

**COMPUTATIONAL PREDICTION OF miRNA TARGETS IN
ESTs OF SORGHUM AND COTTON AND *IN SITU*
DETECTION OF SELECTED DROUGHT RESPONSIVE
miRNAs IN *Arabidopsis thaliana***

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1. INTRODUCTION

MicroRNAs (miRNAs) are small RNAs (21-24 nucleotides long) that regulate gene expression in plants and animals. Like small interfering RNAs (siRNAs), miRNAs get into the post-transcriptional gene silencing pathway, leading either to degradation of the target mRNA or to translational repression. The complementary target region for binding of miRNA is contained within the gene. miRNAs, also called 'killer RNAs' have emerged as an answer for deciphering hitherto unknown ways of gene regulation, which are highly specific to target genes.

The miRNAs are encoded by a distinct class of genes, called MIRGENE; the transcripts of which do not encode proteins. The transcribed RNA is partially self-complementary, folds into a characteristic structure that includes imperfect double-stranded regions from which the miRNAs originate (Bartel *et al.*, 2004). The excision of the mature miRNA is a multi-step reaction that involves trimming the initial precursor several times. In plants, this reaction, at least in part, is performed by the Dicer enzyme DCL1 (Kurihara *et al.*, 2004). Apart from their biosynthesis, miRNAs are characteristically conserved between species. Most families of *Arabidopsis* miRNAs have obvious homologues in rice and other plants (Axtell and Bartel, 2005). However, plant miRNAs only show homology to other plant miRNAs and animal miRNAs to animal miRNAs indicating that this mechanism of gene-regulation has evolved separately in plants and animals (Pasquinelli *et al.*, 2000).

In silico identification of miRNA targets is possible because plant miRNAs generally exhibit a high degree of complementarity to their target sites (Jones-Rhoades *et al.*, 2004). Given the small size of miRNAs, computer-aided target prediction is complicated (Robins *et al.*, 2005). To refine the search for miRNA targets, it is important to test and update the known rules for miRNA and target interaction. Since the first discovery of miRNAs *lin-4* and *let-7* by genetic analysis of *C. elegans* developmental timing (Lee *et al.*, 1993; Reinhart *et al.*, 2000), numerous computational models and tools have been developed to complement biological experiments to design to study the diverse regulatory roles of miRNAs. The bioinformatics approaches have been useful in understanding the complexity of miRNA genes and their targets. Most computational methods used for miRNA studies can be classified into two broad categories; 1) miRNA identification and 2) miRNA target identification (Sungroh and Giovanni, 2006). Methods do vary depending on whether a method is for animals, plants, or viruses, since the biology of miRNAs is somewhat different in each case. A miRNA target detection algorithm can be trained using the properties of known miRNA-mRNA duplexes which can then be used for finding new miRNA-mRNA duplexes (Sungroh and Giovanni, 2006).

Using publicly available nucleotide databases (ESTs, GSSs, etc) numerous miRNAs were identified in diverse plant species with the aid of computational approach as well as expression analysis (Sunkar *et al.*, 2008). These findings will be useful for tracing the evolution of small RNAs by examining their expression in common ancestors of different plant lineages. Many plant miRNAs are evolutionarily conserved from species to species and from angiosperms to mosses (Zhang *et al.*, 2006). Although miRNA precursors display high variability, their mature sequences display extensive sequence complementarity to their target mRNA sequences. Due to differences in the architecture of miRNA-based gene regulation, discovery of miRNAs and their targets are similarly constrained in both plants and animals (Ehrenreich and Purugganan, 2008).

miRNA targets include a large number of transcription factors, suggesting their role in the control of regulatory networks, cellular growth and development. Conserved miRNAs do not necessarily exhibit the same levels of expression, patterns of stage of expression in different species. Therefore, sequence and expression divergence in miRNAs between species may affect miRNA accumulation and target regulation leading to developmental changes and phenotypic variation (Ha and Pang, 2008). The conservation of miRNAs and other small RNAs between the conifers and the angiosperms indicates important RNA silencing processes were highly developed in the earliest spermatophytes (Morin *et al.*, 2008).

A total of 1638 plant miRNAs have been deposited in the miRBase, a database of miRNA (Release 12.0). Many of them were conserved during the evolution of land plants suggesting that they might also retain homologous target interactions (Yin *et al.*, 2008).

Experimental identification of miRNA targets is a difficult and time consuming process. As a consequence several computational prediction methods have been devised in order to predict targets for follow up experimental validation such as Stacking Binding Matrix (SBM) that uses both the information about the miRNAs as well as experimentally validated target sequences in the search for candidate target sequences (Moxon *et al.*, 2008). Recently, 79 miRNA candidates were discovered by using wheat EST sequences (Jin *et al.*, 2008). Similarly, deep sequencing has proved to be an effective strategy for discovering miRNAs of low-abundance and candidate miRNAs (Sunkar *et al.*, 2008) and to recover some of the hits missed by existing methods 454 sequencing can be a valuable tool to assign probability-based scores to them (Vacic *et al.*, 2008).

The impact of a miRNA on gene expression can be complex. In animals, miRNAs can shift the entire transcription profile of a cell, which indicates a pivotal role for miRNAs in establishing and maintaining tissue identity (Lim *et al.*, 2005). In plants, many miRNA targets encode transcription factors, which in turn regulate specific subsets of genes. In addition, the biosynthesis of another class of regulative small RNAs, so called trans-acting siRNAs, has been shown to depend on miRNAs (Allen *et al.*, 2005). Thus a miRNA can be designated as “regulator of regulators” (Jackson *et al.*, 2003).

Having a negative role in regulation of gene expression in both plants and animals, miRNAs are shown to play a critical role in a wide range of developmental and physiological pathways. In plants, miRNAs are primarily involved in regulation of growth and development, although they are also known to be involved in the regulation of response to nutrient stresses (Bartel and Bartel, 2003). Experimental evidence points to the involvement of miRNAs in the regulation of sulfate assimilation and translocation, as well as phosphate homeostasis (Chiou, 2007).

Although hundreds of different miRNAs have now been identified using cloning and computational approaches, characterization of their targets and biological role is limited. Recently, there has been a great interest in the identification of miRNAs, their targets as well as understanding of spatial and temporal regulation of their genes. *In situ* hybridization is an important tool to analyze gene expression and prediction of gene function. *In situ* hybridization can be thought of as a special staining technique for the labeling of the messenger RNA (mRNA) of a specific protein or non-coding regulators of those mRNAs like miRNAs which is being investigated. This method utilizes the complementary nature of nucleic acids to create specific probes, which are complementary and thus specific for the target sequence that is to be localized. The probe can be labeled with radioactive tracer molecules or non-radioactive materials like digoxigenin, biotin etc. Once the probe has bound to the target, the decay of the tracer causes the deposition of a silver granule over the cell that contains the bound probe, allows detection by autoradiography in case of radioactive labeling. In non-radioactive method, after hybridization the sections are treated with the corresponding antibody, allowing detection by substrate reaction which gives rise to development of colors in the tissue sections (Jackson *et al.*, 1992). In this way, it can be determined which cell types synthesize which proteins or which miRNA of interest, and in relationship to what kind of stimuli.

The discovery of hundreds of miRNA genes in animals and plants has provided new challenges for analyzing gene expression and functions. In recent years, the *in situ* hybridization (ISH) technique has found widespread application in both basic science and diagnostic clinical research. The ISH technique has frequently been used to localize specific genes on metaphase chromosomes and to detect viral and bacterial genomes in infected tissues. The miRNA *in situ* hybridization (miRISH) technique for the examination of miRNA expression has gained more attention due to the quite sound expression results though many technical problems associated with this technique. In miRISH technique hybridization specificity is very important as miRNAs differ from each other by only one or two nucleotides in varying position of 19-21 nt length. Therefore, to get that higher specificity Locked Nucleic Acids (LNA) probes are recent choice of researchers. LNA are used nowadays for a diverse application in miRNA research like miRNA profiling (Mirco *et al.*, 2006), real-time expression analysis (Jean *et al.*, 2006), SNP discovery (Joy *et al.*, 2007), *in situ* detection (Valoczi *et al.*, 2006) and others applications.

LNA are a class of nucleic acid analogues in which the ribose ring is “locked” by a methylene bridge connecting the 2'-O atom with the 4'-C atom. LNA nucleosides contain the

six common nucleobases (T, C, G, A, U and methyl C) that appear in DNA and RNA and thus are able to form base-pairs according to standard Watson-Crick base pairing rules. Oligonucleotides incorporating LNA have increased thermal stability and improved discriminative power with respect to their nucleic acid targets. LNA can be mixed with DNA, RNA and other nucleic acid analogs using standard phosphoramidite synthesis chemistry. LNA oligonucleotides 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.

Keeping these in view, the present investigation was undertaken with the following objectives:

1. Computational prediction of miRNA targets in a publically available EST data set of sorghum and cotton and their annotation
2. Optimization of *in situ* detection procedure of specific miRNA in *Arabidopsis thaliana*
3. *In situ* detection of drought responsive selected miRNAs in root and leaf tissues of *Arabidopsis thaliana*

2. REVIEW OF LITERATURE

Gene expression is highly regulated in living organism to ensure proper growth, development and response to the environmental stimuli. One of the most recently discovered regulatory mechanism involves 21-24 nt small RNA molecules, called miRNA. Besides this small RNAs, other small RNA content of plant cells is complex suggesting an extensive regulatory role for these molecules. Since their first discovery a decade ago, miRNAs have attracted the attention of most section of biologist to explore their importance in growth and development. A brief review on various aspects of miRNA is presented in this chapter.

2.1 Origin, conservation, and diversity of miRNAs

In animals, miRNAs are evolutionarily conserved; many miRNAs, such as miR7 and miR18, are conserved from worm to human (Weber, 2005 and Lagos-Quintana *et al.*, 2001). Pasquinelli *et al.* (2000) considered that animal miRNAs existed at least 420 million years ago, with a common metazoan ancestor. miRNA conservation has also been observed in plants (Jones-Rhoades and Bartel, 2004 and Sunkar *et al.*, 2008), however, only mature plant miRNAs are conserved, unlike the conservation of both mature miRNAs and miRNA precursors in animals (Reinhart *et al.*, 2002 and Zhang *et al.*, 2005). Experimental and computational analysis has indicated that many plant miRNAs and their targets are conserved between monocots and dicots, based on limited data for *Arabidopsis* and rice (Sunkar *et al.*, 2008). Axtell and Bartel (2005) employed microarray technology to analyze the expression of several miRNAs in different plant species, and found that some miRNAs existed not only in dicots and monocots but also in ferns, lycipods, and mosses. Recently, a set of 21 abundant miRNAs were found to be conserved among the angiosperms (Axtell and Bowman, 2008), many of which are involved in transcription factor regulation in developmental control. Zhang *et al.* (2006) found that they are ubiquitous based on the analysis of ESTs for miRNA targets in many plant species.

Floyd and Bowman (2004) showed that the class-III homeodomain-leucin zipper (HD-Zip) genes, one of the targets of miR166, have conserved miR166 target regions, although other regions have much lower nucleotide conservation. Both miRNA conservation and miRNA target conservation indicate that plant miRNAs have a very deep origin in plant phylogeny, at least since the last common ancestor of bryophytes and seed plants (Floyd and Bowman, 2004 and Zhang *et al.*, 2005). This suggests that miRNA-mediated gene regulation existed more than 425 million years ago in the plant kingdom. The age of plant miRNA is comparable to the age of miRNA regulation in metazoans; however, no evidence yet shows that plant miRNAs and animal miRNAs have a common ancestor (Reinhart *et al.*, 2002). Recent experiments showed that origin and functional distribution of miRNAs depends on some conserved motif around the transcription start site in different species of *Arabidopsis* (Warthmann *et al.*, 2008). It has been shown that miRNAs in *Arabidopsis* evolved from random sequences by inverted duplication events (Felippes *et al.*, 2008).

A total of 207 miRNAs have been found in *Arabidopsis* and deposited in the miRBase (Release 12.0). These miRNAs can be grouped into 42 miRNA families according to their nucleotide sequence similarity. A majority of these miRNA families have also been found in other species, such as rice, maize, and sorghum (Sunkar *et al.*, 2008). Some plant miRNA families are highly evolutionarily conserved with only a few nucleotide changes among a wide variety of plant species (Sunkar *et al.*, 2008). Axtell and Bartel (2005) found that the miR159/319 family existed in 10 plant species. miRNA families 156/157 and 165/166 exist in 9 plant species of diverse lineages. Zhang *et al.* (2005) reported that about 30 percent of miRNA families existed in at least 10 different plant species. Of these miRNA families, miR156 was found in 36 different plant species (Sunkar and Jagadeeswaran, 2008). The recent findings show that the exon-intron arrangements play a critical role as the source of miRNA genes (Lu *et al.*, 2008).

Based on conservation, miRNAs are classified into 4 groups: highly, moderately, lowly, and non-conserved (Jones-Rhoades and Bartel, 2004 and Zhang *et al.*, 2005). For some miRNAs, for example, miR163 and miR158, Jones-Rhoades and Bartel (2004)

considered as non-conserved miRNAs. Recent discovery of much larger set of weakly expressed, less conserved miRNAs in plants which in turn provided insights into the origin of miRNAs and hypotheses explaining the birth, selection and death of miRNAs in land plants (Axtell and Bowman, 2008).

It is expected that some non-conserved or lowly conserved miRNAs may be re-classified as more miRNAs are discovered. Conservation is an indicator of miRNA function; conserved miRNAs play an important role in conserved gene regulation, such as leaf and flower morphology or signal transduction (Mallory *et al.*, 2006 and Sunkar *et al.*, 2007). Non-conserved miRNAs may play more specific roles in specific plant species, such as the differentiation and elongation of cotton fibres (Cheng, 2007), nodulation regulated miRNAs in soybean root (Subramanian *et al.*, 2008).

2.2 Biogenesis of plant miRNAs

miRNA biogenesis requires multiple steps in order to form mature miRNAs from miRNA genes (Bartel, 2004 and Kurihara and Watanabe, 2004). First, a miRNA gene is transcribed to a primary miRNA (pri-miRNA), which is usually a long sequence of more than several hundred nucleotides. This step is controlled by Polymerase II enzymes (Bartel, 2004 and Lee *et al.*, 2004). Second, the pri-miRNA is cleaved to a stem loop intermediate called miRNA precursor or pre-miRNA. This step is controlled by the Drosha RNase III endonuclease in animals (Lee *et al.*, 2002 and Bartel, 2004) or by Dicer-like 1 enzyme (DCL1) in plants ((Liang *et al.*, 2007). In animals, pre-miRNAs are then transported by exportin 5 from the nucleus into the cytoplasm (Zeng and Cullen, 2004). Only the active miRNA strand of the duplex, but not the passenger strand (miRNA*) is incorporated into the RNA induced silencing complex (RISC). In this step, however, plant miRNAs differ from animals in that they are cleaved into miRNA:miRNA* duplex possibly by dicer-like enzyme 1 (DCL1) in the nucleus rather than in the cytoplasm (Papp *et al.*, 2003), then the duplex is translocated into the cytoplasm by HASTY, the plant orthologue of exportin 5 (Park *et al.*, 2005).

The accumulation of mature miRNAs is thought to be affected by DCL1 in response to different stresses and phytohormone like GA and ABA (Zhang *et al.*, 2008). In the cytoplasm, both plant and animal miRNAs are unwound into single strand, mature miRNAs by helicase. Finally, the mature miRNAs enter a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC) (Schwarz *et al.*, 2002) where they regulate targeted gene expression. This suggests that miRNA biogenesis is required several enzymes for processing of long pri-miRNA to ~20–24 nucleotides mature miRNAs. The mature miRNA is typically 19–22 nucleotides long. Recently identified ABH1/CBP80, a subunit of the mRNA cap binding complex, is necessary to obtain proper mature miRNA levels, which suggests that this protein affects the miRNA-mediated RNA silencing pathway (Brian *et al.*, 2008). Along with this, several proteins like SE (SERRATE) is also required for proper processing of miRNA (Laubinger *et al.*, 2008). Plant miRNAs are mostly involved in transcriptional regulation, developmental timing, cell death, cell proliferation and even nutrient metabolism (Abdel and Pilon, 2008). Recent studies reveal that under stress conditions a specific subset of miRNAs were exported from nucleus to cytoplasm through the karyopherin (importin beta) family of proteins in a Ran-GTPase –dependent pathway (Chinnusamy *et al.*, 2008), which in turn regulated the copies of miRNA in the cytoplasm. Another recent experiment showed that uncapped endogenous transcripts in cytoplasm could become a substrate for small RNA biogenesis (Gregory *et al.*, 2008).

2.3 Molecular mechanisms of plant gene regulation by miRNAs

In the RISC complex, miRNAs bind to target mRNA and inhibit gene expression through its cleavage. This causes gene silencing (Lee *et al.*, 1993); termed RNA interference (RNAi) in animals (Hannon, 2002), quelling in fungi (Cogoni *et al.*, 1996), and post-transcriptional gene silencing (PTGS) in plants (Baulcombe, 2004). In plants, most target mRNAs contain a single miRNA complementary site, and most corresponding miRNAs typically, perfectly complement to these sites and cleave the target mRNAs (Bartel, 2004). Unlike animal miRNA targets, the complementary sites in plants can exist anywhere along the target mRNA rather than at the 3'-UTR. Another mechanism was identified in plant miRNA

regulation such as miR172, where it regulated gene expression by repressing translation possibly, through inhibition of ribosome movement (Aukerman and Sakai, 2003 and Chen, 2004). However, in another study, it was observed that miR172 can also efficiently guide cleavage of its target transcripts (Schwab *et al.*, 2005). This suggests that miRNAs mediated regulation of gene expression in plants is more complex than in animals (Flynt and Lai, 2008).

To provide new insights into the mechanism of plant miRNA function, Schwab *et al.* (2005) employed genome-wide expression profiling to analyze parameters for miRNA-guided mRNA cleavage and deduced a set of empirical parameters for miRNA target recognition and concluded that plant miRNAs have higher specificity than siRNAs. Their study also demonstrated that feedback regulation of miRNA makes the effect of miRNAs on steady-state level of target mRNAs more complicated. Feedback regulation of miRNA targets may provide a sensitive mechanism for fine tuning the expression of target genes.

2.4 Diversity of plant miRNA functions

miRNA is considered as one of the most important post-transcriptional gene regulators. In animals, miRNAs have proven to be involved in many functional processes such as development (Wienholds *et al.*, 2005 and Mead and Tu, 2008), stress response (Ambros *et al.*, 2003), and carcinogenesis (Iorio *et al.*, 2005). Although investigations of plant miRNAs are less extensive, the versatile functions of plant miRNAs are becoming clear (Sunkar *et al.*, 2008). A majority of miRNA functions are currently investigated by over-expression or lowered expression of miRNAs.

2.4.1 miRNAs in plant development

Dicer-like enzyme 1 (DCL1) is an important enzyme that processes pri-miRNA to pre-miRNA then to miRNA:miRNA* duplex. Normal expression of the *dcl1* gene is essential to synthesize and accumulate mature miRNA (Papp *et al.*, 2003). Recently, it was found that a regulatory protein, HYL1 is required for miRNA processing and maturation (Laubinger *et al.*, 2008). Loss-of-function of these genes reduced the level of mature miRNA and consequently caused many developmental abnormalities, such as altered leaf shape, delayed floral transition, arrested embryos at early stages, and female sterility (Dugas and Bartel, 2004 and Laubinger *et al.*, 2008). In addition, two cap binding proteins CBP20 and CBP80 were found to be responsible in processing of primary miRNAs (Kim *et al.*, 2008). HASTY is an orthologue of exportin 5, which is the protein transporting miRNA:miRNA* duplex from the nucleus to the cytoplasm in *Arabidopsis* (Park *et al.*, 2005). Without HASTY, it is impossible to synthesize mature miRNAs in *Arabidopsis*. These findings together indicate that miRNAs are involved in a variety of developmental processes in plants.

Several experiments have demonstrated that many miRNAs regulate various plant development processes, including leaf morphogenesis and polarity (Kim *et al.*, 2005), floral differentiation and development (Chen, 2004), root initiation and development (Guo *et al.*, 2005), vascular development (Kim *et al.*, 2005), and transition of plant growth from vegetative growth to reproductive growth (Itaya *et al.*, 2008). A majority of these miRNAs affect plant traits by regulating the expression of transcription factors that influence cell fate determination (Mallory *et al.*, 2004). Nearly 50 percent of the miRNA targets that have been identified are transcription factors. Of these miRNAs, at least 12 miRNA families target mRNAs encoding transcriptional factors that regulate plant development (Allen *et al.*, 2004). Combined, these studies have illustrated that a majority of miRNAs regulate plant development by controlling the levels of transcription factors that are important in development.

2.4.2 miRNA and leaf development

Plant leaves exhibit an asymmetry pattern along the adaxial/abaxial (upper/lower) axis which is controlled by the polarized expression of class-III homeodomain leucine zipper (HD-ZIP) transcription factor genes (Juarez *et al.*, 2004). PHABULOSA (PHB), PHAVOLUTA (PHV), and REVOLUTA (REV) are three closely related *Arabidopsis* HD-ZIP transcription factors. 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 (Emery *et al.*, 2003). Several experiments have demonstrated that all of these transcription factors are

the targets of miR165 and miR166, and are regulated by these two miRNAs (Bao *et al.*, 2004 and Kim *et al.*, 2005).

The HD-ZIP gene family is conserved in all lineages of land plants, including mosses, ferns, gymnosperms, and angiosperms (Floyd and Bowman, 2004). The miR165 and miR166 targeting the HD-ZIP gene family, are also evolutionarily conserved in all lineages of land plants (Zhang *et al.*, 2005). The developmental abnormality caused by loss-of-function *HD-ZIP* genes in *Arabidopsis* was also observed in other plant species. Juarez *et al.* (2004) found that miR166 directly cleaves *rd1* mRNA and alters leaf polarity in maize. In their experiments, miRNA 166 exhibited a progressively expanding expression pattern in leaves and phloem during leaf development.

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). Overexpression of miR159 resulted in low levels of all tested TCP mRNAs and caused jaw-D phenotypes, including uneven leaf shape and curvature. In contrast, over-expression of miR159-resistant TCP mutants indicated that miR159-guided mRNA cleavage was sufficient to restrict TCP function. miR165/166 and miR159/JAW are essential for controlling the pattern and development of leaves by direct regulation of two classes of transcription factor genes (HD-ZIP and TCP).

2.4.3 miRNA in floral development and vegetative phase change

Flower development is one of the most important stages of plant development, especially for flowering plants. Mature flowers consist of carpels, stamens, petals, and sepals. The origin of the flower can be well explained by the ABC model (Lohmann and Weigel, 2002 and Jack, 2004). According to this model, four flower organs are controlled by the combinatorial actions of three classes (A, B, and C) of transcription factors. APETALA 2 (AP2) is one of the class A genes that play an important role in flowering time and flower morphology. AP2 and AP2-like proteins are required for plant flowering and floral organ identity. Independently in two laboratories it has been demonstrated that AP2 is one of the targets of miR172 (Chen, 2004 and Aukerman and Sakai, 2003). Over expression of miR172 inhibited the translation of the AP2 gene and AP2-like genes and resulted in early flowering and disrupting the specification of floral organ identity similar to loss-of-function *ap2* gene mutants. miR172 regulates *ap2* gene expression through translational inhibition rather than through mRNA cleavage (Chen, 2004).

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

GAMYB is a positive regulator of LEAFY, which is an important factor in floral development (Kaneko *et al.*, 2004). GAMYB is one target of miR159. Studies indicated that miR159 directly guides the cleavage of mRNA encoding GAMYB-related proteins and further affects the GA-promoted activation of LEAFY. Over-expression of miR159 resulted in a reduction of LEAFY transcript levels and further perturbed anther development and delayed flowering in short-day photoperiods (Achard *et al.*, 2004). This indicates that miR159-guided GAMYB cleavage regulates plant anther development and flowering time.

miR156 is also involved in floral development and phase change by targeting members of Squamosa promoter binding protein like (SPL) plant-specific transcription factors. The SPL family has 16 members; some are involved in floral transition and regulating plant flowering (Cardon *et al.*, 1997). Results indicated that over-expression of miR156 affects phase transition from vegetative growth to reproductive growth, including the quickly initiation of rosette leaves, a severe decrease in apical dominance, and a moderate delay in flowering (Schwab *et al.*, 2005).

2.4.4 miRNA in shoot and root development

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). They play important roles in both embryogenic and floral development (Hibara *et al.*, 2003). Loss-of-function of these genes resulted in abnormal floral and shoot development (Mallory *et al.*, 2004). Five members (CUC1, CUC2, NAM, NAC1, and At5g07680) of the NAC-domain gene family in *Arabidopsis* were identified that have complementary sites with miR164 and are targets of miR164 (Rhoades *et al.*, 2002 and Guo *et al.*, 2005). Of these, NAC1 is involved in lateral root development (Xie *et al.*, 2002), whereas CUC1 and CUC2 regulate meristem development and separation of aerial organs (Aida *et al.*, 1997). Over-expression of miR164 resulted in the fusion of vegetative and floral organs, unbalanced floral organ numbers, and reduced lateral root emergence (Laufs *et al.*, 2004 and Guo *et al.*, 2005). In contrast, miR164-resistant mutants of CUC1 and CUC2 resulted in *Arabidopsis* embryonic, vegetative, and floral development defects, including misshaping of rosette leaves, altering flower structure, and changing cotyledon orientation (Mallory *et al.*, 2004). miR164 also control organ boundaries and root formation by regulating NAC1 and CUC2 expression. Loss-of-function miR164 mutants accumulated NAC mRNA, resulting in more lateral root formation. In contrast, over expression of miR164 in wild-type *Arabidopsis* resulted in decreasing NAC1 mRNA levels and decreased lateral root emergence (Guo *et al.*, 2005). miR164 plays an important role in controlling shoot and root development by regulating several NAC transcription factors. It may be possible to increase crop yields and resistance to environmental stress by appropriately controlling miR164 expression.

2.4.6 miRNA in vascular development

HD-ZIP proteins also regulate vascular development as well as lateral organ polarity and meristem formation. ATHB15, a member of the HD-ZIP family, is predominantly expressed in vascular tissues, suggesting that it may play some role in plant vascular development (Kim *et al.*, 2005). ATHB15 is one of the targets of miR166 (Rhoades *et al.*, 2002). Over-expression of miR166a resulted in decreasing ATHB15 mRNA levels and caused accelerated vascular cell differentiation from cambial/procambial cells and consequently produced an altered vascular system with expanded xylem tissue and an inter-fascicular region (Kim *et al.*, 2005). This regulation mechanism may exist in all vascular plant species (Floyd and Bowman, 2004). In addition, some miRNAs may function in the biosynthesis of cell wall metabolites (Lu *et al.*, 2005b) or cotton fibre development (Zhang *et al.*, 2005). Recently, a set of miRNAs were identified and characterized from *Brassica napus* which were showing stress specific expression in phloem (Buhtz *et al.*, 2008).

2.4.7 miRNAs regulate miRNA and siRNA biogenesis and function

Regardless of the regulation mechanism (mRNA cleavage or translational repression), miRNA-guided gene regulation requires that miRNA form a complex with RISC. RISC contains many components; loss-of-function of each component affects the function of RISC and also affects the function of miRNAs. ARGONAUTE proteins are a core component of RISC. ARGONAUTE proteins contain a single-stranded RNA binding site (Yan *et al.*, 2003). This characteristic allows ARGONAUTE proteins to directly associate with miRNAs before and after RISC recognizes their mRNA targets (Vaucheret *et al.*, 2004).

There are 10 proteins in the *Arabidopsis* ARGONAUTE protein family (Morel *et al.*, 2002). Several experiments have indicated that ARGONAUTE1 (AGO1) is required for stem cell function and organ polarity (Kidner and Martienssen, 2005). Computational analysis and 5'RACE experiments demonstrated that only ARGONAUTE 1 (AGO1) mRNA is the target of miR168 (Rhoades *et al.*, 2002 and Vazquez *et al.*, 2004). miR168-guided cleavage of AGO1 mRNA is crucial for the post-transcriptional gene regulation of the *ago1* gene. Vaucheret *et al.* (2004) demonstrated that changing the nucleotides of AGO1 mRNA and making them less complementary with miR168 resulted in increasing levels of AGO1 mRNA and consequently caused developmental defects similar to *dcl1* or *hen1* mutants. Increased target mRNA suggested that non-functional RISCs were formed in the mutant plants. These defects were rescued by expressing a compensatory miRNA that is complementary to the mutant AGO1 mRNA.

miRNA not only regulates the expression of genes controlling plant development but also regulates its own biogenesis and/or function. Five miRNAs (miR162, miR168, miR173, miR390, and miR398) are known to regulate miRNA biogenesis or function (Sunkar *et al.*, 2008).

2.4.9 miRNAs involved in signal transduction

Several independent laboratories observed that miRNAs regulate key components of hormone signalling pathways and further regulate hormone homeostasis and related developmental processes (Sorin *et al.*, 2005, Mallory *et al.*, 2005 and Guo *et al.*, 2005). Several miRNAs affect signal transduction, especially hormone signalling pathways. Plant hormones, especially auxin, are important regulators of plant growth and development, particularly plant cell division, elongation, and differentiation (Palme *et al.*, 1991 and Woodward and Bartel, 2005). Plant hormones also play important roles in organ formation and response to various environmental stresses (Palme *et al.*, 1991 and Woodward and Bartel, 2005). After analysis of a large number of ESTs, Zhang *et al.* (2005) found that several miRNAs (miR159, miR160, miR164, and miR167) were shown in tissues induced by abscisic acid (ABA), GA, jasmonic acid (JA), salicylic acid (SA), and other phytohormones. miR159 is a modulator of GAMYB through the miRNA-guided cleavage of mRNA encoding GAMYB-related proteins that are transcription factors regulating LEAFY (Achard *et al.*, 2004). Achard *et al.* (2004) demonstrated that miR159 was positively regulated by the plant hormone gibberellin (GA). Overexpression of miR159 resulted in decreased LEAFY mRNAs, delayed flowering time, and altered floral development (Achard *et al.*, 2004).

Auxin (principally indole-3-acetic acid) is an important hormone in plants. It affects many aspects of plant growth and development by influencing AUXIN RESPONSE FACTORS (ARF), a plant-specific family of DNA binding proteins (Hagen and Guilfoyle, 2002 and Weijers and Jurgens, 2005). ARFs regulate the expression of auxin-inducible genes, such as GH3 and auxin/indole-3-acetic acid (Aux/IAA) by binding auxin response promoters (AREs) (Hagen and Guilfoyle, 2002). Out of 23 ARFs in *Arabidopsis*, at least 5 ARFs have complementary sites with miRNAs. ARF10, ARF16, and ARF17 are potential targets of miR160, and ARF6 and ARF8 are potential targets of miR167 (Rhoades *et al.*, 2002 and Mallory *et al.*, 2005). Mallory *et al.* (2005) demonstrated that disrupting miR160 function increased ARF10, ARF16, and ARF17 mRNA levels, altered GH3-like gene expression, and led to severe developmental defects. It has also been observed that miR160-resistant ARF plants have dramatic developmental abnormalities (Mallory *et al.*, 2005 and Sorin *et al.*, 2005).

NAC1 is a transcriptional activator that transduces auxin signals for lateral root initiation by regulating transport inhibitor response 1 (TIR1) (Xie *et al.*, 2002 and Guo *et al.*, 2005). Guo *et al.* (2005) demonstrated that loss-of-function miR164 mutants blocked the auxin signalling pathway, resulting in increased NAC1 mRNA levels and more lateral root production. In contrast, overexpression of miR164 resulted in downregulation of NAC1 gene expression and reduced lateral root initiation (Guo *et al.*, 2005). The expression of miR164 and NAC1 can be regulated by auxin through a signalling pathway (Guo *et al.*, 2005). The induction of miR164 at least partially by auxin indicated an autoregulatory loop in which miRNA-guided NAC1 mRNA cleavage attenuated and terminated auxin signalling (Guo *et al.*, 2005). miR164-guided NAC mRNA cleavage downregulates auxin signals for lateral root development.

In addition to miR160, miR164, and miR167, other miRNAs, such as miR393, may also be involved in signalling pathways by regulating TIR1, an important component of a SCF E3 ubiquitin ligase which degrades Aux/IAA proteins in response to auxin (Gray *et al.*, 2001 and Mallory *et al.*, 2005). Studies show, TIR1 and its three most closely related F-box proteins are predicted to be targets of miR393 (Sunkar and Zhu, 2004 and Jones-Rhoades and Bartel, 2004). This suggests that miRNA also can target F-box proteins and affect the activity of the E3 enzyme. All of these studies indicate that miRNAs are involved in hormone-mediated signal transduction. miRNAs can either directly target ARF and NAC1 or be induced by plant hormones.

2.5 miRNAs involved in stress response

Plants have evolved complicated mechanisms to overcome a variety of environmental stresses, where miRNAs also play an important role (Zhang *et al.*, 2008). About 25.8 percent of ESTs containing miRNAs were found in stress-induced plant tissues (Zhang *et al.*, 2005) and many studies have provided supportive evidence for this hypothesis (Liu *et al.*, 2008 and Sunkar *et al.*, 2008). Using microarray platform, a set of stress responsive miRNAs were identified in *Arabidopsis*. Among them, 10 high-salinity, 4 drought and 10 cold-regulated miRNAs were detected (Liu *et al.*, 2008). They also found that there is a cross-talk among the high salinity, drought, and cold stress signalling pathways, which extend the current view about miRNA as ubiquitous regulators under stress conditions. Recently, Sunkar *et al.* (2008) discovered 23 low-abundance new miRNAs and 40 candidate miRNAs in rice. Most of them were found drought and salt stress responsive. All the 23 new miRNAs were derived from a unique locus in rice genome.

miRNAs also regulate the nutrient starvation in plants (Bartel, 2004). Evidence comes from the role of miR399 (Bikram *et al.*, 2008) and miR395 (Daniel *et al.*, 2007). miR399 regulates phosphate homeostasis, and accumulates in higher copies in phloem and root tissues (Bikram *et al.*, 2008). Similarly, in sulphate limited conditions, miR395 was over expressed quantities to regulate the nutrient signalling pathways in plants (Daniel *et al.*, 2007). At low-sulfur conditions, the ATP sulfurylase APS4 and the sulfate transporter AST68 are accumulated, which are the targets of miR395 (Allen *et al.*, 2005).

Drought, cold, and salinity are the other environmental stresses for plants; all of these conditions strongly induce miR402 over expression but miR319 was expressed highly in cold stress (Sunkar and Zhu, 2004). Lu *et al.* (2005b) identified 48 miRNA sequences from the *Populus* genome and found that a majority of these *Populus* miRNAs target developmental- and stress/defense-related genes. They also found that plant miRNAs can be induced by mechanical stress and may function in critical defence systems for structural and mechanical fitness.

Abscisic acid (ABA) and gibberellin (GA) are two kinds of phytohormones which are involved in plant responses to different environmental stresses (Sunkar and Zhu, 2004). Two independent studies revealed that ABA and GA treatment regulates miR159 expression (Sunkar and Zhu, 2004 and Achard *et al.*, 2004). Recently, ABA was found to be involved in the accumulation of mature miRNAs in cells (Zhang *et al.*, 2008). Therefore, miRNAs appear to be involved in plant responses to a variety of biotic and abiotic stresses which induces certain miRNAs to be over or under-expressed, or plants may have evolved some mechanism to synthesize certain miRNAs to regulate the developmental stages and metabolic pathways (Lu *et al.*, 2008).

2.6 Identification of miRNAs

Methods for identifying miRNAs are based on their major characteristics: (A) all miRNAs are small non-coding RNAs, usually consisting of ~20–22 nucleotides for animals and ~20–24 nucleotide for plants (Reinhart *et al.*, 2002 and Bartel, 2004). (B) All miRNA precursors have a well-predicted stem loop hairpin structure, and this fold-back hairpin structure has a low free energy (Reinhart *et al.*, 2002 and Bartel, 2004) as predicted by MFOLD (Zuker, 2003) or other computational programs. Statistical analysis shows that a majority of miRNA precursors have considerably lower free energy (–32 to –57 kcal/mol) than transfer RNAs (tRNAs) (–26 to –29 kcal/mol) or ribosomal RNAs (rRNAs) (–33 kcal/mol) (Bonnet *et al.*, 2004). (C) Many miRNAs are evolutionarily conserved, some from worm to human (Weber, 2005 and Altuvia *et al.*, 2005), or from ferns to higher plants (Llave *et al.*, 2002 and Zhang *et al.*, 2005). The mature miRNAs are conserved in plants rather than miRNA precursors that are usually conserved in animals (Bartel, 2004). However, some of these characteristics are not unique to miRNAs (Ambros *et al.*, 2003). For example, tRNAs also have stem loop structures. Therefore, when a small RNA is considered a miRNA, all of these major characteristics should be included. To avoid designating other small RNAs or fragments of other RNAs as miRNAs, Ambros *et al.* (2003) developed combined criteria to identify new miRNAs. These combined criteria include both biogenesis and expression

criteria, neither of which on its own is sufficient for identifying a candidate gene for a new miRNA (Ambros *et al.*, 2003). So far, all newly predicted/identified miRNAs have confirmed to these rules.

There are four approaches for identifying miRNAs: 1) genetic screening (Lee *et al.*, 1993 and Wightman *et al.*, 1993), 2) direct cloning after isolation of small RNAs (Mead and Tu, 2008), 3) computational strategy (Mathews *et al.*, 1999), and 4) expressed sequence tags (ESTs) analysis (Zhang *et al.*, 2006). In 1993, two research groups led by Ambros and Ruvkun found that a small 22 nucleotides RNA derived from a gene called *lin-4* suppressed the expression of *lin-14*, which controls the timing of *Caenorhabditis elegans* larval development (Lee *et al.*, 1993 and Wightman *et al.*, 1993). *lin-4* is recognized as the founding member of a new class of small RNAs called miRNAs (Lee and Ambros, 2001, Bartel, 2004). Since *lin-4* was recognized as a miRNA in 2001, thousands of miRNAs have been identified from various organisms including *C. elegans*, *Drosophila melanogaster*, fish, mouse, rat, and human (Lai *et al.*, 2003 and Shunmin, 2008).

Plant miRNAs were identified 10 years later. In 2002, several groups found plant miRNAs by cloning small RNAs (Park *et al.*, 2002). Since then, different approaches have been employed to identify new miRNAs. Initially, miRNAs were identified by genetic screening (Lee *et al.*, 1993 and Wightman *et al.*, 1993). This method was similar to methods for identifying other traditional genes. Although this method was useful for identifying some miRNAs, such as *lin-4* and *lin-7*, the application of this method was limited because it is expensive, time consuming, and dominated by chance (Bartel, 2004).

An experimental approach described for isolating and identifying new miRNAs involves direct cloning after isolation of small RNAs (Lu *et al.*, 2005a and Fu *et al.*, 2005). In this approach, small RNA molecules are first isolated by size fractionation. Then these small RNAs are ligated to RNA adapters at their 5' and 3' ends. Finally, they are reverse transcribed into cDNA, which is then amplified and sequenced. Because only small RNAs are isolated and screened by this method, it is a more efficient way to obtain miRNAs than general genetic screening. The approach has been used to identify new miRNAs in several organisms including animals (Edward and Zhijian, 2008) and plants (Xie *et al.*, 2005 and Sunkar *et al.*, 2005). Lu *et al.* (2005a) further refined this method by combining it with massively parallel signature sequencing (MPSS) to study *Arabidopsis* miRNAs. Besides these, high throughput pyrosequencing was also found suitable to identify novel miRNAs in plants (Sunkar *et al.*, 2008).

The third approach is the computational approach. Computational approaches are successful in identifying conserved miRNAs in many plants and animals, but they require knowledge of the complete genome sequence, which is unavailable for most plant species. However, large genomic data in the form of genome survey sequences (GSSs), high-throughput genomics sequences (HTGSs) and nonredundant nucleotides (NRs), are available for several plant species and can be used for identification of conserved miRNAs. GSS and HTGS of GeneBank represent short stretches of genomic sequence, still providing a broader sampling of unfinished genomes. The NR database contains finished genomic sequences and cDNAs. This approach is based on a genome sequence (Jones-Rhoades and Bartel, 2004, Sunkar *et al.*, 2005). Several laboratories have designed computational programs, such as MIRscan (Lim *et al.*, 2003a and Lim *et al.*, 2003b) and MiRAlign (Wang *et al.*, 2005), and successfully predicted miRNA genes in *Arabidopsis* (Jones-Rhoades and Bartel, 2004; Adai *et al.*, 2005), rice (Li *et al.*, 2005), *Brassica* sp. (Xie, *et al.*, 2007), human (Bentwich *et al.*, 2005), *C. elegans* (Grad *et al.*, 2003), and other animals (Du and Zamore, 2007). At present, a majority of the miRNAs are deposited in miRNA databases. However, the actual computational approaches used are slightly inefficient and certainly not comprehensive (Li *et al.*, 2005). The predicted miRNAs need to be confirmed by experiments such as cloning or Northern blotting.

The fourth approach is an expressed sequence tag (EST) analysis approach. It is well recognized that several miRNAs are evolutionarily conserved from species to species (Reinhart *et al.*, 2002; Axtell and Bartel, 2005). Zhang *et al.* (2006) identified conserved miRNAs in plants using ESTs alone. This suggests a powerful approach to predict homologues or orthologues of previously known miRNAs. Weber *et al.* (2005) used this

principle to find 35 new human and 45 new mouse putative miRNA genes. Using ESTs and GSS miRNA genes and their putative targets were identified in cotton (Cheng, 2007). More importantly, this approach is very useful for predicting miRNAs in multiple species and recently a set of miRNA and their targets were reported using the largest data set of triticeae ESTs (Dryanova *et al.*, 2008). In rice, six new miRNAs were identified which are conserved in other monocots (Sunkar *et al.*, 2008). Allowing not more than 3 mis-matches between miRNA and its target mRNA Sunkar *et al.* (2008) predicted 20 targets for 9 of the new miRNAs.

2.7 Identification of miRNA targets

Since the first discovery of miRNAs *lin-4* and *let-7* by genetic analysis of *C. elegans* developmental timing (Lee *et al.*, 1993; Reinhart *et al.*, 2000), numerous computational models and tools have been developed which complement biological experiments to understand the diverse regulatory roles of miRNAs. The bioinformatics approaches have been useful in understanding the complexity of miRNA genes and their targets. Most computational methods used for miRNA studies can be classified into two broad categories; 1) miRNA identification and 2) miRNA target identification (Sungroh and Giovanni, 2006). Methods do vary depending on whether a method is for animals, plants, or viruses, since the biology of miRNAs is somewhat different in each case. A miRNA target detection algorithm can be trained using the properties of known miRNA-mRNA duplexes and can then be used for finding new miRNA-mRNA duplexes (Sungroh and Giovanni, 2006).

Using all publicly available nucleotide databases, 682 miRNAs were identified in 155 diverse plant species by computational approach as well as the expression analysis (Sunkar *et al.*, 2008). These findings will be useful for tracing the evolution of small RNAs by examining their expression in common ancestors of different plant lineages. Many plant miRNAs are evolutionarily conserved from species to species and from angiosperms to mosses (Zhang *et al.*, 2006). Although miRNA precursors display high variability, their mature sequences display extensive sequence complementarity to their target mRNA sequences. Despite differences in the architecture of miRNA-based regulation, miRNAs and their targets are similarly conserved in both plants and animals (Ehrenreich, 2008).

There is abundance of transcripts that do not code for any particular protein and remain functionally uncharacterized. fRNAdb, a novel, newly introduced database service (Kin *et al.*, 2007) contains the entire set of non-coding transcripts including annotated/non-annotated sequences from H-inv database, NONCODE and RNAdb. This could be a potential source to analyse RNA secondary structure motif discovery, EST support evaluation and others. Recently, MotifModeler informatics program has been introduced which can efficiently identify miRNA and their target transcription factors (Guohua *et al.*, 2008).

To date, a total of 1638 plant miRNAs have been deposited in the miRNA miRBase database (Release 12.0). Many of them are conserved during the evolution of land plants, suggesting that the well-conserved miRNAs may also retain homologous target interactions (Yin *et al.*, 2008). Experimental identification of miRNA targets is a difficult and time consuming process. As a consequence several computational prediction methods have been devised in order to predict targets for follow up experimental validation such as Stacking Binding Matrix (SBM) that uses both the information about the miRNAs as well as experimentally validated target sequences in the search for candidate target sequences (Moxon *et al.*, 2008). Recently, 79 miRNA candidates were discovered by using wheat EST sequences (Jin *et al.*, 2008). Similarly, deep sequencing proved to be an effective strategy that allowed the discovery of low-abundance new miRNAs and candidate miRNAs (Sunkar *et al.*, 2008) and to recover some of the hits missed by 454 sequencing can be a valuable tool to assign probability-based scores to them (Vacic *et al.*, 2008).

2.8 miRNA and *in situ* hybridization

In situ hybridization techniques allow specific nucleic acid sequences to be detected in morphologically preserved chromosomes, cells or tissue sections in temporal and spatial manner. In combination with immunocytochemistry, *in situ* hybridization can relate microscopic topological information to gene activity at the DNA, RNA and protein level.

The technique was independently developed by Pardue and Gall (1969) and John *et al.* (1969). Then radioisotopes were the only labels available for nucleic acids, and autoradiography was the only means of detecting hybridized sequences. Furthermore, as molecular cloning was not possible in those days, *in situ* hybridization was restricted to those sequences that could be purified and isolated by conventional biochemical methods (e.g., mouse satellite DNA, viral DNA, ribosomal RNAs). Molecular cloning of nucleic acids and improved radiolabeling techniques has changed this picture dramatically. For example, DNA sequences a few hundred base pairs long can be detected in metaphase chromosomes by autoradiography (Schroeder *et al.*, 1984). Radioactive *in situ* techniques can also detect low copy number mRNA molecules in individual cells (Harper *et al.*, 1986). In spite of the high sensitivity and wide applicability of *in situ* hybridization techniques, their use has been limited to some research laboratories. This is largely due to the problems associated with radioactive probes, *viz.*, safety measures required, limited shelf life, time required for autoradiography, the scatter inherent in radioactive decay limiting the spatial resolution etc.

2.8.1 Non-radioactive probes for *in situ* hybridization

Preparing nucleic acid probes with a stable nonradioactive label removes the major obstacles which hinder general application of *in situ* hybridization. It also opens new opportunities for combining different labels in one experiment. Many sensitive antibody detection systems available for such probes further enhance the flexibility of this method.

There are two types of nonradioactive hybridization methods: direct and indirect. In the direct method, the detectable molecule (reporter) is bound directly to the nucleic acid probe so that probe-target hybrids can be visualized under a microscope immediately after the hybridization reaction. For such methods it is essential that the probe-reporter bond survives the rather harsh hybridization and washing conditions. Perhaps more important is that the reporter molecule does not interfere with the hybridization reaction. The terminal fluorochrome labeling procedure of RNA probes developed by Bauman *et al.* (1984), and the direct enzyme labeling procedure of nucleic acids described by Renz and Kurz (1984) meet these criteria. Roche Applied Science has introduced several fluorochrome-labeled nucleotides that can be used for labeling and direct detection of DNA or RNA probes. If antibodies against the reporter molecules are available, direct methods may also be converted to indirect immunochemical amplification methods (Pinkel *et al.*, 1986). Indirect procedures require the probe to contain a reporter molecule, introduced chemically or enzymatically, that can be detected by affinity cytochemistry. Again, the presence of the label should not interfere with the hybridization reaction or the stability of the resulting hybrid. The reporter molecule should, however, be accessible to antibodies. A number of such hapten modifications have been described (Raap *et al.*, 1989). One of the most popular systems is offered by Roche Applied Science: the Digoxigenin (DIG) System has wide uses and acceptability in different research areas (Valoczi *et al.*, 2006; Maria Cartolano *et al.*, 2007). Many years ago, the chemical synthesis of oligonucleotides containing functional groups (e.g., primary aliphatic amines or sulfhydryl groups) was described. These can react with haptens, fluorochromes or enzymes to produce a stable probe which can be used for *in situ* hybridization experiments (Haralambidis *et al.*, 1987). Modified oligonucleotides can also be obtained with the DIG system (Mühlegger *et al.*, 1990). Such oligonucleotide probes will undoubtedly be widely used as automated oligonucleotide synthesis makes them available to researchers not familiar with DNA recombinant technology.

2.8.2 Digoxigenin in *in situ* hybridization

The Digoxigenin (DIG) System was developed and continues to be expanded by Roche Applied Science (Höltke *et al.*, 1992). The DIG labeling method is based on a steroid isolated from digitalis plants (*Digitalis purpurea* and *Digitalis lanata*). 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. Nucleic acids can also be labeled chemically with DIG-ester or with DIG Chem-Link. Hybridized DIG-labeled probes may be detected with high affinity anti-digoxigenin (anti-DIG) antibodies that are conjugated to alkaline phosphatase, peroxidase, fluorescein, rhodamine, or colloidal gold. Alternatively, unconjugated anti-digoxigenin antibodies and conjugated secondary antibodies may be used.

Detection sensitivity depends upon the method used to visualize the anti-DIG antibody conjugate. For instance, an anti-DIG antibody conjugated to alkaline phosphatase is visualized with colorimetric (NBT and BCIP) or fluorescent (HNPP) alkaline phosphatase substrates.

2.8.3 miRNA and LNA probes

When the experiment is designed to detect the small non-coding RNA like miRNA in different tissues of plants and animals it's better to go for very specific probes like Locked Nucleic Acid (LNA) probes, which can differentiate even in single nucleotide difference in a sequence while hybridizing. Valoczi *et al.* (2006) demonstrated that miRNAs accumulated spatially and temporally in a highly restricted manner in *Nicotiana benthamiana* and *Arabidopsis thaliana*. Their results imply that the spatially and temporally organized accumulation of miRNAs plays a pivotal role in fine-tuning of target gene expression in plant development and also in their detection. A conserved miRNA module that exerts homeotic control over *Petunia hybrida* and *Antirrhinum majus* floral organ identity was confirmed by *in situ* hybridization (Maria *et al.*, 2007). To exploit the improved hybridization properties of LNA with their target RNA molecules, Valoczi *et al.* (2004) designed several LNA-modified oligonucleotide probes for detection of different miRNAs in animals and plants.

Modified DNA oligonucleotides with LNAs using a design, in which every third nucleotide position is substituted by LNA, they could be used as the probes in northern blot analysis employing standard end-labelling techniques and hybridization conditions. The sensitivity in detecting mature miRNAs by LNA northern blots was increased by at least 10-fold compared to DNA probes, while being highly specific, as demonstrated by the use of different single and double mismatched LNA probes. Besides being highly efficient as northern probes, the same LNA modified oligonucleotide probes would also be useful for miRNA *in situ* hybridization and miRNA expression profiling by LNA oligonucleotide microarrays.

LNAs show remarkable affinity and specificity against native DNA and RNA targets. Effects of LNA modifications on mismatch discrimination were studied as a function of sequence context and identity of the mismatch using ultraviolet (UV) melting experiments (You *et al.*, 2006). A triplet of LNA residues centered on the mismatch was generally found to have the largest discriminatory power. An exception was observed for G–T mismatches, where discrimination decreased when the guanine nucleotide at the mismatch site or even the flanking nucleotides were modified. Fluorescence experiments using 2-aminopurine suggest that LNA modifications enhance base stacking of perfectly matched base pairs and decrease stabilizing stacking interactions of mismatched base pairs (You *et al.*, 2006). New guidelines are available for design of LNA probes, which significantly improve mismatch discrimination in comparison with unmodified DNA probes.

A novel microarray platform for genome-wide profiling of mature miRNAs (miChip) using LNA-modified capture probes was developed (Mirco *et al.*, 2006). The biophysical properties of LNA were exploited to design probe sets for uniform, high-affinity hybridizations, yielding highly accurate signals, able to discriminate between single nucleotide differences and, hence, between closely related miRNA family members. The superior detection sensitivity eliminates the need for RNA size selection and/or amplification. miChip will greatly simplify miRNA expression profiling of biological and clinical samples.

Low signal intensity due to poor probe hybridization efficiency is one of the major drawbacks of rRNA targeted *in situ* hybridization. There are two major factors affecting the hybridization efficiency: probe accessibility and affinity to the targeted rRNA molecules. A remarkable improvement in efficiency was achieved by applying LNA-incorporated oligodeoxynucleotide probes (LNA/DNA probes) without compromising specificity of *in situ* hybridization. The fluorescence intensity was increased by 22-fold after three LNA bases were substituted for DNA bases (Kengo *et al.*, 2006). This enabled several research groups to analyze the expression patterns of miRNAs in both plant and animal tissues (Guy *et al.*, 2007).

LNA are now used nowadays for diverse applications in miRNA research viz., miRNA profiling (Mirco *et al.*, 2006), real-time expression analysis (Jean *et al.*, 2006), SNP discovery (Joy *et al.*, 2007) and many others. Single nucleotide polymorphisms (SNPs) are single nucleotide mutations in the genome that are used to diagnose several diseases and traits in an organism. Generally, the methods used in SNP detection are elaborate and time consuming (Chen and Sullivan, 2003; Kirk *et al.*, 2002). Thus, new, rapid, sensitive, and inexpensive methods of SNP detection are important to improve SNP genotyping. Due to the high affinity and excellent mismatch discrimination, LNA is an efficient tool in SNP detection (Mouritzen *et al.*, 2003).

3. MATERIAL AND METHODS

The current investigation on 'Computational prediction of miRNA targets in ESTs of sorghum and cotton and *in situ* detection of selected drought responsive miRNAs in *Arabidopsis thaliana*' was undertaken in two separate experiments; 1) Computational prediction of miRNA targets in publically available EST data sets of sorghum and cotton and their annotation, 2) Functional assessment of selected miRNAs through *in situ* hybridization in drought stress and well watered roots and leaf tissues of *Arabidopsis thaliana*.

Experiment 1: Computational identification of miRNA targets in sorghum and cotton EST data sets

3.1 Mining of sorghum and cotton EST dataset

3.1.1 Downloading EST data sets

All Sorghum ESTs from dbEST database of the National Centre for Biotechnology Information (NCBI) and Fungen (<http://www.fungen.org/>) were downloaded (last accessed on July 2008). First, 'sorghum AND EST' was used as a string of keyword to search nucleotide sequences at the NCBI databases (<http://www.ncbi.nlm.nih.gov>). All matched sequences were downloaded by changing the 'display' dropdown menu to FASTA, and the 'send to' dropdown menu to FILE. After downloading, the file containing all the available 2, 41,562 sequences were saved as a text file. The major part of the ESTs was contributed by the Laboratory for Genomics and Bioinformatics (known as 'Fungen'), University of Georgia, Department of Plant Biology, USA.

EST sequences of cotton were downloaded from the GenBank at NCBI (www.ncbi.nlm.nih.gov). Batch files of EST sequences were downloaded in FASTA format. At the time of download 4, 93, 741 ESTs of cotton (last accessed on March, 2008) were available at NCBI. Batch files of cotton EST sequences were downloaded from GenBank in FASTA format using 'cotton AND EST', as search key for cotton ESTs.

All the available EST sequences of sorghum representing a cereal system and cotton representing a dicot system were processed and subjected for miRNA target mapping. The sorghum ESTs contains a wide range of biotic and abiotic transcriptomes from various tissues such as leaf, root, pollen, stem and so on. Similarly the cotton ESTs also contains a diverse set of transcription factors corresponding to different stimuli such as osmotic stress, cold, pathogenesis and others. There are ESTs which belongs to gene required for cotton fiber development and different signal transduction molecules which are involved in metabolic pathways.

3.1.2 miRNA data source

The miRNAs were taken from miRBase (<http://microrna.sanger.ac.uk>), a growing repository of viruses, animals and plants miRNAs. The miRBase Sequence Database is a searchable database of published miRNA sequences and annotation. The data were previously provided by the miRNA Registry. The miRBase Registry continues to provide gene hunters with unique names for novel miRNA genes prior to publication of results. Each entry in the miRBase represents a predicted hairpin portion of a miRNA transcript (termed mir in the database), with information on the location and sequence of the mature miRNA sequence (termed miR). Both hairpin and mature sequences are available for BLAST, and entries can also be retrieved by name, keyword, references and annotation. All sequence and annotation data are also available for download. Starting from 120-200 bases stem-loop precursor to mature 20-21 bases long sequences are available in FASTA format for download. Along with miRNA ID, other information like accession number, chromosomal location, sense or anti-sense strand, start and end point etc. are available. A total of 207 *Arabidopsis* miRNA was available (last accessed September, 2008) in the database (Release 12.0). These sequences

were downloaded in FASTA format and saved in a text file to find their potential targets in sorghum and cotton EST data sets.

3.1.3 Curing of EST data sets

The ESTs sequences were separated in different text files in FASTA format. Redundancy in downloaded ESTs was cured by importing the text files in batches using “Contig Express”, a sequence assembler tool in Vector NTI 10 windows version (Invitrogen Inc.). Clustering was done in criteria of at least 29 bases overlap and 85% identity between one end of read and other end of the other read. The contigs and the singletons generated were saved as unigenes in FASTA-formatted files.

3.1.4. Construction of local database for standalone BLAST

The FASTA formatted text files were uploaded to create local nucleotide database using “Accessory application” available in BioEdit (version 7.0.9.0) software. The database was constructed separately for sorghum and cotton ESTs as “SOR local database” and “COT local database” which were used for target identification by standalone blasts.

3.1.5 Processing of miRNA sequences

The miRNA sequences in text format were pasted in Fast PCR (version 5.3.64) window and by selecting the “Reverse antisense” option the output sequences were generated. The output files were saved in text format to identify the potential miRNA targets in the sorghum and cotton EST data sets.

3.1.6 Prediction of miRNA targets

The reverse complementary miRNAs were used one by one as query sequence and standalone blast was performed against the respective “SOR local database” and “COT local database”. The output file generated was imported and saved in Microsoft Excel as “*.xls” file. An E-value cut off was used as criteria for the presence of any homology in the query and subject sequence. Most of the miRNA target hits showed 18-21 nucleotide complementarities on an average. The targets were finally selected by discarding those target ESTs having more than 3 mismatches.

3.1.7 Annotation of miRNA target ESTs

BLAST similarity comparison was used to reveal possible functional annotation of each EST hit containing miRNA sequences. The blast search was performed against the redundant data set. An E-value cut off and repeat position was used as criteria to assign identity to any sequence. The hits were selected on the basis of E-value. Homology based functional annotation was done using blastX (<http://ncbi.nlm.nih.gov/blast/Blast.cgi>) to reveal possible relationships of each EST containing miRNA target in the non redundant data set against all non redundant Genbank CDS translations, PDB, SwissProt, PIR and PRF. Various kinds of target proteins like transcription factors, signal transduction molecules, splicing factors, protein involved in stress responses, metabolism and development were having target site in their corresponding mRNA. The schematic representation of miRNA target identification in sorghum and cotton is presented in Fig. 1.

3.1.8 Phylogenetic analysis

The miRNAs having target in sorghum were named as “SOR”, those having target in cotton are named as “COT” and “SORCOT” for those having targets in both the crops. The evolutionary history was inferred using the UPGMA method and the bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. All

positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Kumar *et al.*, 2004).

3.1.9 Software packages used

In this study different softwares were used like Vector NTI 10 windows version (Invitrogen Inc.) was used for clustering of redundant ESTs of sorghum and cotton, for multiple alignments, profile alignment of nucleotide and amino acid sequences. Similarly, BioEdit (version 7.0.9.0) was utilized to construct the local nucleotide database of sorghum and cotton EST sequences, user-friendly software for biological sequence alignment and analysis (Hall, 1999). Offline software Fast PCR (version 5.3.64) was used to get the reverse complementary sequence of miRNAs, for primer and probe design, clustering, *in silico* PCR, restriction, LTRs search and clustering, SSR search and so on.

Experiment 2: Functional assessment of selected miRNAs through *in situ* hybridization

3.2 *In situ* hybridization in *Arabidopsis*

In situ hybridization techniques allow specific nucleic acid sequences to be detected in morphologically preserved chromosomes, cells or tissue sections. In combination with immunocytochemistry, *in situ* hybridization can relate microscopic topological information to gene activity at the DNA, mRNA, and protein level. Some *Arabidopsis* miRNAs were selected which are known to differentially express in soybean with varying level of external stimuli, with a hypothesis that they might express in *Arabidopsis* with respect to those stimuli.

3.2.1 Growing of *Arabidopsis thaliana*

Arabidopsis thaliana Columbia ecotype (col-0) seeds obtained from University of Missouri (Columbia) were used. Seeds were subjected to cold treatment of 4 days to break the dormancy before sowing. Plants were grown in perlite-vermiculite mixture (2:1) in pots at 22°C. A light intensity of 1000 lux with 16/8 hr light duration was maintained.

3.2.2 Exposure of *Arabidopsis thaliana* to drought stress

Plants were grown normally for 4 weeks and then subjected to drought stress for 6 (S1), 11 (S2) and 14 days (S3) as mild, moderate and severe stress respectively. Plants were grown separately with 5 replicates each. Well watered plants were maintained as control (C). The relative water content (RWC) was measured as in (Agnes *et al.*, 2004) for each of these stresses along with the control plants. The formula used is as follows,

$$RWC = (FW - DW / TW - DW) \times 100$$
where FW= fresh weight, DW= dry weight and TW= turgid weight of samples.

3.2.3 Preparation of RNase free water

Diethyl pyrocarbonate (DEPC) (Sigma Aldrich, USA) was used to treat millipure water for preparation of solutions. DEPC (0.1%) was added to required quantity of water and kept overnight with gentle shaking. Next day it was autoclaved to degrade DEPC and finally the DEPC free watered was used for the preparation of reagents.

3.2.4 Tissue harvesting

Plant samples were harvested carefully in glass vials (Borosil Glass Works Limited, India) which were labeled properly to determine the tissue type and their respective treatments. Different tissues were harvested and placed quickly in freshly prepared fixative on ice in 15 ml glass vials.

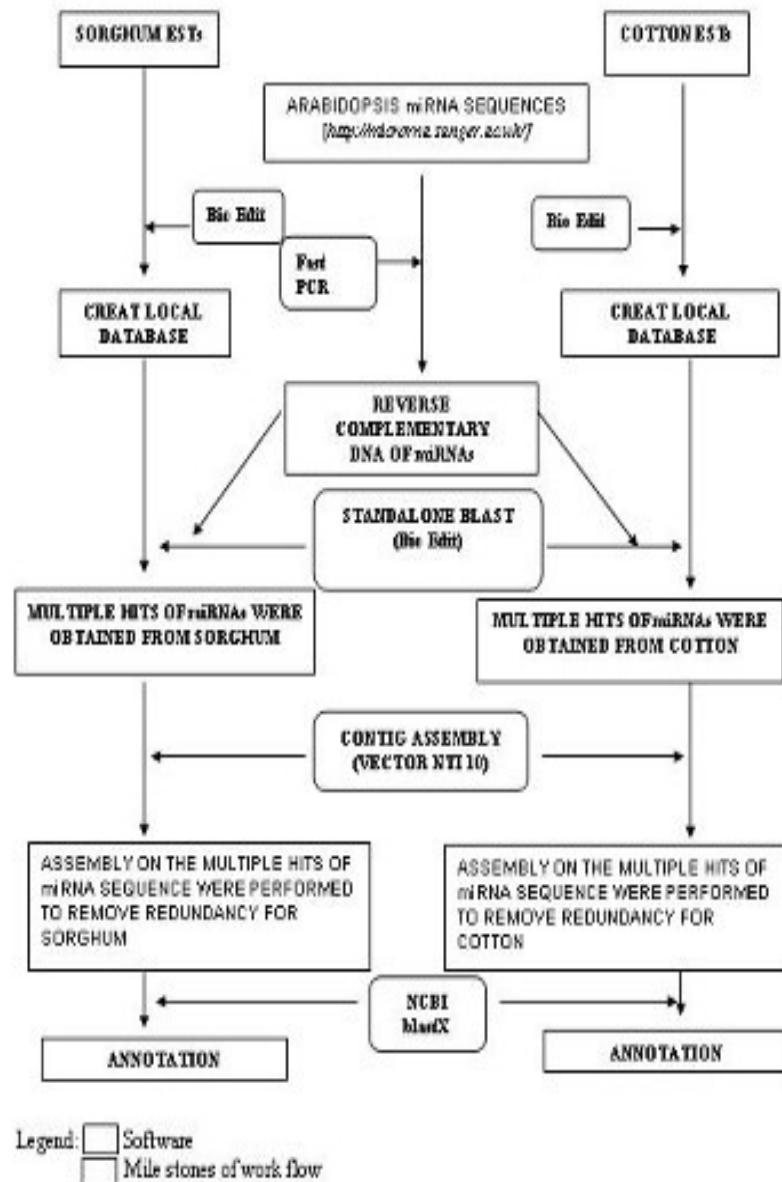


Fig. 1: Schematic representation for the identification of miRNA targets in sorghum and cotton

Fig.1:Schematic representation for the identification of miRNA targets in sorghum and cotton

3.2.5 Fixation of tissues

The tissues were fixed in paraformaldehyde (PFA) (Sigma Aldrich, USA). It was freshly prepared in phosphate buffer saline (PBS) in required quantities. PBS (Sigma Aldrich, USA) was prepared and pH was adjusted to 11.0 with NaOH. Then the solution was heated to 65°C. In a fume hood 4% PFA was added and mixed thoroughly until dissolved.

After harvesting tissues were placed in freshly prepared PFA and vacuum infiltrated at 450 mm Hg on ice. Small bubbles were released from the tissues. Vacuum was held for 20 minutes and released slowly. This step was repeated until tissues sank. PFA was replaced with fresh fixative and gently shaken overnight at 4°C.

3.2.6 Embedding of tissues

Phosphate buffer saline (1X) treatment was given twice 30 minutes each, followed by an increasing series of ethanol (Hayman Limited, England) 60 minutes each. 0.1% Eosin (Sigma Aldrich, USA) was added to 95% ethanol to visualize samples when sectioning and kept overnight at 4°C with gentle shaking.

Samples were transferred to fresh glass vials before the histochoice (Sigma Aldrich, USA) was added. 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 to which replaces the ethanol step by step. At the stage of 100% histochoice, ¼ volume paraplast chips (Fisher Scientific, USA) were added and kept overnight. Then the samples were placed at 42°C until chips completely melted.

More chips were added and placed to 60°C. After 8 hours wax/ histochoice was replaced with freshly melted wax (Merck Specialist Private Limited, India) and left the vials open overnight at 60°C. The wax was replaced twice everyday for 4-5 days and vials were left open overnight at 60°C. Small amount of paraplast chips were placed into moulds (Electron Microscopy Science, USA) and warmed forceps were used to arrange the tissue samples in the mould. The moulds were placed on ice for quick setting and tissue blocks were formed.

3.2.7 Sectioning of tissues

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

Tissue blocks were trimmed and mounted onto the microtome (Leica Microsystem, Germany). About 7-8µm thick sections were taken using disposable microtome blades (Leica Microsystem, Germany) in the form of ribbon and floated on Probe-On-Plus slide covered with DEPC-H₂O. The slides were left in a tilted slide rack at 42°C, overnight, so that tissues adhered to slides.

3.2.8 Preparation of miRNA probes

Digoxigenin (DIG)-labeled miRCURY™ Locked Nucleic Acid (LNA) *in situ* hybridization probes (Exiqon, Vedbaek, Denmark) were designed by taking the reverse complementary sequence for each miRNA (using FastPCR software) as per the information from an unpublished data [In collaboration with ABI (USA) and University of Missouri (Columbia), through a high throughput qRT-PCR platform, using a proprietary techniques, which targets specific miRNA using Taqman technology. A set of *Arabidopsis* miRNAs identified to be expressing several folds in root and leaf tissues in Soybean under drought condition] were used. These are as follows:

miRNA	Probe sequence(5'-3')	Target tissue
ath-miR399	CCGGGCAAATCTCCTTTGGCA	Leaf
ath-miR397a	CATCAACGCTGCACTCAATGA	Leaf
ath-miR168	TTCCCGACCTGCACCAAGCGA	Leaf
ath-miR399e	CGAGGCAAATCTCCTTTGGCA	Root
ath-miR395b	GAGTCCCCCACAACACTTCAG	Root
ath-miR161	CCCCGATGTAGTCACTTTCAA	Root
ath-miR171	GATATTGGCGCGGCTCAATCA	Control

LNA DIG- labelled miRCURY™ antisense probes which are extremely specific and will not bind if there is any single mismatch were used. Three leaf and three root specific and one control (constitutive) miRNA probes were selected for this experiment.

3.2.9 Pre-treatment of tissue sections

For better hybridization, sections were pre-treated to optimize the specificity and signal intensity. The tissue sections were first deparaffinised by histochoice (Sigma Aldrich, USA) treatment for 10 minutes twice and rehydrated through a decreasing ethanol (Hayman Limited, England) series for 1 min each. Sodium citrate saline (SSC) treatment was given for 15-20 minutes. Then Tris-EDTA (TE) (Sigma Aldrich, USA) with proteinase K (1µg/ml) treatment was given for 30 minutes at 37°C followed by 0.2% Glycine (Sigma Aldrich, USA) in PBS for 2 minutes. The slides were washed in fresh PBS twice for 2 min each. PFA (4%) pH 7.0 was prepared freshly in PBS and slides were treated for 10 minutes 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.

3.2.10 Hybridization of tissues

At first hybridization solution was prepared in required quantities. The slides were dried completely and marked with Tissue-Tek Marking pencil (Electron Microscopy Sciences, USA) for respective probes to be hybridised. For each pair of slides, probe was added to 50% formamide (Sigma Aldrich, USA) such that the total volume was 40 µl. This probe-formamide mixture was heated to 80°C for 2 minutes and immediately chilled on ice, spun down completely and kept on ice.

For each pair of slides 160 µl of hybridization solution was added such that the volume made to 200 µl. The probe concentration used was 0.1 ng/ µl. To each slide 100 µl of probe solution was added and spreading it over the entire slide and the two slides were slowly sandwiched together. Slide sandwich was placed on slide rack at 50°C overnight for hybridization.

3.2.11 Post-hybridization treatment

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

The slides pairs were dipped in pre-warmed 0.2X SSC to separate them. Before placing in a rack and the slides were rinsed in 0.2X SSC at 55°C twice for 60 minutes with gentle shaking. NTE (NaCl-Tris-EDTA) solution was used to wash the slides at 37°C twice for 5 minutes. Then the slides were incubated with RNase A (20 µg/ml) in NTE solution with gentle shaking at 37°C for 30 minutes. Again slides were washed twice in fresh NTE at 37°C for 5 minutes. Then the slides were washed in 0.2X SSC for 60 minutes at 55°C with gentle shaking and the slides were placed in PBS for 5 minutes at room temperature.

3.2.12 Antibody treatment

One per cent blocking reagent (Roche Diagnostics, Germany) in 200 ml TBS was prepared freshly just before use. The slides were first blocked to prevent non-specific cross-reaction with the anti-DIG antibody. For that blocking reagent (Roche Diagnostics, Germany) was used and 1% blocking solution was poured to cover the slides and kept over rocking platform for 45 minutes.

The slides were placed on the bottom of a large flat plastic container. During this time 500ml 1% Bovine serum albumin (Sigma Aldrich, USA), 0.3% Triton X-100 (Sigma Aldrich, USA) in TBS was prepared. Volume was made up 500 ml with TBS. Blocking solution was replaced with 1% BSA, 0.3% Triton X-100 in TBS and washed for 45 minutes in a rocking platform. Meanwhile 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.

3.2.13 Substrate treatment

Slides were arranged on racks of plastic pipettes above wet tissue in a tightly sealed plastic container and left overnight at 4°C. Solution was drained from slides on Kimwipes (Kimtech Science, Roswell, GA) and separated. 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 minutes.

Then slides were washed in TN solution for 10 minutes. Each slide was dipped in TN solution to ensure that all detergent was washed off and the pH in the sections was raised to 9.5 for optimum alkaline phosphatase activity. Substrate solution was prepared immediately before use 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.

3.2.14 Detection of hybridization signal

Slides were placed in plastic container above wet tissue paper in total darkness for 1-5 days. Colour development was checked through a dissection microscope (OLYMPUS, SZX9) and the substrate solution was replaced every 48 hr by adsorbing old solution onto kimwipes and fresh substrate solution was brought in place by capillary action.

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

4. EXPERIMENTAL RESULTS

The present investigation was carried out for prediction of miRNA targets in a publically available EST data set of sorghum and cotton through computational approach and to standardize *in situ* hybridization protocol for miRNAs and subsequent detection of selected miRNAs in drought stressed root and leaf tissues of *Arabidopsis thaliana*. The results are presented below.

Experiment 1: Computational identification of miRNA targets in sorghum and cotton EST data sets

4.1.1 Curing of sorghum and cotton EST datasets

A total of 2, 41, 562 and 4, 93, 741 EST sequences, respectively, in sorghum and cotton were available for analysis. Contig analysis was done to remove redundancy. As a result, a set of 2, 33, 817 ESTs in sorghum and 4, 27, 342 ESTs in cotton were found to be non-redundant. The flow chart for EST processing, leading to the identification of miRNA targets is presented in Fig. 2.

4.1.2 Identification miRNA targets

Using available 207 *Arabidopsis* miRNAs a set of 125 miRNA targets were identified in sorghum EST data sets. A set of 29 miRNAs belong to 24 different miRNA families were found to have targets uniquely in sorghum. It was found that *OCL5* protein was targeted by two different miRNAs, miR853 and miR822. Similarly, miR846 and miR835-3p were found to target putative GAG-POL precursor-Orf1 protein. The details on miRNAs and their target genes of sorghum are presented in Table 1.

In cotton EST data sets a total of 138 miRNA targets were detected when tested against available miRNA sequences. A set of 42 miRNAs belonging to 34 miRNA families were uniquely present in cotton. The number of miRNA targets varied from a single to multiple genes depending on miRNA in question. miR403 was found to have a single target while others like miR396a and miR447c recorded five and nine different targets, respectively. miR447c targets included; alpha-tubulin, DREB 3A, endo-1, 4-beta glucanase and a proline-rich protein. The details on individual miRNAs and their respective target genes are presented in Table 2. Out of 207 miRNAs, 96 miRNAs registered common targets in sorghum and cotton. These miRNAs possessed single to many targets in sorghum and cotton (Table 3).

4.1.3 Annotation of miRNA targeted ESTs

All the ESTs targeted by miRNA identified in sorghum and cotton were annotated using NCBI blastX to compare their respective functions. In most cases the annotated protein targets were transcription factors, some of them were involved in signal transduction and different metabolic pathways. The annotated protein targets of 96 conserved miRNA are presented in Table 4.

Proline rich cell wall protein in cotton was found to have target site for miR868 but, in sorghum the same miRNA was having target site for putative non-LTR retro element reverse transcriptase which shows the functional diversities of miRNAs in different plant species. On the other hand, miR172 had DREB 2 in sorghum and EREBP in cotton as target genes, which are reported to be functionally same and belongs to AP2 transcription factor family.

4.1.4 Phylogenetic analysis of miRNAs

Among 167 miRNAs were having targets in either in sorghum or cotton a set of 96 miRNAs were found to have gene targets both in sorghum and cotton and the remaining 71 had unique targets in sorghum (29) and cotton (42). The phylogenetic analysis of 167 miRNAs with a bootstrap of 1000 was carried out and the phylogram contained 15 major clades (Fig. 3), which represent similarities and divergence of miRNAs in cotton and sorghum.

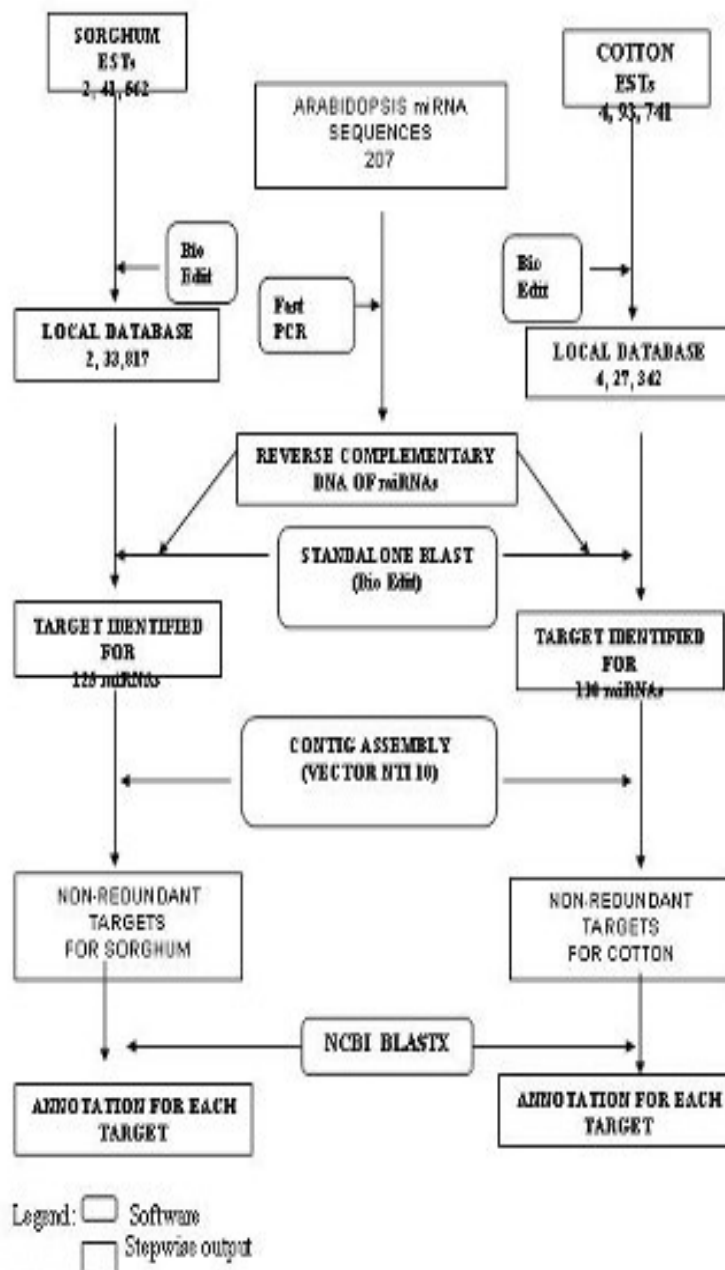


Fig. 2: Scheme employed for miRNA target identification in sorghum and cotton

Fig.2: Scheme employed for miRNA target identification in sorghum and cotton

Table 1: Potential miRNA targets uniquely identified in sorghum ESTs using *Arabidopsis* miRNAs from miRBase

Sl. No.	Sanger miRNA ID	miRNA Sequences (5'-3')	No. of target EST (s)	Gene ID (GI)	Putative Protein Target (S)
	1	2	3	4	5
1.	ath-mir846 mimat0004269	UUGAAUUGAAGUGCUUGAAUU	1	30940844	Putative GAG-POL precursor -Orf1 Protein
2.	ath-mir774 mimat0003933	UUGGUUACCCAUUUGGCCAUC	1	14823519	TNP2-like protein
3.	ath-mir400 mimat0001001	UAUGAGAGUAUUUAAGUCAC	2	9306654 33107592	Hypothetical protein S250 Putative vegetative storage protein
4.	ath-mir853 mimat0004276	UCCCCUCUUUAGCUUGGAGAAG	1	12692231	OCL5 protein
5.	ath-mir822 mimat0004239	UGCGGGAAGCAUUUGCACAUG	2	11922066 1339293	OCL5 protein Receptor-like kinase
6.	ath-mir782 mimat0003941	ACAAACACCUUGGAUGUUCUU	2	33110633 57807333	Phytochrome B Sucrose non-fermenting related protein kinase
7.	ath-mir404 mimat0001005	AUUAACGCUGGCGGUUGCGGCAGC	3	33110147 45956938 57809829	PC Protein A Hypothetical protein S250_18C08.34 Hypothetical protein SB20O07.12
8.	ath-mir173 mimat0000206	UUCGCUUGCAGAGAGAAAUCAC	1	30951819	MADS box protein

Table 1 contd....

	1	2	3	4	5
9.	ath-mir828 mimat0004244	UCUUGCUUAAAUGAGUAUUGCA	1	31333772	Polyprotein
				45961499	Gamma-Kafirin protein
10.	ath-mir831 mimat0004249	UGAUCUCUUCGUACUCUUCUUG	3	57811636	Cytochrome C biogenesis FN
				9304068	Sucrose non-fermenting related protein kinase
11.	ath-mir780.2 mimat0003939	UUCUUCGUGAAUAUCUGGCAU	1	12508668	Putative cleavage stimulation factor subunit1
12.	ath-mir406 mimat0001009	UAGAAUGCUAUUGUAAUCCAG	1	6674955	Serine carboxypeptidase-like protein
13.	ath-mir168a mimat0000198	UCGCUUGGUGCAGGUCGGGAA	1	45963803	Teosinte branched1 protein
14.	ath-mir783 mimat0003942	AAGCUUUGCUCGUUCAUGUUC	1	34445110	Glycosyltransferase
15.	ath-mir447a mimat0002113	UUGGGGACGAGAUGUUUUGUUG	1	18052826	Starch synthase IIc precursor
				30963325	Glycosyltransferase
16.	ath-mir854d mimat0004283	GAUGAGGAUAGGGAGGAGGAG	3	34516127	Putative anthocyanin regulatory C1
				45972180	Glycine-rich RNA-binding protein 2
				61099249	Putative copia reverse transcriptase
17.	ath-mir781 mimat0003940	UUAGAGUUUUCUGGAUACUUA	2	12509678	Photosystem I P700 apoprotein A1
18.	ath-mir823 mimat0004240	UGGGUGGUGAUCAUAUAAGAU	1	30967761	Hypothetical protein
19.	ath-mir777 mimat0003936	UACGCAUUGAGUUUCGUUGCUU	1	5042940	Serine/Threonine kinase
20.	ath-mir863-5p mimat0004309	UUGAGAGCAACAAGACAUAAU	1	45951008	GTP-binding protein-like
				45992517	Hypothetical protein
21.	ath-mir778 mimat0003937	UGGCUUGGUUUAUGUACACCG	2	45994727	Plastid division regulator
22.	ath-mir835-3p mimat0004256	UGGAGAAGAUACGCAAGAAAG	1	30952939	Putative GAG-POL precursor -Orf1 protein
23.	ath-mir779.2 mimat0004329	UGAUUGGAAAUUCGUUGACU	1	30974427	Rp1-like protein
24.	ath-mir869.1 mimat0004320	AUUGGUUCAAUUCUGGUGUUG	1	45961974	Hypothetical protein

The “k” clade contained a cluster of miRNAs including miR156, miR157, miR394 and miR415 present in both the crops. Similarly clade “g” contained all the members of miR169 family which are conserved in sorghum and cotton.

On the other hand, clade “d” constituted different miRNAs viz., miR828, miR859, miR829.1, miR845a and miR845b which have unique targets in cotton. Some miRNAs unique to sorghum are in “c” clade and were related to miR397a and miR397b of cotton.

Under clade “j”, miR172 was found to cluster with miR419, uniquely identified in cotton. Here, miR172b and miR172d were closer to each other similar to miR172a and miR172c whereas miR172e was in a different branch.

4.1.5 Conservation of miR172 target domain in sorghum and cotton

miR172 showed targets onto orthologous genes in the cotton and sorghum, as it targets two different transcription factors belonging to the same transcription family. Thus a study for degree of conservation of miR172 can give out interesting information. Recognition of target site in mRNA sequence of DREB2 and EREB in sorghum and cotton respectively is shown in Fig. 4a. To study such possibility of conservation an alignment using the peptide sequence of the target site of miR172a was carried out.

The profile alignment study using the peptide sequence of the target site revealed conservation of 2 and 4 amino acids of 7 amino acids sequence of miR172 target sites, respectively, for sorghum and cotton (Fig. 4b). Further blocks analysis on the BLOCKS server (<http://www.blocks.fhcrc.org>) was done taking APETALA 2 of *Arabidopsis* as the reference sequence (Fig. 4c). The analysis followed by the annotation of the blocks shown in Fig.4c revealed the presence of profile for the target site in block B that contains the ethylene responsive element binding protein signature.

The ethylene responsive element binding protein block (block B) was taken for annotation and it was found that 1-17 amino acid of block B having a conserved signature for EREBP domain (Fig. 4d). The miR172 target site was also conserved in DREB2 and EREBP, respectively, in sorghum and cotton.

Experiment 2: Functional assessment of selected miRNAs through *in situ* hybridization

4.2.1 Optimization of *in situ* hybridization parameters in *Arabidopsis*

In situ detection protocol for miRNA though technically demanding is very sensitive and requires bench level optimization for its successful application. This is particularly true for detecting RNA species which differ in a few bases. The optimizations of critical steps are presented in Table 5.

4.2.1.1 Probe concentration

For *in situ* hybridization of miRNAs the probe concentration plays an important role on hybridization specificity. The effect of different concentration of probes was tried to get the optimum intensity of hybridization (Fig. 5). The results indicated that the probe concentration of 1.0ng/μl was suitable for hybridization of miRNAs in the respective tissues.

4.2.1.2 Post-hybridization treatment

The second important consideration was washing of tissues after hybridization with miRNA probes. Four different concentrations of saline buffer were tried. Among concentrations tried 0.2 X SSC was found suitable to get the optimum hybridization signal (Fig. 6). In 0.1 X SSC the signal was absent and other higher concentrations gave signal intensities of same pattern. Hence, 0.2 X SSC was used in subsequent experiments.

Table 2: Potential miRNA targets uniquely identified in cotton ESTs using *Arabidopsis* miRNAs from miRBase

Sl. No.	Sanger miRNA ID	miRNA Sequences (5'-3')	No. of Target EST (S)	Gene ID (GI)	Putative protein target (s)
	1	2	3	4	5
1.	ath-mir864-3p mimat0004312	UAAAGUCAAAUAAUACCUUGAAG	1	164345980	AI-induced protein
2.	ath-mir472 mimat0003931	UUUUUCCUACUCCGCCCAUACC	1	84168871	Proline-rich protein
3.	ath-mir845b mimat0004317	UCGCUCUGAUACCAAAUUGAUG	1	11200642	3-ketoacyl-coA reductase 2
4.	ath-mir171b mimat0000920	UUGAGCCGUGCCAAUAUCACG	1	109878446	Long chain fatty acid elongation enzyme
5.	ath-mir447c mimat0002115	UUGGGGACGACAUCUUUUGUUG	9	31407399	Alpha-tubulin
				13245709	Proline-rich protein
				13245710	Endo-1,4-beta-glucanase
				13245728	Endo-1,4-beta-glucanase
				13246258	Alpha-tubulin
				13355660	Alpha-tubulin
				164345459	DREB 3A
				3326328	Alpha-tubulin
				84174498	Gibberellin 20-oxidase
6.	ath-mir158a mimat0000176	UCCCAAUGUAGACAAAGCA	5	13245709	Proline-rich protein
				13245710	Endo-1,4-beta-glucanase
				13245728	Endo-1,4-beta-glucanase
				164345459	DREB 3A
				84174498	Gibberellin 20-oxidase

Table 2 contd...

	1	2	3	4	5
7.	ath-mir172b* mimat0000204	GCAGCACCAUUAAGAUUCAC	1	109849994	Putative cellulose synthase catalytic subunit
8.	ath-mir837-5p mimat0004258	AUCAGUUUCUUGUUCGUUUA	1	2 1095798	Fasciclin-like arabinogalactan protein 16
9.	ath-mir162b mimat0000183	UCGAUAAACCUCUGCAUCCAG	3	109864995	Isopentenyltransferase
				164241038	Proline-rich protein
				3326118	P-glycoprotein
10.	ath-mir396a mimat0000944	UUCCACAGCUUUCUUGAACUG	5	109873901	Ovule/fiber cell elongation protein ghfe1
				13350496	RNA-dependent RNA polymerase
				48739322	RNA polymerase beta subunit
				5048455	AP2/EREBP transcription factor erf-1
				78346825	Auxin-induced basic helix-loop-helix
11.	ath-mir419 mimat0001327	UUAUGA AUGCUGAGGAUGUUG	2	21097636	Adenylyl cyclase associated protein
				164244859	Proline-rich cell wall protein
12.	ath-mir395b mimat0000939	CUGAAGUGUUUGGGGGGACUC	2	109872367	Transcription factor DREB 2
				48739711	Hypothetical chloroplast rf1

Table 2 contd....

	1	2	3	4	5
13.	ath-mir865-3p mimat0004314	UUUUUCCUCAAUUUAUCCAA	4	164320714	Putative beta-mannosidase
				164330196	NADH dehydrogenase
				164347745	ACC synthase
				54120047	Vacuolar ATP synthase subunit b 2
14.	ath-mir775 mimat0003934	UUCGAUGUCUAGCAGUGCCA	1	48775380	Ethanolamine-phosphate cytidyltransferase 2
15.	ath-mir417 mimat0001325	GAAGGUAGUGAAUUUGUUCGA	1	6462170	Kinesin
16.	ath-mir839 mimat0004261	UACCAACCUUUAUCGUUCCC	1	13350355	Putative g-protein beta
17.	ath-mir829.1 mimat0004245	AGCUCUGAUACCAAAUGAUGGAAU	3	11200642	3-ketoacyl-coa reductase 2
				3325943	P-glycoprotein
				84166780	Central motor kinesin 1
18.	ath-mir405a mimat0001006	AUGAGUUGGGUCUAACCCAUAACU	1	164257527	Translation elongation factor
19.	ath-mir859 mimat0004303	UCUCUCUGUUGUGAAGUCAA	1	24976903	Dehydrin
20.	ath-mir407 mimat0001010	UUUAAAUCAUAUACUUUUGGU	2	164356271	Trans-2-enoyl-coA reductase
				164368662	Hypothetical chloroplast rf1
21.	ath-mir869.2 mimat0004321	UCUGGUGUUGAGAUAGUUGAC	1	21103458	Rubisco large sub unit
22.	ath-mir163 mimat0000184	UUGAAGAGGACUUGGAACUUCGAU	1	109846405	Auxin-induced basic helix-loop-helix transcription factor
23.	ath-mir780.1 mimat0004218	UCUAGCAGCUGUUGAGCAGGU	1	5050556	3-ketoacyl-coA synthase 6
24.	ath-mir843 mimat0004265	UUUAGGUCGAGCUUCAUUGGA	1	21093673	Acyltransferase-like protein

Table 2 contd

	1	2	3	4	5
25.	ath-mir832-3p mimat0004251	UUGAUUCCCAAUCCAAGCAAG	4	21092148	Cyclopropane fatty acid synthase
				164258279	Malate synthase
				109874991	CDPK-related protein kinase
				13349798	Sterol delta-7 reductase dwf5
26.	ath-mir827 mimat0004243	UUAGAUGACCAUCAACAAACU	5	109847887	Photosystem ii protein d2
				164345196	Proline-rich protein
				21091136	PR protein class 10
				48779624	3-ketoacyl-coa synthase 6
				78352026	MYB 1
27.	ath-mir855 mimat0004279	AGCAAAAGCUAAGGAAAAGGAA	2	109851112	MYB9
				164338470	MADS box protein ghmad5-1
28.	ath-mir167d mimat0000905	UGAAGCUGCCAGCAUGAUCUGG	2	164367807	Auxin response factor 3
				24977477	Sterol delta-7 reductase dwf5
29.	ath-mir397a mimat0000946	UCAUUGAGUGCAGCGUUGAUG	3	109859739	Putative growth regulator
				109861021	Glutathione s-transferase
				48815677	MYB-like DNA-binding protein
30.	ath-mir865-5p mimat0004313	AUGAAUUUGGAUCUAAUUGAG	6	78339488	Putative CCCH -type zinc finger transcription factor
				13243934	Endo-1,4-beta-glucanase
				13354664	Putative CCCH-type zinc finger transcription factor
				164278944	Putative leucine-rich repeat trans-membrane protein
				164292632	Putative CCCH -type zinc finger transcription factor
				48778935	Putative CCCH -type zinc finger transcription factor

Table 2 contd

	1	2	3	4	5
31.	ath-mir403 mimat0001004	UUAGAUUCACGCACAAACUCG	1	109878229	Chlorophyll a-b binding protein 151
32.	ath-mir863-3p mimat0004310	UUGAGAGCAACAAGACAUAU	2	13350410	40s ribosomal protein
				5045969	Late embryogenesis abundant protein
33.	ath-mir865-5p mimat0004313	AUGAAUUUGGAUCUAAUUGAG	6	78339488	Putative CCCH -type zinc finger transcription factor
				13243934	Endo-1,4-beta-glucanase
				13354664	Putative CCCH -type zinc finger transcription factor
				164278944	Putative leucine-rich repeat transmembrane protein
				78349071	Transcription factor DREB 2
				164292632	Putative CCCH -type zinc finger transcription factor
34.	ath-mir841 mimat0004263	UACGAGCCACUUGAAACUGAA	1	48778935	Putative CCCH -type zinc finger transcription factor

Table 3: Conserved miRNA targets identified in ESTs of sorghum and cotton using *Arabidopsis* miRNAs from miRBase

Sl. No.	Sanger miRNA ID	Sorghum		Cotton	
		No. of target EST (s)	Gene ID (GI)	No. of target EST (s)	Gene ID (GI)
	1	2	3	4	5
1.	ath-miR169a MIMAT0000200	3	15724508,31385382, 45982177	6	13245082,73851526,11205296,48805913, 5049177, 6462282
2.	ath-miR169g* MIMAT0000912	2	30967341, 30966775	1	48751342
3.	ath-miR165a MIMAT0000187	2	45975730, 45950947	4	164264305,164345241, 109873171, 48800635
4.	ath-miR156a MIMAT0000166	8	14365557, 7553383, 11680294, 30974373, 45962897, 59862473, 59864559, 7660905	25	164345361,109872376,164251838,164260183,164336398,48750683,7386 2958,78332943,78337780,109870990,109875775,109879497,11204743,1 3356818,164257520,164272834,48791585,48796122,48796123,48804656 ,5045402,73861547,78336422,78343421,78351702
5.	ath-miR838 MIMAT0004260	4	33110181,31330863, 45975043, 30947062	3	21089249, 21094771, 21094936
6.	ath-miR398a MIMAT0000948	1	45982081	2	109869080, 48740168
7.	ath-miR160a MIMAT0000178	1	30963790	4	164303952,164331396, 48804885, 78329822
8.	ath-miR418 MIMAT0001326	3	7551612,18052604, 7553219	8	164298701,48793705,48793705, 13354743,73861503,2109901, 31073820, 73860684,
9.	ath-miR868 MIMAT0004319	1	18069014	2	48805180, 7627183
10.	ath-miR166a MIMAT0000189	3	45975730, 30973106, 45950947	3	164317275,164345241, 109873171

Table 3 contd....

	1	2	3	4	5
11.	ath-miR847 MIMAT0004278	4	30943549, 31384961, 34510119, 31385307	5	164267349,164339372, 164316722,109890704,48813742
12.	ath-miR172a MIMAT0000203	3	10422016, 13469201, 18069027	9	11203047,48738994, 109826614, 11207416, 31073450, 41578634, 48749699,164352093, 48804582
13.	ath-miR847 MIMAT0004278	4	30943549, 31384961, 34510119, 31385307	5	164267349,164339372, 164316722,109890704,48813742
14.	ath-miR172a MIMAT0000203	3	10422016, 13469201, 18069027	9	11203047,48738994, 109826614, 11207416, 31073450, 41578634, 48749699,164352093, 48804582
15.	ath-miR834 MIMAT0004254	2	12509777, 31377318	1	72272245
17.	ath-miR159a MIMAT0000177	2	30967438, 45983526	3	78327597,164305589,164325775
18.	ath-miR399a MIMAT0000951	2	9852079, 33110997	1	109880371
19.	ath-miR870 MIMAT0004322	5	37706806, 45950328, 45974680, 31331041, 31346839	3	109865381,13248962,164351011
20.	ath-miR167a MIMAT0000196	2	33108029, 57807880	2	164367807, 24977477
21.	ath-miR171a MIMAT0000202	2	14366282, 59869803	7	164337208,164347837,48878235, 78349823, 109878446, 164315690, 164335723
22.	ath-miR393a MIMAT0000934	3	37710341, 59864670, 9308697	9	164248961,164333677, 84173040,164305629, 164280794,164319949, 48828843, 73866133, 84174476
23.	ath-miR394a MIMAT0000936	1	11678997	3	48751915,109888392,164325301
24.	ath-miR413 MIMAT0001321	1	14513058	2	113007346, 21093730
25.	ath-miR319a MIMAT0000511	4	126636101, 6676383, 37708329, 75739088	1	84171179
26.	ath-miR415 MIMAT0001323	2	31329817, 18059595	1	8381823

Table 3 contd....

	1	2	3	4	5
27.	ath-miR861-3p MIMAT0004306	1	18060064	2	48739918, 18101199
28.	ath-miR845a MIMAT0004268	1	9299914	4	109851617, 48749456, 73861948, 73862072
29.	ath-miR830 MIMAT0004248	1	33109309	2	164278584, 31406966
30.	ath-miR830* MIMAT0004247	1	15724656	3	48795116, 21092948, 7625224
31.	ath-miR390a MIMAT0000931	2	18064598, 33107926	5	164296092, 164318918, 48745147, 48746801, 109873684
32.	ath-miR773 MIMAT0003932	2	9299543, 30936960	1	11203489
33.	ath-miR779.1 MIMAT0003938	1	59862825	1	6461835
34.	ath-miR408 MIMAT0001011	5	30963442, 57812756, 9299451, 31346033, 34515950	1	109841047
35.	ath-miR164a MIMAT0000185	12	30951002, 45990312, 45955020, 57804517, 14569844, 31329202, 31333896, 31376674, 31377050, 31385206, 45993790, 57815130	3	48745579, 84172492, 89497563
36.	ath-miR825 MIMAT0004241	1	45993354	1	84151469
37.	ath-miR395a MIMAT0000938	1	31346916	4	109872367, 78347294, 84173071, 48739711
38.	ath-miR837-3p MIMAT0004259	3	30164655, 59864560, 95004750	1	48813657
39.	ath-miR867 MIMAT0004318	1	9852658	1	164250227
40.	ath-miR835-5p MIMAT0004255	1	31330247	3	11205379, 3326113, 78345719
41.	ath-miR836 MIMAT0004257	3	8090320, 34516912, 45977927	1	13247929

Table 3 contd....

	1	2	3	4	5
43.	ath-miR414 MIMAT0001322	43	10421654, 11678273, 12505027, 14592381, 57810930, 18069209, 30164122, 6859697, 30966033, 9302434, 45984354, 31346808, 33108930, 57817425, 45956016, 57803880, 45980780, 49573794, 57803447, 57812723, 61099073, 57809740, 45993362, 59861318, 7659417, 45993250, 15045659, 30933339, 30937178, 31348452, 33110385, 34441633, 34446352, 34518103, 45966472, 45977349, 45980685, 45996954, 57816263, 59862733, 61115258, 7658532, 8088674	114	164297325,109827465,109837732,164334313,10984795 0,109848152,164316169,109850706,109851948,1098529 24,109854681,109855617,109857051,109882268,109865 747,109871748,109875563,109885462,109886190,16425 7485,164359416,164246240,164290239,164319863,7834 0457,164291407,164350157,164355700,84173562,16436 1550,164361929,164286116,164335354,109851253,1642 95563,164340775,164277841,78354888,164250502,1643 61080,164243221,164363491,164253473,164367888,109 871936,164341356,54117243,73851551,73851445,73864 143,73862767,73865278,78323278,78329318,78345850, 78345682,78351141,78346855,164284151,109865029,10 9873481,109877882,109878659,109884719,11205279,13 245596,164247296,164253176,164255444,164259341,16 4264943,164276338,164277346,164282410,164286069,1 64288209,164297325,164309930,164312688,164316768, 164325301,164341744,164345199,164348350,16436866 2,21091211,21092188,21093822,21097241,21097608,21 101107,30841131,31072716,3325612,48740178,4874925 9,48751499,48811900,48812439,48817112,48820920,48 830681,48881524,5047114,5050452,54116400,72271420 ,78331036,78331607,84150683,84154950,84156398,841 64461,84173613

Table 4: Annotated conserved miRNA targets identified in sorghum and cotton ESTs

Sl.No.	Sanger miRNA ID	Sequences (5'-3')	Sorghum		Cotton	
			GI	Target protein (s)	GI	Target protein (s)
	1	2	3	4	5	6
1.	ath-miR418 MIMAT0001326	UAAUGUGAUGAUGAACUGACC	7551612	Cysteine proteinase-like	164298701	Putative calcium-binding protein
2.	ath-miR169a MIMAT0000200	CAGCCAAGGAUGACUUGCCGA	15724508	Polyprotein	13245082	DREB protein
3.	ath-miR169g* MIMAT0000912	UCCGGCAAGUUGACCUUGGCU	30966775	DREB2	48751342	Fasciclin-like arabinogalactan protein 17
4.	ath-miR165a MIMAT0000187	UCGGACCAGGCUUCAUCCCCC	45975730	OCL5 protein	109873171	Homeodomain protein GL2-like 1
5.	ath-miR838 MIMAT0004260	UUUUCUUCUACUUCUUGCACA	33110181	P-type R2R3 Myb protein	21094771	Cytochrome b6
6.	ath-miR398a MIMAT0000948	UGUGUUCUCAGGUCACCCCUU	45982081	Hypothetical protein	48740168	MYB 10
7.	ath-miR156a MIMAT0000166	UGACAGAAGAGAGUGAGCAC	14365557	RAMOSA1 C2H2 zinc-finger transcription factor	164345361	SBP transcription factor
			7660905	Putative GAG-POL precursor	164260183	Promoter binding protein
			59862473	Sbcbf5	48746333	GHMYB 25
8.	ath-miR868 MIMAT0004319	CUUCUUAAGUGCUGAUAAUGC	18069014	Putative retroelement transcriptase	7627183	Proline-rich cell wall protein
9.	ath-miR166a MIMAT0000189	UCGGACCAGGCUUCAUCCCCC	45975730	OCL5 protein	164317275	Homeodomain protein GL2-like 1

Table 4 contd....

	1	2	3	4	5	6
10.	ath-miR160a MIMAT0000178	UGCCUGGCUCCCUGUAUGCCA	30963790	Hypothetical protein	164303952	Auxin response factor 3
11.	ath-miR399a MIMAT0000951	UGCCAAAGGAGAUUUGCCCUG	33110997	Fertilization-independent endosperm protein	109880371	No hits found
12.	ath-miR159a MIMAT0000177	UUUGGAUUGAAGGGAGCUCUA	37708329	Typical P-type R2R3 Myb protein	164325775	Vacuolar ATP synthase 16 kda proteolipid subunit
			30967438	Glutamate/malate translocator	78327597	Hyprp1
			45983526	Hypothetical protein	164343365	CDPK-related protein kinase
13.	ath-miR157a MIMAT0000172	UUGACAGAAGAUAGAGAGCAC	34515794	O-methyltransferase 1	164345361	SBP transcription factor
14.	ath-miR847 MIMAT0004278	UCACUCCUCUUCUUCUUGAUG	34510119	Putative zinc finger protein	164316722	Auxin-induced basic helix-loop-helix transcription factors
			31384961	NADH-plastoquinone oxidoreductase subunit 2	48813742	Proline-rich cell wall protein
15.	ath-miR172a MIMAT0000203	AGAAUCUUGAUGAUGCUGCAU	10422016	DRE-binding protein 2	11203047	Ethylene-responsive element-binding factor
16.	ath-miR870 MIMAT0004322	UAAUUUGGUGUUUCUUCGAUC	37706806	Putative glutathione peroxidase	164351011	Putative calcium-binding protein
17.	ath-miR167a MIMAT0000196	UGAAGCUGCCAGCAUGAUCUA	57807880	Putative actin depolymerizing factor	164367807	Auxin response factor 3
18.	ath-miR171a MIMAT0000202	UGAUUGAGCCGCGCCAAUAUC	14366282	NADH-plastoquinone oxidoreductase subunit J	164337208	DELLA protein GAI (Gibberellic acid- insensitive mutant protein)

Table 4 contd....

	1	2	3	4	5	6
19.	ath-miR393a MIMAT0000934	UCCAAAGGGAUCGCAUUGAUCC	9308697	Fatty acid desaturase	164248961	Transport inhibitor response 1
20.	ath-miR394a MIMAT0000936	UUGGCAUUCUGUCCACCUC	11678997	Putative copia polyprotein	109888392	Putative MYB transcription factor
21.	ath-miR413 MIMAT0001321	AUAGUUUCUCUUGUUCUGCAC	14513058	Hypothetical protein	21093730	Phospholipase D beta 1 isoform 1a
22.	ath-miR319a MIMAT0000511	UUGGACUGAAGGGAGCUC	37708329	Typical P-type R2R3 MYB protein	84171179	Central motor kinesin 1
23.	ath-miR415 MIMAT0001323	AACAGAGCAGAAACAGAACA	18059595	Leucine-rich repeat-containing extracellular glycoprotein	8381823	Zinc finger protein-like protein
24.	ath-miR861-3p MIMAT0004306	GAUGGAUAUGUCUUAAGGAC	18060064	Receptor-like kinase	48739918	IAA 16 protein
25.	ath-miR845a MIMAT0004268	CGGCUCUGAUACCAAUUGAUG	9299914	Cytochrome c biogenesis FN	109851617	2S albumin storage protein
26.	ath-miR830 MIMAT0004248	UAACUAUUUUGAGAAGAAGUG	33109309	Cytochrome P450 71E1	164278584	Putative growth regulator
27.	ath-miR830* MIMAT0004247	UCUUCUCCAAAUAGUUUAGGUU	15724656	Hyp-rich glycoprotein	48795116	Putative serine carboxypeptidase precursor
28.	ath-miR390a MIMAT0000931	AAGCUCAGGAGGGAUAGCGCC	18064598	Putative protein serine/threonine kinase	164296092	RNA polymerase alpha subunit
29.	ath-miR773 MIMAT0003932	UUUGCUUCCAGCUUUUGUCUC	9299543	Unknown	11203489	ADP,ATP carrier protein 1, mitochondrial precursor
30.	ath-miR414 MIMAT0001322	UCAUCUUCAUCAUCGUCA	57812723	Typical P-type R2R3 Myb protein	109852924	Putative leucine-rich repeat trans-membrane protein

Table 4 contd....

	1	2	3	4	5	6
			30937178	Typical P-type R2R3 Myb protein	109886190	Non-expresser of pathogenesis-related 1
			49573794	O-methyltransferase	164319863	MYB9
			7658532	Serine/threonine kinase	164335354	Zinc finger protein
			45977349	Hypothetical protein S250	109851253	Ethanolamine-phosphate cytidyltransferase 2
			61115258	Putative precursor GAG-POL	13245596	Myb transcription factor MYB30
			30933339	Hypothetical protein	164259341	AG-like protein
			45996954	Hypothetical protein	164282410	Beta-tubulin 13
			8088674	Phytochrome B	164348350	3-ketoacyl-coa synthase
			14592381	Methionine synthase protein	48751499	DREB1-like transcription factor
			45980780	NADH-plastoquinone oxidoreductase subunit 1	109851948	Auxin-induced basic helix-loop-helix
			45993362	Fertilization-independent endosperm protein	164361550	Salt inducible-like protein
			12505027	Serine carboxypeptidase-like protein	164295563	Transcription factor TF2
			57803880	Putative transcriptase reverse	164340775	Ribulose-1,5-bisphosphate carboxylase/oxygenase
31.	ath-miR779.1 MIMAT0003938	UUCUGCUAUGUUGCUGCUCAU	59862825	Putative transferase glucosyl	6461835	Probable phospholipid hydroperoxide glutathione peroxidase
32.	ath-miR408 MIMAT0001011	AUGCACUGCCUCUUCCCUGGC	30963442	Predicted protein	109841047	Protease

Table 4 contd....

	1	2	3	4	5	6
33.	ath-miR164a MIMAT0000185	UGGAGAAGCAGGGCACGUGCA	30951002	Hypothetical protein	48745579	TTG1-like protein
34.	ath-miR825 MIMAT0004241	UUCUCAAGAAGGUGCAUGAAC	45993354	RNA polymerase beta subunit	84151469	GHDEL 65
35.	ath-miR395a MIMAT0000938	CUGAAGUGUUUGGGGAACUC	31346916	Glycosyltransferase	10987236 7	DRE-binding factor 2
36.	ath-miR837-3p MIMAT0004259	AAACGAACAAAAACUGAUGG	30164655	Putative stripe rust resistance protein Yr10	48813657	Bacterial-induced lipoxygenase
38.	ath-miR867 MIMAT0004318	UUGAACAUGGUUUUUUAGGAA	16425022 7	Putative stripe rust resistance protein Yr10	16425022 7	Putative leucine-rich repeat family protein
39.	ath-miR835-5p MIMAT0004255	UUCUUGCAUAUGUUCUUUAUC	31330247	Putative receptor kinase	21091211	Putative WRKY transcription factor
40.	ath-miR836 MIMAT0004257	UCCUGUGUUUCCUUUGAUGCGUG G	8090320	Hypothetical URF265	13247929	Cellulose synthase

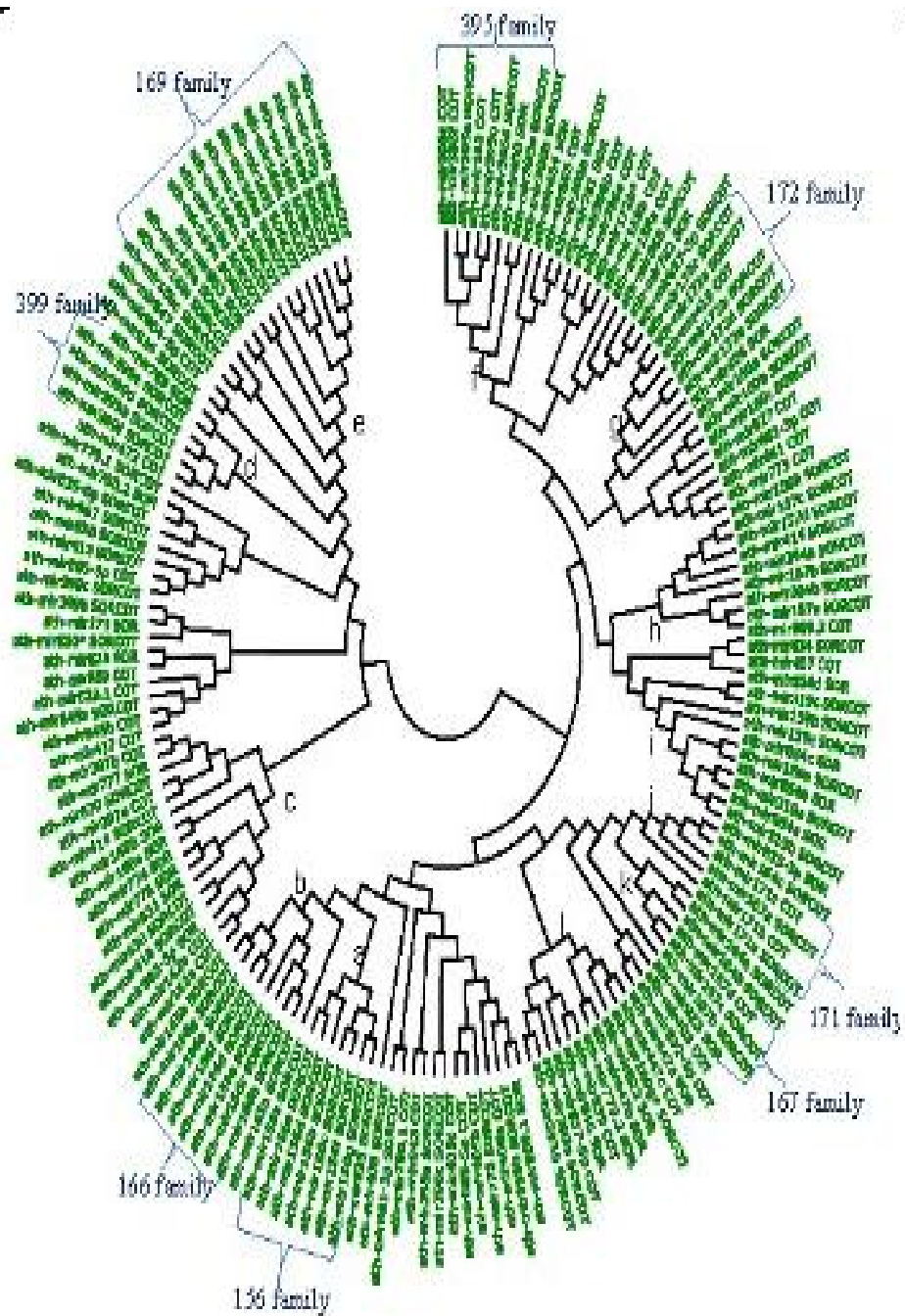


Fig.3: Phylogenetic tree of 167 miRNAs using MEGA4 software, shows the conservation pattern of miRNAs in sorghum and cotton. Different clades on the tree were named as a-1 and conserved miRNA families were marked. Bootstrap were done for 1000 replicates

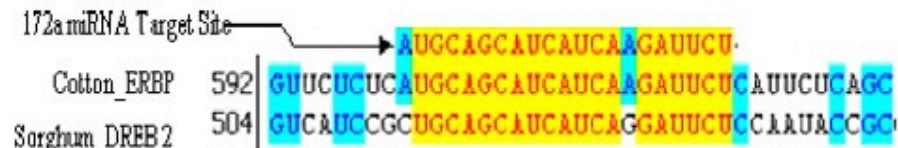


Fig. 4a. Target recognition in cotton and sorghum hits. Numbers on the left hand side of sequence represent position of start nucleic acid for this window .

Fig.4a:Target recognition in cotton and sorghum hits. Numbers on the left hand side of sequence represent position of start nucleic acid for this window

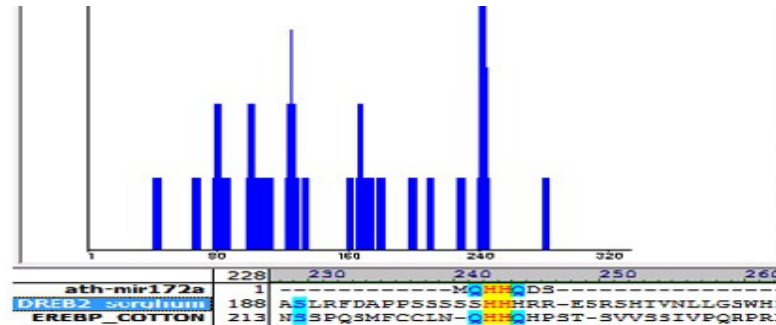


Fig. 4b. A profile study to see the probable degree of conservation of miRNA 172 target site in proteins of its mRNA Targets. Numbers on the left hand side of sequence represent position of start amino acid for this window .

Fig.4b: A profile study to see the probable degree of conservation of miRNA 172 target site in proteins of its mRNA Targets. Numbers on the left hand side of sequence represent position of start amino acid for this window

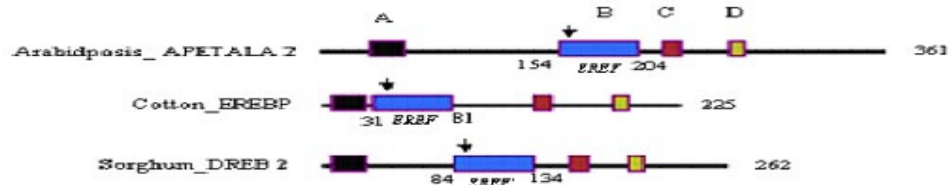


Fig. 4c. Output of block analysis to find the probable existence of miR172 target in functional or conserved regions of protein sequences of its mRNA targets in cotton and sorghum. Arabidopsis APETALA 2 transcription factor is taken as the reference sequence as miR172a shows target site recognition in its mRNA and fall into the same family as DREB and EREBP. Arrows indicated the position of miR172 target site .The numbers at the right hand side being the protein length. The size of block (block B) of interest is 51.

Fig.4c: Output of block analysis to find the existence of miR172 target in functional or conserved regions of protein sequences of its mRNA targets in cotton and sorghum. Arabidopsis APETALA 2 transcription factor is taken as the reference sequence as miR172a shows target site recognition in its mRNA and fall into the same family as DREB and EREBP. Arrows indicated the position of miR172 target site. The numbers at the right hand side being the protein length. The size of block (block B) of interest is 51.

Ara_APETALA2 : 154 RGVTLHKCGRWEARMGQFLGKCYVYLGFLD TEVEAARAYDKAAIKC NGKDA 204

Cotton_ERBP : 31 RGVKRPWGRFAAEIRDPLKKTRVWLGTF DSAEEAARAYDAAITFRNK 81

Sorghum_DREB2: 84 RGVQRQRTWVKWVAEIREPNRGRRLWVLSFPTAVEAAHAYDEAAKAMYGPKA 134

Fig. 4d. Ethylene responsive element binding protein signature form 1-17 . Annotation of block B shows the presence of an Ethylene responsive element binding protein signature colored in red and the one colored in green the profile hit of miR172 target site.

Fig.4d: Ethylene responsive element binding protein signature form 1-17. Annotation of block B shows the presence of an Ethylene responsive element binding protein signature colored in red and the one colored in green the profile hit of miR11172 target site

Table 5: Optimization of *in situ* hybridization protocol

Signal intensities	Probe concentration (ng/μl)				
	0.1	0.5	1.0	1.5	2.0
	+	++	++++	++++	++++
	Buffer concentration (X)				
	0.1	0.2	0.5	1.0	
	+	++++	++++	++++	
	Washing time (minutes)				
	15		45	60	
	+		++++	++++	

Note:

- +
 - ++
 - +++
 - ++++
- : Absent (0-9%)
- : Very low (10-25%)
- : Moderate (25-75%)
- : High (100%)

4.2.1.3 Pre-antibody treatment

Another most important consideration of *in situ* hybridization is BSA treatment just prior to anti-DIG antibody incubation. Here, three different washing times were tried (Fig. 7). The results indicated that 15min washing period gave very poor signal intensity, whereas, 45 and 60 min washing period resulted in high signal intensities. Therefore, 45 min washing period was used to get good quality of hybridization signal.

4.2.2 Drought induction in *Arabidopsis*

The induction of drought was done by following the procedure of Agnes *et al.* (2004). The relative water content (RWC) of leaves were measured for each levels of stress and well watered control plants which are presented in Fig. 8.

4.2.3 Expression of miR171 in different tissues

The miR171 was expressed throughout different parts of leaves. It was accumulated in the cuticle, lower and upper epidermis, spongy and palisade mesophyll tissues and in vascular bundle (Plate 4b). Longitudinal sections of shoot apex when hybridized with miR171 showed its accumulation in the axillary buds, apical leaves along with young vascular tissues. The expression of miR171 was clearly observed in root tissues irrespective of treatments (Plate 5b). A differential expression pattern observed in all sections of root tissues irrespective of treatments (Plate 6a and b)

The higher accumulation was observed in the apical meristem, leaf primordia and axillary buds. Apart from these, its expression was very clear in young vascular tissues throughout the zone of differentiation and elongation (Plate 5a). The longitudinal section of floral meristem showed the presence of miR171 in the developing ovary (Plate 4a). The transverse section of mature flowers showed distinct expression of miR171 in sepals, petals and stamens. Its presence was also detected in the ovary and anthers (Plate 1b). In longitudinal section it showed accumulation in the placenta wall, immature seeds, tip portions of sepals and petals (Plate 3b).

The flower sections of different developmental stages were taken to detect the presence of miR171 in stage specific manner. In sepals it accumulates clearly as in petals and ovary (Plate 1a). The expression was observed in the developing ovules and young vascular bundles. In successive stage, miR171 was highly expressed in ovary, anther and petals (Plate 3a). In anthers miR171 was expressed in its different parts like tapetum, filament and pollen sac (Plate 2a). In case of ovary miR171 was accumulated in the developing ovule and placenta. Its differential expression was also observed in funiculus which connects ovule to placenta. The ovary walls also accumulate miR171 but lesser than the funiculus (Plate 2b).

In developing silique sections its expression was observed. In young developing silique miR171 was expressed in fused carpels and pods. But in case of mature silique the expression was very less or absent in seeds but still continuously expressed in pod and it was confirmed in both the transverse and longitudinal section of silique (Plate 7a, b and c).

4.2.4 *In situ* detection of drought responsive miRNAs in root tissues

Root samples (approximately 12 cm long) were taken and divided into three zones,

1. The zone of differentiation or basal zone, BZ (2-5 cm below the base),
2. The zone of elongation or middle zone, MZ (5-8 cm below the base) and
3. The zone of cell division or terminal zone, TZ (9-12 cm below the base).

After fixation, embedding and sectioning step the tissues on slides were hybridized with respective probes.

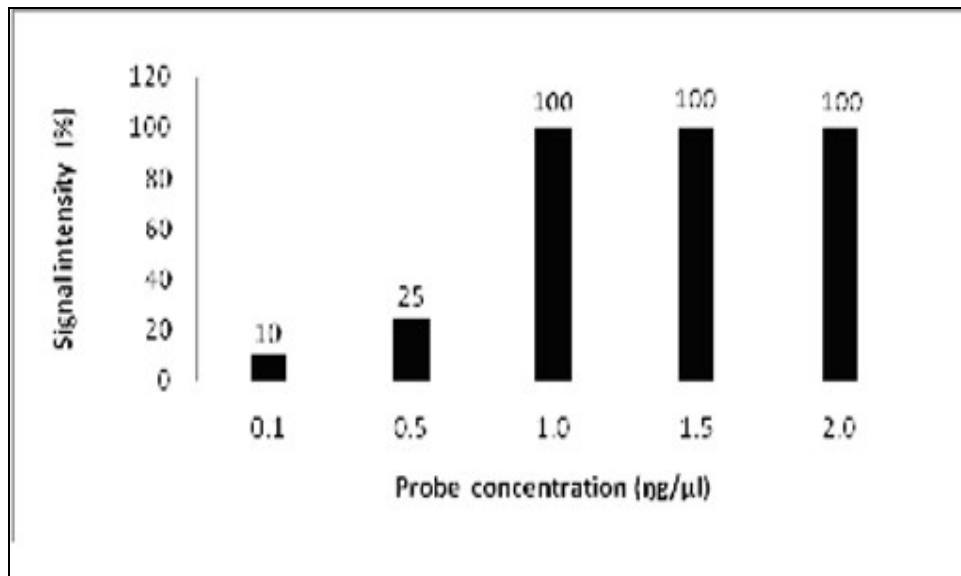


Fig.5: Optimization of probe concentration. Five different probe concentrations (0.1 ng/μl, 1.0 ng/ μl, 1.5 ng/ μl and 2.0 ng/ μl) were tried for optimization of hybridization signal intensity. For 0.1 ng/ μl and 0.5 ng/ μl probe concentration the signal intensity was low while from 1.0 ng/ μl probe concentration showed maximum signal intensity. Probe concentration of 1.0 ng/ μl was considered optimum

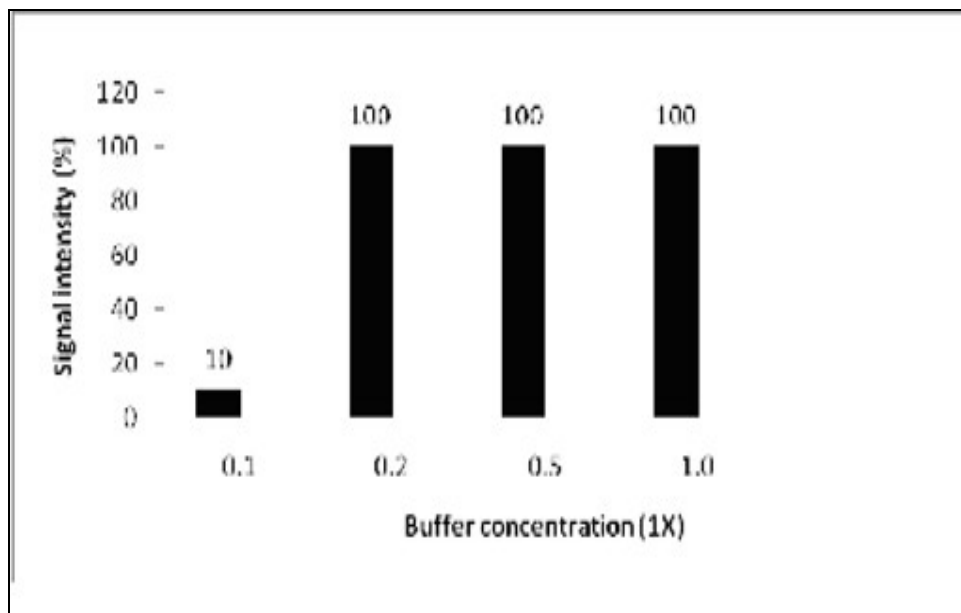


Fig.6: Optimization of buffer concentration. Four different buffer concentrations (0.1X, 0.2X, 0.5X, and 1.0X) were tried in post-hybridization step for highly stringent wash to get good quality hybridization signals. Among these, 0.1X buffer wash showed very low signal intensity whereas, remaining have given maximum signal intensity. Buffer concentration of 0.2X was considered suitable.

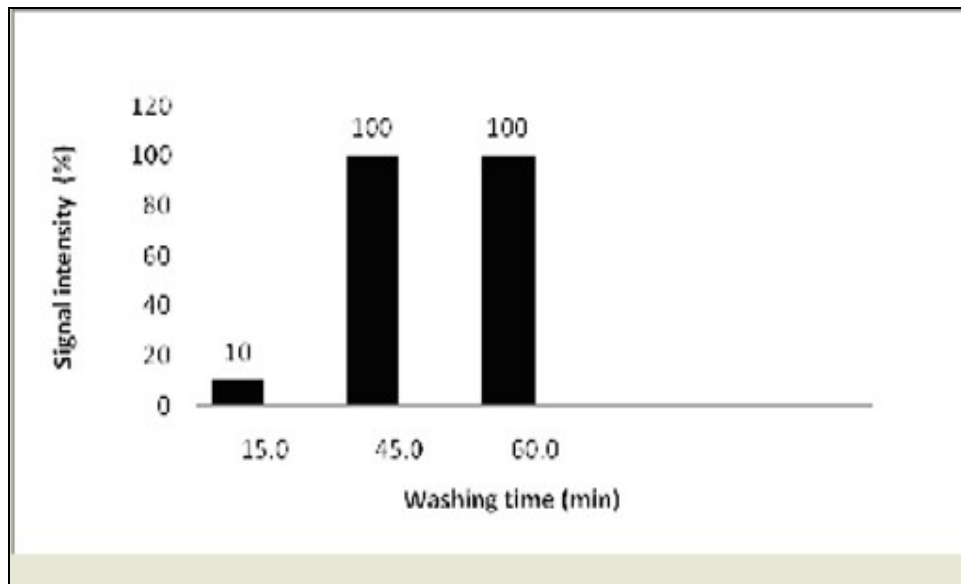


Fig.7: Optimization of washing time. In post-antibody treatment three different washing time (15 min, 45 min and 60 min) were tried after antibody treatment. Among these, 15 min wash resulted in poor signal intensity while, 45 min and 60 min wash showed maximum hybridization signal. Washing time for 45 min was considered as optimum for good signal intensity.

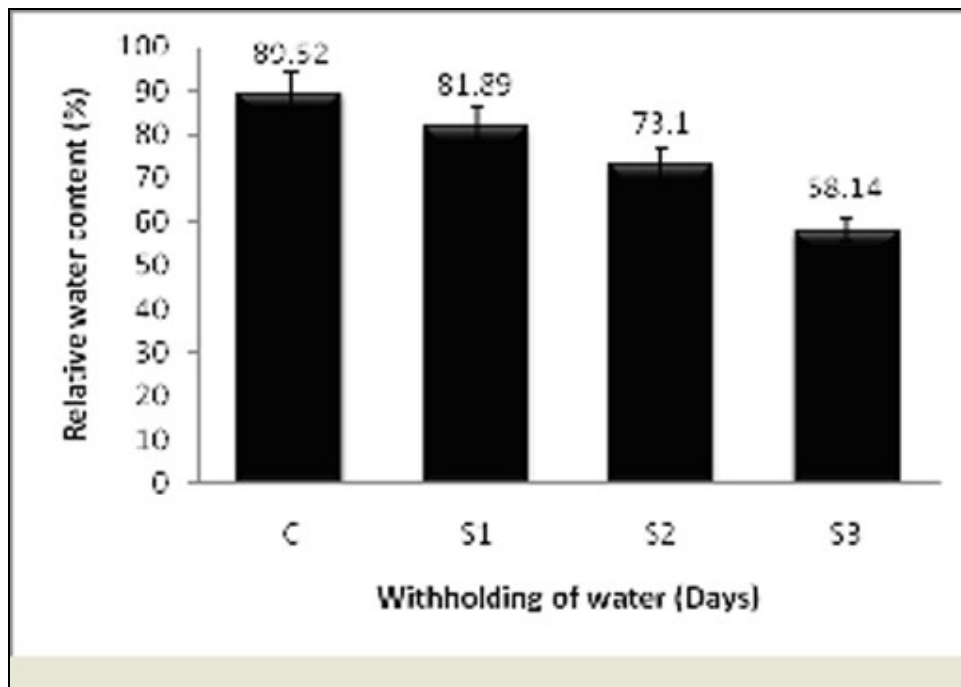


Fig.8: Relative water content of leaves measured in different stress level. Control (C) - well watered, stress1 (S1) -6 day withholding of water, stress 2 (S2) -11 day withholding of water and Stress 3 (S3) -14 day withholding of water. The relative water content (RWC) was measured as described by Agnes et al., (2004) for each of these stresses along with the well watered plants. The formula used is as follows, $RWC = \frac{FW - DW}{TW - DW} \times 100$, where FW= fresh weight, DW= dry weight and TW= turgid weight of samples. (at 5% level of significance p value 5.27E-32)

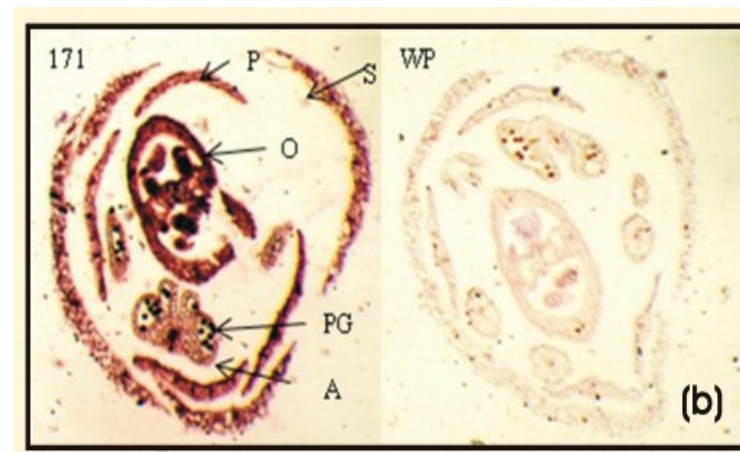
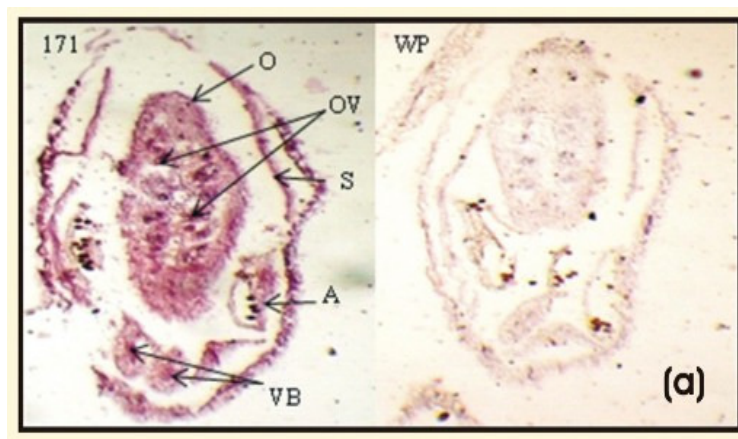


Plate1: Transverse sections of young flower (a) and mature flower (b) hybridized with DIG-labelled LNA modified miR171

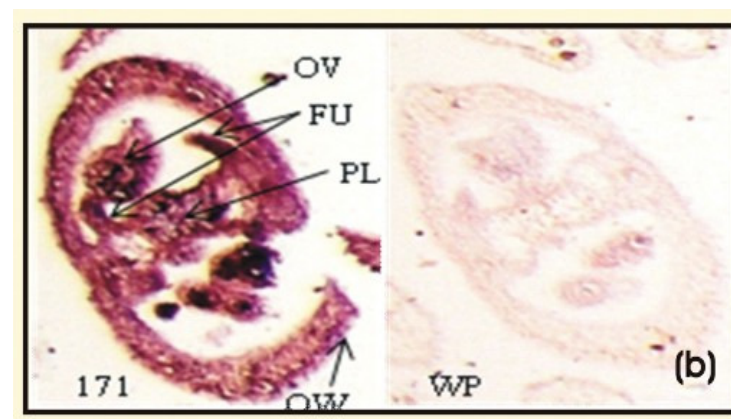
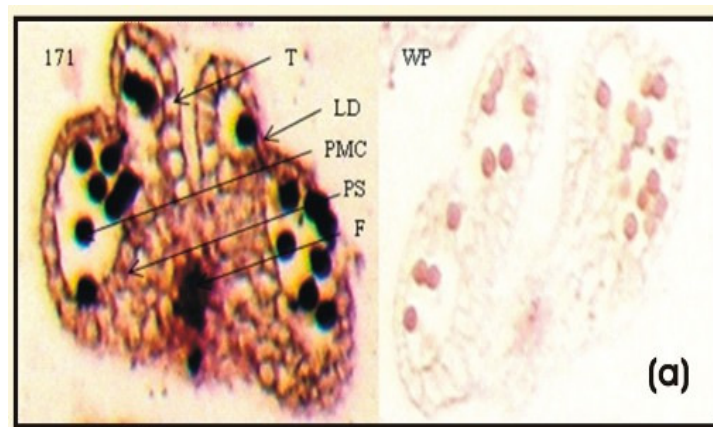


Plate2: Transverse sections of anther (a) and ovary (b) hybridized with DIG- labeled LNA modified miR171

Note: O-Ovary, OV-Ovule, S-Sepal, A-Anther, P-Petal, PG-Pollen Grain, T-Tapetum, LD-Line of Dehiscence, PMC-Pollen Mother Cell, F-Filament, PS-Pollen Sac, PI-Placenta, FU-Funiculus and OW-Ovary Wall



Plate3: Longitudinal sections of young flower (a) and mature flower (b) hybridized with DIG- labeled LNA modified miR171

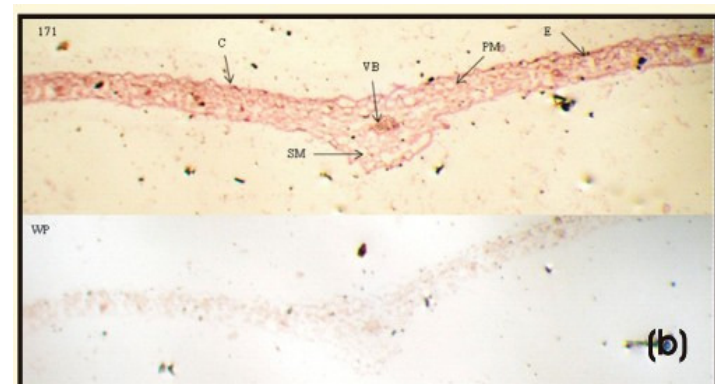
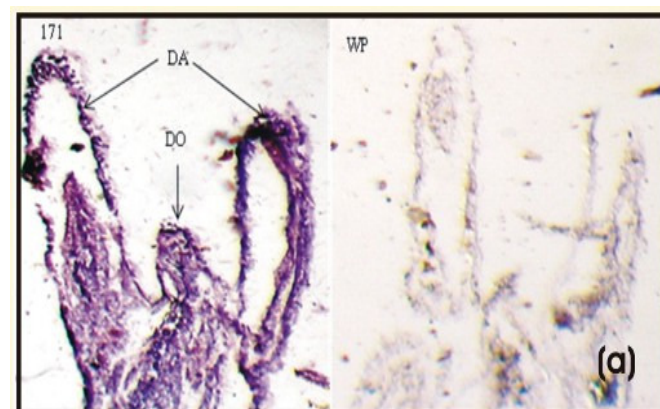


Plate.4: Longitudinal sections of floral primordial (a) and leaves (b) hybridized with hybridized with DIG- labeled LNA modified miR171

Note: O-Ovary, A-Anther, P-Petal, ST-Stigma, IS-Immature Seed, R-Receptacle, PD-Pedicel, PL-Placenta, DO- Developing Ovary, DA- Developing Anther, C-Cuticle, VB-Vascular Bundle, PM-Pallisade Mesophyll, SM-Spongy Mesophyll, E-Epidermis

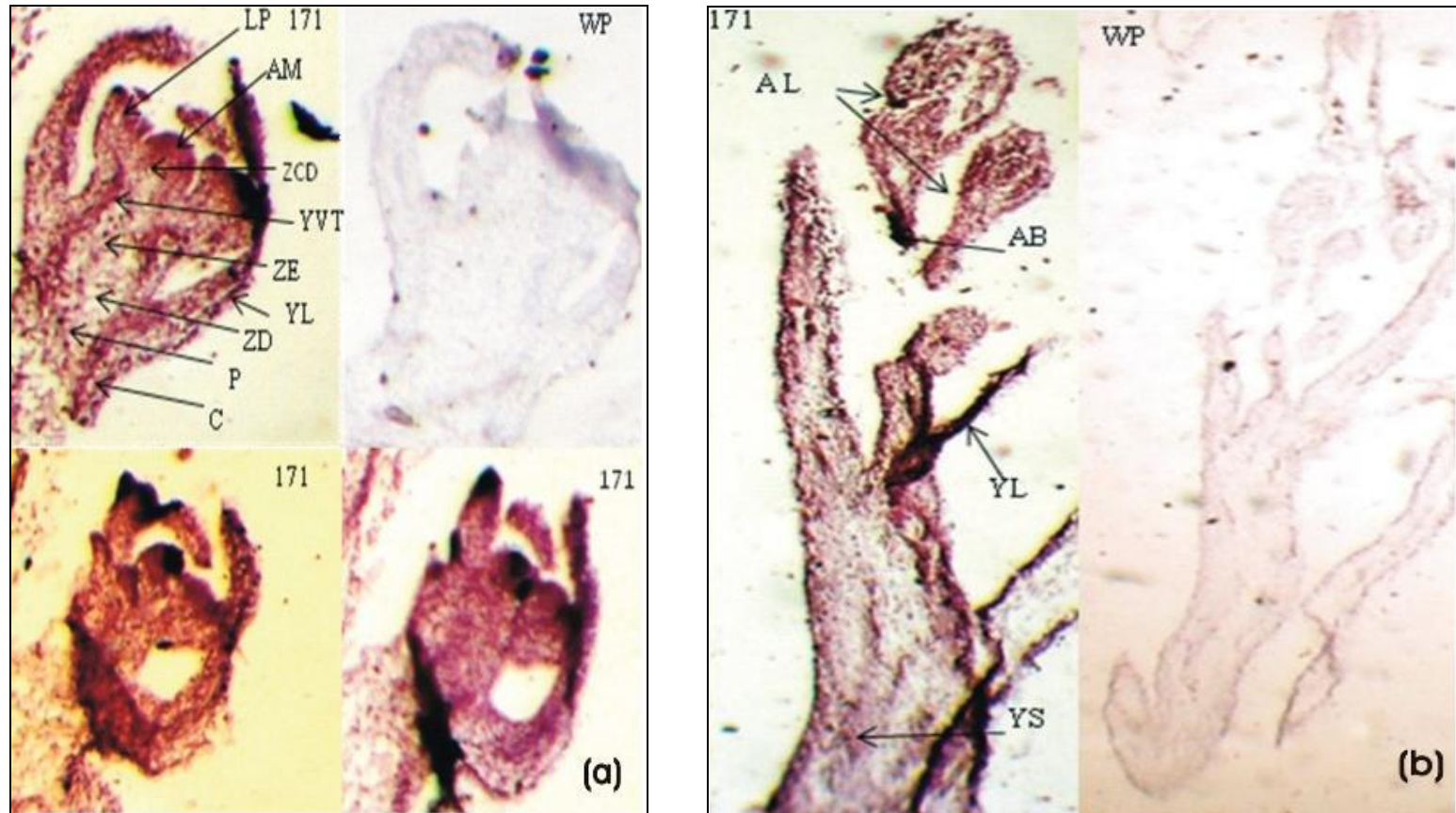


Plate5: Longitudinal sections of apical meristem (a) and shoot apex (b) hybridized with DIG-labelled LNA modified miR171

Note: LP-Leaf Primordia, AM-Apical Meristem, ZCD-Zone of Cell Division, YVT-Young Vascular Tissue, ZE-Zone of Elongation, YL-Young Leaf, ZD-Zone of Differentiation, P-Pith, C-Cortex, AL-Apical Leaf, AB-Axillary Bud, YL-Young Leaf and YS-Young Stem

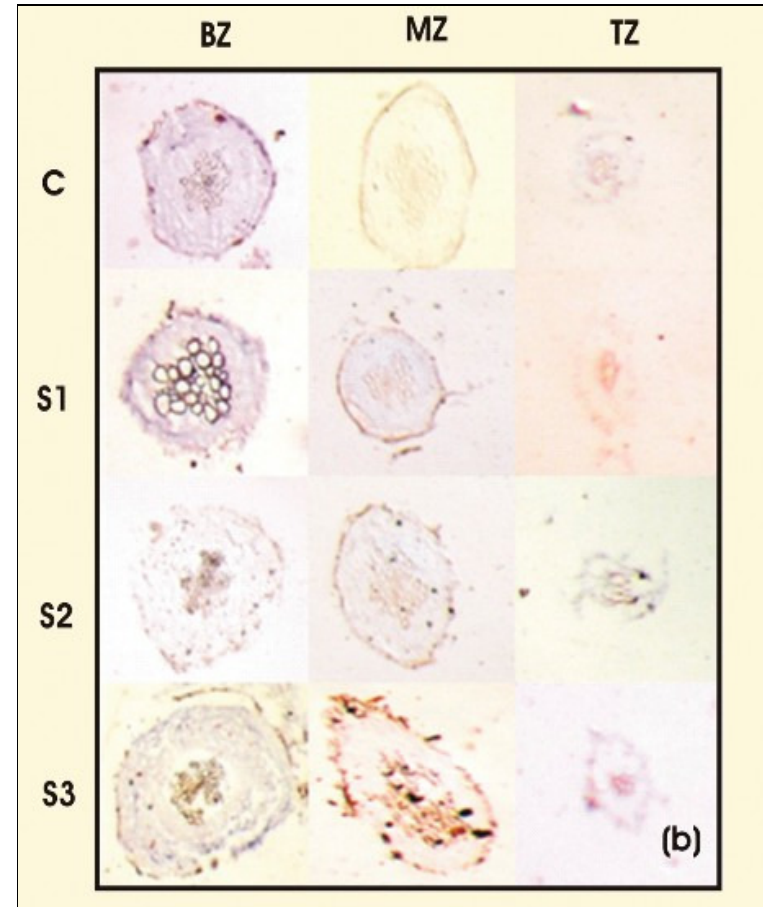
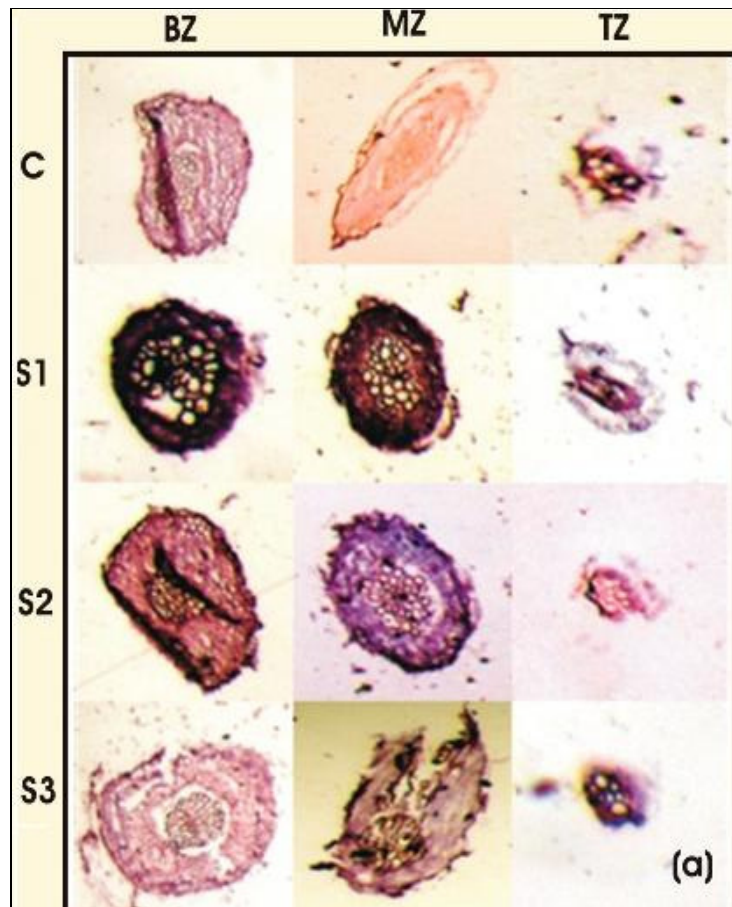


Plate.6: Root Sections hybridized with miR171 (a) and WP (B) probes for different stress in all 3 Zones

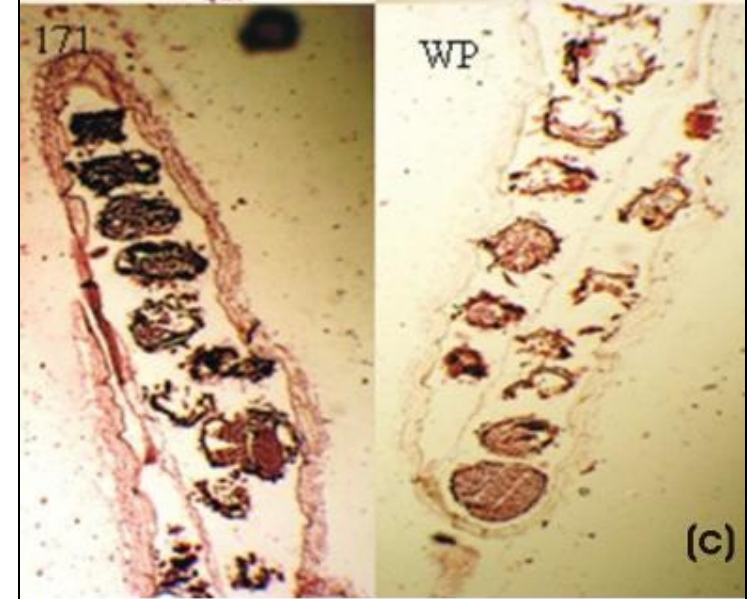
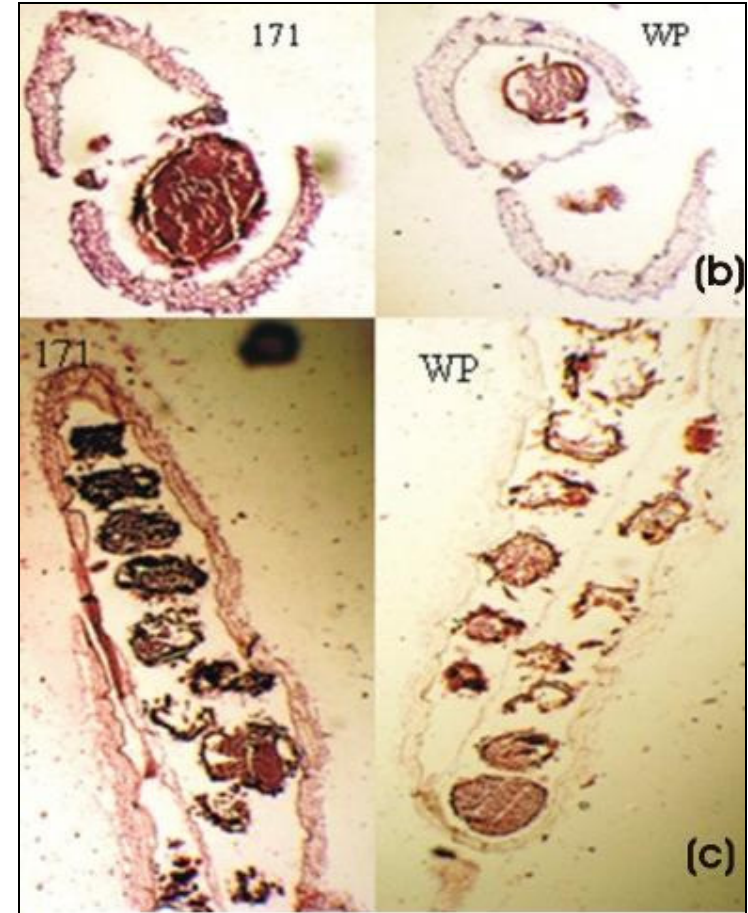
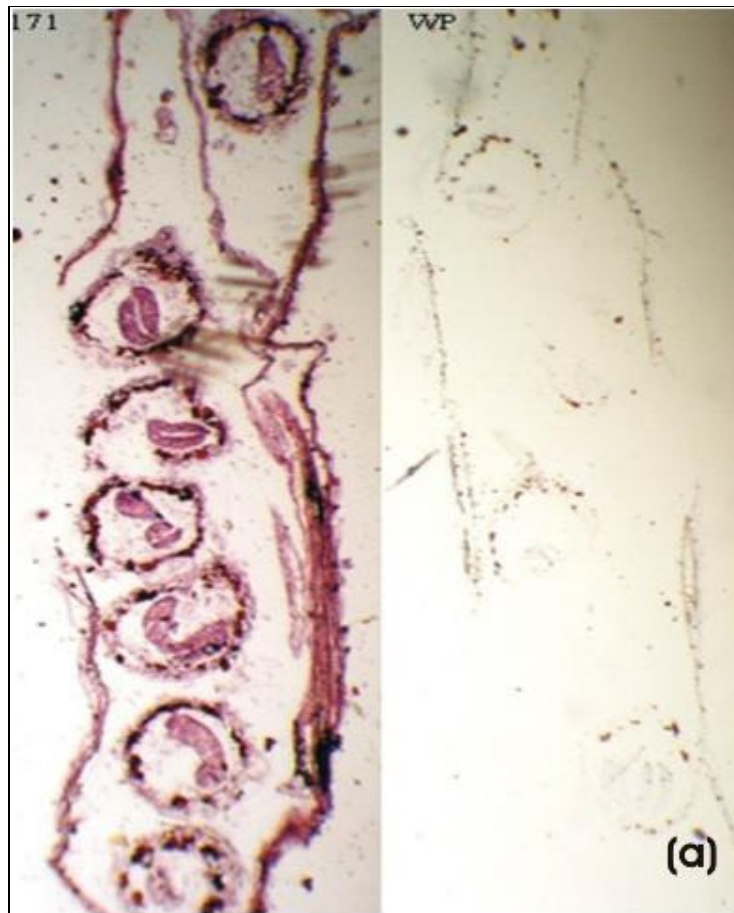


Plate.7: Transverse sections of mature silique (a) and longitudinal sections of young (b) and mature silique (c) hybridized with DIG-labelled LNA modified miR171

In all three drought levels (S1, S2 and S3) and well watered (control) condition miR171 expression was observed in all the tissues in a constitutive manner. In this study miR171 was used as a positive control and the negative control was set up without the probe (WP) as summarized in Table 6.

4.2.4.1 Expression of miRNAs in basal zone

In well watered root tissues the expression was observed only for miR397a and miR171. After first stress treatment, miR399, miR399e, miR395b, miR161, miR397a and miR171 were found to express in the basal zone and comparatively higher expression was seen for miR395b and miR171. In case of stress2 root tissues all miRNAs were found to express, except miR168 where miR395b was expressed little lower quantities compare to others. On severe stress the all miRNAs were showing a clear hybridization signal except the miR168. The expression was typically more for the miR171 compared to others at the same level (Plate 12).

4.2.4.2 Expression of miRNAs in middle zone

Only miR395b and miR171 were found expressing in well watered root tissues. In stress1 root tissues were showing expression only for miR395b, miR397a and miR171 where higher expression was observed for the miR171 and miR397a compare to others. After moderate stress treatment (S2) it was found that most of them were not expressing and only miR397a and miR171 were giving hybridization signals. When severe stress was given the miR168 was still undetected where others were expressed differentially (Plate 13).

4.2.4.3 Expression of miRNAs in terminal zone

In well watered root tissues the clear expression were observed for miR397a, miR399e miR161 and miR171. Lower expression was detected for the miR399 and miR395b where miR168 was completely absent. In stress1 root tissues miR399, miR399e, miR397a and miR171 were showing expression where others were undetected as like miR168. The expressions of all the miRNAs except miR168 were very much clear and prominent in stress2 level. But in severe stress miR171, miR397a, miR161 and miR395b were showing expression signal whereas miR399 and miR399e were undetected (Plate 14).

4.2.5 *In situ* detection of drought responsive miRNAs in leaf tissues

Longitudinal sections of leaves were taken on probe-on plus slides and hybridization was performed for each probe. In all three drought (S1, S2 and S3) and well watered (control) condition a differential pattern of expressions was observed with respect to tissue specificity, zonal distribution of miRNAs as well as expression pattern in a constitutive manner. In our study the miR171 was used as a positive control and without probe (WP) was used as a negative control in all the tissues under consideration.

4.2.5.1 Expression of miRNAs in well watered (C) leaf tissues

In our studies only 4 miRNA (miR168, miR161, miR397a and miR171) were expressed in well watered condition where others (miR399, miR399e and miR395b) were not at all expressed (Plate 18A). miR171 was showing its ubiquitous expression where the level of expression was little low for miR161 under well watered leaf tissues.

4.2.5.2 Expression of miRNAs in S1 leaf tissues

With little change in environment by withholding watering it was observed that miR399 and miR399e were strongly expressed in the leaf tissues. Similarly, miR168 showed the same expression pattern as like miR399. But miR397a was found absent in leaf tissue which is just reverse of well watered condition. In case of miR395b it was found completely absent as like the well watered leaf tissues. The constitutive expression of miR171 was continued in this stress condition (Plate 18B).

Table 6 : Expression of seven miRNA in root and leaf tissues of *Arabidopsis* in well watered and moisture stress regime

Root tissues									
Treatments	Zones	399	399e	168	395b	161	397a	171	WP
CONTROL	BZ	-	-	-	-	-	+++	+++	-
	MZ	-	-	-	+	-	+	++	-
	TZ	+	++	-	+	+++	++	+++	-
STRESS 1	BZ	+++	++	-	++++	+++	++	+++	-
	MZ	-	-	-	++	-	++	+++	-
	TZ	++	++	-	-	-	+	++	-
STRESS 2	BZ	+++	++	-	++	+++	++	+++	-
	MZ	-	-	-	-	-	+++	+++	-
	TZ	++	++	-	+++	+++	++	++	-
STRESS 3	BZ	+++	++	-	++	+++	++++	++++	-
	MZ	++	+	-	++	++	++	++	-
	TZ	+	+	-	++	++++	+++	+++	-
Leaf tissues									
Treatments		399	399e	168	395b	161	397a	171	WP
CONTROL		-	-	++	-	++	+	++	-
STRESS 1		+++	+++	+++	-	+++	+	+++	-
STRESS 2		++	++	++	-	++	+	++	-
STRESS 3		++	++	++	-	++	+	++	-

Note:

- : Absent
 - +
 - ++
 - +++
 - ++++
- : Very low (0-25%)
: Moderate (>25-50%)
: High (>50-75%)
: Very high (>75-100%)

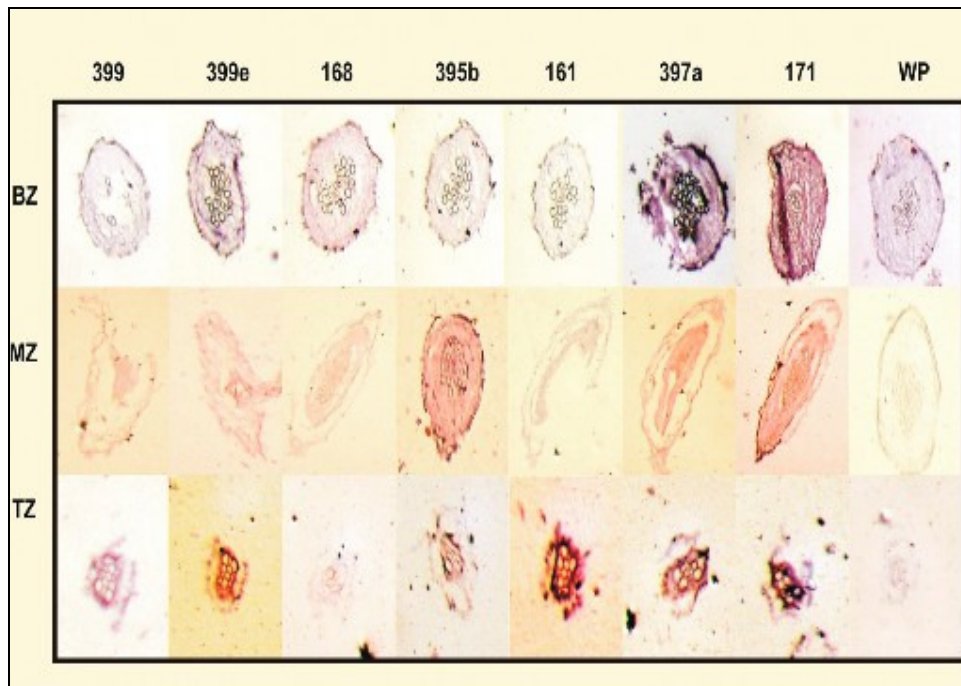


Plate8: Well watered root sections hybridized with selected DIG- labelled LNA modified probes in different zones

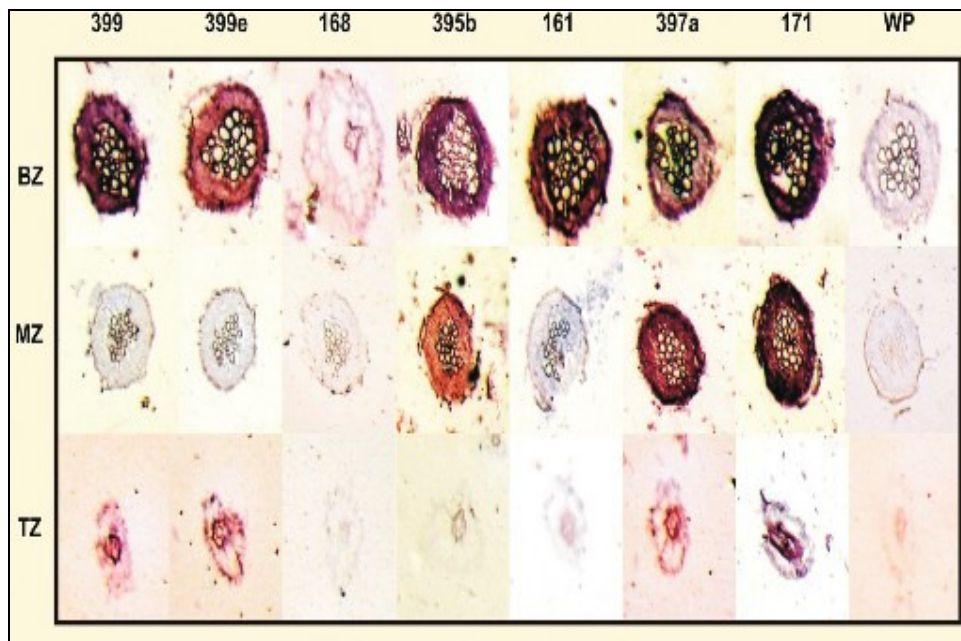


Plate.9: Mild stressed (S1) root sections hybridized with selected DIG- labeled LNA modified probes in different zones

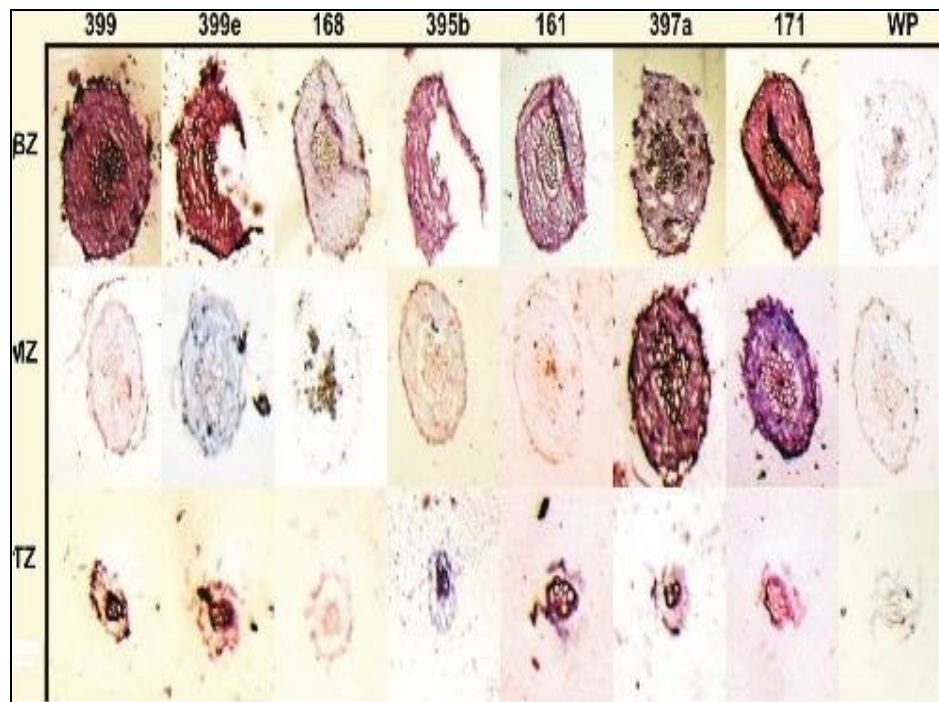


Plate10: Moderate stressed (S2) root sections hybridized with selected DIG-labelled LNA modified probes in different zones

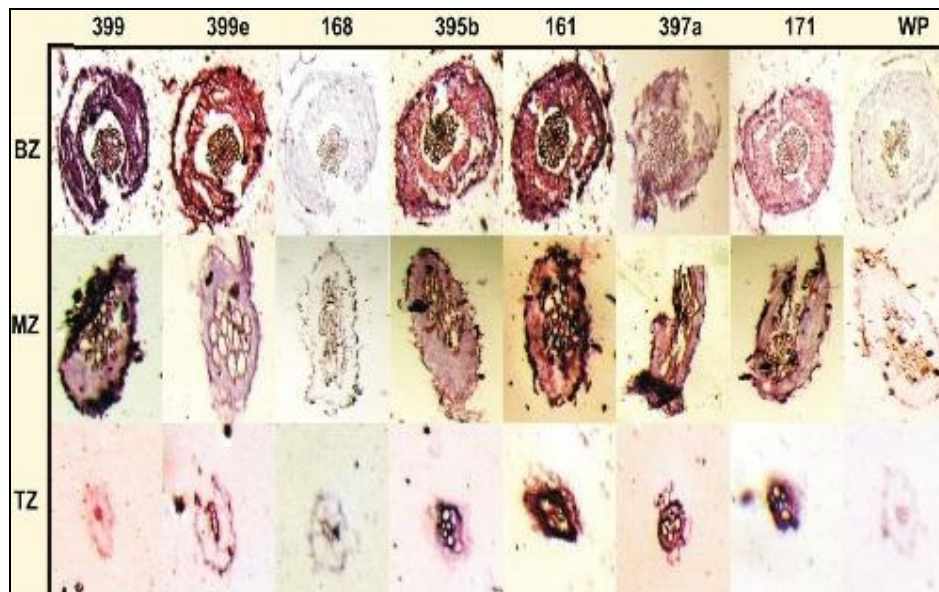


Plate11: Severe stressed (S3) root sections hybridized with selected DIG- labeled LNA modified probes in different zones

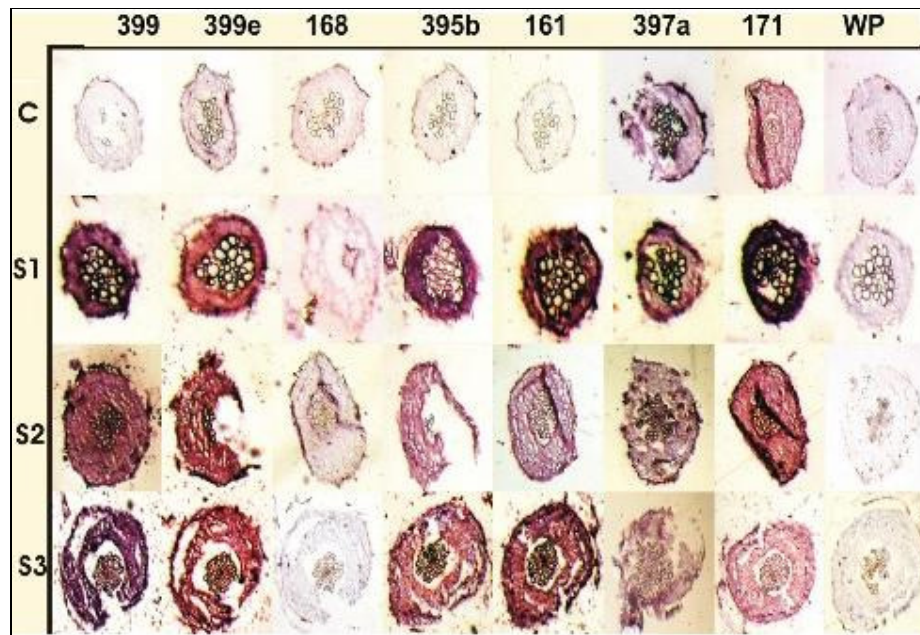


Plate.12: Basal zone root sections hybridized with selected DIG-labelled LNA modified probes for different stress

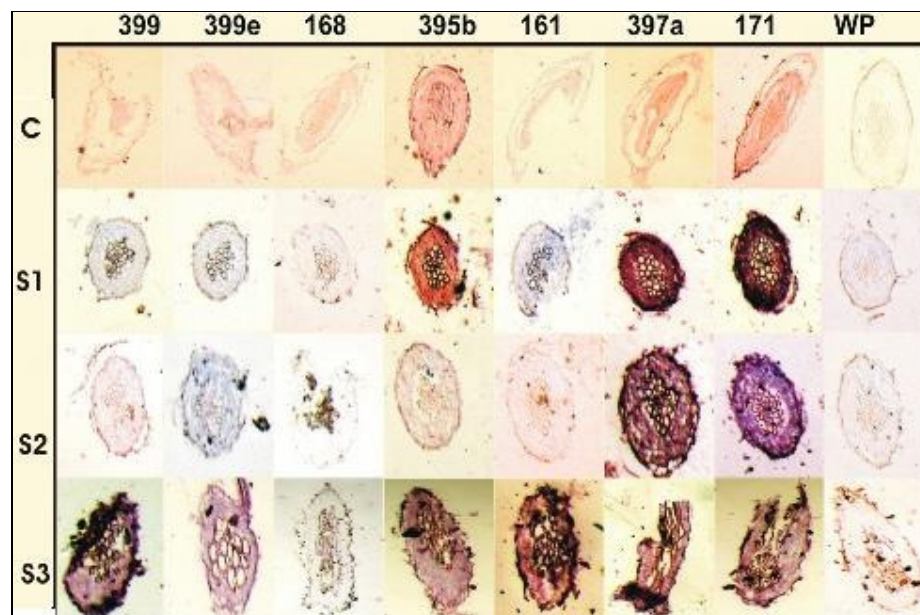


Plate.13: Middle zone root sections hybridized with selected DIG- labelled LNA modified probes for different stress

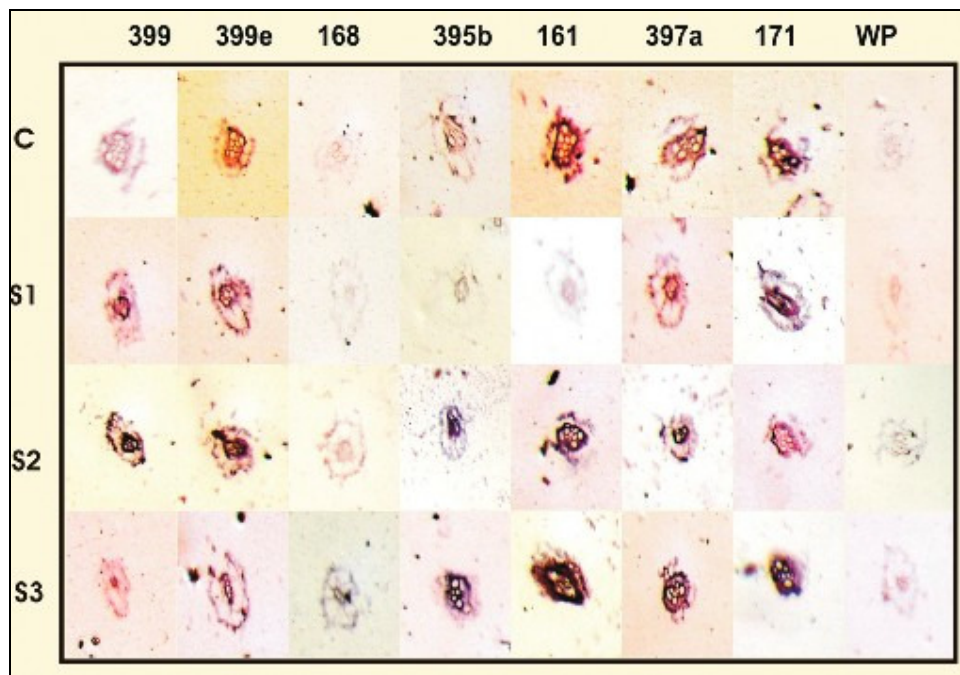


Plate.14: Terminal zone root sections hybridized with selected DIG-labelled LNA modified probes for different stress

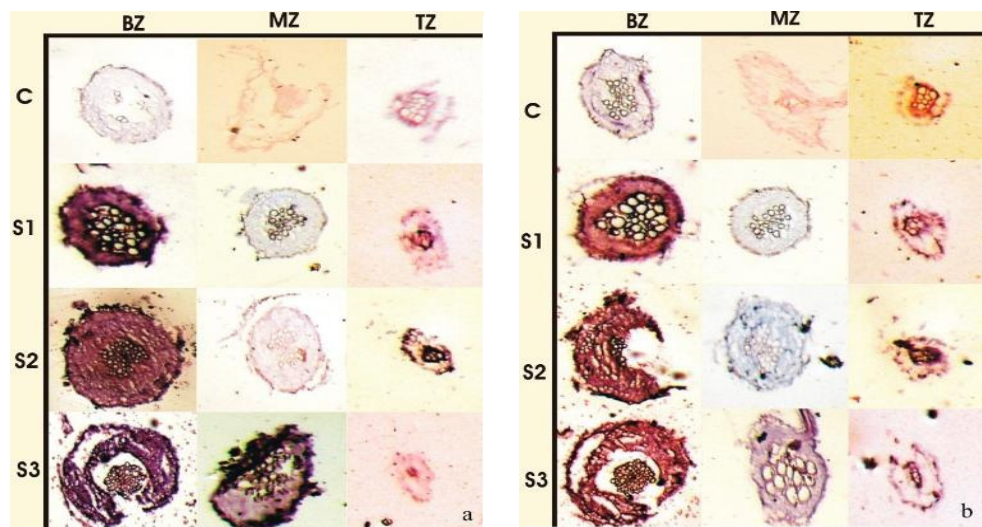


Plate15: Root sections hybridized with miR399 (a) and miR399e (b) probes for three different zones

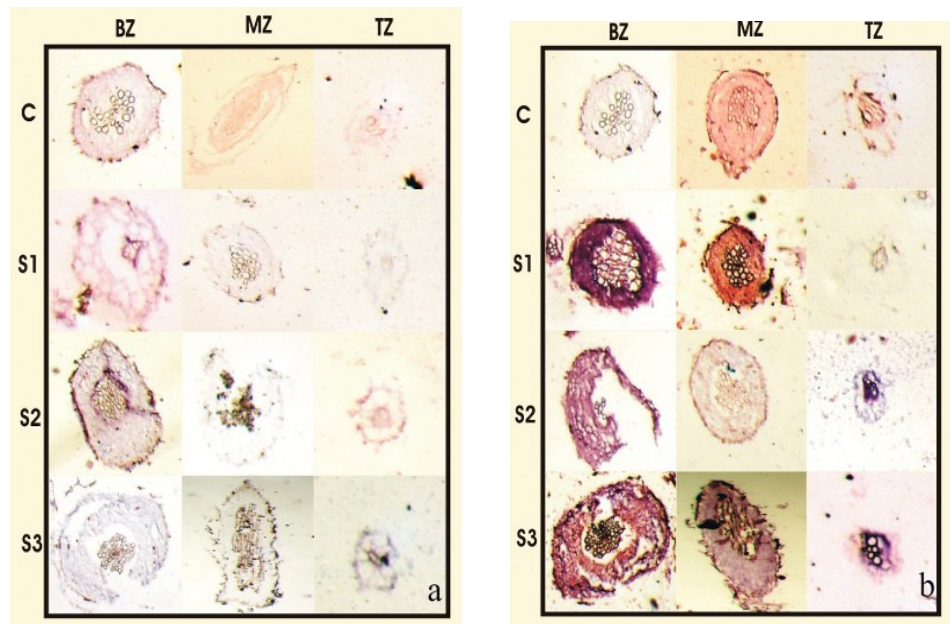


Plate.16: Root sections hybridized with DIG- labeled LNA modified miR168(a) and miR395b (b) probes for three different zones

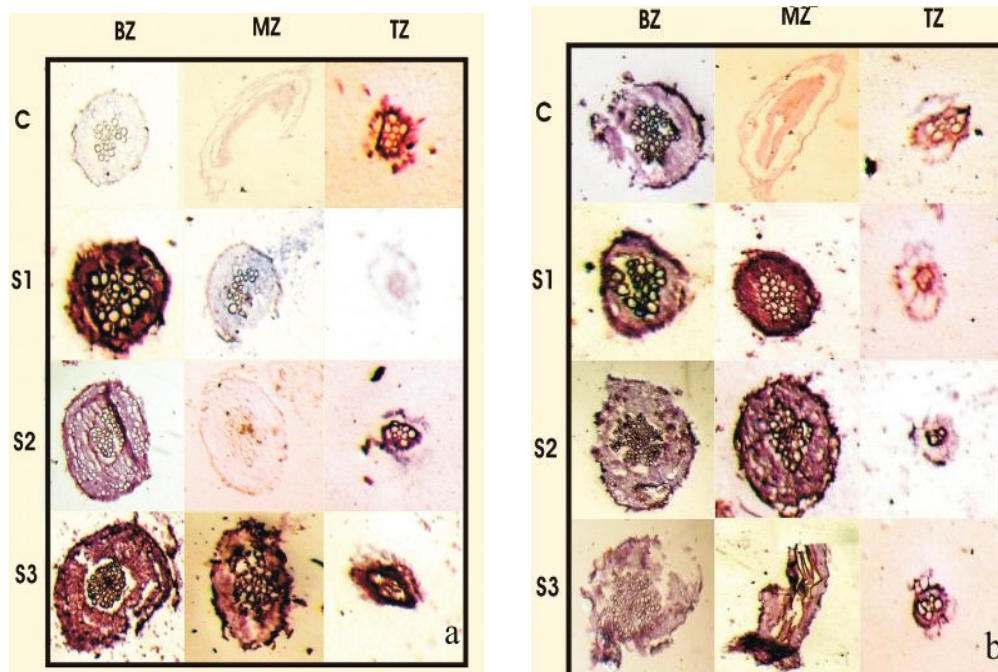


Plate.17: Root sections hybridized with DIG-labelled LNA modified miR161(a) and miR379a(b) probes for three different zones

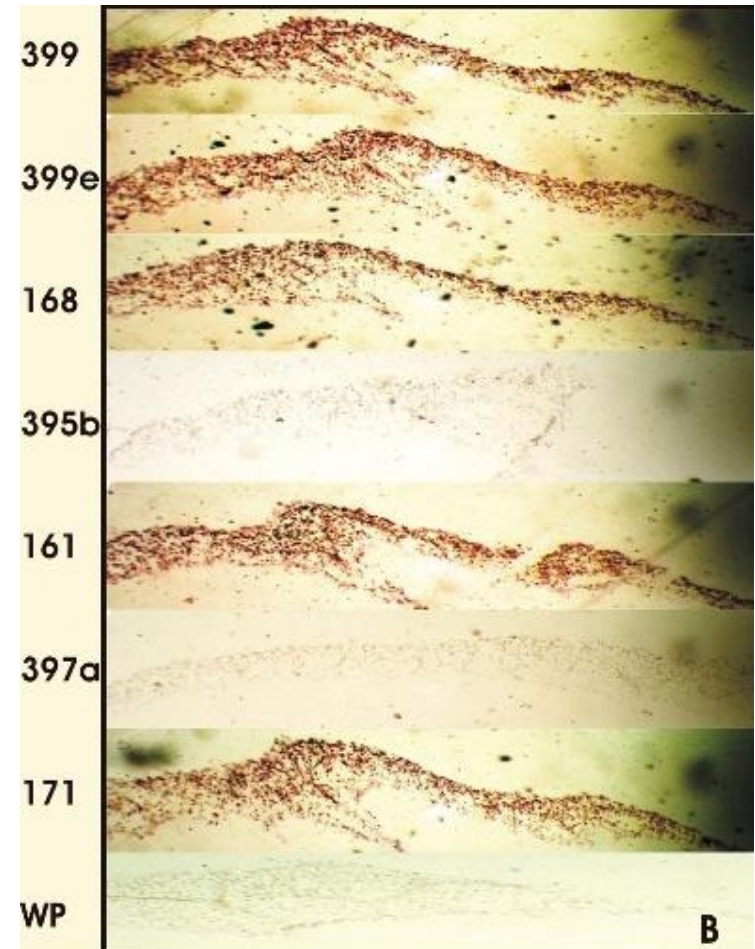
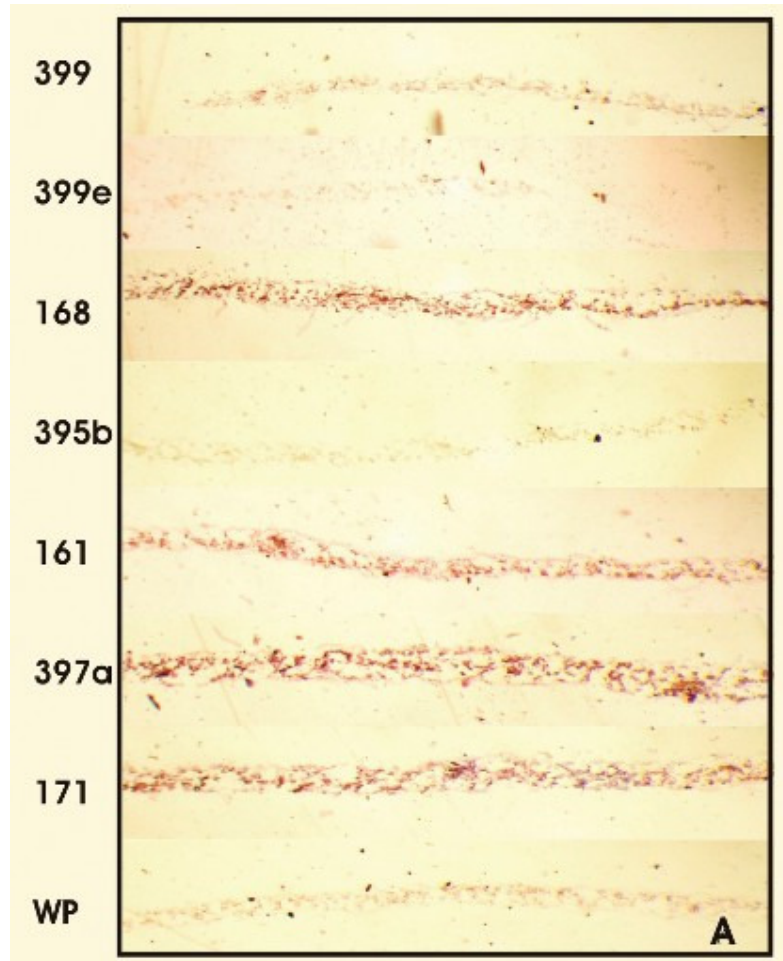


Plate.18: LS of well watered leaves (A) and stressed 1 leaves (B) hybridized with selected DIG-labelled LNA modified probes

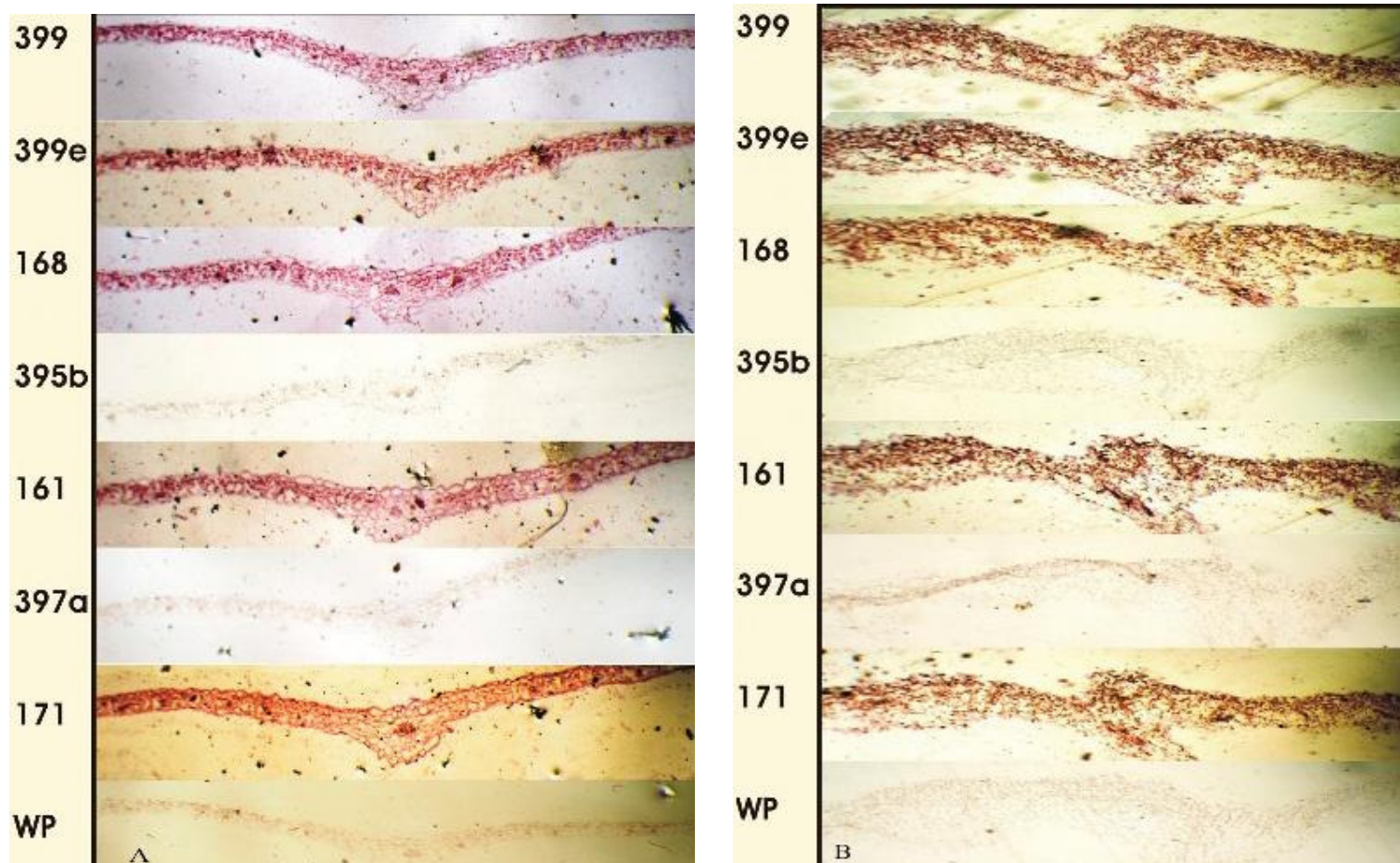


Plate: LS of stressed 2(A) and stressed 3 leaves (B) hybridized with selected DIG-labelled LNA modified probes

4.2.5.3 Expression of miRNAs in S2 leaf tissues

The expression of miR168 was detected and similarly, miR399 and miR399e were showing distinct hybridization signals in different region of leaf tissue. The signal was also prominent for miR161 and miR171. Expression was completely absent in case of miR395b and miR397a as like the stress1 leaf tissues (Plate 19A).

4.2.5.4 Expression of miRNAs in S3 leaf tissues

In this case watering was stopped for 14 days which showed the continuous expression of miR399 and miR399e. But the level of signal intensity was found more than the stress2 leaf tissues. Here also miR395b and miR397a was found completely absent. The expression of miR168 was very clear and intensity of expression was found bit higher. The miR171 was found expressing as per the expectation (Plate 19B).

5. DISCUSSION

A vast majority of miRNAs are having diverse regulatory role in plants and animals development. New methods identifying miRNAs and their targets have been developed recently. The computational method of miRNA target prediction is especially works well in plants as the plant miRNAs are fully complementary to their targets. Experiments in miRNA gene prediction, target analysis and their functional assessment show a wide range of distribution and their biological diversity to conserved regulatory networks in biological systems. The identification of a near complete set of miRNAs in any organism is of fundamental importance to understanding miRNA-mediated gene regulation and the diversity of miRNAs. It lays the necessary foundation for unraveling the complex miRNA-mediated regulatory networks controlling development, nutrient responses and tolerance to biotic and abiotic stresses in plants.

In silico predictions of plant miRNAs and their putative targets in ESTs is possible in computational genomic approach. ESTs are most widely sequenced nucleotide commodity of the plant genomes in terms of the number of sequences and the total nucleotides. EST resources provide a robust sequence resource that can be exploited for gene discovery, genome annotation and comparative genomics. Currently, to identify new miRNAs and their targets, many algorithms have been developed by different groups around the world. For plant miRNA target identification normal BLAST search against EST datasets is a simple and accepted method as plant miRNAs are fully complementary to their target transcripts. Successful identification and validation of a set of miRNAs, which are specific to cold, drought and salinity has been done. Therefore, identification of miRNA targets in diverse crop species to know the conservation pattern of miRNA targets which would help in understanding foundation of gene regulation mechanisms.

miRNAs are very difficult to detect accurately as they differs in a few nucleotide from each other. Using normal DNA and RNA probes the specificity of detection was reported to be very low (Valoczi *et al.*, 2006). To overcome this problem, newly emerged locked nucleic acid (LNA) probes which are very specific and have higher thermal stability as compared to conventional DNA or RNA probes, made the possibilities for accurate interpretation of their expression patterns. LNA probes are very specific to their complementary targets for hybridizations like *in situ*, microarray, chip-on-chip, northern, etc. due to their locked furanose ring; the puckered structure is restricted in 3' endo-conformation. LNA was mainly suitable to detect SNPs. Therefore, to detect miRNAs in tissues they are also found suitable as they differ from each other by a few bases. This technique has enabled pinning down the specific role of miRNAs in various gene regulatory networks in a far more precise manner than before through *in situ* hybridization.

5.1 Identification of miRNA targets in sorghum and cotton EST data sets

Using available 207 *Arabidopsis* miRNAs a set of 125 miRNA targets were identified in sorghum ESTs. Among these, 29 miRNAs belong to 24 different miRNA families were found to have targets uniquely in sorghum. Annotation of those targets revealed a clear distinction of monocot specific functional proteins such as starch synthase IIc precursor, sucrose non-fermenting related protein kinase, etc. Most of the unique miRNAs identified in sorghum EST datasets showed single target transcript while rest had multiple targets. Putative vegetative storage protein was found as one of the targets of miR400 whose homolog was completely undetected in cotton EST dataset. Similarly, miR782 was found to have two target transcripts *viz.*, phytochrome B and sucrose non-fermenting related protein kinase. The same sucrose non-fermenting related protein kinase was also found to be regulated by miR831, which point to the diverse regulation pattern of miRNA in phylogenetically much distant species. One of the interesting predicted target transcript was MADS box protein which is the only target of miR173. Many putative protein targets were identified in sorghum EST dataset whose functions are yet to assigned. Besides these, plastid division regulator, and GTP-binding protein were found to be regulated by miR778 and miR863-5p respectively in sorghum.

In cotton EST data sets a total of 138 miRNA targets were detected when tested against available *Arabidopsis* miRNA sequences. A set of 42 miRNAs belonging to 34 miRNA families were uniquely identified in cotton. As cotton and *Arabidopsis* belong to dicot and phylogenetically close relatives of each other, it was expected that most of the functional target transcript would have more or less similar regulatory role in developmental and metabolic pathways for both the plants. Annotation of the putative target transcript of cotton includes transcription factors such as DREB, MYB, AP2/EREBP, ARF3, zinc finger protein, bHLH, etc., regulatory proteins such as RNA polymerase, PR protein, RUBISCO large subunit, NADH dehydrogenase, etc., fiber elongation protein such as alpha-tubulin, ACC synthase, cellulose synthase, etc. and a member of other metabolic proteins.

This study revealed one of the dehydration responsive element binding protein, DREB 3A, was targeted by two important miRNAs, viz., miR447c and miR158a, which recorded nine and five potential targets respectively in cotton. Different fiber elongation factor like alpha-tubulin and cellulose synthase had potential complementary binding site in respective transcripts as they were found to be targeted by miR447c and 172b* respectively. miR172b* is the antisense transcript of miR172b which is found to be regulating cellulose synthase transcript by antisense regulatory mechanism. Different members of the MYB family were found to be targeted by miR827, miR855 and miR397a which decipher the cumulative regulatory control of miRNAs in plant development. CDPK-related protein kinase, a class of proteins implicated in calcium signaling was found to be targeted by miR832-3p among the four identified targets. Therefore, apart from other regulatory role, the involvement of miRNAs in cell signaling is also apparent in cotton.

In plants miRNA families are well conserved between dicots and monocots. Of these, 7 miRNA families, viz., miR156/157, miR160, miR159, miR319, miR165/166, miR390 and miR408 have been also found in primitive land plants such as *Physcometrella* and *Selaginella* suggesting that these are deeply conserved and possibly involved in developmentally plastic process across taxonomic lineages. In addition, *Arabidopsis*, rice, *Populus* and *Physcometrella* possess many non-conserved lineage- or plant species-specific miRNA families have been reported (Axtell and Bartel, 2005). In the present study, it was interesting to note that all the above mentioned 7 miRNA families, viz., miR156/157, miR160, miR159, miR319, miR165/166, miR390 and miR408 were also detected to have their respective targets in sorghum and cotton. miR156, a plant miRNA family predicted to target the 3'UTR of several genes encoding SQUAMOSA promoter binding proteins (Reinhart *et al.*, 2002; Rhoades *et al.*, 2002). Our study revealed SBP transcription factor is targeted by miR156 family of miRNA in cotton and C2H2 zinc-finger protein in sorghum. Similarly, miRNA157 was found to target SBPs whereas in sorghum it targets O-methyltransferase. SBPs are thought to regulate the *Antirrhinum* floral meristem identity gene Squamosa (Klein *et al.*, 1996). Therefore, it can be assumed that this family miRNAs are sharing similar kind of regulatory network with respect to the dicot and monocot system.

There are five class of homeodomain (HD)-Zip genes in *Arabidopsis* genome, including *REVOLUTA* (*REV*), *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *ATHB8* AND *ATHB15* (Emery *et al.*, 2003). *PHB*, *PHV*, and *REV* are expressed in the adaxial domains of lateral organs, in vascular tissues, and in the apical meristem (McConnell *et al.*, 2001), while *ATHB8* and *ATHB15* appear to be expressed exclusively in the vascular tissues (Emery *et al.*, 2003). Loss-of-function *rev* alleles exhibits aberrant axillary and flower meristem formation and alterations in the position of inter-fascicular fibers in the stem (Otsuga *et al.*, 2001). In our experiment miR165a was found to target HD protein GL2-like 1 in cotton and OCL5 in sorghum respectively.

miR393 predicted to target on TIR1 (transport inhibitor response 1) like protein. It has been revealed that TIR1 together with auxin signaling F-box function as auxin receptors. The TIR1 family links auxin binding directly to transcription factor abundance and the control of auxin-specific gene expression (Badescu and Napier, 2006). TIR1-like protein as a miRNA target suggests that miRNAs may regulate auxin responsiveness at multiple points (Jones-Rhoades and Bartel, 2004). On the other hand, miRNA393 targets fatty acid desaturase in sorghum which were typically related to cereals metabolism and development. This shows the diversified regulatory role of miRNA in monocot and dicot system.

Auxin (principally indole-3-acetic acid) is an important hormone in plants. It affects many aspects of plant growth and development by influencing AUXIN RESPONSE FACTORS (ARF), a plant-specific family of DNA binding proteins (Hagen and Guilfoyle, 2002 and Weijers and Jurgens, 2005). ARF10, ARF16, and ARF17 are potential targets of miR160, and ARF6 and ARF8 are potential targets of miR167 (Rhoades *et al.*, 2002 and Mallory *et al.*, 2005). Mallory *et al.* (2005) demonstrated that disrupting miR160 function increased ARF10, ARF16, and ARF17 mRNA levels, altered GH3-like gene expression, and led to severe developmental defects. It was clear from the present results that in cotton miR160 and miR167 had the same target site in ARF transcripts. But in sorghum, putative actin depolymerizing factor and hypothetical protein was identified as the putative targets of miR167 and miR160 respectively. Hence, it is likely that these mRNAs regulate different hormonal pathways and metabolic signals with diversified functions in sorghum and cotton.

The regulatory function of miRNA159 found to be conserved between *Arabidopsis* and sorghum as the R2R3 type MYB was the only target of miRNA159 which revealed in present study. *Arabidopsis* plants with mutations in genes involved in miRNA mediated gene regulation have much higher steady state levels of MYB33 transcripts, whereas the plants, over-expressing miRNA159 and having decreased levels of MYB33, are male sterile and have delayed flowering time (Achard *et al.*, 2004). The MYB transcription factors play important roles in a variety of biological functions including plant development, hormone signaling and metabolism (Suo *et al.*, 2003). Reports indicated that three *Arabidopsis* miRNAs, miR159a, miR159b and miR159c, have complementarity and a highly conserved motif in the coding region of the GAMYB-like genes (Park *et al.*, 2002 and Rhoades *et al.*, 2002). Millar and Gubler (2005) demonstrated that the miRNA159 target motif restricted MYB expression, and when this motif is absent, aberrant MYB gene expression occurred, resulting in the defects in plant growth and development. miRNA 414 can also perfectly complement with the mRNA encoding MYB9 protein, suggesting that the different miRNAs can regulate expression of same gene family.

In phylogram of 167 miRNAs two clear patterns were observed; 1) family wise clustering of miRNAs and 2) 'SORCOT' conservation clustering. miR169 family contains 15 respective members including miR169*. As the phylogram was done on the basis of nucleotide sequences, the 14 members of miR169 family formed a single clade and miR169g* separated to another distant clade. Similarly, different miRNA families, viz., miR166 family, miR156 family, miR395 family, etc. showed a family wise clustering pattern. Beside these, the miRNAs have uniquely identified in sorghum and cotton formed separate clades and those were found conserved in both the crops showed a clear pattern of similarities with each other in different clades. So, through phylogenetic analysis of those miRNAs a conservation pattern in respective crops were detected which can be related to target domain conservation of miRNAs in different crop species by doing further analysis.

In case of osmotic regulation in plants, there are two independent pathways described by Yamaguchi-Shinozaki and Shinozaki (2006), where miR172 showed its target domain conservation in monocot and dicot having DREB2 and EREBP as target transcription factors. Both of them belongs to AP2 transcription factor family and follows ABA-independent pathways in osmotic stress (Fig. 9). This showed the target domain conservation of miRNAs in dicot and monocot system throughout the evolution.

In ABA-dependent pathways MYB family of transcription factors were found to have eight miRNA (miR156, miR157, miR319, miR394, miR398, miR834, miR848, and miR870) target site and bZIP in the similar pathway found to targeted by miR414, miR156, miR847 and miR415. But, there were no target detected for MYC by any of the *Arabidopsis* 207 miRNA. Therefore, with these basic studies the diverse role of miRNAs and its target conservation in sorghum and cotton were clearly deciphered and identification of these predicted targets that are implicated in diverse biological processes expands the breadth of processes and pathways that are under miRNA regulation.

5.2 Functional assessment of selected miRNAs through *in situ* hybridization

To study the expression of miRNAs in different tissues *in situ* hybridization technique was found suitable though many technical challenges were associated. In this study seven miRNAs were taken for their functional assessment.

5.2.1 Expression analysis of miR171

We conducted our study to explore the possible involvement of miR171 in flower development; we found its differential expression pattern in developing flower tissues. In transverse section (TS) of young and mature flower it was detected in sepals, petals, and stamen. But in ovary, specifically in funiculus and placenta, it was expressed in higher. Therefore, it might have some role in ovary development and further maturation. In TS of anther it was detected moderately in tapetum cells and in higher frequencies in filament

In longitudinal section (LS) of mature flower it was accumulated in developing ovules, vascular bundles, sepals and petals. A clear expression was observed in placenta of mature flower of *Arabidopsis*. In young developing flower a strong expression was detected in petals and zonal accumulation was noticed in developing ovary and anther. Our results confirmed that miR171 has a definite role in development of shoot apical meristems as it was detected in higher intensities in apical buds, developing young leaves, apical meristem and leaf primordia. In young vascular tissues, miR171 was showed a clear expression pattern which might be required for vascular development. In developing shoot apex a strong expression was detected in apical buds and leaves. In developing tender leaves miR171 accumulated typically in higher intensities.

In LS of leaf miR171 was detected in cuticles and vascular bundle along with the epidermal cell layer. On the other hand miR171 was also detected in developing anther and ovary of floral primordia. The expression was also found in case of TS of root tissues. The expression levels were strong in the epidermis and moderately in the cortex, endodermis and stele. Therefore, it can be assumed that it might have some developmental regulatory role in roots.

Results of the experiments in silique to study the expression of miR171 confirmed that in young developing silique the accumulation seen in the fused ovule and pod cover but in mature silique hybridization signal was detected mainly in pod cover which was cross confirmed by TS and LS of mature silique. A clear shift in miR171 expression was observed between young and mature siliques which might be temporarily required for seed development and permanently needed for pod cover maintenance. 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; Kamiya *et al.*, 2003). Three members of the *Arabidopsis* SCR-like putative transcription factors (SCL) were reported to possess perfectly complementary target sites for miR171 (Reinhart *et al.*, 2002).

5.2.2 Expression analysis of miR399 and 399e

Expression of miR399 and miR399e was relatively more in terminal root zone in well watered, mild stress (S1) and moderate stress (S2) and nearly absent in severe stress (S3) condition. In middle zone of the root, accumulation of these miRNA was observed in the severe stress alone which suggests their mobility or shifting in root zones. In basal zone the expression was completely absent in well watered root tissues but expressed differentially in successive stresses. Taken together, these miRNAs are drought specific in roots.

In case of leaf tissues both the miRNAs were found absent in well watered condition. However, successive stress levels from S1 to S3 induced its expression in leaves. All the leaf sections taken from stressed conditions showed a strong hybridization signals. Therefore, in leaf also these two miRNAs were found drought responsive.

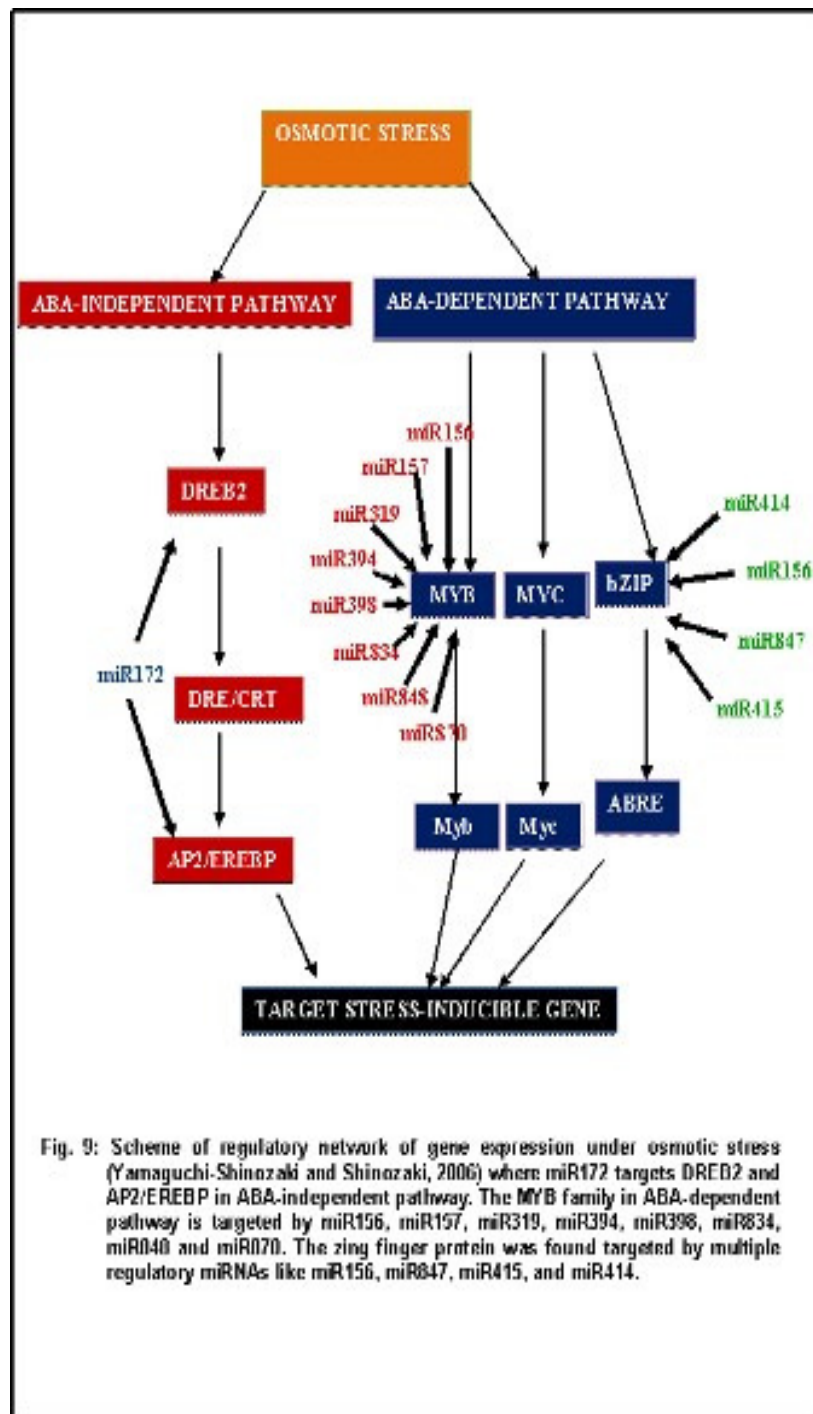


Fig.9: Scheme of regulatory network of gene expression under osmotic stress (Yamaguchi-shinozaki and Shinozaki,2006) where miR172 targets DREB2 and AP2/ERF in ABA- independent pathway. The MYB family in ABA-dependent pathway is targeted by miR156, miR157, MiR319, miR394, miR398, miR834, miR848 and miR870. The zing finger protein was found targeted by multiple regulatory miRNAs like miR156, miR847, miR415, and miR414.

miR399 and 399e grouped under miR399 family (Bartel, 2004). Recent studies showed that miR399 regulates phosphate homeostasis and accumulates in higher copies in phloem and root tissues. It was predicted to target two genes belonging to different families: a phosphate transporter (Jones-Rhoades *et al.*, 2004) and a putative ubiquitin conjugating enzyme (UBC24) possibly involved in protein degradation (Sunkar and Zhu, 2004). Hence, it could be assumed that due to water stress condition the nutrient (phosphorous) might be unavailable to plant and it induces phosphate homeostasis pathway and therefore, these two miRNAs were giving differential expression pattern in the *Arabidopsis* root and leaf tissues.

5.2.3 Expression analysis of miR395b

In well watered root tissues expression of miR395b was very low. But very strong signal was detected in basal zone of S1 root where in S2 and S3 the expression was moderate. In middle zone it was found very low or nearly absent in well watered and S2 conditions respectively. The expression was detectable in middle zone of S1 and S2 roots. In terminal zone strong expression was observed in S2 and S3 roots. Therefore, a differential expression pattern was evident and it was found to be water stress responsive in root tissues. In all the leaf tissues irrespective of treatment, expression of miR395b was completely absent. Therefore, it is considered as root specific and stress responsive.

miR395 targets ATP sulfurylases enzymes that catalyzes the first step of the sulfur assimilation pathway (Lappartient, 1999). miR395 also targets AST68, a low-affinity sulfate transporter (Allen *et al.*, 2005). In sulfur limiting condition it was found to express in *Arabidopsis* (Phillips *et al.*, 2007). Therefore, it might be possible that due to osmotic stress the sulfur was limiting for the plant which intern regulates sulfur assimilation pathway and induces the expression of this stress responsive miRNA.

5.2.4 Expression analysis of miR397a

Expression of miR397a was present in the entire root zone except in the middle zone of well watered root tissues. But, in leaves, the expression of mir397a was only observed in well watered leaf tissues and found completely absent in all the successive stresses. Therefore, it was found stress responsive in leaves and ubiquitous in root tissues.

Out of 17 annotated *LACCASE* genes or laccase-like multicopper oxidases (McCaig *et al.*, 2005), 16 are expressed in the roots. *LACCASE* was suggested to be closely associated with lignin synthesis in vascular tissues and thus important for water transport (Croteau *et al.*, 2000; Boerjan *et al.*, 2003). The miR397a targets this *LACCASE* gene and imply its role in root physiology. Gene expression analysis showed an increase in *AtLAC2* transcript level in roots treated with PEG in addition to NaCl treatment (Xiaoning *et al.*, 2006). Therefore, it can be assumed that miR397a is required in roots to fine tune the *LACCASE* gene expression and development of root tissues whereas in leaf it was typically stress responsive.

5.2.5 Expression analysis of miR161

miR161 was differentially expressed in the entire basal zone, except in the well watered root tissues. In the middle zone very low expression was observed in stress1 roots, where a strong signal was detected in the severe stress condition. In terminal zone, it was expressed in very high intensity where a complete absence was found in S1 condition. In leaves it was found constitutive under normal and stress condition.

Post-transcriptional regulation of gene expression is a dominant mode of controlling gene activity in mitochondria. PPR (Pentatricopeptide Repeat) proteins are implicated in the regulation of organellar gene expression by controlling diverse aspects of RNA metabolism, such as RNA splicing, editing, processing, and translation (Meierhoff *et al.*, 2003 and Andres *et al.*, 2007). The PPR domain protein family is particularly large in plants. In the *Arabidopsis* genome, 441 putative PPR genes were identified (Lurin *et al.*, 2004), but only six of them have been characterized in detail (Andres *et al.*, 2007). Altogether, the biological function of no more than 20 plant PPR proteins is known (Andres *et al.*, 2007). PPR protein was targeted by the miR161 (Howell *et al.*, 2007 and Chen *et al.*, 2007). Therefore, it can be concluded

that miR161 might play some role in the leaf vegetative phase change and root physiology by regulating organellar gene expression.

5.2.6 Expression analysis of miR168

miR168 was found completely devoid of its expression in all the root zones irrespective of treatments. Not a single region in different root sections was showing the expression signal for this miRNA. In leaf tissues it maintained constitutive expression both under stress and well watered condition.

AGO1 is the founding member of the ARGONAUTE protein family and the ARGONAUTE proteins (also known as PPD proteins because they carry PAZ and PIWI domains) have a β -barrel core that together with a side appendage weakly bind to single-stranded RNAs at least 5 bases in length and also to double-stranded RNA (Yan *et al.*, 2003), suggesting that ARGONAUTE proteins could be directly associated with siRNAs/miRNAs before and after they recognize their mRNA targets. AGO1 is a particularly attractive candidate to act in the miRNA RISC owing to the possible feedback regulation of *AGO1* mRNA by miR168 (Rhoades *et al.*, 2002). Targeting of *AGO1* mRNA by miR168 is needed for proper plant development, illustrating the importance of feedback control by this miRNA. miR168 is typically a leaf specific and expressed constitutively to regulate leaf developmental biology.

Using these miRNA target information it is possible to modulate some important genes and further can be confirmed by *in situ* hybridization to know the exact location of expression in respective tissues. Cross- species conservation of miRNAs can be done by using precursor miRNA sequences from miRBase which will be more useful to identify new miRNAs in different species. In conclusion, the expression assays by *in situ* hybridization revealed that miRNAs differentially express in *Arabidopsis* leaf and root tissues and possibly play a vital diverse regulatory role in *Arabidopsis* development. It is also thought that they are involved in stabilizing cross-talks between different genes targeted by them. Though miRNAs are tiny, they seem to play a significant role in genome biology to maintain system response to different abiotic stresses.

6. SUMMARY AND CONCLUSIONS

With the advent of new sequencing technologies and high throughput assays the miRNA databases are accumulating a lot of resources in important crops like rice, wheat, sorghum, cotton, soybean and others. In the present study, available ESTs of sorghum and cotton were analyzed for miRNA targets and they were functionally annotated. The summary of the present investigation is presented here;

1. A total of 2, 41,562 and 4, 93, 741 available ESTs of sorghum and cotton were analyzed and a set of 125 and 138 miRNAs were identified to have potential targets in sorghum and cotton respectively.
2. Among these 125 (sorghum) and 138 (cotton) miRNAs, 96 miRNAs were found common to both the crops and the remaining 29 and 42 were unique, which were found to have potential targets, respectively in sorghum and cotton only.
3. Annotation of each target EST revealed that majority of the identified targets were different transcription factor families and rest were involved in the signal transduction, metabolic pathways, and developmental regulation.
4. Different transcription factors like bZIP (miR414, miR156, miR846 and miR415), MYB (miR156, miR157, miR319, miR394, miR398, miR834, miR848 and miR870), DREB and EREBP (miR172) were found to be targeted by a set of miRNAs indicating their possible involvement in the regulation of respective gene expression during osmotic stress.
5. Functional analysis of miR172 showed the target conservation pattern of miRNAs in monocot and dicot species like sorghum and cotton. AP2 transcription factor of *Arabidopsis* as reference for profile alignment, which revealed the conservation of target domain in DREB2 and EREBP, respectively in sorghum and cotton. Interestingly, the DREB2 and EREBP come under the AP2 transcription factor family, which are found to have similar regulatory network.
6. Different miRNAs were found to have single to multiple targets in both sorghum and cotton, among which some targets had similar functions and others were showing functional diversity with respect monocot and dicot system.
7. Phylogenetic analysis of 167 miRNAs showed a clear pattern of divergence and similarities with respect to their potential targets in sorghum and cotton. The miRNAs formed different clade (s) with respect to different families and also with the targets conserved in sorghum and cotton.
8. The *in situ* hybridization protocol was optimized for miRNA detection in *Arabidopsis thaliana*, by standardizing the probe concentration for hybridization, washing buffer concentration and pre-antibody treatment.
9. The optimized protocol was used to detect miR171 in leaf, root, silique, developing flowers, shoot apical meristem, flower primordia, etc. which showed differential expression pattern with respect to developmental stages and zonal accumulation.
10. Drought induction was carried out by using leaf relative water content measurements and tissues were selected for *in situ* hybridization to analyze the expression pattern of selected six miRNAs in leaf and root tissues.
11. The miR395 was found completely absent in leaf tissues irrespective of all the treatments. But in roots it was showing stress response and expressed differentially in all the three root zones.
12. In leaf and root tissues miR399 and miR399e were showing clear pattern of drought responsiveness as they were absent in well watered condition in both root and leaves. The mobility of these miRNAs was observed in all the root zones with respect to different treatments.
13. The miR161 was found expressing constitutively in leaves, irrespective of all the treatments but, in roots it showed differential expression pattern in three selected zones.
14. The miR168 was not detected in any of the three root zones with respect to all the stress treatments, but expressed constitutively in all the leaf tissues. So, miR168 is leaf specific and expresses ubiquitously.
15. Laccase gene targeting miR397a was differentially expressed. In leaf, its expression was prominent but under stressed condition it was undetected. But in root its expression was

very prominent and in high intensities throughout the zones irrespective of stresses. Therefore, miR168 expression is stress specific in leaves.

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APPENDIX

Appendix I : Phosphate buffer saline

a. 10X Phosphate buffer saline (PBS) (1000ml) pH 7.0	
130mM Sodium chloride	130 ml
7mM Na ₂ HPO ₄	70 ml
3mM NaH ₂ PO ₄	30 ml
DEPC-water (RNase free)	770 ml

b. Paraformaldehyde (PFA) in PBS (500ml) 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	50ml	50 ml
60 % Ethanol	60 ml	40 ml
70 % Ethanol	70 ml	30 ml
85 % Ethanol	85 ml	15 ml
95 % Ethanol	95 ml	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	75ml	25 ml
100 % Histochoice	100 ml	-

e. Decreasing Ethanol series (100 ml)	Ethanol	DEPC-water
100 % Ethanol	100 ml	-
95 % Ethanol	95 ml	5 ml
90 % Ethanol	90ml	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 : Sodium-Sodium Citrate

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

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

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
Protinase k (10 mg/ml)	60 µl
Acetic anhydride	4 ml

Appendix III : In situ salts

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 table:				
Pairs of slide	Probe (µl)	50% Formamide (µl)	Hybridization solution (µl)	Final volume (µl)
1	2	38	160	200
5	10	190	720	1000

Appendix IV : NTE

a. 5X NTE (1000 ml)	
2.5M NaCl	416.66 ml
50mM Tris pH 8.0	50 ml
5mM EDTA	50 ml
DEPC-water (RNase free)	483.34 ml

b. Tris buffer saline (TBS) 1000 ml	
100mM Tris pH 8.0	100 ml
150mM 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 (Fab fragments) for 25 pairs of slides	
Anti-DIG antibody	10 µl
1% BSA, 0.3% Triton X-100 in TBS	12.5 ml

Appendix V : Tris-Sodium chloride

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	

COMPUTATIONAL PREDICTION OF miRNA TARGETS IN ESTs OF SORGHUM AND COTTON AND *IN SITU* DETECTION OF SELECTED DROUGHT RESPONSIVE miRNAs IN *Arabidopsis thaliana*

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ABSTRACT

Increasing scientific interest on gene regulatory network microRNAs (miRNAs) are getting importance as they are found to have diverse regulatory roles in plant and animal development. Potential miRNA targets were identified in sorghum and cotton EST datasets to explore the regulatory network of miRNAs in plant development. A total of 2, 41,562 and 4, 93, 741 available ESTs were mined and a set of 125 and 138 miRNAs were identified to have potential targets in sorghum and cotton, respectively. Among these, 96 miRNAs were common for both species and the remaining 29 (sorghum) and 42 (cotton) were unique. Annotation of each target EST revealed that majority of the targets were from different transcription factor families and rest were involving in signal transduction, metabolic pathways, and developmental regulation.

In situ hybridization protocol was optimized for miRNA detection using Locked Nucleic Acid (LNA)-modified miR171 probe in various organs of *Arabidopsis* which showed differential expression pattern with respect to developmental stages and zones within a tissue. The LNA probe was very specific and sensitive to its target miRNA sequence. miR168 and miR395b were found to be leaf and root specific, respectively. miR171 and miR397a were constitutive and the remaining, miR161, miR399 and miR399e were found drought responsive in *Arabidopsis*. In conclusion, the expression assays by *in situ* hybridization revealed that miRNAs differentially express in *Arabidopsis* leaf and root tissues and possibly play a diverse regulatory role in *Arabidopsis* development. Taken together the results of EST mining for mRNA target & *in situ* detection of selected miRNAs seem to play a significant role in plant development and their response to moisture stress in *Arabidopsis*.