEPC (0.1%) was added to required quantity of water and kept overnight with gentle shaking. Overnight DEPC treated water was autoclaved next day in order to degrade the DEPC and this DEPC free water was used for the preparation of reagents.

3.5.2 Tissue collection

With the maximum care the *Arabidopsis thaliana* plant samples were harvested in glass vials (Borosil Glass Works Limited, India) which were properly labeled for their tissue type and treatments (Table 1). The fixative was prepared freshly and the harvested tissues were immediately placed in the 15ml glass vials containing freshly prepared fixative on ice. The minimum time gap was maintained during tissue harvesting and its fixation.

3.5.3 Fixation of tissues

The harvested tissues were fixed in paraformaldehyde (PFA) (Sigma Aldrich, USA). PFA was freshly prepared in phosphate buffer saline (PBS) (Sigma Aldrich, USA) in required quantities. PBS (Sigma Aldrich, USA) was prepared and pH was adjusted to 11.0 with NaOH. The solution was heated to 65 °C in a fume hood, 4% PFA was added and mixed gently until it dissolves completely. The pH of 4% PFA in PBS was adjusted to 7.0 with conc.H_2SO_4. Later Dimethyle sulphoxide (DMSO) was added in the solution and mixed properly.

Harvested tissues were placed in freshly prepared 4% PFA solution which was placed on ice and vacuum infiltrated at 450 mm Hg. Vacuum was held for 20 min. and released slowly. This step was repeated until tissues sunk in the solution. Later, the PFA solution was replaced with fresh fixative and kept on gentle shaking condition overnight at 4°C.

3.5.4 Embedding of tissues

Tissues were treated with phosphate buffer saline (1X) twice for 30min. each, followed by an increasing series of ethanol (Hayman Limited, England) treatments for 60 minutes each. 0.1% Eosin (Sigma Aldrich, USA) in 95% ethanol treatment was given for visualization of tissue samples at the time of sectioning and kept overnight at 4 °C with gentle shaking.

Before giving histochoice (Sigma Aldrich, USA) treatment the samples were transferred to fresh glass vials. 100% ethanol with 0.1% eosin treatment was given for 45 minutes at room temperature thrice. The increasing series of histochoice treatment was given for 30 minutes each. An increasing series of histochoice treatment was given for 30 min. each. At the stage of 100% histochoice, ¼ volume paraplast chips (Fisher Scientific, USA) were added and kept overnight.

Next day the samples containing vials were placed at 42 °C until chips completely melted. More chips were added and temperature of incubator was raised to 60 °C. After 8 hr wax/ histochoice was replaced with the freshly melted wax (Merck Specialist Pvt. Ltd., India) and the vials were left open overnight at 60 °C.

The wax was replaced twice everyday for 4-5 days and the vials were left open overnight at 60 °C. Blocks of tissues in wax were formed by placing small amount of paraplast chips moulds (Electron Microscopy Science, USA). Warmed forceps were used to arrange the tissue samples in the mould. The moulds were placed on ice for quick setting. The blocks were transferred to cold room at 4 °C for long term storage.

3.5.5 Sectioning of tissues

Slides used in this experiment were Probe-On-Plus slide from Fisher Scientific, USA. The slides were pre-cleaned and charged.

Table 1: Low temperature and salt regimes and tissue types selected for *in situ* hybridization of seven selected miRNAs in *Arabidopsis thaliana*

Treatment	Duration	Tissue type	
25 ±1 °C		Leaf	Root
	1hr	Leaf	Root
Low temperature (4 ±1 °C)	2hr	Leaf	Root
	3hr	Leaf	Root
Control (normal water)		Leaf	Root
	3hr	Leaf	Root
Salt (Water with 300 mM NaCl)	6hr	Leaf	Root
	12hr	Leaf	Root
	24hr	Leaf	Root

The slides also have white paint label on them that makes a capillary space when two are sandwiched together. The painted white space was used for labeling the slides.

The tissue blocks were trimmed and mounted onto the microtome (Leica Microsystem, Germany). About 6-7 µm thick sections were taken using disposable microtome blades (Leica Microsystem, Germany) in the form of ribbon and slides were pre-warmed at 42 °C and placed on RNase free slide warmer. The ribbon was floated on DEPC free water and slowly taken on the Probe-On- Plus slide. By using Kim-wipe tissues excess of water present in the slides was removed and it was ensured that there was no presence of water bubble on the slide. In order to facilitate adhering of the tissues on to the slide surface, the slides were left in a tilted slide rack at 42 °C, for overnight.

3.5.6 Pre-treatment of tissue sections

To facilitate the better hybridization the sections were pre-treated to optimize the specificity and signal intensity. The tissue sections were first deparaffinised by 100% histochoice (Sigma Aldrich, USA) treatment and tissues were rehydrated through a decreasing ethanol (Hayman Limited, England) series for 1 min each. Two times of sodium citrate saline (SSC) treatment was given for 15-20 min. The proteinase K treatment was given with Tris-EDTA (TE) (Sigma Aldrich, USA) buffer solution containing proteinase K (1 µg/ml) for 30 min. at 37 °C. The 0.2% Glycine (Sigma Aldrich, USA) in PBS (1X) treatment was given to the sectioned tissues for 2 min. The slides were washed in fresh PBS twice for 2 min each. 4% PFA solution of pH 7.0 was prepared freshly in PBS (1X) and slides were treated for 10 min. with PFA. Again PBS wash was given for 5 min twice, followed by acetic anhydride treatment for 10 minutes. The slides were finally washed in PBS for 5 minutes twice, followed by an increasing series of ethanol treatment for dehydration of tissues on the slides.

3.5.7 Digoxygenin labeled LNA modified microRNA probes

Digoxygenin (DIG)-labeled miRCURY™ Locked Nucleic Acid (LNA) probes (Exiqon, Vedbaek, Denmark) were used for *in situ* hybridization. These probes were designed by taking the reverse complementary sequence of microRNA (using FastPCR software). A set of seven microRNAs previously tested for their up/down regulation in water and heat stressed *Arabidopsis thaliana* were used in the present study. The sequence information of these microRNA probes are presented in table 2.

LNA DIG- labeled miRCURY™ antisense probes which are extremely specific and will not bind if there is any single mismatch were used. The ath-miR171, which is ubiquitously expressing in all tissues, was used as a control.

3.5.8 Hybridization of tissues

The slides were dried completely and marked with Tissue-Tek marking pencil (Electron Microscopy Sciences, USA) for respective probes to be hybridized. In required quantity hybridization solution was prepared. Probe solution was prepared by adding probe to 50% formamide (Sigma Aldrich, USA) solution. For each pair of slides 40 µl probe-formamide solution was used. This probe-formamide mixture was heated to 80 °C for 2 min. and immediately chilled on ice, spun down completely and kept on ice.

For each pair of slides 160 µl of hybridization solution was added for each pair of slides in such a way that the probe-hybridization solution volume was made up to 200 µl. The probe concentration 0.1 ng/µl was used. To each slide the probe-hybridization solution was added at the middle of the one slide and slowly massaged with other one on the top until the two slides came closer. The slide sandwich was placed elevated above wet paper towel in plastic container in order to avoid drying of solution placed in between the slides. The container was placed at 50°C overnight for hybridization.

3.5.9 Post-hybridization treatment

The slides were washed gently to remove the probe-hybridization solution and subsequently treated with RNase A (Roche Diagnostics, Germany) to remove single stranded RNA and to increase the specificity of the probe. The 0.2X SSC solution was pre-warmed at 55 °C and NTE solution pre-warmed at 37 °C were used for washing the slides.

The slides pairs were dipped in pre-warmed 0.2X SSC and separated slowly. Before placing them in a rack, the slides were rinsed in 0.2X SSC at 55 °C twice for 60 min. with gentle shaking. The pre-warmed NTE (NaCl-Tris-EDTA) solution was used to wash the slides at 37 °C twice for 5 min. each.

Table 2: The miRNAs and their nucleotide sequence information used for probe preparation and expressional assessment through *in situ* hybridization

Sl. No.	miRNA	Probe sequence (5′-3′)
1	ath-miR399	CCGGGCAAATCTCCTTTGGCA
2	ath-miR397a	CATCAACGCTGCACTCAATGA
3	ath-miR168	TTCCCGACCTGCACCAAGCGA
4	ath-miR399e	CGAGGCAAATCTCCTTTGGCA
5	ath-miR395b	GAGTCCCCCCAAACACTTCAG
6	ath-miR161	CCCCGATGTAGTCACTTTCAA
7	ath-miR171	GATATTGGCGCGGCTCAATCA

The slides were incubated with RNase A (20 µg/ml) in NTE solution with gentle shaking at 37 °C for 30 min. then slides were treated with fresh NTE solution at 37 °C twice for 5 min. The slides were washed in 0.2X SSC for 60 min. at 55 °C with gentle shaking and placed in PBS for 5 min. at room temperature.

3.5.10 Antibody treatment

1% blocking reagent (Roche Diagnostics, Germany) was freshly prepared in 200 ml TBS just before its actual use. The slides were blocked with blocking reagent (Roche Diagnostics, Germany) in order to prevent non-specific cross-reaction with the anti-DIG antibody. Then the slides were placed on the bottom of large plastic container. During this time 500 ml 1% Bovine serum albumin (Sigma Aldrich, USA), 0.3% Triton X-100 (Sigma Aldrich, USA) in TBS was prepared. The blocking solution was replaced with 1% BSA, 0.3% Triton X-100 in TBS and washed for 45 min. in a rocking platform.

The anti-DIG antibody (Roche Diagnostics, Germany) was diluted (1:1250) in 1% BSA, 0.3% Triton X-100 in TBS solution. 10 µl antibodies in 12.5 ml 1% BSA, 0.3% Triton X-100 in TBS were added. The anti-DIG antibody solution was allowed to get applied on tissues by sandwiching slides together and allowing capillary action to pull solution. Then the slides were arranged on racks of plastic pipettes above the wet paper towel and kept at room temperature for 2 hr. A tightly sealed plastic container was left overnight at 4 °C.

3.5.11 Substrate treatment

The anti-DIG antibody solution was drained out from slides on Kim-wipes (Kimtech Science, Roswell, GA, USA) and separated gently. The slides were placed on the bottom of a large flat plastic container and washed in 1% BSA, 0.3% Triton X-100 in TBS solution on a rocking platform at room temperature for 45 min. for 3-4 times.

The slides were washed in TN (Tris-NaCl) solution for 10 min. it was ensured that each slide should get dipped in TN solution so that all detergent get washed off and the pH in the sections was raised to 9.5 for optimum alkaline phosphatase activity. The substrate solution was prepared freshly and immediately by adding 200 µl of pre-mixed NBT/BCIP (Sigma Aldrich, USA) to 10 ml of TN solution. The slides were sandwiched together and dipped in the solution, allowing capillary action to pull up the solution mixture.

3.5.12 Detection of hybridization signal

The slides were placed in plastic container above wet tissue paper in total darkness for 1- 5 days. The colour development was checked through a dissection microscope (OLYMPUS, SZX9). After every 48 hrs the old substrate solution was replaced by adsorbing onto the Kim-wipes fresh substrate solution was brought in place by capillary action.

When the signal was clear, solution was drained and slides were rinsed in TE (Tris- EDTA) solution to stop the alkaline phosphatase activity. The sections were dehydrated through an increasing ethanol series followed by 100% histochoice treatment for 2 min. twice. The slides were allowed to dry in a fume hood. After drying, the slides were mounted with a few drops of Cytoseal (Electron Microscopy Science, USA). The slides were left overnight for drying.

The observations for signal development were made for microRNA expression and based on the intensity of stain development microRNA expression was categorized into low (+), moderate (++), high (+++), very high (++++) and no (-) expression. Observations were taken on a visual basis. Observations were taken by placing specimen on the microscope stage. Initially interpupillary distance was adjusted followed by diopter adjustment. Zooming knob was set to lowest zoom magnification and microscope was brought into focus by rotating the coarse focus adjustment knob. Zooming knob was rotated to the desired magnification and microscope was precisely focused on specimen with the help of coarse focus adjustment knob and fine focus adjustment knob.

3.6 Expressional quantitation of selected miRNAs and their cognate genes in leaf and root tissues of *Arabidopsis thaliana*

3.6.1 Tissue collection

The *Arabidopsis thaliana* Columbia ecotype (col-0) plants were used to collect leaf and root tissues. Plants were as described in section 3.1. Salinity and low temperature stress was induced as described in section 3.2 and 3.3 respectively.

Table 3: Selected *Arabidopsis thaliana* miRNAs and their target genes used for functional analysis

Sl. No.	Selected miRNAs	Cognate gene	
		Gene ID	Gene name
1	ath-miR399	NM_179887.2	*UBC24*
2	ath-miR397a	NM_128470.4	*LAC2*
		NM_125395.3	*LAC17*
3	ath-miR168	NM_001198240.1	*AGO1*
4	ath-miR399e	NM_179887.2	*UBC24*
5	ath-miR395b	NM_128651.4	*APS1*
		NM_179507.1	*APS4*
6	ath-miR161	NM_117745.3	*PPR*
7	ath-miR171	EU550749.1	*SCL6-III*
		EU550777.1	*SCL6-IV*

Normal watered ambient temperature grown plants were used as controls. Stressed and control tissues (leaf and root) were harvested from each treatments separately. Details of treatments given are presented in table 1.

3.6.2 Total RNA isolation

The total RNA was extracted from leaf and root tissues of NaCl (300mM) treated, low temperature stressed and control tissues using TRIZOL reagent (Invitrogen, San Diego USA) following bench level protocol which was previously optimized in our laboratory. Approximately 2-3 g of tissue sample was weighed and grounded into fine powder in a DEPC treated autoclaved pre-chilled mortar and pestle. The ground tissue was transferred to a pre-chilled 50ml conical bottom tube and TRIZOL reagent (1 ml TRIZOL/100mg tissue) was added. The contents were mixed well with vortex and 1 ml of liquid phase was transferred into labeled RNase-free 1.5 ml tubes and incubated at room temperature for 5min. after incubation, 200 µl of chloroform per 1 ml of TRIZOL was added and vortexed for 20 sec. and this was incubated at room temperature for 10 min. and samples were centrifuged at 13,200 rpm for 10 min at 4 °C.

After centrifugation, the aqueous phase containing RNA was carefully transferred into a new RNase-free 1.5 ml tube without disturbing the other phases and tubes were immediately placed on ice. An equal volume of isopropyl alcohol (isopropanol) was added and mixed gently by inverting (2-3 times). The samples were incubated on ice for 30 min for precipitating the RNA and centrifuged at 13,200 rpm for 20 min at 4°C. A very small pellet was visible at the bottom of the tube which was the total RNA. The supernatant was discarded and the tubes were let to stand upside down on Kimwipe (Kim Tech Science, Canada) for 5 min. The pellet was washed with 1 ml of 75 per cent ethanol gently by vortexing and centrifuging. The liquid was decanted and the inside of the tube was wiped to dry with a clean kimwipe, without touching the pellet. The pellet was resuspended in 50 µl of RNase-free water and this was incubated on ice for at least an hour and pipette occasionally for dissolving the pellet and centrifuged at 13,200 rpm for 20 min at 4°C.

Sodium acetate precipitation was done by transferring to a new *RNase*-free 1.5 ml tubes. Precipitation was achieved by adding 10% volume of 3M sodium acetate and equal volume of isopropanol. The contents were mixed well and incubated on ice for overnight at -80°C. Next day, contents were centrifuged at 13,200 rpm for 20 min at 4°C. The supernatant was decanted and the pellet was washed with 1 ml of 75 per cent ethanol. Finally, the pellet was resuspended in 50 µl of RNAase-free water. This was stored at -80°C till further use.

3.6.3 Purification of total RNA

The total RNA was treated to remove the DNA contamination using TURBO DNAfree™ kit (Cat#AM1907 Ambion, USA) as per the instructions in the manual supplied by the company. To eliminate the genomic DNA, 10 µg of total RNA was digested with RNase-free DNaseI. Finally, the total RNA was precipitated into desired volume of water. The overall quantity and quality of total RNA was checked using NanoDrop ND- 1000 spectrophotometer (NanoDropTechnologies, USA). In order to check quality of RNA preparation, the samples were fractionated over a formaldehyde agarose gel (Appendix VI). The absence of genomic DNA was confirmed by PCR with total RNA as a template.

3.6.4 Stem-loop RT primers for miRNA quantitation

In order to design the stem loop primer the pre-miRNA sequences were downloaded from miRBase database of microRNAs (*http://microrna.sanger.ac.uk*). The position of mature miRNAs were located in the pre-miRNA sequences based on nucleotide sequence similarity and stem-loop primer was designed in such a way that the 3′ end of stem-loop primer forms complementarity with 3′ end of mature miRNA sequence in pre-miRNA. The stem-loop backbone was retained same for all the target miRNAs as given by Chen *et al*. (2005). The stem-loop properties of designed primers were verified using Mfold RNA secondary structure analysis tool (http://mfold.rna. albany.edu/). The primers were also checked for the stem-loop energy content which was approximately -27.The designed stem-loop primers for selected four miRNAs are presented in appendix VII. The same set of primers was used for cDNA synthesis.

3.6.5 Preparation of cDNA for quantitation of expression levels of selected miRNAs

Single stranded cDNA was prepared by using HighCapacity™ cDNA Reverse Transcription kit (Cat#4374966, Ambion, USA) along with a special type of stem loop primers and cDNA was prepared as per the manufacturer's instructions. Stem loop primers were specific to each of selected miRNA. All the reagents were thawed and stored on ice.

Upon thawing, the reagents were mixed thoroughly and spun down before pipetting. About 2 µg of total RNA in a 20 µl reaction was quantitatively converted to single-stranded cDNA using thermal condition mentioned below;

Composition of components used for the cDNA synthesis

Sl. No.	Components	Volume/reaction (µl)
1	10X RT buffer	2.00
2	25X dNTP (100 mM)	0.80
3	Stem loop RT primers	2.00
4	MultiScribeTM reverse transcriptase	1.00
5	RNase inhibitor	1.00
6	Nuclease free water	3.20
7	Total RNA (2 µg)	10.0
	Total volume	20.0

Thermal conditions for single stranded cDNA conversion

	Step 1	Step 2	Step 3	Step 4
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 min	120 min	5 sec	hold

3.6.6 Stem-loop qRT primers for miRNA quantitation

The mature miRNA sequences were downloaded from miRBase database of miRNAs (*http://microrna.sanger.ac.uk*) to design stem-loop qRT primers. The 5′ end nucleotides of mature miRNA sequences were used to design the forward qRT primer. By using same pre-miRNA sequence for all RT primer a universal reverse primer was designed. All the stem-loop RT primers were designed to according to Chen *et al*. (2005). List of primers used for amplification of mature miRNAs are listed in appendix VII.

3.6.7 Selection of miRNAs and their cognate genes

Seven different miRNAs (miR399, miR397, miR399e, miR395b, miR168, miR161 and miR171) were selected based on the information available from previous studies which were conducted in IABT UAS, Dharwad to see their expression pattern under water stress and heat shock regimes in *Arabidopsis thaliana*.

Based on the available information and annotated functions in *Arabidopsis thaliana*, the cognate genes of miRNAs were selected. Details of selected miRNAs and their cognate genes are presented in table 3.

3.6.8 Preparation of cDNA for quantitation of miRNA cognate genes

The RNA which was free from DNA contamination was used to prepare cDNA. Single stranded cDNA was prepared by using HighCapacity™ cDNA Reverse Transcription kit (Cat#4374966, Ambion, USA) as per the manufacturer's instructions. All the reagents were thawed and stored on ice. Upon thawing, the reagents were mixed thoroughly and spun down before pipetting. About 2 µg of total RNA in a 20 µl reaction was quantitatively converted to single-stranded cDNA using thermal condition mentioned below;

Composition of components used for the cDNA synthesis

Sl. No.	Components	Volume/reaction (µl)
1	10X RT buffer	2.00
2	25X dNTP (100 mM)	0.80
3	10X RT random primers	2.00
4	MultiScribe™ reverse transcriptase	1.00
5	RNase inhibitor	1.00
6	Nuclease free water	3.20
7	Total RNA (2 µg)	10.0
	Total volume	20.0

Thermal conditions for single stranded cDNA conversion

	Step 1	Step 2	Step 3	Step 4
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 min	120 min	5 sec	hold

3.6.9 Selection of miRNA cognate genes primer pairs

The primer pairs specific to miRNA cognate genes were designed using Primer3plus software (http//frodo.wi.mit.edu/cgibin/ primer3plus/primer3pluswww.cgi) and the primers were synthesized at IDT Pvt. Ltd. (USA). A predicted melting temperature (Tm) of 55 ± 2 °C, 20-24 nucleotides of primer lengths, guanine-cytosine (GC) contents of 45-55 per cent and PCR amplicon length of 100-200 bp were adopted for designing the primer pairs. The specificity of primer pairs was confirmed through BLAST analysis. The primer sequences are given in appendix VIII.

3.6.10 Standardization of real time conditions

Primer concentration: primer concentration is one of the key factors in real-time quantitation of gene expression. Primer concentrations ranging from 100 nM, 150 nM, 200 nM, 250 nM, 300nM and 300 nM were used to optimize the amplification. At a primer concentration of 250 nM a single melting curve, low Ct value, high fluorescence value and no primer dimer when loaded on gel was observed. Finally this concentration was selected and used for rest of the experiment.

qRT-PCR reaction: the reaction mixture containing 4 ng of cDNA, 250 nM of gene specific primer and 5 µl of 2X SYBR® Green reagents (Cat.#4368706, Ambion, USA) were used in each reaction. Each component of the reaction mixture was standardized for 10 µl reaction volume. The master mix of different components of real-time PCR was prepared fresh to avoid handling errors.

3.6.11 Setting baseline and threshold level

Prior to significant accumulation of the target amplicon, the background signal was most evident during the initial cycles of PCR. During these initial PCR cycles, the background signal in all wells is used to determine the “baseline fluorescence” across the entire reaction plate. For analysis data, more accurate measurements of fluorescence are needed and which are attained when the target amplification is sufficiently above the background signal. The Eppendrof Mastercycler® ep *realplex* detection software sets the default baseline from 3-15 cycles.

Once the baseline has been set correctly, the software automatically sets the threshold at 10 standard deviations above the mean baseline fluorescence.

It was ensured that threshold line would be placed in the exponential phase to increase the precision and quality of the experimental data and same baseline and threshold default setting was used for all PCR reactions.

3.6.12 Internal controls

For data normalization, the transcripts of stably expressed (reference genes) genes are commonly used. In Arabidopsis, the house keeping genes like *18S rRNA*, *EF-1*α, β - *actin* and *ubiquitin (UBQ)* have served as good reference genes (Caldana *et al*., 2007 and Czechowski *et al*., 2004). In our experiment we selected *Arabidopsis thaliana* housekeeping gene *actin* as an internal control.

3.6.13 Real time conditions

An Eppendorf Mastercycler® ep *realplex* instrument (Eppendorf Pvt. Ltd., Germany) was used for all real-time PCR amplifications. The optimal PCR conditions used for amplification are presented below:

Stage	Step	Temperature °C	Duration	No. of cycles
1	Initial denaturation	95	10 min.	1
2	Denaturation	95	30 sec.	
3	Annealing	55	30 sec.	40
4	Extension	60	30 sec.	
5	Melting curve	95	20 min.	1

3.7 Statistical analysis

3.7.1 Quantitation of relative gene expression

Absolute quantification and relative quantification are the two most commonly used methods to analyze data from real-time PCR experiments. Absolute quantification method determines the copy number of a gene by relating the PCR signal to a standard curve. The relative quantification relates the PCR signal of the target transcript in a treatment group to that of other untreated control and determines relative fold change in gene expression. In this study, relative quantification approach was selected and used to measure the expression level of selected miRNAs and their cognate genes in NaCl (300mM) treated, low temperature stressed and normal watered ambient temperature grown leaf and root tissues of *Arabidopsis thaliana*. The mathematical model delta-delta Ct method (Livak and Schmttgen, 2001) was used to determine relative change in gene expression (fold change).

In the present study, the fold change expression of selected miRNA and their cognate genes were calculated by using relative quantification with PCR efficiency correction. PCR efficiency of all the TF genes was obtained from the exponential phase of each individual amplification plot using the equation $(1+E) = 10^{slope}$ (Ramakers et al., 2003). To calculate initial concentration of mRNA and PCR efficiencies for each sample, the LinRegPCR (http://www.bioinfo@amc.uva.nl; subject: LinRegPCR) software based on the above equation proposed a linear regression on the log fluorescence per cycle number data as an assumption-free method was used. The log-linear part of the PCR data was determined for each sample by selecting a lower and an upper limit of a 'window of linearity'. Linear regression analyses was used to calculate the intercept and the slope, log (No) and log (eff.) respectively, from the straight line that best fits to the included data points. In order to ensure unambiguous selection of data point within the 'window of linearity', the lines with the highest R^2 value (0.99) were selected.

The raw fluorescence data was extracted from the Eppendorf Mastercycler® ep *realplex* using extract PCR (Eppendorf Pvt. Ltd., Germany) software. Generally the background is resulted due to residual fluorescence of the dye, differences in tube transparency, dust, and noise of the electronics. A variable background makes a near-linear contribution to the curves generated by the amplifier and it should be subtracted from the raw fluorescence without distorting the data considerably. The baseline fluorescence data was collected from 3-15 cycles for background correction.

The fluorescence increments (raw fluorescence – Yo) were normalized to reaction fluorescence background (Yo) for each sample reaction as Normalized fluorescence = raw fluorescence – Yo/ Yo (Pfaffl *et al*., 2004).

The background corrected or normalized fluorescence data was used to calculate PCR efficiency by LinRegPCR software. The calculated PCR efficiency was used to derive fold expression of miRNAs and their cognate genes using the following method:

$$\text{Ratio} = \frac{E\,(\text{target})^{-\Delta Ct}}{E\,(\text{control})^{-\Delta Ct}}$$

E_{target} =PCR efficiency of target gene in sample.

$E_{control}$ = PCR efficiency of target gene in control.

Δ Ct = (Ct of target gene - Ct of reference gene)

The statistical data analysis of gene expression quantitation was performed on the basis of classical standard parametric tests such as t-test and standard error. The technical precision or reproducibility of real-time PCR measurement was assessed by performing replicate measurements in separate PCR runs (technical and biological replicate). Microsoft Excel programme was used to calculate above statistical parameters.

EXPERIMENTAL RESULTS

The present study was carried out to analyze the expression pattern of selected miRNAs in response to low temperature and NaCl (300 mM) treatments. The expression patterns of selected miRNAs were previously characterized for their water stress and heat shock response in *Arabidopsis thaliana* at Department of Biotechnology (Ghosh, 2008; Mahale, 2010).

4.1 Expression pattern of selected miRNAs

The *Arabidopsis thaliana* plants, which were grown under ambient temperature with normal culture conditions showed differential up and down regulations for tested microRNAs.

4.1.1 Expression pattern of selected miRNAs in ambient temperature control leaf tissues

The expression of four different miRNAs such as, miR397a, miR168, miR161 and miR171 was noticed in leaf tissues of *Arabidopsis thaliana* plant grown under ambient temperature (25 °C) with normal culture conditions. The expression of miRNAs was observed all over the leaf including in cuticle, lower and upper epidermis, spongy and palisade mesophyll tissues and also in vascular bundle. From the *in situ* stained sections, it was clear that miR171 and miR161 were accumulated at higher levels when compared with that of miR397a and miR168 (Plate 2). But, the expression of miR399, miR399e and miR395b was not found in leaf tissues of *Arabidopsis thaliana* plant grown under ambient temperature with normal culture conditions (Plate 2).

4.1.2 Expression pattern of selected miRNAs in ambient temperature control root tissues

The expression of miR397a and miR171 was recorded in root tissues of *Arabidopsis thaliana* grown in ambient temperature with normal culture conditions. The expression of miRNAs was observed all over the root including vascular bundle, cuticle layer *etc*. The intensity of *in situ* hybridization signals was found to be similar in both miR397a and miR171 (Plate 3). On the other hand, two miRNAs, miR161 and miR168 did not show their expression in any part of the root tissues. However, the accumulation of miR399, miR399e and miR395b was not found in both leaf and root tissues of *Arabidopsis thaliana*.

4.2 Expression pattern of selected miRNAs in leaf tissues of *Arabidopsis thaliana* upon low temperature (4 ±1 °C) stress

A set of seven miRNAs were analyzed for their expression upon low temperature (4 ± 1 °C) with different durations (1 hr, 2hr and 3 hr) of exposure of *Arabidopsis thaliana* leaf tissues.

Exposure of *Arabidopsis thaliana* shoot to low temperature (4±1 °C) for 1 hr and 2 hr duration recorded accumulation of only miR171 and miR397a (Plate 4 and 5). However, the expression of four miRNAs such as, miR171, miR397, miR399 and miR399e was observed at 3 hr exposure of *Arabidopsis thaliana* to low temperature (Plate 6). But, the expression of two miRNAs *viz*., miR399 and miR399e was not recorded at 1 and 2hr exposure. A set of three miRNAs such as, miR395b, miR168 and miR161 did not express upon low temperature (4 ±1 °C) in *Arabidopsis thaliana* leaf tissues. Increase in intensity of expression of miR171 and miR397 was recorded with increasing duration of exposure (Plate 4, 5 and 6).

4.3 Expression pattern of selected miRNAs in ambient root tissues of *Arabidopsis thaliana* whose shoot was exposed to low temperature (4±1 °C) stress

In order to maintain the ambient temperature (27±1 °C) at root zone, a double layered thermocol box filled with warmed ice packs was made use. Exposure of *Arabidopsis thaliana* plant shoot to low temperature (4±1 °C) for 1 hr, 2 hr and 3 hr resulted in the accumulation of miR171 and miR397a in ambient root tissues. But, the expression of miR399 and miR399e was not recorded in ambient root tissue who's only shoot part was exposed to low temperature. However, the expression of miR395b, miR161 and miR168 was absent in both shoot and ambient root tissues (Plate 7).

4.4 Expression pattern of selected miRNAs upon NaCl treatments

4.4.1 Expression of selected miRNAs in well watered control leaf and root tissues

In normal watered leaf tissues of *Arabidopsis thaliana* the upregulation of four miRNAs such as miR397a, miR168, miR161 and miR171 was noticed. The expression was recorded all over the leaf tissue including cuticle, lower and upper epidermis, spongy and palisade mesophyll tissues *etc*.

miR399f

miR397a

miR168

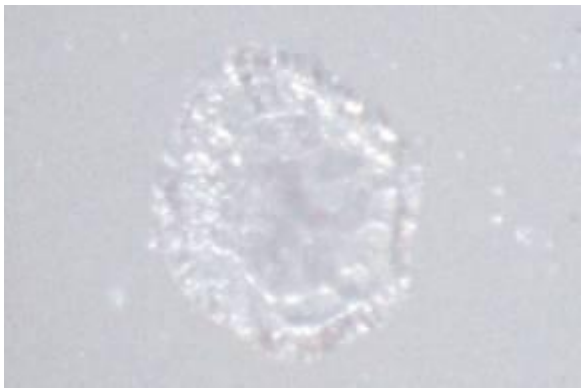
miR399e

miR395b

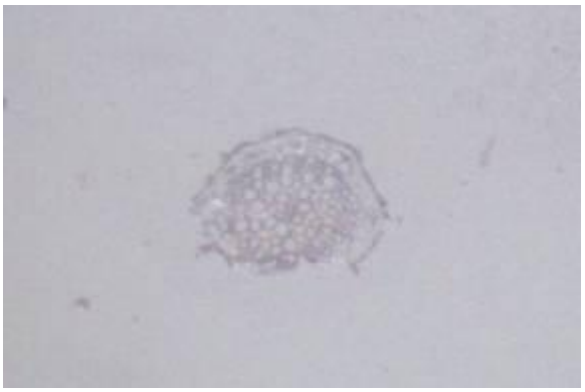
miR16

miR171

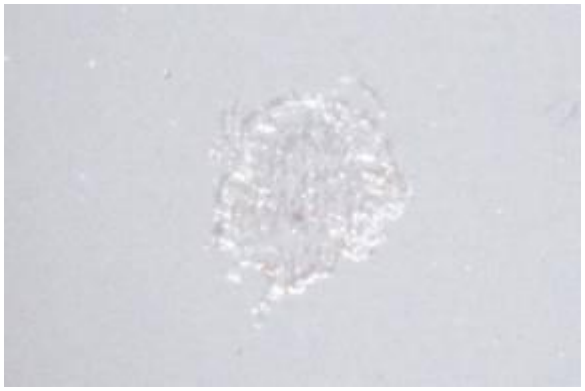
Plate 2: Expression pattern of selected miRNAs in control (25±1 °C) leaf Tissues of Arabidopsis thaliana assessed through LNA mediated ISH



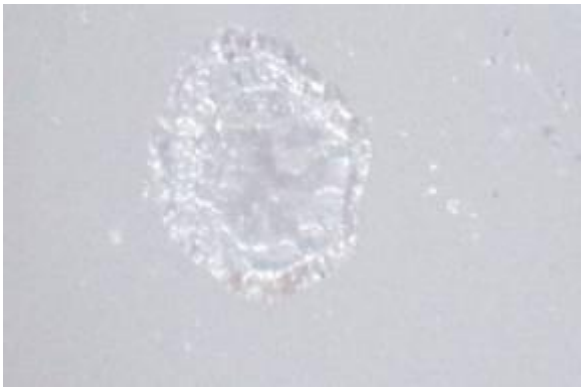
miR399f



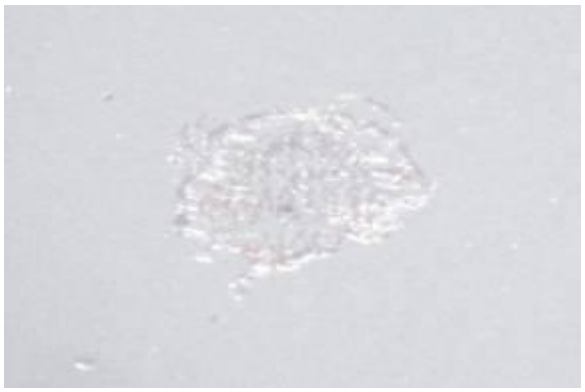
miR397a



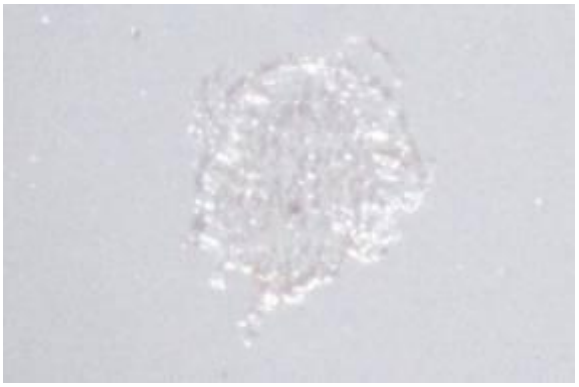
miR168



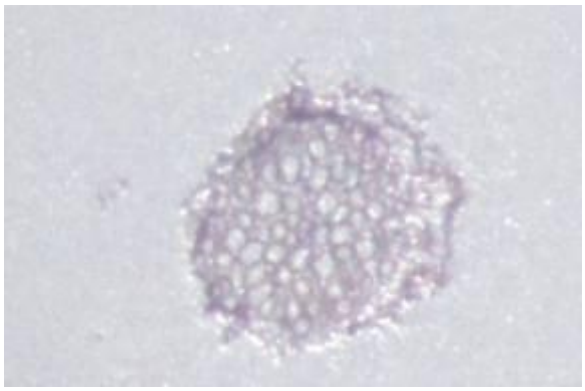
miR399e



miR395b



miR16



miR171

Plate 3: Up and down regulation of selected mirnas in control root tissues of Arabidopsis thaliana

miR399f

miR367a

miR168

miR399e

miR335b

miR16

miR171

Plate 4: Expression pattern of selected miRNAs in leaf tissues of Arabidopsis Thaliana exposed to 1 hr low temperature (4±1 °C)

miR399f

miR397a

miR198

miR399e

miR395b

miR16

miR171

Plate 5: Expression pattern of selected miRNAs in leaf tissues of Arabidopsis Thaliana exposed to 2 hr low temperature (4±1 °C)

miR399f

miR397a

miR168

miR399e

miR395b

miR16

miR171

Plate 6: Expression pattern of selected miRNAs in leaf tissues of Arabidopsis Thaliana exposed to 2 hr low temperature (4±1 °C)

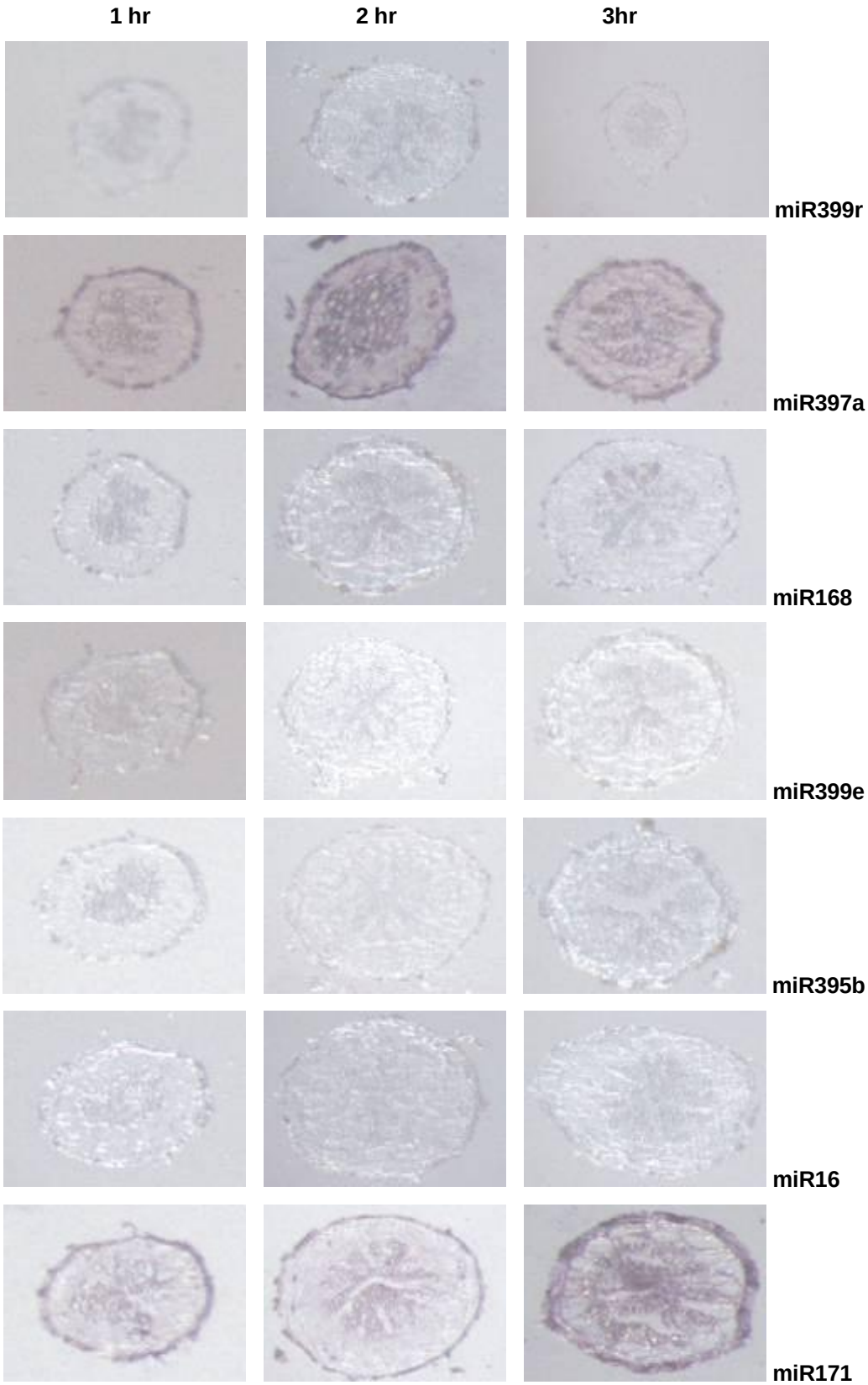


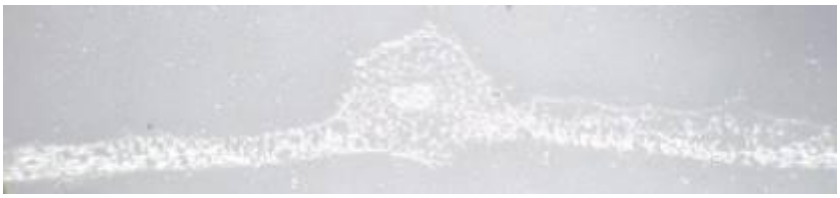
Plate 7: Expression pattern of selected miRNAs in ambient root tissues of Arabidopsis Thaliana whose shoot portion was exposed to low temperature (4±1 °C) for 1, 2 and 3 hr durations

Table 4: Expression of selected microRNAs in leaf and root tissues of *Arabidopsis thaliana* exposure to shoot low temperature regimes assessed through *in situ* hybridization

Treatment	Duration	Tissue			microRNAs expression levels				
			miR399	miR397a	miR168	miR399e	miR395b	miR161	miR171
Control (25±1°C)		Leaf	-	+	+	-	-	++	++
	1hr	Leaf	-	+	-	-	-	-	+
Low temperature (4 ±1°C)	2hr	Leaf	-	++	-	-	-	-	++
	3hr	Leaf	+	+++	-	+	-	-	++
Control (25±1°C)		Root	-	+	-	-	-	-	++
	1hr	Root	-	+	-	-	-	-	+
Cold (4 ±1°C)	2hr	Root	-	++	-	-	-	-	++
	3hr	Root	+	+++	-	+	-	-	++

Scale:

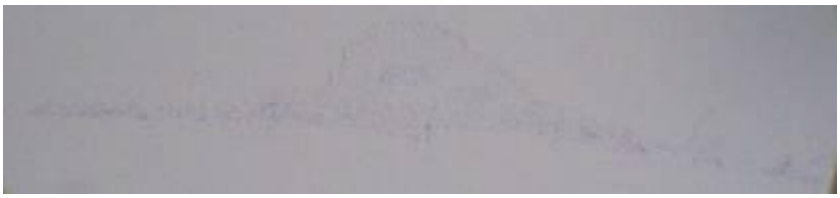
-	Absent
+	Very low (0-25%)
++	Moderate (25-50%)
+++	High (50-75%)
++++	Very high (>75%)



miR399f

miR397a

miR168



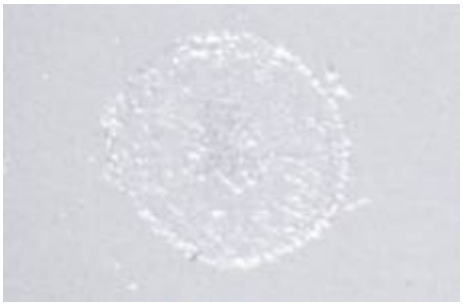
miR399e

miR395b

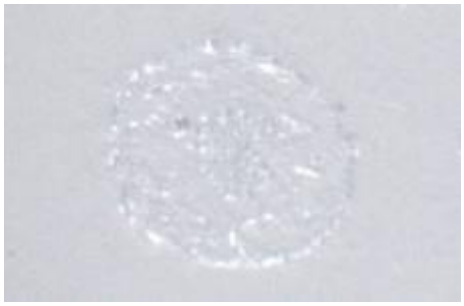
miR16

miR171

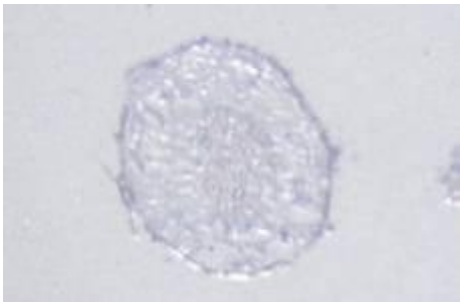
Plate 8: Expression pattern of selected miRNAs in normal watered control leaf tissues of Arabidopsis thaliana assessed through LNA mediated ISH



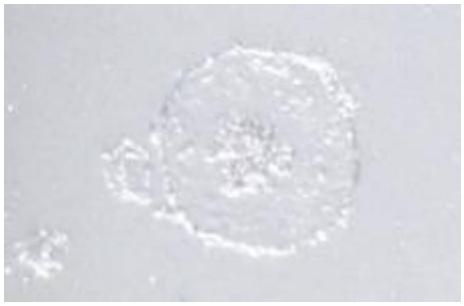
miR399



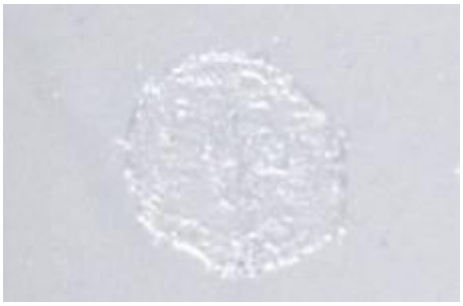
miR399e



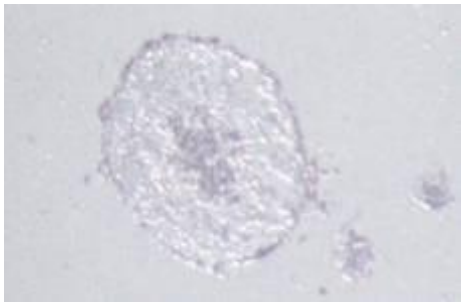
miR397a



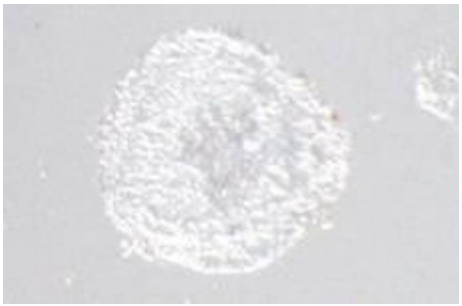
miR395b



miR168



miR171



miR168

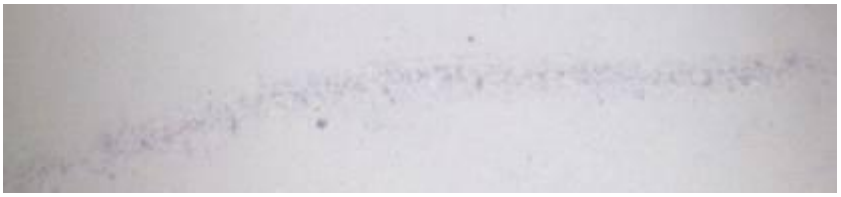
Plate 9: Expression pattern of selected miRNAs in normal watered control root tissues of Arabidopsis thaliana assessed through LNA mediated ISH

miR399f

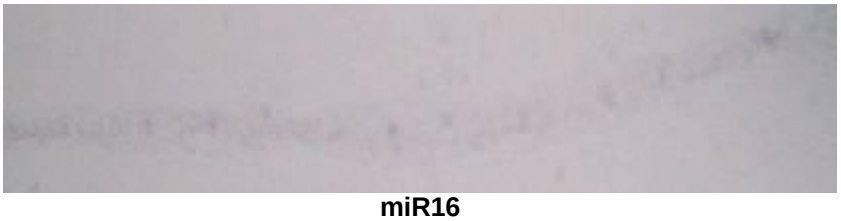
miR397a

miR168

miR399e



miR395b

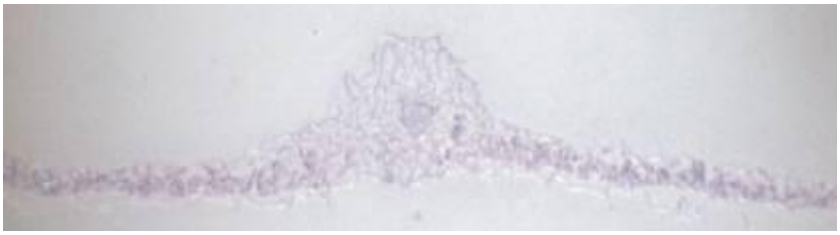


miR16

miR171

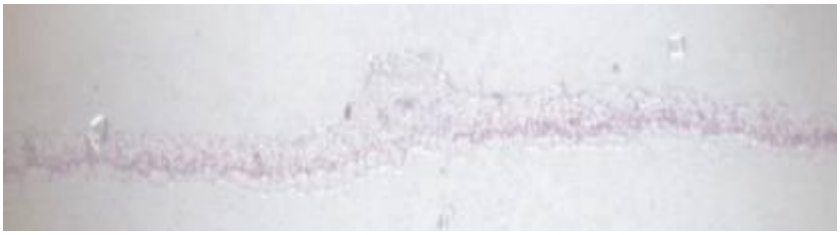
Plate 10: Expression pattern of selected miRNAs in leaf tissues of Arabidopsis Thaliana response to 24 hr NaC1 (300 mM) treatment

miR399f



miR399e

miR397



miR395b

miR171

miR168

Plate 11: Expression pattern of selected miRNAs in leaf tissues of Arabidopsis thaliana exposed to 12 hr NaC1 (300 mM) treatment

miR399f

miR399e

miR397

miR395b

miR171

miR168

Plate 12: Expression pattern of selected of miRNAs in leaf tissues of Arabidopsis Thaliana exposed to 6 hr NaC1 (300 mM) treatment

miR399f

miR399e

miR397

miR395b

miR171

miR168

Plate 13: Expression pattern of selected miRNAs in leaf tissues of Arabidopsis thaliana exposed to 3 hr NaC1 (300 mM) treatment

Comparatively higher level of accumulation of miR171 and miR161 was recorded over miR397a and miR168 from *in situ* stained sections (Plate 8). However, the expression of only miR397a and miR171 was found in root tissues of well watered *Arabidopsis thaliana*. The uniform expression was recorded all over the root tissues including vascular bundle cuticle layer *etc*. In contrast, the miR168 and miR161 did not express in root tissues, whereas their expression was recorded in leaf tissues of normal watered *Arabidopsis thaliana* plant (Plate 9). A set of three miRNAs such as miR399, miR399e and miR395b did not accumulate either in leaf or in root tissues of normal watered *Arabidopsis thaliana* plant as revealed through *in situ* hybridization signals (Plate 8 and 9).

4.4.2 Expression pattern of selected miRNAs in leaf tissues of *Arabidopsis thaliana* subjected for NaCl treatments

The expression pattern of seven selected miRNAs was analyzed in leaf tissues of *Arabidopsis thaliana* plants upon NaCl (300 mM) treatments with different duration regimes of 3, 6, 12 and 24 hr.

Expression of a set of five miRNAs such as miR399, miR399e, miR395b, miR168 and miR171 was recorded in leaf tissues of *Arabidopsis thaliana* exposed to 3 hr, 6 hr, 12 hr and 24 hr durations of NaCl (300 mM) treatments. The increase in intensity of expression was observed with increasing duration of exposure. The expression was recorded all over the leaf tissue. Interestingly, the expression of miR397a was not found in the leaf tissues of *Arabidopsis* plants exposed to different durations (3, 6, 12 and 24 hr) of NaCl (300 mM) treatments (Plate 10, 11, 12 and 13).

4.4.3 Expression pattern of selected miRNAs upon NaCl (300 mM) treatments in root tissues of *Arabidopsis thaliana*

A set of four miRNAs such as miR399, miR399e, miR395b and miR171 were noticed upregulated in root tissues of *Arabidopsis thaliana* exposed to different durations (3, 6, 12 and 24 hr) of NaCl (300 mM) treatments. But, the expression of miR168 was not recorded in root tissues, whereas its upregulation was observed in leaf tissues of *Arabidopsis thaliana*. The uniform expression was found all over the root tissues. However, the expression of miR397a was not found in either leaf or root tissues of *Arabidopsis thaliana* plants upon NaCl (300 mM) treatments (Plate 14).

4.5 Expressional quantitation of relative levels of selected miRNAs and their cognate genes through real time PCR analysis

4.5.1 Real-time quantification of miRNAs by stem-loop RT-PCR

Based on the *in situ* hybridization results, a stem-loop RT-PCR approach was applied to detect four mature miRNAs *viz*., miR399e/f, miR397 and miR171 showing their differentional regulations in both leaf and root tissues of *Arabidopsis thaliana* exposed for different durations of low temperature (4± 1 °C) and NaCl (300 mM) treatments.

4.5.2 Total RNA isolation and its integrity

A total RNA was isolated from leaf and root tissues of low temperature and NaCl (300 mM) treated *Arabidopsis thaliana* plants separately by using TRIZOL reagent. The integrity of total RNA was examined by electrophoresing the individual RNA samples on denaturing, formaldehyde 1% agarose gel stained with Ethidium bromide. The presence of two bright bands corresponding to ribosomal *28S rRNA* and *18S rRNA* with a ratio of intensities of ~ 2:1 was noticed from electrophoresis (Plate 15). The quantity of RNA was checked on NanoDrop ND-1000 spectrophotometer™ which recorded a ratio of ~1.8 at 260/280 absorbance indicating the purity of RNA for further use in downstream reactions.

The genomic DNA contamination was eliminated by treating total RNA with *DNase I* enzyme. The complete elimination of genomic DNA from RNA preparations was confirmed through PCR amplification where RNA was used as template. The observation of absence of PCR amplification from RNA template clearly indicated the complete elimination of genomic DNA from RNA preparations (Plate 16).

4.5.3 Specificity and precision of real-time RT PCR

The qRT-PCRs were performed in an optical PCR strips with an eppendorf mastercycler ep realplex detection system, using the Power SYBR® Green master mix to monitor DNA synthesis (Plate 17a and 17b). The specificity of the PCR reactions for miRNAs and their cognate genes were determined by loading the PCR products on a 4 per cent agarose gel (Plate 18a and 18b). It was noticed that all the miRNAs and their cognate genes yielded specific sized amplicon as expected.

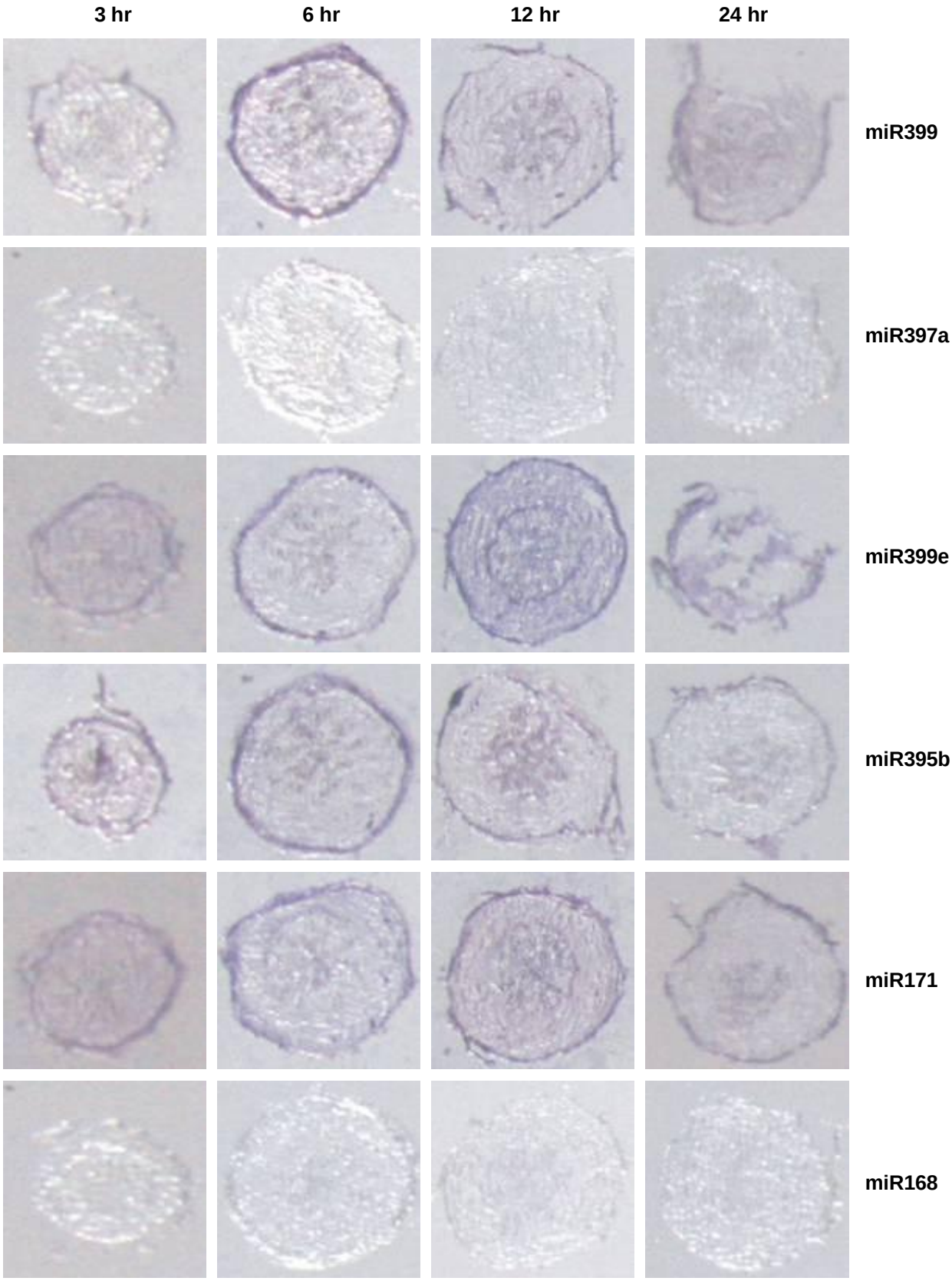


Plate 14: Expression pattern of selected miRNAs in root tissues of Arabidopsis thaliana in response to 3, 6, 12 and 24 hr NaC1 (300 mM) treatments

Table 5: Expression of selected miRNAs in leaf and root tissues of *Arabidopsis thaliana* in normal watered control and NaCl treatments assessed through *in situ* hybridization

Treatment	Duration	Tissue			microRNAs expression levels				
			miR399	miR397a	miR168	miR399e	miR395b	miR161	miR171
Control (water)		Leaf	-	+	+	-	-	++	++
	3hr	Leaf	+	-	+	+	+	-	++
Salt (300mM NaCl)	6hr	Leaf	++	-	++	++	++	-	+++
	12hr	Leaf	+++	-	+++	+++	+++	-	+++
	24hr	Leaf	+++	-	++++	+++	++++	-	+++
Control (water)		Root	-	+	-	-	-	-	++
	3hr	Root	+	-	-	+	+	-	+++
Salt (300mM NaCl)	6hr	Root	++	-	-	++	++	-	+++
	12hr	Root	+++	-	-	+++	+++	-	+++
	24hr	Root	+++	-	-	+++	++++	-	+++

Scale:

-	Absent
+	Very low (0-25%)
++	Moderate (25-50%)
+++	High (50-75%)
++++	Very high (>75%)

The occurrence of specific amplification peaks and an absence of primer-dimer formation clear from the dissociation curve analysis (Plate 19a and 19b).

The technical precision or reproducibility of real-time PCR was assessed by performing replicate measurements in separate PCR runs using the same pool of cDNA (technical replicates) and two different pools of cDNA obtained independent two different batches of total RNA samples under same conditions (biological replicates). It was found that precision, as reflected by the correlation coefficient (r) was 0.98 for technical replicate and 0.96 for biological replicates. Fig 1a and 1b represents the correlation coefficient between duplicate measurements of cDNA levels from same and different pool of total RNA harvested from same treatment.

4.5.4 Expression profiling of miRNAs in leaf and root tissues of *Arabidopsis thaliana* whose shoot part was exposed to low temperature (4±1 °C) stress

Among four selected miRNAs accumulation of two miRNAs (miR171 and miR397a) were recorded in both leaf and root tissues at 1, 2 and 3 hr durations of exposure of *Arabidopsis thaliana* exposed to low temperature (4± 1 °C). The gradual increase in the expression of these miRNAs with increase in duration of exposure to low temperature was noticed with the highest of 4.28 and 3.49 fold changes in miR171 and miR397, respectively. There was a down regulation of expression of miR399e/f with its lowest 0.6 fold change in both leaf and root tissues at 1 and 2 hr durations of exposure. Interestingly the same set of miRNAs reported up regulation of miR399e/f with 1.06 fold change in leaf tissues but not in root tissues at 3 hr duration of exposure (Table 6, 7 and Fig. 2, 3).

4.5.5 Expression levels of selected miRNAs in leaf and root tissues of *Arabidopsis thaliana* in response to NaCl (300 mM) treatments

There was an up/down regulation of selected miRNAs in both leaf and root tissues as a response to NaCl (300 mM) treatment in *Arabidopsis thaliana* plant. Among the selected miRNAs, miR171 showed highest expression level of upregulation of 6.5 and 6.2 folds in leaf and root tissues, respectively. The miR397 reported their lowest expression level of 0.8 and 0.9 in both leaf and root tissues, respectively. The expression of miR399e/f was noticed elevated with increase in duration of exposure with the maximum of 3.4 and 3.5 fold up regulation in leaf and root tissues, respectively at 24 hr durations of exposure (Table 8, 9 and Fig. 4, 5).

4.5.6 Expression pattern of selected miRNA cognate genes

Based on the information available on miRNAs and their cognate genes, a set of nine miRNA cognate genes *viz*., *UBC24, LAC2, LAC17, AGO1, APS1, APS4, PPR, SCL6-III* and *SCL6-IV* were selected for functional analysis of selected miRNAs in response to low temperature and NaCl (300 mM) treatments in leaf and root tissues of *Arabidopsis thaliana*.

The expression patterns of each selected miRNA cognate genes were analyzed separately in low temperature (4 ± 1 °C) and NaCl (300 mM) treated leaf and root tissues of *Arabidopsis thaliana* plant. The up and down regulation of selected miRNA cognate genes in both leaf and root tissues upon low temperature and NaCl (300 mM) treatments were recorded in real time RT-PCR quantification.

4.5.6.1 Expression level of selected miRNA cognate genes in *Arabidopsis thaliana* leaf and root tissues in response to low temperature (4±1 °C)

A set of nine miRNA cognate genes were analyzed for their expression levels in leaf and root tissues of *Arabidopsis thaliana* plants whose shoot part was exposed to low temperature (4±1 °C) with different durations regimes of 1, 2 and 3 hr.

One and two hour exposure of *Arabidopsis thaliana* shoot to low temperature resulted in up regulation of five miRNA cognate genes such as *UBC24, AGO1, APS1*and *APS4,* and *PPR* which are predicted to be targets of miR399, miR168, miR395b and miR161 respectively in both leaf and root tissues. However, the up regulation of four miRNA cognate genes such as *AGO1, APS1*and *APS4,* and *PPR* was recorded in leaf tissues exposed for at 3 hr low temperature. The down regulation of four miRNA cognate genes such as *LAC2* and *LAC17*, and *SCL6-III* and *SCL6-IV* which are predicted to be targets of miR397a and miR171, respectively were noticed in both leaf and root tissues commonly at 1, 2 and 3 hr duration of exposure. The down regulation of *UBC24* which is predicted target of miR399 was recorded in leaf tissues but not in root tissues of 3 hr duration of exposure plant (Table 10, 11 and Fig. 6, 7).

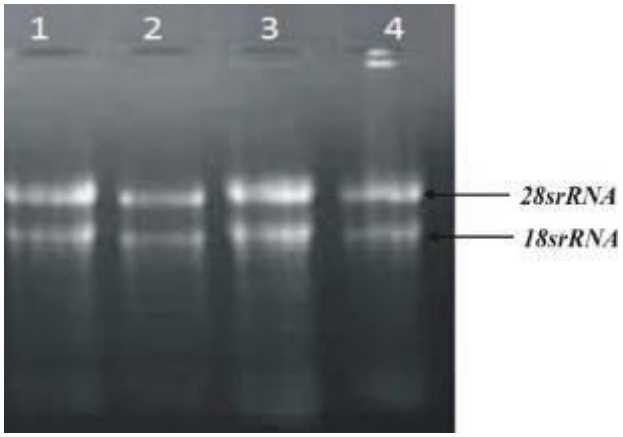


Plate 15: Confirmation of integrity and quality of total RNA on formaldehyde Denaturing agarose gel

Plate 16: PCR confirmation of target genes with total RNA for complete elimination of genomic DNA upon DNase I treatment

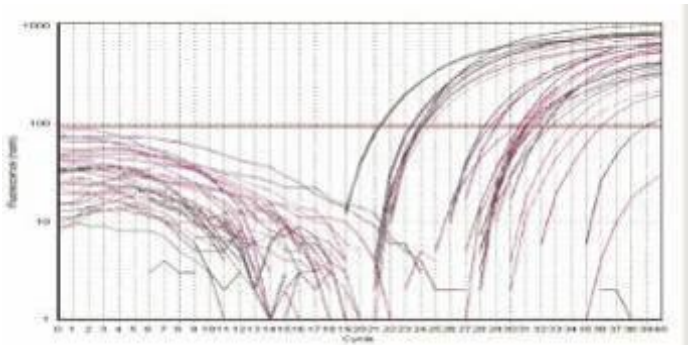


Plate 17a: Real time RT PCR amplification plots of selected miRNA showing increase in SYBR green flurescence with PCR cycle number

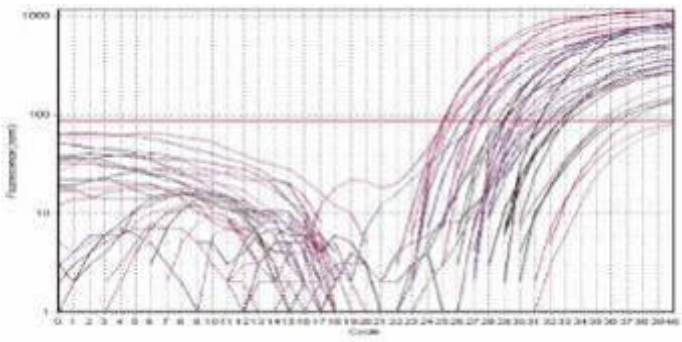


Plate 17b: Real time RT PCR amplification plots of miRNA cognate genes showing increase in SYBR green fluorescence with PCR cycle number

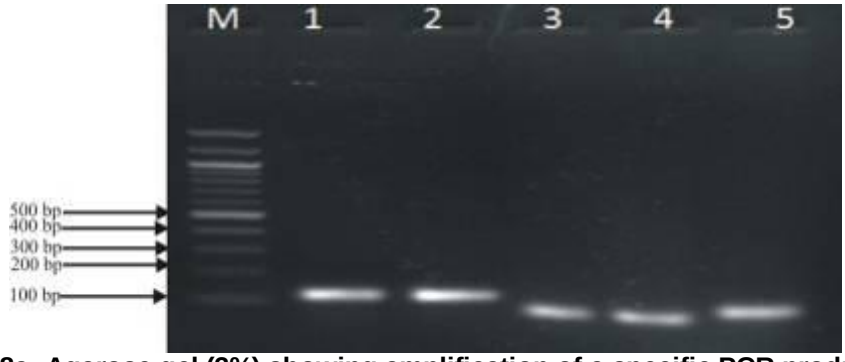


Plate 18a: Agarose gel (3%) showing amplification of a specific PCR product of expected Size for each miRNA cognate gene tested in the study

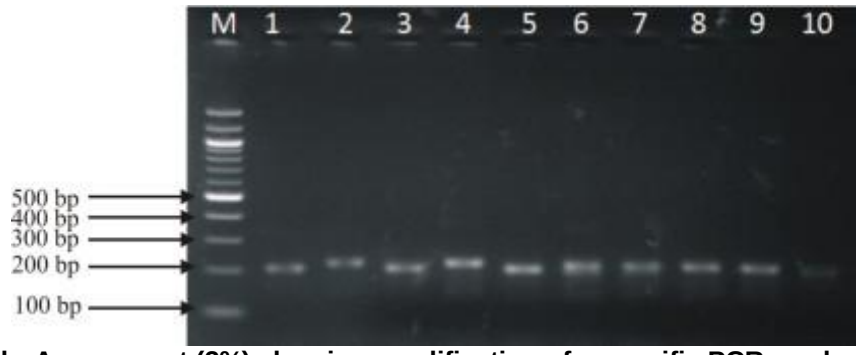


Plate 18b: Agarose get (3%) showing amplification of a specific PCR product of expected size For each miRNA cognate gene tested in the study

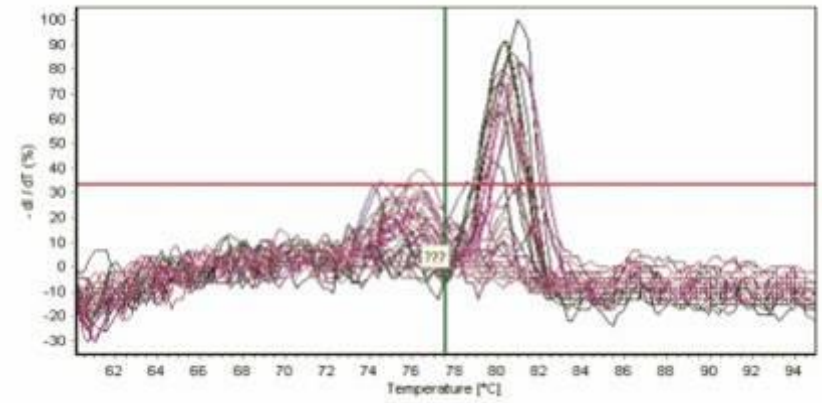


Plate 19a: Melting curve analyses of selected miRNAs confirming the occurrence of specific Amplification peaks and an absence of prime-dimer formation

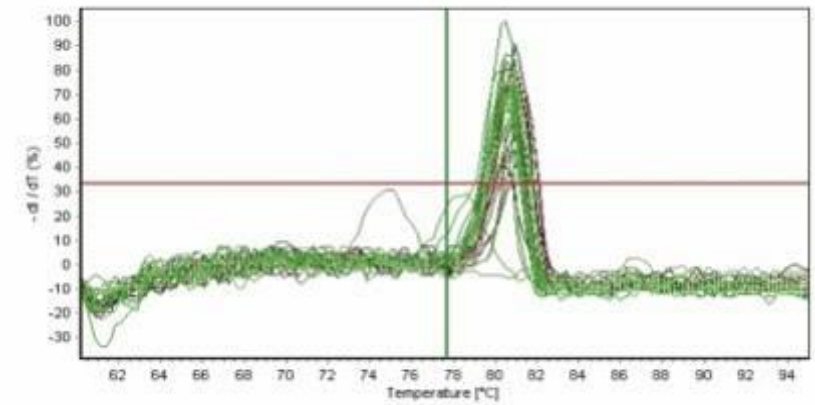


Plate 19b: Melting curve analyses of miRNA cognate confirming the occurrence of specific Amplification peaks and an absence of primer-dimer formation

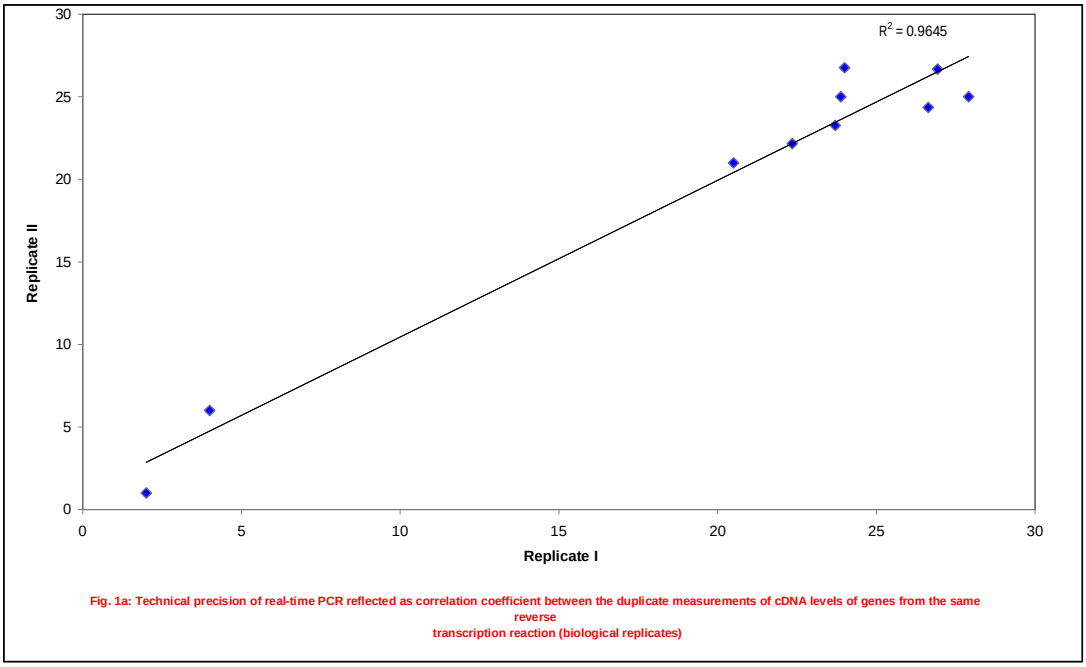


Fig. 1a: Technical precision of real-time PCR reflected as correlation coefficient between the duplicate measurements of cDNA levels of genes from the same reverse
transcription reaction (biological replicates)

Fig. 1a: Technical precision of ral-time PCR reflected as correlation coefficient between the duplicate measurements of cDNA levels of genes from the separate reverse transcription reaction (technical replicates)

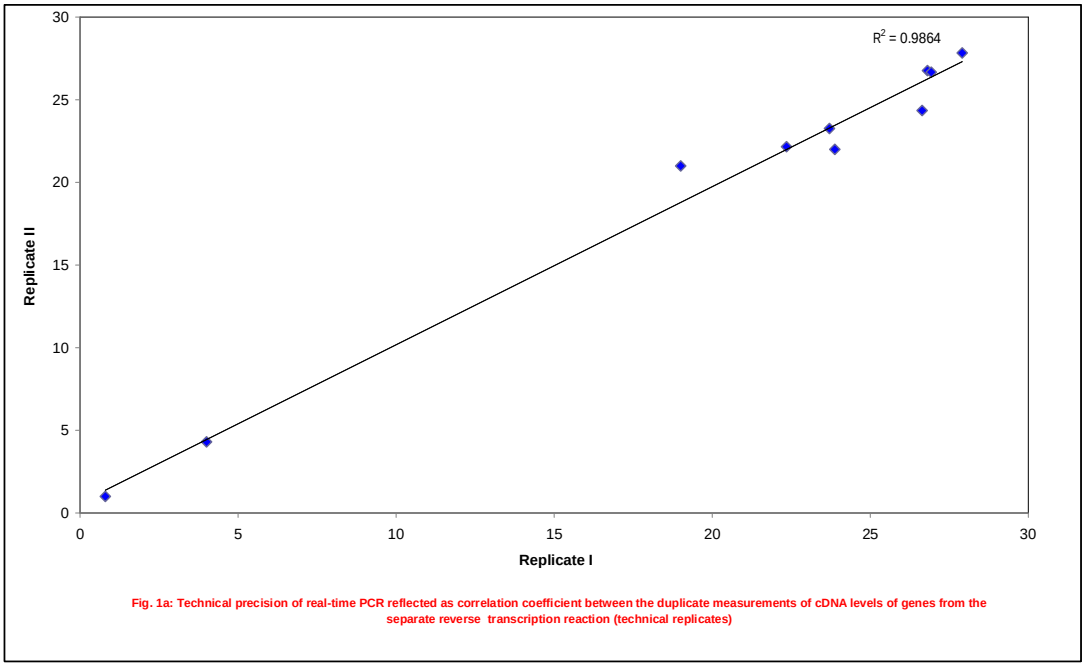


Fig. 1a: Technical precision of real-time PCR reflected as correlation coefficient between the duplicate measurements of cDNA levels of genes from the separate reverse transcription reaction (technical replicates)

Fig. 1a: Technical precision of real-time PCR reflected as correlation coefficient between the duplicate measurements of cDNA levels of genes from the same reverse transcription reaction (biological replicates)

Table 6: Relative change in the expression pattern of selected miRNAs in 1, 2 and 3 hr low temperature (4±1 °C) stressed leaf tissues compared to ambient temperature controls (25 ±1 °C) in *Arabidopsis thaliana*

Sl. No.	Selected miRNAs			Normalized Ct values		Fold expression				Calculated t value	
		CntL_ΔCt	CS1L_ΔCt	CS2L_ΔCt	CS3L_ΔCt	CS1L	CS2L	CS3L	CS1L	CS2L	CS3L
1	miR171	9.405	8.47	7.5	7.305	1.911	3.7321	4.287	0.3553	0.2678	0.1374
2	miR397a	10.79	6.88	6.35	5.985	1.872	2.703	3.4942	0.2861	0.2561	0.0793
3	miR399e/f	3.71	3.89	4.4	3.625	0.879	0.619	1.0606	0.1611	0.8670	0.0489

- CntL_ΔCt: Normalized Ct value of control leaf
- CS3L_ΔCt: Normalized Ct value of 3hr salt stressed leaf
- CS6L_ΔCt: Normalized Ct value of 6hr cold stressed leaf
- CS12L_ΔCt: Normalized Ct value of 12hr cold stressed leaf
- ΔCt: (Ct of target gene-Ct of reference gene)

Table 7: Relative change in the expression pattern of selected miRNAs in ambient root tissues of *Arabidopsis thaliana* exposure to shoot low temperature (4±1 °C) regimes

Sl. No.	Selected miRNAs			Normalized Ct values		Fold expression			Calculated t value		
		CntR_∆Ct	CS1R_∆Ct	CS2R_∆Ct	CS3R_∆Ct	CS1R	CS2R	CS3R	CS1R	CS2R	CS3R
1	miR171	7.98	7.11	6.525	6.21	1.821	2.741	3.410	0.547	0.438993	0.446
2	miR397a	8.47	7.535	7.35	6.59	1.911	2.173	3.680	0.095	0.0486	0.193
3	miR399e/f	8.73	9.105	9.375	9.46	0.771	0.639	0.602	0.025	0.175739	0.329

- CntL_∆Ct: Normalized Ct value of control leaf
- CS3L_∆Ct: Normalized Ct value of 3hr salt stressed leaf
- CS6L_∆Ct: Normalized Ct value of 6hr cold stressed leaf
- CS12L_∆Ct: Normalized Ct value of 12hr cold stressed leaf
- ∆Ct: (Ct of target gene-Ct of reference gene)

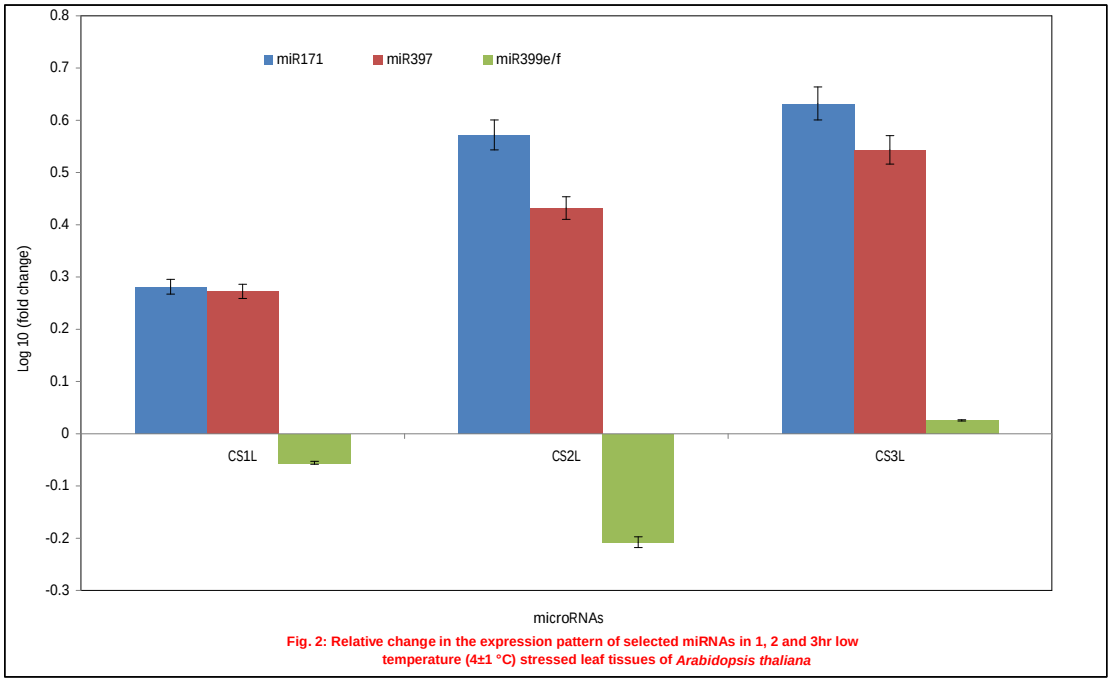


Fig. 2: Relative change in the expression pattern of selected miRNAs in 1, 2 and 3hr low temperature (4±1 °C) stressed leaf tissues of *Arabidopsis thaliana*

Fig. 2: Relative change in the expression pattern of selected miRNAs in 1, 2 and 3hr low temperature (4±1 °C)

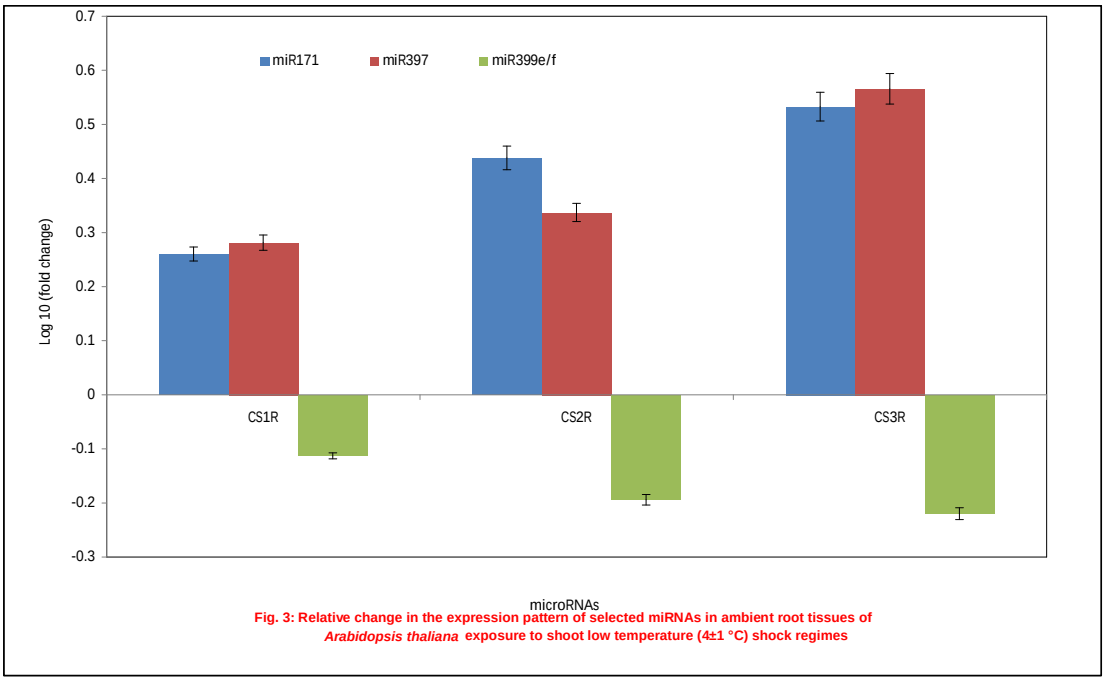


Fig. 3: Relative change in the expression pattern of selected miRNAs in ambient root tissues of *Arabidopsis thaliana* exposure to shoot low temperature (4±1 °C) shock regimes

Fig. 3: Relative change in the expression pattern of selected miRNAs in ambient

Table 8: Relative change in the expression pattern of selected miRNAs in normal watered control, 3, 6, 12 and 24 hr 300 mM NaCl treated leaf tissues of *Arabidopsis thaliana*

Sl. No.	miRNAs			Normalized Ct values			Fold expression				Calculated t value			
		CntL_ΔCt	SS3L_ΔCt	SS6L_ΔCt	SS12L_ΔCt	SS24L_ΔCt	SS3L	SS6L	SS12L	SS24L	SS3L	SS6L	SS12L	SS24L
1	ath-miR171	9.405	9.05	8.525	8.465	8.07	3.340	4.807	5.011	6.589	0.099	0.585	0.595	0.079
2	ath-miR397	10.79	9.415	9.535	9.555	9.57	0.993	0.914	0.901	0.892	0.210	0.801	0.514	0.029
3	ath-miR399e/f	3.71	2.745	2.565	2.025	1.94	1.952	2.211	3.215	3.411	0.131	0.912	0.903	0.274

- CntL_ΔCt: Normalized Ct value of control leaf
- CS3L_ΔCt: Normalized Ct value of 3hr salt stressed leaf
- CS6L_ΔCt: Normalized Ct value of 6hr cold stressed leaf
- CS12L_ΔCt: Normalized Ct value of 12hr cold stressed leaf
- CS24L_ΔCt: Normalized Ct value of 24hr salt stressed leaf
- ΔCt: (Ct of target gene-Ct of reference gene)

Table 9: Relative change in the expression pattern of selected miRNAs in normal watered control, 3, 6, 12 and 24 hr 300 mM NaCl treated root tissues of *Arabidopsis thaliana*.

Sl. No.	miRNAs			Normalized Ct values				Fold expression				Calculated t value		
		CntR_ΔCt	SS3R_ΔCt	SS6R_ΔCt	SS12R_ΔCt	SS24R_ΔCt	SS3R	SS6R	SS12R	SS24R	SS3R	SS6R	SS12R	SS24R
1	ath-miR171	8.98	7.305	6.775	7.1	6.82	4.485	6.476	5.169	6.277	0.031	0.839	0.027	0.455
2	ath-miR397	9.47	9.065	9.06	9.1	9.09	0.943	0.946	0.920	0.927	0.077	0.511	0.015	0.144
3	ath-miR399e/f	8.15	7.245	6.84	6.3	6.34	1.873	2.479	3.605	3.506	0.458	0.186	0.019	0.492

- CntL_ΔCt: Normalized Ct value of control leaf
- CS3L_ΔCt: Normalized Ct value of 3hr salt stressed leaf
- CS6L_ΔCt: Normalized Ct value of 6hr cold stressed leaf
- CS12L_ΔCt: Normalized Ct value of 12hr cold stressed leaf
- CS24L_ΔCt: Normalized Ct value of 24hr salt stressed leaf
- ΔCt: (Ct of target gene-Ct of reference gene)

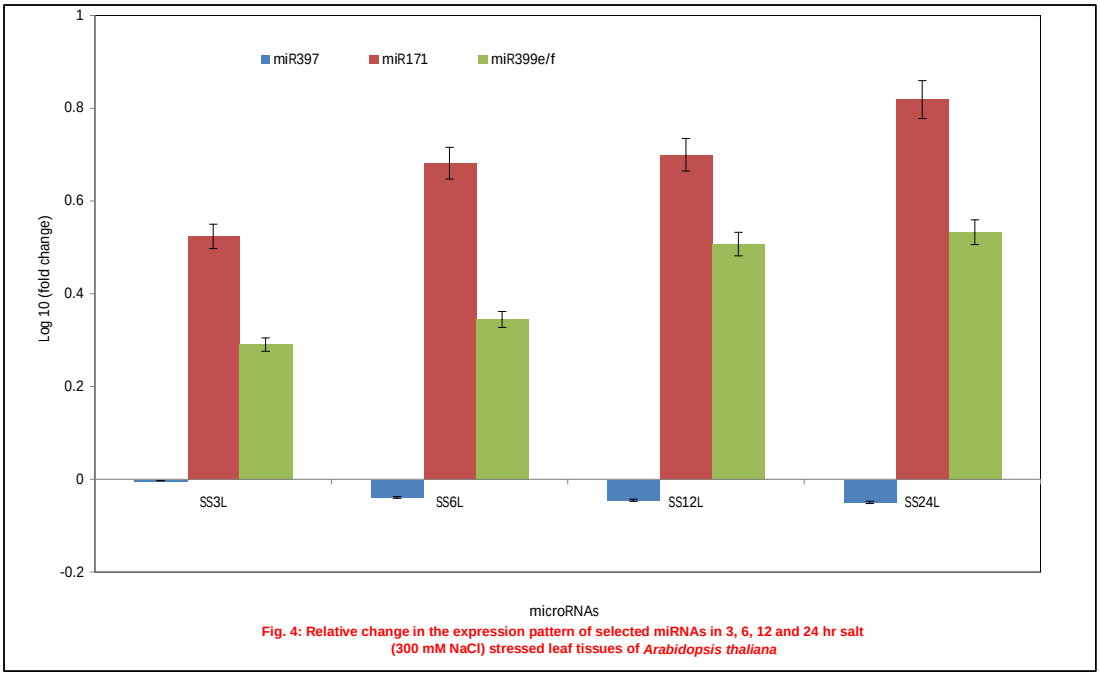


Fig. 4: Relative change in the expression pattern of selected miRNAs in 3, 6, 12 and 24 hr salt (300 mM NaCl) stressed leaf tissues of *Arabidopsis thaliana*

Fig. 4: Relative change in the expression pattern of selected miRNAs in 3, 6, 12 and 24 hr salf (300 mM NaCl) stressed leaf tissues of Arabidopsis thaliana

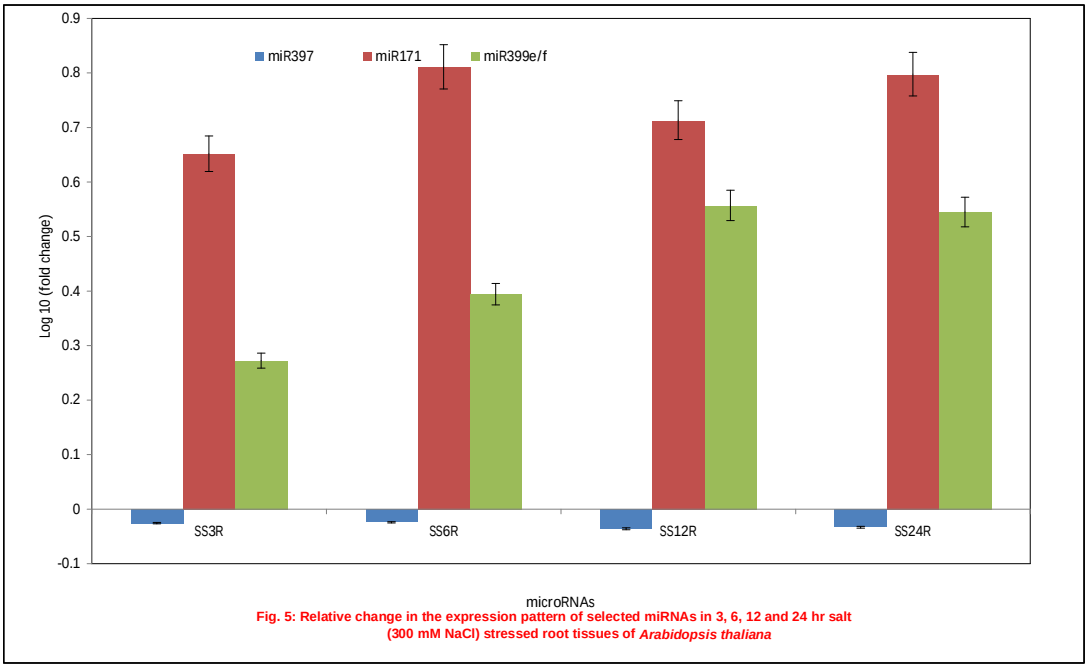


Fig. 5: Relative change in the expression pattern of selected miRNAs in 3, 6, 12 and 24 hr salt (300 mM NaCl) stressed root tissues of *Arabidopsis thaliana*

Fig. 5: Relative change in the expression pattern of selected miRNAs in 3, 6, 12 and 24 hr Salf (300 mM NaCl) stressed root tissues of Arabidopsis thaliana

4.5.6.2 Expression levels of selected miRNA cognate genes in leaf and root tissues of *Arabidopsis thaliana* in response to NaCl (300 mM) treatments

The expression levels of nine miRNA cognate genes were analyzed in leaf and root tissues of *Arabidopsis thaliana* plants upon NaCl (300 mM) treatments with different durations (3, 6, 12 and 24 hr) of exposure.

Expression levels of selected miRNA cognate genes were compared in NaCl (300 mM) treated leaf and root tissues with respective normal watered controls of *Arabidopsis thaliana*. The up regulation of three miRNA cognate genes such as *LAC2* and *LAC17*, and PPR which are predicted targets of miR397 and miR161 respectively was recorded in leaf and root tissues. A set of six miRNA cognate genes such as UBC24 (predicted target of miR399), *APS1* and *APS4* (predicted target of miR395b) and *SCL6-III* and *SCL6-IV* (predicted target of miR171) were found to be down regulated in both leaf and root tissues *Arabidopsis thaliana* plants exposed for different durations (3, 6, 12 and 24 hr) of NaCl treatment. However, the up regulation of AGO1 (predicted target of miR168) was noticed in root tissues whereas, its down regulation was recorded in leaf tissues (Table 12, 13 and Fig. 8, 9). It was noticed that there was no expressional changes for rest of miRNA cognate genes as revealed by real time transcript analysis which was obvious well.

Table 10: Relative change in the expression pattern of selected miRNA cognate genes in 1, 2 and 3 hr low temperature (4±1 °C) stressed leaf tissues compared to ambient temperature controls (25 ±1 °C) in *Arabidopsis thaliana*

Sl. No.	Cognate genes		Normalized Ct values				Fold expression			Calculated .t value		
	Gene ID	Gene name	CntL_ΔCt	CS1L_ΔCt	CS2L_ΔCt	CS3L_ΔCt	CS1L	CS2L	CS3L	CS1L	CS2L	CS3L
1	NM_179887.2	UBC24	4.05	3.884	4.185	4.397	1.122	0.911	0.786	0.235	0.202	0.266
2	NM_128470.4	LAC2	4.14	4.205	4.255	4.32	0.956	0.923	0.883	0.150	0.059	0.550
3	NM_125395.3	LAC17	6.29	6.41	6.45	6.49	0.920	0.895	0.871	0.347	0.598	0.256
4	NM_001198240.1	AGO1	6.555	5.545	5.355	5.28	2.014	2.297	2.420	0.187	0.261	0.152
5	NM_128651.4	ASP1	12.005	10.785	10.745	10.735	2.329	2.395	2.412	0.279	0.133	0.919
6	NM_179507.1	ASP4	5.965	4.665	4.715	4.755	2.462	2.378	2.313	0.136	0.082	0.219
7	NM_117745.3	PPR	2.925	2.595	2.695	2.589	1.257	1.173	1.262	0.155	0.003	0.455
8	EU550749.1	SCL6-III	3.145	3.295	3.765	4.085	0.901	0.651	0.521	0.234	0.163	0.345
9	EU550777.1	SCL6-IV	10.18	10.205	10.22	10.27	0.983	0.973	0.940	0.396	0.308	0.498

- CntL_ΔCt: Normalized Ct value of control leaf
- CS1L_ ΔCt: Normalized Ct value of 1hr cold stressed leaf
- CS2L_ ΔCt: Normalized Ct value of 2hr cold stressed leaf
- CS3L_ ΔCt: Normalized Ct value of 3hr cold stressed leaf
- ΔCt: (Ct of target gene-Ct of reference gene)

Table 11: Relative change in the expression pattern of selected miRNA cognate genes in root tissues of *Arabidopsis thaliana* exposure to shoot low temperature (4±1 °C) regimes

Sl. No.	Cognate genes		Normalized Ct values				Fold expression				Calculated t value	
	Gene ID	Gene name	CntL_ΔCt	CS1L_ΔCt	CS2L_ΔCt	CS3L_ΔCt	CS1R	CS2R	CS3R	CS1R	CS2R	CS3R
1	NM_179887.2	UBC24	4.02	3.72	3.53	3.52	1.24	1.40	1.41	0.13	0.43	0.48
2	NM_128470.4	LAC2	4.04	5.66	5.89	6.49	0.33	0.28	0.18	0.37	0.49	0.61
3	NM_125395.3	LAC17	6.26	6.31	6.36	6.63	0.97	0.93	0.78	0.42	0.48	0.53
4	NM_001198240.1	AGO1	4.955	4.81	4.75	4.69	1.11	1.15	1.20	0.19	0.94	0.30
5	NM_128651.4	ASP1	7.125	5.71	5.71	5.82	2.68	2.68	2.47	0.66	0.46	0.27
6	NM_179507.1	ASP4	4.935	4.93	4.62	4.32	1.01	1.24	1.53	0.82	0.72	0.51
7	NM_117745.3	PPR	2.75	1.73	1.71	0.95	2.03	2.06	3.48	0.17	0.41	0.92
8	EU550749.1	SCL6-III	2.815	2.92	4.00	4.57	0.93	0.44	0.30	0.38	0.92	0.46
9	EU550777.1	SCL6-IV	8.985	9.09	9.29	9.79	0.93	0.81	0.57	0.54	0.47	0.44

- CntL_ΔCt: Normalized Ct value of control leaf
- CS1L_ ΔCt: Normalized Ct value of 1hr cold stressed leaf
- CS2L_ ΔCt: Normalized Ct value of 2hr cold stressed leaf
- CS3L_ ΔCt: Normalized Ct value of 3hr cold stressed leaf
- ΔCt: (Ct of target gene-Ct of reference gene)

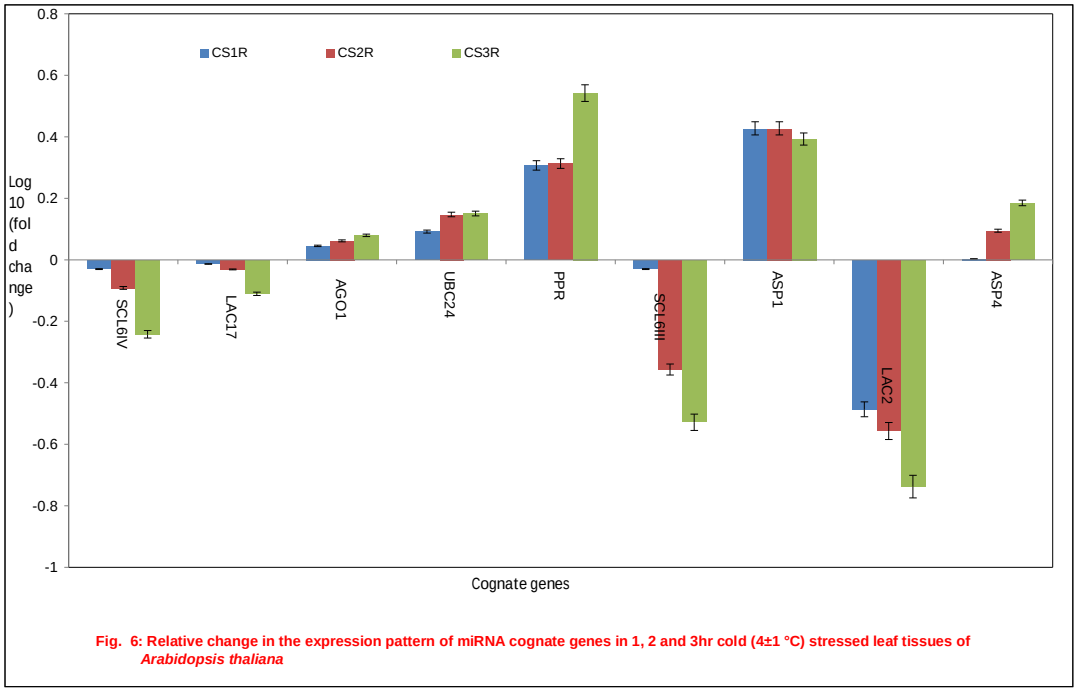


Fig. 6: Relative change in the expression pattern of miRNA cognate genes in 1, 2 and 3hr cold (4±1 °C) stressed leaf tissues of *Arabidopsis thaliana*

Fig. 6: Relative change in the expression pattern of miRNA cognate genes in 1, 2 and 3 hr cold (4±1 °C) stressed leaf tissues of Arabidopsis thelians

Fig. 7: Relative change in the expression pattern of miRNA cognate genes in root tissues of *Arabidopsis thaliana* exposure to shoot cold (4±1 °C) shock regimes

Fig. 7: Relative change in the expression pattern of miRNA cognate genes in root tissues of Arabidopsis thaliana exposure to shoot cold **(4±1 °C) shock regimes**

Table 12: Relative change in the expression pattern of selected miRNA cognate genes in normal watered control, 3, 6, 12 and 24 hr 300 mM NaCl treated leaf tissues of *Arabidopsis thaliana*

Sl. No.	Cognate genes				Normalized Ct values			Fold expression					Calculated t value		
	Gene ID	Gene name	CntL_ΔCt	SS3L_ΔCt	SS6L_ΔCt	SS12L_ΔCt	SS24L_ΔCt	SS3L	SS6L	SS12L	SS24L	SS3L	SS6L	SS12L	SS24L
1	NM_179887.2	UBC24	4.15	4.66	4.67	4.695	4.8	0.7022	0.6974	0.6854	0.6373	0.0129	0.4994	0.4872	0.0182
2	NM_128470.4	LAC2	4.14	3.265	3.285	3.245	3.11	1.834	1.8088	1.8596	2.042	0.506	0.4344	0.4079	0.4461
3	NM_125395.3	LAC17	6.09	5.89	5.7	5.69	5.45	1.1487	1.3104	1.3195	1.5583	0.4291	0.9812	0.3379	0.5024
4	NM_001198240.1	AGO1	5.81	10.605	11.455	12.35	13.05	0.04	0.039	0.029	0.0266	0.0962	0.574	0.325	0.7501
5	NM_128651.4	ASP1	10.505	10.595	10.675	10.955	10.96	0.9395	0.8888	0.732	0.7295	0.0166	0.5128	0.494	0.0421
6	NM_179507.1	ASP4	4.91	6.045	6.015	5.965	6.42	0.4553	0.4649	0.4813	0.3511	0.1886	0.6788	0.3285	0.0392
7	NM_117745.3	PPR	2.025	0.875	0.835	0.645	0.5	2.2191	2.2815	2.6027	2.8779	0.3851	0.4857	0.4606	0.3291
8	EU550749.1	SCL6-III	2.81	3.435	4.305	4.225	4.27	0.6484	0.3548	0.375	0.3635	0.3941	0.4805	0.475	0.1293
9	EU550777.1	SCL6-IV	10.06	11.38	11.25	11.34	11.38	0.4005	0.4383	0.4118	0.4005	0.4517	0.5835	0.58	0.5357

- CntL_ΔCt: Normalized Ct value of control leaf
- CS3L_ΔCt: Normalized Ct value of 3hr salt stressed leaf
- CS6L_ΔCt: Normalized Ct value of 6hr cold stressed leaf
- CS12L_ΔCt: Normalized Ct value of 12hr cold stressed leaf
- CS24L_ΔCt: Normalized Ct value of 24hr salt stressed leaf
- ΔCt: (Ct of target gene-Ct of reference gene)

Table 13: Relative change in the expression pattern of selected miRNA cognate genes in normal watered control, 3, 6, 12 and 24 hr 300 mM NaCl stressed root tissues of *Arabidopsis thaliana*

Sl. No.	Cognate genes				Normalized Ct values				Fold expression				Calculated t value			
	Gene ID	Gene name	CntR_ΔCt	SS3R_ΔCt	SS6R_ΔCt	SS12R_ΔCt	SS24R_ΔCt	SS3R	SS6R	SS12R	SS24R	SS3R	SS6R	SS12R	SS24R
1	NM_179887.2	UBC24	2.86	3.045	3.43	3.53	3.93	0.88	0.674	0.629	0.476	0.013	0.263	0.468	0.753
2	NM_128470.4	LAC2	4.2	2.35	2.48	2.29	2.37	3.605	3.294	3.758	3.555	0.452	0.714	0.528	0.010
3	NM_125395.3	LAC17	6.04	3.955	3.76	3.46	3.49	4.243	4.857	5.979	5.856	0.078	0.855	0.685	0.020
4	NM_001198240.1	AGO1	5.1	1.885	1.45	0.755	0.055	4.64	6.27	8.25	12.38	0.013	0.199	0.155	0.015
5	NM_128651.4	ASP1	7.03	7.215	7.29	7.116	7.12	0.88	0.835	0.942	0.940	0.544	0.240	0.440	0.740
6	NM_179507.1	ASP4	5	5.065	5.14	5.43	5.985	0.956	0.908	0.742	0.505	0.048	0.884	0.603	0.029
7	NM_117745.3	PPR	2.8	2.555	2.48	2.28	2.43	1.185	1.248	1.434	1.292	0.027	0.968	0.949	0.026
8	EU550749.1	SCL6-III	2.575	2.805	2.82	2.805	2.835	0.853	0.844	0.853	0.835	0.019	0.618	0.343	0.051
9	EU550777.1	SCL6-IV	8.2	9.105	8.99	9.075	9.065	0.534	0.578	0.545	0.549	0.096	0.964	0.076	0.009

- CntL_ΔCt: Normalized Ct value of control leaf
- CS3L_ΔCt: Normalized Ct value of 3hr salt stressed leaf
- CS6L_ΔCt: Normalized Ct value of 6hr cold stressed leaf
- CS12L_ΔCt: Normalized Ct value of 12hr cold stressed leaf
- CS24L_ΔCt: Normalized Ct value of 24hr salt stressed leaf
- ΔCt: (Ct of target gene-Ct of reference gene)

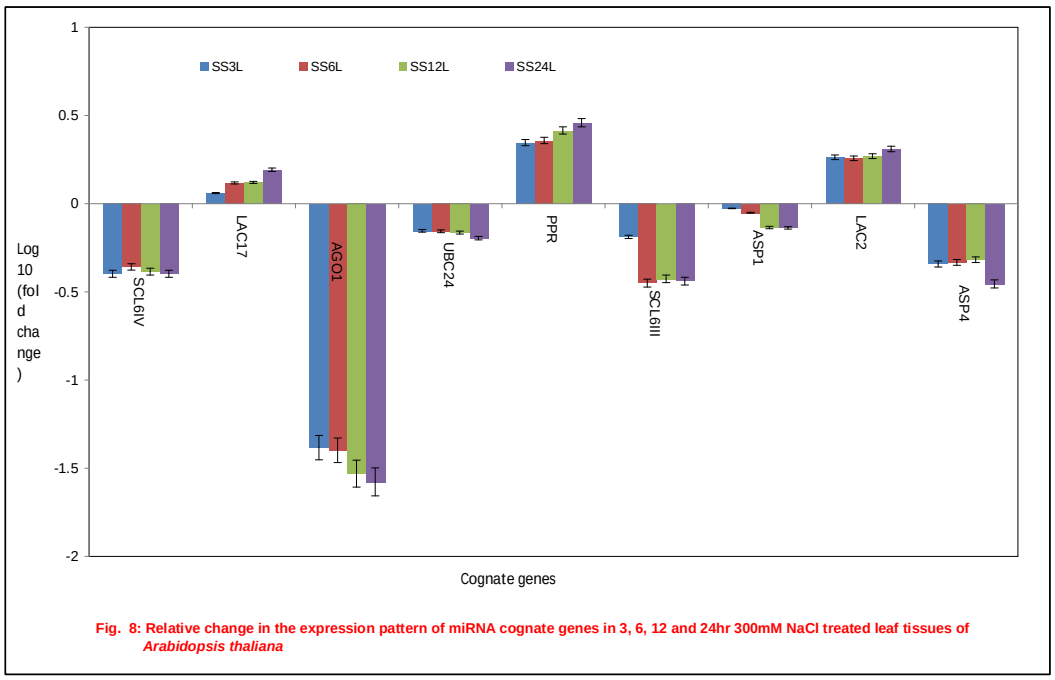


Fig. 8: Relative change in the expression pattern of miRNA cognate genes in 3, 6, 12 and 24hr 300mM NaCl treated leaf tissues of *Arabidopsis thaliana*

Fig. 8: Relative change in the expression pattern of miRNA cognate genes in 3, 6, 12 and 24 hr 300mM NaCl treated leaf tissues of Arabidopsis thaliana

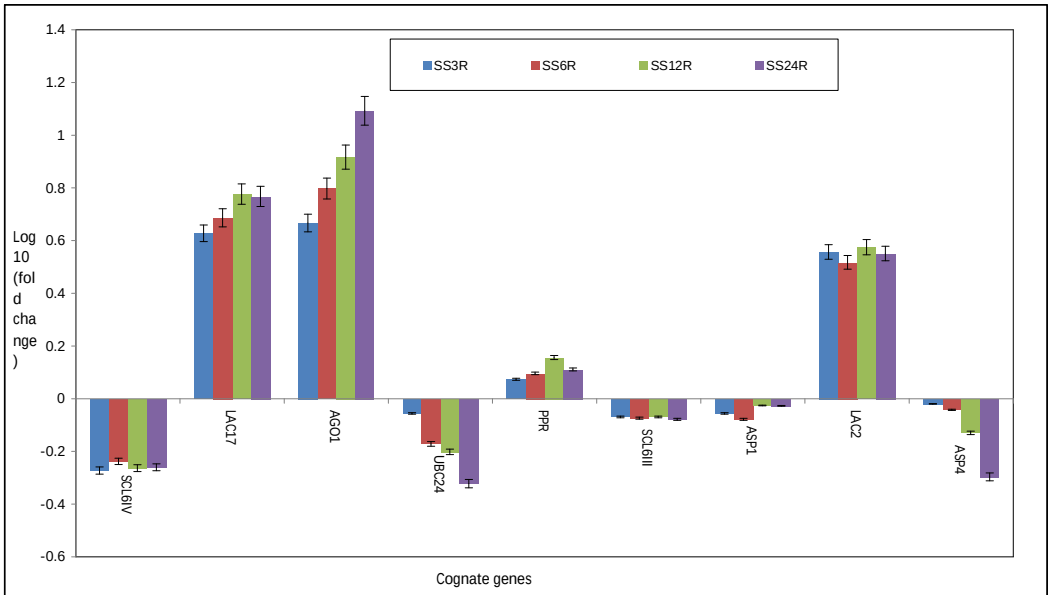


Fig. 9: Relative change in the expression pattern of miRNA cognate genes in 3, 6, 12 and 24hr 300mM NaCl treated root tissues of *Arabidopsis thaliana*

Fig. 9: Relative change in the expression pattern of miRNA cognate genes in 3, 6, 12 and 24 hr 300mM NaCl treated root tissues of Arabidopsis thaliana

DISCUSSION

Plants are exposed to serious biotic and abiotic stresses such as drought, salinity, alkalinity, cold, pathogen infections and diseases, which are the predominant cause of decreased crop yields. Plants use adaptive responses operating at the transcriptional, post-transcriptional, translational and post-translational levels to cope with these environmental challenges. To cope with these stresses, plants respond by reprogramming gene expression, which results in osmolyte accumulation for osmotic adjustment, up-regulation of antioxidant pathways for reactive oxygen species (ROS) homeostasis, minimizing, as well as repairing the damage caused to the cellular constituents including DNA, proteins and membranes, and maintaining processes that sustains cellular homeostasis under stress. As a post-transcriptional gene regulator, a number of miRNAs play roles in multiple stress responses in plants. The roles of microRNAs in plants are quite diverse and involves in plant development, nutrient homeostasis and stress responses. Recent evidences showed that there is a direct link between miRNA regulation and stress response in plants.

Regulation of gene expression is one of the essential mechanisms, which operate in all living organism. There are two major mechanisms *viz*., target mRNA cleavage and translational repression through which miRNAs regulate their target gene expression. In plants, most of the miRNAs have significant complementarity to their target genes and regulate their targets by directing mRNA cleavage at single sites in the coding regions. The miRNA represent a relatively abundant class of transcripts and their expression levels vary greatly among different cells and tissues. Less abundant miRNAs routinely escape detection with technologies such as cloning, northern hybridization and microarray analysis. Further, the specificity of detection of such microRNAs was reported to be very low if normal DNA and RNA probes are used (Valoczi *et al.*, 2006). Hence, to avoid the sensitivity problem of miRNA ISH technology locked nucleic acid (LNA)-modified oligonucleotide probes were used to enhance the efficiency of hybridization. LNA probes found very specific to their complementary targets due to their locked furanose ring; the puckered structure is restricted in 3′ endo-conformation. This technique has pinned down the specific role of microRNAs in various gene regulatory networks in a far more precise manner than before through *in situ* hybridization.

The miRNA and target mRNA expression level measurement presents valuable information about the miRNAs functions. For a true target of a specific miRNA, the modulation of miRNA concentration should correspond to a predictable change in the amount of protein encoded by the target mRNA (Ding *et al*., 2012). The downstream effects of differential miRNAs have been identified at the mRNA level by qRT-PCR. Sensitive qRT-PCR techniques are available for sensitive and specific detection of miRNAs. Stem loop RT-PCR method is one of the novel methods which detect and quantify mature miRNAs in a fast, specific, accurate and reliable manner (Chen *et al*., 2005). In the present study, seven microRNAs, miR399, miR397a, miR168, miR399e, miR395b, miR161 and miR171 were selected for their expression assessment in shoot (leaf) and root tissues of *Arabidopsis thaliana* plants experiencing low temperature and NaCl (300 mM) stress. The miRNA probes were LNA modified and used in the *in situ* hybridization, the real time stem-loop quantitation approach was used for the detection of mature miRNAs transcripts and real time expression levels of selected miRNA cognate genes were analyzed using RT-PCR.

5.1 Expression pattern of selected miRNAs in leaf and root tissues of *Arabidopsis thaliana* plants in response to shoot low temperature (4±1 °C) stress

Each plant has its unique set of temperature requirements, which are optimum for its proper growth and development. Any deviations in the optimum temperature have adverse effects on plant growth and development. Cold stress, which includes chilling (<20 °C) and/or freezing (< 8 °C) temperatures, adversely affects the growth and development of plants and significantly constraints the spatial distribution of plants and agricultural productivity. Cold stress prevents the expression of full genetic potential of plants owing to its direct inhibition of metabolic reactions and indirectly, through cold-induced osmotic (chilling-induced inhibition of water uptake and freezing-induced cellular dehydration), oxidative and other stresses. Plant cells can sense cold stress through low temperature-induced changes in membrane fluidity, protein and nucleic acid conformation and/or metabolite concentration. Cold acclimation involves the remodeling of cell and tissue structures and the reprogramming of metabolism and gene expression. Characterization of cold-regulated miRNAs helps to understand the role of post-transcriptional regulation of mRNA stability in cold stress response of plants.

In present study, four miRNAs *viz.,* miR171, miR168, miR161 and miR397a recorded their expression in leaf tissues, whereas the expression of three miRNAs *viz*., miR399, miR399e and miR395b was absent in both leaf and root tissues and also the expression of miR168 and miR161 was not detected in root tissues of *Arabidopsis thaliana* plants grown under ambient temperature conditions. The expression was observed in longitudinal sections (LS) of leaf and root tissues through *in situ* hybridization.

The *SCARECROW* like transcription factors are the potential targets of miR171. *SCARECROW* (*SCR*) is a member of the plant-specific *GRAS* gene family encoding putative transcription factors, and plays a significant role in the radial patterning of both roots and shoots and hormone signaling (Helariutta *et al.,* 2000 and Kamiya *et al*., 2003). In this study, to assess the role of miR171 in response to low temperature stress, tissue sections (leaf and root) representing various durations (1, 2 and 3 hr) of low temperature stress was hybridized with miR171 specific LNA modified oligonucleotide probes. Longitudinal sections of both leaf and root tissues revealed that accumulation of miR171 increased with increasing durations of exposure to stress. Further, this was confirmed by stem-loop real-time RT-PCR analysis, which demonstrated elevated up regulation of miR171 by 4.2 fold increase in miRNA transcript levels in leaf tissues whereas there was 3.8 fold increases in root tissues which were experiencing 3 hr durations of low temperature stress when compared with plants grown in ambient temperature conditions.

Previous studies demonstrated that three members of the *Arabidopsis* SCR-like putative transcription factors (SCL), *SCL6-II*, *SCL6-III* and *SCL6-IV* possess perfectly complementary target sites for miR171 (Llave *et al*., 2002; Reinhart *et al*., 2002). In *Arabidopsis thaliana*, Northern and qRT-PCR expression analysis revealed increased levels of miR171 target *SCL6-III* in miR171_3B decoy plants (Ivashuta *et al*., 2011). In present study, the real time quantification revealed increased down regulation of two SCR genes such as *SCL6-III* (0.5 fold) and *SCL6-IV* (0.9 fold) in both leaf and root tissues of *Arabidopsis thaliana* plants subjected at maximum 3 hr durations of low temperature stress when compared with control plants grown in ambient culture conditions. Zhou *et al*. (2008) reported up-regulation of miR171 in response to Cd stress by using qRT-PCR based assay. Our result strengthens the assumption of involvement of miR171 functioning not only as a developmental regulator but also in wide range of stress responsiveness in *Arabidopsis thaliana*.

The miR397 is a short RNA molecule and which is predicted to be target mRNAs coding for laccases. Laccases are multi copper containing glycoproteins and found to be widely distributed in higher plants including both monocots and dicots. In this study, the accumulation pattern of miR397 obtained by *in situ* hybridization revealed that up regulation of miR397 increased with increasing durations of low temperature stress in leaf and root tissues *Arabidopsis thaliana* plants. The expression was uniform all over the leaf, especially in vascular tissues, spongy tissues, palisade and cuticle. The elevated up regulation of miR397 of miRNA transcript levels by 3.4 fold increase in leaf tissues and 3.6 fold increase in root tissues when compared with plants grown in ambient temperature conditions was confirmed by using stem-loop real-time RT-PCR.

A bioinformatics analysis of the *Arabidopsis* genome and target validation analysis reported that miR397 family targets *LAC2, LAC4* and *LAC17* genes (Rhoades and Bartel, 2004). In the present study, the real time RT-PCR analysis revealed down regulation of two *LACCASE* genes such as *LAC2* and *LAC17* (0.8fold) in leaf tissues whereas *LAC2*(0.3 fold) and *LAC17* (0.42 fold) in root tissues when compared with control plants in response to low temperature. It was reported that copper-related microRNAs play systemic roles in plants to regulate the abundance of number of copper enzymes in response to fluctuations in copper availability (Salah *et al*., 2008). Quantitative real time PCR analysis revealed that the expression of *LAC2*, *LAC4* and *LAC17* were negatively correlated with the expression pattern of miR397 in response to copper limitation (Abdel-Ghany and Pilon, 2008).

The miR399 is a microRNA it has been identified in both *Arabidopsis thaliana* and *Oryza sativa* by using computational approaches. The mature sequence of miR399 is excised from the 3' arm of the hairpin. It has been also identified that each plant genome contains multiple copies of miR399. In the present study, the accumulation pattern of miR399 and miR399e obtained by *in situ* hybridization revealed that it was undetectable or poorly detectable in leaf tissues of *Arabidopsis* plants which are subjected to 1 and 2 hr durations of low temperature stress and predominantly accumulated in leaf tissues of *Arabidopsis* plants experiencing 3 hr durations of low temperature stress. The accumulations of these two miRNAs were not detected in root tissues of *Arabidopsis* plants which are exposed to 1, 2 and 3 hr durations of low temperature stress.

The stem-loop real-time RT-PCR analysis of miR399/399e revealed elevated up regulation of miR399 and miR399e miRNA transcript levels by 1.06 fold increases in leaf tissue and 0.6 fold decreases in root tissues of Arabidopsis plants subjected to maximum 3 hr durations of low temperature stress when compared with plants grown in ambient temperature conditions.

It was reported that, this family of microRNA targets two genes belonging to two different gene families; one is phosphate transporter (Jones- Rhoades *et al*., 2004) and another is ubiquitin conjugating enzyme (*UBC24*) which is involved in protein degradation pathway (Fujii *et al*., 2005). In present study, miR399 and miR399e mediated cleavage of *UBC24* was analysed by using real-time RT-PCR approach. The real time quantification showed elevated down regulation of *UBC24* (0.78 fold) in leaf tissues whereas there was *UBC24* (1.41 fold) up regulation in root tissues when compared with plants grown in ambient temperature conditions. The up regulation of miR399 and miR399e in response to water stress and heat shock was found through in situ detection method (Ghosh, 2008; Mahale, 2010).

Through *in situ* hybridization the expression of miR161 was detected in control leaf tissues of *Arabidopsis* plants but not in leaf and root tissues of *Arabidopsis* plants subjected to different regimes of low temperature stress, indicating its down regulation upon low temperature stress. However the expression of this miRNA was not detected in root tissues of Arabidopsis plants grown under ambient culture conditions. Studies were reported that miR161 targets pentatrico peptide repeat (*PPR*) protein gene in *Arabidopsis* (Howell *et. al.,* 2007 and Chen, 2010). In present study, the real time quantification analysis showed elevated up regulation of PPR (1.2 fold) in leaf and 3.4 fold in root tissues of *Arabidopsis* plants experiencing low temperature stress. With this consideration, miR161 expression in vegetative tissues of plant under normal physiological conditions seems to be required.

The miR168 has been identified as the regulator of function of all miRNAs by targeting AGO1 expression, therefore modulating its actual levels and consequently RISC activity (Xie *et al*., 2003). In this study, the role of miR168 upon low temperature stress, tissue sections (leaf and root) representing various durations (1, 2 and 3 hr) of low temperature stress was hybridized with miR168 specific LNA modified oligonucleotide probes. *In situ* hybridization of sections from leaf and root tissues of *Arabidopsis* plants experiencing low temperature stress revealed down regulation of miR168 when compared to leaf tissues of *Arabidopsis* plants grown under normal temperature conditions. The miR168 targets *ARGONAUTE* 1 (*AGO1*), one of 10 AGO proteins in *Arabidopsis* which mediate siRNA directed post-transcriptional gene silencing and microRNA-directed regulation (Rhoades *et al*., 2002; Vaucheret *et al*., 2004; Vaucheret, 2008).

In present study, the expression levels of *AGO1*, predicted target of miR168 was analyzed by using real-time RT-PCR approach. The real-time quantification showed up regulation of *AGO1* (2.4 fold) in leaf tissue whereas there was 1.2 fold increase in root tissues as compared to control leaf tissues of *Arabidopsis thaliana* plants. This observation implicating that miR168 seems to have no direct role upon low temperature stress.

In *Arabidopsis thaliana* four different APS genes (*APS1, APS2, APS3* and *APS4*) have been identified and which encodes ATP sulfurylase isoforms. ATP sulfurylase is the first enzyme in the sulfate assimilation pathway, which provides reduced sulfate for the synthesis of cysteine, methionine and glutathione. In this study, hybridization with an LNA modified miR395b specific probe revealed that this miRNA was not accumulated neither in leaf tissues nor in root tissues of *Arabidopsis* plants subjected to different durations of low temperature stress as well as *Arabidopsis* plants grown at ambient culture conditions.

The miR395 family of microRNAs have direct role in sulfate nutrition in *Arabidopsis*. Sulfate is taken up by plant roots mainly as inorganic form (Nikiforova *et al*., 2006). The sulfate starvation induces many responsive genes expression which result in the changes of physiological and biological aspects such as the alterations in the expression levels of sulfate assimilation enzymes so that sulfate acquisition can be sustained to some extent and sulfate assimilation is suspended (Sunkar *et al*., 2007). Studies have been reported that miR395 targets mRNAs coding for ATP sulfurylases encoded by APS genes (*APS1* and *APS4*). In present study, miR395b mediated cleavage of two APS genes (*APS1* and *APS4*) were analyzed by using real-time RT-PCR approach. The real time quantification showed up regulation of two APS genes such as *APS1* (2.4 fold) and *APS4* (2.3 fold) in leaf whereas there was (*APS1* 2.6 fold) and *APS4* (1.2 fold) in root tissues when compared with control plants in response to low temperature. Recently, deep sequencing results in *Arabidopsis* showed that repressed expression of miR395 in response nitrogen starvation (Liang *et al.,* 2012).

5.2 Expression pattern of selected miRNAs in leaf and root tissues of *Arabidopsis thaliana* plants upon NaCl (300 mM) treatments

Salt stress is one of the most severe and wide-ranging environmental stress that significantly affect crop growth and productivity. A better understanding of the mechanisms underlying the plant response to salinity is essential to confront this agronomic problem. It has been determined that the detrimental effects of salinity occur as the result of osmotic stress, the interruption of metabolic activities by ionic excesses and imbalances and the interference of salt ions with the uptake of essential macronutrients and micronutrients (Tester and Davenport 2003). These adverse effects are manifested in the inhibition of germination, reduction of growth and disturbance of development (Verslues *et al*., 2006). However, many genes and pathways of plants are differentially regulated between salt and drought stress (Golldack *et al*., 2011). In addition to protein-coding genes, research in the last several years has established that small non-coding RNAs including microRNAs (miRNAs) and short interfering RNAs (siRNAs) are also critical regulators of plant growth, development and stress responses.

In this study, LNA- modified oligonucleotide probes were used for sensitive and specific detection of selected miRNAs in leaf and root tissues of control *Arabidopsis* plants through in situ hybridization. The plants irrigated with normal water were considered as control plants. A set of four miRNAs *viz.,* miR171, miR168, miR161 and miR397a recorded their expression in leaf tissues, whereas the expression of three miRNAs *viz*., miR399, miR399e and miR395b was absent in both leaf and root tissues under ambient temperature conditions. The expression was observed in longitudinal sections (LS) of leaf and root tissues through *in situ* hybridization.

The miR171 is a well conserved miRNA family known to regulate members of the SCARECROW-LIKE (SCL) transcription factor family. The SCLs belong to a subclass of the highly conserved GRAS family composed of homologs of GAI, RGA and SCR (Bolle, 2004). In present study, the role of miR171 upon NaCl (300 mM) treatments was analyzed through *in situ* hybridization. *In situ* hybridization of sections from leaf and root tissues of *Arabidopsis* plants subjected for different durations (3, 6, 12 and 24 hr) of NaCl treatments revealed the increased levels of miR171 expression with increasing durations of exposure to NaCl. Stem-loop real-time RT-PCR approach was applied to confirm the results obtained through *in situ* hybridization. The Stem-loop real-time RT-PCR results showed elevated up regulation of miR171 miRNA transcript levels by 6.5 fold increase in leaf and 6.3 fold in root tissues at maximum of 24 hr duration of exposure.

In *Arabidopsis*, there are three MIR171 genes (a, b and c) which are predicted to regulate three SCL6 genes (SCLII, III, IV, also known as HAIRY MERISTEM (HAM) and LOST MERISTEMS (LOM) (Engstrom *et al.,* 2011). The expression domains of the miR171 family members and the SCL6-II/III mRNAs overlap, suggesting a redundant function for both miRNA and target mRNAs (Wang *et al.,* 2010). In present study, the real time RT-PCR analysis revealed down regulation of two SCL6 genes such as SCL6-III (0.4 fold) and SCL6-IV (0.36 fold) in leaf tissues whereas SCL6-III (0.8 fold) and SCL6-IV (0.5 fold) in root tissues of *Arabidopsis* plants irrigated with solution containing NaCl (300mM). Upregulation of miR171, which target *GRAS* gene family indicates possible modulation of these genes. In *Arabidopsis*, Northern and qRT-PCR expression analysis revealed increased levels of miR171 target *SCL6-III* in miR171_3B decoy plants (Ivashuta *et al*., 2011).

The miR397a targets *LACCASE* genes regulation. The *LACCASE* are four copper atom containing glycoproteins which catalyze the oxidation of a suitable substrate molecule with the production of water and oligomers. In *Arabidopsis*, there are 17 annotated *LACCASE* genes that can be divided into four sub-groups (McCaig *et al.,* 2005). In present study, the role of miR397 in response to different durations of NaCl treatments in leaf and root tissues of *Arabidopsis* plants was hybridized with miR397a specific LNA modified oligonucleotide probes. Longitudinal sections of both leaf and root tissues revealed increased accumulation of miR171 with increasing durations of exposure to stress. Further, this was confirmed by stem-loop real-time RT-PCR analysis, which demonstrated elevated up regulation of miR397 by 6.5 fold increase in miRNA transcript levels in leaf tissues whereas there was 6.2 fold increases in root tissues of *Arabidopsis* plants which were irrigated with solution containing 300 mM NaCl for 24 hr duration when compared with plants irrigated with normal water.

The target gene validation of miR397 through bioinformatics approaches reported that miR397 family targets *LAC2, LAC4* and *LAC17* genes (Rhoades and Bartel, 2004). Different functions of laccases in plants have been proposed including, lignin synthesis (Ranocha *et al*., 2002), wound healing (Dean and Eriksson, 1994).

Iron acquisition (Hoopes and Dean, 2004), response to stress (Liang *et al*., 2006), and maintenance of cell wall structure and integrity (Ranocha *et al*., 2002). In this study, the real time RT-PCR analysis revealed up regulation of two *LACCASE* genes such as *LAC2* (2.0 fold) and *LAC17* (1.5 fold) in leaf tissues whereas *LAC2* (3.5 fold) and *LAC17* (5.8 fold) in root tissues of *Arabidopsis* plants irrigated with solution containing NaCl (300 mM) for maximum durations of 24 hr when compared with control plants irrigated with normal water. The up regulation of miR397 was found under water stress in *Arabidopsis thaliana* (Ghosh, 2008). However, miR397a was found down regulated under heat stress in *Arabidopsis thaliana* (Mahale, 2010).

The ubiquitin conjugation E2 and phosphate transporter are potential targets for miR399 and miR399e. Ubiquitin conjugating enzyme E2 catalytic domain is an important part of ubiqutin mediated protein degradation pathway. The miR399 and miR399e belong to the family miR399 (Bartel, 2004). In this study, through *in situ* hybridization approach the increased expression levels of miR399 and miR399e was detected in both leaf and root tissues with increasing durations of NaCl treatments. This was further confirmed by using stem-loop real-time RT PCR analysis. The stem-loop real-time RT PCR analysis revealed 3.4 fold increases in miR399 and miR399e in both tissue types at maximum of 24 hr duration of exposure to NaCl treatments when compared to Arabidopsis plants irrigated with normal water.

Ubiquitin conjugating enzymes performs the second step in the ubiquitylation reaction which targets a protein for degradation via the proteasome. It was reported that ubiquitylation plays important functions in many aspects of plant growth and development, including phytohormone and light signalling, embryogenesis, organogenesis, leaf senescence and plant defence (Dreher and Callis, 2007). In *Arabidopsis thaliana*, 37 proteins with a UBC domain and active-site cysteine has been predicted (Kraft *et al*., 2005). Among the 37 proteins, *UBC24* was reported for its involvement in phosphate signalling (Bari *et al*., 2006). Recent reports showed that *UBC 24* is targeted by miR399 and the expression of *UBC24* down regulated whereas the level of miR399 is increased in response low phosphate conditions (Chiou *et al*., 2006). In the present study, miR399 and miR399e mediated cleavage *UBC24* was of analyzed by using real-time RT-PCR approach. The real time quantification analysis resulted in 0.6 fold decreased levels of *UBC24* in leaf tissues whereas there was 0.47 fold decrease in root tissues at maximum 24 hr durations of exposure to NaCl treatments when compared to *Arabidopsis* plants treated with normal water. Previous studies reported that under low-Pi stress, the expression of miR399 is induced and *UBC24* expression is suppressed: this result in alleviation of Pi transporter expression and thus alteration of root architecture-which is very important for maintaining of Pi level in plants. Under normal phosphorus supply condition, miR399 expression is suppressed and *UBC24* expressed and presumably participates in an ubiquitin or proteosome pathway that negatively regulates the expression of Pi transporters and controls hormonal signaling for root growth regulation to prevent the over-absorption of Pi from media which is very toxic for plants (Lin *et al*., 2009).

The pentatricopeptide repeat (PPR) is a helical repeat motif found in an exceptionally large family of RNA–binding proteins that functions in mitochondrial and chloroplast gene expression. PPR proteins harbor between 2 and 30 repeats and typically bind single-stranded RNA in a sequence-specific fashion (Barkan *et al*., 2012). In this study, the expression pattern of miR161 in leaf and root tissues of *Arabidopsis* plants irrigated with solution containing 300 mM NaCl was assessed through *in situ* hybridization approach. The in situ hybridization of miR161 revealed there was no expression of miR161 either in leaf or in root tissues of NaCl treated plants. But, the expression of miR161 in control leaf tissues indicated role of this miRNA in normal growth and development of *Arabidopsis* plants but not in plants response to stress. The miR161 targets pentatrico peptide repeat (*PPR*) protein gene in *Arabidopsis* (Howell *et. al.,* 2007 and Chen *et. al*., 2007). In present study, the real time quantification showed elevated up regulation of PPR in leaf (2.8 fold) and root (1.2 fold) tissues of NaCl treated *Arabidopsis* plants.

The miR168 is the one of the most commonly detected stress inducible miRNA. miR168 is critical for modulating the level of AGO1 by miR168-targeted cleavage of the AGO1 mRNA and translational repression of AGO1 (Vaucheret, 2009). Homologs of MIR168 exist in various plant species, including dicots such as poplar (*Populus trichocarpa*), tobacco (*Nicotiana tabacum*), and Arabidopsis and monocots such as maize (*Zea mays*) and rice (*Oryza sativa*). These homologs have been found to also respond to salt, drought and cold stresses or ABA treatment (Liu *et al*., 2008; Jia *et al*., 2009; Zhou *et al*., 2010). In this study, the role of miR168 upon NaCl stress was assessed in tissue sections (leaf and root) representing various durations (3, 6, 12 and 24 hr) NaCl stress was hybridized with miR168 specific LNA modified oligonucleotide probes.

In situ hybridization of sections from leaf and root tissues of *Arabidopsis* plants subjected to NaCl stress revealed up regulation of miR168 when compared to leaf tissues of *Arabidopsis* plants irrigated with normal water. Whereas, the expression of miR168 was not detected in root tissues of neither NaCl treated nor normal water treated *Arabidopsis* plants.

The miR168 is one of the miRNA which control its own expression and also the other miRNAs by targeting specific proteins involved in the posttranscriptional gene silencing pathway. In particular, miR168 regulates the function of all miRNAs by targeting *AGO1* expression, therefore modulating its actual levels and consequently RISC activity (Xie *et al*., 2003). In present study, miR168 mediated cleavage of *AGO1* was analyzed by using real-time RT-PCR approach. The real time quantification analysis resulted in 0.02 fold decreased levels of *AGO1* mRNA transcripts in leaf tissues whereas there was 12.38 fold increase in root tissues at maximum 24 hr durations of exposure to NaCl treatments when compared to *Arabidopsis* plants treated with normal water.

The mir395 is a microRNA which has been identified in both *Arabidopsis thaliana* and *Oryza sativa* by using computational approaches. Studies reported that miR395 targets mRNAs coding for ATP sulfurylases encoded by APS genes (*APS1* and *APS4*). Significant upregulation of miR395 expression have been identified during sulfate limitation (Liang *et al*., 2010). In present study, the accumulation pattern of miR395b obtained by *in situ* hybridization revealed that up regulation of miR395b increased with increasing durations of NaCl treatments in leaf and root tissues *Arabidopsis thaliana* plants compared to the plants irrigated with normal water. The expression was uniform all over the leaf, especially in vascular tissues, spongy tissues, palisade and cuticle.

Recently, deep sequencing results in *Arabidopsis* revealed the repressed expression of miR395 in response nitrogen starvation (Liang *et al.,* 2012). Studies identified that miR395 regulates the accumulation of sulfate by targeting ASP genes (Kawashima *et al*., 2009). Quantitative RT-PCR data showed that decreased level of *APS1* transcripts in both shoot and root tissues in response to sulfate limitation (Liang *et al*., 2010). In present study, the miR395b mediated cleavage of two ASP genes such as *APS1* and *APS4I* was analyzed using real-time RT-PCR approach. The results obtained from real-time RT-PCR analysis revealed the decresed level of two ASP genes transcripts *APS1* (0.7 fold) and *APS4* (0.4 fold) in leaf tissues whereas there was *APS1* (0.9 fold) and *APS4* (0.5 fold) decrease in root tissues. Up-regulation of miR395b observed in water stressed and heat shock in *Arabidopsis thaliana* plants, confirms its role in drought stress and heat shock response by plant (Ghosh, 2008 and Mahale, 2010).

From the present study it is evident that, the LNA mediated hybridization can precisely and effectively detect microRNAs which differ in a few bases through *in situ* hybridization. Gene specific experimental validation of individual miRNA: mRNA interactions are important for functional analysis of miRNAs. Real-time qPCR approach was used to detect the expression levels of miRNA cognate genes upon low temperature (4 ± 1 °C) and NaCl (300 mM) stress responses. Stem-loop real time RT-PCR was efficient and reliable detection and quantification method for miRNA expression analysis. This approach was employed in detection of some of the selected miRNAs in leaf and root tissues of *Arabidopsis thaliana*. Among selected microRNAs, under ambient conditions miR161, miR168, miR171 and miR397a expressed in leaf tissues while miR171 and miR397a expressed in root tissues. Upon low temperature to shoot portion of *Arabidopsis* miR397a and miR171 upregulated at 1, 2 and 3 hr duration of exposure in leaf and root tissues, where root tissues were in ambient conditions. Further the expression of miR399 and miR399e got upregulated in leaf tissues at 3 hr duration of exposure. In response NaCl (300 mM) treatment, miR171, miR395b, miR399 and miR399e upregulated commonly in leaf and root tissues. Whereas, miR168 upregulated only in leaf tissues but not in root tissues upon NaCl treatment. Silencing of target genes of these microRNAs could permit the direct molecular modulation of plant traits, which could in turn be applied to the breeding of crop species.

SUMMARY AND CONCLUSIONS

Plants exposed to stress use multiple gene regulatory mechanisms, including post-transcriptional regulation of gene expression, to restore and re-establish cellular homeostasis. The miRNAs have emerged as ubiquitous post-transcriptional gene regulatory molecules in plants. The expression of microRNAs is faster than that of protein-coding genes, because microRNAs cannot be restricted or influenced by the process of translation and can efficiently regulate target genes and potentially play a role in long distance communication within plant system. The important role of plant microRNAs involved in gene expression and regulation under stress conditions provides new opportunities for detailed and deeper understanding of complex biological mechanisms involved in plant abiotic stress tolerance. In the present study the selected miRNAs were tested for their responsiveness under low temperature (4 ± 1 °C) and NaCl (300 mM) treatments in *Arabidopsis thaliana* by using *in situ* and real time quantification approaches. The summary of present investigation is presented below:

1. The 30 days old healthy *Arabidopsis thaliana* plants grown in culture room conditions were used for low temperature and NaCl stress response of selected microRNAs.
2. The *Arabidopsis thaliana* plants were exposed to 1, 2 and 3 hr durations of low temperature stress in order to identify the expression pattern of selected miRNAs in response to low temperature stress. From each treatment leaf and root tissues were harvested and fixed immediately for further processing.
3. In order to identify the salt stress responsive miRNAs among selected miRNAs The *Arabidopsis thaliana* plants were exposed to 3, 6, 12 and 24 hr durations NaCl (300 mM) treatments. From each treatment leaf and root tissues were harvested and fixed immediately for further processing.
4. A thermocol box containing warmed ice packs were used to maintain the ambient root zone temperature when shoot portion was exposed to different durations of low temperature.
5. *Arabidopsis thaliana* plants grown under ambient temperature conditions and irrigated with normal water were used as control plants. From control plant, leaf and root tissues harvested and fixed immediately for further processing.
6. A set of seven microRNAs *viz.*, miR399, miR399e, miR397a, miR395b, miR171, miR168 and miR161, previously characterized for their water stress and heat shock response in *Arabidopsis thaliana* at Department of Biotechnology were LNA modified and used *in situ* hybridization. Assays comprised of technical and biological replications along with positive and negative controls as check.
7. Differential up and down regulation among tested microRNAs was observed in shoot and root tissues of *Arabidopsis thaliana* plants grown under ambient temperature conditions. The miR171, miR161, miR168 and miR397a were up-regulated in leaf tissues, whereas, miR171 and miR397a up-regulated in root tissues.
8. The up regulation of miR171, miR397, miR399 and miR399e was recorded in low temperature stressed leaf tissues where miR171 and miR397a got up regulated in root tissues of *Arabidopsis thaliana* plants whose shoot portion was exposed to low temperature.
9. An increase in hybridization signals of up regulated miRNAs was observed in both leaf and root tissues of *Arabidopsis thaliana* plants whose shoot portion was exposed to increasing durations of low temperature.
10. The up regulation of a set of five miRNAs *viz.*, miR171, miR395b, miR399 and miR399e was recorded in leaf and root tissues of *Arabidopsis thaliana* plants exposed to different durations of NaCl (300 mM) treatments.
11. The up regulation of miR168 was recorded in leaf tissues where as its down regulatin recorded in root tissues of *Arabidopsis thaliana* plants exposed to different durations of NaCl (300 mM) treatments.
12. Based on the *in situ* hybridization results, a stem-loop RT-PCR approach was applied to detect four mature miRNAs *viz.*, miR399e/f, miR397 and miR171 showing their differentional expressions in both leaf and root tissues of *Arabidopsis thaliana* plants exposed to low temperature and NaCl stress.
13. Among four selected miRNAs accumulation of two miRNAs (miR171 and miR397a) were recorded in both leaf and root tissues at 1, 2 and 3 hr durations of exposure of *Arabidopsis thaliana* exposed to low temperature (4± 1 °C).
14. The down regulation of expression of miR399e/f in both leaf and root tissues at 1 and 2 hr durations of exposure to low temperature stress was confirmed through stem-loop real-time RT-PCR.

15. The stem-loop real-time RT-PCR analysis of miR399e/f reported up regulation of miR399e/f with 1.06 fold change in leaf tissues but not in root tissues at 3 hr duration of exposure.
16. The up regulation of three miRNAs such as miR171 miR399 and miR399e was assessed through stem-loop real-time RT-PCR approach in leaf and root tissues of *Arabidopsis thaliana* plants exposed to different durations of NaCl treatments.
17. The down regulation of miR397a was assessed through stem-loop real-time RT-PCR approach in leaf and root tissues of *Arabidopsis thaliana* plants exposed to different durations of NaCl treatments.
18. The real time RT-PCR approach was used to analyze expression levels of selected miRNA cognate genes in leaf and root tissues of low temperature and NaCl treated *Arabidopsis thaliana* plants.
19. One and two hour exposure of *Arabidopsis thaliana* shoot to low temperature resulted in up regulation of five miRNA cognate genes such as *UBC24, AGO1, APS1*and *APS4,* and *PPR* which are predicted to be targets of miR399, miR168, miR395b and miR161 respectively in both leaf and root tissues.
20. The up regulation of four miRNA cognate genes such as *AGO1, APS1*and *APS4,* and *PPR* was recorded in leaf tissues exposed for at 3 hr low temperature.
21. The down regulation of four miRNA cognate genes such as *LAC2* and *LAC17*, and *SCL6-III* and *SCL6-IV* which are predicted to be targets of miR397a and miR171, respectively were noticed in both leaf and root tissues commonly at 1, 2 and 3 hr duration of exposure to low temperature stress.
22. Expression levels of selected miRNA cognate genes were compared in NaCl (300 mM) treated leaf and root tissues with respective normal watered controls of *Arabidopsis thaliana*.
23. The up regulation of 3 miRNA cognate genes such as *LAC2* and *LAC17*, and *PPR* which are predicted targets of miR397 and miR161 respectively was recorded in leaf and root tissues of *Arabidopsis thaliana* plants irrigated with solution containing 300 mM NaCl.
24. A set of six miRNA cognate genes such as UBC24 (predicted target of miR399), *APS1* and *APS4* (predicted target of miR395b) and *SCL6-III* and *SCL6-IV* (predicted target of miR171) were found to be down regulated in both leaf and root tissues *Arabidopsis thaliana* plants exposed for different durations (3, 6, 12 and 24 hr) of NaCl treatment.
25. The up regulation of AGO1 (predicted target of miR168) was noticed in root tissues whereas, down regulation was recorded in leaf tissues.

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APPENDIX I

a. 10X Phosphate buffer saline (PBS) (1000 ml) pH 7.0	
130 mM Sodium chloride	130 ml
7 mM Na2HPO4	70 ml
3 mM NaH2PO4	30 ml
DEPC-water (RNase free)	770 ml

b. Paraformaldehyde (PFA) in PBS (500 ml) pH 7.0	
4% Paraformaldehyde	20 g
4% DMSO	20 ml
Before adding PFA adjust the pH to 11.0 and heat the solution at 60-70°C to dissolve PFA. Then bring down the pH to 7.0 and add DMSO	

c. Increasing Ethanol series (100 ml)	**ethanol**	**DEPC - free water**
30 % Ethanol	30 ml	70 ml
40 % Ethanol	40 ml	60 ml
50 % Ethanol	50 ml	50 ml
60 % Ethanol	60 ml	40 ml
70 % Ethanol	70 ml	30 ml
85 % Ethanol	85 ml	15 ml
95 % Ethanol	95ml	5 ml
100 % Ethanol	100 ml	-
Addition of 0.1 % Eosin to 95-100 % ethanol helps to visualize samples when sectioning.		

d. Increasing Histochoice series (100 ml)	Histochoice	Ethanol
25 % Histochoice	25 ml	75 ml
50 % Histochoice	50 ml	50 ml
75 % Histochoice	75 ml	25 ml
100 % Histochoice	100 ml	

c. Decreasing Ethanol series (100 ml)	ethanol	DEPC - free water
100% Ethanol	100 ml	-
95% Ethanol	95 ml	5 ml
90 % Ethanol	90 ml	10 ml
80 % Ethanol	80 ml	20 ml
60 % Ethanol	60 ml	40 ml
45 % Ethanol	45 ml	55 ml
30 % Ethanol	30 ml	70 ml
DEPC-free water	100 ml	-

APPENDIX II

a. 20X Sodium-Sodium Citrate (SSC) (1000 ml)	
3 M Sodium chloride	500 ml
300 mM Sodium Citrate	300 ml
DEPC-water (RNase free)	200 ml

b. Proteinase K in Tris-EDTA buffer (600 ml) pH 8.0	
1 M Tris pH 8.0	60 ml
500 mM EDTA	60 ml
DEPC-water (RNase free)	480 ml
Protinase k (10 mg/ml)	60µl

c. Glycine (0.2 %) in 10X PBS (500 ml)	
Glycine	1 g in 500 ml PBS

d. Acetic anhydride in 0.1 M Triethanolamine buffer ph 8.0 (800 ml)	
Triethanolamine	10.4 ml
Conc. HCl	3.2 ml
DEPC-water (RNase free)	786.4 ml
Proteinase k (10 mg/ml)	60 µl
Acetic anhydride	4 ml

APPENDIX III

a. 10X in situ salts (100 ml)	
3M Sodium chloride	30 ml
100mM Tris pH 8.0	10 ml
100mM Na Phosphate pH 6.8	10 ml
50mM EDTA	10 ml
DEPC-water (RNase free)	40 ml

b. Hybridization solution (for 5 slide pairs) (800 µl)	
10X in situ salts	100 µl
Deionized formamide	400 µl
50% Dextran Sulfate	200 µl
50X Denhardts solution	20 µl
tRNA (100 mg/ml)	10 µl
DEPC-water (RNase free)	70 µl

c. Probe dilution	
Probe (stock)	1 µl
Nuclease free water	170 µl

d. Hybridization Solution tal

Pairs of slides	Probe(µl)	50% Formaldehyde	Hybridization solution (µl)	Final volume (µl)
5	10	190	720	1000

APPENDIX IV

a. 5X NTE (1000 ml)	
2.5 M NaCl	416.66 ml
50 mM Tris pH 8.	50 ml
5 mM EDTA	50 ml
DEPC-water (RNase free)	483.34 ml

b. Tris buffer saline (TBS) 1000 ml	
100 mM Tris pH 8.0	100 ml
150 mM Sodium chloride	150 ml
DEPC-water (RNase free)	750 ml

c. BSA, Triton X-100 in TBS (500 ml)	
1% BSA	5 g
0.3% Triton X-100	1.5 ml
Make up the volume 500 ml with TBS	

d. 1% Blocking Solution in TBS (200 ml)	
Blocking reagent	2 g
Make up the volume 200 ml with TBS and stirred at 60°C for at least 1 hour	

e. Anti-DIG alkaline phosphate solution for 25 pairs of slides	
Anti-DIG antibody	10 µl
1% BSA, 0.3% Triton X-100 in TBS	12.5 ml

APPENDIX V

a. Tris-Sodium chloride (TN) solution (250 ml)	
100mM Tris pH 9.5	50 ml
100mM NaCl	25 ml
DEPC-water (RNase free)	175 ml

b. NBT/BCIP mix in TN for 25 pairs of slides	
NBT/BCIP	200 µl
TN	10 ml

c. Tris-EDTA (TE) buffer pH 8.0 (500 ml)	
10mM Tris pH 8.0	10 ml
1mM EDTA	5 ml
Add some amount DEPC-water and adjust the pH to 8.0 And make up the volume 500 ml	

Appendix VI

List of RNA loading solutions used in experiment

a) RNA loading dye composition (5X)		
Sl. No.	**Components**	**Volume (µl)**
1	Saturated *a*queous bromophenol blue solution	16.00
2	EDTA (0.5M; pH: 8.0)	80.00
3	Formaldehyde	720.00
4	Glycerol (100 %)	2000.00
5	Formamide	3048.00
6	10X FA gel buffer	4000.00
7	RNase free water	100.00
	Total	10 ml
b) Ethidium bromide		10 mg/ml in *RNase* free water.
c) Recipe for 1.2 % formaldehyde agarose gel (60 ml)		:720 mg Agarose : 1 ml Formaldehyde :3 µl Ethidium bromide (10 mg/ml) : 60 ml 1X MOPs buffer
d) 10X MOPs electrophoresis buffer		: 41.8 g MOPs : 20 ml Sodium Acetate (DEPC-treated) :20 ml EDTA (DEPC-treated; pH: 8.0) Total volume 1000 ml with DEPC treated water

APPENDIX VII

List of stem-loop RT primers and qRT primers used for quantification of mature miRNAs

miRNA	miRNA sequence (5′- 3′)	RT Primer (5′- 3′)	Forward primer (5′- 3′)	Reverse primer (5′- 3′)
miR171a	UGAUUGAGCCGCGCCAAUAUC	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATATT	ATTGAGCCGCGCCAATA	GTGCAGGGTCCGAGGT
miR397a	UCAUUGAGUGCAGCGUUGAUG	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCATCAA	TGAACATCATTGAGTGCAG	GTGCAGGGTCCGAGGT
miR399f	UGCCAAAGGAGAUUUGCCCGG	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCGGG	CTCTGCCAAAGGAGATTTGC	GTGCAGGGTCCGAGGT
miR399e	UGCCAAAGGAGAUUUGCCUCG	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGAGGC	CTCTGCCAAAGGAGATTTGC	GTGCAGGGTCCGAGGT

APPENDIX VIII

List of qRT prmers used for quantification of miRNA cognate genes

Sl. No.	Primer name	Forward sequence (5'-3')	Reverse sequence (5'-3')
1	*AGO1*	GAGGCTGTTTGCTCAGAACC	CGAGAAGTGCCCTGAATACC
2	*ASP1*	TTAATGTTGGTGTGGGAGCA	TATTGTCCCCATAGGCAAGC
3	*ASP4*	TAACCCCACAGGAGTTGACC	TCAAACATTCACCTCCATCG
4	*LAC2*	ACGGTGACTGGACAAAGAGG	ACCACTGCTTGAGGATCAGC
5	*LAC17*	CGTTTCATAGAGCAGCTTCG	CATCACCAACTCCACACTGG
6	*PPR*	GTTCTTTACTTGGGGCTTGC	GAGGCAGCTTCACGTAATCC
7	*UBC24*	TTGAGCGTGTCTCTGTCACC	TCTTTGCCCTGGGTAATACG
8	*SCL6IV*	TGGTTAAGGCAGCAGAGGTC	TTAGGGTTTGGGAGACGTTG
9	*SCL6III*	CGTTTCATAGAGCAGCTTCG	GGTTTGCTGTGAAGTTGACG

FUNCTIONAL AND EXPRESSIONAL QUANTITATION OF SELECTED microRNAs IN RESPONSE TO LOW TEMPERATURE AND NaCl STRESS CONDITIONS IN LEAF AND ROOT TISSUES OF *Arabidopsis thaliana*

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ABSTRACT

The discovery of miRNAs has led to a fundamental change in the understanding of complex biological mechanisms involved in plant responses to stress tolerance. In this study, the expression of seven selected miRNAs was studied in *Arabidopsis thaliana* plants experiencing low temperature and salt stress separately. The selected miRNAs were *in situ* hybridized with Locked Nucleic Acid (LNA)-modified oligonucleotide probes. Among the tested miRNAs, expression of miR161, miR168, miR171 and miR397a in leaf tissues and miR171 and miR397a in root tissues was recorded in control plants. Elevated expression of miR171 and miR397a was recorded in both tissue types of low temperature treated plants. A set of four miRNAs *viz*., miR171, miR395b, miR399e and miR399 showed their up regulation in both tissue types upon NaCl (300 mM) treatment. Expression of miR168 was recorded only in leaf tissues, and on the other hand, down regulation of miR397a was recorded in both tissue types in response to NaCl stress. The miRNA stem-loop RT-PCR assay indicated gradual increase in the expression of miR171 and miR397 with the highest of 4.28 and 3.49 fold changes in leaf tissues of *A. thaliana* plants experiencing low temperature stress and 6.5 and 6.3 fold up-regulation of miR171, 0.8 and 0.9 fold down-regulation of miR397a and 3.4-3.5 fold up-regulation of miR399 and miR399e in leaf and root tissues, respectively, at 24 hrs of exposure to salt stress. The RT-qPCR assay recorded reduced levels of miRNA target gene transcripts *viz*., *SCL6 III*, *SCL6 IV*, *LAC2*, and *LAC17* in response to low temperature and *SCL6 III*, *SCL6 IV*, *APS1, APS4* and *AGO1* transcripts in response NaCl treatment in both tissue types of *Arabidopsis thaliana* plants. The study points at the possibility of modulating low temperature and salt tolerance in plants through the down regulation of specific cognate genes.