MicroRNAs of *Hevea brasiliensis*: Role in abiotic stress responsive gene regulation

Thesis Submitted to

MAHATMA GANDHI UNIVERSITY

KOTTAYAM

For the award of the degree of

DOCTOR OF PHILOSOPHY

In

BIOTECHNOLOGY

(Faculty of Science)

By

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INDIA-686 009

January 2017

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This is to certify that the thesis entitled "**MicroRNAs of *Hevea brasiliensis*: Role in abiotic stress responsive gene regulation**" is an authentic record of original research work carried out by **Smt. Linu Kuruvilla**, at Rubber Research Institute of India, Kottayam-9, under my supervision and guidance for the award of the degree of **Doctor of Philosophy in Biotechnology,** under the faculty of Science, Mahatma Gandhi University, Kottayam. It is also certified that the work presented in this thesis have not been published or submitted earlier for any degree, diploma or any other similar titles of any university.

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Dedicated to my father

ACKNOWLEDGMENT

I would like to express my immense gratitude to my guide **Dr. M.B. Mohamed Sathik**, for helping me to successfully complete my research work. His valuable advice and splendid supervision improved the quality of my work at all stages and his constant encouragement enabled me to present the work in this format. I sincerely thank him for the patience, confidence and faith he had in me during the course of my doctoral thesis.

I sincerely express my deep sense of gratitude to **Dr. James Jacob**, Director of Research, for allowing me to conduct this doctoral research at Rubber Research Institute of India, Kottayam and his encouragement and support throughout my work.

I wish to express my heartfelt sense of gratitude to **Dr. Molly Thomas** Principal Scientist, Crop Physiology Division, RRII, for the immense help and encouragement rendered during my work. Her motherly affection was always a source of comfort for me and will be remembered with gratitude.

I convey my thanks to **Dr. K. Annamalainathan**, Joint Director, Crop Physiology Division, RRII, for his valuable and timely suggestions and providing all the facilities during the course of this study.

I would like to thank **Dr. R. Krishnakumar**, Joint Director (Retired), Climate Change and Ecosystem Studies, RRII, for all the help rendered to me during this work.

I am deeply indebted to **Dr. K. V. Sumesh**, Scientist B, Crop Physiology Division, RRII for his timely help and support in my research.

The timely support given by **Dr. Shaji Philip, Dr. Shammi Raj, Dr. Kavitha K. Mydin, Dr. A. Thulaseedharan**, and **Dr. R.G. Kala** were invaluable on academic and personal level for which I am grateful.

*I wish to express my gratitude to **Dr. T.R. Keerthi**, Director, School of Biosciences, M.G University, Kottayam, **Dr. J.G. Ray** Professor, School of Biosciences, M.G University, Kottayam, **Dr. K. Jayachandran**., Associate Professor, School of Biosciences, M. G. University, Kottayam and all the Faculty members of School of Biosciences, M.G University.*

*It is a great pleasure to express my deep sense of gratitude to **Dr. S. Sreelatha, Dr. Jayasree Gopalakrishnan, Dr. D. Bhuvanendran Nair, Dr. Jayantha Sarkar, Dr. Sheela P. Simon and Mr. Harikumar** of Crop physiology Division, Rubber Research Institute of India, Kottayam.*

*I owe my thanks to **Rajan Mathew, P.M. Sebastian, M.B. Rajan** and all other members of Crop Physiology Division for their timely help in different stages of this work.*

*I wish to express my gratitude to **Dr. K. V. Thomas** and **Mr.A Ananthanaramanan** of Genome Analysis, **Dr. Karunaichami** and **Dr. Rajagopal** of Latex Harvest Technology Division for their valuable advices and suggestions.*

*My heartfelt thanks are due for the support, encouragement and constant companionship rendered by **Mrs. Lisha P. Luke, Mrs Ambily P.K., Mrs. Smitha M. Xavier, Mrs. Anu Mary Joseph, Dr. Mrudula P Musthapha, Dr. Satheesh P. R., Dr. Amith Abraham** and **Mr. S. Pramod** during my wonderful days in RRII. A special thanks to Dr. Satheesh for the cover photo.*

*I am grateful to **Mr. Aneesh**, Assistant Statistician, Rubber Research Institute of India, for the help in statistical data analysis. I convey my thanks to staff members of library, computer section and other staff members of RRII, Kottayam.*

I am grateful to Council for Scientific and Industrial Research, New Delhi vii
for the Research Fellowship grant offered to me.

I bow down to my parents and my in-laws for their moral support, pport, *immense patience, loving care and the personal sacrifices that enabled me to complete this venture successfully.*

Words cannot express how grateful I am to my husband Mr. Binu Philip and my son Ivin for their constant unconditional support; incredible patience and encouragement that made me to complete this work.

Above all, I bow my head before the ***God Almighty*** *whose blessings were always with me enabling to undertake this endeavor successfully.*

Linu Kuruvilla

ABSTRACT

Plant respond to abiotic stresses by precisely regulating expression of stress responsive genes through several mechanisms such as transcriptional, post-transcriptional, and posttranslational regulations at different levels. MicroRNAs (miRNAs) are single-stranded non-coding RNAs that play critical roles in regulating gene expression at the post-transcriptional level by repressing translation or by enhancing degradation of specific target mRNAs. A large number of miRNA sequences are evolutionarily conserved across species boundaries and have near perfect complementarities with their specific targets which are messenger RNAs (mRNA). Regulation of gene expression through sequence specific interaction between miRNAs and their target mRNAs offers an accurate and inheritable mechanism for plant's response to environmental stimuli.

Hevea brasiliensis which is the major commercial source of natural rubber performs well in Kerala and Kanyakumari District of Tamil Nadu which experience favourable weather parameters like optimum sun shine hours, rain fall, humidity, etc. Due to the increasing demand for natural rubber coupled with non-availability of land in traditional rubber growing regions, cultivation of natural rubber is being extended to non-traditional regions which experience adverse climatic conditions which limit the growth and productivity of rubber. So it is highly imperative to identify or develop clones that can withstand such extreme weather factors. As miRNAs are known to be involved in regulating the abiotic stress responsive gene expression, their level of expression may vary in stress tolerant/susceptible clones of *Hevea*. If the miRNAs that are involved in regulating the stress tolerant genes can be identified, it would enable the plant breeders to identify or develop clones with improved stress tolerance. Hence the present work on identification and expression analysis of abiotic stress responsive miRNAs of *H. brasiliensis* was conducted to identify miRNAs associated with drought/ cold tolerance in *Hevea brasiliensis*.

In this study, attempts were made to identify drought and cold responsive miRNAs from *H. brasiliensis* through both conventional as well as by next generation sequencing method. Both drought and cold responsive miRNAs were identified from which differentially expressed miRNAs were selected for further validation. Expression of miRNAs was analyzed in various clones of *H. brasiliensis* with contrasting levels of drought and cold tolerance which led to the identification of miRNAs that are strongly associated with drought/cold tolerance. Further, their expression was validated in various germplasm accessions with different levels of tolerance in order to confirm their association with tolerance. In addition to this, targets of both known and novel miRNAs were predicted followed by expression analysis of selected miRNAs and their putative targets in order to evaluate their relationship.

From this study, miRNAs such HbmiRn_63, HbmiRn_42, miR168 and miR160 were found strongly associated with drought tolerance in *H.brasiliensis*. This study also revealed miR169, miR482 and miR159 to have strong association with cold tolerance. This study indicates the possibility of using these miRNAs as markers for drought/cold tolerance in *H. brasiliensis*. These miRNAs can be futher utilized in the crop improvement programmes by the breeders to identify or develop drought/cold tolerant genotypes of *H. brasiliensis*.

Key words: *Hevea brasiliensis*, miRNAs, drought tolerance, cold tolerance, expression analysis, qPCR

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ABBREVIATIONS

ABA	:	Abscisic acid
AREB2	:	Abscisic Acid Responsive Element Binding Protein 2
ARF	:	Auxin response factor
ATP	:	Adenosine triphosphate
BLAST	:	Basic local alignment search tool
bp	:	base pair
COR	:	cold-responsive
CSD	:	Cu/Zn-superoxide dismutase
DGE	:	Digital gene expression
dNTPs	:	deoxyribonucleoside triphosphates
HD-Zip	:	Homeodomain-leucine zipper
HMGR	:	HMG-CoA reductase
kcal	:	kilocalorie
LSD	:	Least significant difference
MFE	:	Minimal folding free energy
miRNA	:	microRNA
mRNA	:	messenger RNA
NBS-LRR	:	Nucleotide Binding Site-leucine-rich-repeat receptor
NFY	:	Nuclear factor Y
NR	:	Natural rubber
nt	:	nucleotide
PAGE	:	Polyacrylamide gel electrophoresis
POD	:	Peroxidase
PS II	:	Photosystem II

qPCR	:	quantitative PCR
RH	:	relative humidity
RNA	:	ribonucleic acid
RRII	:	Rubber Research Institute of India
RT-PCR	:	Reverse transcription PCR
SCL	:	Scarecrow-Like
SSR	:	Simple-Sequence Repeats

Units

°C	:	degree Celsius
g	:	gram(s)
hr	:	hour(s)
l	:	litre(s)
M	:	molar
min	:	minutes
mol	:	mole(s)
rpm	:	revolutions per minute

Prefixes

K	:	kilo
M	:	milli
μ	:	micro

Chapter 1

Introduction

Hevea brasiliensis Muell. Arg., a tropical tree native to Amazon rain forests of South America, is the major source of natural rubber (NR) (Wycherley, 1992). The genus *Hevea* belongs to the Euphorbiaceae family, which is comprised of 11 inter-crossable species (Pires *et al.,* 2002). Approximately 2,500 plant species synthesize rubber (Mooibroek and Cornish, 2000), but only a few plants produce high quality natural rubber. The other potential rubber producing plants are *Parthenium argentatum* (guayule) and *Taraxacum koksaghyz* (Russian dandelion) (Gronover *et al.*, 2011). NR is the major constituent of latex and is synthesized in specialized cells or tissues called laticiferous tissue.

Global dependence of NR is likely to increase because of the fast shrinking resources of non-renewable energy sector, the petroleum industry, which is the source of synthetic rubber. NR consists of 94% *cis*-1,4-polyisoprene and 6% proteins and fatty acids (Sakdapipanich, 2007). *Cis*-1,4-polyisoprene biopolymers are made up of C5 monomeric isopentenyl diphosphate (IPP) units and are formed by sequential condensation on the surface of rubber particles. Due to its structure and high molecular weight (> 1 million Dalton), NR has superior properties such as resistance to abrasion and impact, elasticity, efficient heat dispersal, resilience and malleability at low temperature when compared to synthetic rubber. These properties make NR difficult to be replaced by synthetic rubber in many applications, such as medical gloves and heavy-duty tyres for aircrafts and trucks and so on. In addition to NR, rubber trees are used as a source of timber. Rubber wood has become a major export item of Southeast Asia (Prabhakaran, 2010) and due to its timber value, research priorities are being given to develop several superior latex-timber clones.

Depending up on climate, soil condition and management practices, the initial growth phase of rubber tree generally varies from 5-7 years which would have a productive lifespan of 25 to 30 years.The ideal agroclimate for rubber cultivation is the tropical environment with hot humid wet weather and plenty of sunshine. The optimal growth conditions of rubber tree are high temperature around 28 ± 2 °C, high humidity and about 2000-4000 mm rainfall per annum (Webster and Baulkwill, 1989; Priyadarshan *et al.,* 2005). The rubber tree is well adapted to humid tropics between 10° S to 10° N latitudes. Within the rubber tree plantation industry, this latitudinal belt is known as the traditional rubber growing region. Rubber trees were introduced to many tropical/sub-tropical regions of Asia, Africa, and Latin America. In India, the traditional rubber belt encompasses the southern tip of the peninsula, where rubber is being cultivated on a plantation scale for over a century. Because of the decrease in availability of cultivable land in traditional tracts, rubber cultivation in India is being extended to areas of diverse agroclimatic zones where near similar weather conditions prevail (Krishnakumar and Meenattoor, 2000).

Hevea is a diploid (2n=36), highly heterozygous, monoecious, cross-pollinating, perennial tree with a very long breeding cycle. Rubber breeding over the last century has made significant progress through recombination breeding and selection. Being a perennial crop which requires over about five years for attaining the latex harvestable stage and then at least additional seven years to evaluate its yield potential, breeding programmes of rubber requires about 15 years for developing a suitable genotype. Therefore, need for early selection methods for promising clones is often emphasized. Efforts on breeding *Hevea* at molecular level were commenced since 1985 (Low and Bonner, 1985) with an initial approach of global characterization of the nuclear genome of *Hevea*. It was followed by cloning and characterization of latex biosynthesis genes and gene expression studies influenced by various biotic and abiotic stresses, tapping panel dryness (TPD), and ethylene stimulation of latex production. Simultaneously, different genetic markers were established in rubber for understanding the inheritance and diversity of natural variation existing among the Wickham and wild populations. Genetic markers were used successfully to generate linkage map for QTLs involving disease tolerance. During the last decade, transgenic research progressed significantly with the development of transgenic *Hevea* clones designed to over- express MnSOD gene which would impart tolerance to TPD and drought stress (Jayashree *et al.,* 2003). Over the past two decades, there has been an exponential increase in data acquisition pertaining to genomic microsatellite markers (Le Guen *et al.,* 2011; Mantello *et al.,* 2012), expressed sequence tag-simples sequence reapeats (EST-SSRs) (Feng *et al.,* 2008; Triwitayakorn *et al.,* 2011; Li *et al.,* 2012b) linkage maps (Lespinasse, *et al.*, 2000; Souza *et al.*, 2013), and gene expression profiles (Chow *et al.,* 2007; 2012) of rubber. The draft genome of the rubber tree was published by Rahman *et al.,* (2013). High-throughput genomic techniques would facilitate development of superior clones suitable for agroclimatic conditions (Saha and Priyadarshan, 2012). Various studies indicated the occurence of altered level of expression of several abiotic stress responsive genes in *Hevea* clones with contrasting stress tolerance (Thomas *et al.,* 2011; 2012; Sathik *et al.,* 2012; Luke *et al.,* 2015) and have identified genes associated with drought and cold tolerance.

As there are constraints in the availability of cultivable land in the traditional rubber growing regions of India, cultivation of rubber is being extended to regions having suboptimal environments which are known for their adverse climatic conditions. These include North Konkan region where the summer will be hotter and north-eastern regions of India where the temperature during winter is too low. In *Hevea*, drought and cold stresses have been reported to affect the development, latex yield and general performance of rubber trees (Sethuraj *et al.*, 1984; Priyadarshan *et al.*, 2005; Sreelatha *et al.*, 2007; 2011).

Plants have evolved unique adaptation mechanisms to abiotic stresses through fine-tuned adjustment of gene expression and metabolism. Of the different gene regulatory mechanisms, transcriptional regulation, which depends on the action of specific transcription factors that bind to specific *cis*-elements in the promoter region, is relatively a better understood phenomenon. Although post-transcriptional gene regulation was thought to be one of the critical mechanisms of gene regulation, the components that mediate these processes were relatively unknown. The early findings in metazoans and plants were that a certain group of ~22 nucleotide (nt) long small RNA molecules act as key post-transcriptional regulators of gene expression which had revolutionized the understanding of the multitude of gene regulatory pathways (Napoli *et al.*, 1990; Lee *et al.*, 1993). Over the last decade, small RNA molecules have emerged as critical regulators in the expression and functioning of eukaryotic genomes.

MicroRNAs (miRNAs) are extensive class of small endogenous 21-22 nt long non-coding RNAs that regulate gene expression at the post-transcriptional level by mRNA cleavage or translation inhibition. They are present in many eukaryotic organisms including animals (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001) and plants (Llave *et al.*, 2002a; Park *et al.*, 2002; Reinhart *et al.*, 2002). The first plant miRNAs were described in *Arabidopsis* (Park *et al.*, 2002) and later in other species. The existence and importance of miRNAs was completely unknown until two decades ago as the scientific community focused mainly on the discovery and manipulation of protein coding genes (Almeida *et al.*, 2011). Even though miRNAs constitute only a small fraction of the small RNA population, the miRNA-guided post-transcriptional gene regulations have been found one of the most conserved and well characterized gene regulatory mechanisms (Voinnet, 2009; Jones-Rhoades *et al.*, 2006). The first miRNAs *lin-4 and let-7* were discovered in *Caenorhabditis* *elegans* as key regulators of embryonic development timing (Lee *et al.,* 1993). The discovery of *let-7* conservation across species from flies to humans triggered a major revolution in non-coding small RNA's research that led to the discovery of miRNAs in animals, plants and in unicellular organisms (Lagos-Quintana *et al.*, 2001; Lau, *et al.,* 2003; Pasquinelli *et al.,* 2000). They are evolutionarily conserved across species boundaries and are capable of regulating the expression of protein-coding genes in eukaryotes (Jones-Rhoades *et al.,* 2006). They are involved in regulating various developmental and metabolic pathways, signal transduction, response to environmental stresses such as oxidative stress, nutrient stress, dehydration and mechanical-stress (Sunkar and Zhu, 2004; Shukla *et al.,* 2008).

miRNA genes are transcribed by RNA polymerase II in the nucleus and generate primary microRNA transcript (pri-miRNA), which are capable of forming a self-complementary fold-back structures (Fig. 1.1.). The pri-miRNAs are approximately 70 to many hundreds of bases in length (Axtell *et al.,* 2008) which are then processed into pre-miRNA, and subsequently cleaved by Dicer-lke (DCl) enzymes into mature miRNA and the corresponding star molecule. Mature miRNAs are then transported to cytosome by HASTY and incorporated into ARGONAUTE (AGO) containing RNA induced silencing complexes (RISCs). miRNAs recognize and target mRNA transcripts based on sequence complementarity to function as negative regulators in multiple gene regulatory networks existing in plants and animals (Bartel, 2009; Chen, 2009). AGO proteins catalyze ribonucleolytic cleavage of the target at the position opposite to the tenth nucleotide of the small RNA (Filipowicz, 2005; Kim, 2005).

In human, more than 60% of protein coding genes appear to be under selective pressure to maintain pairing with miRNAs (Friedman *et al.,* 2009) whereby a single miRNA can regulate hundreds of genes (Selbach *et al.,* 2008). For most plant miRNAs, their target mRNAs contain motifs that have perfect/near perfect complementarity resulting in a regulatory mechanism that includes RISC-directed slicing (Jones-Rhoades, *et al.*, 2006). Due to these high sequence complementarity requirements, it is relatively easier to bioinformatically predict potential targets of miRNA in plants (Rhoades, *et al.*, 2002). In plants, majority of miRNAs are linked with negative regulation of transcription factors playing central roles in numerous developmental processes, including organ identity, polarity, cell division patterning, cell fate determination and responses to abiotic stresses (Mallory *et al.*, 2004; Guo *et al.*, 2005; Sunkar *et al.*, 2012; Ding *et al.*, 2013; Xie *et al.*, 2015; Zhang *et al.*, 2015; Ferdous *et al.*, 2015). Certain miRNAs have been recently reported to target transcripts related to secondary metabolism in plants (Boke *et al.*, 2015; Bulgakov and Avramenko, 2015).

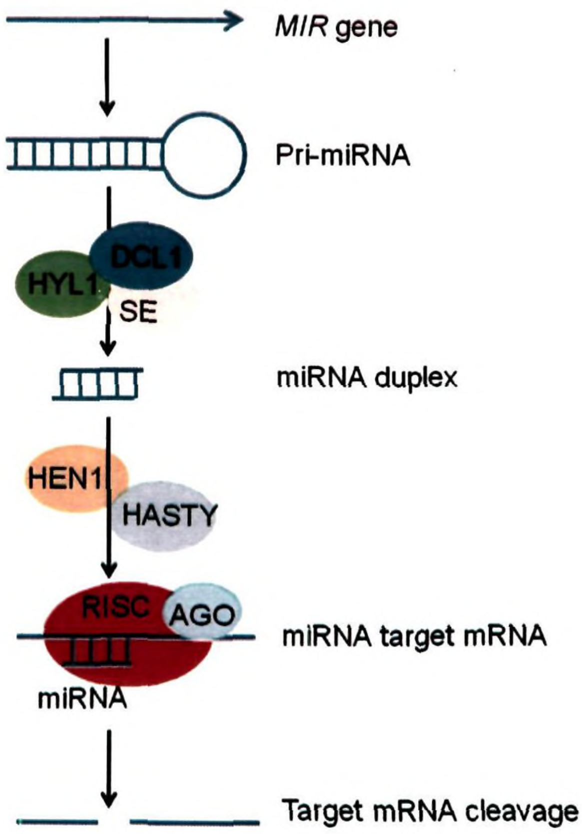


Fig. 1.1. miRNA biogenesis and function

miRNAs have evolved different forms within a family capable of targeting various genes involved in different processes and functions (Martin *et al.,* 2010). In response to abiotic stresses such as drought, salt, cold, heat and nutrient limitations, expression levels of some miRNAs vary resulting in modulation in the expression patterns of their target genes that are associated with stress adaptation. Generally, stress up-regulated miRNAs down-regulate their target mRNAs, whereas, their suppression leads to accumulation and function of positive regulators (Chinnusamy *et al.,* 2007). A better understanding of the regulation of stress responsive miRNAs and their corresponding targets can facilitate breeders to design strategies to improve yield, quality and tolerance to abiotic and biotic stresses in plants. Due to the involvement of miRNA in regulation of gene expression, extensive investigations aiming at discovery of new microRNAs are being carried out in several plant species. Three major approaches are generally being employed for the identification and expression profiling of stress induced miRNAs. The first method involves direct cloning, genetic screening, or expression profiling. The second approach involves computational predictions from genomic or EST loci and the third one involve a combination of both through prediction of miRNAs from High Throughput Sequencing (HTS). Each of these is followed by experimental validations by northern analysis, real time PCR or microarrays.

Introduction of various bioinformatics databases and tools have revolutionized the study of miRNAs and other small RNAs. Next generation sequencing technologies have accelerated the processes of small RNA discovery in many plant species and have increased the recovery of rare miRNA, which together with the completion of more plant genome sequences, allows the identification of new and weakly expressed miRNAs (Meyers *et al.*, 2006). Currently, a total of 48,496 mature plant miRNAs derived from 6992 hairpin precursors reported in 73 plant species have been deposited in the microRNA registry database, miRBase release 21. The plant ncRNA database

(PNRD) contains miRNAs from 150 plant species (Yi *et al.,* 2015). The first involvement of microRNAs in response to stress were described by Rhoades *et al.,* (2002), in *Arabidopsis thaliana*, by predicting miRNA targets such as superoxide dismutase, laccases and ATP sulfurylases (APS). Cloning of small RNAs from *Arabidopsis* under abiotic stress conditions led to the identification of stress responsive miRNAs (Sunkar and Zhu, 2004). According to Zhang *et al.,* (2013b) a total of 1062 differentially expressed miRNAs were reported in 41 plant species under 35 different types of abiotic stresses. Several reports affirmed the involvement of microRNAs in plant's response to abiotic stresses (Jeong and Green, 2013; Zhou and Luo, 2013; Zhang and Wang, 2015; Akdogan *et al.*, 2015; Shriram *et al.,* 2016). The application of miRNAs as novel genetic markers has been developed for genotyping applications in foxtail millet (*Setaria italica* L.) and related crop species (Yadav *et al.*, 2014). SSR markers have also been identified from salt responsive miRNA of *Oryza sativa* (Mondal and Ganie, 2014).

Recent reports have established the role of miRNAs in regulating genes associated with various metabolic as well as abiotic stress responsive pathways in *Hevea* too. Earlier Zeng *et al.,* (2010) studied conservation and diverse expression patterns of twenty three miRNA families during developmental and abiotic stress response in four euphorbiaceous plants (*Ricinus communis, Manihot esculenta, Hevea brasiliensis, Jatropha curcas* L). However, this approach did not allow comprehensive identification of miRNA families in *Hevea*. Gebelin *et al.*, (2012) identified 48 conserved and 10 putative novel miRNAs responsive to various abiotic stress conditions from *Hevea.* Lertpanyasampatha *et al.* (2012) identified 115 miRNAs belonging to 56 families from high yielding (PB 260) and low yielding (PB 217) *Hevea* clones. Gebelin *et al.,* (2013a) reported regulation of microRNAs in response to different types of abiotic stress and hormone treatments in *Hevea.* Gebelin *et al.*, (2013b) reported deep sequencing of TPD associated small RNAs from latex cells. All these reports and findings led to the deposit of 31 mature miRNA sequences in miRBase of *Hevea brasiliensis* till now. Though there were few reports available on abiotic stress responsive expression of miRNAs in *H. brasiliensis*, a clone wise miRNA expression studies with regard to drought and cold stress in contrasting clones of *Hevea* are not available. Clone wise expression studies are necessary to identify the miRNAs that are regulating the expression of drought or cold tolerance associated genes/regulatory elements in *Hevea.*

Under this scenario, this study was initiated with an objective to identify drought and cold responsive miRNAs from *Hevea brasiliensis* and to further select the miRNAs that exhibit much stronger association with stress tolerance/susceptibility. This study would also envisage potential abiotic stress responsive miRNA marker genes and their corresponding target genes which could eventually be employed by the plant breeders to either develop crops with improved stress tolerance or use them as markers to screen germplasm lines for identifying abiotic stress tolerant genotypes.

Objectives

- To identify drought and cold responsive miRNAs of *Hevea brasiliensis*.
- To quantify and validate their association with drought and cold stress tolerance.
- To study the miRNA-target interactions.
- To identify candidate miRNAs that could be further utilized to select/develop drought/cold tolerant varieties of *Hevea brasiliensis*.

Chapter 2

Review of Literature

2.1. *Hevea brasiliensis*

Hevea brasiliensis a native of the Amazonian rain forest in Brazil, is the major source of natural rubber (NR). Commercial rubber cultivation was the result of effective introduction of Wickham germplasm from the Amazonian rain forest to the eastern hemisphere (Wycherley, 1968) which consisted of a limited set of surviving seeds collected by Sir Henry Wickham in 1876. The history of rubber cultivation in India dates back to 1878 when rooted cuttings were imported from the Royal Botanic Gardens, Ceylon (Thomas and Panikkar, 2000). Most of the clones under cultivation today are derived from the Wickham base which represents a very small gene pool compared to the wide variability of the species in its natural habitat (Varghese *et al.*, 2000; Das *et al.*, 2014). This narrow genetic base has further narrowed down through directional selection for yield and wide spread adoption of clonal materials (Varghese *et al.*, 2006). Productivity depends on the genetic potential of the planting material, its adaptability to the existing environment and its ability to respond to improved agro techniques (Mydin, 2014). The perennial nature of *Hevea* makes development of improved variety a tedious and time consuming process.

Tropical environment with hot humid wet weather and plenty of sunshine is the ideal agro-climate for rubber cultivation. Due to non-availability of land in traditional rubber growing regions, NR cultivation is being extended to non-traditional areas of India which are known for their adverse climatic conditions that limit the growth, development and productivity of *Hevea*. These include North Konkan where the summer will be severe and north-eastern regions of India where the temperature during winter is too low. The varieties of *Hevea* being cultivated in traditional regions do not perform well in such regions as they are inherently sensitive to such extreme abiotic stress conditions. It is essential to identify or develop clones that can withstand such extreme weather factors without compromising on yield and productivity. Screening for drought and cold strengthened the crop improvement programmes for the non- traditional regions.

2.2. Abiotic stress responses in plants

Plant growth and development is highly dependent on a variety of environmental conditions such as temperature, light, water availability and soil conditions that strongly affect the growth and productivity of crops worldwide. Abiotic stress can be defined as the negative impact of non-living factors on the living organisms in a specific environment. Abiotic stress conditions may be segregated into 35 different types that can be sorted under 11 groups, *viz.* cold, heat, drought, flooding, radiations (UV and light), wind, salinity, heavy metal toxicity, nutrient deprivation in soil, and oxidative stress (Mahajan and Tuteja, 2005). Abiotic stress inflicts various deleterious effects at the molecular, biological and physiological levels (Yamaguchi-Shinozaki and Shinozaki, 2006). Since abiotic stress disrupts many normal cellular functions, plants resort to a quick and extensive molecular reprogramming both at the transcriptional and post-transcriptional level in order to recover from the stress effects. Response to abiotic stress in plants depends on a number of factors including the developmental stage, severity of stress, age, plant species and the genotype (Le Gall *et al.*, 2015). The most studied abiotic stress conditions are cold, high temperature, salt, and drought stress. The response to abiotic stresses is usually multigenic which involves altering the expression of nucleic acids, proteins and other macromolecules. Plants exhibit a wide range of stress response mechanisms that are usually employed at the whole plant, tissue, cellular and molecular levels for the metabolic adjustment and gene expression regulation to enhance physiological and morphological adaptation. To develop novel effective molecular strategies for enhancing stress tolerance, understanding the mechanism of stress perception and downstream gene regulatory pathways is of paramount importance.

Drought is one of the major environmental stress factors that limits productivity of agricultural crops worldwide (Rivero *et al.,* 2007). Water makes up to 90 % mass of the growing plants and plays an important role in photosynthesis, maintenance of turgor pressure for rigidity, mechanical stability and is a vital component in metabolism, transport of solutes apart from being a key reactant in many biochemical reactions (Wood, 2005). Water availability is therefore a key determinant of plant's survival. In plants, drought stress is aggravated by both high solar radiation and increased atmospheric temperature, which increases the degree of damage even under a short period of drought (Sumesh *et al.,* 2011).

In order to overcome the effects of drought stress, plants employ different morphological, biochemical and physiological responses like drought escape, drought avoidance and/or tolerance. Drought escape is associated with short life cycles allowing the plant to reproduce before the onset of drought (Abdel-Ghany and Pilon, 2008). Drought avoidance is a protective mechanism achieved through morphological changes in plants, such as decreased stomatal conductance, reduced leaf area, formation of cuticular wax to prevent water loss, development of widespread root systems, reduced canopy, and early maturity to escape the effects of drought stress (Levitt, 1980; Rivero *et al.*, 2007; Pardo, 2010). Drought tolerance is achieved by physiological and molecular mechanisms, including osmotic adjustment, and the production of antioxidant and scavenger compounds (Bartels and Sunkar, 2005). At the molecular level, response and adaptation to water deficit is controlled by a cascade of multi-genic regulatory networks which activate stress responsive mechanisms through transcriptional gene expression regulation to protect, repair damaged proteins and membranes and re-establish homeostasis (Wang *et al.,* 2003). Majority of these genes code for functional proteins in stress associated pathways and protection related macromolecules such as compatible solutes accumulation regulators, ion transporters, ROS scavengers, fatty acid metabolism, proteinase inhibitors, ferritin and lipid-transfer proteins, LEA proteins, osmoprotectants and chaperones (Seki *et al.,* 2003).

Low temperature is another major factor limiting productivity and geographical distribution of many species. Cold stress affects virtually all aspects of cellular function in plants. One of the major influences of cold stress is membrane disintegration which adversely affects the growth and development of plants (Yadav, 2010). Cold response is a very complex trait involving many different metabolic pathways, gene regulations and cell compartments (Hannah *et al.,* 2005). Plants from temperate climatic regions are considered to be chilling tolerant with variable degree, which can increase their freezing tolerance by getting exposed to chilling, non-freezing temperatures, a process known as cold acclimation (Levitt, 1980). But, plants of tropical and subtropical origins are sensitive to cold stress and lack cold acclimation mechanism. The discovery of change in the gene expression during cold acclimation was the beginning of exploration of antifreezing molecular mechanisms (Sanghera *et al.,* 2011). During cold acclimation definite regulation of expression of cold-regulated (COR) genes such as transcription factors and effector genes has been found to occur (Thomashow 1999, Viswanathan and Zhu 2002). Significant progress has been made in identifying transcriptional, post-transcriptional and post-translational regulators of cold-induced expression of COR genes.

2.2.1. Drought and cold stress responses in *Hevea*

In *Hevea*, drought stress has been reported to affect its yield and general performance (Sethuraj *et al.,* 1984; Sreelatha *et al.,* 2007; 2011). Drought stress results in growth retardation of both rubber tree seedlings and mature tapping trees, shortening of tapping period, decreased latex yield and dry latex contents, increased TPD incidence, or even tree death at severe conditions (Huang and Pan, 1992). The biochemical investigations indicated severe inhibition in metabolic activity of clone RRII 105 during drought stress (Sreelatha *et al.,* 2007). Gas exchange parameters measured under drought stress indicated the lesser inhibition in clone RRIM 600 while the clone RRII 414 got severely affected (Sumesh *et al.,* 2011). In rubber, few studies have been reported previously on quantification of several abiotic stress responsive transcripts (Thomas *et al.,* 2011; 2012 and Sathik *et al.,* 2012). The association of CRT/DRE binding factor (CRT/DRE bf) and ABC transporter protein with drought tolerance was reported by Thomas *et al.,* (2011). Genes such as peroxidase, WRKY transcription factor and late embryogenesis abundant 5 (LEA 5) proteins were reported to have stronger association with drought tolerance in *Hevea* (Thomas *et al.*, 2012). Luke *et al.,* (2015) analysed the expression pattern of few drought responsive transcripts in young *Hevea* plants experiencing drought stress and MAPK was found to exhibit a strong association with drought tolerance.

In *Hevea* during cold injury plants display symptoms like wilting of leaves followed by withering without abscission, occasional inter-venal chlorosis, black discolouration of green bark and its drying off extending downward, occasional oozing of latex from green bark and dieback of shoots (Meti *et al.*, 2003). Clonal difference in low temperature tolerance has been reported in *Hevea* based on physiological trait like loss of membrane stability (Sathik *et al.*, 1998a). Gene expression analysis in two clones of *Hevea* exposed to cold stress indicated the association of LEA 5 protein, peroxidase, ETR1, ETR2 and NAC transcription factor with cold tolerance (Sathik *et al.,* 2012).

2.3. Small RNAs as regulators of gene expression in plants

Post-transcriptional regulation of gene expression is one of the complex gene regulatory mechanisms employed by plants in response to development, biotic and abiotic stresses. Small-RNA-mediated gene expression regulation has emerged as one of the fundamental principles in cell function (Meister, 2013). Small RNAs are 20-30 nucleotide (nt) non-coding RNAs that guide regulatory processes in a wide range of eukaryotic organisms (Chen, 2009). Based on their size, biogenesis, mode of action and regulatory role, three distinctive types of small RNAs *viz.*, microRNAs (miRNAs), short interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs) have been well characterized in animals and plants (Table 2.1.). Although both miRNAs and siRNAs are products of RNA precursor transcripts by the RNase III endonuclease Dicer-like proteins, the 21-24 nt siRNAs are generated from long double-stranded RNAs, which give rise to multiple siRNA species from both strands while the 21-22 nt miRNAs are derived from single-stranded RNA precursors that form imperfect hairpin structures (Axtell and Bowman, 2008). In contrast, the 26-30 nt piRNAs found only in animals are derived from presumably single-stranded precursors in a Dicer-independent manner (Juliano *et al.,* 2011). In plants, the biogenesis and function of siRNAs and miRNAs are controlled by a group of three protein families *viz.*, RNA-dependent RNA polymerases (RDRs), Dicer-like (DCLs) and ARGONAUTES (AGOs) proteins. The DCL RNAse III endonucleases process the hairpin RNA precursors into 20-24 short double-stranded duplexes with a 2 nucleotide 3' overhangs (Margis *et al.,* 2006) while the RDRs produce dsRNAs by synthesizing the second strand from an RNA template, which is an essential step in the siRNA biogenesis pathway (Zong *et al.,* 2009). The AGO proteins effect the downstream silencing function by forming complexes with the small RNAs to target the mRNA transcripts for slicing or translation repression (Vaucheret, 2008). Although biogenesis and functions of miRNAs and siRNAs share marked similarities, they require distinct set of Dicer-like and AGO proteins for their biogenesis and target recognition (Jones-Rhoades *et al.,* 2006).

Table 2.1. Small RNAs involved in plant's response to abiotic stresses (Mirlohi and He, 2016)

Class	Name	Orginating Loci	Function	Biogenesis
miRNA	microRNA	*MIRNA* genes	Represses target gene expression through mRNA cleavage and translational repression	The fold-back structures of long ssRNA transcripts that are transcribed by RNA polymerase II are cleaved by Dicers
siRNA	Short-interfering RNA	Repeats, transposons, retroelements, transgenes and viral RNAs	Silences repeats and transposons through RNA dependent DNA methylation and chromatin modification	RDR-generated siRNAS are cleaved by Dicers
ta-siRNA	Trans-acting siRNA	TAS loci	Represses target gene expression through miRNA cleavage	TAS transcripts are cleaved by miRNAs, transcribed by RDR into dsRNA, and then processed by Dicers
nat-siRNA	Natural antisense transcript-derived siRNA	Loci producing pairs of sense-antisense transcripts	Stress-induced nat-siRNA to repress target gene expression through mRNA cleavage	The dsRNA derived from overlapping transcripts is cleaved by Dicers
piRNA	Piwi-interacting RNA	Repeats, transposons and retroelements	Germ-line specific piRNA to suppress repeats and transposons in flies and mammals	ssRNA derived from transposons is cleaved by PIWI protein

2.3.1. MiRNAs: Discovery

In 1993, Ambros and colleagues identified a small RNA molecule, called *lin-4* which led to the recognition of a large family of endogenous small RNAs, namely microRNAs (Lee *et al.*, 1993). *lin-4* is a heterochronic gene in *Caenorhabditis elegans* essential for the normal temporal control of developmental timing of larval stages and *lin-4* loss of function (lf) mutations cause reiteration of early larval fates at later developmental stages (Ambros and Horvitz,1987). Cloning of *lin-4* revealed that *lin-4* did not encode a protein, rather, the 693-nt rescue fragment produced at least two small RNAs: a longer, 61-nt species termed lin-4L and a shorter, 22-nt species termed lin-4S (Lee *et al.*, 1993). However, the 22-nt lin-4S RNA was hypothesized to be the functional species because it was more abundant and because the hairpin secondary structure of the lin-4L species was thought to sequester the sequences complementary to the target mRNA. Through this and other studies, *lin-14* was then identified as the first miRNA target gene in *C. elegans*. *lin-4* temporally regulates levels of the LIN-14 protein through the *lin-14* 3UTR containing multiple elements that are partially complementary to *lin-4*, thus leading to the conclusion that *lin-4* regulates *lin-14* through an antisense RNA-RNA interaction (Lee *et al.*, 1993).

In the year 2000, *let-7* was identified in a genetic screen as the second *C.elegans* miRNA gene (Reinhart *et al.*, 2000). *let-7* is a temporally regulated, heterochronic gene that controls the transition between the late larval stage to the adult stage. Similar to *lin-4*, *let-7* did not encode a protein but rather a small RNA, and moreover, the transgene complementation fragment that rescued *let-7*(lf) mutations encodes multiple small RNA species produced from *let-7* (Reinhart *et al.*, 2000). Similar to the interaction between *lin-4* and *lin- 14* interaction, *let-7* was thought to repress *lin-41* by imperfect RNA:RNA base pairing with the *lin-41* 3□UTR (Reinhart *et al.*, 2000). The 21-nt let-7 sequence and its temporal regulation were found to be conserved across a wide range of species, including vertebrates from zebrafish to humans (Pasquinelli *et al.*, 2000). This observation implied that miRNAs were not just a unique phenomenon in *C. elegans* developmental biology, but rather were evolutionarily significant and broadly used gene regulatory molecules. Such initial cloning efforts and bioinformatic analyses resulted in identification of numerous miRNA genes and their conservation in *C. elegans*, *Drosophila*, and humans (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001).

Evidence for the existence of RNA-mediated silencing mechanisms in plants first appeared in the late 1990's, when short antisense RNA molecules were isolated from tomato plants where post-transcriptional gene silencing (PTGS) had been detected (Hamilton and Baulcombe, 1999). In plants, post-transcriptional gene silencing (PTGS) called co-suppression, had been observed during flower patterning upon over-expression of a transgene, and in *C. elegans,* PTGS called RNA interference (RNAi) was caused by the introduction of double-stranded RNA (dsRNA) (Fire *et al.*, 1998; Napoli *et al.*, 1990). Small RNAs from 21-25-nt in length, originating from longer dsRNA species were shown to be the determinants of RNAi, through perfect base-pairing and degradation of the target mRNA (Elbashir *et al.*, 2001b; Hamilton and Baulcombe, 1999; Hammond *et al.*, 2000). Since then, the knowledge on sRNAs has broadened and these molecules have been identified as important players in a wide variety of processes in plants. Reports on plant miRNAs were available only after 10 years of finding of animal miRNAs (Reinhart *et al.*, 2002).When the first set of plant microRNAs (miRNAs) was cloned (Reinhart *et al.*, 2002); there were only 218 entries in the public miRNA database miRBase (Griffiths-Jones, 2004) whereas more than 15 000 entries can be found currently.

Formal naming and recognition of miRNAs as a separate group of RNAs holding regulatory functions were commenced in 2001 (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001). miRNAs were found to be regulating several biological processes of plants development of roots, stems, leaves and floral parts (Bartel, 2004; Chen, 2004; Kim *et al.*, 2005; Liu and Chen, 2009). Several studies show that many miRNA families are evolutionarily conserved across all major lineages of plants, including mosses, gymnosperms, monocots, and eudicots, suggesting that miRNA-mediated gene regulation might to have existed since earlier stages of plant evolution and has been tightly constrained (functionally) for more than 425 million years (Zhang *et al.*, 2006b). At present there are several proposed mechanisms for miRNA origin, including duplication of pre-existing miRNA genes or protein-coding genes, generation from transposable elements and formation of hairpin structure during genome evolution. The first two mechanisms are common in plants (Fahlgren *et al.*, 2007; Fahlgren *et al.*, 2010; Cuperus *et al.*, 2011; Nozawa *et al.*, 2012; Zhou *et al.*, 2013b) while the third mechanisms is more common in animal miRNA origin (Nozawa *et al.*, 2010).

2.3.2. miRNA: Biogenesis

Plant microRNA (MIR) genes are located mainly in intergenic regions throughout the genome (Reinhart *et al.*, 2002) and most plants possess over 100 miRNA genes (MIR) (Nozawa *et al.*, 2012). miRNA pathway evolved before multicellularity and the unicellular algae *Chlamydomonas reinhardtii* have miRNAs with similar characteristics to those of higher plants (Molnár *et al.* 2007). miRNA biogenesis is a multistep process (Fig.2.1.). Most characterized eukaryotic MIR genes possess their own transcriptional unit (Griffiths-Jones *et al*, 2008) and are transcribed by RNA polymerase II (Pol II) (Xie *et al*, 2005a; Kim *et al*, 2011) to yield a long capped and poly(A) tailed primary miRNA transcript called a pri-miRNA. The pri-miRNA typically forms an imperfect fold-back stem-loop structure of partially complimentary double stranded RNA (dsRNA) and further processed into hairpin loop structured pre-miRs (precursor miRNAs) in the D bodies (Dicing bodies) or SmD3-bodies (small nuclear RNA binding protein D3 bodies) (Kurihara *et al.*, 2006; Fang and Spector, 2007) by a protein complex containing the DCL1 (Schauer *et al.,* 2002) and the CBC (Cap-Binding protein Complex) (Kim *et al.,* 2008).

In plants, pri-miRNA stem-loops are processed into short double stranded RNAs (dsRNAs) consisting of mature miRNA guide and passenger (miRNA*) strands with 2-nucleotide 3□overhangs by a family of four DCL RNase III endonucleases (Margis *et al.,* 2006). The regulation of DCL1 mediated pri-miRNA processing and miRNA accumulation is promoted by RNA binding proteins, C_2H_2-zinc finger protein, serrate (SE) (Kurihara *et al.,* 2006; Dong *et al.,* 2008; Manavella *et al.,* 2012a), Double strand RNA-Binding protein (DRB), Hyponastic Leaves 1 (HYL1/DRB1), (Han *et al.,* 2004; Kurihara *et al.,* 2006) and the G-patch domain protein Tough (TGH) (Ren *et al.,* 2012). All the three proteins bind RNA. HYL1 binds double stranded (ds) region on the pri-miR (Hiraguri *et al.,* 2005; Rasia *et al.,* 2010; Yang *et al.,* 2010), TGH binds the single-stranded (ss) RNA region (Ren *et al.,* 2012) and SE binds pri-miRNA at single stranded RNA/dsRNA junctions (Machida *et al.,* 2011). Despite their general roles in miRNA biogenesis, HYL1 and TGH can modulate the accumulation of specific miRNAs (Szarzynska *et al.,* 2009; Ren *et al.,* 2012).

HYL1 is a phospho-protein that directly interacts with C-terminal domain Phosphatase-Like 1 (CPL1) protein to maintain its hypo-phosphorylated state while CPL1 plays a critical role in accurate miR processing, although it is not directly required for DCL1 activity (Manavella *et al.,* 2012). Phosphorylation status of HYL1 had been found affected by SE mutation and CPL1 had been found to interact with SE and gets recruited to the DCL1 complex by SE (Manavella *et al.,* 2012). This suggests a model in which the pri-miR processing has been shown to require association of multiple RNA binding proteins with definite regions to maintain the structural determinants for recruiting and directing DCL1 activity. DAWDLE (DDL) a phosphothreonine binding forkhead-associated domain protein has been shown to bind with RNA and associates with DCL1 (Yu *et al.*, 2008). The DCL1, HYL1, SE, and TGH seem to interact directly (Kurihara *et al.*, 2006; Yang *et al.*, 2006; Qin *et al.*, 2010; Machida *et al.*, 2011; Ren *et al.*, 2012). The hairpin looped pre-miRNAs thus formed are further processed by DCL1 to produce miR/miR* duplex (Xie *et al.*, 2005b). Additionally a proline-rich protein, SIC (Sickle), was identified to co-localize with HYL1 foci which was found to play an important role in the accumulation of mature miR duplex (Zhan *et al.*, 2012). The plant miRNA/miRNA* duplexes are protected from uridylation and degradation by the activity of a methyltransferase protein known as HEN1 (Hua Enhancer1) which covalently attaches a methyl residue at the 3′ ribose of last nucleotide from each strand (Li *et al.*, 2005; Yu *et al.*, 2005). HEN1 methylates miRNAs before the dissociation of the miRNA and miRNA* stands and in the absence of methylation, miRNAs vary in size due to combined 3'-end truncation and oligouridylation (Li *et al*, 2005). The miRNA duplexes can either stay in the nucleus where they are involved in chromatin modification of the genomic locus encoding the target messanger RNA (Axtell and Bowman, 2008) or get transported to the cytoplasm by HASTY protein (HST), the ortholog of Exportin-5 (Park *et al.*, 2005) for post transcriptional gene silencing (PTGS).

In the cytoplasm miRNA duplex unwinds and the mature guide strand loaded into Argonaute1 protein (AGO1) containing RNA-induced silencing complex (RISC) act upon highly or perfectly complementary target transcripts by promoting cleavage or repressing the translation (Llave *et al.*, 2002b; Rhoades *et al.*, 2002; Chen, 2004). AGO1 that has both a small RNA-binding PAZ domain and catalytic PIWI domain mediates miRNA-guided cleavage of complementary target transcripts (Vaucheret *et al.*, 2004; Baumberger and Baulcombe, 2005). *Arabidopsis* encodes 10 AGOs among which AGO1 predominates the miRNA pathway and is involved in the post-transcriptional

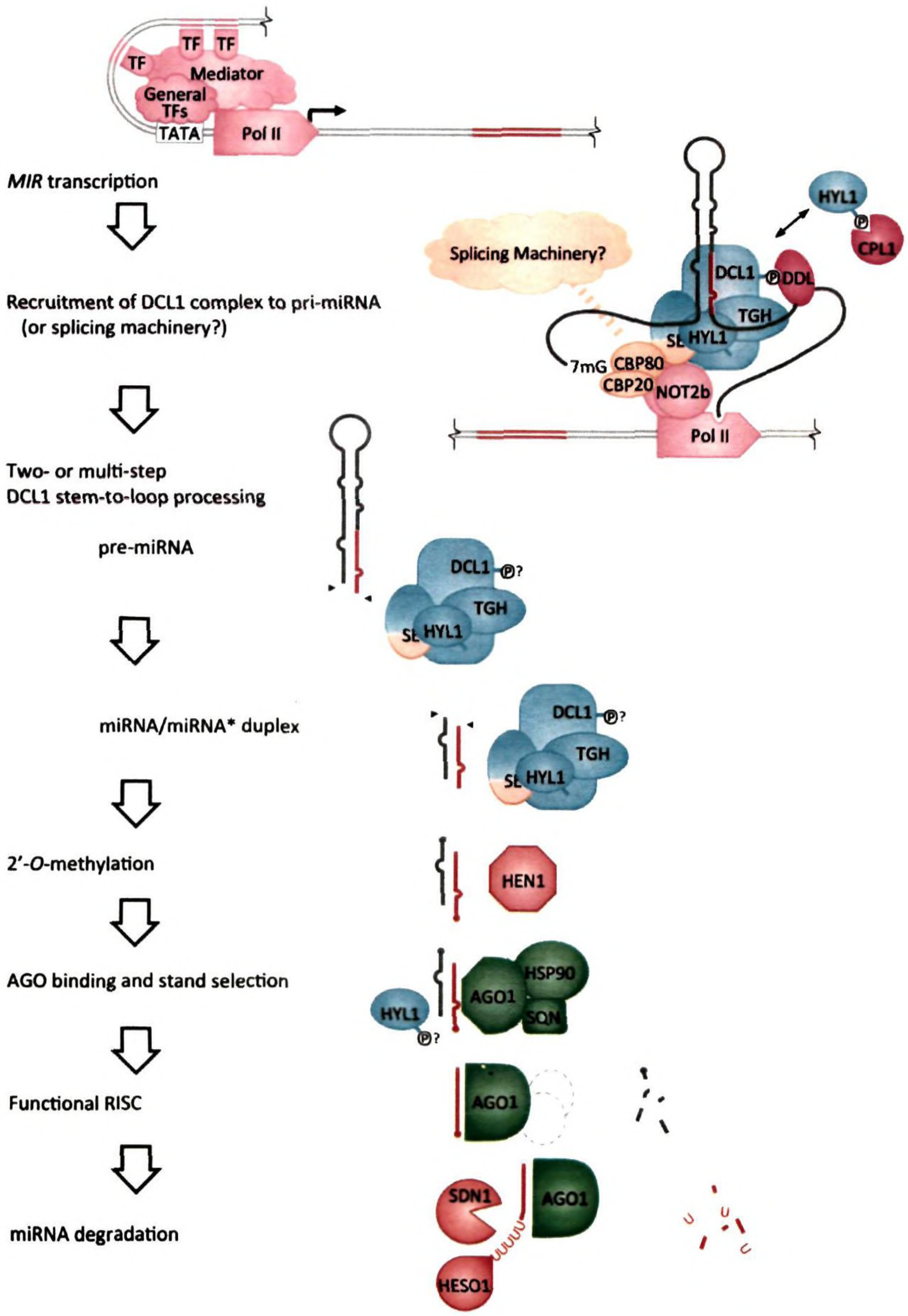


Fig. 2.1. Steps in miRNA Biogenesis and Turnover (Rogers and Chen, 2013).

gene silencing (PTGS) (Baumberger and Baulcombe, 2005). Hsp90, a chaperone involved in protein folding was co-purified with AGO1 and was found to be required for the loading of sRNAs into AGO1, apparently by inducing conformational changes in this protein (Iki *et al.*, 2010). Based on the AGO protein loaded, the miRNA selects their mRNA target in a sequence specific manner through complimentary base pairing. The RISC protein complex represses the expression of the target mRNA either through cleavage of its backbone (Baumberger and Baulcombe, 2005), or translation repression on partial base pairing (Doench *et al.*, 2003; Doench and Sharp, 2004; Brodersen and Voinnet, 2009).

2.3.3. miRNA target recognition

The mode of target recognition of miRNA differs between animals and plants. In animals, miRNA target sites are usually within the mRNA 3'-untranslated region (3′- UTR) (Bartel, 2009) although 5'-UTR and open reading frame (ORF) target sites have been reported to occur less frequently (Grimson *et al.*, 2007). These miRNA target sites form a seven consecutive base pairs "seed" region from position two (2) through eight (8) of the 5′ end of the aligned miRNA. Additional pairings in the miRNA 3′ region, which enhances target recognition has also been reported (Grimson *et al.*, 2007). In plants, majority of miRNAs have target sites in the ORFs and occasionally in the 5'-UTRs, 3'- UTRs, or in non-coding RNAs (Addo-Quaye *et al.*, 2008; German *et al.*, 2008). The miRNA forms extensive complementarity with the target with less than 5 mismatches and a single G: U wobble. The 5' region from position 2 to 13 is important for plant miRNA-mediated target repression with positions 9 to 11 being critical for AGO slicing (Mallory *et al.*, 2004; Schwab *et al.*, 2005).

Despite the difference in target recognition between animals and plants, the targets are similarly repressed through degradation and translational repression. In animals, repression which involves inhibition of translation followed by subsequent deadenylation and decapping of the mRNA is widely common (Iwasaki *et al.,* 2009). In plants, many sites are subjected to AGO1 endonucleolytic cleavage although studies have reported the existence of translational repression in plants (Brodersen *et al.,* 2008; Lanet *et al.,* 2009). Recently, in plants Liu *et al.,* (2014) demonstrated efficacy of low complementarity in target recognition using a luciferase based sensor system to assess miRNA-target complementarity.

In general, the sequences of mature miRNAs and the target genes of miRNA families are conserved across different plant families. However, some nucleotide variations are still found in the miRNA sequences, especially in the "seed sequences", which are usually the highly conserved regions of the miRNA sequences. Moreover, there have been many nucleotide changes among the targets of different plant species also (Axtell and Bartel, 2005; Zhang *et al.,* 2006a; 2006b).

2.3.4. Identification of miRNAs in plants

In order to identify and to elucidate miRNA function in both plant and animal kingdoms, both computational and experimental methods have been widely employed. Genetic screening and direct cloning were among the first approaches (Palatnik *et al.,* 2003; Sunkar and Zhu, 2004). Genetic screening technology was used to identify the first miRNA, lin-4 (Lee *et al.,* 1993). In the year 2000, identification of plant miRNAs began with direct cloning and sequencing (Llave *et al.,* 2002; Park *et al.*, 2002; Reinhart *et al.,* 2002) which is a sequence independent approach where a prior knowledge of miR sequence is not required. It involves creation of a cDNA library followed by six steps: isolation of total RNA from plant tissue, recovery of small RNAs from an acrylamide gel, adaptor ligation, reverse transcription, RT-PCR, cloning and sequencing. The conventional sequencing of relatively small-sized cDNA libraries of plant sRNAs from *Arabidopsis,* rice and poplar with Sanger method had led to the conclusion that plant miRNAs are highly conserved (Axtell and Bartel, 2005). Although direct cloning and genetic approaches had enabled the identification of many miRNAs, it is still difficult to clone low abundance miRNAs. Species specific miRNAs are often expressed at lower levels than that of conserved miRNAs, thus many non-conserved miRNAs cannot be detected in small-scale sequencing studies.

Several studies show that most known mature miRNAs are evolutionary conserved within the plant kingdom, it is possible to perform computational search for new miRNAs homologues or orthologues in other plant species (Wang *et al.,* 2004; Zhang *et al.,* 2006). These conserved mature miRNAs are almost identical or there are only a couple of nucleotide changes among them. Apart from this conservation, pre-miRNAs and mature miRNAs, also have several significant and unique features (Bartel, 2004), such as stem-loop hairpin structure, high negative minimal free folding energy (MFE) and high MFE index (MFEI) (Zhang *et al.,* 2006). Several computational approaches have been designed to identify plant miRNAs, particularly conserved miRNAs. One of them is homologue-based comparative genome approach in which miRNAs are identified against all potential nucleotide sequences (including expressed sequence tags [EST], genome sequence survey [GSS], and genome sequences) using currently known miRNA sequences (Zhang *et al.,* 2005; 2006b). This strategy was usually used to identify miRNAs in a new plant species, using already known miRNAs in a model plant species such as *Arabidopsis* or rice. The use of computational algorithms based on the extensive conservation of the miRNAs during their biogenesis has helped in the identification of several new miRNAs and the postulation of many others (Adai *et al.,* 2005; Jones-Rhoades and Bartel, 2004). Search criteria allowed up to three sequence mismatches while looking for conserved miRNAs in heterologous species. Computational approaches have been quite useful in the identification of miRNA in various plant species such as *Arabidopsis* (Wang *et al.*, 2004; Adai *et al.*, 2005), maize (Zhang *et al.*, 2006a), rice (Zhang *et al.*, 2005), foxtail millet (Khan *et al.*, 2014), grape (Carra *et al.*, 2009) tomato (Yin *et al.*, 2008) soybean (Zhang *et al.*, 2008a), and in many other plants. Although numerous miRNAs were identified by computational algorithms, this was not found to be appropriate for species with less annotated genomes (Chen and Xiong, 2012).

Continued technical improvements and decreasing cost of next-generation sequencing technology have made RNA sequencing (RNA-seq) a popular choice for gene expression studies. Currently, deep sequencing approach has become the most commonly used strategy for plant miRNA study which has been extensively used to identify miRNAs in a wide variety of plant species. The deep sequencing technology can generate millions of sequences per run that can be used for the genome-wide identification of all potential miRNAs and their expression levels based on read number. High throughput sequencing of small RNA libraries has also revealed an unexpected diversity and greater abundance of endogenous siRNAs in plants (Sunkar *et al.*, 2005; Rajagopalan *et al.*, 2006). The first release of miRBase in the year 2002 included a total of 15 miRNAs from only 1 plant species, *Arabidopsis thaliana.* This was followed by inclusion of *Oryza sativa* in the year 2003. There after miRNAs were reported from *Medicago truncatula, Glycine max* and *Populus trichocarpa* in the year 2005. The current version of miRBase (release21) includes 48,496 mature plant miRNAs derived from 6992 hairpin precursors reported in 73 plant species. The number of identified plant miRNAs keeps increasing and accordingly their target genes are also being dentified. High throughput sequencing technologies have an important role in identification and characterization of miRNA targets with PARE or Degradome sequencing. This involves sequencing of the entire pool of cleaved targets followed by mapping of the miR-guided cleavage sites (Ding *et al.*, 2012). High throughput sequencing and degradome analysis identified several stress induced miRNAs and their targets in maize (Liu *et al.,* 2014), tomato (Cao *et al.*, 2014), *Raphanus sativus* (Wang *et al.,* 2014), Populus (Chen *et al.,* 2015) rice (Qin *et al.,* 2015), *Phaseolus vulgaris* (Formey, 2015) and barley (Hackenberg *et al.,* 2015). Better understanding of miRNA-guided gene regulations can contribute to improving the abiotic stress tolerance in plants (Sunkar *et al.,* 2006). On the other hand, deep sequencing approaches which generate a large number of sequences and datasets need the involvement of bioinformatics to extract the important information.

2.3.5. miRNA function in plants

Most of the early cloned miRNAs are involved in plant growth and development and were reported to target different transcription factors and hormone related genes (Reinhart *et al.*, 2002). The cloning of miRNAs from different plant species revealed a highly conserved nature of miRNAs across the plant kingdom (Willmann and Poethig, 2007; Groszhans and Filipowicz, 2008). Since miRNA targets the mRNA in a sequence specific manner, it is possible that the miRNAs have a similar functional role across different plant species. Most of the miRNAs target transcription factor genes which are involved in leaf, shoot and root development, floral identity, flower development, flowering time, hormone signaling and vascular development (Llave *et al.,* 2002; Palatnik *et al.,* 2003; Achard *et al.,* 2004; Mallory *et al.,* 2004a; Kim *et al.,* 2005; Jones-Rhoades *et al.,* 2006).

The recent developments and findings of miRNA research indicate the existence of conserved miRNAs in plant species as well as species specific miRNAs. This suggests that conserved miRNAs may regulate common traits in plants, such as plant morphology and phase change, and that species-specific miRNAs may control unique and variable processes in individual plant species, such as fibre initiation and development in cotton (Xie *et al.*, 2015). Both conserved and species-specific miRNAs may be involved plant's response to abiotic stress.The highly conserved miRNAs, miR165 and miR166 targets three homeodomain TFs PHABULOSA (PHB), PHAVOLUTA (PHV), and REVOLUTA (REV) which are involved in leaf development, leaf polarity (Zhang *et al.,* 2006a) and vascular development (Kim *et al.,* 2005). It has also been reported that miR165 is involved in HDZIP-III mediated indeterminacy in apical and vascular meristems (McHale and Koning, 2004). MicroRNA167 negatively regulates *ARF6* and *ARF8* (Rhoades *et al.,* 2002; Xie *et al.,* 2005) and is important in controlling the proper expression pattern of these genes in *Arabidopsis* especially in maintaining the fertility of both ovules and anthers (Wu *et al.,* 2006). Over expression of miR167 results in longer hypocotyls, sterile and smaller flowers compared to wild type plants (Ru *et al.,* 2006). MicroRNA156/157 target mRNAs of Squamosa-promoter Binding Protein (SBP) box genes (Schwab *et al.,* 2005; Wu and Poethig, 2006) which are involved in developmental timing in *Arabidopsis* (Wu and Poethig, 2006). The *Arabidopsis* plants expressing miR156/157 resistant forms of SPL3/4 and SPL5 showed an early flowering whereas constitutive expression of miR156 in *Arabidopsis* prolonged the vegetative phase and delayed flowering (Wu and Poethig, 2006). Root, shoot, floral, and embryo development have all been shown to be regulated by TFs of the type NAM/ATAF/CUC (NAC) (Takada *et al.,* 2001; Hibara *et al.,* 2003) and also auxin response factors (ARF) involved in root patterning (Sorin *et al.,* 2005; Yang *et al.,* 2006a). These TFs were shown to be affected by miR164 expression (Guo *et al.,* 2005), which is coupled to abnormalities in the developmental programs (Mallory, 2004; Guo *et al.,* 2005).

In plants, flowering time may be altered to produce early-transitioning adults by down-regulating APETALA-2 protein (AP2), a regulator of floral-timing and floral-patterning (Lohmann and Weigal, 2002). miR172 down regulates the APETALATA 2 (AP2) like transcription factor genes and controls the flowering time and floral organ pattern in *Arabidopsis* (Aukerman and Sakai, 2003). MicroRNA171 targets a family of putative transcription factors known as scarecrow-like (scl) proteins (Reinhart *et al.*, 2002; Rhoades *et al.*, 2002; Xie *et al.*, 2005), which are involved in radial patterning of roots and hormone signaling (Silverstone *et al.*, 1998; Helariutta *et al.*, 2000). In *Arabidopsis* and *N. benthamiana* a relatively high level of miR171 was detected in the inflorescence and flowers compared to stem and leaf (Llave *et al.*, 2002). MicroRNA319 targets TCP (TEOSINTE BRANCHED1, CYCLOIDEA, and PCF) family of transcription factors involved in leaf morphogenesis (Palatnik *et al.*, 2003). The miR159 family members were predicted as well as validated to target MYB and TCP family gene transcripts in floral organ development (Xie *et al.*, 2005). MYB proteins are known to bind to promoter regions of a number of genes including the floral meristem identity gene LEAFY (Rhoades *et al.*, 2002; Achard *et al.*, 2004). It was also reported that the plant hormone ABA has a regulatory role on the levels of miR159 during seed germination. MicroRNA159 accumulates in response to ABA during the seed germination resulting in the degradation of its target mRNAs (MYB33 and MYB101) to desensitize the hormone signaling during seedling stress in *Arabidopsis* (Reyes and Chua, 2007). MicroRNA168 regulates the expression of AGO1 through an auto-regulatory mechanism to maintain homeostasis of AGO1 for proper development (Vaucheret *et al.*, 2004). Since it regulates the key component of RISC, any variation in this miRNA expression has potential influence on the function of other miRNAs.

2.3.6. miRNAs and abiotic stress responses in plants

During the course of evolution, plants evolved complicated physiological and genetic mechanisms in order to cope with and adapt to the harsh environment. Most of the conserved miRNAs are known to have key roles in plant development and adaptive responses to abiotic stresses by targeting a variety of transcription factors (TFs) (Llave *et al.*, 2002b; Rhoades *et al.*, 2002; Carrington and Ambros, 2003; Sunkar and Zhu, 2004, Sunkar *et al.,* 2006; Todesco *et al.,* 2010). Abiotic stresses causes up or down regulation of synthesis of new miRNAs to cope with the effects of stress (Fig. 2. 2) The abiotic stress responsive role of miRNAs in plants was initially suggested after obtaining data from miRNA target prediction, expression profiling studies of miRNAs during plant response to abiotic stress, and NCBI expressed sequence tags (ESTs) surveys (Zhang, 2015). Plant miRNAs target transcripts in a sequence-specific manner which allowed Jones-Rhoades and Bartel (2004) to predict and validate ATP sulphurylase (APS), the enzyme that catalyses the first step of inorganic sulphate assimilation, as the target of miR395, which is responsive to sulphate levels in plants. Based on this initial result, they further analysed the response of miR395 to cellular sulphate levels and found that expression of miR395 depends on sulphate availability. Expression of miR399 which targets ubiquitin-conjugating enzyme (UBC) was induced during low-phosphate stress and in *Arabidopsis,* UBC mRNA accumulation is decreased during low-phosphate stress for the induction of phosphate transporter gene AtPT1 and attenuation of primary root elongation (Chiou *et al.,* 2006; Fujii *et al.,* 2005). Overexpression of miR399 even under high phosphate conditions led to the down regulation of UBC and induced accumulation of phosphate. Conversely, *mir399*-UBC mutants showed limited induction of AtPT1 under low-phosphate conditions and showed limited attenuation of primary root elongation. Sunkar and Zhu (2004) constructed small RNA libraries from *Arabidopsis* seedling and identified a variety of conserved miRNAs that were differentially expressed under cold stress (0 °C for 24 h), salt stress (300 mM NaCl for 5 h), drought stress (dehydration for 10 h), and hormones [100 μM abscisic acid (ABA) for 3 h], as well as from the untreated controls. After identification of conserved and novel miRNAs, miR393 miR397b and miR402 were found strongly induced by all stress conditions (cold, dehydration, NaCl, and ABA treatments). In contrast, miR389a.1 was inhibited by all of the stress treatments, which was later found to be related to ta-siRNAs

(Allen and Howell, 2010). miR319 was found induced by cold but not by salinity, dehydration, or ABA (Sunkar and Zhu, 2004).

Involvement of miRNAs in plant abiotic stress came from the identification of miR398 which targets two Cu/Zn superoxide dismutases (SODs). Reactive oxygen species (ROS) produced during regular metabolism is converted to less toxic hydrogen peroxide by SODs (cytosolic-*CSD1* and chloroplastic *CSD2*). But during abiotic stress, enhanced production of ROS occurs which results in the accumulation of ROS to toxic levels (Apel and Hirt, 2004; Sunkar *et al.*, 2007) and these highly toxic ROS need to be quickly scavenged. Detailed study on the expression of Cu/Zn SODs during oxidative stress conditions revealed that they are under post-transcriptional control by miR398, indicating the key role of miRNA-mediated regulation of SODs during abiotic stress (Sunkar *et al.*, 2006). In rice, miR169 family members were induced by drought and salinity stress (Zhao *et al.*, 2009) while miR396 was found responsive to high salinity, drought and cold stresses (Liu *et al.*, 2008). Since these initial studies, the role of miRNAs in plant response to environmental stresses has been attracting attention of many researchers.

Most of the studies have investigated the expression profiles of miRNA in the whole plant under stress condition. However, studies conducted on tissue specific expression of miRNAs indicated the differential response occuring in the root and shoot tissues during stress. For example in barley, tissue-specific miRNA profiling found that miR166 was up-regulated in leaves, but down-regulated in roots while miR156a, miR171 and miR408 were induced in leaves, but unchanged in roots (Kantar *et al.*, 2010). Using miRNA array analysis, Jia *et al.* (2009) reported 24 differentially expressed UV-B-radiation responsive miRNAs in *Populus tremula.* In *Arabidopsis*, miR156, miR160, miR165/166, miR167 and miR398 were found induced in response to UV-B radiation (Zhou *et al.*, 2007).

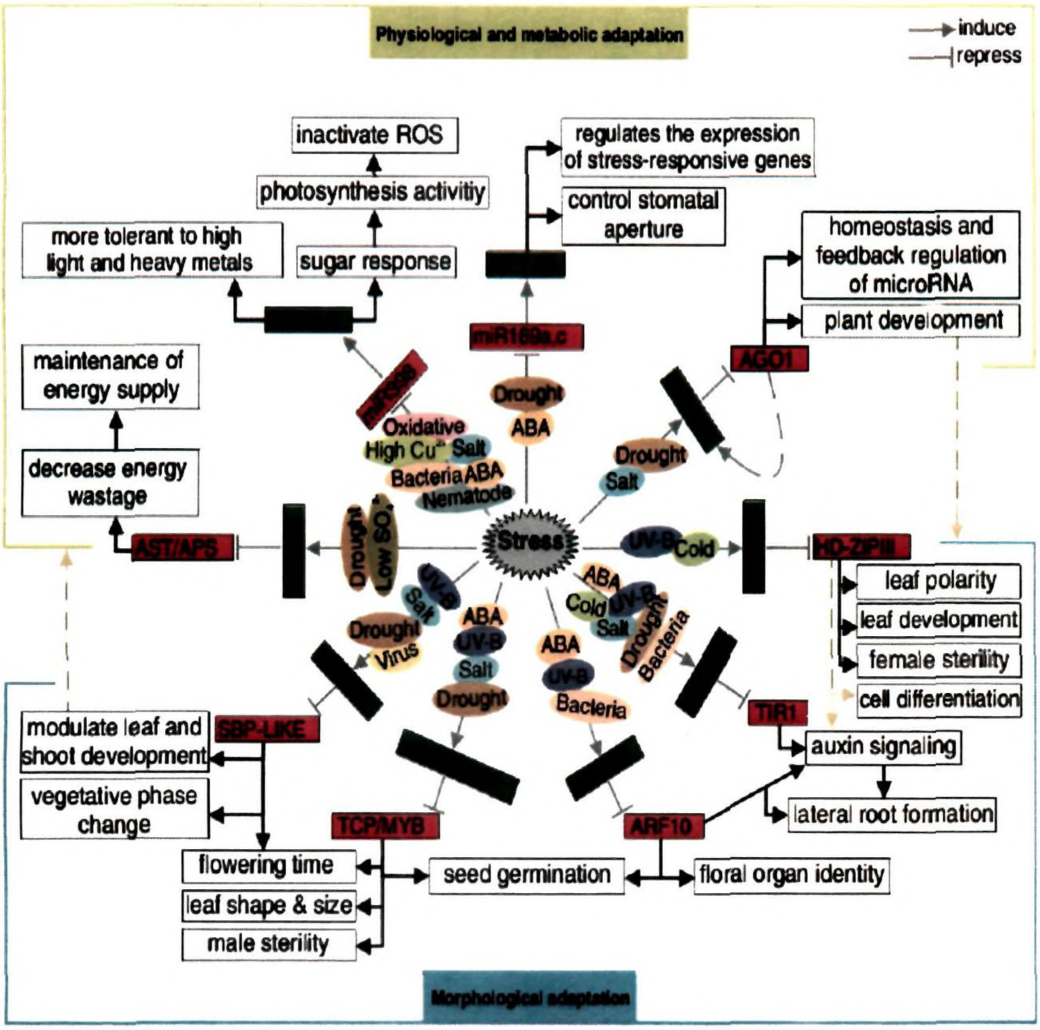


Fig. 2.2. Regulatory network of stress responsive miRNAs (Khraiwesh *et al.*, 2012).

Deep sequencing technologies and miRNA microarrays made it easier to identify the stress responsive miRNAs and their expression levels in various tissues in the same species. Currently deep sequencing is the most efficient approach to study miRNA expression profiles which made it convenient to find new or novel miRNAs that are induced by individual stress particularly in plant species for which no complete genome sequence data are available along with simultaneous surveying of their expression levels.

2.3.7. Genotype-dependent response of miRNAs to abiotic stress

Different genotypes of the same plant species may show differential gene expression due to difference in individual plant growth conditions and due to the human interventions in cultivated crops compared with their wild relatives. Similar to protein-coding genes, many miRNAs also show difference in expression from species to species and also from genotype to genotype under certain conditions. Genotype-dependent response of miRNAs to abiotic stress was evidenced by analysing miRNA expression levels in response to certain stresses among several plant species and cultivars. Deep sequencing, microarrays, quantitative real-time PCR analysis, and the transgenic plants creation, indicated the difference in the miRNA expression profiles among plant species. Reports show that one miRNA may respond to the same stress differently depending on the plant species.

The genotype-dependent response of miRNAs to abiotic stresses is not only different among plant species but also varies among cultivars (genotypes) of the same species (Zhang, 2015). It is well known that the genotypes of one plant species may differ in their capacity to respond to abiotic stress. The impact of drought treatment on two cowpea cultivars (drought-tolerant IT93K503-1 and drought-sensitive CB46) were investigated using deep sequencing (Barrera-Figueroa *et al.*, 2011). Between the two genotypes, 20 miRNAs were found differentially expressed under drought. Of these miRNAs, nine got highly expressed in one of the two genotypes but not in the other. Simultaneously, they also identified 11 drought-regulated miRNAs in one genotype but not in the other. miRNA expression profiles of two cotton cultivars with varying levels of tolerance to salinity (SN-011 with high tolerance to salinity and LM-6 with sensitivity to salinity) (Yin *et al.*, 2012) indicated the expression of 12 miRNAs in a genotype-specific pattern. Under salinity treatment, four miRNAs (miR156, miR169, miR535, and miR827) showed significantly higher expression in LM-6 while expression of three miRNAs (miR167, miR397, and miR399) got significantly inhibited. Mondal and Ganie (2014) identified 12 polymorphic miR-SSRs (simple sequence repeats) by comparing 12 salinity-tolerant and 12 salinity-susceptible genotypes in rice which indicated the lesser variability of miRNA genes in the tolerant cultivars than in the susceptible cultivars, as evidenced by polymorphic index content. All these studies suggest the cultivar-specific response of miRNAs to abiotic stress conditions. Ma *et al.*, 2015, also reported the opposite patterns of expression of 13 miRNAs in two wheat genotypes Hanxuan10, which is drought tolerant, and Zhengyin1, which is drought-susceptible after dehydration stress.

2.3.8. miRNAs and their response to drought stress

Drought as a major environmental stress factor causes detrimental effects to plant metabolic processes including stomatal conductance, nutrient uptake and photosynthesis thus ultimately resulting in yield losses in crops (Neumann, 2008; Shinozaki *et al.,* 2003). Under drought conditions, the expression levels of many genes/metabolites such as dehydrins, glutathione S-transferase (GST), abscisic acid (ABA)-inducible genes, helicase, proline and carbohydrates were found altered (Nezhadahmadi *et al.,* 2013). miRNAs have emerged as important regulators in drought tolerance and avoidance via regulation of drought-inducible genes (Shinozaki and Yamaguchi- Shinozaki, 2007) (Fig. 2.3.). The first direct evidence that miRNA is involved in the stress response came in 2006, demonstrating the repression of miR398 which led to the up-regulation of its target (CSD1 and CSD2) mRNAs under oxidative stress (Sunkar *et al.,* 2006).

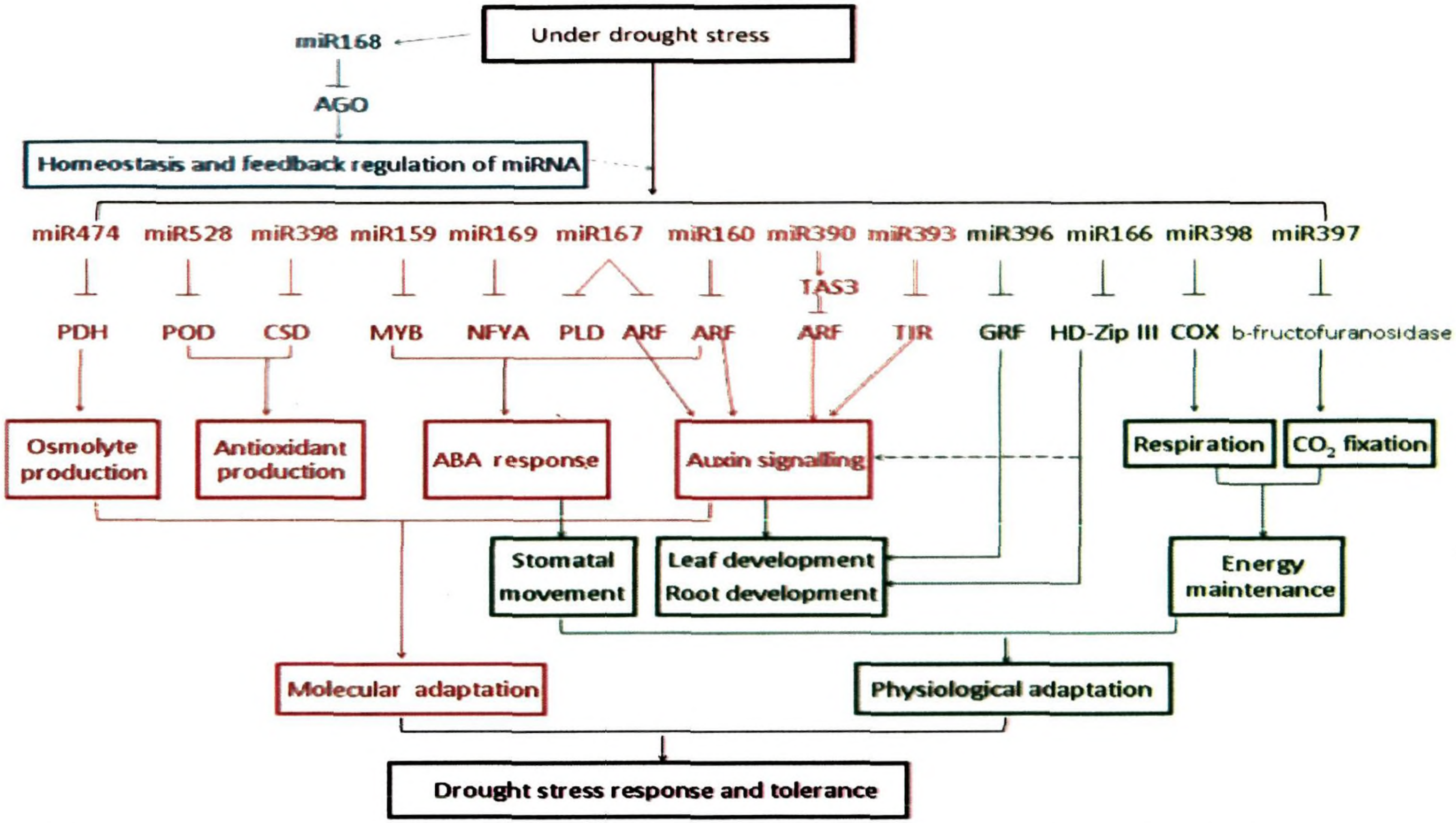


Fig. 2.3. Regulatory networks involving miRNAs and their target genes in drought response of plants (Ding *et al.*, 2013)

There are many reports available on drought associated miRNAs from many plant species such as *Arabidopsis* (Sunkar and Zhu, 2004; Liu *et al.*, 2008), tobacco (Frazier *et al.*, 2011), *Phaseolus vulgaris* (Arenas-Huertero *et al.*, 2009), populus (Li *et al*, 2011a; Shuai *et al*, 2013), cowpea (Barrera-Figueroa *et al.*, 2011), soya bean (Kulcheski *et al.*, 2011), and rice (Zhou *et al.*, 2010) (Table 2.2.). Many miRNAs respond to drought stress via signal transduction pathways such as auxin signalling, ABA-mediated regulation, osmoprotectant biosynthesis and scavenging of antioxidants (Ding *et al.*, 2013). Zhao, *et al.*, 2007 reported the up-regulation of miR169g, miR393 and miR397b in rice seedlings subjected to PEG-mediated dehydration stress. In *Arabidopsis,* miR159, miR156, miR167, miR171,miR168, miR172, miR319, miR393, miR394a, miR395c, miR395e, miR396 and miR397 were up-regulated, while miR161, miR168a, miR168b, miR169, miR171a and miR319c were down-regulated, under drought stress (Liu *et al.*, 2008; Sunkar and Zhu, 2004). Reports shows that up-regulated miRNAs were also involved in different developmental stages (Alonso-Peral *et al.*, 2012; Curaba *et al.*, 2013; Wu and Poethig, 2006; Xie *et al.*, 2014; Zhu and Helliwell, 2011), suggesting that the regulation of drought tolerance and developmental stages by miRNAs is tightly associated, indicating the existence of common mechanism. Under dehydration conditions, miR408 was up regulated in root and shoot tissues of *Medicago truncatula* (Trindade *et al.*, 2010) and in *Hordeum vulgare* leaves (Kantar *et al.*, 2010). In several plant species miR408 was found to target the plantacyanin-like transcripts thus linking it to the control of copper homeostasis suggesting a possible relationship between copper deficiency and water deficit (Abdel-Ghany and Pilon, 2008; Trindade *et al.*, 2010). In the wild emmer wheat *Triticum turgidum ssp. dicoccoides*, miR1432 and miR1867 induced by dehydration stress in both roots and shoots were predicted to target phenylalanine tRNA synthetase and a protein from the DUF1242 super family respectively (Kantar *et al.*, 2010).

In *Arabidopsis*, miR393, miR319 and miR397 were up-regulated in response to drought stress. miR393 also found up-regulated in rice under drought condition.The expression levels of miR1446a-e, miR1444a, miR1447 and miR1450 were significantly reduced in *Populus trichocarpa* (Lu *et al.*, 2008). During drought miR156 got up-regulated in *Arabidopsis, Prunus persica*, barley, *Panicum virgatum* and *Triticum dicoccoides* (Eldem *et al.*, 2012; Kantar *et al.*, 2010, 2011; Sun *et al.*, 2012; Sunkar and Zhu, 2004), but down-regulated in rice and maize (Wei *et al.*, 2009; Zhou *et al.*, 2010). Similarly, expression of miR169 got down regulated during drought stress in *Arabidopsis*, P. *persica, P. virgatum* and *Medicago truncatula* (Li *et al.*, 2008), but got up-regulated in rice, *Glycine max, Populus euphratica* and tomato (Li *et al.*, 2011; Qin *et al.*, 2011; Zhang *et al.*, 2011; Zhou *et al.*, 2010). In tomato, up-regulation of miR169 under drought led to the down regulation of its targets NF-YA1/2/3. In tomato, overexpression of miR169 resulted in enhanced drought tolerance with reduced stomatal opening, transpiration, and leaf water loss (Zhang *et al.*, 2011). Contrarily, in response to drought in *Arabidopsis*, expression of NFYA5 got strongly upregulated while miR169 was down regulated (Li *et al.*, 2008). Generally under abiotic stress MIR169 family members exhibit upregulation in both monocots and dicots except for few cases where downregulation was also reported (Xu *et al.*, 2014).

Over expression of miR168a and its target *AGO1* in loss-of-function mutants of *Arabidopsis* resulted in the hypersensitivity to ABA and drought. In contrast, in the miR168 mutants *of Arabidopsis* under drought *miR168a-2* displayed ABA and drought hypersensitivity (Li *et al.*, 2012a). Artificial miRNA - mediated silencing of CBP80 gene in potato rendered plants drought tolerant and ABA hypersensitive (Pieczynski *et al.*, 2013). Down regulation of CBP80 led to the decreased expression of miR159 and increased expression of MYB33 and MYB101 in the potato transgenic plants and *Arabidopsis cbp80* mutants (Pieczynski *et al.*, 2013).

Table 2.2. Drought-responsive miRNAs in plants (adopted from Ferdous *et al.*, 2015)

miRNA	Target name and function	Species	Reference
miR156	SBP family of transcription factors – promote phase transitions, flowering time	*Ath*↑, *Tdi*↑, *Hvu*↑, Rice↓ *Peu*↑, *Ppe* (slightly)↑, *Pto*↓	Eldem *et al.*, (2012), Kantar *et al.* (2011), Liu *et al.*, (2008), Ren *et al.*, (2012) and Zhou *et al.*, 2010
miR157	SBP family of transcription factors	*Ppe*↑↓	Eldem *et al.*, (2012)
miR159	MYB and TCP transcription factors—ABA response, Nacl stress response, floral asymmetry and leaf development	Ath↑ Rice↓ Ppe↓Ppe↑, Pto↑, Ptc↓	Arenas-Huertero *et al.*, (2009), Eldem *et al.*, (2012), Jones-Rhoades and Bartel (2004), Liu *et al.*, (2008), Reyes and Chua (2007) and Zhou *et al.*, (2010)
miR164	NAC domain TF—lateral root development	Mtr↓, Ptc↓, Bdi↓	Shuai *et al.*, (2013) and Wang *et al.*, (2011)
miR160	ARF 10, ARF 16 and ARF 17—seed germination and postgermination stages	Ppe↑, Pto↑, Ptc↓	Eldem *et al.*, (2012), Jones-Rhoades and Bartel (2004), Liu *et al.*, (2007), Ren *et al.*, (2012) and Shuai *et al.*, (2013)
miR166	HD-ZIPIII transcription factor—axillary meristem initiation, leaf and vascular development	Tdi↓, Gma↑	Kantar *et al.*, (2011), Li *et al.*, (2011a,b), Sun (2012) and Williams *et al.*, (2005)
miR167	ARF6 and ARF8—gynoecium and stamen development	Ath↑, Ppe↓, Pto↑	Eldem *et al.*, (2012), Liu *et al.*, (2008), Ren *et al.*,(2012) and Wu and Poethig (2006)
miR168	ARGONAUTE1, MAPK—miRNA biogenesis and mRNA degradation, plant development	Ath↑ Rice↓ Z. mays↓	Liu *et al.*, (2008), Wei *et al.*, (2009) and Zhou *et al.*, (2010)

miR169	NF-YA transcription factor subunit A-3, NF-YA transcription factor subunit A-10, SlMRP1—Plant development and Flowering timing, response to different abiotic stresses	Ath↓, Tomato↑, Rice↑, Mtr↓, Ppe↓, Gma↑, Pto↓, Peu↑	Eldem *et al.*, (2012), Li *et al.*, (2008), Li *et al.*, (2011a,b), Qin *et al.*, (2011), Ren *et al.*, (2012), Trindade *et al.*, (2010), Wang *et al.*, (2011), Zhang *et al.*, (2011), Zhao *et al.*, (2007) and Zhou *et al.*, (2010)
miR171	GRAS transcription factors—response to abiotic stresses and floral development	Ath↓, Rice↓	Sun (2012) and Zhou *et al.*, (2010)
miR393	TIR1 and AFB2 and AFB3—susceptibility to virulent bacteria	Ath↑ Ppe↓	Liu *et al.*, (2008), Navarro *et al.*, (2006) and Eldem *et al.*, (2012)
miR394	Dehydration-responsive protein and F-box proteins—abiotic stress-response pathway	Pto↑, Ptc↓, Gma↑	Li *et al.*, (2011a,b), Ren *et al.*, (2012) and Shuai *et al.*, (2013)
miR395	Sulphate transporter—response to sulphate deprivation	Rice↑, Ppe↓, Pto↓	Eldem *et al.*, (2012), Liang *et al.*, (2010), Ren *et al.*, (2012) and Zhou *et al.*, (2010)
miR398	Copper superoxide dismutases; cytochrome C oxidase subunit V—Copper homoeostasis, oxidative stress; enzyme involved in respiration	Mtr↑, Tdi↑, Mtr↓, Ppe↓	Eldem *et al.*, (2012), Jones-Rhoades and Bartel (2004), Kantar *et al.*, (2011), Sunkar *et al.*, (2006), Trindade *et al.*, (2010) and Wang *et al.*, (2011)
miR1432	Poly (ADP-ribose) polymerase; calcium binding EF hand domains—activate in signal transduction pathways	Tdi↑	Kantar *et al.*, (2011) and Zhang *et al.*, (2009)

↑, up-regulation by drought; ↓, down-regulation by drought; *Ath, Arabidopsis; Bdi, Brachypodium distachyon; Gma, Glycine max; Hvu, Hordeum vulgare; Mtr, Medicago truncatula; Peu, Populus euphratica; Ptc, Populus trichocarpa; Pto, Populus tomentosa; Ppe, Prunus persica; Tdi, Triticum dicoccoides; Z. mays, Zea mays*

In some plant species, members of the same miRNA families were found to be differently expressed under drought stress. For example, in rice up-regulation of miR319 family members have been found under drought stress condition (Zhou *et al.,* 2010). Within a plant species, miRNA levels may vary and also can exhibit different responses depending upon the nature of the stress. Trindade *et al.,* (2010) reported up-regulation of miR398a/b in M. truncatula under drought whereas in another study it was found repressed (Wang *et al.,* 2011). Under drought conditions, expression of these miRNAs get regulated by their corresponding regulators thus reflecting in the levels of miRNAs and their respective targets (Reyes and Chua, 2007; Trindade *et al.,* 2010). It is also possible to identify the functional role of both the conserved and specific miRNAs in each plant species by target validation.

2.3.9. miRNAs and their response to cold stress

Post-transcriptional regulation of gene expression plays an important role in response to low temperatures (Chinnusamy *et al.,* 2007). Previously in *Arabidopsis,* five miRNAs were reported to be cold responsive (Sunkar and Zhu 2004). Later in *Arabidopsis* seedlings miR168, miR171 and miR396 were shown to be induced by drought, cold and salt stress (Liu *et al.,* 2008), suggesting that miRNAs can be involved in the pathways common to all these stimuli. Zhou *et al.,* (2008) identified four *Arabidopsis MIR* genes that are inducible by cold stress, using a computational approach based on transcriptome and promoter analysis data, coupled with experimental validation. Northern blot analysis revealed the up-regulation of miR165/ miR166, miR169 and miR172 upon cold treatment (Zhou *et al.,* 2008). miR166 family were also found up-regulated in similar conditions in rice, while miR168, miR169 and miR171 showed opposite expression profiles (Lv *et al.,* 2010). These observations indicate the complexity of miRNA expression upon abiotic stress and its dependency on a variety of parameters. Interestingly, most of these conserved cold regulated miRNAs are known to target TFs with known roles in plant development (Jones- Rhoades and Bartel, 2004), suggesting that miRNA-mediated responses to this kind of stress could be mainly at the structural level.

Regulatory motifs associated with cold response such as W-box (TTGAC), ABRE-core (ACGTGG/TC) and LTRE-core (A/GCCGAC) were found in abundance on the promoter region of cold inducible *MIR* genes (Zhou *et al.,* 2008) suggesting that stress-responsive miRNAs can be regulated at the transcriptional level. In *Brachypodium,* 25 cold stress responsive miRNAs were identified of which only three miRNAs (miR397, miR169 and miR172) were upregulated (Zhang *et al.*, 2009). As in drought stress, under cold stress also members of same miRNA family exhibited different response patterns. In cassava, differential expressions of miRNAs were observed between two cultivars (S124 and C4) under cold stress. In SC124 most of the miRNAs were down regulated, but in cultivar C4 only four miRNAs were down regulated and 31 miRNAs were up-regulated (Zeng *et al.,* 2010). These results indicate that miRNAs are not only regulated at species level but also at the level of variety or cultivars. These observations strongly suggest that miRNA family members can be carefully manipulated in germplasm varieties to overcome temperature extremes.

Comparative profiles of miR expression during cold stress among *Arabidopsis, Brachypodium,* and *Populus trichocarpa* revealed the up-regulation of miR397 and miR169 indicating the presence of conserved cold responsive pathways in all the species. Where as the expression of miR172 got triggered in *Arabidopsis* and *Brachypodium* but not in *Populus* (Zhang *et al.,* 2009a). Lv *et al.* (2010) identified eighteen cold-responsive miRNAs in rice with most of them being down regulated under cold stress.

2.3.10. miRNA based genetic modification for developing abiotic stress tolerant plants

The recent developments in miRNA research indicate the possibility of manipulating miRNA mediated gene regulations to engineer plants for enhanced abiotic stress tolerance. (Zhang and Wang, 2015). Due to their vital role in complex gene regulatory networks, miRNAs may prove potent targets for plant improvement, with improved tolerance to abiotic stresses (Zhang and Wang, 2015). miRNA based genetic modification seems most promising since miRNA regulates gene expression at transcriptional or post-transcriptional levels. There are several methods employed for miRNA manipulations including desired over expression/repression of stress-responsive miRNAs and/or their target mRNAs, miRNA-resistant target genes, target-mimics and artificial miRNAs (Zhou and Luo, 2013). Overexpression of *gma-miR394a* in *Arabidopsis* showed enhanced drought tolerance (Ni *et al.,* 2012). Transgenic *Arabidopsis* overexpressing *miR394* as well as *LCR* (*LEAF CURLING RESPONSIVENESS,* a target of *miR394*) *lcr* mutants exhibited enhanced cold stress tolerance, indicating the involvement of *miR394* and its target gene LCR in low-temperature responses in plants (Song *et al.,* 2016). Overexpression of *gma-miR172* in *Arabidopsis* revealed enhanced water deficit and salt tolerance (Li *et al.,* 2016). *MiR156* overexpressing rice plants showed reduced cold tolerance (Cui *et al.,* 2015). Overexpression of *osa-miR319a* in creeping bentgrass (*Agrostis stolonifera*) significantly improved the salt and drought tolerance of transgenic plants (Zhou *et al.,* 2013).Transgenic rice overexpressing *miR319* showed enhanced cold tolerance (Yang *et al.,* 2013).

2.3.11. miRNA based markers

In molecular breeding, DNA-based molecular markers have been explored and implemented in crop improvement programs. miRNA based molecular markers are functional markers that were exploited mainly in animal sciences, but were lesser reported in plants. The higher level of conservation of miRNA sequences provides an opportunity to develop novel molecular markers (Table 2.3.).

Table 2.3. Overexpression of stress responsive miRNA for conferring abiotic stress tolerance. (Shriram *et al.*, 2016)

miRNA	Source of the targeted miRNA gene	Target	Transgenic plant	Expression strategy	Response	Reference
miR156	*Oryza sativa*	SPL	*Oryza sativa*	Overexpression of OsmiR156k	Decreased cold tolerance	Cui *et al.*, 2015
miR172	*Glycine max*	AP2 like Tfs	*Arabidopsis*	Overexpression of gma-miR172c	Increased water deficit and salt tolerance	Li *et al.*, 2016
miR319	*Oryza sativa*	PCF5 and PCF8	*Oryza sativa*	RNAi	Increased cold tolerance	Yang C. *et al.*, 2013
miR319	*Oryza sativa*	TCP	*Agrostis stolonifera*	Constitutive overexpression of osa-miR319a	Increased drought and salt tolerance	Zhou *et al.*,2013
miR390	*Oryza sativa*	SRK	*Oryza sativa*	Overexpression of miR390	Decreased Cd tolerance/ enhanced Cd accumulation	Ding *et al.*, 2016
miR394a	*G. max*	F-box protein	*Arabidopsis*	Overexpression of gma miR394a	Increased drought tolerance	Ni *et al.*, 2012
miR394a	*Arabidopsis thaliana*	LCR	*Arabidopsis*	Overexpression of miR394a/ LCR loss of function mutant	Increased cold tolerance	Song *et al.*, 2016

miR395	*A. thaliana*	*BnSultr*, *BnAPS*	*Brassica napus*	Overexpression of miR395 driven by CaMV35S promoter	Shorten or no surface trichomes with delayed transition from juvenile to adult vegetative stage	Huang *et al.*, 2010
miR398	*A. thaliana*	CSD1,CSD2,CCS	*A. thaliana*	Loss function of CSD1 and CCs, knockdown mutant of CSD2	Increased thermo tolerance	Guan *et al.*, 2013
miR399	*A. thaliana*	IPS-1	*Solanum lycopersicum*	Overexpression of *Ath-miR399d* under control of *rd29A* promoter	Better growth performance under phosphrous deficiency and low temperature	Gao *et al.*, 2015
miR408	*A. thaliana*	Copper related gene	*Cicer arietinum*	Overexpression of Athpre-miR408	Enhanced drought tolerance	Hajyzadeh *et al.*, 2015

Application of miRNA as genetic markers was developed for genotyping of foxtail millet (*Setaria italica* L.) and related grass species (Yadav *et al.,* 2014). When pre-miRNA sequences of foxtail millet and other related crops were retrieved and aligned for the identification of conserved regions, 66 miRNA-based markers could be identified. In order to understand the genetic diversity of salt responsive-miRNA genes in rice, SSR markers were mined from 130 members of salt-responsive miRNA genes and validated in tolerant as well as susceptible rice genotypes (Mondal and Ganie, 2014). Although 12 miR-SSRs were found to be polymorphic, only miR172b-SSR was able to differentiate the tolerant and susceptible genotypes in 2 different groups. miRNAbased molecular markers displayed sufficient level of polymorphism in *Silybum marianum* genotypes (Ražná *et al.,* 2015).

2.3.12. miRNAs in *Hevea*

Zeng *et al.,*(2010) studied conservation and diverse expression patterns of 23 miRNA families during developmental and abiotic stress response in four Euphorbiaceous plants (*Ricinus communis, Manihot esculenta, Hevea brasiliensis and Jatropha curcas* L). However, this approach did not allow comprehensive identification of miRNA families in *Hevea.* Gebelin *et al.,* (2012) identified 48 conserved miRNA families and 10 putatively novel miRNA families by deep sequencing from plantlets subjected to abiotic stress. They also predicted miRNA targets and could identify targets involved in stress response, antioxidant activity and transcription regulation. High throughput sequencing was performed in high yielding (PB 260) and low yielding (PB 217) *Hevea* clones and could identify 115 miRNAs belonging to 56 families as well as could predict 20 novel miRNAs (Lertpanyasampatha *et al.,* 2012). They could predict miRNA targets computationally and identified genes involved in various biological processes including stress responses, and rubber biosynthesis. The regulation of microRNAs in response to different types of abiotic stress and hormone treatments in *Hevea* was reported by Gebelin *et al.,* (2013a). A negative co-regulation between *HbMIR398b* with its *chloroplastic HbCuZnSOD* target messenger was observed in response to salinity in *Hevea.* The expression of *MIR159b* gene was found enhanced in response to cold in leaves and bark, as well as in response to jasmonic acid treatment in leaves of juvenile plantlets. Gebelin *et al.,* (2013b) identified TPD associated miRNAs and their targets from latex cells and found 21nt size small RNAs as abundant class in TPD trees when compared with 24 nt in healthy trees. They reported that there is a decline in small RNAs in TPD-affected trees, due to both RNA degradation and a shift in miRNA biogenesis. They also could observe the enhanced expression of *Hbpre-MIR159b* gene upon TPD occurrence. However there are no reports available on the mechanism by which miRNAs are regulated to confer different levels of stress tolerance in various clones of *Hevea* during drought and cold stress.

Chapter 3

Identification and expression analysis of drought responsive microRNAs of *Hevea*

Abstract

Drought is probably one of the most significant environmental stress factors that restrict the expansion of rubber cultivation to non-traditional areas where the climatic conditions are characterized by long dry periods, high temperatures and low atmospheric humidity for almost half of the year. It is essential to identify or develop clones that can withstand such extreme weather conditions without compromising on yield and productivity. This study was initiated with an objective to identify drought responsive miRNAs from *H. brasiliensis* by conventional and next generation sequencing technology and find miRNAs that are associated with drought tolerance that can be used as markers for selecting drought tolerant clones. By conventional cloning and sequencing four conserved and one novel miRNAs were identified. Next generation sequencing using Illumina HiSeq method revealed the expression of 33 conserved miRNA families and 32 novel miRNAs in the drought treated and control samples altogether. Among the differentially expressed miRNAs identified, selected miRNAs were subjected to quantitative expression analysis. From the results, two novel miRNAs (HbmiRn_63 and HbmiRn_42) as well as miR168 and miR160 were found to have stronger association with drought tolerance. When expression of three selected miRNAs was validated in known tolerant and susceptible clones as well as germplasm accessions, the results matched with their tolerance/susceptibility nature thus strengthening the view that these miRNAs can be used as markers for drought tolerance in *Hevea brasiliensis*.

Key words: *Hevea brasiliensis*, drought tolerance, miRNAs, expression analysis

3.1 Introduction

Drought is one of the limiting factors that affect the plant growth and development. Drought stress has adverse effects on plant metabolic processes including stomatal conductance, nutrient uptake and photosynthetic assimilation and can cause serious damage to yield or complete loss of crops (Shinozaki *et al.*, 2003; Jaleel *et al.*, 2009). Drought tolerance is a quantitative trait, with a complex phenotype. Understanding mechanism of drought tolerance in plant is important for the improvement of crop productivity (Lawlor, 2013). Drought is probably one of the most significant environmental stress factors that restrict the expansion of cultivation of *Hevea brasiliensis* to newer areas in several rubber growing countries (Devakumar *et al.*, 1998). In *Hevea*, drought stress has been reported to severely affect its yield and general performance (Sethuraj *et al.*, 1984; Sreelatha *et al.*, 2007, 2011). In India, its cultivation is extended to non-traditional regions such as North Konkan region of Maharashtra, parts of Madhya Pradesh, Orissa, etc. which experience long dry periods, high temperatures and low atmospheric humidity for almost half of the year. The rainfall distribution in these areas is seasonal with almost no rainfall between November and May. The daily sunshine hour is longer wherein the mean daytime temperature ranges between 37°C and 42°C in summer with occasional days getting as hot as 45°C. The extreme temperature and low relative humidity prevailing in these regions increase the evaporative demand of the atmosphere (Mohanakrishna *et al.*, 1991) which creates atmospheric drought stress on the plant. In this context, various attempts are being made to develop clones by conventional plant breeding methods which are being tested in such regions for assessing their tolerance to abiotic stress conditions. As the selection for drought/stress tolerance is an extensive process it is highly imperative to identify early selection parameters to shorten the selection process. Marker assisted selection is widely employed to identify such varieties. Various studies are being conducted to identify best suitable genetic, physiological, biochemical markers for selecting clones with promising yield along with abiotic stress and disease tolerance.

Plants respond to drought by altering its gene expression (Golldack *et al.*, 2011) which involves a number of gene regulatory networks that require the coordination of multiple factors at several steps (Orphanides and Reinberg, 2002). The recent reports also indicate the involvement of small RNAs in gene regulation under stress conditions (Sunkar *et al.*, 2012; Macovei *et al.*, 2012; Zhang *et al.*, 2015). Expression of microRNAs has been found to be influenced by drought stress. These findings help shed light on drought responsive mechanisms in plants which can potentially be employed in developing of new stress tolerant crops (Kantar *et al.*, 2010; Chen *et al.*, 2012; Ding *et al.*, 2013). There are several reports on drought-responsive miRNAs in *Arabidopsis* (Sunkar and Zhu, 2004; Liu *et al.*, 2008; Li *et al.*, 2008), rice (Zhao *et al.*, 2007; Zhou *et al.*, 2010), maize (Xu *et al.*, 2010), barley (Kantar *et al.*, 2010), soybean (Kulcheski *et al.*, 2011), *Triticum dicoccoides* (Kantar *et al.*, 2011), *Populus trichocarpa* (Lu *et al.*, 2008), *Medicago truncatula* (Trindade *et al.*, 2010), *Phaseolus vulgaris* (Arenas-Huertero *et al.*, 2009), sorghum (Pasini, *et al.*, 2014), potato (Zhang, *et al.*,2014), barley (Hackenberg *et al.*, 2015), tomato (Candar-Cakir, *et al.*, 2015), etc.

miRNAs were previously identified by using conventional method of direct cloning (Park *et al.*, 2002; Reinhart *et al.*, 2002) which is a sequence-independent approach which does not require initial knowledge of miRNA sequence. In addition, this method gives better accuracy with lesser false positives. Several studies led to the establishment of different protocols for small RNA isolation and adaptor mediated synthesis of a cDNA library followed by their amplification and then cloning. The clones are screened and further sequenced to identify the potential miRNAs (Llave *et al.*, 2002a; Reinhart *et al.*, 2002; Sunkar and Zhu, 2004). But it is a time-consuming, low throughput, laborious, and expensive approach. Although direct cloning has enabled the identification of many miRNAs, it is still difficult to clone low abundance miRNAs. Currently, deep sequencing approach has become the most commonly used method for plant miRNA identification which is being extensively used in a wide variety of plant species. This deep sequencing technology can generate millions of sequences per run that can be used for the genome-wide identification of all potential miRNAs. In addition to that deep sequencing approach can also be employed to understand the expression levels of each miRNA based on its read number. High-throughput sequencing is also employed to identify non-conserved miRNAs in several species. It has also opened avenues to identify and quantify miRNAs that are responsive to specific stress (Zhang *et al.*, 2015). This approach is also useful for identification of miRNAs in plants that do not have genome sequence data.

Recent reports have established the role of miRNAs in regulating genes associated with various metabolic as well as abiotic stress responsive pathways in *Hevea* (Lertpanyasampatha *et al.*, 2012; 2013; Gebelin *et al.*, 2012; 2013a; 2013b). But there are no reports on the mechanism by which miRNAs are regulated to confer different levels of stress tolerance in *Hevea* clones during drought stress. This study was initiated with an objective to identify novel as well as conserved miRNAs specifically expressed under drought stress in *Hevea brasiliensis* using conventional and high throughput sequencing method and also to determine their expression levels in different *Hevea* clones with varying levels of drought tolerance in order to identify miRNAs associated with drought tolerance which could be employed in crop improvement programmes.

3.2 Materials and Methods

3.2.1 Plant material and stress induction

In order to isolate and clone drought stress specific miRNAs, six months old polybag grown plants of *Hevea brasiliensis* (clone RRIM 600) were exposed to drought stress in the open field of Rubber Research Institute of India (RRII), Kottayam, Kerala during summer season by withholding irrigation. The plants were generated by budding of seedlings raised from *Hevea* seeds with clonal buds collected from *Hevea* bud wood nursery maintained at RRII. One set of plants was subjected to water stress by withholding irrigation for 10 days and the other set was watered on alternate days to maintain field capacity. Leaf samples were harvested and immersed immediately in liquid N_2 after assessing the drought status of the plant by measuring the net CO_2 assimilation rate (A) and stomatal conductance (g_s) using portable photosynthesis system (LI-6400), LI-COR, U.S.A. Leaf samples were later stored in -80 °C freezer.

3.2.2 Cloning and sequencing of small RNAs by conventional method

3.2.2.1. miRNA isolation

Frozen leaf samples were ground to fine powder followed by isolation of miRNA mirVana miRNA isolation kit (Ambion, USA). The samples were first lysed in ten volumes of denaturing lysis solution which stabilizes RNA and inactivates RNases. One volume of miRNA homogenate was mixed with tissue lysate followed by 10 min incubation on ice. This was followed by mixing with 10 volume of acid–phenol: chloroform gently. The samples were then centrifuged for 5 min at 10000×g at room temperature to separate the aqueous and organic phases. The aqueous phase was mixed with 1/3 volume of absolute ethanol and further transferred on to the filter cartridge and centrifuged at 10000×g for 15s. The filtrate collected was added with 2/3 volume of absolute ethanol and further passed through a second glass filter where the small RNAs would get immobilized. The filter containing the small RNAs was transferred to a new tube after washing and the small RNAs were subsequently eluted in 100 μl of pre-heated nuclease-free water. About 2 μg of small RNAs (measured spectrophotometrically by Nano drop, USA) were resolved on a 12% denaturing (7M urea) polyacrylamide gel. miRNA marker (New England BioLabs, USA) was loaded as size control for the identification of RNAs in 17-25 nucleotide (nt) size range. The Small RNAs were visualized on a UV transilluminaor after staining the gel with Sybr Gold nucleic acid stain. Subsequently RNA fragment(s) of 20-22 nt size were excised from the gel and purified using DTR columns.

3.2.2.2. Reverse Transcription and PCR Amplification

The purified small RNAs that were recovered from the gel were further ligated with a 3' and a 5' linker in two separate reactions. Initially, 3' linker was ligated with the enriched miRNAs. In order to avoid circularization of the RNA fragments, 3' linkers (provided in the IDT cloning kit) were ligated to the small RNAs using T4 RNA ligase in the absence of ATP. The 3' linkered species were resolved on a 12% denaturing (7M urea) polyacrylamide gel. This was followed by ligating the 5' MRS linkers to the 3' linkered small RNAs in the presence of 1.0 mM ATP. Subsequently the 5' and 3' ligated miRNAs were converted to cDNA using SuperScript III Reverse Transcriptase (Invitrogen) and RT/REV primer (IDT miRNA cloning kit). This was followed by PCR amplification of cDNA using linker specific primers, purification of amplicons, cloning and sequencing.

3.2.2.3. Cloning and Sequencing

Purified PCR products were ligated in to pTZ57R/T (Fig.3.1) cloning vector (PCR cloning kit, Fermentas) which was later used to transform JM109 cells by using TransformAid Bacterial Transformation Kit (Fermentas).

The inserts from individual colonies were PCR amplified in order to select the transformants containing the plasmid DNA with the small RNA inserts by adaptor specific forward and reverse primers and subsequently the colonies containing the inserts were selectively sequenced. After trimming the adaptor sequences, small RNA sequences in the range of 18-30 nt length were selected and their sequences were searched against Rfam family database to identify non-coding RNAs, followed by BLAST analysis against miRBase database v20.0. Small RNAs that did not show similarity to any of the known miRNAs in miRBase were analysed by using m-Fold web server with default parameters. The secondary structure of those miRNA precursor's that having a free energy equal or less than -25 kcal per mol were treated as novel miRNAs.

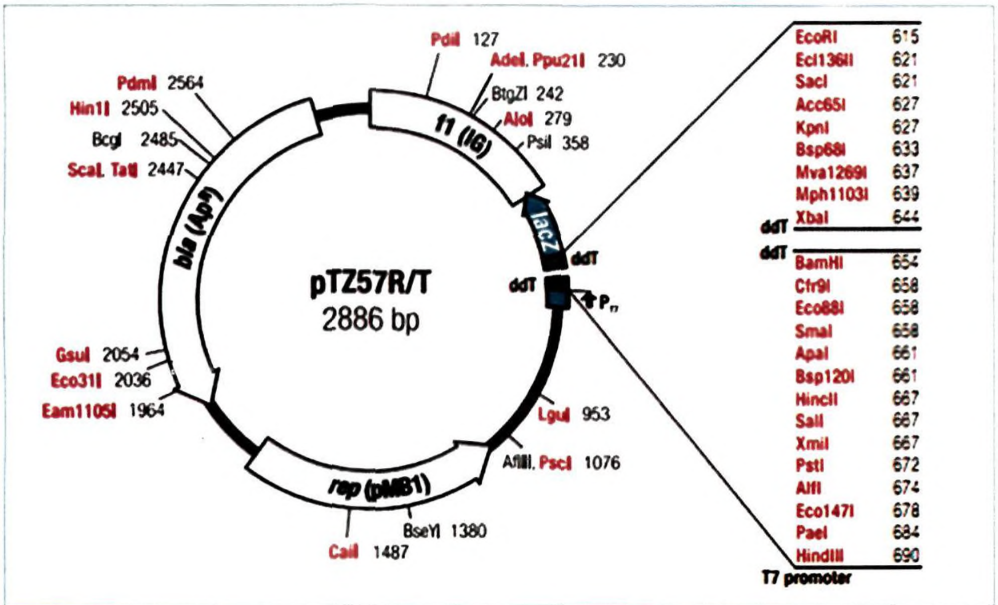


Fig. 3.1. pTZ57R/T cloning vector

3.2.3 Cloning and sequencing of small RNAs by Next Generation Sequencing (NGS)

3.2.3.1. Total RNA isolation

Total RNAs were extracted using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) according to the manufacturer's instructions. Leaf samples (~100 mg) ground to fine powder in liquid nitrogen were mixed with 500 μl of lysis solution: 2 mercaptoethanol mixture and vortexed vigorously for 30 sec. The samples were then incubated at 56°C for 5 min and centrifuged. In order to recover more small RNA, the supernatant was mixed thoroughly after adding 750μl of binding solution. The mixture was later transferred to binding column followed by centrifugation at 12000 rpm for 1min to enable binding of RNA. The RNAs bound to the column were then washed and transferred to a new tube for further elution in 70 μl of nuclease-free water. The total RNAs thus eluted were quantified spectrophotometrically using Nanodrop-1000 and the quality was confirmed by resolving on 1% denatured agarose gel.

3.2.3.2. Small RNA library construction and sequencing

The pair-end cDNA sequencing libraries for small RNA were prepared for control and drought stressed samples using Illumina® TruSeq Small RNA Sample Preparation Kit as per manufacturer's instructions. The library construction involves ligation of 3' adapter with 1 μg total RNA followed by 5' adapter ligation. These adapter ligated mix were reverse transcribed and were PCR amplified. After purification, they were subjected to deep sequencing using Illumina HiSeq 2000 (Xceleris Genomics, Ahemedabad, India).

3.2.3.3. Identification of conserved and novel miRNAs of *Hevea* by NGS

To identify the conserved miRNAs, the data of small RNAs were mapped to the mature plant miRNAs registered in the miRBase (Release 20) database using CLC Workbench (version 6) software allowing two maximum mismatches in the annotation. In order to identify novel miRNAs, draft genome of *Hevea brasiliensis* was used as reference (accession no: AJJZ01, total number of contigs, 1,223,365). Due to the limited size of its draft genome, draft genome sequences of *Ricinus communis* and *Manihot esculenta* were also used as references (ftp://ftp.jgi-psf.org/pub/compgen/ phytozome/ v9.0). The secondary structures for precursor molecules of potential candidate novel miRNAs were predicted by using m-Fold web server. All parameters were set to default values. The miRNAs precursor's with a minimal folding free energy (MFE) equal or less than -25 kcal per mol for its secondary structure were considered as novel miRNAs. Lower the MFE value, higher the thermodynamically stable secondary structure of the miRNAs.

3.2.3.4. Target prediction for miRNAs

Target prediction for known and novel miRNAs were performed using web based psRNA Target program with default parameters and TAPIR. Following parameters were used for psRNA Target program *viz* (1) a maximum expectation value of 3.0 (2) a complementarity scoring length of (hsp size) 20; (3) a target accessibility of 25 or less; and (4) no mismatch at positions 9-11.

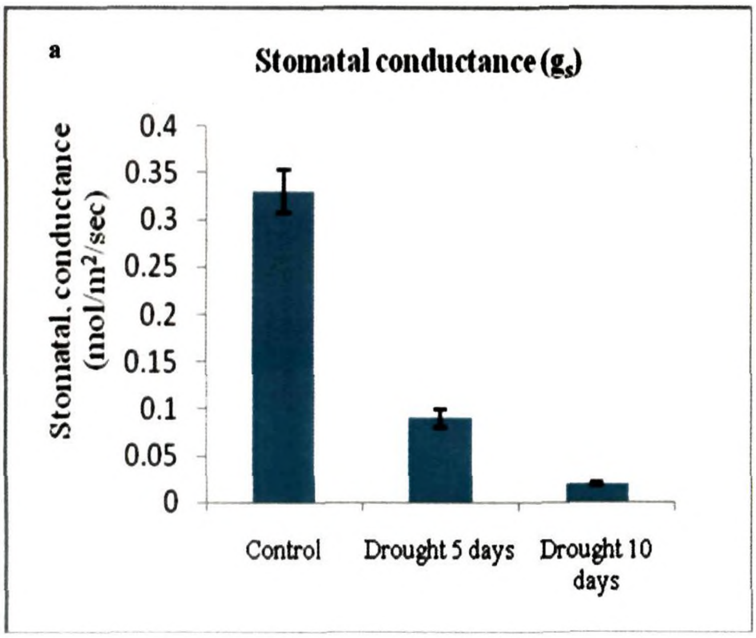
For target prediction using TAPIR (http://bioinformatics.psb. ugent.be/ webtools/tapir/) (Bonnet *et al.,* 2010), score and the free energy ratio were considered for each search. Mismatches and gaps were given a score of 1, while G: U pairs were given a score of 0.5. Mismatches, gaps and G: U pair scores were doubled within the seed region. The default value for the score cutoff was 4.0 and the default value for the free energy ratio was 0.7.

3.2.3.5. Validation of miRNAs and their potential target genes by qPCR

The clones viz. RRIM 600, RRII 430, RRII 208 (drought tolerant), RRII 105, RRII 414 (drought susceptible) and germplasm accessions, RO 3261, AC 612 (drought tolerant) RO 3242 and MT 1619 (drought susceptible) were used for validation. The imposition of drought stress and leaf sample collection was performed as described above.Total RNA (2μg) from each sample were reverse transcribed using Mir-X miRNA first strand c-DNA synthesis kit (Clontech). Small RNAs were polyadenylated and reverse transcribed using poly(A) polymerase and SMART MMLV Reverse Transcriptase. Expression of 16 conserved miRNAs and four novel miRNAs in control and drought imposed plants was validated by qPCR on Light Cycler 480 II (Roche) using SYBR Advantage qPCR Premix (Takara). The reaction consisted of 0.5 μl from 10 times diluted cDNA, 0.1μM of each forward and reverse primers and 5μl of 2x SYBR Advantage qPCR Premix in a 10 μl reaction volume. The reaction conditions included an initial denaturation step of 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Changes in the levels of expression were calculated as normalized fold ratios using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

3.3 Results

In order to identify the drought responsive miRNAs of *Hevea* and to study their role in drought alleviation, polybag plants of clone RRIM 600 grown in open field conditions were subjected to drought stress for 10 days. The impact of stress on the plants was confirmed by measuring the gas exchange parameters. The stomatal conductance of stressed plants got reduced to near zero after 10 days of drought stress compared to irrigated control (0.33 mol m^{-2} s^{-1}) plants (Fig. 3.2.a). Drought stressed plants exhibited significant reduction in net CO_2 assimilation rate (2.7 µmol m^{-2} s^{-1}) than the irrigated control (11.5 µmol m^{-2} s^{-1}) plants (Fig. 3.2.b).



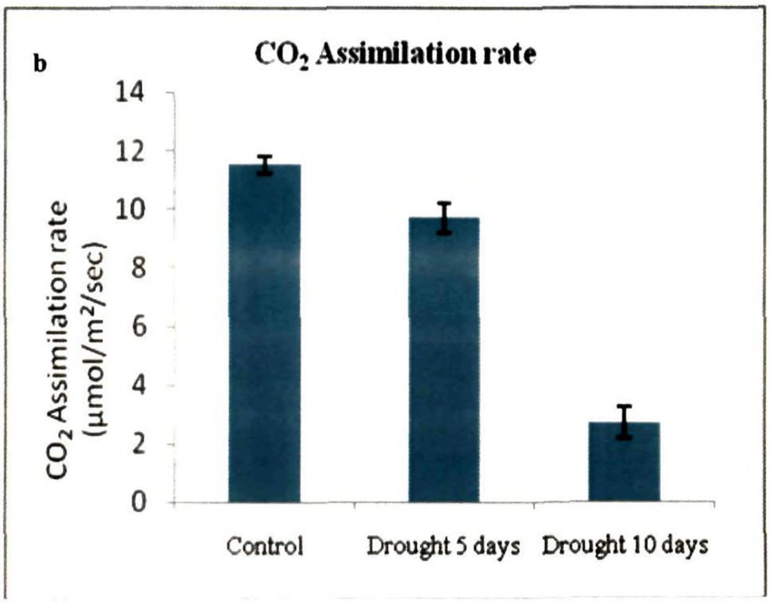


Fig. 3.2. (a & b) a. Stomatal conductance (gs); b. CO_2 assimilation rate (A), of irrigated and drought imposed plants of *Hevea* clone RRIM 600.

3.3.1. Identification of small RNA population by conventional method

The polyacrylamide gel electrophoresis (PAGE) profile of small RNAs from clone RRIM 600 is shown in (Fig. 3.3.a). Purified small RNAs were ligated with 3' linker (Fig.3.3.b) and 5' linker in two independent reactions. Further, small RNAs were reverse transcribed and PCR amplified (Fig. 3.3.c).

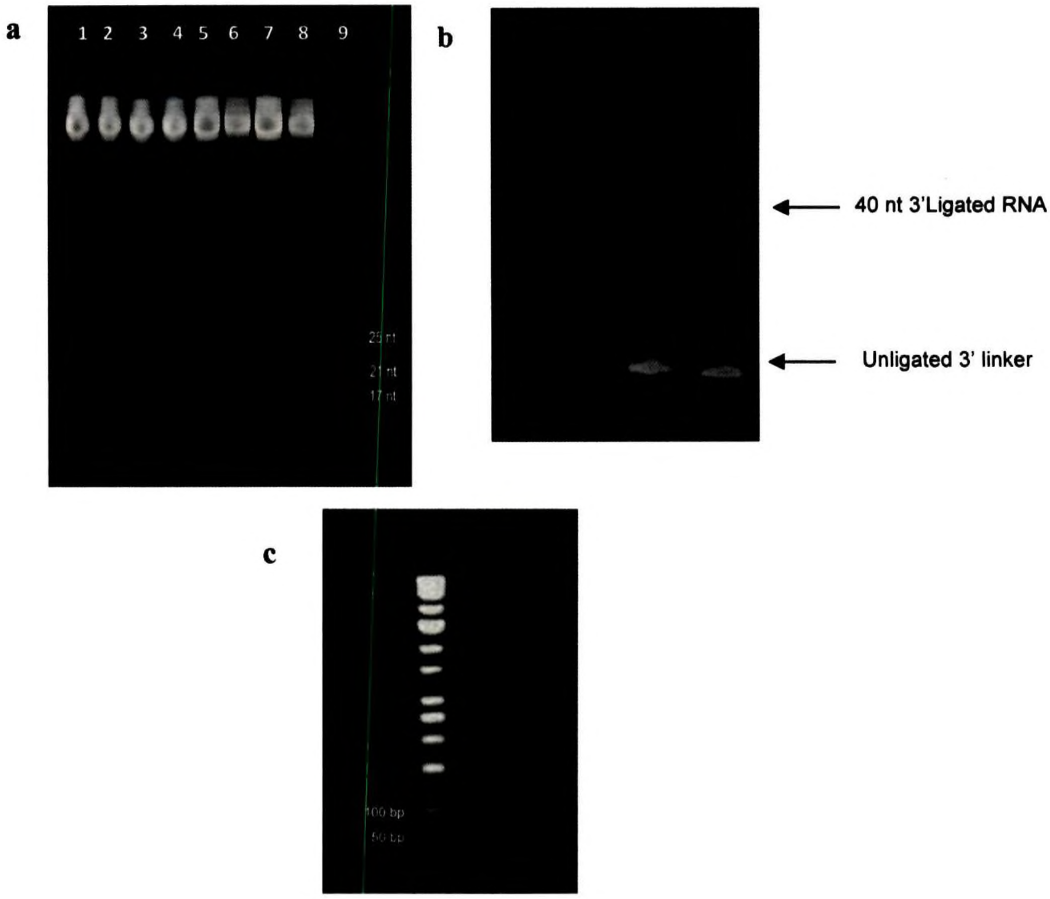


Fig. 3.3. (a-c) Gel images of each stage of miRNA isolation from leaf samples of drought stressed *Hevea* clone RRIM 600. a, PAGE profile of small RNAs (Lanes 1-8, miRNA from 8 samples; Lane 9, miRNA marker); (b), PAGE profile of 3' linkered small RNA species. c. PCR amplified products of 3' and 5' linker adapted small RNAs (Lane 1-DNA marker; Lane 2 &3, linker attached small RNAs).

Transformed colonies were identified by colony PCR (Fig. 3.4.). About 120 clones were sequenced. Four families of conserved miRNAs (miR2911, miR166, miR167 and miR482) were identified after excluding redundancy. Four sequences were found similar to miR2911 and the miR166 was found repeated thrice. Only one sequence was obtained for miR167 while miR482 occurred twice. Among the rest of the sequences, one was confirmed as novel miRNA. The secondary structure of its precursor molecules was predicted using m-Fold tool with default parameters and its free energy was found to be -30.7. The sequence of one novel miRNA (HbmiRn_42) identified is given in Table 3.1. and its stem loop structure is given in Fig. 3.5.

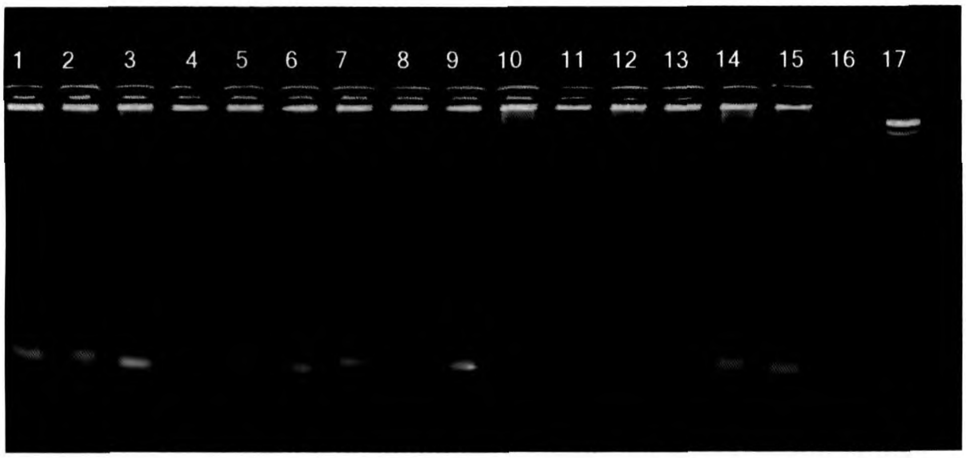


Fig. 3.4. Colony PCR of transformed colonies (Lane1-15, colonies with small RNA insert; Lane 16, NTC; Lane 17, DNA marker)

Table 3.1. The sequence and putative target of miRNAs identified by conventional method

miRNA	Sequence 5'-3'	Putative target
miR166a	UCGGACCAGGCUUCAUUCCCC	HD-ZIP III protein
miR166b	CGGACCAGGCUUCAUUCCCC	HD-ZIP III protein
miR167	CAGAUCAUGCUGGCAGCUUC	Auxin response factors
miR2911	UCCCAGUCCGUCCCCCGGCC	unknown
miR482	GGAAUGGGCGGUGUGGGUAAGA	LRR Protein
HbmiRn_42	CCAGGCGTCGGCCAGCGGGCTC	HMGR3

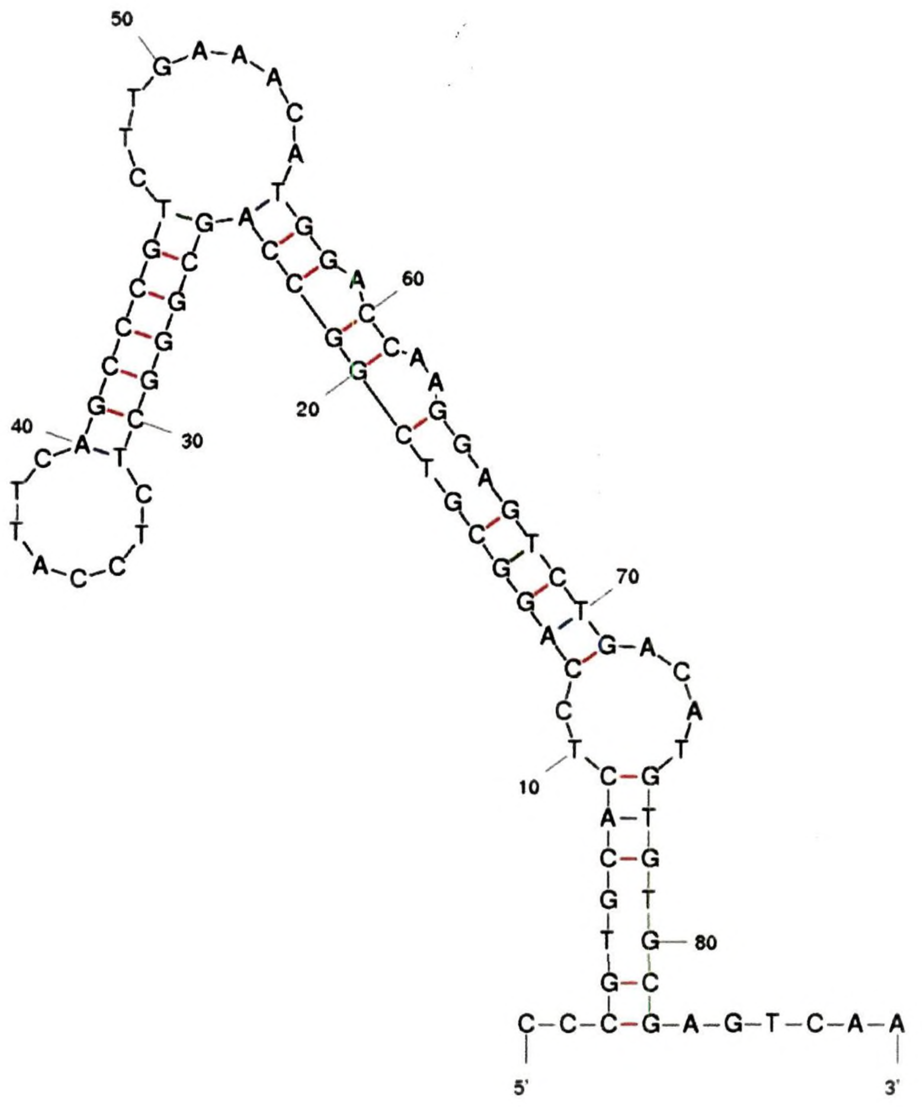


Fig. 3.5. Stem loop structure of novel miRNA (HbmiRn_42).

3.3.2 Identification of small RNA population by NGS

3.3.2.1. Analysis of small RNA population by NGS

To identify drought responsive miRNAs of *Hevea* two small RNA libraries from control and drought stressed leaves were constructed and sequenced independently. Small RNA sequencing results yielded a total of 12,176,240 reads for control and 18,499,616 reads for drought stressed samples. The raw sequences were processed and filtered by applying several criteria to identify conserved and novel miRNAs. After removing adaptor sequences, sequences smaller than 20 nt and larger than 24 nt were discarded. From a total of 324,448 reads in control 52,420 reads were found unique. In drought stressed sample, among the 353,428 reads 53,280 reads were found unique. These unique reads were considered as small RNAs and were used in further analysis. The size distribution pattern was found similar in both the small RNA sequence libraries in which the size class of 24 nt long sequence was found most abundant, followed by 21 nt (Fig.3.6.).

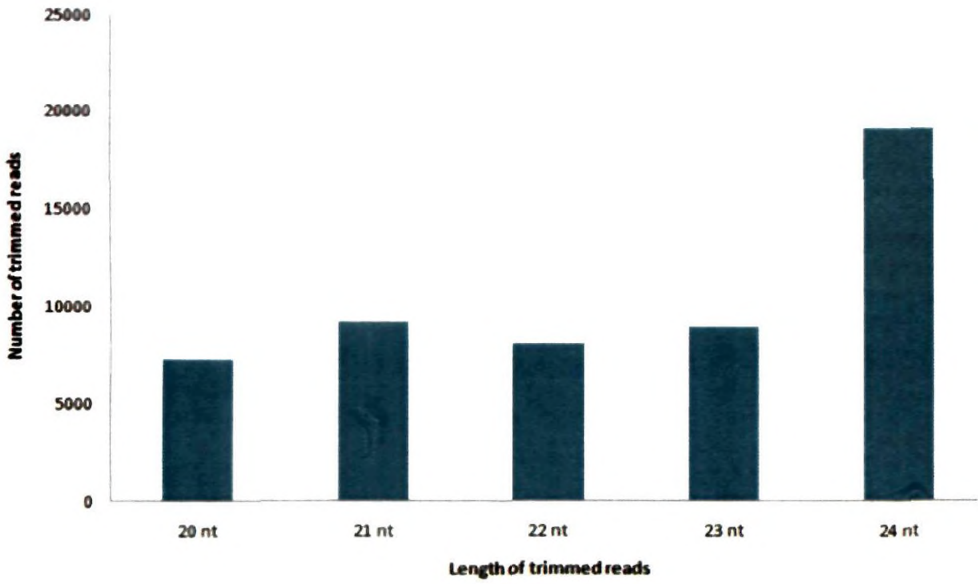


Fig. 3.6. Length of small RNA sequences from drought imposed plants of *Hevea*

3.3.2.2. Identification of conserved and novel miRNAs of *Hevea* by NGS

Sixty four miRNAs belonging to 29 known miRNA families were identified from irrigated control samples and 63 miRNAs belonging to 32 known miRNA families were identified from drought imposed samples (Table 3.2.).

Table 3.2. miRNAs identified from leaves of *Hevea brasiliensis* and their putative targets

Sl. No.	miRNA	Sequence (5' - 3')	Target
1	MIR166	TCGGACCAGGCTTCATTCCCCC	Hypothetical protein
2	MIR482	AGATGGGTGGCTGGGCAAGAAG	Abscisic acid responsive element
3	MIR167	TGAAGCTGCCAGCATGATCTGA	Transmembrane protein,
4	MIR396	TTCCACAGCTTTCTTGAACTG	Regulatory-associated protein of mTOR,
5	MIR156	TGACAGAAGATAGAGAGCAC	nacl-inducible calcium binding,
6	MIR535	TGACAACGAGAGAGAGCACGT	leucine carboxyl methyltransferase, putative
7	MIR397	ATTGAGTGCAGCGTTGATGAA	laccase, putative
8	MIR393	TCCAAAGGGATCGCATTGATCT	hypothetical protein
9	MIR390	AAGCTCAGGAGGGATAGCGCC	zinc finger protein
10	MIR2916	TGGGGACTCGAAGACGATCATAT	kinesin, putative
11	MIR858	TTCGTTGTCTGTTCGACCTGA	Myb domain protein 13
12	MIR4995	AGGCAGTGGCTTGGTTAAGGG	guanosine-3',5'- bis (diphosphate) 3'-pyrophosphohydrolase
13	MIR1310	AGGCATCGGGGGCGCAACGCCC	ribulose-5-phosphate-3- epimerase
14	MIR7767	CCCCAAGCTGAGAGCTCTCCC	Cell wall-associated hydrolase
15	MIR6445	TTCATTCCTCTTCCTAAAATGG	hypothetical protein
16	MIR6478	CCGACCTTAGCTCAGTTGGTG	hypothetical protein
17	MIR157	TTGACAGAAGATAGAGAGCAC	Myosin-9, putative
18	MIR159	TTTGGATTGAAGGGAGCTCTG	MYB transcription factor
19	MIR169	GAGCCAAGAATGACTTGCCGA	Nuclear transcription factor Y subunit A-1
20	MIR399	TGCCAAAGGAGAGTTGCCCTG	2-oxoglutarate/malate translocator, chloroplast precursor, putative
21	MIR894	CGTTTCACGTCGGGTTCACC	40S ribosomal protein S26, putative
22	MIR171	TTGAGCCGCGTCAATATCTCC	SCL protein
23	MIR395	CTGAAGTGTTTGGGGGAACTC	Homeobox protein LUMINIDEPENDENS,
24	MIR1425	TAGGATTCAATCCTTGCTGCT	leucine carboxyl methyltransferase, putative
25	MIR1432	ATCAGGAGAGATGACACCGAC	aminobutyrate aminotransferase
26	MIR164	TGGAGAAGCAGGGCACGTGCA	dtdp-glucose 4-6-dehydratase, putative
27	MIR168	TCGCTTGGTGCAGATCGGGAC	predicted protein [Populustrichocarpa]
28	MIR3627	TCGCAGGAGAGATGGCACTGTC	conserved hypothetical protein
29	MIR444	TGCAGTTGTTGTCTCAAGCTT	Beclin-1, putative
30	MIR528	TGGAAGGGGCATGCAGAGGAG	Conserved hypothetical protein
31	MIR6476	TCAGTGGAGATGAAACATGA	Photosystem I reaction centre subunit IV A chloroplast precursor
32	MIR2118	GAAATGGGTGGATGGGAGTGA	Rhicadhesin receptor precursor putative
33	MIR160	TGCCTGGCTCCCTGTATGCCA	Auxin response factor

Of the 33 conserved miRNAs, miR166 was found abundant in both the samples followed by miR482. A significant difference in the number of members of each conserved miRNA family was detected among which miR393, the largest family among the miRNAs obtained was found to have eight members while the miR156, and the second largest family had 7 members. Of the remaining families, 15 miRNAs were represented by a single member while others comprised between 2 and 6 members. Seventeen and 25 novel miRNAs were identified in control and drought samples respectively (Table 3.3.). The secondary structures of novel miRNAs are represented in Appendix 1.

Table 3.3. Novel miRNAs in both control and drought samples by NGS method

Species	No. of novel miRNAs in control	No. of novel miRNAs in drought
Hevea brasiliensis	7	**13**
Ricinus communis	2	4
Manihot esculenta	8	8
Total	**17**	**25**

3.3.2.3. Differential expression analyses of miRNAs

When a total of 33 conserved miRNA families were used for differential expression analysis by DESeq package, 29 and 32 miRNA families were identified in control and drought treated samples respectively. 28 miRNA families were found commonly present in both the samples. microRNA family, miR166 was found abundant in both the samples. miR1432 was found only in irrigated samples while miR160, miR2118, miR528 and miR6476 were found only in drought stressed samples. While five conserved miRNAs (miR164, miR168, miR3627, miR395, miR6478) got significantly up-regulated, 5 others (miR1310, miR156, miR169, miR393, miR858) got significantly down-regulated in drought stressed samples (Fig. 3.7.).

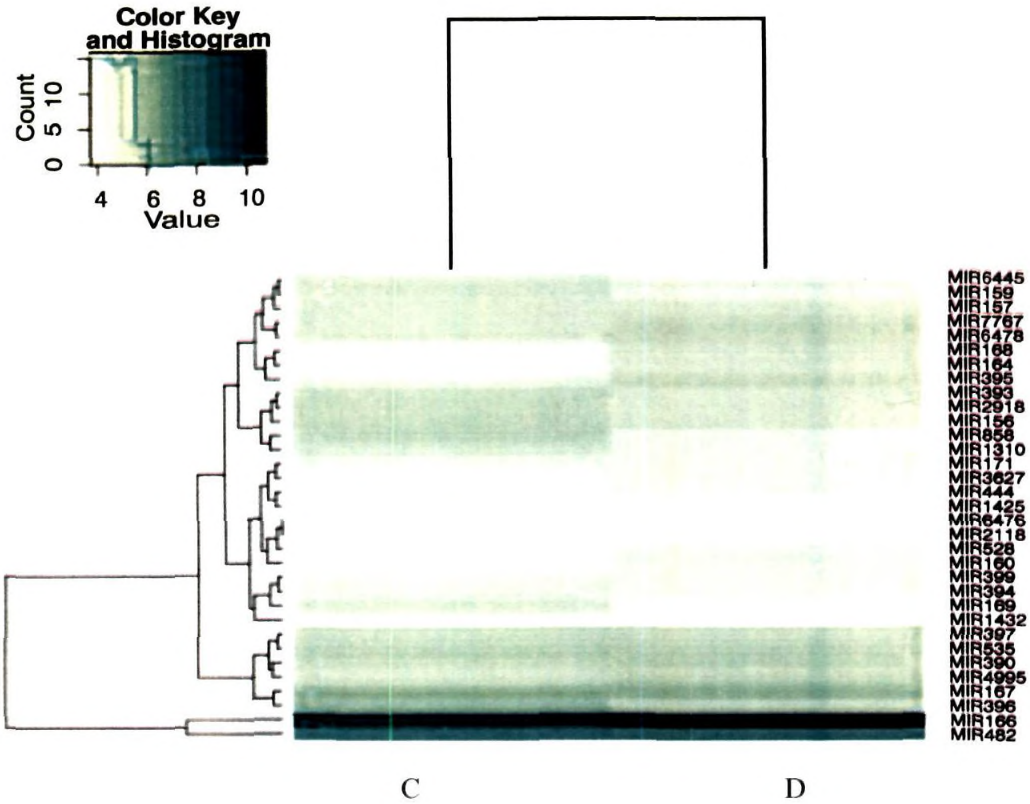


Fig. 3.7. Heatmap of conserved miRNAs from control (C) and drought stressed (D) samples

Apart from these, 17 and 25 novel miRNAs were identified in the control and the drought treated samples respectively. A total of 10 novel miRNAs were found common to both the samples. When digital gene expression analysis was carried out for these 10 novel miRNAs, three miRNAs (HbmiRn_26, HbmiRn_42 and HbmiRn_48) were found down-regulated and (HbmiRn_20) got significantly up-regulated (Fig 3.8.).

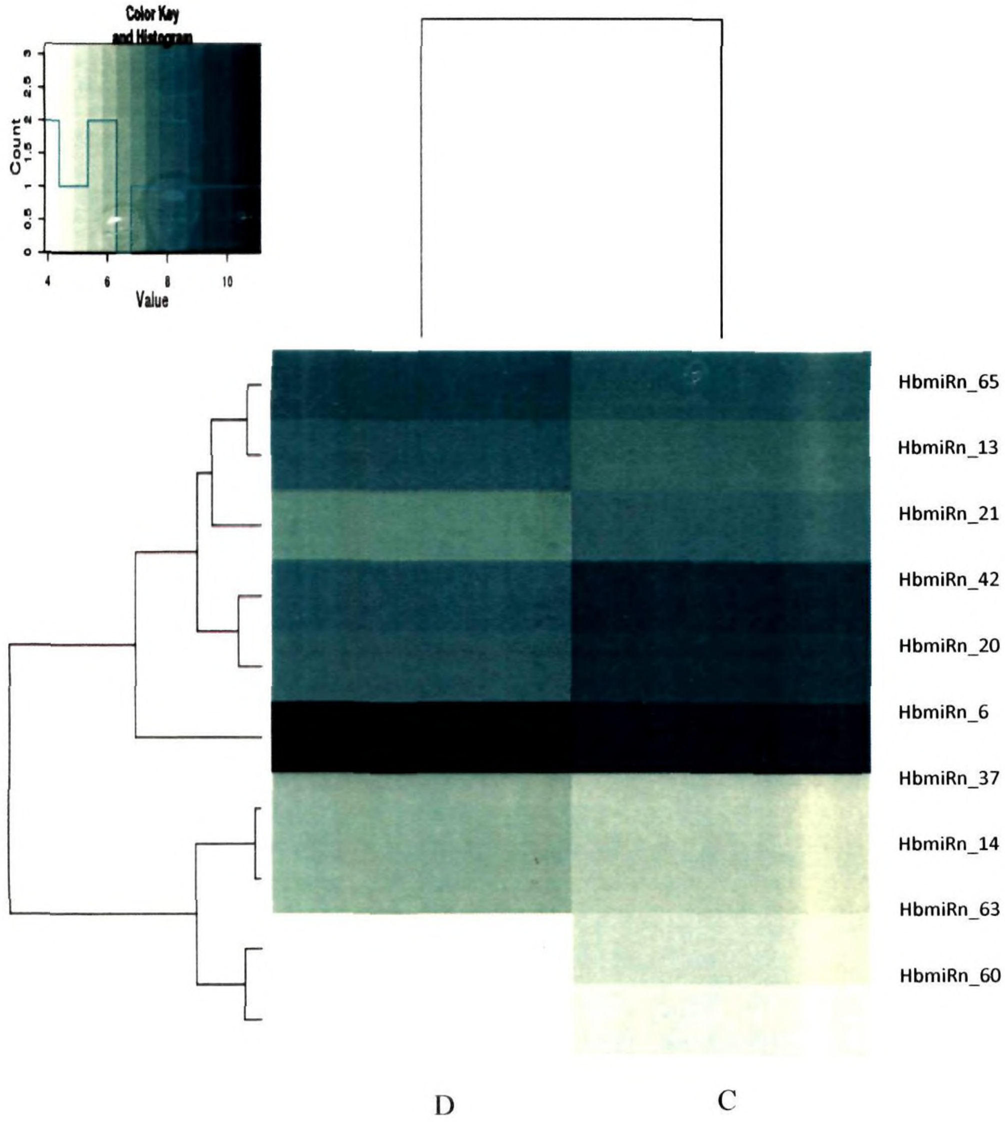


Fig.3. 8. Heat map of novel miRNAs from drought stressed and control sample

3.3.2.4. Target prediction for Conserved and novel miRNAs of *H. brasiliensis*

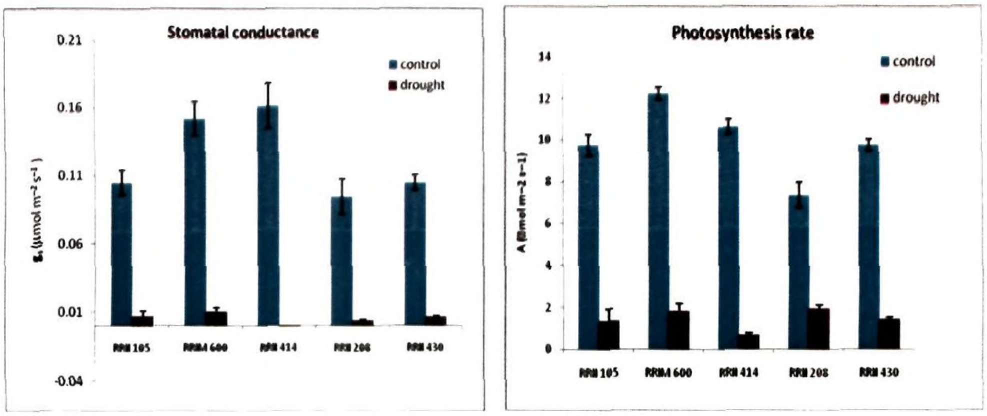
In order to understand the functional role of the identified miRNAs their targets also have to be predicted primarly. psRNA Target program, an open source web server was used with its default parameters to predic conserved and novel miRNAs (Dai and Zhao,2011). All 33 conserved miRNA families were searched for targets against ESTs or gene sequences of *Ricinus communis*, *Hevea brasiliensis* and *Manihot esculenta*. There were 27 known miRNA families out of 33 found to have targets in *Hevea brasiliensis*, 28 known miRNA families had targets in *Ricinus communis* and 27 known miRNA families had targets in *Manihot esculenta* (Appendix 1, Table 1.3.). These target sequences were further annotated against non-redundant (nr) protein database for functional identification using the program blastx. Several regulatory proteins such as auxin response factor (ARF), nuclear transcription factor Y subunit A-1(NFYA -1), MYB transcription factor, zinc finger protein, Homeobox protein LUMINIDEPEDENS and regulatory associated protein of mTOR were found as targets of miR160, miR169, miR858, miR390, miR395 and miR396 respectively. Beside this, ribulose-5-phosphate-3-epimerase, azetidine-2-carboxylic acid resistant 1 family protein, Myosin-9, dtdp-glucose 4-6 dehydratase, transmembrane protein, electron transporter, kinesin, hypothetical protein POPTR, Beclin 1, ascorbate peroxidase, protein binding protein, chloroplast precursor protein and cell wall associated hydrolase were found targets of miR1310, miR156, miR157, miR164, miR167, miR2118, miR2916, miR399, miR444, miR4995, miR535, miR6476 and miR7767. Apart from these, there were about five conserved hypothetical proteins found as targets of miR3627, miR393, miR528, miR6478, miR6445.

From control samples, four miRNA-target pairs were obtained for seven novel miRNAs of *H. brasiliensis* and two miRNA-target pairs were obtained for two novel miRNAs of *Ricinus*, while six miRNA-target pairs were obtained for eight novel miRNAs of *Manihot* (Appendix 1, Table 1.4.). Among the four novel miRNAs, HbmiRn_31 and HbmiRn_32 were found to target ubiquitin and WLM domain-containing protein and HbmiRn_48 and HbmiRn_49 were found to target putative DNA binding protein.

From drought stressed samples, five miRNA-target pairs were obtained for 13 novel miRNAs using *H. brasiliensis* database and three miRNA-target pairs were obtained for four novel miRNAs using *Ricinus* database, while five miRNA-target pairs were obtained for eight novel miRNAs using *Manihot* database(Appendix 1, Table 1.4). Among the five novel miRNAs obtained from drought samples, HbmiRn_10, HbmiRn_37 and HbmiRn_65 were found to target ARM repeat superfamily protein, ubiquitin and WLM domain containing protein and Tar1p respectively while both the HbmiRn_60 and HbmiRn_63 were found to target Tubulin beta-7 chain.

3.3.2.5. Validation of miRNAs and their potential target genes by qPCR

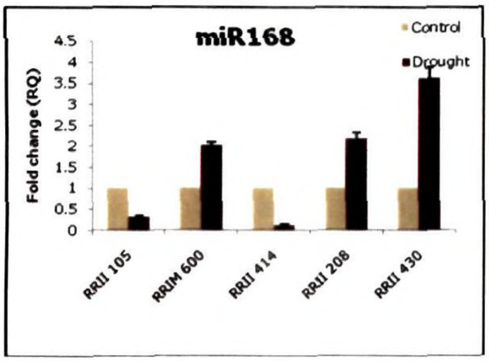
Quantitative real time PCR (qPCR) was performed to determine the expression levels of conserved and *Hevea*-specific miRNAs in five clones of *Hevea* with varying levels of drought tolerance and to validate the results obtained through deep sequencing data of *Hevea* miRNAs. Reduction in stomatal conductance was noticed in all the clones under drought stress, while it was maximum in RRII 414 (Fig.3.9.a). Similarly, reduction in photosynthetic assimilation rate was found in all the clones that were under drought stress and it was maximum in clone RRII 414 (Fig.3. 9. b).

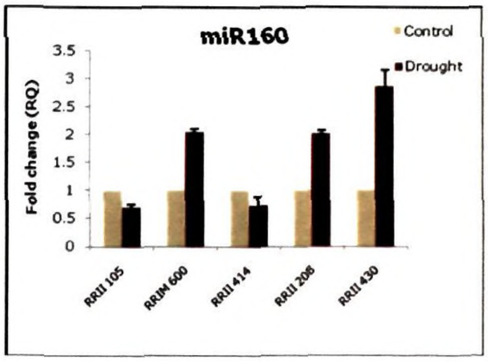


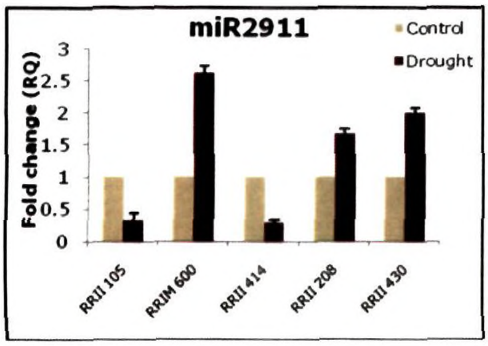
a b

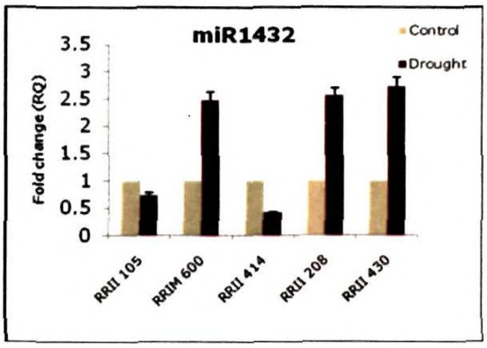
Fig. 3.9. (a&b). Stomatal conductance and CO2 assimilation rate in irrigated and drought imposed polybag plants of *Hevea*.

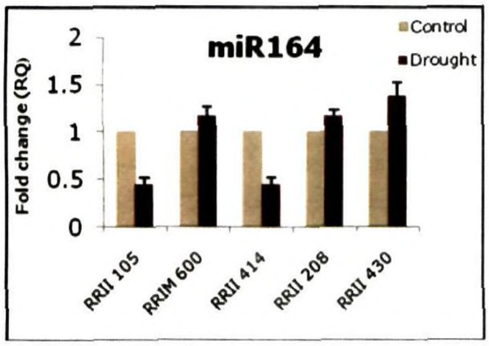
In this study, 16 conserved and four novel miRNAs were selected (Table 3.4). All the miRNAs showed differential expression under drought stress condition though their level of expression varied among the clones studied (Fig.3.10.). Among them, miR168, miR160 and miR1432 got up regulated in relatively tolerant clones (RRIM 600, RRII 208 and RRII 430) and got down regulated in relatively susceptible clones (RRII 105 and RRII 414). Expression of miR6478 got significantly repressed in susceptible clones RRII 414 and RRII 105 while there was no significant change in tolerant clones. The expression level of miR858a was significantly lower in RRII 105, RRIM 600 and RRII 414 while there was no significant change in RRII 208 and RRII 430. Expression of miR858b got reduced in all the five clones studied. miR482 got down regulated in clones RRIM 600 and RRII 208 whereas there was no significant change in RRII 430, RRII 105 and RRII 414. miR164 and miR167 were found down regulated in RRII 105 and RRII 414 while there was no much change relatively tolerant clones. The expression of miR169 and miR6476 got reduced in clones RRII 105, RRIM 600 and RRII 414, but it got up-regulated in RRII 208 and RRII 430 which are relatively tolerant clones. Expression of miR3627 was found significantly down regulated in susceptible clones and higher in tolerant clones except in the case of RRII 208. In the case of miR398, down regulation was much evident in RRII 208 whereas no such trend was seen in other tolerant clones. miR395 showed significant down regulation in the susceptible clone RRII 414 and significant up regulation in RRII 430. miR166 did not show any significant change in its expression level among the clones studied. Expression of novel miRNAs HbmiRn_42 and HbmiRn_63 was up-regulated in tolerant clones, while they were found down regulated in drought susceptible clones. Expression of HbmiRn_48 got down regulated in all the clones except in RRII 430 in which it got up- regulated. In contrast, HbmiRn_11 got up-regulated in all the clones except RRII 414 in which it was found significantly down regulated.

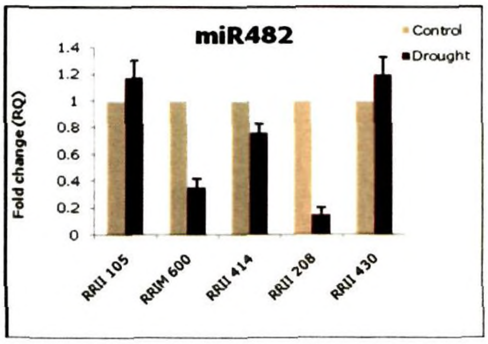

miR168
Control
Drought
Fold change (RQ)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430

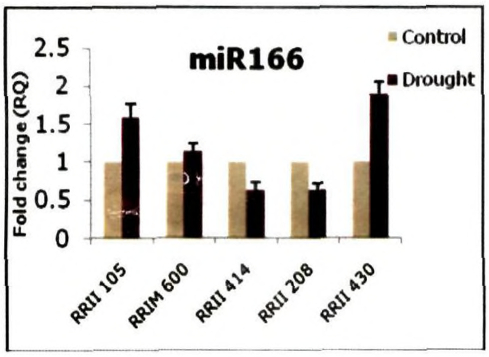

miR160
Control
Drought
Fold change (RQ)
3.5
3
2.5
2
1.5
1
0.5
0
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430

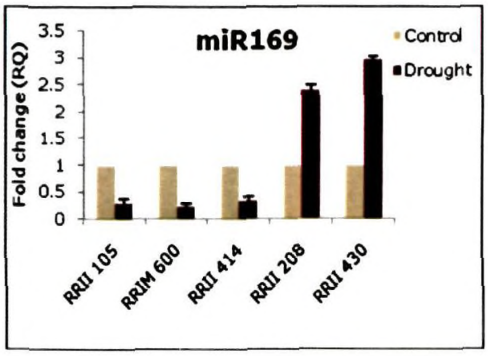

miR2911
Control
Drought
Fold change (RQ)
3
2.5
2
1.5
1
0.5
0
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430

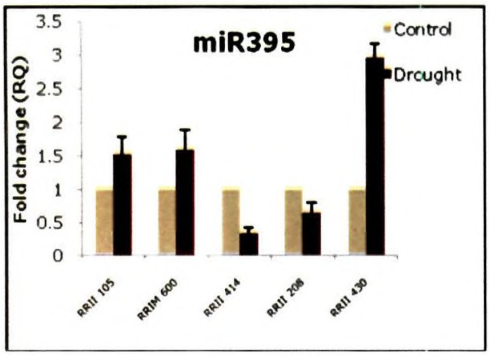

miR1432
Control
Drought
Fold change (RQ)
3.5
3
2.5
2
1.5
1
0.5
0
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430

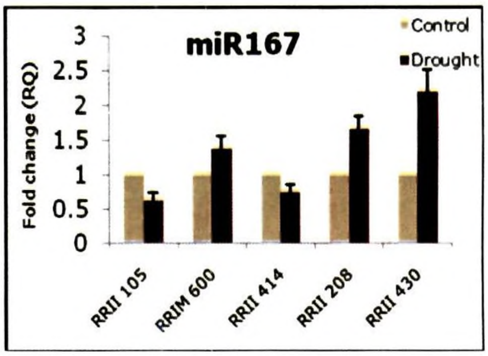

miR164
Control
Drought
Fold change (RQ)
2
1.5
1
0.5
0
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430

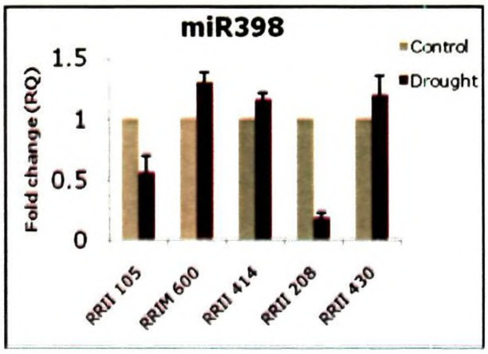

miR482
Control
Drought
Fold change (RQ)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430

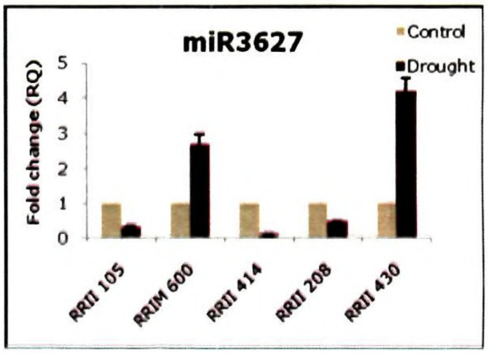

miR166
Control
Drought
Fold change (RQ)
2.5
2
1.5
1
0.5
0
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430

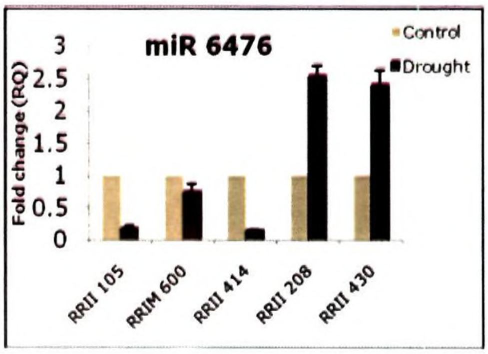

miR169
Control
Drought
Fold change (RQ)
3.5
3
2.5
2
1.5
1
0.5
0
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430

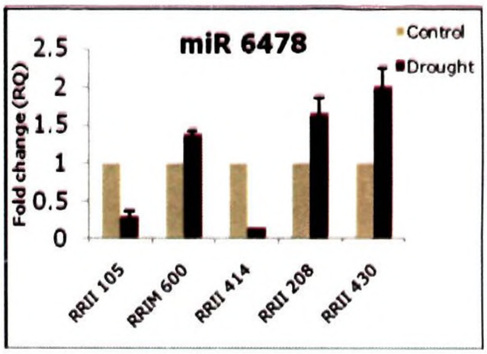

miR395
Control
Drought
Fold change (RQ)
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430

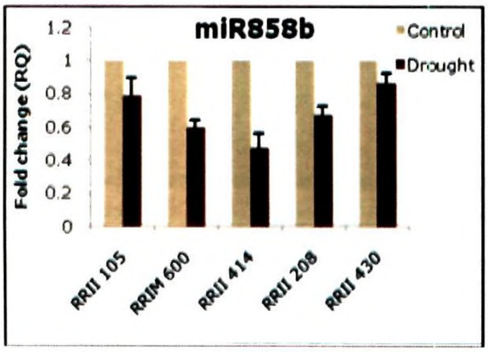

miR167
Control
Drought
Fold change (RQ)
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430

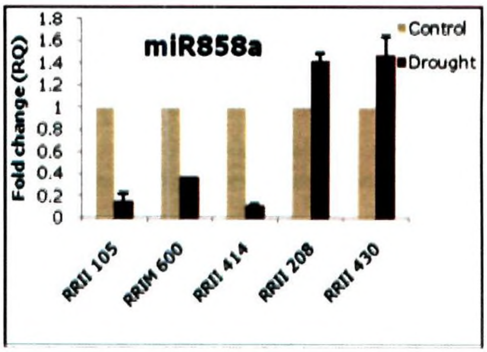

miR398
Control
Drought
Fold change (RQ)
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430


miR3627
Control
Drought
Fold change (RQ)
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430


miR 6476
Control
Drought
Fold change (RQ)
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430


miR 6478
Control
Drought
Fold change (RQ)
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430


miR858b
Control
Drought
Fold change (RQ)
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430


miR858a
Control
Drought
Fold change (RQ)
RRII 105
RRIM 600
RRII 414
RRII 208
RRII 430

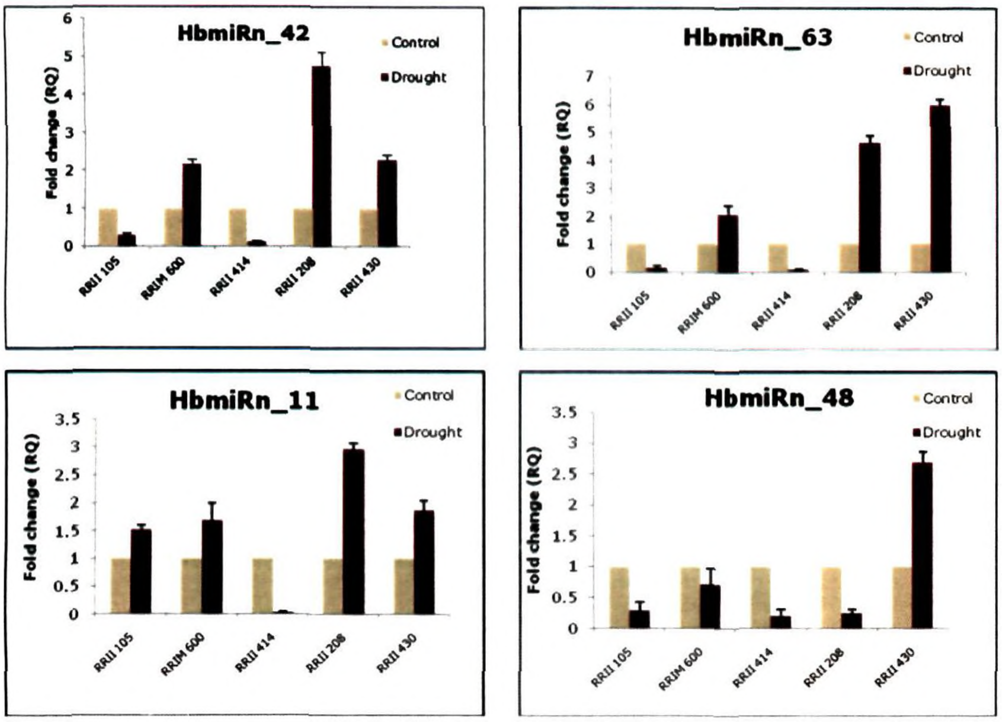


Fig. 3.10. Expression analysis of twenty microRNAs in five clones of *Hevea* under drought condition. [Error bars indicate standard error of three biological replicates]

From the miRNA-target pairs obtained from this study, three conserved miRNAs as well as one novel miRNA and their corresponding targets were selected for further miRNA-target pair expression analysis (Fig. 3.11. & 3.12.). The expression patterns of these corresponding target genes, namely MYB (miR858), NFY A-1 (miR169), ARF (miR160) and HMGR3 (HbmiRn_42) were examined to ascertain the correlation between the corresponding miRNAs under drought stress. Novel miRNA HbmiRn_42 and its target HMGR3 showed a negative correlation in all the clones studied. In the case of MYB transcription factor and miR858a, a negative correlation was noticed in all the clones except RRII 414. No significant negative correlation could be seen in the case of miR160 and its corresponding target ARF as well as in the case of miR169 and its target NFYA-1.

Table 3.4. Relative quantification (fold change) of microRNAs in five clones of *Hevea* under drought condition using its own irrigated control as calibrator

miRNA	RRII 105	RRIM 600	RRII 414	RRII 208	RRII 430
miR3627	0.284	2.674	0.098	0.433	4.202
miR6476	0.186	0.751	0.149	2.541	2.392
miR6478	0.286	1.353	0.133	1.615	1.985
miR168	0.309	2.012	0.125	2.178	3.619
miR858a	0.154	0.367	0.1102	1.415	1.4641
miR858b	0.784	0.595	0.471	0.667	0.859
miR395	1.540	1.602	0.343	0.662	2.969
miR164	0.448	1.169	0.454	1.176	1.379
miR167	0.630	1.3733	0.739	1.654	2.197
miR166	1.585	1.127	0.615	0.621	1.886
miR398	0.551	1.302	1.15	0.175	1.189
miR482	1.175	0.352	0.762	0.151	1.193
miR169	0.294	0.228	0.341	2.395	2.956
miR160	0.704	2.050	0.740	2.017	2.850
miR1432	0.725	2.472	0.416	2.557	2.725
miR2911	0.334	2.625	0.287	1.667	1.998
HbmiRn_42	0.261	2.141	0.090	4.736	2.259
HbmiRn_11	1.526	1.681	0.0337	2.958	1.867
HbmiRn_63	0.146	1.991	0.081	4.615	5.969
HbmiRn_48	0.291	0.712	0.2035	0.249	2.686

MYB

```
# Search parameters
    # score <= 4.0
    # mfe ratio >= 0.7

Target          gi|445335854
miRNA           miR858
score           2
mfe_ratio       0.79
start           308
seed_gap        0
seed_mismatch   0
seed_gu         0
gap             0
mismatch        2
gu              0
miRNA_3'        UUCCAGCUUGUCUGUUGCUUU
aln             .||||.|||||||||||||||
target_5'       CAGGUAGAACAGACAACGAAA
```

NFYA

```
# Search parameters
    # score <= 4.0
    # mfe ratio >= 0.7

Target          isotig23816
miRNA           miR169
score           3.5
mfe_ratio       0.72
start           412
seed_gap        0
seed_mismatch   0
seed_gu         0
gap             0
mismatch        3
gu              1
miRNA_3'        AGCCGUUCAGUAAGAACCGAG
aln             ..|||||.||||||||||||o
target_5'       CAGGCAAUUCAUUCUUGGCUU
//
```

HMGR3A

```
# score <= 4
# mfe ratio >= 0.7

Target          gb|M74798.1
miRNA           HbmiRn_42
score           4
mfe_ratio       0.75
start           821
seed_gap        0
seed_mismatch   1
seed_gu         0
gap             0
mismatch        2
gu              0
miRNA_3'        CUCGGGCGACCGGCUGCGGACC
aln             .|||||.|||||||.|||||||
target_5'       CAGCCCACUGGCCGCCGCCUGG
```

ARF

```
# score <= 4.0
   # mfe ratio >= 0.7

Target          isotig06533
miRNA           miR160
score           1
mfe_ratio       0.91
start           259
seed_gap        0
seed_mismatch   0
seed_gu         0
gap             0
mismatch        1
gu              0
miRNA_3'        ACCGUAUGUCCCUCGGUCCGU
aln             .||||||||||||||||||||
target_5'       AGGCAUACAGGGAGCCAGGCA
```

Fig . 3.11. Target prediction for plant microRNAs using TAPIR software

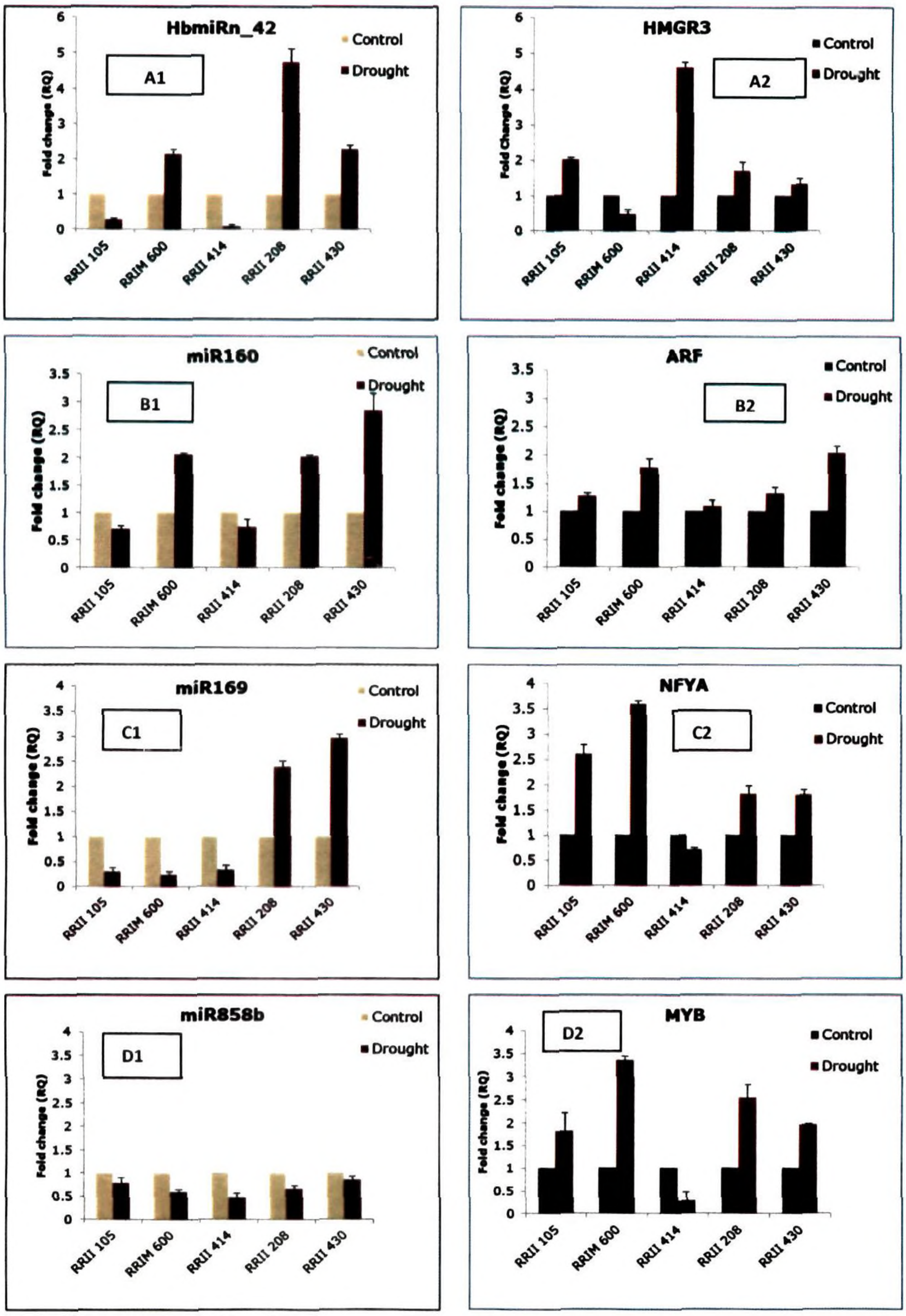


Fig. 3.12. Expression analysis of four miRNAs and their corresponding target genes. Expression of HbmiRn_42 (A1) and its putative target HMGR3 (A2); miR160 (B1) and its putative target ARF (B2); miR169 (C1) and its putative target NFYA (C2); miR858b (D1) and putative target MYB.

3.3.2.6. Statistical analysis of miRNA expression

Statistical analysis was performed with single factor ANOVA using normalized expression data of both tolerant and susceptible clones of *Hevea*. When the analysis at 0.05 and 0.1 level for both tolerant and susceptible clones together was performed, significant F value could not be obtained. But when the analysis was performed in tolerant and susceptible clones separately, significant difference at 0.05 levels was found in drought tolerant clones. Eventually when Fisher's Least Significant difference was performed on this data set, HbmiRn_63 was found to display much stronger association with drought tolerance followed by HbmiRn_42 (Table 3.5.).

Table 3.5. Fisher's least significant difference analysis of relative quantification values of drought tolerant clones

miRNA	RQ value	
HbmiRn_63	4.3270	a
HbmiRn_42	3.0457	ab
miR168	2.6047	bc
miR1432	2.5821	bc
miR3627	2.3820	bcd
miR160	2.3691	bcd
HbmiRn_11	2.2024	bcde
miR2911	2.0995	bcdef
miR 6476	1.8946	bcdef
miR169	1.8603	bcdef
miR395	1.7445	bcdef
mirl67	1.7418	bcdef
miR 6478	1.6511	bcdef
miR858a	1.5453	bcdef
miR164	1.2214	cdef
miR166	1.2120	cdef
HbmiRn_48	1.1872	cdef
miR398	0.8710	def
miR858b	0.7073	ef
mir482	0.5432	f

The miRNAs such as miR168, miR1432, miR3627, miR160 and HbmiRn_11 were found on par with the HbmiRn_42 and can be treated as highly associated with drought tolerance whereas miR2911, miR6476, miR169, miR395, miR167, miR6478, miR858a were found to be associated with drought tolerance to a lesser degree. The miRNAs such as miR164, miR166, HbmiRn_48, miR398, miR858b and miR482 did not show any association with drought tolerance.

3.3.2.7. Validation of miRNAs in *Hevea* germplasm accessions

In order to ascertain the association of miRNAs with drought tolerance, expression analyses of two conserved miRNAs (miR160 and miR168) and one novel miRNA (HbmiRn.42) which exhibited strong association with drought tolerance was carried out in two relatively drought tolerant (RO 3261 and AC 612) and two susceptible (RO 3242 and MT 1619) germplasm accessions along with check clones after imposing drought for 10 days (Table 3.6.). The miRNAs showed up-regulation in both the tolerant check clones and the tolerant germplasm accessions while it got down regulated in susceptible clones. No significant change was observed in susceptible germplasm accessions (Fig. 3.13.).

Table 3.6. Quantification of miR160, miR168 and HbmiRn_42 in *Hevea* germplasm

miRNA	RRIM 600	RRII 414	RO3261	AC612	RO3242	MT1619
miR168	2.207	0.216	9.196	2.340	1.098	1.352
miR160	2.461	0.202	2.657	1.896	1.163	1.757
HbmiRn_42	2.833	0.337	4.819	2.001	1.585	0.997

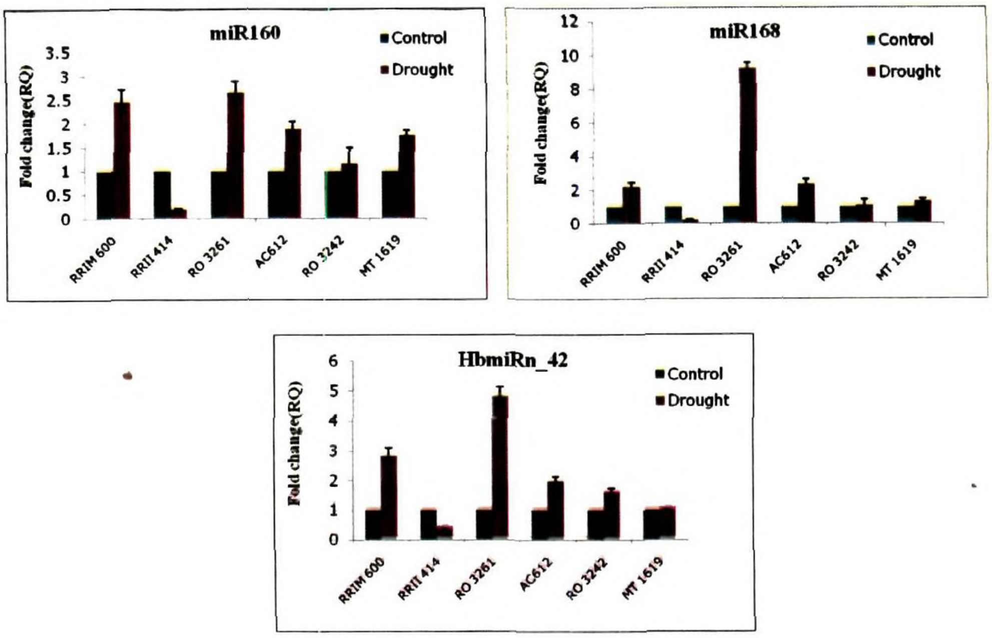


Fig. 3.13. Quantification of miR160, miR168 and HbmiRn_42 in *Hevea* germplasm

3.4 Discussion

Tolerant clones exhibit several inherent adaptive mechanisms to manage or escape the adverse effects of extreme climate. In the recent years, various molecular biological approaches were adopted to develop *Hevea* clones with improved stress tolerance (Leclercq *et al.*, 2012). miRNAs were reported to have roles in almost all aspects of plant development and stress response (Shriram *et al.*, 2016; Zhang *et al.*, 2015; Ferdous *et al.*, 2015). Recent reports have established the role of miRNAs in regulating genes associated with abiotic stress responsive pathways in *Hevea.* miRNA families related to developmental and abiotic stress response (Zeng *et al.*, 2010) and conserved miRNAs and putative novel miRNAs associated with abiotic stress in *Hevea* have been reported (Gebelin *et al.*, 2012; Gebelin *et al.*, 2013b). Identification of drought stress specific miRNAs of *Hevea* and validation of their role in drought tolerance was attempted in this study.

Isolation of miRNAs is a pre-requisite for identification and characterization of abiotic stress responsive miRNAs of *Hevea*. In tree species, especially in *Hevea* which contains high levels of phenolic compounds, carbohydrates and other unidentified compounds (Thomas *et al.*, 2002; Sathik *et al.*, 2005), isolation of good quality miRNA in large quantities is a difficult task. During tissue homogenization, phenolic compounds get immediately oxidized to form covalently linked quinines (Loomis, 1974) that readily bind with nucleic acids making the RNA unusable for downstream purposes. Yew *et al.*, 2012 reported miRNA isolation from recalcitrant tissues. For the identification of drought specific miRNAs, sequencing of small RNAs was performed by conventional cloning and also by high-throughput sequencing.

Isolation and identification of miRNAs from matured leaves of drought stressed *Hevea brasiliensis* were successfully achieved by conventional cloning techniques. Standardization of various issues related to the isolation of small RNA, gel elution, adapter linkering, cloning, sequencing and identification of microRNAs from *Hevea brasiliensis* was accomplished which yielded four families of conserved miRNAs and one novel miRNA. Using Next Generation Sequencing method (Illumina) 64 miRNAs from control (29 families) and 63 miRNAs (32 known miRNA families) from drought were identified. Seven and 13 novel miRNAs were identified in control and drought samples respectively. Differential expression analyses showed that 28 conserved miRNAs were common to both the samples. Five conserved miRNAs got significantly up-regulated and six got significantly down regulated during drought stress.

Genotype-dependent response of miRNAs to abiotic stress was evidenced by analysing miRNA expression levels in response to certain stresses among several plant species and cultivars. To understand the role and level of expression of these miRNAs, five clones of *Hevea* with varying levels of drought tolerance were exposed to drought condition. CO_2 assimilation rate was found inhibited in drought imposed plants of all the clones while it was much severe in susceptible clone RRII 414. Similarly, stomatal conductance also declined significantly in all the clones under drought stress while it was almost zero in clone RRII 414. These gas exchange parameters monitored on drought treated plants confirmed the drought impact on these plants.

The expression of miR168 was up regulated in tolerant clones of *Hevea* (RRIM 600, RRII 208 and RRII 430) and got down regulated in relatively susceptible clones (RRII 105 and RRII 414). miR168 is a conserved miRNA which has been detected in 30 species is one of the most commonly detected stress-inducible MIR genes. Homologs of MIR168 exist in various plant species, including monocots such as maize, rice and dicots such as poplar, tobacco and *Arabidopsis*. These homologs have been found to respond to salt, drought, and cold stresses or ABA treatment (Liu *et al.*, 2008; Zhou *et al.*, 2010). miR168 targets ARGONAUTE1 which is a core component of the RNA-induced silencing complex that associates with miRNAs to inhibit target genes by mRNA cleavage and/or translational repression (Vaucheret *et al.*, 2004; Vaucheret, 2008; Voinnet, 2009). Mutations in *AGO1* cause increased accumulation of miRNA targets (Vaucheret *et al.*, 2004; Kurihara *et al.*, 2009). Loss of miR168 function has been found to cause developmental defects in *Arabidopsis* (Vaucheret *et al.*, 2004). In *Arabidopsis, MIR168a*-overexpressing plants and its target *ago1* loss-of-function mutants showed ABA hypersensitivity and drought tolerance, while the *mir168a* mutants showed ABA hyposensitivity and drought hypersensitivity (Li *et al.*, 2012a). The promoter region of *MIR168a* gene contains the ABRE cis element that could influence drought tolerance mechanism. *MIR168a* is activated by abscisic acid-responsive element (ABRE)-binding transcription factors ABF1, ABF2, ABF3, and ABF4. A typical ABRE motif within the *MIR168a* promoter (which can be bound by the four ABRE-binding transcription factors) is highly conserved in the miR168 homologs of many plant species.

These results imply a common and conserved mechanism of miR168 transcriptional control in plant stress response (Li *et al.*, 2012a).

The expression of miR160 got up regulated in tolerant clones of *Hevea* when the plants were exposed to drought while there was a reduction in the susceptible clones. Similar results were found in drought-tolerant cowpea cultivar (Barrera-Figueroa *et al.*, 2011) and also in peach root during drought stress (Eldem *et al.*, 2012). miR160 regulates the expression of *Auxin Response Factors* (*ARF10*, *ARF16* and *ARF17*). Various reports indicate the existence of possible link between auxin signaling and miR160 expression (Sunkar *et al.*, 2012). Hevamin A, one of the genes encoding Hevamine has also been predicted to be targeted by miR160 in *Hevea* (Lertpanyasampatha *et al.*, 2012).

The expression of miR1432 got up-regulated in tolerant clones of *Hevea* when the plants were exposed to drought. miR1432 identified by high throughput sequencing, was predicted to target aminobutyrate aminotransferase and orf36 gene product in *Hevea*. Differential expression of miR1432 was reported in drought tolerant and susceptible cultivars of sugarcane under drought stress (Gentile *et al.*, 2013). miR1432 have been found to be induced by drought in *Triticum dicoccoides* (Kantar *et al.*, 2011). In *Phyllostachys edulis* it is reported that under drought treatment miR1432 got up regulated (Lili *et al.*, 2015).

The expression of miR167 was higher in drought tolerant clones than in relatively susceptible clones. miR167 has been reported to regulate auxin response factors (ARF) such as ARF6 and ARF8 under drought condition (Liu, *et al.*, 2008). The miR167 guides the regulation of ARF6 and ARF8 which are reported to negatively regulate free IAA levels by interfering with the GH3-like gene expression (Mallory, *et al.*, 2005; Teotia *et al.*, 2008)). Under drought condition, expression of miR167 has been found induced in *Arabidopsis* (Liu, *et al.*, 2008).

miR482 are known to suppress the expression of Nucleotide Binding Site-leucine-rich-repeat receptor protein (NBS-LRR protein) (Shivaprasad *et al.*, 2012., Zhu and Luo, 2013) and in *Hevea* it is reported to target Abscisic Acid Responsive Element Binding Protein 2 (AREB2) which is abiotic stress responsive (Lertpanyasampatha *et al.*, 2012; Arenas-Huertero *et al.*, 2009). miR482 family is reported to have more variable sequences than other miRNA families (Shivaprasad *et al.*, 2012). The miR482 obtained by conventional method was found similar to csmiR482b (Xu *et al.*, 2010) while the one obtained by NGS method was similar to the one reported (HbmiR482) in *Hevea brasiliensis* (Gebelin *et al.*, 2012). When quantified, its expression in tolerant clones (RRIM 600 and RRII 208) got reduced to 0.2 fold (with an exception of clone RRII 430) while there was no much change in the relatively susceptible clones like RRII 105 and RRII 414. In cotton plant, miR482 has been reported to be down-regulated under high-temperature stress conditions (Wang *et al.*, 2016). The results of this study indicate that miR482 is drought responsive and do have role in imparting drought tolerance.

The expression analysis of two miRNAs viz. miR858a and miR858b of the miR858 family studied, the expression level of miR858a was found significantly lower in susceptible clones and in one tolerant clone RRIM 600 while there was no significant change in other tolerant clones. miR858b got down-regulated under drought condition irrespective of clones evaluated. miR858 is reported to target MYB genes in plants (Xia *et al,* 2012; Guan *et al,* 2014) which are the largest transcription factor gene family playing vital roles in plant growth and development and also in plant responses to various biotic and abiotic stresses. miR2911 was found significantly down regulated in susceptible clones and up-regulated in tolerant clones. In cowpea, expression of miR2911 was induced during drought stress (Barrera-Figueroa *et al.,* 2011). It is reported to target cytochrome p450 like tbp (TATA box binding protein) in *Camellia sinensis* which is involved in stress response (Zhu and Luo, 2013).

miR2911 is reported in *Populus euphratica*, *Nicotiana tabacum* and *Helianthus annuus* (Li *et al.*, 2009; Tang *et al.*, 2012; Barozai *et al.*, 2012). This is an atypical miRNA as it is derived from ribosomal RNA (rRNA) and does not follow classical miRNA biogenesis (Gregory *et al.*, 2004; Lee *et al.*, 2003; Denli *et al.*, 2004). miR2911 is known to exist stably in honeysuckle decoction (HS decoction) due to its special high G-C content. It has been reported to target the genes of *Influenza A viruses* (IAVs) in humans and mice (Zhou *et al.*, 2014). Plant miR2911 can directly bind to the target genes *PB2* and *NS1*, which are essential for influenza replication, thereby inhibiting their amplification. The results of this study show that miR2911 might have direct association with drought tolerance.

miR398 targets two closely related Cu/Zn SODs (CSD1 and CSD2) which are known to involve in oxidative stress detoxification (Sunkar *et al.*, 2006). miR398 was down regulated under drought stress in *Medicago truncatula* (Wang *et al.*, 2011) and in maize (Wei *et al.*, 2009). This leads to increased activity of CSDs rendering oxidative stress tolerance. The expression analysis data indicate that its level did not alter in clones RRIM 600, RRII 430 and RRII 414 while it got reduced significantly in RRII 208 and to some extent in RRII 105. Probably, the ROS scavenging enzyme Cu/Zn SOD levels would have been up-regulated in RRII 208. From the results it can be presumed that free radical scavenging activities must have been much higher in RRII 208 when compared to other clones studied. The expression of *chloroplastic HbCu/ZnSODs* under saline stress has been reported to be induced in *Hevea* while its corresponding miR398a and miR398b got significantly repressed (Gebelin *et al.*, 2013).

miR169 is a conserved miRNA family that regulates a homologous target, and it appears to behave in contradictory ways in different plant species, because of differences in plant developmental stages, growth conditions and the duration and strength of the applied stress (Ding, *et al.*, 2013). miR169 targets the NFYA5 mRNA, encoding a subunit of the nuclear factor Y (NF-Y) transcription factor (Liu *et al.*, 2008) which are plant specific transcription factors playing important role in plant development and in coping up with the environmental stresses (Kumimoto *et al.*, 2008). miR169 was reported to be down regulated under drought in *Arabidopsis* (Li *et al.*, 2008), *Medicago truncatula* (Wang *et al.*,2011) and peach (Eldem *et al.*,2012). miR169 was down-regulated by drought stress through an ABA-dependent pathway (Li *et al.*, 2008). In contrary, it was found up-regulated in rice (Zhao *et al.*, 2007) and tomato (Zhang *et al.*, 2011). In this study, miR169 was found down-regulated in drought susceptible clones and also in RRIM 600 which is a tolerant one. In contrast, RRII 430 and RRII 208 presumed to be drought tolerant clones displayed up-regulation of miR169.

miR166 is reported to be drought responsive and is known to regulate class III homeodomain-leucine zipper (*HD-Zip III*) transcription factors which are important for lateral root development, axillary meristem initiation and leaf polarity (Hawker, *et al.*, 2004). In barley and *Triticum dicoccoides*, miR166 has been found down- regulated in response to drought (Kantar *et al.*, 2010, 2011). In *Medicago truncatula*, it was found up-regulated in roots while being suppressed in seedlings and shoots under drought stress (Trindade *et al.*, 2010). In this attempt, there is no significant difference in miR166 expression among the tolerant and susceptible clones.

miR164 is reported to be involved in regulating the post-transcriptional processing of NAC transcription factors (Guo *et al.*, 2005). Expression of NAC proteins in response to abiotic stresses in various plants and their possible role is well known (Puranik e*t al.*, 2012). A rice stress responsive NAC gene, SNAC1, confers drought resistance under field drought conditions by promoting stomatal closure (Hu *et al.*, 2006). On the contrary, the recent report in rice indicated the association of miR164 targeted NAC genes with drought susceptibility (Fang *et al.*, 2014). In this study, expression of miR164 was found down regulated in relatively susceptible clones under drought stress, while there was not much change in relatively tolerant clones. In deep sequencing data, it was found highly induced in RRIM 600 during drought stress in contrary to what was expected.

miR3627 reported to be highly conserved among the fruit trees, poplar and in other non-woody plant species (Solofoharivelo *et al.*, 2014). In apple amino acid transporter was predicted as target of miR3627 (Xia, *et al.*, 2012) while this study predicted conserved hypothetical protein as its target. When quantified, its expression was found down regulated in susceptible clones and up regulated in tolerant clones except in RRII 208. miR6478 is less conserved and present only in some of the plant species (Liu *et al.*, 2014). A protein of unknown function was predicted as its target in *Accasia crassicarpa* (Liu *et al.*, 2014) and this study predicted conserved hypothetical protein as its target. The result of this study indicates the down regulation of miR6478 significantly in susceptible clones (RRII 414 and RRII 105) while there was an increase in all tolerant clones studied.

There was a significant reduction in the expression of miR6476 in susceptible clones and up-regulation in tolerant clones under drought condition. This study predicted photosystem I reaction center subunit IV A, chloroplast precursor as its target in *Hevea brasiliensis.* In tomato, amino acid transporter and TPR Domain containing protein have been predicted as its target (Din, *et al.*, 2014). From the results it could be inferred that miR6476 might target proteins associated with stress amelioration.

miR395 targets two families of genes, ATP sulfurylases (encoded by APS genes) and sulfate transporter 2;1 (SULTR2;1, also called AST68), both of which are involved in the sulfate metabolism pathway. Their transcripts are suppressed strongly in miR395-over-expressing transgenic *Arabidopsis*, which over-accumulates sulfate in the shoot but not in the root (Liang *et al.*, 2010). Zhou *et al.*, (2010) reported that during drought stress in *Oryza sativa* miR395 got significantly up-regulated. In tobacco miR395 was most sensitive to both drought and salinity stress and got up-regulated during both stresses (Frazier *et al.,* 2011). In this study, expression of miR395 was found significantly reduced in the susceptible clone RRII 414 and significantly up-regulated in drought tolerant clone RRII 430

The higher level expression of novel miRNAs HbmiRn_42 and HbmiRn_63 in tolerant clones and their down regulation in drought susceptible clones indicates its strong association with drought tolerance. Probably, it might be controlling the expression of its target gene which might be a negative regulator of drought tolerance. Expression of HbmiRn_48 got down regulated in all the clones except in RRII 430 where it got up regulated. In contrast, HbmiRn_11 got up regulated in all the clones except in RRII 414 in which it was found significantly down regulated.

The attempts made to ascertain the association of selected miRNAs with drought tolerance using the germplasm accessions with known tolerant levels (Thomas *et al.,* 2015) indicated the existence of similar trend. When the quantification of three miRNAs (miR160, miR168 and HbmiRn.42) which exhibited stronger association with drought tolerance in two tolerant (RO 3261 and AC 612) and two susceptible (RO 3242 and MT 1619) germplasm accessions, they were found up-regulated in both the tolerant check clone (RRIM 600) and germplasm accessions. But in susceptible check clone (RRII 414), it was *vice versa* while there was no significant change in the susceptible germplasm accessions. These results also confirm the association of the above miRNAs with drought tolerance as well as confirm the tolerance/susceptibility of the germplasm accessions evaluated using physiological and biochemical parameters (Thomas *et al.,* 2015). Lack of significant change in the levels of these miRNAs in susceptible germplasm accessions indicates the inherent drought tolerance of these accessions when compared to the susceptible clones validated in this study.

In order to understand the functional importance of the identified miRNAs, their corresponding targets were predicted using psRNA target finder server. All 33 conserved miRNA families when searched for targets against ESTs or gene sequences of *Ricinus communis*, *Hevea brasiliensis* and *Manihot esculenta*, 27 known miRNA families out of 33 were found to have targets in *Hevea* brasiliensis. Target prediction revealed that many targets were transcription factors including MYB, NFYA, ARFs which are known to be involved in regulating stress responsive genes. Apart from this, stress responsive and stress amelioration related genes, hypothetical proteins and cell wall associated and signalling related proteins were also found as targets of these miRNAs. Target prediction carried out for the novel miRNAs revealed four miRNA-target pair from control samples and five miRNA-target pair from *Hevea brasiliensis* database. Among the five miRNA-target pairs predicted in drought samples, HbmiRn_10 targets ARM repeat superfamily protein which interact with numerous other proteins and regulate a variety of cellular processes (Mudgil *et al.,* 2004). ARM repeat superfamily proteins are also involved in protein degradation pathways as E3 ubiquitin. HbmiRn_37, HbmiRn_31 and HbmiRn_32 target the ubiquitin and WLM domain-containing protein. The WLM (WSS1-like metalloprotease) domain belonging to the zincin-like superfamily of Zn-dependent peptidase functions as a specific de-SUMOylating domain of distinct protein complexes in the nucleus and the cytoplasm (Iyer *et al.,* 2004). HbmiRn_65 targets Tarlp (Transcript Antisense to Ribosomal RNA) while both the HbmiRn_60 and HbmiRn_63 were found to target Tubulin beta-7 chain. HbmiRn_48 and HbmiRn_49 target the putative DNA binding protein.

The expression patterns of four corresponding target genes, namely MYB, NFYA3, ARF and HMGR3 of miRNAs *viz.* miR858, miR169, miR160 and HbmiRn_42 respectively were quantified to confirm the association between the miRNAs and their target genes under drought stress. In the case of MYB, its expression got induced in all the drought exposed clones except

RRII 414 which is a susceptible clone while the corresponding miRNA got reduced in all the clones. Expression of MYB has been reported to be significantly higher in tolerant clones like RRII 208 and RRIM 600 and moderately up-regulated in RRII 105 while it got significantly down-regulated in clone RRII 414 (Luke *et al.*, 2015). Though the expression of miR858 in clone RRII 414 was at lower level in this study, the level of MYB in this clone was also found down-regulated indicating that the expression of this particular target gene in susceptible clone is not being directly regulated by this particular miRNA.

Novel miRNA HbmiRn_42 and its target HMGR3 (HMG-CoA reductase) showed a negative correlation in all the clones studied. Its higher expression in tolerant clones resulted in down-regulation of its target protein HMGR3. Down regulation of this miRNA in drought susceptible clones during drought condition led to up-regulation of its corresponding protein. Probably, it might be controlling the expression of its target gene which might be a negative regulator of drought tolerance. Plant HMGR is a key regulatory enzyme of the MVA pathway for isoprenoid biosynthesis which is controlled by various endogenous signals and environmental factors (Antolín-Llovera *et al.*, 2011). HMGR3's up-regulation in susceptible clones under drought conditions indicates the existence of continued metabolic activity that might restrict diverting the resources for stress amelioration thus resulting in susceptibility when compared to the tolerant clones.

No significant correlation could be seen in the case of miR160 and its corresponding target ARF which are key regulators of physiological and morphological processes mediated by auxins by binding to specific *cis*-element in the upstream regions of auxin-inducible genes that may contribute to stress adaptation (Guilfoyle and Hagen, 2007). miR160 got up regulated under drought conditions in tolerant clones while there was a no significant reduction in the expression levels of ARF. In a similar study in *Hevea*, through experimental validation miR160 has been found to target ARF (Gebelin *et al.*, 2012). Shuai *et al.*, (2013) reported that the down-regulation of miR160 in drought-stressed *Populus trichocarpa* allowed increased expression of their target, ARF.

Nuclear factor Y (NF-Y) associated with drought tolerance is a ubiquitous transcription factor which is induced by drought stress at both transcriptional and post-transcriptional levels. Overexpression of NFYA5 and NFYB1 in *Arabidopsis* has been found to impart drought tolerance (Li *et al.*, 2008). miR169 has been reported to direct NFYA3 mRNA cleavage in Glycine max (Ni *et al.*, 2013). Over-expression of GmNFYA3 in Arabidopsis resulted in reduced leaf water loss and enhanced drought tolerance (Ni *et al.*, 2013). Down-regulation of miR169a under drought condition resulted in the increased levels of *NFYA5* (Li *et al.*, 2008). In clone RRII 105, RRII 414 and RRIM 600, miR169 was found down regulated, while its target gene was found up-regulated significantly in clones RRII 105 and RRIM 600 only. Though the reduction in the NFYA levels in clone RRII 414 is associated with its drought susceptibility, a correlation with its corresponding miRNA could not be seen. Similarly, correlation could not be obtained from the comparatively higher level of NFYA found in clone RRII 208 and RRII 430 and the higher level of its corresponding miRNA. A recent report in *H. brasiliensis* (Luke *et al.*, 2015) also confirms the abundant expression of NFYA in tolerant and moderate clones (RRIM 600 and RRII 105 respectively) while its level was at minimal in susceptible clone (RRII 414).

The quantitative expression analysis data of drought tolerant clones when subjected to single factor ANOVA resulted in significant difference between the miRNA expression levels at 0.05 levels. The Fisher's LSD test further conducted revealed two miRNAs such as HbmiRn_63 and HbmiRn_42 to have much stronger association with drought tolerance when compared to other miRNAs. Another set of five miRNAs (miR168, miR1432, miR3627, miR160 and HbmiRn_11) were found to be on par with the above ones. Though another set of seven miRNAs (miR2911, miR6476, miR169, miR395, miR167, miR6478 and miR858a) had a positive trend with drought tolerance, they may not merit as marker for drought tolerance. The rest of the six miRNAs did not show any trend with drought tolerance. The novel miRNAs HbmiRn_63 and HbmiRn_42 and the other five miRNAs (miR168, miR1432, miR3627, miR160 and HbmiRn_11) which displayed stronger association with drought tolerance can be further utilized in crop improvement programmes after validation in more number of tolerant/susceptible clones.

3.5 Conclusions

This study aimed to identify drought-responsive microRNAs from *Hevea brasiliensis* through both conventional and Next Generation Sequencing. Sixty four miRNAs belonging to 29 conserved miRNA families from control samples and 63 miRNAs belonging to 32 conserved miRNA families from drought stressed samples were identified. Targets of both conserved and novel miRNAs were also predicted. Validation of selected miRNAs resulted in identification of novel miRNAs *viz.* HbmiRn_63 and HbmiRn_42 exhibiting stronger association with drought tolerance. Another set of five miRNAs was also found equally contributing for the tolerance. Validation of selected miRNAs in germplasm accessions with varying levels of drought tolerance also confirmed with the results obtained, thus strengthening the association of these miRNAs with drought tolerance. This study opens up the possibility of employing the identified miRNAs as marker for drought tolerance in the crop improvement programmes of *Hevea brasiliensis*.

Chapter 4

Identification and expression analysis of cold responsive microRNAs of *Hevea*

Abstract

Cold stress is one of the major abiotic factors that adversely affect the productivity and geographical distribution of many agriculturally important crops like *Hevea*. Developing cold tolerant *Hevea* clones is a primary requisite to maximize the productivity under such challenging environmental conditions. The present study was initiated with an objective to identify and characterize cold stress responsive miRNAs from *Hevea* in order to find miRNAs that show stronger association with cold tolerance. Next generation sequencing using Illumina HiSeq method revealed the expression of 21 and 29 conserved miRNA families in cold treated and control samples, respectively (clone RRIM 600). Forty two novel miRNAs were identified. From the differential expression analysis, eight conserved miRNAs were found commonly expressed in both the samples. When expression analyses performed subsequently with six selected miRNAs in two *Hevea clones* (*viz.* RRII 105 and RRIM 600), miR169 showed a strong association with cold tolerance. miR482 and miR159 were the other miRNAs that showed association with cold tolerance. These miRNAs can be employed as markers for cold tolerance after extending the validation to larger number of clones with varying levels of cold tolerance.

Key words *Hevea brasiliensis*, Cold tolerance, miRNAs, qPCR, Expression analysis, High throughput sequencing

4.1. Introduction

Cultivation of *Hevea* in India is being extended to regions having suboptimal environments like north-eastern regions where the temperature during winter is too low for its survival and optimum productivity and has been reported to affect the development and latex biosynthesis (Priyadarshan *et al.,* 2005; Jacob *et al.,* 1999). Cold damage to rubber trees is a complex phenomenon which involves differential response of clones, age and vigour of the plant. Hence it is imperative to select clones/varieties with enhanced tolerance to low temperature stress in order to achieve sustainable productivity in the cold prone regions. However, factors like lack of techniques for early evaluation for cold tolerance in the pipeline clones or in the newly developed hybrid clones and the time required to assess their tolerance in field conditions are the real constraints in selecting clones for such abiotic stress prone regions. In order to maximize the productivity of *Hevea* and to identify best performing clones for stress prone agroclimatic zones, attempts have to be made to breed suitable clones for such regions.

In general, plants respond to cold stress by adjusting their metabolism and by effecting various physiological and molecular changes in order to acquire enhanced cold tolerance (Thomashow *et al.,* 1999). Cold stress induces changes in membrane fluidity and protein conformation. The plants respond to cold stress by re-arranging its cytoskeleton followed by activation of Ca^{2+} channels which lead to increased cytosolic Ca^{2+} levels eventually triggering the expression of COR genes. The COR genes are known to be involved in altering the metabolism, protein stability and cell structure by regulating hundreds of COR genes related to signal transduction, defence against pathogens and transcription factors. Cold stress also induces the expression of C-repeat binding transcription factors (CBF), which play vital role in regulation of genes such as late-embryogenesis abundant (LEA) type protein and osmoprotectant biosynthesis in plants. Under cold stress, regulation of cold signalling is effected by MAP (mitogen activated protein) Kinase cascade. MAP Kinase Kinase (MAPKK) is involved in phosphorylation of MPKs under cold stress which triggers further the cold signalling pathway (Chinnusamy, 2006).

Gene expression studies carried out in low temperature exposed *Hevea* clones RRII 105 and RRIM 600 revealed the existence of stronger association between genes such as NAC transcription factor, LEA 5 protein and peroxidase with cold tolerance (Sathik *et al.,* 2012). Among them, processes such as repression of genes, mRNA export and mRNA degradation have been found to be of central importance for the cold-stress response (Zhu *et al.,* 2007). Various reports on a wide range of species have beyond doubt proven that gene regulation by microRNAs is essential for coordinating plant's responses to cold stress. miRNAs have been found to play main role in regulating the cold responsive genes and are directly associated with cold tolerance. Cold responsive miRNAs have been reported in *Arabidopsis* (Sunkar and Zhu, 2004; Liu *et al.,* 2008; Zhou *et al.,* 2008), poplar (Lu *et al.,* 2008; Chen *et al.,* 2012), *Brachypodium distachyon* (Zhang *et al.,* 2009), rice (Lv *et al.,* 2010), wheat (Tang *et al.,* 2012), tomato (Cao *et al.,* 2014) and potato (Ou *et al.,* 2015). The effect of cold on miRNA expression is species, tissue or developmental stage dependent (Sunkar *et al.,* 2012). Gebelin *et al.,* (2013) identified eight cold specific *MIR* genes in *Hevea*, of which seven *MIR* genes were found significantly down-regulated under cold stress conditions in clone PB 260. As reports were not available on cold responsive miRNAs of *Hevea* in different clones with varying levels of cold tolerance, finding miRNAs strongly associated with cold tolerance is not possible. Hence, in this study, attempts were made to identify cold responsive miRNAs and to find miRNAs having stronger association with cold tolerance by validating in two clones of *Hevea* with contrasting levels of cold tolerance with an aim to identify miRNA markers for cold tolerance.

4.2. Materials and methods

4.2.1. Plant material and stress induction

Six-months-old polybag plants of clone RRII 105 (cold susceptible) and RRIM 600 (cold tolerant) were acclimatized in a growth chamber for three days with a minimum temperature of 15 °C during night (for 3 h) and a gradual rise in maximum temperature up to 25 °C in the day time. Fourth day onwards, cold treatment was imposed by reducing the temperature to 8 °C during night followed by a gradual increase in maximum temperature up to 15 °C in the day time for five consecutive days. Light intensity regime ranging between a minimum of 200 to a maximum of 800 μ mol m^{-2} s^{-1} with RH in the range of 60 to 70% were provided. Control plants were allowed to grow at stress free and ambient weather conditions.

4.2.2. Gas exchange measurements

Leaf samples were harvested after assessing the stress response of the plants by measuring the net CO_2 assimilation rate (A) and stomatal conductance (gs) using a portable photosynthesis system (LI-6400), LI-COR, U.S.A. All the gas exchange parameters were measured at a constant CO_2 concentration of 360 ppm using a CO_2 injector and at 500 µmol m^{-2} s^{-1} of light intensity using red LED source (with 10% blue light) attached with the leaf chamber (LI-6400). On the same leaves chlorophyll fluorescence was also measured using a fluorescence monitoring System (Hansatech, UK). Twenty minutes of dark adaptation was done by clamping aluminium clips over the leaf for the subsequent measuring of the maximum potential quantum yield. Minimal fluorescence (F_0) and maximum fluorescence (F_m) were measured in dark adapted leaves by giving saturating flash of light. The flash of light allowed transient closure of PSII reaction centres. The ratio [$(F_v/F_m) = (F_m-F_0)/F_m$] reflected the potential quantum efficiency of PSII (Maxwell and Johnson 2000).

4.2.3. Small RNA library construction and sequencing

Total RNAs were extracted from leaves of cold treated plants of clone RRIM 600 using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) according to the manufacturer's instructions. The quantity and quality of total RNA was determined using Nanodrop-1000 and resolving on 1% denatured agarose gel. The pair-end cDNA sequencing library for small RNA were prepared for control and drought stressed samples using Illumina® TruSeq Small RNA Sample Preparation Kit (Illumina) as per manufacturer's instructions. For the library preparation, 1 μg total RNA was first ligated with 3' adapter followed by 5' adapter ligation. Reverse transcription followed by PCR was performed to create cDNA constructs based on the small RNAs ligated with 3' and 5' adapters. The final PCR products were purified and subjected to deep sequencing by employing Illumina HiSeq 2000 at Xcelris Genomics, Ahemedabad, India.

4.2.4. Identification of conserved and novel miRNAs

To identify the conserved miRNAs, small RNAs were annotated against miRBase database (version 21) by using CLC Workbench (Version 6). A maximum of two mismatches were allowed in the annotation. To identify novel miRNAs from *Hevea,* sequences ranging from 20 to 24 nt were used for further analysis using stringent criteria for miRNA prediction. The small RNAs were mapped to the draft genome of *Hevea brasiliensis* (accession no: AJJZ01, total number of contigs,1,223,365), *Ricinus communis* and *Manihot esculenta* to identify novel miRNAs using miRanalyzer Version 3 with default parameters and the precursor molecules were extracted from their genome sequences.

4.2.5. Target prediction for miRNAs

Target prediction for known and novel miRNAs was performed using web based psRNA target program with default parameters *viz* (1) a maximum expectation value of 3.0 (2) a complementarity scoring length of (hsp size) 20; (3) a target accessibility of 25 or less; and (4) no mismatch at positions 9-11.

4.2.6. Validation of miRNAs by qPCR

Total RNAs were extracted from control and cold stressed samples of *Hevea* clones RRII 105 and RRIM 600 using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) according to the manufacturer's instructions. Total RNA (2 μg) from each sample was then reverse transcribed using Mir-X miRNA first strand c-DNA synthesis kit (Clontech). In a single reaction small RNAs were poly-adenylated and reverse transcribed using poly(A) polymerase and SMART MMLV Reverse Transcriptase. Validation of six conserved miRNAs (Table 4.1.) in control and cold stressed plants was performed by qPCR on Light Cycler 480 II (Roche) using SYBR Advantage qPCR Premix (Takara). The reaction consisted of 0.5 μl of 10 times diluted cDNA, 0.1μM of each forward and reverse primers and 5 μl of 2x SYBR Advantage qPCR Premix in a 10 μl reaction volume. The reaction conditions included an initial denaturation step of 95 °C for 30 sec, followed by 40 cycles of 95 °C for 5 sec and 60 °C for 30 sec. Changes in expressions were calculated as normalized fold ratios using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 4.1. List of miRNAs and their sequences for qPCR analysis

miRNA	Sequence (5'-3')
miR169	GAGCCAAGAATGACTTGCCGA
miR482	AGATGGGTGGCTGGGCAAGAAG
miR858	TTTCGTTGTCTGTTCGACCTT
miR171	TCTATAATCACGCCAAGTTAG
miR159	AAACCTAACTTCCCTCGAGAC
miR166	AGCCTGGTCCGAAGTAAGGAG

4.3 Results

4.3.1. Gas exchange parameters

The plants grown under growth chamber conditions showed cold stress responsive syndromes after cold treatment at 8 °C during night and at 15 °C during day time for five days. The physiological parameters such as stomatal conductance (gs), net CO_2 assimilation rate (A) and quantum efficiency of PS II indicated the incidence of stress in both the clones. The stomatal conductance in the susceptible clone RRII 105 came down drastically from about 0.11 mol m^{-2} s^{-1} to near zero under cold stress while the tolerant clone RRIM 600 could maintain the gs at about 0.04 mol m^{-2} s^{-1} (from 0.13 mol m^{-2} s^{-1} in control condition) (Fig. 4.1.a). While both the clones maintained A at about 6 to 7 µmol m^{-2} s^{-1} in control conditions, RRIM 600 maintained a better A (3 µmol m^{-2} s^{-1}) than RRII 105 (near 0) (Fig. 4.1.b). Similarly, the F_v/F_m ratio also was found better in RRIM 600 (0.6) than RRII 105 (0.3) under low temperature condition (Fig. 4.1.c).

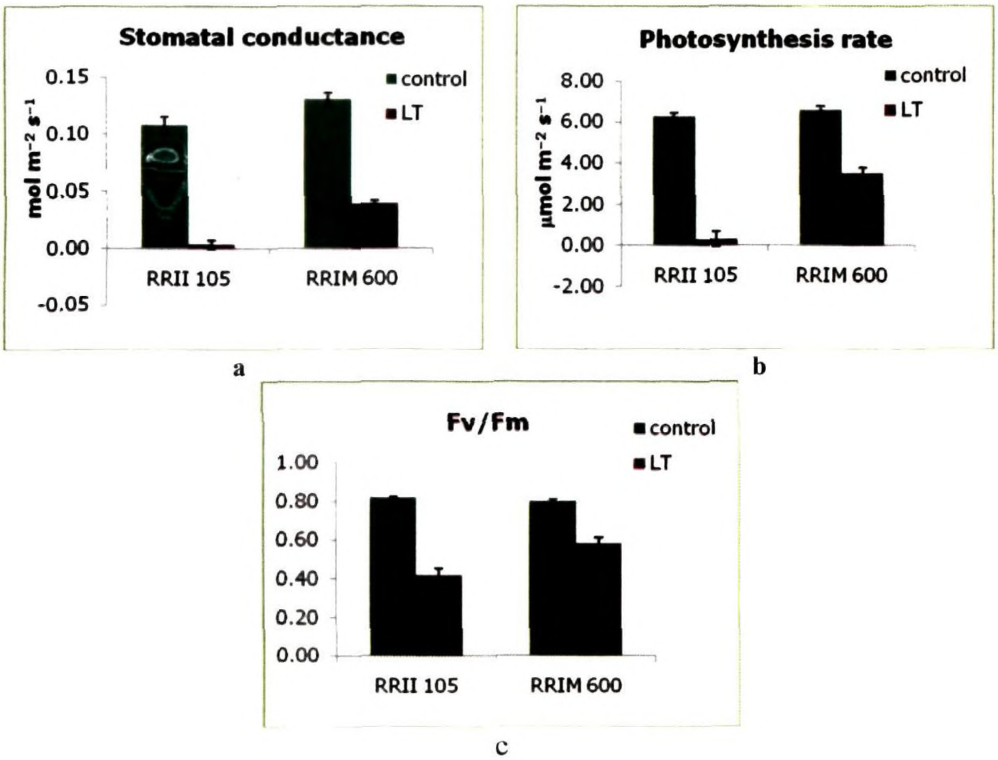


Fig. 4. 1. (a) Stomatal conductance (g_s), (b) CO_2 assimilation rate (A), (c) Fv/Fm of control and low temperature (LT) treated plants of RRII 105 and RRIM 600

4.3.2. Analysis of small RNA population

Small RNA libraries from cold treated leaf samples and control samples were constructed and sequenced using Illumina HiSeq2000 platform. A total number of 11,383,272 reads were generated from the cold treated library. After removing the 5' and 3' adaptor, the reads smaller than 20 bp and greater than 24 bp were avoided. A total number of 1,162,006 clean reads corresponding to 170,743 unique reads were obtained. Among the unique sequences, 22 nt small RNA was found abundant (Fig 4.2.).

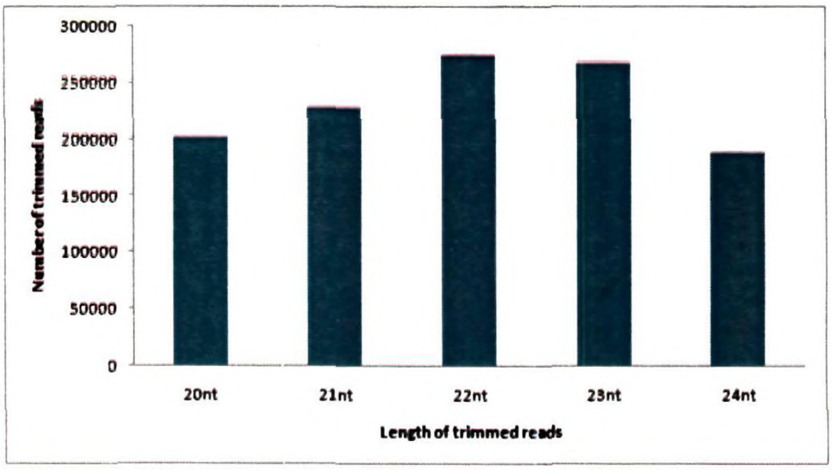


Fig. 4. 2. Length of small RNA sequences in cold treated *Hevea brasiliensis*

4.3.3. Identification of conserved and novel miRNAs

218 miRNAs belonging to 21 conserved miRNA families were identified, among which miR166 got highly expressed with an expression value of 17295 followed by miR159 and miR9386 with expression value of 1410 and 1377 respectively. The number of members varied among miRNA families with largest being the miR166 with 141 members followed by the miR159 with 45 members. Thirteen miRNA families were represented by only one member (Appendix 2, Table 2.1.). This study could identify a set of 13 conserved miRNAs which were not reported previously in miRBase (Release 21). The miRNAs identified and reported in this study are *viz.* miR2275, miR3630, niR399, miR4995, miR5021, miR535, miR5368, miR5658, miR7760, miR7782, miR827, miR858 and miR8175 (Table 4. 2).

Table 4.2. Cold-responsive miRNAs of *Hevea brasiliensis* and their putative targets

miRNA	Expression Value	Sequence	Target ID	Target name
miR9386	1377	UUUGCAGUUCGAAAGUGGAAGC	gi|164375933|gb|DB925992.1|DB925992	Phospholipase C 4 precursor,
			41674.m000014	putative[*Ricinus communis*]
				Aldehyde dehydrogenase family
miR159	1410	UUUGGAUUGAAGGGAGCUCUG		Not identified
miR166	17295	UCGGACCAGGCUUCAUUCCUC	isotig04051	DNA binding protein,
			contig10287	Homeobox-leucine zipper family protein / lipid-binding START domain-containing protein isoform 6 [*Theobroma cacao*]
miR171	96	AGAUAUUAGUGCGGUUCAAUC	contig509309	beta-1,3-galactosyltransferase 2 [*Jatropha curcas*]
miR2275	25	AGGAUUAGAGGGACUUGAACC		
miR3630	44	UGCAAGUGACGAUAUCAGACA	gi|164377982|gb|DB948752.1|DB948752	phospholipase d delta, putative [*Ricinus*

				communis]
miR399	132	UGCCAAAGGAGAUUUGCCCUG		Not identified
miR476	67	UAAUCCUUCUUUGCAAAGUC		Not identified
miR482	442	UCUUCCCUACUCCUCCCAUUCC	isotig03914 gi|164397563|gb|DB933070.1|DB933070	hypothetical protein arsenical pump-driving ATPase, putative [*Ricinus communis]*
miR4995	78	AGGCAGUGGCUUGGUUAAGGG		Not identified
miR5021	23	UGAGAAGAAGAAGAAGAAAA		Not identified
miR535	31	UGACAACGAGAGAGAGCACGC		Not identified
miR5368	601	GGACAGUCUCAGGUAGACA		Not identified
miR5658	14	AUGAUGAUGAUGAUGAUGAAA		Not identified
miR6173	164	AGCCGUAAACGAUGGAUACU		hypothetical protein [*Populus]*
miR7760	18	CAGCGGACAGAAUGGAGCAAGCAG		Not identified
miR7782	11	ACCUGCUCUGAUACCAUGUUGUGA		Not identified
miR8175	13	GAUCCCCGGCAACGGCGCCA		Not identified
miR827	204	UUAGAUGACCAUCAACAAACU		Not identified
miR858	36	UUCGUUGUCUGUUCGACCUUG	gi|164397853|gb|DB937467.1|DB937467	Myb domain protein 7

A large group of potential candidate novel miRNAs were also obtained based on database of *Hevea brasiliensis*, *Ricinus communis* and *Manihot esculenta* in the cold treated samples. Consequently, secondary structures were predicted for precursors of such candidate novel miRNAs by using m-Fold web server with default parameters. miRNA precursors possessing secondary structure with a free energy of equal or less than -25 kcal per mol were considered as novel miRNAs (Table 4.3.) (Appendix 2, Fig. 2.1.)

Table 4.3. Novel miRNAs identified from cold stressed *Hevea brasiliensis*

Species	No. of novel miRNAs
Hevea brasiliensis	18
Ricinus communis	7
Manihot esculenta	17

4.3.4. Targets for miRNAs

All 218 conserved miRNAs were searched for targets against ESTs or genes of Ricinus, *Hevea* and *Manihot*. Among the 218 conserved miRNAs, 203 miRNAs had 399 targets in *Ricinus*, 165 miRNA had 739 targets in *Hevea* and 14 miRNAs had 16 targets in *Manihot* (Appendix 2, Table 2.2.). Twenty six miRNA-target pairs were obtained for six novel miRNAs out of 18 in *Hevea brasiliensis* and five miRNA-target pairs were obtained for four novel miRNAs out of seven in *Ricinus communis* while 27 miRNA-target pairs were obtained for six novel miRNAs out of 17 in *Manihot esculenta* (Appendix 2, Table 2.3.).

4.3.5. Differential expression analysis of cold stressed and control samples

A total of 29 and 21 miRNA families were identified in control and cold stressed samples respectively. From the differential expression analysis, carried out by digital gene expression (DGE) method, eight miRNAs were found commonly expressed in both the samples (Fig. 4.3.). From this analysis, miR166 was found highly expressed in both the samples. miR159 and miR171 were found highly up-regulated in cold stressed than in control samples while miR482 were found more in control than cold stressed samples.

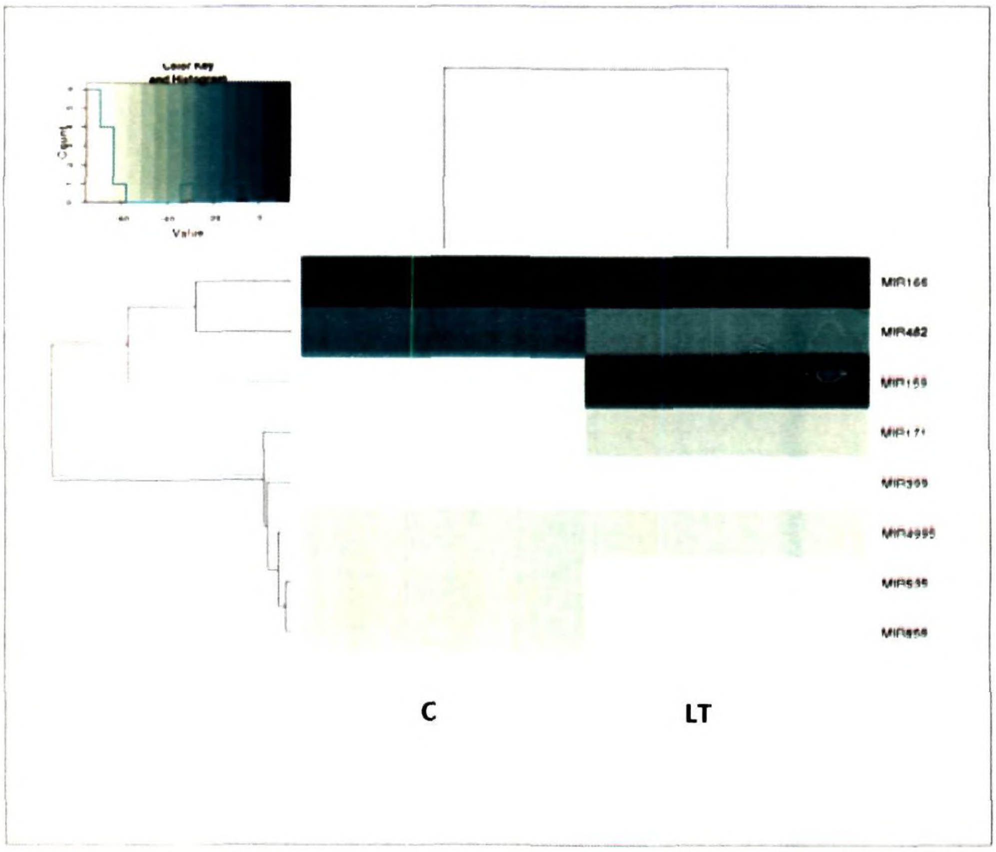


Fig. 4.3. Digital gene expression analysis of control (C) and low temperature (LT) stressed samples

4.3.6. Validation of miRNAs by qPCR

In order to reconfirm the DGE results of miRNAs, cold stress treated plants of clones RRII 105 and RRIM 600 were used for qPCR analysis of six conserved miRNAs (Fig.4.4.). The qPCR results were found matching with the deep sequencing results. miR166, miR159 and miR171 got up-regulated in tolerant clone while no significant change could be noticed in the susceptible one. Expression level of miR858 got reduced in RRII 105 while there was no significant change in RRIM 600. The expression of miR482 in RRIM 600 got significantly down regulated while there was no much change in clone RRII 105. Interestingly, expression of miR169 was found significantly reduced in tolerant clone while there was a significant up-regulation in susceptible clone.

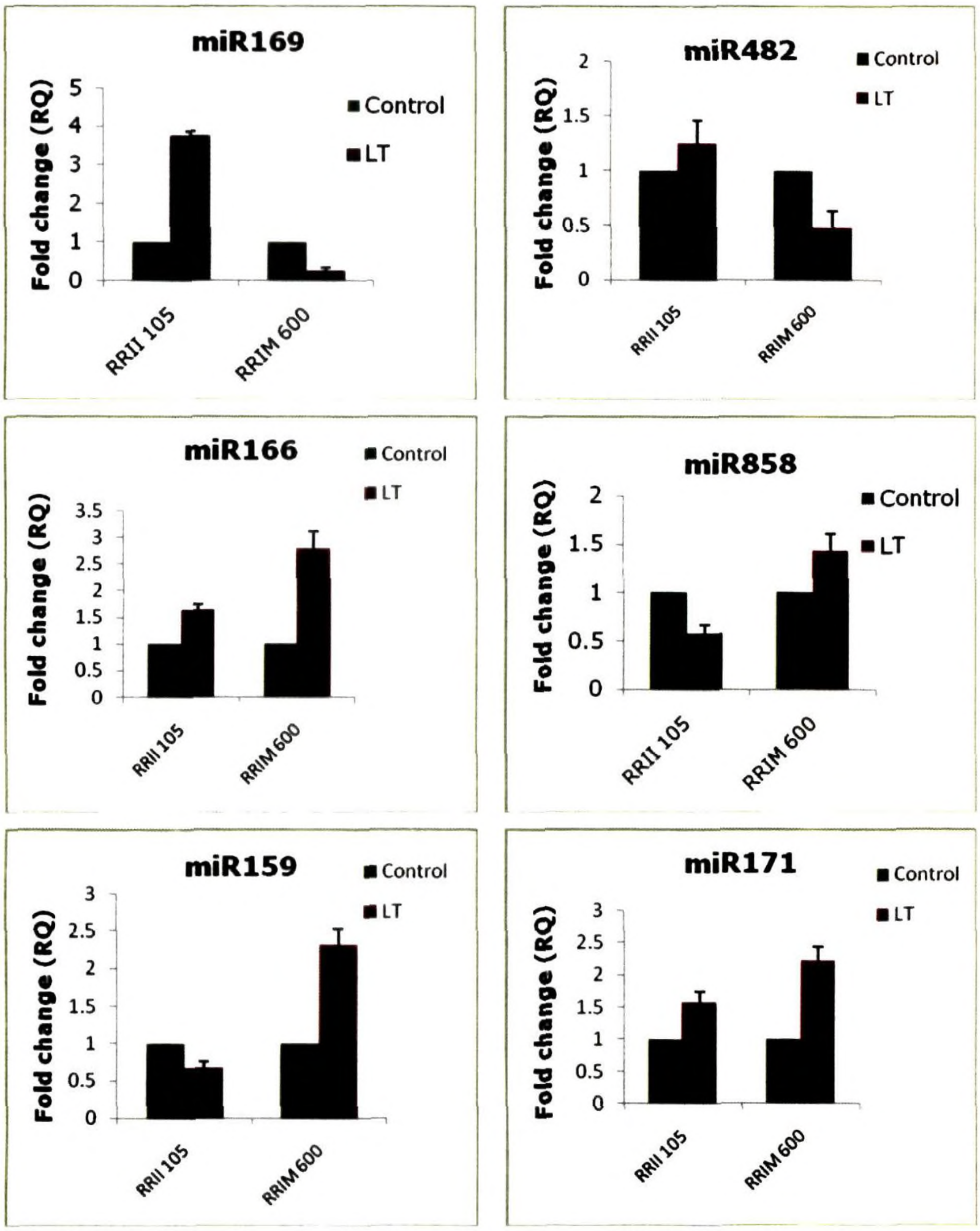


Fig. 4.4. Relative quantification of six miRNAs in cold stressed plants of *Hevea brasiliensis*. Error bars indicate standard error of three biological replicates.

4.4. Discussion

The plant's response to cold stress involves altering different metabolic pathways and regulation of genes involved in stress alleviation. During cold stress, photosynthetic processes are often primarily inhibited. In tropical trees, photo-assimilation occurs at an optimum level when the ambient temperature is between 15 and 45 °C (Sage and Kubien 2007). At temperatures below 15 °C, with high light intensity, the major components of photosynthetic apparatus get damaged due to the increase in the levels of reactive oxygen species (ROS). The plants that do not produce sufficient ROS scavenging enzymes may succumb to cold stress in the absence of protective mechanism to save the plants from photodamage (Foyer and Harbinson, 1994). Cold stress or cold damage to the plants or the cold tolerance of the cultivar is in general assessed by analysing chlorophyll fluorescence parameters along with net gas exchange data (Maxwell and Johnson 2000).

In general, clone RRIM 600 is known as cold tolerant and is being widely cultivated in the cold prone regions of North East India (Meti *et al.*, 2003; Reju *et al.*, 2003). High light during day time combined with cold stress in the previous nights during winter season lead to severe inhibition of photosynthesis and chlorophyll bleaching (Jacob *et al.*., 1999; Devakumar *et al.*, 2002; Ray *et al.*, 2004) in cold susceptible clones like RRII 105 whereas the cold tolerant clones like RRIM 600 were proven to display better photosynthesis and lesser membrane permeability. Alam *et al.*, (2003) reported that the percentage reduction in yield due to winter stress was lesser in clone RRIM 600 than PB 235. Mai *et al.*, (2010) compared eight *Hevea* clones for their tolerance towards cold stress and found clone RRIM 600 as the most tolerant clone. The results of the physiological parameters recorded in this study also indicated the effect of cold stress by way of reduction in stomatal conductance (gs), net CO_2 assimilation rate (A) and quantum efficiency of PS II (Fv/Fm) in both the clones. The severe reduction in gs and A observed in clone RRII 105 indicated its susceptible nature while the reduction was minimal in RRIM 600, indicating its inherent tolerance. The higher F_v/F_m ratio found in clone RRIM 600 under cold stress also supported its tolerance nature. The results obtained in this study are also in conformity with the previous reports of its field performance (Meti *et al.,* 2003; Reju *et al.,* 2003).

In order to identify cold responsive miRNAs from *Hevea,* small RNA libraries from plants subjected to cold stress were constructed and sequenced. High-throughput sequencing approach was employed to identify both the conserved and novel miRNAs. A total number of 1,162,006 clean reads corresponding to 170,743 unique reads were obtained. Following filtering, 218 miRNAs belonging to 21 conserved miRNA families were identified. When differential expression analysis was performed using DGE analysis in control and cold treated samples of clone RRIM 600, eight miRNAs were found common to both the cold treated and control samples. For estimating the expression levels of miRNAs, the abundance of miRNAs was treated as an index. miR159, miR171 and miR166 were found expressing significantly at higher levels in cold stressed than control samples. Expression of miR482 and miR535 were found significantly down-regulated under cold stress in clone RRIM 600.

When expression analyses were performed subsequently with six selected miRNAs in two *Hevea* clones *viz.* RRII 105 and RRIM 600, up-regulation of miR166 was noticed in tolerant clone RRIM 600 under cold stress. In *Solanum lycopersicum,* miR166 was found up regulated while its target HD-Zip III got suppressed under cold stress (Valiollahi *et al.,* 2014). Similarly in cotton too, under cold condition (4°C) the miR166 was found expressed at higher levels (Wang *et al.,* 2016) which have been predicted to negatively regulate its target HD-Zip III transcription factor. In this study also, HD-ZIP III transcription factor was predicted as its target. Probably, in *Hevea* also it might negatively regulate HD-ZipIII transcription factor under cold stress.

miR171 is a widely distributed and highly conserved miRNA family in plants which is known to play an important role in plant growth and development by regulating the expression of *SCARECROW-LIKE* (SCL) transcription factors. In the present study the expression of miR171 got induced in tolerant clone RRIM 600 while there was no significant change in RRII 105. Similar trend had been reported in tea in which miR171 family was found significantly up-regulated in cold tolerant cultivar whereas it got down-regulated in cold sensitive cultivar (Zhang *et al.,* 2014). In *Arabidopsis,* miR171 has also been reported to target *SCL6-II*, *SCL6-III*, and *SCL6-IV* (*SCL6*) which play important roles in plant root and leaf development, gibberellin response, photochrome signalling, lateral organ polarity, meristem formation, vascular development, and stress response (Llave *et al.,* 2002a, 2002b; Lee *et al.,* 2008; Wang *et al.,* 2010). SCLs play an important role in suppressing chloroplast development in dividing cells during early leaf growth (Ma *et al.,* 2014). In *Hevea,* the target prediction performed using TAPIR, indicated the probability of β-1,3-galactosyltransferase 2 as its target. In *Arabidopsis,* it has been reported to be involved in synthesis of hemicellulose which are basic components of cell wall synthesis and had been reported to be down-regulated under water deficit conditions (Bray 2004). It may be presumed that the up-regulation of miR171 in the tolerant clone might be directly involved in suppression of its target β-1,3-galactosyl transferase.

miR169 regulates the expression of sub-unit A of NF-Y in many plants (Rhoades *et al.,* 2002; Ni *et al.,* 2013) which in turn play key roles in development and is expressed in response to adverse environmental conditions like drought, cold, salinity, ABA, etc. (Lee *et al.,* 2003). miR169 which had been found associated with drought tolerance from our previous study was also included in this analysis. Its expression was significantly reduced in tolerant clone RRIM 600 while there was a significant up-regulation in susceptible clone RRII 105, thus confirming its role in cold tolerance also.

Expression of miR169 has also been reported to be at higher levels under cold stress in other plants like *Arabidopsis* (Sunkar and Zhu, 2004), *Brachypodium* (Zhang *et al.*, 2009), etc. Over-accumulation of miR169 under cold stress was found correlated with reduction in NF-YA transcripts in *Arabidopsis* (Zhou *et al.*, 2008; Lee *et al.*, 2010). The lower levels of miR169 found in tolerant clone RRIM 600 in this study might be involved in positively regulating the accumulation of its target NF-YA thus contributing for cold tolerance.

MIR482 is a highly diverse miRNA gene that has been found ubiquitously distributed across gymnosperm, monocot, and dicot plants (Zhao *et al.*, 2012). In *Hevea* it has been reported to target abiotic stress responsive Abscisic Acid Responsive Element Binding Protein 2 (AREB2) (Lertpanyasampatha *et al.*, 2012; Arenas-Huertero *et al.*, 2009). The expression of miR482 was found significantly reduced in tolerant clone RRIM 600 while there was no much change in RRII 105. The down-regulation in tolerant clone might be indirectly promoting the function of its target gene AREB2 which in turn might be imparting cold tolerance in clone RRIM 600.

miR159 is one of the most conserved miRNAs in land plants (Reinhart *et al.*, 2002). In this study, miR159 was found expressed at higher levels in RRIM 600 under cold conditions while there was no change in RRII 105. In tea, miR159 was reported to be down-regulated in cold-sensitive cultivar (Zhang *et al.*, 2014). In *Hevea*, miR159 was predicted to target genes involved in rubber biosynthesis, antioxidant activity and transcription regulation (Gebelin *et al.*, 2012, 2013b). Expression of *HbMIR159a* in leaves and roots was found antagonistic. In leaves, *HbMIR159* genes displayed a significant up-regulation while in root it displayed a significant down regulation in response to cold stress (Gebelin *et al.*, 2013a). Hence based on the available reports, it could be presumed that up-regulation of miR159 under cold stress condition might possibly suppress its target genes associated with rubber biosynthesis, antioxidant activity and transcription regulation.

Reduction in the levels of miR858 was found in clone RRII 105 while there was no significant change in RRIM 600. In this study, its target was predicted as MYB transcription factor. miR858 has been found to regulate the homologous *MYB2* gene during both *Arabidopsis* trichome and cotton fibre development (Guan, 2014). However the results of this study did not show any consistent trend with either cold susceptibility or tolerance.

4.5. Conclusion

The cold responsive small RNA data of *H. brasiliensis* generated on Illumina platform revealed the expression of 21 conserved miRNA families and 42 novel miRNAs. The gene expression analysis indicated the distinct association between miR169 and cold tolerance. miR169 has been found to regulate its target NF-YA which is known to play main role in imparting abiotic stress tolerance in many plants. miR482 which targets AREB2, a known stress responsive factor and miR159 which targets a set of cold stress and rubber biosynthesis related genes were also found to have stronger association with cold tolerance. Though this study could identify miRNAs associated with cold tolerance from two contrasting clones, it would be more appropriate to carryout further validation experiments in more number of clones with wide range of tolerance/susceptibility levels to identify miRNAs that would have stronger association with cold tolerance in order to use as markers in the crop improvement programmes.

Chapter 5

Summary and Conclusions

MicroRNAs are an extensive class of endogenous small non-coding single stranded RNAs that are found in almost all eukaryotes. Recent advancements on miRNA research have revealed their significant role in regulation of numerous developmental and stress responsive pathways in plants. They regulate gene expression either through post-transcriptional degradation or translational repression of their target mRNAs in a sequence specific manner. Mostly, targets of miRNAs encode various transcriptional factors or functional enzymes that are having important role in abiotic stress response. Several studies have confirmed the changes in their expression levels under abiotic stress conditions in plants. In recent years, due to the advent of high throughput sequencing and computational approaches, a large number of stress-related miRNAs have been identified. Various studies conducted on these miRNAs indicated the possibility of employing them as potential biomarkers in developing abiotic stress tolerant plants.

Hevea brasiliensis is the primary source of natural rubber. Drought and cold stresses are the most significant environmental stress factors that restrict the expansion of rubber cultivation to non-traditional areas in India. Genotypes which can withstand such extreme climatic conditions without compromising on yield and productivity have to be identified or developed through breeding techniques. However, lack of methods for early evaluation of stress tolerance of the newly developed clones and the extensive time required for assessing their tolerance in the original field conditions are the major constraints for clonal selection. Hence, this study was conducted to identify drought and cold responsive miRNAs from *H. brasiliensis* and to select miRNAs associated with drought/cold tolerance which can eventually be used as markers for selection of clones for abiotic stress tolerance.

In this study, identification of drought responsive miRNAs from *Hevea* was performed by both conventional and next generation sequencing methods. By conventional method, isolation, cloning and sequencing of miRNAs led to the identification of four conserved and one novel miRNAs. The next generation sequencing (Illumina HiSeq) method revealed expression of 33 conserved miRNA families and 32 novel miRNAs in the drought treated and control samples altogether. The secondary structures of novel miRNAs were also predicted computationally followed by prediction of targets of both conserved and novel miRNAs which could reveal transcription factors and stress responsive genes as targets. By digital gene expression analysis, miRNAs that are specifically expressed under drought condition and irrigated control were identified. Among the differentially expressed miRNAs identified, a set of drought responsive miRNAs were selected and subjected to quantitative expression analysis in irrigated and drought imposed plants of *Hevea* clones with varying levels of drought tolerance. Two novel miRNAs (HbmiRn_63 and HbmiRn_42) as well as two conserved miRNAs (miR168 and miR160) were found to have much stronger association with drought tolerance. When expression of three selected miRNAs (miR160, miR168 and HbmiRn_42) was validated in known drought tolerant and susceptible clones as well as in germplasm accessions, the results corroborated with their tolerance/ susceptibility evaluated based on biochemical parameters. The target prediction and miRNA-target expression analysis of selected miRNAs and their putative targets performed to assess their relationship indicated the existence of significant correlation between HbmiRn_42 and its target *HMGR3*.

Identification of cold responsive miRNAs performed by next generation sequencing revealed the expression of 21 conserved miRNA families and 42 novel miRNAs in cold treated samples. Targets of both conserved and novel miRNAs were predicted. From the differential expression analysis eight miRNAs were found common to both cold and control samples. When expression analyses of selected miRNAs were performed in cold tolerant and cold susceptible clones of *Hevea*, miR169 was found to have stronger association with cold tolerance. miR482 and miR159 were the other miRNAs that were found associated with cold tolerance.

This study could reveal a set of miRNAs (HbmiRn_63, HbmiRn_42, miR168 and miR160) strongly associated with drought tolerance in *H.brasiliensis* which can be used as markers for early selection for drought tolerance. The study also revealed miRNAs (miR169, miR482 and miR159) that are strongly associated with cold tolerance which could be used as markers for cold tolerance. By identifying a set of drought and cold tolerance associated miRNAs, this investigation opens up the possibility of employing them further in crop improvement programmes of *Hevea brasiliensis*.

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APPENDICES

Appendix 1- Identification of drought responsive miRNAs from *Hevea brasiliensis*

Table.1.1 Conserved miRNAs in of *Hevea brasiliensis*

Table 1.1. Conserved miRNAs in control samples

miRNA family	Expression value (Reads)	Sequence (5'-3')	length (nt)	miRNA
MIR166	916	TCGGACCAGGCTTCATTCCCC	21	Pvu-MIR166a
		TCGGACCAGGCTTCATTCCCCC	22	Ctr-MIR166
		GGAATGTTGTCTGGCTCGAGG	21	Aly-MIR166a
		GGAATGTTGTCTGGCTCGAGG	21	Aly-MIR166c
		GGAATGTTGTCTGGCTCGAGG	21	Aly-MIR166d
		TCGGACCAGGCTTCATTCCCT	21	Csi-MIR166d
MIR482	248	AGATGGGTGGCTGGGCAAGAAG	22	Hbr-MIR482
MIR167	39	TGAAGCTGCCAGCATGATCTG	21	Csi-MIR167a
		TGAAGCTGCCAGCATGATCTG	21	Csi-MIR167c
		TGAAGCTGCCAGCATGATCTGA	22	Bdi-MIR167c
		TGAAGCTGCCAGCATGATCTGA	22	Bdi-MIR167d
		TGAAGCTGCCAGCATGATCTGA	22	Bdi-MIR167e
MIR396	30	TTCCACAGCTTTCTTGAACTG	21	Aau-MIR396
		TCCACAGGCTTTCTTGAACTG	21	Bdi-MIR396a
		TTCCACAGCTTTCTTGAACTT	21	Aca-MIR396b
		TTCCACAGCTTTCTTGAACT	20	Vvi-MIR396b
MIR156	22	TGACAGAAGATAGAGAGCAC	20	Aca-MIR156b
		TGACAGAAGAGAGTGAGCAC	20	Aly-MIR156d
		TGACAGAAGAGAGTGAGCAC	20	Cme-MIR156d

		TGACAGAAGAGAGCGAGCAC	20	Sbi-MIR156e
		TTGACAGAAGAGAGTGAGCAC	21	Gma-MIR156k
		TTGACAGAAGAGAGTGAGCAC	21	Gma-MIR156n
		TTGACAGAAGAGAGTGAGCAC	21	Gma-MIR156o
MIR535	21	TGACAACGAGAGAGAGCACGT	21	Mes-MIR535a
		TGACAACGAGAGAGAGCACGG	21	Mes-MIR535b
		TGACGACGAGAGAGAGCACGC	21	Ppe-MIR535b
		TGACGACGAGAGAGAGCACGC	21	Mdo-MIR535d
MIR397	20	ATTGAGTGCAGCGTTGATGAA	21	Bdi-MIR397b
MIR393	19	TCCAAAGGGATCGCATTGATCT	22	Ghi-MIR393
		TCCAAAGGGATCGCATTGATCC	22	Cpa-MIR393
		TCCAAAGGGATCGCATTGATC	21	Bdi-MIR393a
		TCCAAAGGGATCGCATTGATC	21	Bdi-MIR393b
		TTCCAAAGGGATCGCATTGATC	22	Gma-MIR393h
		TTCCAAAGGGATCGCATTGATC	22	Gma-MIR393i
		TTCCAAAGGGATCGCATTGATC	22	Gma-MIR393j
		TTCCAAAGGGATCGCATTGATC	22	Gma-MIR393k
MIR390	18	AAGCTCAGGAGGGATAGCGCC	21	Aly-MIR390a
		AAGCTCAGGAGGGATAGCGCC	21	Aly-MIR390b
		AAGCTCAGGAGGGATAGCGCC	21	Bdi-MIR390a
MIR2916	16	TGGGGACTCGAAGACGATCATAT	23	Peu-MIR2916
MIR858	15	TTCGTTGTCTGTTCGACCTGA	21	Mdo-MIR858
		TTTCGTTGTCTGTTCGACCTT	21	Aly-MIR858
MIR4995	13	AGGCAGTGGCTTGGTTAAGGG	21	Gma-MIR4995
MIR1310	10	AGGCATCGGGGGCGCAACGCCC	22	Han-MIR1310
MIR7767	9	CCCCAAGCTGAGAGCTCTCCC	21	Bdi-MIR7767

MIR6445	7	TTCATTCCTCTTCCTAAAATGG	22	Ptr-MIR6445a
		TTCATTCCTCTTCCTAAAATGG	22	Ptr-MIR6445b
MIR6478	7	CCGACCTTAGCTCAGTTGGTG	21	Ptr-MIR6478
MIR157	6	TTGACAGAAGATAGAGAGCAC	21	Aly-MIR157a
		TTGACAGAAGATAGAGAGCAC	21	Aly-MIR157b
		TTGACAGAAGATAGAGAGCAC	21	Aly-MIR157c
MIR159	6	TTTGGATTGAAGGGAGCTCTG	21	Hvu-MIR159b
MIR169	6	GAGCCAAGAATGACTTGCCGA	21	Csi-MIR169
MIR399	5	TGCCAAAGGAGAGTTGCCCTG	21	Pvu-MIR399a
		TGCCAAAGGAGAATTGCCCTG	21	Csi-MIR399e
MIR894	4	CGTTTCACGTCGGGTTCACC	20	Ppa-MIR894
MIR171	2	TTGAGCCGCGTCAATATCTCC	21	Ctr-MIR171
MIR395	2	CTGAAGTGTTTGGGGGAACTC	21	Lus-MIR395d
MIR1425	1	TAGGATTCAATCCTTGCTGCT	21	Osa-MIR1425
MIR1432	1	ATCAGGAGAGATGACACCGAC	21	Osa-MIR1432
MIR164	1	TGGAGAAGCAGGGCACGTGCA	21	Aly-MIR164a
MIR168	1	TCGCTTGGTGCAGATCGGGAC	21	Bdi-MIR168
MIR3627	1	TCGCAGGAGAGATGGCACTGTC	22	Ppe-MIR3627
MIR444	1	TGCAGTTGTTGTCTCAAGCTT	21	Osa-MIR444b

Table 1.1.2. Conserved miRNAs in drought stressed samples

miRNA family	Expression value (Reads)	Sequence (5'-3')	length (nt)	miRNA
MIR858	2	TTTCGTTGTCTGTTCGACCTT	21	Aly-MIR858
		TTCGTTGTCTGTTCGACCTGA	21	Mdo-MIR858
MIR1425	1	TAGGATTCAATCCTTGCTGCT	21	Osa-MIR1425
MIR156	7	TTGACAGAAGAGAGTGAGCAC	21	Gma-MIR156k
		TTGACAGAAGAGAGTGAGCAC	21	Gma-MIR156o
		GTTGACAGAAGAGAGTGAGCAC	22	Cme-MIR156j
		TTGACAGAAGAGAGTGAGCAC	21	Gma-MIR156n
MIR157	8	TTGACAGAAGATAGAGAGCAC	21	Aly-MIR157a
		TTGACAGAAGATAGAGAGCAC	21	Aly-MIR157b
		TTGACAGAAGATAGAGAGCAC	21	Aly-MIR157c
MIR159	5	TTTGGATTGAAGGGAGCTCTG	21	Hvu-MIR159b
MIR160	5	TGCCTGGCTCCCTGTATGCCA	21	Aca-MIR160b
		TGCCTGGCTCCCTGAATGCCA	21	Ahy-MIR160
		TGCCTGGCTCCCTGAATGCCA	21	Htu-MIR160a
MIR164	8	TGGAGAAGCAGGGCACGTGCA	21	Aly-MIR164a
		TGGAGAAGCAGGGCACGTGCA	21	Aly-MIR164b
MIR166	1756	TCGGACCAGGCTTCATTCCCC	21	Pvu-MIR166a
		TCGGACCAGGCTTCATTCCCCC	22	Ctr-MIR166
		TCGGACCAGGCTTCATTCCCT	21	Csi-MIR166d
		GGAATGTTGTCTGGCTCGAGG	21	Aly-MIR166d

		GGAATGTTGTCTGGCTCGAGG	21	Aly-MIR166a
		GGAATGTTGTCTGGCTCGAGG	21	Aly-MIR166c
MIR167	29	TGAAGCTGCCAGCATGATCTGA	22	Bdi-MIR167c
		TGAAGCTGCCAGCATGATCTGA	22	Bdi-MIR167d
		TGAAGCTGCCAGCATGATCTGA	22	Bdi-MIR167e
		TGAAGCTGCCAGCATGATCTG	21	Csi-MIR167c
		TGAAGCTGCCAGCATGATCTG	21	Csi-MIR167a
MIR168	11	TCGCTTGGTGCAGATCGGGAC	21	Bdi-MIR168
		TCGCTTGGTGCAGGTCGGGA	20	Cme-MIR168
		TCGCTTGGTGCAGGTCGGGAA	21	Aly-MIR168a
		TCGCTTGGTGCAGGTCGGGAA	21	Aly-MIR168b
MIR169	1	GAGCCAAGAATGACTTGCCGA	21	Csi-MIR169
MIR171	4	TTGAGCCGCGTCAATATCTCC	21	Ctr-MIR171
MIR2118	1	GAAATGGGTGGATGGGAGTGA	21	Hbr-MIR2118
MIR2916	10	TGGGGACTCGAAGACGATCATAT	23	Peu-MIR2916
MIR3627	4	TCGCAGGAGAGATGGCACTGTC	22	Ppe-MIR3627
MIR390	15	AAGCTCAGGAGGGATAGCGCC	21	Aly-MIR390a
		AAGCTCAGGAGGGATAGCGCC	21	Aly-MIR390b
MIR393	9	TCCAAAGGGATCGCATTGATCT	22	Ghi-MIR393
		TCCAAAGGGATCGCATTGATC	21	Bdi-MIR393a
MIR395	17	CTGAAGTGTTTGGGGGAACTC	21	Lus-MIR395d
MIR396	25	TTCCACAGCTTTCTTGAACTG	21	Aau-MIR396

		TTCCACAGCTTTCTTGAACT	20	Vvi-MIR396b
		TCCACAGGCTTTCTTGAACTG	21	Bdi-MIR396b
		TCCACAGGCTTTCTTGAACTG	21	Bdi-MIR396a
		TTCCACAGCTTTCTTGAACTT	21	Aca-MIR396b
MIR397	26	ATTGAGTGCAGCGTTGATGAA	21	Bdi-MIR397b
MIR399	4	TGCCAAAGGAGAATTGCCCTG	21	Csi-MIR399e
MIR444	2	TGCAGTTGTTGTCTCAAGCTT	21	Osa-MIR444b
		TGCAGTTGTTGTCTCAAGCTT	21	Osa-MIR444c
MIR482	266	AGATGGGTGGCTGGGCAAGAAG	22	Hbr-MIR482
MIR4995	20	AGGCAGTGGCTTGGTTAAGGG	21	Gma-MIR4995
MIR528	2	TGGAAGGGGCATGCAGAGGAG	21	Bdi-MIR528
MIR535	22	TGACGACGAGAGAGAGCACGC	21	Ppe-MIR535b
		TGACGACGAGAGAGAGCACGC	21	Mdo-MIR535d
		TGACAACGAGAGAGAGCACGT	21	Mes-MIR535a
MIR6445	5	TTCATTCCTCTTCCTAAAATGG	22	Ptr-MIR6445b
		TTCATTCCTCTTCCTAAAATGG	22	Ptr-MIR6445a
MIR6476	1	TCAGTGGAGATGAAACATGA	20	Ptr-MIR6476c
MIR6478	15	CCGACCTTAGCTCAGTTGGTG	21	Ptr-MIR6478
MIR7767	14	CCCCAAGCTGAGAGCTCTCCC	21	Bdi-MIR7767
MIR894	3	CGTTTCACGTCGGGTTCACC	20	Ppa-MIR894
MIR1310	4	AGGCATCGGGGGCGCAACGCCC	22	Han-MIR1310

Table 1.2. Novel miRNAs in control and drought stressed samples

Table 1.2.1. Novel miRNAs in control samples

Novel miRNAs in Ricinus				
mature_miRNA_id	MFE	mature_miRNA	miRNA length	Pri-miRNA
HbmiRn_ 1	-30.4	TTCAAATCTGGTTCCTGGCACA	22	GTTGAAAATGGTCGGGATAGCTCAGCTGGTAGAGCAGAGGACTGAAAATCCTCGTGTCACCAGTTCAAATCTGGTTCCTGGCACATGATTAATTT
HbmiRn _5	-25.9	ATGGTACTTACTTTCATACAGG	22	CCTTAACGGGATGGTACTTACTTTCATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTTGGTCAAGTCCTATAACGAGC
Novel miRNAs in Manihot				
mature_miRNA_id	MFE	mature_miRNA	miRNA length	Pri-miRNA
HbmiRn _18	-27.6	TTCAAATCTGGTTCCTGGCATA	22	GTCGGAAATGGTCGGGATAGCTCAGCTGGTAGAGCAGAGGACTGAAAATCCTCGTGTCACCAGTTCAAATCTGGTTCCTGGCATATGATTAATAT
HbmiRn _20	-34.14	GGGATTGTAGTTCAATTGGTCAGA	24	TTACCGCCCCGGGATTGTAGTTCAATTGGTCAGAGCACCGCCCTGTCAAGGCGGAAGCTGCGGGTTCGAGCCCCGTCAGTCCCGACGGATCCAA
HbmiRn _26	-29.9	AACCGGGACGTGGCGGCTGACGGC	24	ACGAGGTGCGAACCGGGACGTGGCGGCTGACGGCGACGTTAGGGAGTCCGGAGACGTCGGCGGGGGCCTCGGGAAG
HbmiRn _28	-38.4	GTCGCGGTTCCACATCCGACCGG	23	CGCTGGAATCCGCGTCGGTCCGCGGCCCGAGCCGATCGGTGAACCGGCTAGTCGCGGTTCCACATCCGACCGGATGCAGAATT
HbmiRn _42	-30.7	CCAGGCGTCGGCCAGCGGGCTC	22	CCCGTGCACTCCAGGCGTCGGCCAGCGGGCTCTCCATTCAGCCCGTCTTGAAACATGGACCAAGGAGTCTGACATGTGTGCGAGTCAA
HbmiRn _48	-38.5	AGGAGGGCGCGGCGGTCGCTGCA	23	CCCGATGAGTAGGAGGGCGCGGCGGTCGCTGCAAAACCTGGGG

				CGCGAGCCCGGCGGAGCGGCCGTCGGTGCACATCTTGGTG
HbmiRn _64	-31	CCGGCCCCCAAAGGCACGTGCCGT	24	GCCAGAAGCGACGCCTGTGTCCGCCGCCCATTTGCCGACCCTCAGTAGGGGCAGTCCGGCCCCCAAAGGCACGTGCCGTTGGCCAAGCC
HbmiRn _46	-30.9	GCCAGGCCCCGATGAGTAGGAGG	23	ATGGATGGCGCTTAAGCGCGCGACCTATACTCGGCCGTTAGGGCAAGAGCCAGGCCCCGATGAGTAGGAGGGCGCGGCGGT
Novel miRNAs in Hevea				
mature_miRNA_id	**MFE**	**mature_miRNA**	**miRNA length**	**Pri-miRNA**
HbmiRn _11	-31.2	CGCTTCTGGCCCGGATTCTGACTT	24	CCTACTGCGGGTCGGCAAGCGGGCGCCGGACACGGGCGTCGCTTCTGGCCCGGATTCTGACTTAGAGGCGTTC
HbmiRn _20	-30.15	GCTCGGGACGTCTGACAATGGGGG	24	GCTGGGCGGAGGAAGAAGGCTGTCCCGGCTCACCTTTGCCGATTCCGACTTCGGGAACGCGCTCGGGACGTCTGACAATGGGGGCTAGCCAAAA
HbmiRn _31	-26.9	TTCAAATCTGGTTCCTGGCATT	22	GTCGAAAATGGTCGGGATAGCTCAGCTGGTAGAGCAGAGGACTGAAAATCCTCGTGTCACCAGTTCAAATCTGGTTCCTGGCATTGTGTATGAGT
HbmiRn _32	-26.9	TTCAAATCTGGTTCCTGGCATT	22	GTCGAAAATGGTCGGGATAGCTCAGCTGGTAGAGCAGAGGACTGAAAATCCTCGTGTCACCAGTTCAAATCTGGTTCCTGGCATTGTGTATGAGT
HbmiRn _48	-28.6	CAGGACTCGAGGAAGAAGCCCC	22	GCGATCATGACAGGACTCGAGGAAGAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAAGACGGGGGGGGCAAGTGTTCTTCGGA
HbmiRn _49	-28.6	CAGGACTCGAGGAAGAAGCCCC	22	GCGATCATGACAGGACTCGAGGAAGAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAAGACGGGGGGGGCAAGTGTTCTTCGGA
HbmiRn _50	-35.7	TCGGATCGCGGCGACGTGGGC	21	GGTGAAGTGTTCGGATCGCGGCGACGTGGGCGGTTCGCCGCCGGCGACGTCGCGAGAAGTCCACTG

Table 1.2.2. Novel miRNAs in drought stressed samples

Novel miRNAs in Ricinus				
mature_miRNA_id	MFE	mature_miRNA	miRNA length	Pri-miRNA
HbmiRn _6	-30.4	TTCAAATCTGGTTCCTGGCACA	22	GTTGAAAATGGTCGGGATAGCTCAGCTGGTAGAGCAGAGGACTGAAAATCCTCGTGTCACCAGTTCAAATCTGGTTCCTGGCACATGATTAATTT
HbmiRn _12	-26.3	AAGGTAGGCTCAAGCTAAGATTC	23	ACAATATGAGGTGGGCAGTTTATCTGGGGCGGATGCCTCCTAAAGAGTAACAGAGGTGTGTGAAGGTAGGCTCAAGCTAAGATTCTGCTCGTGAG
HbmiRn _13	-32.2	TTCGAGCCCCGTCAGTCCCGAC	22	TAATTTTCCTGGGATTGTAGTTCAATCGGTCAGAGCACCGCCCTGTCAAGGCGGAAGCTGCGGGTTCGAGCCCCGTCAGTCCCGACGGATCAAAAT
HbmiRn _15	-31.32	GAATGACTGGGCGTAAAGGGCA	22	GGCTAACTCCGTGCCAGCAGCCGCGGTAAGACGGGGGGGCAAGTGTTCTTCGGAATGACTGGGCGTAAAGGGCACGTAGGCGGT
Novel miRNAs in Manihot				
mature_miRNA_id	MFE	mature_miRNA	miRNA length	Pri-miRNA
HbmiRn_5	-29.7	CATGCTGATCTTCCCCAAGAGCTC	24	CTAGAGATAACATGCTGATCTTCCCCAAGAGCTCACATCGATGGGAAGGTTTGGCACTTCGATGTCTGCTCTTCGCCACCTGGGGCTGTAGTATG
HbmiRn _6	-25.3	TTATCATTACGATAGGTGTCAAG	23	GATGAGCCGTTTATCATTACGATAGGTGTCAAGTGAAAGTGCAGTGATGTATGCAGCTGAGGCATCCTAACATATTGATAGACT
HbmiRn_14	-27.6	TTCAAATCTGGTTCCTGGCAT	21	GTCGGAAATGGTCGGGATAGCTCAGCTGGTAGAGCAGAGGAC

				TGAAAATCCTCGTGTCACCAGTTCAAATCTGGTTCCTGGCATATGATTAATA
HbmiRn _20	-29.9	AACCGGGACGTGGCGGCTGACGGC	24	ACGAGGTGCGAACCGGGACGTGGCGGCTGACGGCGACGTTAGGGAGTCCGGAGACGTCGGCGGGGGCCTCGGGAA
HbmiRn_33	-30.7	CCAGGCGTCGGCCAGCGGGCTC	22	CCCGTGCACTCCAGGCGTCGGCCAGCGGGCTCTCCATTCAGCCCGTCTTGAAACATGGACCAAGGAGTCTGACATGTGTGCGAGTCAACGG
HbmiRn_35	-26.44	CGAAGCTACTGTGCGCTGGATTAT	24	ACCGTTGATTCGCACAATTGATCATCGCGCTTGGTTGAAAAGCCAGTGGCGCGAAGCTACTGTGCGCTGGATTATGACTGAACGC
HbmiRn_43	-31.1	GACGGGGTATTGTAAGTGGCAGA	23	TTAAATACGCGACGGGGTATTGTAAGTGGCAGAGTGGCCTTGCTGCCACGATCCACTGAGATTCAGCCCTTTGTCGCTTCGATT
HbmiRn _53	-38.09	GGCGGATGTAGCCAAGTGGATCAA	24	TAGTAGTAGGGGCGGATGTAGCCAAGTGGATCAAGGCAGTGGATTGTGAATCCACCATGCACGGGTTCAATTCCCGTCATTCGCCCACCTATTAT
Novel miRNAs in Hevea				
mature_miRNA_id	**MFE**	**mature_miRNA**	**miRNA length**	**Pri-miRNA**
HbmiRn_10	-29.6	CCGAGGAGGGGCTTGCGTCTGAT	23	CCGAGGAGGGGCTTGCGTCTGATTAGCTAGTTGGTGAGGCAATAGCTTACCAAGGCGATGATCAGTAGCTGGTCCGAGAGGATGAT
HbmiRn _19	-34.1	AGCGCGCGACCTATACCCGGCCG	23	ATGGCGCTTAAGCGCGCGACCTATACCCGGCCGTCGGGGCAAGAGCCAGGCCCCGATGAGTAGGAGGGCGCGGCG
HbmiRn_21	-34.1	AAGCGCGCGACCTATACCCGGCCG	24	GATGGCGCTTAAGCGCGCGACCTATACCCGGCCGTCGGGGCAAGAGCCAGGCCCCGATGAGTAGGAGGGCGCGGCG
HbmiRn_28	-42.1	GAATAGTACTTCAAGGCGGCCCGC	24	CGCCCGGTGGGAATAGTACTTCAAGGCGGCCCGCGCGGCTCGTCCGCCGCGGGGGCTTGGCCAACGGCACGTGCCTCTGG

				GG
HbmiRn_37	-26.9	TTCAAATCTGGTTCCTGGCAT	21	GTCGAAAATGGTCGGGATAGCTCAGCTGGTAGAGCAGAGGACTGAAAATCCTCGTGTCACCAGTTCAAATCTGGTTCCTGGCATTGTGTATGAG
HbmiRn_38	-33.39	GGCGGATGTAGCCAAGTGGATCAA	24	TAGTATTAGGGGCGGATGTAGCCAAGTGGATCAAGGCAGTGGATTGTGAATCCACCATGCGCGGGTTCAATTCCCGTCATTCGCCCATAGTCATA
HbmiRn_39	-33.39	GGCGGATGTAGCCAAGTGGATCAA	24	TAGTATTAGGGGCGGATGTAGCCAAGTGGATCAAGGCAGTGGATTGTGAATCCACCATGCGCGGGTTCAATTCCCGTCATTCGCCCATAGTCATA
HbmiRn_40	-33.39	GGCGGATGTAGCCAAGTGGATCAA	24	TAGTATTAGGGGCGGATGTAGCCAAGTGGATCAAGGCAGTGGATTGTGAATCCACCATGCGCGGGTTCAATTCCCGTCATTCGCCCATAGTCATA
HbmiRn_41	-33.39	GGCGGATGTAGCCAAGTGGATCAA	24	TAGTATTAGGGGCGGATGTAGCCAAGTGGATCAAGGCAGTGGATTGTGAATCCACCATGCGCGGGTTCAATTCCCGTCATTCGCCCATAGTCATA
HbmiRn_51	-38.2	TCGACTGTTCGACGCCCGGGGA	22	CAGTTCTGAGTCGACTGTTCGACGCCCGGGGAAGGCCCCCGGAGGGGCCGTTCCCAGTCCGTCCCCCGGCCGGCACGCGGCG
HbmiRn_60	-31.32	GAATGACTGGGCGTAAAGGGCA	22	GGCTAACTCCGTGCCAGCAGCCGCGGTAAGACGGGGGGGCAAGTGTTCTTCGGAATGACTGGGCGTAAAGGGCACGTAGGCGGT
HbmiRn_63	-31.32	GAATGACTGGGCGTAAAGGGCA	22	GGCTAACTCCGTGCCAGCAGCCGCGGTAAGACGGGGGGGCAAGTGTTCTTCGGAATGACTGGGCGTAAAGGGCACGTAGGCGGT
HbmiRn_65	-29.54	GACACCGCCCGTCGCTCCTACCGA	24	CCTCGTTGAAGACACCGCCCGTCGCTCCTACCGATTGAATGGTCCGGTGAAGTGTTCGGATCGCGGCGACGTGGGCGGT

Table 1.3. Targets of conserved miRNAs

Table 1.3.1 Targets of conserved miRNAs in *Hevea brasiliensis*

miRNA Id	Target Id	Protein name	Alignment
MIR1310	isotig20755	ribulose-5-phosphate-3-epimerase, putative [Ricinus communis]	miRNA 20 CGCAACGCGGGGGCUACGGA 1 ::::: .:::.:::::::: Target 858 GCGUUCUGCCUCCGAUGCCA 877
MIR156	isotig08477	azetidine-2-carboxylic acid resistant 1 family protein	miRNA 20 ACGAGUGAGAGAAGACAGUU 1 ::::::::::: :::::.:: Target 2578 GGCUCACUCUCUCCUGUUAA 2597
MIR157	isotig08459	Myosin-9, putative [Ricinus communis]	miRNA 20 ACGAGAGAUAGAAGACAGUU 1 : :::: :::.::::::.:: Target 2860 UCCUCUGUAUUUUCUGUUAA 2879
MIR160	isotig06533	Auxin response factor, putative [Ricinus communis]	miRNA 20 CCGUAUGUCCCUCGGUCCGU 1 :::::::::::::::::::: Target 1460 GGCAUACAGGGAGCCAGGCA 1479
MIR164	isotig18417	dtdp-glucose 4-6-dehydratase, putative [Ricinus communis]	miRNA 20 CGUGCACGGGACGAAGAGGU 1 ..: ::::::::::::::: Target 325 UUGCUUGCCCUGCUUCUCCA 344
MIR166	isotig26453	hypothetical protein Moror_6019 [Moniliophthora roreri MCA 2997]	miRNA 21 CCCCUUACUUCGGACCAGGCU 1 ::::::::::::::::::::: Target 333 GGGGAAUGAAGCCUGGUCCGA 353
MIR167	isotig24325	Transmembrane protein, putative [Ricinus communis]	miRNA 20 UCUAGUACGACCGUCGAAGU 1 ::.:: :::: ::::.:::: Target 485 AGGUCUUGCUUGCAGUUUCA 504
MIR168	isotig27392	unknown [Populus trichocarpa]	miRNA 20 AGGGCUAGACGUGGUUCGCU 1 :::::: :::::::::::: Target 354 UCCCGACCUGCACCAAGCGA 373

MIR169	isotig23816	Nuclear transcription factor Y subunit A-1, putative [Ricinus communis]	miRNA 20 GCCGUUCAGUAAGAACCGAG 1 ::::: :::::::::::::. Target 413 AGGCAAUUCAUUCUUGGCUU 432
MIR2118	isotig23693	electron transporter, putative [Ricinus communis]	miRNA 21 AGUGAGGGUAGGUGGGUAAAG 1 :: :::::: :::.:::::::. Target 656 UCUCUCCCACCCAUCCAUUUU 676
MIR2916	isotig01679	kinesin, putative [Ricinus communis]	miRNA 23 UAUACUAGCAGAAGCUCAGGGGU 1 : :: : .:::::.::::::::: Target 285 ACAUAAGUGUCUUUGAGUCCCCA 307
MIR3627	isotig14309	conserved hypothetical protein [Ricinus communis]	miRNA 20 GUCACGGUAGAGAGGACGCU 1 ::::: ::::::::::.:. Target 2066 AAGUGCAAUCUCUCCUGUGG 2085
MIR390	isotig25145	zinc finger protein, putative [Ricinus communis]	miRNA 20 CGCGAUAGGGAGGACUCGAA 1 :: ::::::.:.::::.:.:: Target 53 GCACUAUCCUUUCUGGGUUU 72
MIR393	isotig19491	conserved hypothetical protein [Ricinus communis]	miRNA 21 CUAGUUACGCUAGGGAAACCU 1 :::::: :::: ..::::::: Target 128 GAUCAACGCGAAUUCUUUGGA 148
MIR395	isotig15115	Homeobox protein LUMINIDEPENDENS, putative [Ricinus communis]	miRNA 20 UCAAGGGGGUUUGUGAAGUC 1 :::::::::::: :.::: : Target 235 AGUUCCCCCAAAAAUUUCUG 254
MIR396	isotig27529	Regulatory-associated protein of mTOR, putative [Ricinus communis]	miRNA 20 UCAAGUUCUUUCGACACCUU 1 :::.:.:::::.:::::.: Target 4 UGUUUAGGAAAGUUGUGGGA 23
MIR397	isotig13410	laccase, putative [Ricinus communis]	miRNA 20 AGUAGUUGCGACGUGAGUUA 1 : :::::.:::::::::: Target 227 UGAUCAAUGCUGCACUCAAC 246
MIR399	isotig21072	hypothetical protein POPTR_0018s05070g	miRNA 20 UCCCGUUAAGAGGAAACCGU 1 :::: :::::::.: :::::: Target 321 AGGGAAAUUCUUCCUUGGCA 340

MIR444	isotig15824	Beclin-1, putative [Ricinus communis]	miRNA 21 UUCGAACUCUGUUGUUGACGU 1 :::::::: .::.:::.:::. Target 759 AAGCUUGAAGCAGCAAUUGCG 779
MIR4995	isotig18846	ascorbate peroxidase [Hevea brasiliensis]	miRNA 21 GGGAAUUGGUUCGGUGACGGA 1 :: : :::::: ::::::::: Target 211 CCAUCAACCAACCCACUGCCU 231
MIR528	isotig17682	conserved hypothetical protein [Ricinus communis]	miRNA 20 AGGAGACGUACGGGGAAGGU 1 ::.:::::::: ::::::::: Target 721 UCUUCUGCAUUCCCCUUCCA 740
MIR535	isotig15163	protein binding protein, putative [Ricinus communis]	miRNA 20 GCACGAGAGAGAGCAGCAGU 1 : : .:::::::.::::::: Target 1972 CUUCUUCUCUCUUGUCGUCA 1991
MIR6445	isotig16386	conserved hypothetical protein [Ricinus communis]	miRNA 20 UAAAAUCCUUCUCCUUACUU 1 :::::::: .:.::::.::.: Target 1438 AUUUUAGGUGGGGGAGUGGA 1457
MIR6476	isotig13412	photosystem I reaction center subunit IV A, chloroplast precursor [Ricinus communis]	miRNA 20 AGUACAAAGUAGAGGUGACU 1 :.:::::::::.::..::: Target 578 UUAUGUUUCAUUUCUGCUGC 597
MIR6478	isotig20025	conserved hypothetical protein [Ricinus communis]	miRNA 20 UGGUUGACUCGAUUCCAGCC 1 : ::::::::::::::.: Target 575 AGCAACUGAGCUAAGGUUGA 594
MIR7767	isotig00130	Cell wall-associated hydrolase [Medicago truncatula]	miRNA 20 CCUCUCGAGAGUCGAACCCC 1 ::::::: :::: ::::::: Target 778 GGAGAGCACUCAUCUUGGGG 797
MIR858	isotig23970	Myb domain protein 13, putative [Theobroma cacao]	miRNA 20 UCCAGCUUGUCUGUUGCUUU 1 ::: :.:::::::::::.: Target 35 AGGAAGGACAGACAACGAGA 54

Table 1.3.2. Targets of conserved miRNAs in *Ricinus communis*

miRNA Id	Target Id	Protein name	Alignment
MIR1425	30005.m001301	starch synthase, putative [Ricinus communis]	miRNA 21 UCGUCGUUCCUAACUUAGGAU 1 ::::: ::::::::::::..: Target 1284 AGCAGAAAGGAUUGAAUUUUC 1304
MIR156	30138.m003942	LIGULELESS1 protein, putative [Ricinus communis]	miRNA 20 ACGAGUGAGAGAAGACAGUU 1 ::::: ::::::::::::: Target 2952 UGCUCUCUCUCUUCUGUCAU 2971
MIR157	30138.m003942	LIGULELESS1 protein, putative [Ricinus communis]	miRNA 20 ACGAGAGAUAGAAGACAGUU 1 :::::::: :::::::::: Target 2952 UGCUCUCUCUCUUCUGUCAU 2971
MIR159	27956.m000358	transcription initiation factor ia, putative [Ricinus communis]	miRNA 21 GUCUCGAGGGAAGUUAGGUUU 1 .::::::::: ::::::.::: Target 834 UAGAGCUCCCAUCAAUUCAAU 854
MIR160	29908.m005953	Auxin response factor, putative [Ricinus communis]	miRNA 21 ACCGUAUGUCCCUCGGUCCGU 1 ::::::.:::::::::::::: Target 2600 UGGCAUGCAGGGAGCCAGGCA 2620
MIR164	29866.m000624	transcription initiation factor ia, putative [Ricinus communis]	miRNA 20 CGUGCACGGGACGAAGAGGU 1 : : ::::::::.::::::: Target 3845 GGAAGUGCCCUGUUUCUCCA 3864
MIR166	29625.m000702	DNA binding protein, putative [Ricinus communis]	miRNA 20 CCCUUACUUCGGACCAGGCU 1 : :.::::::..:::::.:: Target 403 GUGGAUGAAGUUUGGUCUGA 422
MIR167	29447.m000032	transcription factor, putative [Ricinus communis]	miRNA 22 AGUCUAGUACGACCGUCGAAGU 1 :::: .:::.::::::::::: Target 357 UCAGCCUAUGUUGGCAGCUUCA 378
MIR168	29677.m000188	eukaryotic translation initiation factor 2c, putative [Ricinus communis]	miRNA 20 AGGGCUAGACGUGGUUCGCU 1 :::::: :::::::::: :

			Target 1147 UCCCGAGCUGCACCAAGCAA 1166
MIR169	29637.m000726	hypothetical protein VITISV_017108 [Vitis vinifera]	miRNA 20 GCCGUUCAGUAAGAACCGAG 1 :::.::::::::.::::.:. Target 9014 CGGUAAGUCAUUUUUGGUUU 9033
MIR171	29916.m000544	conserved hypothetical protein [Ricinus communis]	miRNA 20 CUCUAUAACUGCGCCGAGUU 1 :.:::::::.:::::::::: Target 1721 GGGAUAUUGGCGCGGCUCAA 1740
MIR2118	30128.m008995	Rhicadhesin receptor precursor, putative [Ricinus communis]	miRNA 20 GUGAGGGUAGGUGGGUAAAG 1 : ::::.::.::..:::::: Target 82 CUCUCCUAUUCAUUCAUUUC 101
MIR3627	30147.m014274	retrotransposon 4 protein	miRNA 20 GUCACGGUAGAGAGGACGCU 1 .::::::.:: :.:.:::::: Target 6144 UAGUGCUAUAUUUUCUGCGA 6163
MIR390	29762.m000488	Cell differentiation protein rcd1, putative [Ricinus communis]	miRNA 20 CGCGAUAGGGAGGACUCGAA 1 :. .::::.::.:::::::: Target 3711 GUAUUAUCUCUUCUGAGCUU 3730
MIR393	30169.m006350	Ribosomal RNA assembly protein mis3, putative [Ricinus communis]	miRNA 22 UCUAGUUACGCUAGGGAAACCU 1 : .:::::: :::.:::::::: Target 2398 AUGUCAAUGAAAUCUCUUUGGA 2419
MIR395	30167.m000865	phytoene dehydrogenase, putative [Ricinus communis]	miRNA 20 UCAAGGGGGUUUGUGAAGUC 1 :::::..:::::.:::: :: Target 5418 AGUUCUUCCAAAUACUUAAG 5437
MIR396	30073.m002312	conserved hypothetical protein [Ricinus communis]	miRNA 21 GUCAAGUUCUUUCG-ACACCUU 1 : :::::::::::: ::::::: Target 279 CCGUUCAAGAAAGCCUGUGGAA 300
MIR397	29933.m001443	conserved hypothetical protein [Ricinus communis]	miRNA 21 AAGUAGUUGCGACGUGAGUUA 1 :::: ::.::::::::.::::: Target 16 UUCACCAGCGCUGCAUUCAAU 36
MIR399	29917.m002001	conserved hypothetical protein [Ricinus communis]	miRNA 21 GUCCCGUUAAGAGGAAACCGU 1 :::::. ::: ::.::::::: Target 3790 CAGGGUUAUUGUCUUUUGGCA 3810

MIR444	29588.m000852	metal transporter, putative [Ricinus communis]	miRNA 21 UUCGAACUCUGUUGUUGACGU 1 : ::: ::::..:::::::::: Target 5691 AUGCUGGAGAUGACAACUGCA 5711
MIR482	30174.m008712	conserved hypothetical protein [Ricinus communis]	miRNA 22 GAAGAACGGGUCGGUGGGUAGA 1 .:::::::::.::: :::::: : Target 3333 UUUCUUGCCUAGCAACCCAUAU 3354
MIR4995	29904.m003044	triacylglycerol lipase, putative [Ricinus communis]	miRNA 20 GGAAUUGGUUCGGUGACGGA 1 .:::. ::::::.::::::: Target 3234 UCUUGUCCAAGCUACUGCCU 3253
MIR528	29654.m000304	transcription factor, putative [Ricinus communis]	miRNA 20 AGGAGACGUACGGGGAAGGU 1 :::::::::::: .::::: Target 4469 UCCUCUGCAUGCAUCUUCCC 4488
MIR535	29706.m001273	Exportin-T, putative [Ricinus communis]	miRNA 20 GCACGAGAGAGAGCAGCAGU 1 : ::::::::.:::::: :: Target 6304 CUUGCUCUCUUUCGUCGCCA 6323
MIR6445	30147.m014147	DNA binding protein, putative [Ricinus communis]	miRNA 21 GUAAAAUCCUUCUCCUUACUU 1 ::::: :::::::::: :::: Target 436 CAUUUAAGGAAGAGGAUUGAA 456
MIR7767	29637.m000727	multidrug resistance-associated protein 2, 6 (mrp2, 6), abc-transoprter, putative [Ricinus communis]	miRNA 20 CCUCUCGAGAGUCGAACCCC 1 ::: : ::::::::::::: Target 7352 GGAUAAGUCUCAGCUUGGGG 7371
MIR858	28117.m000016	unnamed protein product	miRNA 20 UCCAGCUUGUCUGUUGCUUU 1 ::: :::::::::::.:::: Target 520 AGGACGAACAGACAAUGAAA 539

Table 1.3.3. Targets of conserved miRNAs in *Manihot esculenta*

miRNA Id	Target Id	Protein name	Alignment
MIR1310	gi|67214320|gb|DR087001.1|DR087001	hypothetical protein	miRNA 22 CCCGCAACGCGGGGGCUACGGA 1 :::::::::::::::::::::: Target 304 GGGCGUUGCGCCCCCGAUGCCU 325
MIR1425	gi|119015131|gb|DV458686.1|DV458686	leucine carboxyl methyltransferase, putative [Ricinus communis]	miRNA 21 UCGUCGUUCCUAACUUAGGAU 1 .::::::: ::::::::::: Target 15 GGCAGCAAUUAUUGAAUCCUA 35
MIR156	gi|164399275|gb|DB937629.1|DB937629	inosine triphosphate pyrophosphatase, putative [Ricinus communis]	miRNA 20 ACGAGUGAGAGAAGACAGUU 1 : ::::::.:::::::::.: Target 447 UUCUCACUUUCUUCUGUCGA 466
MIR157	gi|119013820|gb|DV455740.1|DV455740	Squamosa promoter-binding protein, putative [Ricinus communis]	miRNA 20 ACGAGAGAUAGAAGACAGUU 1 :::::::::::::::::::: Target 448 UGCUCUCUAUCUUCUGUCAA 467
MIR159	gi|164394232|gb|DB945841.1|DB945841	actin depolymerizing factor, putative [Ricinus communis]	miRNA 20 UCUCGAGGGAAGUUAGGUUU 1 :::::.::::::::..::: Target 414 CGAGCUUCCUUCAAUUUAAA 433
MIR160	gi|56920075|gb|CK644412.1|CK644412	hevamine-A precursor, putative [Ricinus communis]	miRNA 20 CCGUAUGUCCCUCGGUCCGU 1 :::::::: ::::::::: Target 25 AGCAUACAGCAAGCCAGGCA 44
MIR164	gi|56920221|gb|CK644558.1|CK644558	putative nucleotide sugar epimerase [Cucumis sativus]	miRNA 20 CGUGCACGGGACGAAGAGGU 1 ..: ::::::::::::::: Target 14 UUGCCUGCCCUGCUUCUCCA 33
MIR168	gi|119008432|gb|DV444850.1|DV444850	glyceraldehyde 3-phosphate dehydrogenase, putative [Ricinus communis]	miRNA 20 AGGGCUAGACGUGGUUCGCU 1 :..:::::::::::::.:. Target 114 UUUCGAUCUGCACCAAGUGG 133

MIR169	gi|119010381|gb|DV448363.1|DV448363	Nuclear transcription factor Y subunit A-3, putative [Ricinus communis]	miRNA 20 GCCGUUCAGUAAGAACCGAG 1 .:: ::::::: :::::::: Target 449 UGGGAAGUCAUCCUUGGCUC 468
MIR171	gi|164405945|gb|DB929878.1|DB929878	signal transducer, putative [Ricinus communis]	miRNA 20 CUCUAUAACUGCGCCGAGUU 1 :::::::::: :: :::::. Target 380 GAGAUAUUGAAGCUGCUCAG 399
MIR2118	gi|119004485|gb|DV445920.1|DV445920	hypothetical protein POPTR_0008s03160g [Populus trichocarpa]	miRNA 21 AGUGAGGGUAGGUGGGUAAAG 1 ::::::.: ::::::::: :: Target 133 UCACUCUCUUCCACCCAUCUC 153
MIR2916	gi|164377119|gb|DB930738.1|DB930738	conserved hypothetical protein [Ricinus communis]	miRNA 21 UACUAGCAGAAGCUCAGGGGU 1 :::: :::::: :::::::: Target 70 AUGAACGUCUUGGAGUCCCCU 90
MIR3627	gi|164382630|gb|DB939881.1|DB939881	peroxidase [Populus kitakamiensis]	miRNA 21 UGUCACGGUAGAGAGGACGCU 1 : : ::::::.:::.:::::: Target 216 AAAUUGCCAUUUCUUCUGCGA 236
MIR390	gi|56925664|gb|CK650001.1|CK650001	PREDICTED: ribonuclease P protein subunit p29-like [Loxodonta africana]	miRNA 21 CCGCGAUAGGGAGGACUCGAA 1 ::.:::::::. ::::::::: Target 168 GGUGCUAUCCUACCUGAGCUU 188
MIR393	gi|119006126|gb|DV449616.1|DV449616	TRANSPORT INHIBITOR RESPONSE 1 protein, putative [Ricinus communis]	miRNA 20 UAGUUACGCUAGGGAAACCU 1 ::::::::::::::::: Target 156 GACAAUGCGAUCCCUUUGGA 175
MIR395	gi|67215065|gb|DR087360.1|DR087360	sulfate adenylyltransferase, putative [Ricinus communis]	miRNA 21 CUCAAGGGGGUUUGUGAAGUC 1 :::::::.:::::: ::::: Target 363 GAGUUCCUCCAAACUCUUCAU 383
MIR396	gi|56925157|gb|CK649494.1|CK649494	2,3-bisphosphoglycerate-independent phosphoglycerate mutase [Ricinus communis]	miRNA 20 UCAAGUUCUUUCGACACCUU 1 ::::.::::: ::::: ::: Target 110 AGUUUAAGAAUGCUGUUGAA 129
MIR397	gi|67215690|gb|DR087678.1|DR087678	laccase, putative [Ricinus communis]	miRNA 20 AGUAGUUGCGACGUGAGUUA 1 : ::::::::::.:::::: Target 350 UAAUCAACGCUGCGCUCAAU 369

MIR482	gi|164405877|gb|DB947720.1|DB947720	aldo-keto reductase, putative [Ricinus communis]	miRNA 22 GAAGAACGGGUCGGUGGGUAGA 1 :::::::::.:: :.::::::: Target 324 CUUCUUGCUCAUACGCCCAUCU 345
MIR4995	gi|164389999|gb|DB940991.1|DB940991	amino acid adenylation enzyme/thioester reductase family protein, partial [Synechocystis sp. PCC 7509]	miRNA 21 GGGAAUUGGUUCGGUGACGGA 1 .:: ::::::: ::::::::: Target 112 UCCAUAACCAAACCACUGCCU 132
MIR528	gi|164391487|gb|DB923025.1|DB923025	conserved hypothetical protein [Ricinus communis]	miRNA 21 GAGGAGACGUACGGGGAAGGU 1 ::::::::: :::: :::::: Target 118 CUCCUCUGCUUGCCGCUUCCA 138
MIR6445	gi|119012267|gb|DV452242.1|DV452242	Red chlorophyll catabolite reductase, chloroplast precursor, putative [Ricinus communis]	miRNA 22 GGUAAAAUCCUUCUCCUUACUU 1 ..:::: : ::::::::.::::. Target 502 UUAUUUGAAGAAGAGGGAUGAG 523
MIR6476	gi|119011063|gb|DV449585.1|DV449585	Early nodulin, putative [Ricinus communis]	miRNA 20 AGUACAAAGUAGAGGUGACU 1 :::: ::::::: :..:::: Target 107 UCAUAUUUCAUCACUGCUGA 126
MIR6478	gi|219275167|gb|FG805050.1|FG805050	zinc finger protein, putative [Ricinus communis]	miRNA 20 UGGUUGACUCGAUUCCAGCC 1 ..::::: :::::::::.:: Target 191 GUCAACUCAGCUAAGGUUGG 210
MIR858	gi|56920059|gb|CK644396.1|CK644396	Myb domain protein 13, putative [Theobroma cacao]	miRNA 20 UCCAGCUUGUCUGUUGCUUU 1 ::: ::::::::::::.:::: Target 382 AGGACGAACAGACAAUGAAA 401
MIR894	gi|164389745|gb|DB955332.1|DB955332	40S ribosomal protein S26, putative [Ricinus communis]	miRNA 20 CCACUUGGGCUGCACUUUGC 1 : :::::..:::::::.:.: Target 481 GAUGAACUUGACGUGAGAUG 500
MIR1432	gi|282661194|gb|GH571913.1|GH571913	orf36 gene product (mitochondrion) [Daucus carota subsp. sativus]	miRNA 20 AGCCACAGUAGAGAGGACUA 1 :: ::::::.::::.:::: Target 185 UCUAUGUCAUUUCUCUUGAU 204

Table 1.4. Targets of novel miRNAs

Table 1.4.1 Targets of novel miRNAs in *Hevea*

miRNA Id	Target Id	Protein name	Alignment
HbmiRn_10	isotig16596	ARM repeat superfamily protein [*Arabidopsis thaliana*]	miRNA 20 UCUGCGUUCGGGGAGGAGCC 1 ::.: :::::::.::::.:: Target 817 AGGCACAAGCCCUUCCUUGG 836
HbmiRn_31	isotig15623	Ubiquitin and WLM domain-containing protein [*Medicago truncatula*]	miRNA 20 ACGGUCCUUGGUCUAAACUU 1 :::::.::::::::::::: Target 907 AUCCAGGGACCAGAUUUGAA 926
HbmiRn_32	isotig15623	Ubiquitin and WLM domain-containing protein [*Medicago truncatula*]	miRNA 20 ACGGUCCUUGGUCUAAACUU 1 :::::.::::::::::::: Target 907 AUCCAGGGACCAGAUUUGAA 926
HbmiRn_37	isotig15623	Ubiquitin and WLM domain-containing protein C1442.07c, putative [*Theobroma cacao*]	miRNA 20 ACGGUCCUUGGUCUAAACUU 1 :::::.::::::::::::-: Target 907 AUCCAGGGACCAGAUUUGAA 926
HbmiRn_48	isotig10355	DNA binding protein, putative [Ricinus communis]	miRNA 21 CCCGAAGAAGGAGCUCAGGAC 1 : :::::::..:: ::::::: Target 1064 GUGCUUCUUUUUCUAGUCCUG 1084
HbmiRn_49	isotig10355	DNA binding protein, putative [*Ricinus communis*]	miRNA 21 CCCGAAGAAGGAGCUCAGGAC 1 : :::::::..:: ::::::: Target 1064 GUGCUUCUUUUUCUAGUCCUG 1084
HbmiRn_60	isotig16178	Tubulin beta-7 chain [*Theobroma cacao*]	miRNA 22 ACGGGAAAUGCGGGUCAGUAAG 1 ::.:::::: ::::::::.:::: Target 1349 UGUCCUUUA-GCCCAGUUAUUC 1369
HbmiRn_63	isotig16178	Tubulin beta-7 chain [*Theobroma cacao*]	miRNA 22 ACGGGAAAUGCGGGUCAGUAAG 1 ::.:::::: ::::::::.:::: Target 1349 UGUCCUUUA-GCCCAGUUAUUC 1369
HbmiRn_65	isotig25694	Tar1p [*Medicago truncatula*]	miRNA 24 AGCCAUCCUCGCUGCCCGCCACAG 1 ::::::: :::::::::::::::: Target 122 UCGGUAGUAGCGACGGGCGGUGUG 145

Table 1.4.2. Targets of novel miRNAs in *Ricinus*

miRNA Id	Target Id	Protein name	Alignment
HbmiRn_1	30138.m003905	conserved hypothetical protein [Ricinus communis] Sequence ID: ref|XP_002514589.1|	miRNA 22 ACACGGUCCUUGGUCUAAACUU 1 : ::::::::: :: :::::: Target 1621 UCUGCCAGGAAACAUAUUUGAU 1642
HbmiRn_5	29646.m001072	protein phosphatase 2c, putative [Ricinus communis]	miRNA 21 GACAUACUUUCAUUCAUGGUA 1 .:: ::::::::::::::. : Target 1848 UUGGAUGAAAGUAAGUACUUU 1868
HbmiRn_6	30138.m003905	conserved hypothetical protein [Ricinus communis]	miRNA 22 ACACGGUCCUUGGUCUAAACUU 1 : ::::::::: :: :::::: Target 1621 UCUGCCAGGAAACAUAUUUGAU 1642
HbmiRn_12	51971.m000051	hypothetical protein MTR_6g043000 [Medicago truncatula]	miRNA 20 AGAAUCGAACUCGGAUGGAA 1 :::::::: ::.:::.::: Target 641 GCUUAGCUUCAGUCUAUCUU 660
HbmiRn_15	30190.m011164	protein binding protein, putative [Ricinus communis]	miRNA 22 ACGGGAAAUGCGGGUCAGUAAG 1 : ::.::::.:..::::::::. Target 3422 UUCCUUUUAUGUUCAGUCAUUU 3443

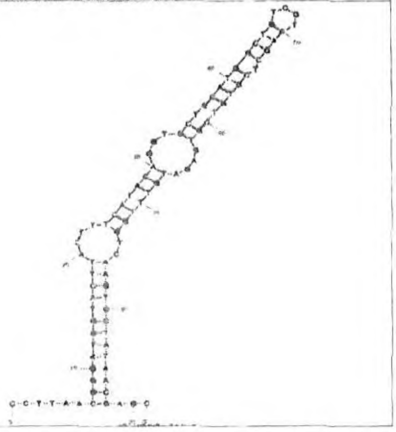
Table 1.4.3. Targets of novel miRNAs in *Manihot*

HbmiRn_14	gi|164388836|gb|DB936430.1|DB936430	conserved hypothetical protein [Ricinus communis]	miRNA 19 CGGUC-CUUGGUCUAAACUU 1 ::::: :::::::::::::: Target 84 GCCAGAGAACCAGAUUUGAA 103
HbmiRn_35	gi|54414382|gb|CK901174.1|CK901174	putative senescence-associated protein [Pisum sativum]	miRNA 24 UAUUAGGUCGCGUGUCAUCGAAGC 1 :::::::::::::.::::::::: Target 18 AUAAUCCAGCGCACGGUAGCUUCG 41
HbmiRn_43	gi|164407878|gb|DB925348.1|DB925348	succinate dehydrogenase, putative [Ricinus communis]	miRNA 20 CGGUGAAUGUUAUGGGGCAG 1 :.: ::::.:::::.::::. Target 60 GUCUCUUAUAAUACUCCGUU 79
HbmiRn_5	gi|119019209|gb|DV456844.1|DV456844	sedoheptulose-1,7-bisphosphatase, chloroplast, putative [Ricinus communis]	miRNA 24 CUCGAGAACCCCUUCUAGUCGUAC 1 :. .::::: :::.:: ::::::: Target 333 GGCUUCUUGUGGAGGAACAGCAUG 356
HbmiRn_6	gi|119019136|gb|DV456719.1|DV456719	protein phosphatase 2C [Hevea brasiliensis]	miRNA 20 CUGUGGAUAGCAUUACUAUU 1 ::.::::: :::.:::::.. Target 214 GAUACCUACCGUGAUGAUGG 233
HbmiRn_18	gi|164388836|gb|DB936430.1|DB936430	conserved hypothetical protein [Ricinus communis] Sequence ID: ref|XP_002529308.1|	miRNA 19 CGGUC-CUUGGUCUAAACUU 1 ::::: :::::::::::::: Target 84 GCCAGAGAACCAGAUUUGAA 103
HbmiRn_20	gi|164396327|gb|DB923733.1|DB923733	conserved hypothetical protein [Ricinus communis] Sequence ID: ref|XP_002523496.1|	miRNA 24 AGACUGGUUAACUUGAUGUUAGGG 1 :.: ::::::::::: :.::::: Target 46 UUUCUCCAAUUGAACUUCGAUCCC 69

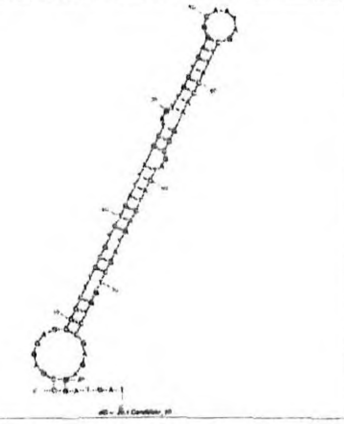
HbmiRn_26	gi|164378374|gb|DB926160.1|DB926160		miRNA 23 GGCAGUCGGCGGUGCAGGGCCAA 1 .: ::: ::::: : :::::::: Target 83 UCCUCAACCGCCUCCUCCCGGUU 105
HbmiRn_28	gi|164381293|gb|DB921768.1|DB921768	methylmalonyl-CoA mutase large subunit [Sphaerobacter thermophilus DSM 20745]	miRNA 20 CAGCCUACACCUUGGCGCUG 1 ::::.::::::::.:::::. Target 461 UUCGGGUGUGGAAUCGCGAU 480
HbmiRn_46	gi|119010062|gb|DV447784.1|DV447784	solute carrier family 31 (copper transporters), member 1 [Danio rerio]	miRNA 23 GGAGGAUGAGUAGCCCCGGACCG 1 :. :.::::. :.:::::.:::: Target 149 CUACUUACUUUUUGGGGCUUGGC 171
HbmiRn_48	gi|164401711|gb|DB924889.1|DB924889	lupus la ribonucleoprotein, putative [Ricinus communis]	miRNA 21 GUCGCUGGCGGCGCGGGAGGA 1 :::::.::::::: :::::: Target 360 CAGCGGCCGCCGCCACCUCCU 380
HbmiRn_64	gi|119011831|gb|DV451261.1|DV451261	translation initiation factor, putative [Ricinus communis]	miRNA 20 GUGCACGGAAACCCCCGGCC 1 :: :::::::::: :::.:: Target 561 CAGGUGCCUUUGGUGGCUGG 580

Fig. 1.1 Stem loop structure of novel miRNA from *Hevea brasiliensis*

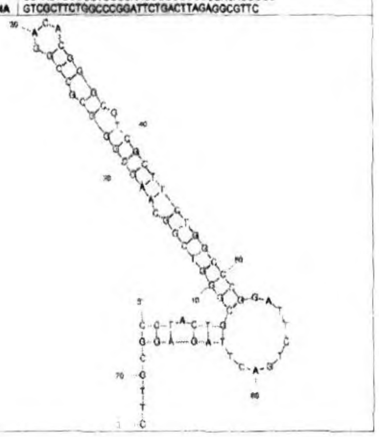
mature_miRNA_id	Candidate_5
MFE	-25.9
Chromosome	50775
Chr_start	3580
Chr_end	3670
mature_miRNA	ATGGTACTTACTTTCATACAGG
miRNA length	22
Pri-miRNA	CCTTAACGGGATGGTACTTACTTTCATACAGG TGCTGCATGGCTGTCGTCAGCTCGTGTCGTG AGATGTTTGGTCAAGTCCTATAACGAGC



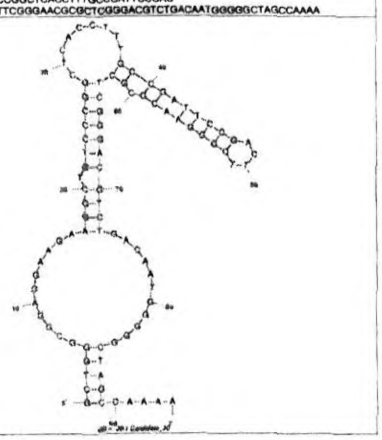
mature_miRNA_id	Candidate_10
MFE	-29.6
Chromosome	contig00066
Chr_start	2
Chr_end	87
mature_miRNA	CCGAGGAGGGGCTTGCGTCTGAT
miRNA length	23
Pri-miRNA	CCGAGGAGGGGCTTGCGTCTGATTAGC TAGTTGGTGAGGCAATAGCTTACCAAGG CGATGATCAGTAGGTGGTCCGAGAGGATGAT



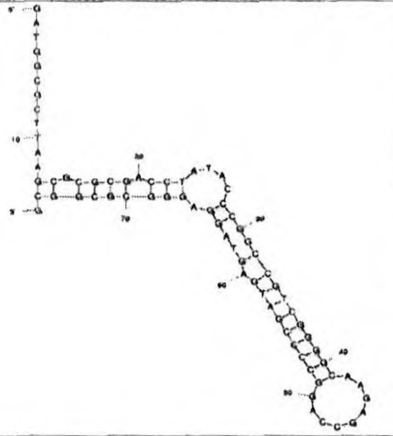
mature_miRNA_id	Candidate_11
MFE	-31.2
Chromosome	isotig27223
Chr_start	223
Chr_end	295
mature_miRNA	CGCTTCTGGCCCGGATTCTGACTT
miRNA length	24
Precursor miRNA	CCTACTGCGGGTCGGCAAGCGGGCGCCGGACACGGGC GTCGCTTCTGGCCCGGATTCTGACTTAGAGGCGTTC



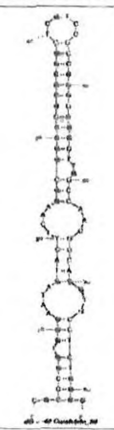
mature_miRNA_id	Candidate_20
MFE	-30.15
Chromosome	isotig11066
Chr_start	500
Chr_end	593
mature_miRNA	GCTCGGGACGTCTGACAATGGGGG
miRNA length	24
Pri-miRNA	GCTGGGCGGAGGAAGAAGGCTGTC CCGGCTCACCTTTGCCGATTCCGAC TTCGGGAACGCGCTCGGGACGTCTGACAATGGGGGCTAGCCAAAA



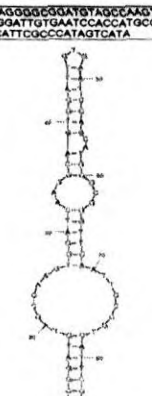
_miRNA_id	Candidate_21
	-34.1
osome	isotig14137
rt	358
d	431
_miRNA	AAGCGCGCGACCTATACCCGGCCG
length	24
NA	GATGGCGCTTAAGCGCGCGACCTATACCCGGCCG TCGGGGCAAGAGCCAGGCCCCGATGAGTAGGAGGGCGCGGCG



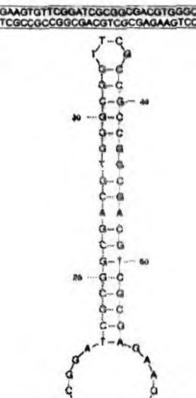
mature_miRNA_id	Candidate_28
MFE	-42.1
Chromosome	isotig27223
Chr_start	130
Chr_end	211
mature_miRNA	GAATAGTACTTCAAGGCGGCCCGC
miRNA length	24
Pri-miRNA	CGCCCGGTGGGAATAGTACTTCAAGGCGGCCCGCGC GGCTCGTCCGCCGCGGGGGCTTGGCCAACGGCACGT GCCTCTGGGG



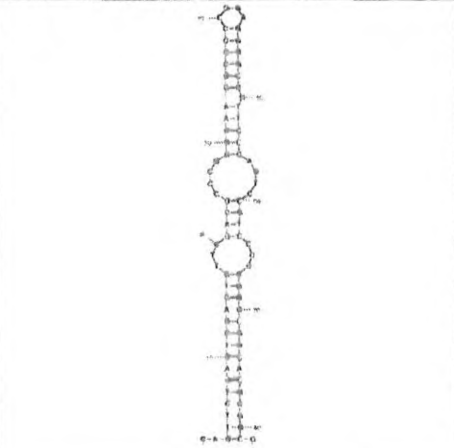
ure_miRNA_id	Candidate_38
	-33.39
omosome	isotig00036
_start	5437
_end	5531
ure_miRNA	GGCGGATGTAGCCAAGTGGATCAA
NA length	24
miRNA	TAGTATTAGGGGGCGGATGTAGCCAAGTGGATCAA GGCAGTGGATTGTGAATCCACCATGCGCGGGTTCAAT TCCCGTCATTCGCCCATAGTCATA



mature_miRNA_id	Candidate_50
MFE	-35.7
Chromosome	isotig28211
Chr_start	130
Chr_end	196
mature_miRNA	TCGGATCGCGGCGACGTGGGC
miRNA length	21
Pri-miRNA	GGTGAAGTGTTCGGATCGCGGCGACGTGGGC GGTTCGCCGCCGGCGACGTCGCGAGAAGTCCACTG

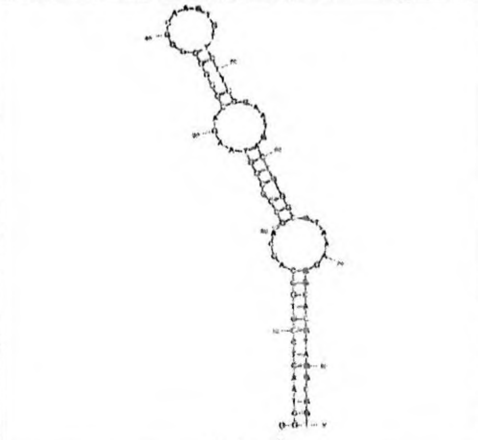


mature_miRNA_id	Candidate_51
MFE	-38.2
Chromosome	isotig28130
Chr_start	127
Chr_end	208
mature_miRNA	TCGACTGTTCGACGCCCGGGGA
miRNA length	22
Pri-miRNA	CAGTTCTGAGTCGACTGTTCGACGCCCGGGGA AGGCCCCCGGAGGGGCCGTTCCCAGTCCGTC CCCCGGCCGGCACGCGGCG



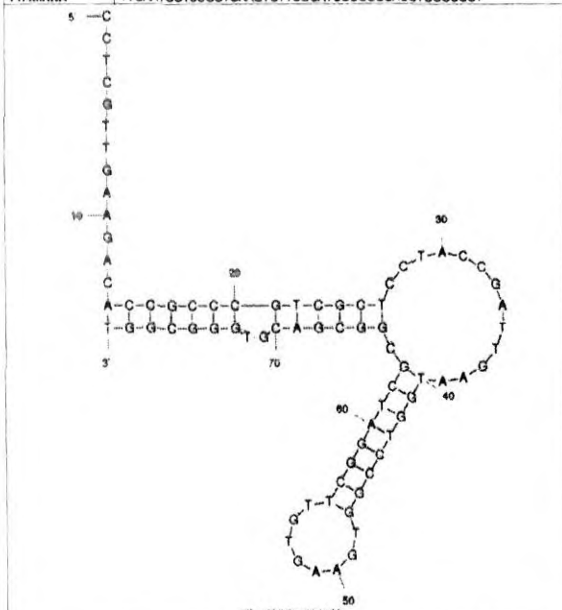
dG = -38 Candidate_51

mature_miRNA_id	Candidate_63
MFE	-31.32
Chromosome	isotig03565
Chr_start	1309
Chr_end	1393
mature_miRNA	GAATGACTGGGCGTAAAGGGCA
miRNA length	22
Pri-miRNA	GGCTAACTCCGTGCCAGCAGCCGCG GTAAGACGGGGGGGGCAAGTGTTCT TCGGAATGACTGGGCGTAAAGGGCACGTAGGCGGT



dG = -31.3 Candidate_63

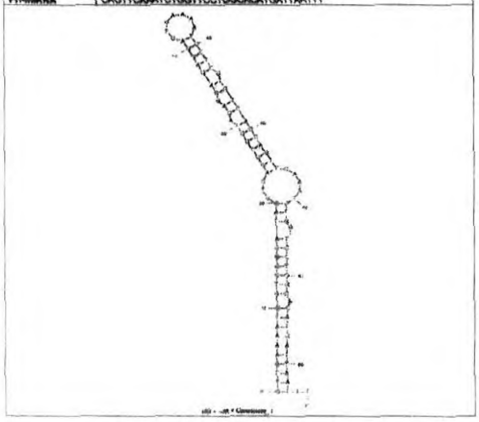
mature_miRNA_id	Candidate_65
MFE	-29.54
Chromosome	isotig28211
Chr_start	65
Chr_end	163
mature_miRNA	GACACCGCCCGTCGCTCCTACCGA
miRNA length	24
Pri-miRNA	CCTCGTTGAAGACACCGCCCGTCGCTCCTACCGA TTGAATGGTCCGGTGAAGTGTTCGGATCGCGGCGACGTGGGCGGT



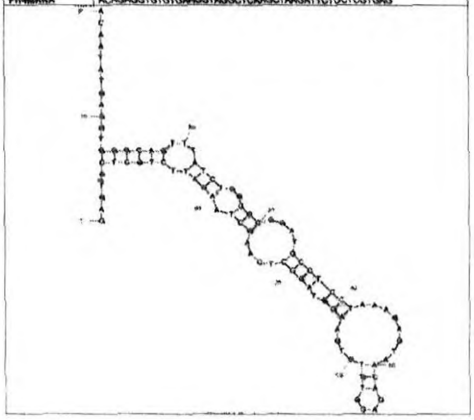
dG = -29.5 Candidate_65

Novel miRNAs from Ricinus communis

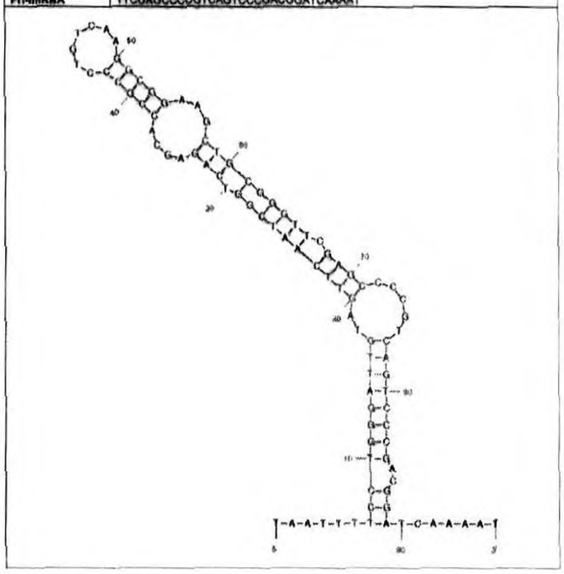
mature_miRNA_id	Candidate_1
MFE	-30.4
Chromosome	60625
Chr_start	52119
Chr_end	52213
mature_miRNA	TTCAAATCTGGTTCCTGGCACA
miRNA length	22
Pri-miRNA	GTTGAAAATGGTCGGGATAGCTCAGCTGGT AGAGCAGAGGACTGAAAATCCTCGTGTCAC CAGTTCAAATCTGGTTCCTGGCACATGATTAATTT



mature_miRNA_id	Candidate_12
MFE	-26.3
Chromosome	30160
Chr_start	34156
Chr_end	34250
mature_miRNA	AAGGTAGGCTCAAGCTAAGATTC
miRNA length	23
Pri-miRNA	ACAATATGAGGTGGGCAGTTTATCT GGGGCGGATGCCTCCTAAAGAGTA ACAGAGGTGTGTGAAGGTAGGCTCAAGCTAAGATTCTGCTCGTGAG

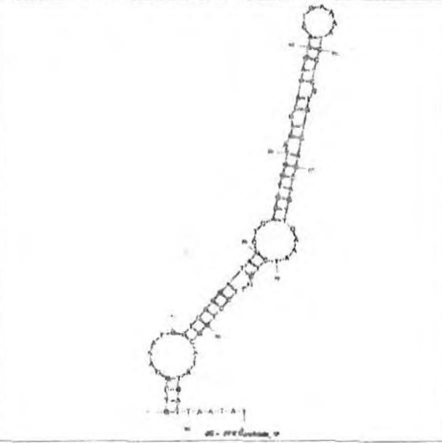


mature_miRNA_id	Candidate_13
MFE	-32.2
Chromosome	44525
Chr_start	6496
Chr_end	6591
mature_miRNA	TTCGAGCCCCGTCAGTCCCGAC
miRNA length	22
Pri-miRNA	TAATTTTCCTGGGATTGTAGTTCAA TCGGTCAGAGCACCGCCCTGTCAA GGCGGAAGCTGCGGG TTCGAGCCCCGTCAGTCCCGACGGATCAAAAT

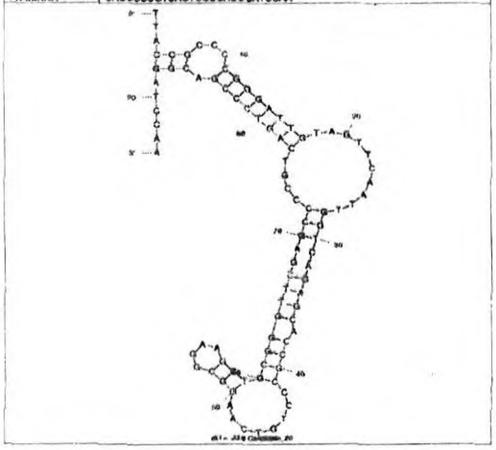


miRNAs from *Manihot esculenta*

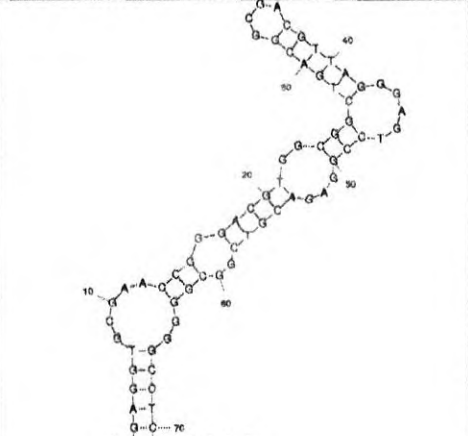
mature_miRNA_id	Candidate_16
MFE	-27.8
Chromosome	scaffold04355
Chr_start	70122
Chr_end	70216
mature_miRNA	TTCAAATCTGGTTCCTGGCATA
miRNA length	22
Pri-miRNA	GTCGGAAATGGTCGGGATAGCTCAGCT GGTAGAGCAGAGGACTGAAAATCCTCG TGTCACCAGTTCAAATCTGGTTCCTGGCATATGATTAATAT



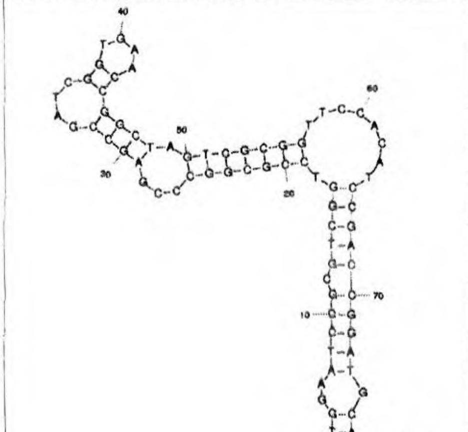
mature_miRNA_id	Candidate_20
MFE	-33.6
Chromosome	scaffold04355
Chr_start	109402
Chr_end	109495
mature_miRNA	GGGATTGTAGTTCAATTGGTCAGA
miRNA length	24
Pri-miRNA	*TTACCGGCCCCGGGATTGTAGTTCAATTGGTCAGA* GCACCGCCCTGTCAAGGCGGAAGCTGCGGGTTC GAGCCCCGTCAGTCCCGACGGATCCAA



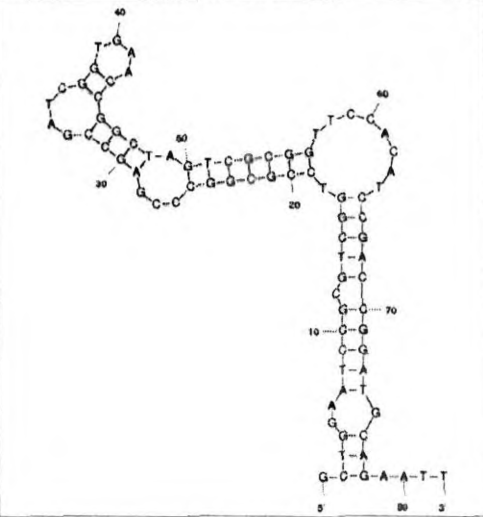
mature_miRNA_id	Candidate_26
MFE	-29.9
Chromosome	scaffold10870
Chr_start	11269
Chr_end	11364
mature_miRNA	AACCGGGACGTGGCGGCTGACGGC
miRNA length	24
Pri-miRNA	ACGAGGTGCGAACCGGGACGTGGCGGCTGACGGC GACGTTAGGGAGTCCGGAGACGTCGGCGGGGCC TCGGGAAG



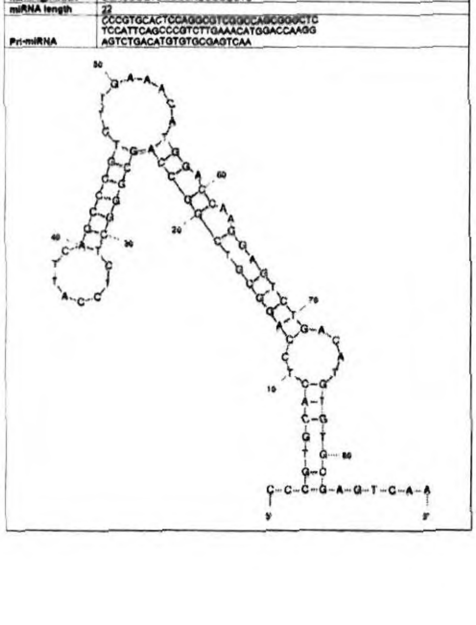
mature_miRNA_id	Candidate_28
MFE	-38.4
Chromosome	scaffold09151
Chr_start	629844
Chr_end	629926
mature_miRNA	GTCGCGGTTCCACATCCGACCGG
miRNA length	23
Pri-miRNA	CGCTGGAATCCGCGTCGGTCCGCG GCCCGAGCCGATCGGTGAACCGGC *TAGTCGCGGTTCCACATCCGACCGGATGCAGAATT*



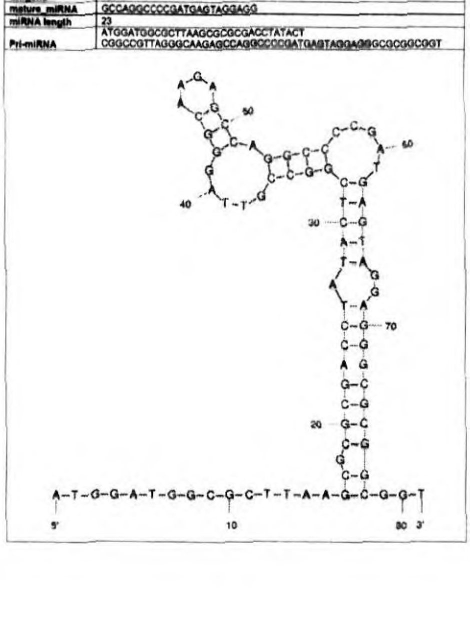
mature_miRNA_id	Candidate_26
MFE	-38.4
Chromosome	scaffold09151
Chr_start	629844
Chr_end	629926
mature_miRNA	GTCGCGGTTCCACATCCGACCGG
miRNA length	23
Pri-miRNA	CGCTGGAATCCGCGTCGGTCCGCG GCCCGAGCCGATCGGTGAACCGGC TAGTCGCGGTTCCACATCCGACCGGATGCAGAATT



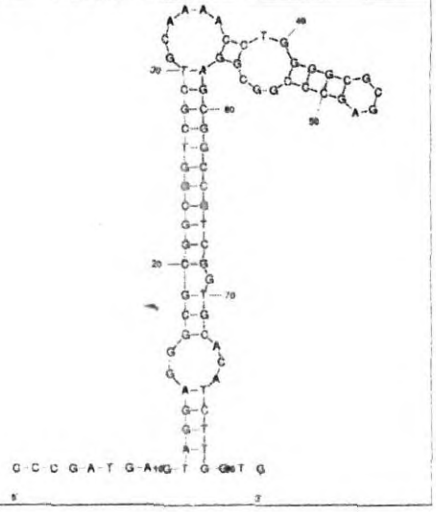
mature_miRNA_id	Candidate_42
MFE	-30.7
Chromosome	scaffold00183
Chr_start	1128
Chr_end	1215
mature_miRNA	CCAGGCGTCGGCCAGCGGGCTC
miRNA length	22
Pri-miRNA	CCCGTGCACTCCAGGCGTCGGCCAGCGGGCTC TCCATTCAGCCCGTCTTGAAACATGGACCAAGG AGTCTGACATGTGTGCGAGTCAA



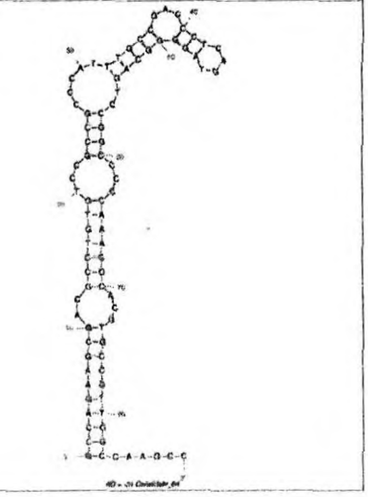
mature_miRNA_id	Candidate_46
MFE	-30.6
Chromosome	scaffold00183
Chr_start	1837
Chr_end	1917
mature_miRNA	GCCAGGCCCCGATGAGTAGGAGG
miRNA length	23
Pri-miRNA	ATGGATGGCGCTTAAGCGCGCGACCTATACT CGGCCGTTAGGGCAAGAGCCAGGCCCCGATGAGTAGGAGGGCGCGGCGGT



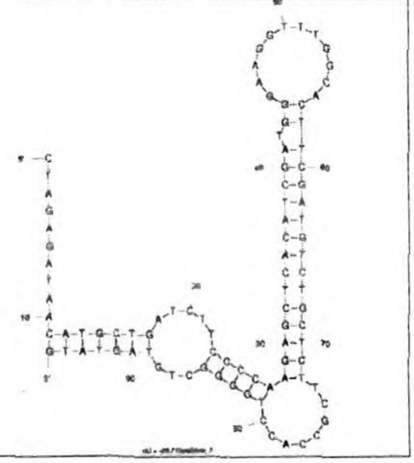
mature_miRNA_id	Candidate_48
MFE	-38.5
Chromosome	scaffold00183
Chr_start	1892
Chr_end	1974
mature_miRNA	AGGAGGGCGCGGCGGTCGCTGCA
miRNA length	23
Pri-miRNA	CCCGATGAGTAGGAGGGCGCGGCGGTCGCTGCA AAACCTGGGGCGCGAGCCCGGCGGAGCGGCCG TCGGTGCACATCTTGGTG



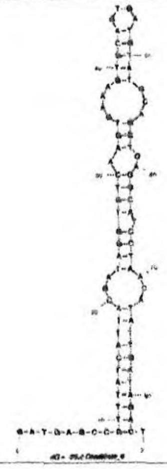
mature_miRNA_id	Candidate_64
MFE	-31
Chromosome	scaffold02369
Chr_start	29532
Chr_end	29620
mature_miRNA	CCGGCCCCCAAAGGCACGTGCCGT
miRNA length	24
Pri-miRNA	GCCAGAAGCGACGCCTGTGTCCGCCG CCCATTTGCCGACCCTCAGTAGGGGCA GTCCGGCCCCCAAAGGCACGTGCCGTT GGCCAAGCC



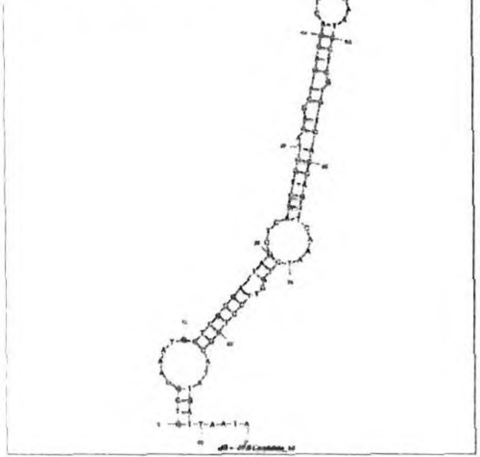
mature_miRNA_id	Candidate_5
MFE	-29.7
Chromosome	scaffold07536
Chr_start	55726
Chr_end	55820
mature_miRNA	CATGCTGATCTTCCCCAAGAGCTC
miRNA length	24
Pri-miRNA	CTAGAGATAACATGCTGATCTTCCCCAAGAGCTC ACATCGATGGGAAGGTTTGGCACTTCGATGTCTG CTCTTCGCCACCTGGGGCTGTAGTATG



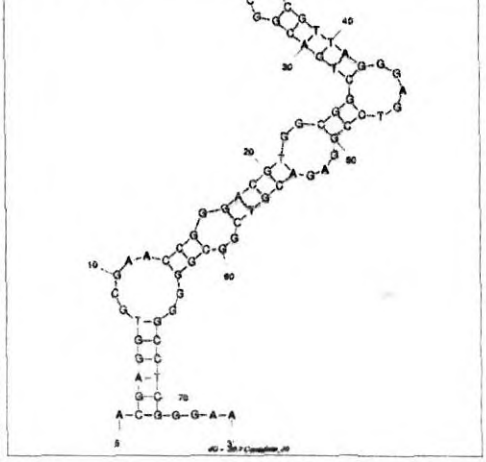
mature_miRNA_id	Candidate_6
MFE	-25.3
Chromosome	scaffold07536
Chr_start	56168
Chr_end	56251
mature_miRNA	TTATCATTACGATAGGTGTCAAG
miRNA length	23
Pri-miRNA	GATGAGCCGTTTATCATTACGATAGGTGTCAAG TGAAAGTGCAGTGATGTATGCAGCTGAGGCATC CTAACATATTGATAGACT



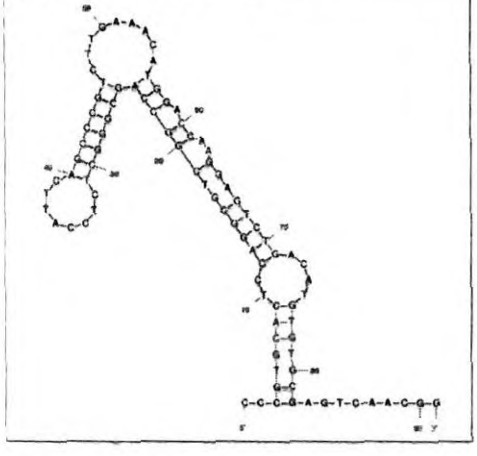
mature_miRNA_id	Candidate_14
MFE	-27.6
Chromosome	scaffold04355
Chr_start	70123
Chr_end	70216
mature_miRNA	TTCAAATCTGGTTCCTGGCAT
miRNA length	21
Pri-miRNA	GTCGGAAATGGTCGGGATAGCTCAGCTG GTAGAGCAGAGGACTGAAAATCCTCGTGT CACCAGTTCAAATCTGGTTCCTGGCATATGATTAATA



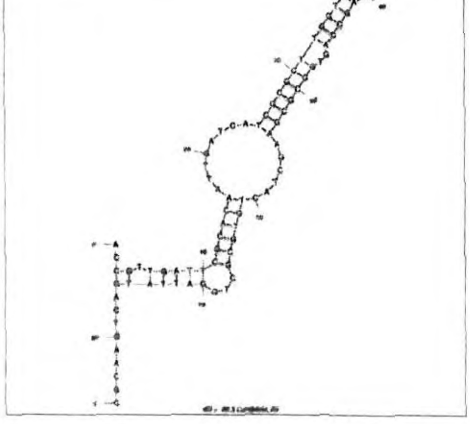
mature_miRNA_id	Candidate_20
MFE	-29.9
Chromosome	scaffold10670
Chr_start	11290
Chr_end	11364
mature_miRNA	AACCGGGACGTGGCGGCTGACGGC
miRNA length	24
Pri-miRNA	ACGAGGTGCGAACCGGGACGTGGCGGCTGACGGC GACGTTAGGGAGTCCGGAGACGTCGGCGGGGGCC TCGGGAA



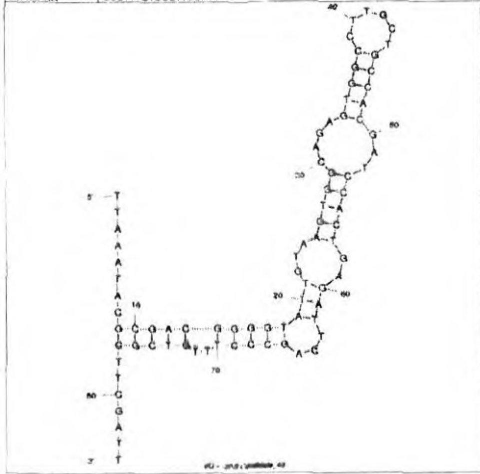
mature_miRNA_id	Candidate_33
MFE	-30.7
Chromosome	scaffold0183
Chr_start	1128
Chr_end	1216
mature_miRNA	CCAGGCGTCGGCCAGCGGGCTC
miRNA length	22
Pri-miRNA	CCCGTGCACTCCAGGCGTCGGCCAGCGGGCTC TCCATTCAGCCCGTCTTGAAACATGGACCAAGG AGTCTGACATGTGTGCGAGTCAACGG



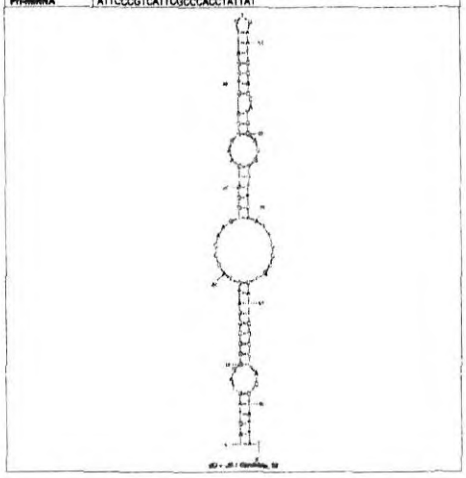
mature_miRNA_id	Candidate_35
MFE	-26.44
Chromosome	scaffold06829
Chr_start	89
Chr_end	173
mature_miRNA	CGAAGCTACTGTGCGCTGGATTAT
miRNA length	24
Pri-miRNA	ACCGTTGATTCGCACAATTGATCATC GCGCTTGGTTGAAAAGCCAGTGGCG CGAAGCTACTGTGCGCTGGATTATGACTGAA CGC



mature_miRNA_id	Candidate_43
MFE	-30.9
Chromosome	scaffold12053
Chr_start	108644
Chr_end	108727
mature_miRNA	GACGGGGTATTGTAAGTGGCAGA
miRNA length	23
Pri-miRNA	TTAAATACGCGACGGGGTATTGTAAGTGGCAGA GTGGCCTTGCTGCCACGATCCACTGAGATTCAG CCCTTTGTCGCTTCGATT



mature_miRNA_id	Candidate_53
MFE	-38.09
Chromosome	scaffold01755
Chr_start	38494
Chr_end	38588
mature_miRNA	GGCGGATGTAGCCAAGTGGATCAA
miRNA length	24
Pri-miRNA	TAGTAGTAGGGGCGGATGTAGCCAAGTGGATCAA GGCAGTGGATTGTGAATCCACCATGCACGGGTTCA ATTCCCGTCATTCGCCCACCTATTAT



Appendix 2 Cold responsive miRNAs of *Hevea brasiliensis*

Table 2.1 Cold-responsive conserved miRNAs

miRNA family	Expression value (Reads)	length (nt)	Sequence (5' - 3')	miRNA
miR9386	1377	22	UUUGCAGUUCGAAAGUGGAAGC	hbr-miR9386
miR159	63	21	UUUGGAUUGAAGGGAGCUCUG	mes-miR159b
	55	21	UUUGGAUUGAAGGGAGCUCUA	htu-miR159a
	53	21	UUUGGAUUGAAGGGAGCUCUA	cpa-miR159a
	53	21	UUUGGAUUGAAGGGAGCUCUA	mtr-miR159a
	52	21	UUUGGAUUGAAGGGAGCUCUA	ath-miR159a
	50	21	CUUGGAUUGAAGGGAGCUCCC	nta-miR159
	49	21	CUUGGAUUGAAGGGAGCUCCC	csi-miR159
	47	21	UUUGGAUUGAAGGGAGCUCUG	hbr-miR159b
	47	21	UUUGGAUUGAAGGGAGCUCUA	mes-miR159a
	46	21	UUUGGAUUGAAGGGAGCUCUA	bra-miR159a
	44	21	CUUGGAUUGAAGGGAGCUCCC	atr-miR159
	43	21	AUUGGUUUGAAGGGAGCUCCA	gma-miR159e-3p
	43	21	UUUGGAUUGAAGGGAGCUCUA	hbr-miR159a
	43	21	UUUGGAUUGAAGGGAGCUCUG	pvu-miR159a.1
	42	21	CUUGGAUUGAAGGGAGCUCCC	ahy-miR159
	42	21	UUUGGAUUGAAGGGAGCUCUA	gma-miR159a-3p
	41	21	CUUGGAUUGAAGGGAGCUCCC	ppe-miR159
	41	21	CUUGGAUUGAAGGGAGCUCCC	sly-miR159
	40	21	CUUGGAUUGAAGGGAGCUCCC	bna-miR159
	39	21	UUUGGAUUGAAGGGAGCUCUA	cme-miR159a
	38	21	UUUGGAUUGAAGGGAGCUCUA	aly-miR159a-3p
	35	21	CUUGGAUUGAAGGGAGCUCCC	rco-miR159
	26	21	UUUGGAUUGAAGGGAGCUCUU	aly-miR159b-3p
	26	21	UUUGGAUUGAAGGGAGCUCCU	lus-miR159c
	25	21	UUUGGAUUGAAGGGAGCUCCU	vvi-miR159c
	24	21	UUUGGAUUGAAGGGAGCUCUG	hvu-miR159b
	22	21	UUUGGAUUGAAGGGAGCUCUA	sbi-miR159a
	20	21	UUUGGAUUGAAGGGAGCUCUU	ath-miR159b-3p
	19	21	UUUGGAUUGAAGGGAGCUCUA	hvu-miR159a
	19	21	UUUGGAUUGAAGGGAGCUCUA	zma-miR159a-3p
	19	21	UUUGGAUUGAAGGGAGCUCUG	zma-miR159k-3p
	18	21	CUUGGAUUGAAGGGAGCUCCC	far-miR159
	17	21	AUUGGAUUGAAGGGAGCUCCG	sof-miR159d
	17	21	UUUGGAUUGAAGGGAGCUCUG	zma-miR159j-3p
	16	21	UUUGGAUUGAAGGGAGCUCUG	bdi-miR159b-3p.1
	16	21	UUUGGAUUGAAGGGAGCUCUG	lus-miR159b
	16	21	UUUGGAUUGAAGGGAGCUCUA	ssp-miR159a
	16	21	UUUGGAUUGAAGGGAGCUCUA	tae-miR159a
	15	21	UUUGGAUUGAAGGGAGCUCUG	osa-miR159a.1
	14	21	UUUGGAUUGAAGGGAGCUCUG	osa-miR159b
	14	21	UUUGGAUUGAAGGGAGCUCUU	zma-miR159b-3p
	13	21	UUUGGAUUGAAGGGAGCUCUG	zma-miR159f-3p
	11	21	UUUGGAUUGAAGGGAGCUCUG	sof-miR159b
	11	21	UUUGGAUUGAAGGGAGCUCUG	tae-miR159b
	10	21	UUUGGAUUGAAGGGAGCUCUA	sof-miR159a

miR166	1187	21	UCGGACCAGGCUUCAUUCCUC	gma-miR166h-3p
	1140	21	UCGGACCAGGCUUCAUUCCCC	gma-miR166k
	1134	21	UCGGACCAGGCUUCAUUCCC	atr-miR166b
	130	21	UCGGACCAGGCUUCAUUCCC	vvi-miR166e
	124	21	UCGGACCAGGCUUCAUUCCCC	gma-miR166g
	124	21	UCGGACCAGGCUUCAUUCCCC	mes-miR166d
	123	21	UCGGACCAGGCUUCAUUCCC	cpa-miR166a
	122	22	UCGGACCAGGCUUCAUUCCCCC	ctr-miR166
	122	21	UCGGACCAGGCUUCAUUCCCC	mes-miR166g
	122	21	UCGGACCAGGCUUCAUUCCCC	osa-miR166f
	121	21	UCGGACCAGGCUUCAUUCCC	mtr-miR166a
	119	21	UCGGACCAGGCUUCAUUCCC	nta-miR166e
	119	21	UCGGACCAGGCUUCAUUCCCC	osa-miR166c-3p
	119	21	UCGGACCAGGCUUCAUUCCCC	smo-miR166c
	118	21	UCGGACCAGGCUUCAUUCCC	rco-miR166a
	117	21	UCGGACCAGGCUUCAUUCCC	cpa-miR166b
	116	21	UCGGACCAGGCUUCAUUCCC	nta-miR166a
	116	21	UCGGACCAGGCUUCAUUCCCC	rco-miR166c
	115	21	UCGGACCAGGCUUCAUUCCCC	mes-miR166f
	114	21	UCGGACCAGGCUUCAUUCCCC	cme-miR166d
	114	21	UCGGACCAGGCUUCAUUCCCC	osa-miR166b-3p
	113	21	UCGGACCAGGCUUCAUUCCCC	ata-miR166d-3p
	113	21	UCGGACCAGGCUUCAUUCCCC	ath-miR166e-3p
	113	21	UCGGACCAGGCUUCAUUCCCC	bdi-miR166c-3p
	113	21	UCGGACCAGGCUUCAUUCCCC	lus-miR166c
	112	21	UCGGACCAGGCUUCAUUCCCC	ath-miR166c
	112	21	UCGGACCAGGCUUCAUUCCCC	gma-miR166f
	112	21	UCGGACCAGGCUUCAUUCCCC	lus-miR166h
	112	21	UCGGACCAGGCUUCAUUCCC	ppe-miR166a
	111	21	UCGGACCAGGCUUCAUUCCCC	ath-miR166g
	111	21	UCGGACCAGGCUUCAUUCCC	bna-miR166e
	111	21	UCGGACCAGGCUUCAUUCCC	pvu-miR166a
	110	21	UCGGACCAGGCUUCAUUCCC	hvu-miR166b
	110	21	UCGGACCAGGCUUCAUUCCCC	mes-miR166c
	110	21	UCGGACCAGGCUUCAUUCCCC	mtr-miR166e-3p
	110	21	UCGGACCAGGCUUCAUUCCCC	stu-miR166a-3p
	109	21	UCGGACCAGGCUUCAUUCCCC	ata-miR166a-3p
	109	21	UCGGACCAGGCUUCAUUCCC	cme-miR166b
	109	21	UCGGACCAGGCUUCAUUCCCC	cme-miR166f
	109	22	UCGGACCAGGCUUCAUUCCCC	lus-miR166i
	109	21	UCGGACCAGGCUUCAUUCCCC	osa-miR166a-3p
	109	21	UCGGACCAGGCUUCAUUCCCC	osa-miR166j-3p
	109	21	UCGGACCAGGCUUCAUUCCC	rco-miR166e
	108	21	UCGGACCAGGCUUCAUUCCUC	mtr-miR166g-3p
	107	21	UCGGACCAGGCUUCAUUCCCC	aly-miR166b-3p
	107	21	UCGGACCAGGCUUCAUUCCCC	rco-miR166d
	107	21	UCGGACCAGGCUUCAUUCCCCC	ssp-miR166
	106	21	UCGGACCAGGCUUCAUUCCCC	atr-miR166d
	106	21	UCGGACCAGGCUUCAUUCCCC	nta-miR166d
	106	21	UCGGACCAGGCUUCAUUCCCC	nta-miR166h

		105	21	UCGGACCAGGCUUCAUUCCCC	aly-miR166f-3p
		105	21	UCGGACCAGGCUUCAUUCCCC	bna-miR166d
		105	21	UCGGACCAGGCUUCAUUCCCC	csi-miR166e-3p
		105	21	UCGGACCAGGCUUCAUUCCCC	lus-miR166j
		105	21	UCGGACCAGGCUUCAUUCCC	pta-miR166b
		105	21	UCGGACCAGGCUUCAUUCCCC	vvi-miR166f
		104	21	UCGGACCAGGCUUCAUUCCCC	aly-miR166a-3p
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		103	21	UCGGACCAGGCUUCAUUCCC	lus-miR166a
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		103	21	UCGGACCAGGCUUCAUUCCCC	vvi-miR166d
		103	21	UCGGACCAGGCUUCAUUCCCC	vvi-miR166g
		102	21	UCGGACCAGGCUUCAUUCCCC	ath-miR166d
		102	21	UCGGAUCAGGCUUCAUUCCUC	bdi-miR166i-3p
		102	21	UCGGACCAGGCUUCAUUCCC	gma-miR166e
		102	21	UCGGACCAGGCUUCAUUCCC	mes-miR166e
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		99	21	UCGGACCAGGCUUCAUUCCCC	aly-miR166e-3p
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		95	21	UCGGACCAGGCUUCAUUCCCC	atr-miR166c
		95	21	UCGGACCAGGCUUCAUUCCCC	vvi-miR166h
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		90	21	UCGGACCAGGCUUCAUUCCCC	cpa-miR166c
		90	21	UCGGACCAGGCUUCAUUCCCC	hvu-miR166c
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		88	21	UCGGACCAGGCUUCAUUCCCC	ata-miR166e-3p
		85	21	UCGGACCAGGCUUCAUUCCCC	aly-miR166c-3p
		85	21	UCGGACCAGGCUUCAUUCCC	mes-miR166b
		84	21	UCGGACCAGGCUUCAUUCCUU	gma-miR166o
		83	21	UCGGACCAGGCUUCAUUCCCC	stu-miR166c-3p
		79	21	UCGGACCAGGCUUCAUUCCCC	nta-miR166c
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		1143	21	UCGGACCAGGCUUCAUUCCCC	bdi-miR166f
		39	21	UCGGACCAGGCUUCAUUCCCC	mes-miR166i
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		15	21	UCGGACCAGGCUUCAUUCCCC	csi-miR166d
		13	21	UCGGACCAGGCUUCAUUCCCC	bdi-miR166e-3p
		12	20	UCGGACCAGGCUUCAUUCCCC	csi-miR166c
		11	21	GGAAUGUUGUCUGGCACGAGG	bdi-miR166e-5p
		10	22	UCGGACCAGGCUUCAUUCCC	csi-miR166b
		10	20	UCGGACCAGGCUUCAUUCCC	gma-miR166r
		10	20	UCGGACCAGGCUUCAUUCCC	gma-miR166t
		10	21	AAUGGAGGCUGAUCCAAGAUC	mtr-miR166g-5p
miR171		59	23	AGAUAUUAGUGCGGUUCAAUC	gma-miR171b-5p
		16	21	GAGGUGAGCCGAGCCAAUAUC	mtr-miR171g
		11	21	UGAUUGAGCCGUGCCAAUAUC	ppe-miR171d-3p
		10	21	GUGAGCCGAACCAAUAUCACU	mtr-miR171h
miR2275		13	21	AGGAUUAGAGGGACUUGAACC	zma-miR2275c-5p
		12	21	AGAGUUGGAGGAAAGAAAACU	zma-miR2275d-5p
miR3630		26	22	UGCAAGUGACGAUAUCAGACA	han-miR3630-5p
		18	22	UUUGGGAAUCUCUCUGAUGCAC	vvi-miR3630-3p
miR399		13	21	UGCCAAAGGAGAUUUGCCCUG	ppe-miR399a
miR476		67	20	UAAUCCUUCUUUGCAAAGUC	hbr-miR476
miR482		129	22	UCUUCCCUACUCCUCCCAUUCC	hbr-miR482a
		97	22	UCUUCCCUACUCCUCCCAUUCC	ghr-miR482b

	81	22	UCUUUCCUACUCCUCCCAUUCC	mes-miR482
	38	21	UCUUCCCUACUCCUCCCAUUCC	hbr-miR482b
	34	22	UCUUCCCUACACCUCCCAUACC	stu-miR482d-3p
	33	22	UCUUCCCUACUCCUCCCAUUCC	sly-miR482b
	30	22	UCUUUCCUACUCCACCCAUUCC	ppe-miR482f
miR4995	78	21	AGGCAGUGGCUUGGUUAAGGG	gma-miR4995
miR5021	23	20	UGAGAAGAAGAAGAAGAAAA	ath-miR5021
miR535	31	21	UGACAACGAGAGAGAGCACGC	ppe-miR535b
miR5368	601	19	GGACAGUCUCAGGUAGACA	gma-miR5368
miR5653	26	24	UGGGUUGAGUUGAGUUGAGUUGGC	ath-miR5653
miR5658	14	21	AUGAUGAUGAUGAUGAUGAAA	ath-miR5658
miR6173	164	20	AGCCGUAAACGAUGGAUACU	hbr-miR6173
miR7760	18	24	CAGCGGACAGAAUGGAGCAAGCAG	bdi-miR7760-5p
miR7782	11	24	ACCUGCUCUGAUACCAUGUUGUGA	bdi-miR7782-3p
miR8175	13	20	GAUCCCCGGCAACGGCGCCA	ath-miR8175
miR827	112	21	UUAGAUGACCAUCAACAAACU	ath-miR827
	19	21	UUAGAUGACCAUCAACAAACA	ghr-miR827b
	19	21	UUAGAUGACCAUCAACAAACU	ppe-miR827
	15	21	UUAGAUGACCAUCAACAAACU	mes-miR827
	14	21	UUAGAUGACCAUCAACAAACA	ghr-miR827c
	13	21	UUAGAUGACCAUCAACAAACA	ghr-miR827a
	12	21	UUAGAUGACCAUCAACAAACU	csi-miR827
miR858	22	21	UUCGUUGUCUGUUCGACCUUG	ath-miR858b
	14	21	UUUCGUUGUCUGUUCGACCUU	aly-miR858-5p

Table 2.2 Targets of conserved miRNAs in *Hevea brasiliensis*

S.No.	miRNA	Target Id	Hit Acc.	Protein Name
1	gmamiR166h3p	isotig04052	CBI36079.3	unnamed protein product [Vitis vinifera]
2	gmamiR166h3p	isotig04050	CBI36079.3	unnamed protein product [Vitis vinifera]
3	gmamiR166h3p	isotig04053	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
4	gmamiR166h3p	isotig04051	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
5	gmamiR166h3p	contig10287	EOY25497.1	Homeobox-leucine zipper family protein / lipid-binding START domain-containing protein isoform 6 [Theobroma cacao]
6	bdimiR166f	isotig04052	CBI36079.3	unnamed protein product [Vitis vinifera]
7	bdimiR166f	isotig04050	CBI36079.3	unnamed protein product [Vitis vinifera]
8	bdimiR166f	isotig04053	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
9	bdimiR166f	isotig04051	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
10	bdimiR166f	contig10287	EOY25497.1	Homeobox-leucine zipper family protein / lipid-binding START domain-containing protein isoform 6 [Theobroma cacao]
11	gmamiR166k	isotig04052	CBI36079.3	unnamed protein product [Vitis vinifera]
12	gmamiR166k	isotig04050	CBI36079.3	unnamed protein product [Vitis vinifera]
13	gmamiR166k	isotig04053	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
14	gmamiR166k	isotig04051	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
15	gmamiR166k	contig10287	EOY25497.1	Homeobox-leucine zipper family protein / lipid-binding START domain-containing protein isoform 6 [Theobroma cacao]
16	atrmiR166b	isotig04052	CBI36079.3	unnamed protein product [Vitis vinifera]
17	atrmiR166b	isotig04050	CBI36079.3	unnamed protein product [Vitis vinifera]
18	atrmiR166b	isotig04053	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
19	atrmiR166b	isotig04051	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
20	atrmiR166b	contig10287	EOY25497.1	Homeobox-leucine zipper family protein / lipid-binding START

				domain-containing protein isoform 6 [Theobroma cacao]
21	hbrmiR6173	isotig14360	ERP59677.1	hypothetical protein POPTR_0006s19580g [Populus trichocarpa]
22	vvimiR166e	isotig04052	CBI36079.3	unnamed protein product [Vitis vinifera]
23	vvimiR166e	isotig04050	CBI36079.3	unnamed protein product [Vitis vinifera]
24	vvimiR166e	isotig04053	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
25	vvimiR166e	isotig04051	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
26	vvimiR166e	contig10287	EOY25497.1	Homeobox-leucine zipper family protein / lipid-binding START domain-containing protein isoform 6 [Theobroma cacao]
27	hbrmiR482a	isotig03914	XP_002515202.1	conserved hypothetical protein [Ricinus communis]
28	hbrmiR482a	isotig03915	XP_002515202.1	conserved hypothetical protein [Ricinus communis]
29	hbrmiR482a	isotig03916	XP_002515202.1	conserved hypothetical protein [Ricinus communis]
30	hbrmiR482a	isotig03917	XP_002515202.1	conserved hypothetical protein [Ricinus communis]
31	gmamiR166g	isotig04052	CBI36079.3	unnamed protein product [Vitis vinifera]
32	gmamiR166g	isotig04050	CBI36079.3	unnamed protein product [Vitis vinifera]
33	gmamiR166g	isotig04053	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
34	gmamiR166g	isotig04051	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
35	gmamiR166g	contig10287	EOY25497.1	Homeobox-leucine zipper family protein / lipid-binding START domain-containing protein isoform 6 [Theobroma cacao]
36	mesmiR166d	isotig04052	CBI36079.3	unnamed protein product [Vitis vinifera]
37	mesmiR166d	isotig04050	CBI36079.3	unnamed protein product [Vitis vinifera]
38	mesmiR166d	isotig04053	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
39	mesmiR166d	isotig04051	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
40	mesmiR166d	contig10287	EOY25497.1	Homeobox-leucine zipper family protein / lipid-binding START domain-containing protein isoform 6 [Theobroma cacao]
41	cpamiR166a	isotig04052	CBI36079.3	unnamed protein product [Vitis vinifera]
42	cpamiR166a	isotig04050	CBI36079.3	unnamed protein product [Vitis vinifera]
43	cpamiR166a	isotig04053	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
44	cpamiR166a	isotig04051	XP_002515977.1	DNA binding protein, putative [Ricinus communis]

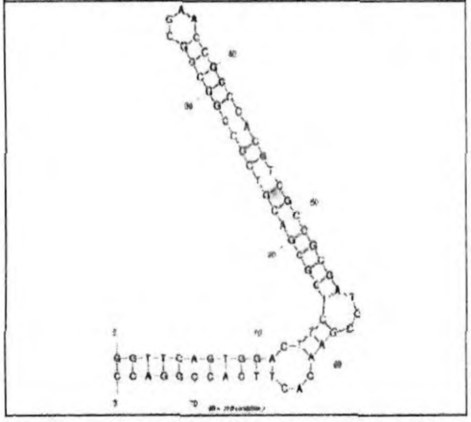
45	cpamiR166a	contig10287	EOY25497.1	Homeobox-leucine zipper family *protein / lipid-binding* START domain-containing protein isoform 6 [Theobroma cacao]
46	ctrmiR166	isotig04052	CBI36079.3	unnamed protein product [Vitis vinifera]
47	ctrmiR166	isotig04050	CBI36079.3	unnamed protein product [Vitis vinifera]
48	ctrmiR166	isotig04053	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
49	ctrmiR166	isotig04051	XP_002515977.1	DNA binding protein, putative [Ricinus communis]
50	ctrmiR166	contig10287	EOY25497.1	Homeobox-leucine zipper family protein / lipid-binding START domain-containing protein isoform 6 [Theobroma cacao]

Table 2.3 Targets of novel miRNAs in *Hevea brasiliensis*

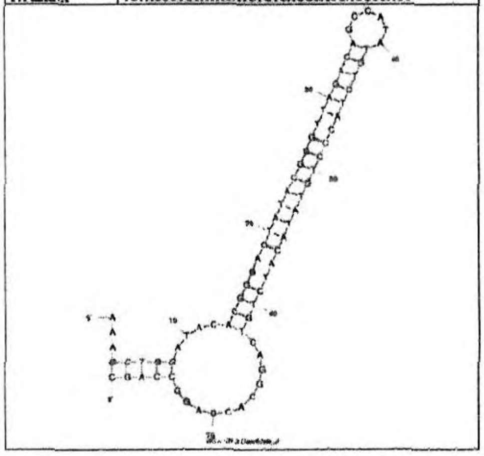
S.No.	miRNA	Target Id	Hit Acc.	Protein Name
1	Candidate_6	isotig21997	XP_002517775.1	cinnamoyl-CoA reductase, putative [Ricinus communis]
2	Candidate_6	isotig05745	XP_002272130.1	PREDICTED: E3 ubiquitin-protein ligase SINAT5 [Vitis vinifera]
3	Candidate_6	isotig05746	XP_002272130.1	PREDICTED: E3 ubiquitin-protein ligase SINAT5 [Vitis vinifera]
4	Candidate_6	isotig14453	XP_002521786.1	Disease resistance protein RPP13, putative [Ricinus communis]
5	Candidate_7	isotig24433	XP_002529780.1	sphingosine-1-phosphate phosphohydrolase, putative [Ricinus communis]
6	Candidate_7	isotig19535	EOX96433.1	Uncharacterized protein TCM 005685
				[Theobroma cacao]
7	Candidate_15	isotig12189	O65812.1	RecName: Full=Profilin-1; AltName: Full=Pollen allergen Hev b 8.0101; AltName: Allergen=Hev b 8.0101
8	Candidate_15	isotig03961	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
9	Candidate_15	isotig03959	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
10	Candidate_15	isotig03960	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
11	Candidate_15	isotig03958	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
12	Candidate_16	isotig12189	O65812.1	RecName: Full=Profilin-1; AltName: Full=Pollen allergen Hev b 8.0101; AltName: Allergen=Hev b 8.0101
13	Candidate_16	isotig03961	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
14	Candidate_16	isotig03959	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
15	Candidate_16	isotig03960	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
16	Candidate_16	isotig03958	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
17	Candidate_17	isotig12189	O65812.1	RecName: Full=Profilin-1; AltName: Full=Pollen allergen Hev b 8.0101; AltName: Allergen=Hev b 8.0101
18	Candidate_17	isotig03961	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
19	Candidate_17	isotig03959	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
20	Candidate_17	isotig03960	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
21	Candidate_17	isotig03958	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
22	Candidate_18	isotig12189	O65812.1	RecName: Full=Profilin-1; AltName: Full=Pollen allergen Hev b 8.0101; AltName: Allergen=Hev b 8.0101
23	Candidate_18	isotig03961	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
24	Candidate_18	isotig03959	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
25	Candidate_18	isotig03960	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]
26	Candidate_18	isotig03958	XP_002522372.1	GTP-binding protein alpha subunit, gna, putative [Ricinus communis]

Fig.2.1 Stem-loop structure of novel miRNAs of *Hevea brasiliensis*

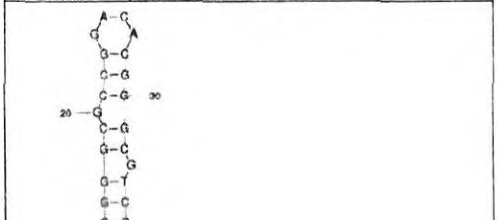
mature_miRNA_id	Candidate_1
MFE	-32.8
Chromosome	isotig28211
Chr_start	125
Chr_end	199
mature_miRNA	CACGTCGCCGCGATCCGAACACT
miRNA length	23
Precursor miRNA	GGTTCAGTGGACTTCTCGCGACGTCGCCGGCGGCGAACCGCC CACGTCGCCGCGATCCGAACACTTCACCGGACC



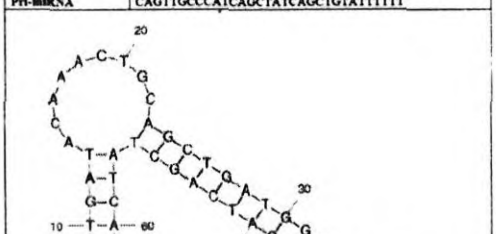
mature_miRNA_id	Candidate_2
MFE	-31.3
Chromosome	isotig13585
Chr_start	296
Chr_end	373
mature_miRNA	ACCCTGTATACATCTGTCAGGCA
miRNA length	23
Pri-miRNA	AAAGCTGGATACACGGGAGTATACGGGTTAGACAGCCATATG TCTACCCTGTATACATCTGTCAGGCACGAGGCCAGC



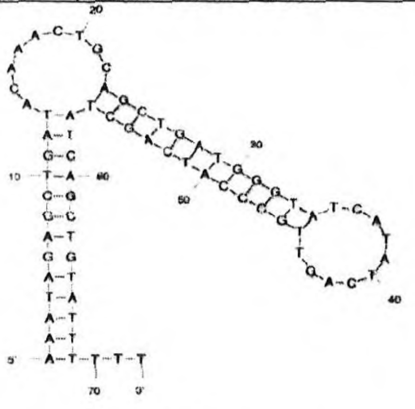
mature_miRNA_id	Candidate_3
MFE	-30.5
Chromosome	*isotig*27223
Chr_start	228
Chr_end	289
mature_miRNA	CGTCGCTTCTGGCCCGGATTC
miRNA length	21
Pri-miRNA	TGCGGGTCGGCAAGCGGGCGCCGGACACGGGCGTCGCTT CTGGCCCGGATTCTGACTTAGAG



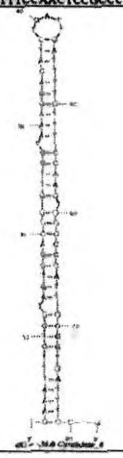
mature_miRNA_id	Candidate_4
MFE	-31.6
Chromosome	isotig12442
Chr_start	588
Chr_end	659
mature_miRNA	GATACAAACTGCAGCTGATGGGTA
miRNA length	24
Pri-miRNA	AAATAGAGCTGATACAAACTGCAGCTGATGGGTATCATAT CAGTTGCCCATCAGCTATCAGCTGTATTTTTT



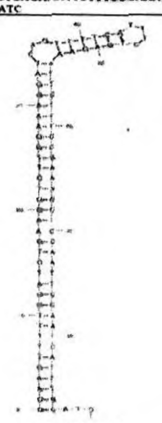
mature_miRNA_id	Candidate_5
MFE	-31.6
Chromosome	motig12443
Chr_start	565
Chr_end	636
mature_miRNA	GATACAAACTGCAGCTGATGGGTA
miRNA length	24
Pri-miRNA	AAATAGAGCTGATACAAACTGCAGCTGATGGGTATCATATC AGTTGCCCATCAGCTATCAGCTGTATTTTTT



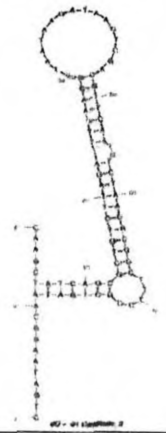
mature_miRNA_id	Candidate_6
MFE	-33.6
Chromosome	isotig19436
Chr_start	16
Chr_end	97
mature_miRNA	TTTCCAACTCCGCCCATTCCGG
miRNA length	22
Pri-miRNA	TGAAATTTCTGGTAGTGGACGGGTTGGTAAAGGACATCTG ATTGATTTTCTTTCCAACTCCGCCCATTCCGGTGATTTCCTG



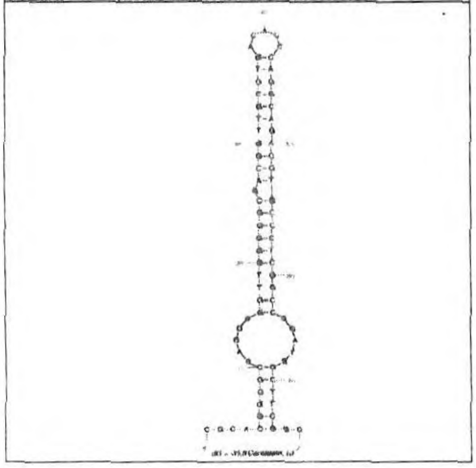
mature_miRNA_id	Candidate_7
MFE	-36.5
Chromosome	isotig19436
Chr_start	201
Chr_end	290
mature_miRNA	TTTCCGAAACCTCCAATTCCAAT
miRNA length	23
Pri-miRNA	GCAAGTTTTTGGATCTGAAGGTCTTGGAAAGGATCAGTTTT CATTTCTGAGAAATTCTTTCCGAAACCTCCAATTCCAATCA TTTGCATC



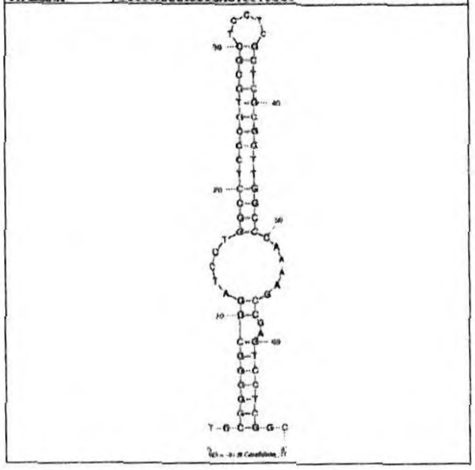
mature_miRNA_id	Candidate_9
MFE	-31
Chromosome	isotig23551
Chr_start	114
Chr_end	203
mature_miRNA	GCGCTGTCTTAGATTGAACGG
miRNA length	21
Pri-miRNA	CAAGCTATCAGCGCTGTCTTAGATTGAACGGTAATAAGATAAGTC GACGTTCAATCTCTAAGGCGGCTTTCCGCTGATAACGGAATAGTC



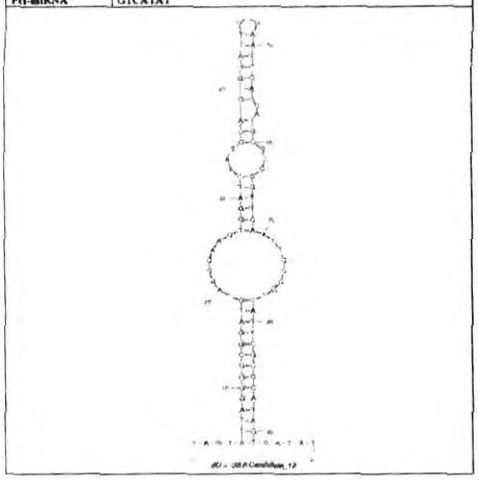
mature_miRNA_id	Candidate_10
MFE	-35.8
Chromosome	contig00023
Chr_start	538
Chr_end	613
mature_miRNA	GAGGGGGTTGGGGGCGACGGTT
miRNA length	22
Pri-miRNA	CGCACGGGGCGAGGGGGTTGGGGGCGACGGTTGCGTGACACCC AGGCAGACGTGCCCTCGGCCGGATGGCTTCGGG



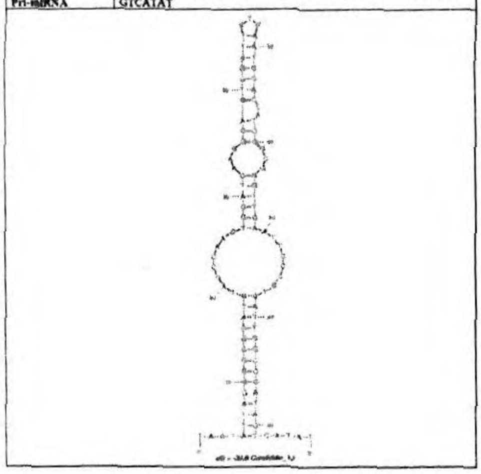
mature_miRNA_id	Candidate_11
MFE	-31.9
Chromosome	contig00023
Chr_start	466
Chr_end	533
mature_miRNA	GCTCGCGGTTGGCCCAAAAGCCG
miRNA length	23
Pri-miRNA	TGCGGGGGCGGATCCTGGCCTCCCGTGCGCTCCTCGCTCGCGGTT GGCCCAAAAGCCGAGTCCTCGGC



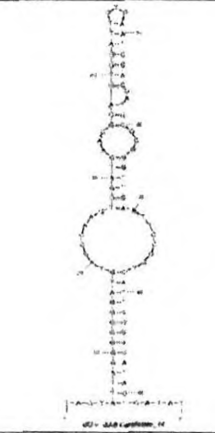
mature_miRNA_id	Candidate_12
MFE	-33.6
Chromosome	contig00036
Chr_start	5437
Chr_end	5532
mature_miRNA	GGCGGATGTAGCCAAGTGGATCAA
miRNA length	24
Pri-miRNA	TAGTATTAGGGGCGGATGTAGCCAAGTGGATCAAGGCAGTGGAT TGTGAATCCACCATGCGCGGGTTCAATTCCCGTCATTCGCCCATA GTCATAT



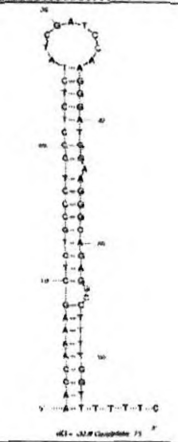
mature_miRNA_id	Candidate_13
MFE	-33.6
Chromosome	contig00038
Chr_start	5437
Chr_end	5532
mature_miRNA	GGCGGATGTAGCCAAGTGGATCAA
miRNA length	24
Pri-miRNA	TAGTATTAGGGGCGGATGTAGCCAAGTGGATCAAGGCAGTGGAT TGTGAATCCACCATGCGCGGGTTCAATTCCCGTCATTCGCCCATA GTCATAT



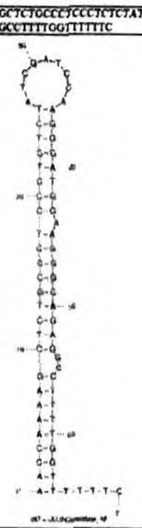
mature_miRNA_id	Candidate_14
MFE	-33.6
Chromosome	isotig00041
Chr_start	4525
Chr_end	4620
mature_miRNA	GGCGGATGTAGCCAAGTGGATCAA
miRNA length	24
Pri-miRNA	TAGTATTAGGGGCGGATGTAGCCAAGTGGATCAAGGCAGTGGATTGTGAATCCACCATGCGCGGGTTCAATTCCCGTCATTCGCCCATAGTCATAT



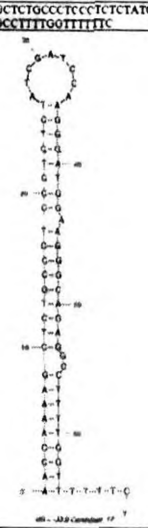
mature_miRNA_id	Candidate_15
MFE	-33.9
Chromosome	isotig00118
Chr_start	5
Chr_end	73
mature_miRNA	GATGGAAGGGCAGAGGCCTTT
miRNA length	21
Pri-miRNA	AACCAAAAGCTCTGCCCTCCCTCTCTATCGATCCAAGGGATGGAAGGGCAGAGGCCTTTTGGTTTTTTC



mature_miRNA_id	Candidate_16
MFE	-33.9
Chromosome	isotig00122
Chr_start	5
Chr_end	73
mature_miRNA	GATGGAAGGGCAGAGGCCTTT
miRNA length	21
Pri-miRNA	AACCAAAAGCTCTGCCCTCCCTCTCTATCGATCCAAGGGATGGAAGGGCAGAGGCCTTTTGGTTTTTTC



mature_miRNA_id	Candidate_17
MFE	-33.9
Chromosome	isotig00129
Chr_start	1188
Chr_end	1256
mature_miRNA	GATGGAAGGGCAGAGGCCTTT
miRNA length	21
Pri-miRNA	AACCAAAAGCTCTGCCCTCCCTCTCTATCGATCCAAGGGATGGAAGGGCAGAGGCCTTTTGGTTTTTTC



mature_miRNA_id	Candidate_18
MFE	-33.9
Chromosome	contig00126
Chr_start	5
Chr_end	73
mature_miRNA	GATGGAAGGGCAGAGGCCTTT
miRNA length	21
Pri-miRNA	AACCAAAAGCTCTGCCCTCCCTCTCTATCGATCCAAGGGATGGAAGGGCAGAGGCCTTTTGGTTTTTTC