EXPRESSION PROFILING OF microRNAs ASSOCIATED WITH WATER STRESS IN BANANA (Musa spp.)

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KERALA, INDIA

2020

DECLARATION

I, hereby declare that this thesis entitled "**EXPRESSION PROFILING OF microRNAs ASSOCIATED WITH WATER STRESS IN BANANA (*Musa* spp.)**" is a bonafide record of the research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other university or society.

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Certified that this thesis entitled "**EXPRESSION PROFILING OF microRNAs ASSOCIATED WITH WATER STRESS IN BANANA (*Musa* spp.)**" is a record of research work done independently by Mr. Sayooj P. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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LIST OF SYMBOLS AND ABBREVIATIONS

A genome	*Musa acuminata* genome
A_{260}	Absorbance at 260 nm wave length
A_{280}	Absorbance at 280 nm wave length
ABA	Abscisic acid
APX	Ascorbate peroxidase
B genome	*Musa balbisiana* genome
BLAST	Basic local alignment search tool
bp	Base pairs
cDNA	Complementary DNA
Cl^-	Chloride ion
CPN20	Chaperonin 20
DNA	Deoxyribonucleic acid
DREs	Dehydration-responsive elements
dsRNA	Double-stranded RNA
EST	Expressed sequence tags
FP	Forward primer
gm	Gram
GPX	Glutathione peroxidase
GST	Glutathione s-transferase
h	Hour
HEN1	Hua Enhancer 1
HST	Exportin-5 homolog named Hasty
INV	invariant method
ISH	*In situ* hybridization

m	Meter
M	Molar
M. acuminata	*Musa acuminata*
M. balbisiana	*Musa balbisiana*
Mb	Mega bases
MFE	Minimal folding energy
mg	Milligram
min	Minute
miRNA	MicroRNA
miRNA*	MicroRNA star stand
ml	Millilitre
mM	Millimolar
MPSS	Massively parallel signature sequencing
mRNA	Messenger RNA
mRNA-Seq	mRNA sequencing
Na^+	Sodium ion
NaCl	Sodium chloride
NAT-siRNAs	Natural antisense siRNAs
NCBI	National centre for biotechnology information
ng	Nanogram
NGS	Next generation sequencing
nM	Nanomolar
nt	Nucleotides
NYFA5	Nuclear factor Y subunit A
PCR	Polymerase chain reactions
PMRD	Plant MicroRNA Database
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA

PTGS	Post-transcriptional gene silencing
QTL	Quantitative trait loci
RFU	Raw fluorescence unit
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT-PCR	Reverse-transcriptase PCR
RT-qPCR	Reverse-transcription quantitative PCR
siRNA	Small interfering RNA
snoRNA	Small nucleolar RNA
SOD	Superoxide dismutase
sp.	Species (singular)
spp.	Species (plural)
sRNA-Seq	Small RNA sequencing
SSR	Short sequence repeat
Tm	Melting temperature
TMM	Trimmed Mean of Method
tRNA	Transfer RNA
U	Unit
V	Volt
VSN	variance stabilization
XEH	Xyloglucan endohydrolase
XET	Xyloglucan endotransglucosylase
µl	Microliter

Introduction

1. INTRODUCTION

Bananas are the most important crops cultivated and consumed across the world and take the fourth position in the developing world in terms of gross production value and it is a commodity that carries high export volume (Perrier *et al*., 2011), with an average production of 115.73 million tonnes. India produces 29-31 million tonnes of bananas annually and ranks first in the production and productivity around the world (FAOSTAT, 2018). Only a few numbers of genotypes contribute towards the majority of global banana production as bananas have a narrow as well as poor genetic base. Hence cultivated bananas are more susceptible to various biotic stresses like pests and diseases such as fungi, bacteria, viruses, nematodes and insects and abiotic stresses like as drought, salinity, heat, flood, radiation and poor soil fertility (Perrier *et al*., 2011).

Soil moisture deficit stress has the most devastating effect among the abiotic stresses, which limits and reduces the production and productivity of bananas. Water stress is experienced by plants when the supply of water to the roots retards or due to an intense rise in the transpiration rate.

Drought stress is a performance limiting factor as well as a great threat to the successful production of crops. It negatively affects plant growth and development in banana, leading to a sharp decline in fruit productivity. The effect depends on the stage of the crop, as well as the duration and intensity of the stress (Muthusamy *et al*., 2014). Moisture stress is a complex trait involving physiochemical processes in which a large number of biomolecules are involved (Bartels, 1996). Transcriptional and posttranscriptional levels of regulatory responses are shown during drought stress.

Presently the role of small RNAs in abiotic and biotic stresses in plants has become the focus of research and the RNA interference mechanism of siRNA and miRNA has proved to be successful in providing tolerance towards various abiotic

stress in plants. microRNAs take part in drought stress induced gene expression regulation as post-transcriptional gene regulators.

The miRNAs are 18-22-nt short single stranded functional RNAs that are transcribed from a distinct class of genes and regulate the expression of other genes at

the post-transcriptional level. In plants, post-transcriptional gene silencing (PTGS) is the mechanism involved in miRNA mediated gene regulation, which operates through an RNA interference (RNAi) pathway. Responsive mechanisms in the plant depend on specific miRNA expression mediated regulation of gene regulatory networks and transcription factors (Zhou *et al*., 2010). As suggested by Sunkar *et al*. (2012) and Kantar *et al*. (2011) the expression profiles of protein-coding genes fluctuate greatly in response to drought stress due to the expression of specific miRNAs. So, the information on water stress responsive miRNAs and their targets will be helpful in developing strategies to impart stress tolerance in banana.

Several plant miRNAs are known to regulate responses to plant development and environmental stresses, including drought, salinity, and nutrient deficiency (Ding *et al*., 2009). Several miRNAs associated with abiotic stress conditions are identified in the banana (Wang *et al*., 2017). Approximately 25 percent of the miRNAs identified to date have been found in stress induced ESTs.

Water stress induces a unique set of miRNAs which are expressed in banana leaves, thereby regulate several biological processes such as stress induced signalling, transcriptional as well as translational regulations, stress defense and tolerance, cellular transport and homeostasis, metabolic as well as other stress-related functions. Several genes that are coding for proteins responsive to salt stress were predicted and found to be regulated by miRNAs. Even though 37 families have been identified in banana which consists of 235 miRNAs in banana (D'Hont *et al*., 2012), their roles in abiotic stress involved responses, mainly during moisture-deficit stress in banana remain unclear.

In a study conducted in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2016-18, 52 miRNAs were predicted in banana genome using computational tools and their 124 target genes were identified. From this information generated, five miRNAs and their predicted targets were selected in the present-day study with an objective to identify the miRNAs associated with water stress in banana var. Nendran from the expression profile of selected miRNAs.

Review of literature

2. REVIEW OF LITERATURE

2.1 BANANA; ORIGIN AND GEOME CONSTITUTION

Banana is one among the foremost important commercial crops, cultivated throughout the world. It's the world's most favourite and starchy staple food crop, consumed and cultivated in over 130 countries, within the tropics and subtropical regions. Bananas and plantain are large perennial monocotyledonous herbs, belonging to the *Musa* genus that belongs to the order Zingiberales.

There are two types of bananas, dessert (*Musa acuminata*) and cooking type (*Musa balbisiana*). The wild bananas are normally seeded and sexually propagated, whereas present day edible bananas are generally sterile and polyploids of two triploid species, *Musa acuminate* and *Musa balbisiana* and their hybrids. All these varieties are parthenocarpic and are propagated through suckers.

The banana varieties include diploid (AA, BB, AB), triploid (AAA, AAB, ABB) and synthetic tetraploids (AAAA, AAAB, AABB and ABBB). The origin of banana dates back to 7^{th}century AD within the humid tropical rain forest of south east Asia, present day bananas are cultivated throughout the humid tropical regions between 30^{o} north and 30^{o} of the equator.

Average production of banana is estimated to be 115.73 million tonnes in over 120 countries and India ranks first with 30.8 million tonnes, which accounts for about 13percent of the area and 33percent of worlds production (FAOSTAT, 2018).

Sequencing of whole genome of wild banana (*Musa acuminata*) has revealed the distribution and genome structure of coding and non-coding sequences, transposable elements, regulatory elements, transcription factors, repetitive DNA etc. within the genome of banana (D'Hont, *et al*., 2012). Composition, structure and function of genes in bananas are often well understood by various genetic and cytogenetic investigations (Roux *et al*., 2008). In the haploid stage, *Musa acuminate* genome has 11 chromosomes and 600Mb size (Heslop-Harrison and Schwarzacher, 2007). The genome sequencing of *Musa acuminate* ssp. showed genome value 1C = 523Mb for

double-haploid genotype of this wild subspecies as estimated in flow cytometry (D'Hont., *et al*., 2012). Transcriptomic studies help to elucidated expressed sets of genes or transcripts and their expression levels in specific growth and development stages of banana plants, and their response to various environmental factors. The progress and advancement occurred in *Musa* genomics, transcriptomics and genetics were due to major contributions from many *Musa* research groups, organizations, institutes and collaboration networks all over the world, mostly by the collaborations and sharing of resources at international level.

Knowledge on genetic and molecular changes in banana plant is useful in formulation of genetic improvement programmes in plants to develop desirable characteristics in plants, such as resistance and tolerance to abiotic and biotic stresses, herbicide resistance, insect resistance and improved yield, because the global banana production is a battle against biotic and abiotic stress. Banana production is hampered by pathogens, insects and by suboptimal cultivation environments and, as with other crops, is to be adversely affected by global climate change and related biotic and abiotic stress.

2.2 ABIOTIC STRESS

In biological terms, stress is defined as an adverse force or a condition which inhibits the normal functioning and well-being of a biological system (Mahajan and Tuteja, 2005). Abiotic and biotic stresses are the major threats to the production and productivity of banana worldwide resulting in the major reduction of economic yield of the plant. The varied abiotic stresses that causes adverse effects in plant growth and productivity are drought, flooding, salinity, cold, heat, radiation, chemicals and pollutants, oxidative stress, wind, and nutrient stress (Mahajan and Tuteja, 2005). Abiotic stresses lead to reduction in crop productivity, and thereby causes agricultural and economical loss of nearly hundreds of million rupees annually by which sustainable agriculture and food security are threatened (Mahajan and Tuteja, 2005).

2.2.1 water stress and its effects on plants

Plants experience water stress when the water supply to their roots becomes limiting or when the transpiration rate becomes intense. Water stress is primarily caused by the water deficit, *i*e. drought or soil salinity. The condition where plants are not capable of absorbing water albeit it is present within the soil solution in the case of high salinity in the soil, periods of inundation and low soil temperature is called "physiological drought" (Lisar *et al.*, 2012).

Drought stress is considered as one of the crop's performance limiting factors that threatens successful crop production. Soil moisture deficit stress negatively affects plant growth and development in banana, resulting in a sharp decline in fruit production and productivity. The effect depends on the stage of the crop, as well as the intensity and duration of the stress (Muthusamy *et al*., 2014). The weight of the bunch is affected due to the effect of water deficit during the flowering period, which reduces the number of fingers produced (Fermont *et al.*, 2011).

2.2.2 Water stress response and regulation

A better understanding on the mechanisms at molecular level involved in stress induced response is highly helpful for engineering or imparting tolerance in plants towards stress by enhancement or introduction of stress tolerance mechanisms with the help of molecular and genetic engineering techniques (Mizoi and Yamaguchi-Shinozaki, 2013; Nakashima *et al*., 2014).

Accelerated progress is achieved in understanding abiotic stress responses with recent advancement in plant molecular biology, and many genes have been discovered which are found to be having association with adaptation to stress conditions (Hirayama and Shinozaki, 2010; Mizoi and Yamaguchi-Shinozaki, 2013). Those genes consist of regulatory genes, for transcription factors and protein kinases; functional genes, for enzymes like biosynthesis enzymes and antioxidant enzymes for protective metabolites production, transporter and particular protective proteins; and stress tolerance associate quantitative trait loci (QTLs) (Mizoi and Yamaguchi-Shinozaki, 2013).

Cellular metabolisms during drought conditions is regulated by the reduction in water potential and surge in cells ABA content. Elevated levels of proline, betaine and glycine can be considered as the major responses at molecular level to drought stress (Matysik *et al*., 2002).

Osmotic adaptation is the accumulation of solutes in cells under stress conditions, in order to compensate cell volume against the loss of water (Fathi and Tari, 2016). A common physiological survival mechanism used by plants in response to different stresses involve low-molecular weight organic compounds accumulation which are highly water soluble and nontoxic even at high concentrations. These compounds are often called osmoprotectants or compatible solutes which protect plants from stress by osmotic adjustment as well as by acting as ROS scavengers to protect cell structures from any kind of damage and denaturation and also as low-molecular-weight chaperones that protect membrane integrity and stabilize proteins.

Lipid peroxidation and membrane deterioration are caused by free radicals induced during drought stress in plants (Nair *et al*, 2008). An imbalance is created between antioxidant defences and amount of Reactive Oxygen Species (ROS) due to drought stress, resulting in oxidative stress. ROS which are necessary for intracellular signalling at optimal concentrations can cause damage at its high concentration at various levels of organization including chloroplasts and mitochondria (Smirnoff, 1993). The destructive capacity of ROS ranges from initiation of lipid peroxidation to degradation of proteins, lipids and nucleic acids (Tian *et al.*, 2012). The lipid peroxidation retardation Mechanism consists of enzymes that scavenge free radical, such as superoxide dismutase, catalase and peroxidase (Fridovich *et al*., 2000). In chloroplasts there are number of enzymatic and nonenzymatic antioxidants that serve in preventing ROS accumulation (Srivalli *et al*., 2003). The increased ROS accumulation during water stress is regulated by the antioxidant system and it protects the cell by regulating the ROS concentration at intracellular level. increased lipid peroxidation in the cell membrane is one of the most damaging cellular responses observed during water stress due to cellular build-up of ROS (Thankamani *et al*., 2003). The amount of lipid peroxidation can be considered as one of the factors indicating the severity of stress experienced by a plant (Chowdhury and Chowdhury, 1985).

2.3 STRATEGIES TO COMBAT WATER STRESS

2.3.1 Drought tolerant plants

The adaptation of the plant to different abiotic stress conditions includes a series of cell physiological processes including the production of metabolites and proteins that are involved in protective and regulatory functions. Therefor many of the strategies used for obtaining plants with increased stress tolerance are aimed at the modulation of the expression of genes involved in the biosynthesis of these metabolites, as well as those coding for those proteins that play a direct part in the mechanisms of protective function of the plant cell.

One of the main strategies for achieving greater tolerance to abiotic stress is to improve the capacity of plants to re-establish both ionic and osmotic homeostasis that are altered under stressful environments. Transporter proteins involved in the transport of solutes can be manipulated with the help of genetic engineering to develop stress tolerant transgenic plants. Overexpression of aquaporins has resulted in improvement of tolerance to drought stress (Lian *et al*., 2004).

Plants overproducing Biosynthesis enzymes are involved in the synthesis of protective metabolites or compatible solutes such as amino acid, proline which acts as an osmolyte and contributing to the maintenance of cell turgor or osmotic balance, other roles have been proposed for proline in plant stress response, such as molecular chaperone, being a metal chelator, forming part of the antioxidative defines mechanism, or even playing a role in signalling. The accumulation of proline in plants under stress is caused either by the induction of expression of proline biosynthesis genes or by the repression of the genes of its degradation pathway (Bhatnagar-Mathur *et al*., 2008).

Since the majority of abiotic stresses induce the accumulation of active ROS, which in turn cause oxidative Boosting cell enzymatic and nonenzymatic ROS-scavenging systems (Gill and Tuteja 2010; Miller *et al*. 2010), an improved stress tolerance has been observed in several transgenic plants that overexpress several antioxidant enzymes such as GPX, SOD, APX, and GST (John *et al*., 2010).

Another transgenic strategy for obtaining tolerance against stresses in plants to modulate the expression of genes encoding the regulatory proteins that are part of those signal transduction pathways, in order to mimic or even enhance the plant adaptation mechanisms (Šamajová *et al*., 2013).

2.3.2 RNA silencing mechanism

Fire *et al*. (1998) discovered that RNA silencing is a remarkable type of gene regulation based on sequence-specific targeting and degradation of RNA, which help to develop new concepts to combat various abiotic and biotic stresses in plants. It occurs during transcription and translational phases and it is otherwise known as gene knockdown. The process of gene silencing is carried out by two small RNAs which are siRNA and miRNA. In plants the RNAi technology in is called as post transcriptional gene silencing (Khare *et al.,* 2018).

There are three main pathways of RNA silencing in plants among which, the first pathway is cytoplasmic siRNA Silencing (Hamilton *et al.,* 1999), the second pathway is the silencing of endogenous messenger RNAs by miRNAs, and the third pathway is associated with DNA methylation and suppression of transcription. Among these pathways miRNA mediated gene silencing assists the entry of dsRNA into the cytoplasm, which is then divided into smaller fragments with the help of dicer like enzymes (DCL 1). These double stranded fragments can be either siRNA or miRNA.

The miRNA mediated gene silencing pathway involves the silencing of endogenous messenger RNAs by miRNAs. These miRNAs negatively regulate gene expression by base pairing to specific mRNAs, resulting in either RNA cleavage or arrest of protein synthesis by translation. The prototype miRNAs in plants were considered as a subset of the short RNA population, with the molecular characteristics of the heterochronic RNAs *let-7* and *lin-4* in *Caenorhabditis elegans*. miRNAs are derived from an inverted repeat precursor RNA with partially double-stranded regions, and they target a complementary single-stranded mRNA.

2.4 microRNA

MicroRNAs (miRNAs) class of non-coding, endogenous small single stranded riboregulatory RNAs, which exist in plants, animals, as well as viruses, and play important roles in silencing of genes. Studies proved that these miRNAs take part in in various biological processes, including vegetative cell differentiation, organ development, phase transition, signalling, disease, cancer, and response to biotic and abiotic environmental stresses.

miRNA was first observed in 1991, as small RNA produced from the *lin-4* transcript which was found to be having an impact on *lin-14* mRNA, in modulation of a temporal developmental switch in *Caenorhabditis elegans*. In the year 2001, these small RNAs were named as 'microRNA' as its biogenesis, origin and mode of action were identified. The discovery of genes coding miRNA in *Caenorhabditis elegans* (Lee *et al*., 1993) was followed by an enormous increment in identification of further more miRNA families in plants, which later became a hotspot of research (Cao *et al*., 2014). miRNAs are an important group of regulatory molecules, which regulate gene expression at the post transcriptional levels. Mechanism of miRNA-guided gene regulation at the post-transcriptional levels include Translational repression, mRNA cleavage, and mRNA degradation initiated by miRNA-directed deadenylation of targeted mRNAs.

Many efforts are dedicated to the identification of conserved as well as lineage-specific miRNAs. Currently, it's known that, in developmental processes, lot of plant miRNA target genes are involved and thus any kind of disruption of biogenesis of such miRNAs generally lead to developmental abnormalities like the accelerated growth of lateral roots in the early developmental stages of seedling (Guo *et al*., 2005). The miRNAs directed cleavage of mRNAs was demonstrated in Arabidopsis (Llave *et al*., 2002).

The advancement in the high-throughput sequencing has facilitated the identification of abiotic stress-responsive miRNAs. For instance, it was reported that the expression level of miR398 was decreased due to Cu+, Fe+, ozone, and salt treatments (Jagadeeswaran *et al*., 2009). The identification of the cleavage targets of

miR398 has enabled the understanding of the functions of this miRNA under abiotic stresses (Bouché, 2012).

2.4.1 Biogenesis of microRNA and RNAi machinery

The miRNAs are short single stranded functional RNA species of 18-22-nts long which are formed from stem-loop precursors that are transcriptionally synthesised from endogenous genomic DNA by RNA polymerase II enzyme action (Bartel, 2004; Lee *et al*., 2004). Further capping, splicing, polyadenylation and folding of this non-coding RNA transcripts respectively leads to the formation of precursor miRNA (pre-miRNA) which is a long hairpin stem-loop. A shorter hairpin structure known as primary microRNA (pri-miRNA) is formed by the further processing of the structure by a Dicer-like enzyme, RNAse III in plants (Kurihara and Watanabe, 2004; Liu *et al*., 2005; Chen, 2005b). A shorter double-stranded RNA (dsRNA) is formed by further trimming of the pri-miRNA by Dicer-like enzyme, and form a miRNA:miRNA duplex which is then transported to the cytoplasm from the nucleus (Papp *et al*., 2003). Incorporation of the active strand of the miRNA duplex into a RNA induced silencing complex (RISC or miRISC) occurs in cytoplasm, and these miRISC guides the mature miRNA to the target mRNA which is complementary to the miRNA and there it causes the degradation of the target mRNA, translational inhibition of the target mRNA or transcriptional inhibition by the depositing repressive chromatin marks (Zhang *et al*., 2006c; Cuperus *et al*., 2011; Meng *et al*., 2011; Khraiwesh *et al*., 2012; Axtell, 2013). Plant miRNAs have been found to be having extensive sequence complementarities to their target mRNAs with imperfect paired bases less than 4 (Jones-Rhoades, 2012).

Axtell (2013) recommended the classification of plant small RNAs into two groups via, small interfering RNAs (siRNAs) as well as hairpin RNAs (hpRNAs). The class of hpRNAs are further grouped into ‘miRNAs’ and ‘other hpRNAs’ whereas siRNAs are further classified into heterochromatic siRNAs, secondary siRNA and natural antisense siRNAs (NAT-siRNAs), according to their biogenesis and function. Endogenous origin and hairpin stem-loop RNA structure formation for further processing into miRNA:miRNA duplexes distinguish miRNAs from other categories

of small RNAs. Generally, miRNAs are of 21 nts in size, whereas 20 or 22 nt sized are also there. Some miRNAs that function similar to heterochromatic siRNAs are 23-24 nt in size (Axtell, 2013). But siRNAs have both endogenous as well as exogenous origin and a dsRNA precursor compared to ssRNA derived hpRNA like miRNA precursor. During biogenesis, RNA stem-loop hairpin structure is formed by the folding of miRNA (20-22 nt) and long miRNA (23-24 nt), which are transcribed from genomic DNA by RNA polymerase II. For maturation, miRNA require DCL1-clade DCL and for functioning through slicing, miRNA require AGO1-clade AGO. But in case of long miRNA, they require DCL3-clade DCL for maturation and later leads to heterochromatic siRNA effector pathway and modify the chromatin in their target genes (Axtell, 2013), while AGO 4 bound to siRNA causes histone modification as well as methylation of DNA.

Some of the proteins that take part in miRNA biogenesis and RNA silencing actions in plants include: Hyponastic Laves 1 (HYL1) which is a double-stranded RNA binding protein, protein that assist DCL1 in cleaving nuclear pri-miRNA called ethylene zinc finger protein Serrate (SE), proteins that methylates miRNA:miRNA duplexes called ; sRNA-specific methyltransferase Hua Enhancer 1 (HEN1), protein that exports miRNA:miRNA duplex from the nucleus to the cytoplasm called Exportin-5 homolog named Hasty (HST) (Rogers and Chen, 2013).

miRNA mediated gene regulation mechanism follows (RNAi) pathway, which involve post-transcriptional gene silencing (PTGS) mainly in plants and in fungi it is quelling. RNA silencing process, RNA interference or RNAi consist of a group of effector proteins which carries hairpin or longer double-stranded RNA derived small regulatory RNA. Preliminary discovery of RNAi phenomenon was from *Caenorhabditis elegans* in response to double-stranded RNA (dsRNA) resulting in sequence-specific gene silencing (Fire *et al*., 1998).

2.4.2 Role of miRNAs in abiotic stress responses in plants

Several miRNAs are identified to be abiotic stress regulated in important in many crops and/or model plants under different variable abiotic stress conditions. Khraiwesh, *et al*. (2012) suggested a network of interaction of biotic and abiotic stress

factors with miRNA-mRNA modules that triggers physiological, metabolic and morphological adaptation like that of stomatal regulation, ROS mediated inactivation and lateral and seminal root formation in Arabidopsis. miR398 is a stress responsive miRNA that was found to be directly linked to the regulation of stress in plants and widely take part in regulation of stresses like oxidative stress, abscisic acid stress, ultraviolet stress, water deficit, salt stress, copper and phosphate deficiency, high sucrose and bacterial infection (Zhu *et al*., 2011). Stress induced down-regulation of miR398 cause up-regulation of its corresponding targets, Cu/Zn superoxide dismutases (CSD1 and CSD2) that are involved in detoxification of reactive oxygen species. Drought as well as ABA stress induced down-regulation of miR169 leads to the accumulation of NFYA5 causing stomatal closure. Biotic and abiotic stress may be enhanced by the growth attenuation caused by the up-regulation of miR393 and miR160 respectively due to the Repression of TIR1 and ARF10, that control auxin perception and signalling (Sunkar *et al*., 2012).

Several miRNA families were found to be involved in various biotic and abiotic stress induced responses and nutrient homeostasis in plants.

2.4.2.1 Role of miRNAs in Drought stress responses

So far, the Role of miRNAs in Drought stress responses in many plants have been studied. Four drought-regulated miRNAs, miR167, miR168, miR171 and miR396 were identified and reported by Liu *et al*. (2008) and Liu *et al*. (2010) in Arabidopsis using microarray analysis, where their promoter's region shown the presence of stress-related elements, whereas the same assay also identified 30 differentially expressed miRNAs under drought stress condition in rice (Zhou *et al*., 2010). miR156, miR166, miR171 and miR408 which are found to have a positive correlation in case of expression of miRNAs and their corresponding target mRNAs, which are suppressed during dehydration-stress in barley were detected as dehydration stress-responsive (Kantar *et al*., 2010). Among the miR169 family in rice, miR169g was the only one miRNA which is found to be induced by drought and the upstream of the miR169g promoter was imparted with dehydration-responsive elements (DREs) (Zhao *et al*, 2007a). ABA-dependent pathway was found to downregulate miR169, that targets

nuclear factor Y subunit A (NYFA5) during drought stress in Arabidopsis. Li *et al*. (2008) reported an enhanced drought tolerance in response to the overexpression of an miR169-resistant NYFA mutant by enhancing stomatal closure under drought stress. Zhang *et al*. (2011b) indicated the increased expression of miR169 in drought-stressed affected tomato plants. miR169 targets three nuclear factor Y subunit genes (SINF-YA1/2/3) and one multidrug resistance-associated protein gene (SIMRP1) and responds to drought stress by negative regulation of stomatal movement (Zhang *et al*., 2011b). Similar study conducted in soyabean shown that the GmNFYA3 target gene overexpression leads to reduced water loss and enhanced drought tolerance (Ni *et al*., 2013). An F-box gene transcript level was reduced by the overexpression of miR394a in soybean and was found to confer tolerance to drought in transgenic Arabidopsis (Ni *et al*., 2012).

As reported by Frazier *et al*. (2011) miR395 is a highly drought stress sensitive miRNA found in tobacco which shown 616-fold upregulation induced by 1percent PEG. *Medicago truncatula* showed up-regulation of miR398 and miR408 in response to water deficit stress. miR162 targeting DCL1 mRNA and miR168 targeting AGO1 mRNA) were down regulated in the roots of *Medicago truncatula* under drought and their targets were upregulated (Capitao *et al*., 2011).

Several miRNAs were identified with the advancement of next generation sequencing, high-throughput sequencing as well as small RNA sequencing, with application of these advanced techniques, many drought-responsive miRNAs were identified in plants such as cowpea (Barrera-Fig.ueroa *et al*., 2011), *Medicago truncatula* (Wang *et al*., 2011e) and *Populus euphratica* (Li *et al*., 2011a).

2.4.3 prediction of microRNAs

2.4.3.1 Experimental approaches for miRNA prediction

Several studies were conducted towards the identification, characterisation and validation of miRNAs in different plants. Random mutation induction based forward genetics approach, also called Genetic screening which is used to inspect genes that cause phenotypic change on mutation, is an approach used for the discovery of miRNAs. Founding members of miRNA, lin-4 and let-7 were discovered by this

approach from *Caenorhabditis elegans* (Lee, *et al*., 1993; Wightman, *et al*., 1993). This is a relatively expensive approach when it comes to resources and labour and also the miRNA number that can be discovered is limited.

cDNA library construction of small RNA followed by direct cloning and sequencing approach (conventional Sanger sequencing) is a practicable way to discover miRNAs and other small RNA sequences which are found to be expressed in different tissues, developmental stages and environmental conditions of particular organism (Reinhart *et al*., 2002). Many cloning techniques are required for this approach including the T4 RNA ligase-based ligation of oligo-nucleotide adapters to the 5' and 3' ends of small RNAs isolated followed by amplification by RT-PCR. Either direct ligation of the amplified products can be done or it can be ligated into a cloning vector after concatemerizing it and then subjecting to DNA sequencing (Wang *et al*., 2004a). Sequencing the short inserts of clones in the cDNA library in the former approach is an expensive task, whereas in case of later approach, multiple steps are required to produce concatemers to be used in cloning for Sanger sequencing. A method based on hybridization for high-throughput as well as parallel quantitative identification of an array of particular DNA or RNA sequences is Microarray. Solid surface over a chip which is immobilized probes of known sequences, where samples under study having complementary sequence will bind and quantitative detection of signals released from the labelled targets are done. Oligonucleotides having sequence complementary with target miRNAs acts as probes for detection of miRNA in miRNA profiling. In plant, for the first time, Liang *et al*., (2005) reported microarray approach for studying miRNA profile.

Several miRNA studies in various plant revealed the application of microarray approach via, rice under drought stress (Zhao *et al*., 2007a), salt stress in maize roots (Ding *et al*., 2009) and cotton (Yin *et al*., 2012), maize root under submergence stress (Zhang *et al*., 2008b), predicted miRNAs validation in tomato (Zhang *et al*., 2008a), phosphorous deficiency (Zeng *et al*., 2010), disease resistance (Guo *et al*., 2011) and shoot apical meristem (Wong *et al*., 2011) in soybean, cadmium and low-N stress in rice (Ding *et al*., 2011; Nischal *et al*., 2012) and *Brassica juncea* under arsenic stress (Srivastava *et al*., 2013).

Incorporation of high-throughput sequencing as well as quantitative PCR analysis along with microarray analysis are helpful in expression, comparison and validation of miRNAs (Lu *et al*., 2008), however miRNA microarray has limitation in profiling of miRNAs with unknown sequences.

Thousands to millions of sequences tags or reads of small RNA cDNA libraries can be produced in parallel with help of High-throughput or the next-generation sequencing like MPSS, 454 Life Sciences and Illumina (Barakat *et al.,* 2007*)*. Option for deep sequencing of small RNA cDNA libraries at affordably lower cost, less time and less effort has been offered by Next generation high-throughput sequencing approach (Sun, 2012). Possibility of recovering rare transcripts is higher with the help of NGS technologies with a capacity to deep sequence sRNAomes ans generate more than 107 sequences even in single experiment (Jones-Rhoades, 2012).

NGS application in sequencing sRNAome of plant was first reported by Lu *et al*. (2005a). More than two million small RNA reads were produced from four seedlings and inflorescence libraries of *Arabidopsis thaliana* by the application of massively parallel signature sequencing (MPSS). Wide application of NGS technology is reported now a days for sequencing sRNAomes of various plant species, tissues, stages and growing environment. Extensive computational analyses along with dependence on available genomic or transcriptomic resources enables next generation sequencing technologies to derive huge amount of data.

The sequences of small RNAs generated from next generation sequencing approach are annotated with small RNA sequence databases, mainly miRBase (Griffiths-Jones *et al*., 2006), which is a miRNA registry consisting of many sequences irrespective of species. PMRD which is a plant microRNA database (Zhang *et al*., 2010c) and Rfam, an annotated non-coding RNAs and structured RNA elements containing database (Gardner *et al*., 2011). Thus, generated sRNA-Sequences reads are further mapped with respect to a reference genome or transcriptome to find novel as well as homologous miRNAs, Where, characteristic miRNA precursors or hairpin stem-loop structures are formed by the reads which are mapped to the genome regions.

Comparative study was made by Metpally *et al*. (2013) on most cited software packages used for mapping reads to reference genome, such as miRDeep2,

miRNAKey.and.miRExpress, and revealed that more miRNAs were detected and aligned by miRDeep2 compared to the rest of the two tools. Tool used for the prediction of secondary structures in RNA in miRDeep2 is RNAfold tool and evaluation of structures and signature of every miRNA precursor is done by assigning of particular score reflecting the genuine miRNA based on its likelihood (Metpally *et al*., 2013).

A significant step involved in the analysis of NGS data to assess the relative miRNA expression levels of different samples comparable is Normalization. Standard procedure for normalizing sRNA-Seq experiments now a days is scaling to library size (Meyer *et al*., 2010). Till now, no such normalization method is described which can be considered as best (Zhou *et al*., 2013b), and the primary consideration while normalization is the difference in sequencing depths (McCormick *et al*., 2011). As suggested by Garmire and Subramaniam (2012), Lowess and quantile normalization methods is found to perform best compared to other commonly tested miRNA-Seq normalization methods such as global normalization, Trimmed Mean of Method (TMM), scaling normalization, quantile normalization, variance stabilization (VSN) and invariant method (INV).

For the differential expression analysis of sRNA-Seq, commonly used packages involve DESeq and EdgeR (Metpally. *et al*., 2013). Negative binomial model is used for normalization in DESeq, whereas M-values trimmed mean (TMM) method is used by EdgeR for computation of differentially expressed miRNAs (Garmire and Subramaniam, 2012).

2.4.3.2 Computational approaches for miRNA prediction

2.4.3.2.1 Homology based analysis

Annotated complete genome sequences or gene sequences sets like, EST and GSS can be analysed to identify particular miRNA genes of a species without any annotated genome with the help of computational approaches (Bonnet *et al*., 2004a). Homologous miRNAs of any species of plants can be identified with the help of computational prediction methods, based on known miRNA conservation (Zhang *et al*., 2006a).

The BLAST analysis is compared against known unique miRNAs sequences of plants downloaded from EST database miRBase or gene bank. In addition, extra filtering criteria can also be applied in prediction algorithm of miRNA which include 1) they must be 18 nts long 2) only 2-3 mismatches in sequence compared with all other known miRNA 3) in secondary structure and hairpin prediction analysis they should have 3.2 minimal folding energy (MFE) (Unver *et al*., 2009). This approach has been used for miRNA prediction from EST and GSS databases for various plant species as it is comparatively fast and affordable (Bonnet *et al*., 2004a; Zhang *et al*., 2005a). Even though EST analysis data can give indication of gene expression, representation of miRNAs were observed at lower levels around 1-10 per 10,000 EST (Zhang *et al*., 2005a,).

2.4.3.2.2 Bioinformatic prediction studies

Majority of the mature plant miRNAs are 21–24 nts long. In the analyses of eight plant species, 84percent of plant miRNA loci are seen in clusters at intergenic regions, with a couple of exceptions seen in intronic regions (Nozawa *et al.,* 2012). Contrary to animal miRNAs which are frequently clustered together, only 20percent of plant miRNA genes are found to be clustered together, and those clustered genes often encode miRNAs belonging to the same family or targeting genes of the same protein family (Merchan *et al.,* 2009). The common characteristic actions of miRNAs in plants in *trans* and the hairpin structure of their precursors are hallmarks often employed by common miRNA prediction tools.

Bioinformatic tools predict miRNAs with very high efficiency and accuracy (Dong *et al*., 2012), given the well-developed and relatively standard prediction pipeline, an overwhelming number of miRNAs has been documented in different plant species. These conventional prediction tools are mirCoS, microHARVESTER, miRAlign, miRDeep, MiRCheck and NOVOMIR. NOVOMIR is widely used and popularized tool that uses a set of criterions to screen miRNAs from the genome of an organism. The process is followed by a sophisticated statistical model which can predict pre-miRNAs from other miRNAs, here the genome become the input. The standard

used here is *Arabidopsis thaliana* miRNA (positive control), tRNA, mRNA, ncRNA and DNA sequence is negative control.

In a study conducted in College of Agriculture Vellayani during 2016-18, 52 mature miRNAs were predicted using NOVOMIR software and about 124 corresponding target genes were predicted using psRNA target tool in banana genome (Mathew, 2018). These in silico predicted results were utilized for the present study by validating its expression through Real-Time PCR.

2.5 DETECTION AND EXPRESSION MEASUREMENTOF miRNAs

Usually the relative quantity of a target RNA transcripts in transcriptome is determined by the help of Northern blot analysis. In the case of miRNA studies common use of northern blotting comes at detecting the miRNA presence, including mature miRNA, miRNA and pre-miRNA sequences, in order to confirm the presence of computationally predicted miRNAs. (Molnar *et al*., 2007). Size and expression levels of miRNAs can be indicated with help of northern blot analysis

In situ hybridisation (ISH) is another method for studying miRNA which is based on hybridization (Eldem *et al*., 2013). Expression of spatial and temporal miRNAs can be detected and their relative expression levels can be determined with the help of Synthetic probes that shows sequence complementary to the target miRNAs within particular cells, tissues, organs and even for whole organisms (Eldem *et al*., 2013). Quantitative RT-PCR (RT-qPCR) the most commonly used method for measuring or comparing of expression of miRNAs quantitatively and accurately by regular monitoring. miRNA expression derived from computational prediction, microarray and high-throughput sequencing data can be validated with this method. RT-qPCR based miRNA expression profiling can be carried out in two levels; mature miRNAs (Raymond *et al*., 2005) and miRNA precursors. Poly(A)-tailing RT-qPCR, miQPCR and stem-loop RT-qPCR are some of the RT-qPCR based methods for miRNA quantification (Benes and Castoldi, 2010). In poly(A)-tailing RT-qPCR, RNA isolated are reverse transcribed to cDNA, and then mature miRNA poly(A)-tailing take place followed by binding of an oligo-d(T) primer and quantitative PCR is carried out. In case of miQPCR T4 RNA ligase are used to universal linker to the miRNA, and

followed by reverse transcription and quantitative PCR (Benes and Castoldi, 2010). Most popular approach is the stem-loop RT-qPCR where, stem-loop primer containing complementary sequence of the target miRNA are used for reverse transcription and followed by quantitative PCR (Chen *et al*., 2005a). SYBR Green or TaqMan chemistries are usually used for doing RT-qPCR for miRNAs (Chen *et al*., 2005a; Raymond *et al*., 2005). TaqMan based stem-loop RT-qPCR miRNA is advantageous over SYBR Green based RT-qPCR as the former help discriminating mature miRNAs that differ in a single nucleotide and also the contamination by genomic DNA will not affect expression quantification (Chen *et al*., 2005a).

2.5.1 Expression profile analysis of predicted miRNAs

Identification and prediction of miRNA using computer software and bioinformatic tools is a vital approach in finding miRNA in plants and confirmation of these computationally predicted miRNAs using wet lab procedure is essential. There are generally two processes in which we can confirm miRNA, they are: evaluation of host miRNA target sequence and creation of a miRNA sequence (Watanabe *et al*., 2007). The experimental validation of miRNAs includes creation of gene specific target primers and stem-loop primers for miRNAs. RNA from plants are isolated and are then converted to its corresponding cDNA. Real time PCR is conducted using cDNA samples with the gene specific primers, this process will give a comprehensive knowledge regarding the expression profile of miRNAs. The study focuses on the correlation between miRNA and their corresponding target gene during abiotic stress condition.

2.5.2 Expression profile analysis

The expression profile of miRNA helps to understand the regulatory behaviour and metabolic activities of plants. By studying the genes expressed under extreme situations, their biological role in plants during stress can be identified (Chen *et al*., 2005). The expression level of genes can be confirmed and quantified with the help of RT-qPCR. It is highly sensitive and can provide an accurate result during quantification

process even though the miRNA extraction is difficult due to its small size (Chen *et al*., 2005).

In real-time PCR, expression analysis is done through comparative or relative analysis between a stable gene like β-Actin(control) and gene of our choice. The results are demonstrated as fold change (increase or decrease) in protein, cytochrome oxidase subunit 5B protein glyceraldehyde-3-phosphate dehydrogenase respectively (Mathew., 2018).

2.6 SELECTION OF ABIOTIC STRESS RESPONSIVE miRNA

For the current study "expression profiling of microRNA related to water stress in banana *Musa.* Nendran, five miRNAs were selected from the predicted list of 52 miRNAs. The selection was based on the abiotic stress responsiveness of the target genes; miR-3900-5p(target; Nitrate transporter 1.1), miR-67 (target: Probable xyloglucan endotransglucosylase /hydrolase protein 33), miR-971-5p (target: 20 kDa chaperonin), miR-2293 (target: Putative Zinc finger protein VAR3 chloroplastic), miR-449c-3p (target: ABC transporter C family member 13) were selected miRNAs.

2.6.1 Expression of proteins during abiotic stress

Plants respond to abiotic stress once it is subjected to environmental stress condition. Significant change in expression profiles of genes were found due to abiotic stresses during different developmental stages; these change in expression regulate plant developmental and timing plasticity ultimately (Fernandez, 2014; Mathur *et al.*, 2014). In response to abiotic stresses, several prominent genes consisting of those encode transcription factors were implicated, and the plants such as *Arabidopsis*, as well as other important crops in which these genes were found to be overexpressed were found to be more significantly tolerant towards different stresses significantly (Ganesan *et al.*, 2012).

ROS activated response ROS have emerged as an important regulators of plant stress responses. Many unfavourable environmental conditions lead to oxidative stress due to increased ROS production or/and impaired ROS detoxification (Mittler, 2010). Accumulation of ROS is observed during cold, heat, drought, high-light, heavy-metal stress and exposure to fungal toxins (Gechev, 2004). In addition, H_2O_2 is a secondary messenger during wound responses and various biotic interactions (Bai, 2003). The stress tolerance achieved by transient elevations in ROS levels can be explained by pre-activation of defence mechanisms, including kinases, transcription factors and other components of the signalling network, antioxidant enzymes, dehydrins, low-temperature-induced, heat-shock and pathogenesis-related proteins (Moon, 2003). Transcription factors play an important role in various environmental stress induced plant responses.

2.6.1.1 Nitrate transporter 1.1

Several unique functional properties are displayed by NRT1.1 (Gojon *et al*., 2011): it is a phosphorylation regulated dual-affinityNO_3 transporter which is also involved in auxin transport (Krouk *et al*., 2010). Initially characterized as an influx transporter participating in the uptake of NO_3 from the external medium, NRT1.1 has also been shown to display a NO_3 sensing function governing expression of NO_3 responsive genes and NO_3 induced changes in root development. The NRT1.1 gene is expressed in both roots and shoots. Apart from its role in NO_3 uptake and NO_3regulation during root development, its expression is also seen in cortex, apex of primary root, base of lateral roots, young emerging lateral roots, and lateral root primordia (Remans *et al*., 2006). NRT1.1 is also expressed in the central cylinder: endodermis and stele in more mature parts of the roots (Remans *et al*., 2006). The role of Nitrate transporter 1.1 (target of miR-3900-5) in water stress was analysed in the study.

2.6.1.2 Probable xyloglucan endotransglucosylase /hydrolase protein 33

Being a major structural component of cell wall matrix at different developmental stages it takes part in cell plate formation in dividing cells (Moore and Staehelin, 1988). The key enzymes responsible for xyloglucan metabolism are XTHs. These enzymes may have xyloglucan endohydrolase (XEH) and/or xyloglucan endotransglucosylase (XET) activities, which mediate splitting and reconnection of the xyloglucan crosslinks in the cell wall. Due to their potential enzymatic actions, XTHs are thought to play a pivotal role in the construction, remodelling and disassembly of the xyloglucan/cellulose framework in type I cell walls during cell growth and differentiation (Nishitani and Vissenberg, 2007). XET activities which mediate splitting and reconnection of the xyloglucan crosslinks in the cell wall. The role of Probable xyloglucan endotransglucosylase /hydrolase protein 33 (target of miR-67) in water stress was analysed in the study.

2.6.1.3 Putative Zinc finger protein VAR3 chloroplastic

Putative Zinc finger protein VAR3 chloroplastic is a part of a protein complex required for normal chloroplast and palisade cell development. The *VAR3* gene encode the novel VAR3 zinc-finger protein. Genetic, molecular and biochemical analyses indicate that VAR3 interacts with, and may thereby affect the activity of, chloroplast localized enzymes. The role of Putative Zinc finger protein VAR3 chloroplastic (target of miR-2293) in water stress was analysed in the study.

2.6.1.4 20 kDa chaperonin

Chaperonins are characteristic protective proteins which help in the folding of other proteins and thus preventing the aggregation and misfolding of proteins. Superoxide dismutase (SOD) acts as a primary defence against ROS by converting O^{-}_{2} to O_2 and H_2O_2 where chloroplast-localized co-chaperonin CHAPERONIN20 (CPN20) acts as a mediator of FeSOD activation by direct interaction. CPN20 mediates FeSOD

activity in chloroplasts, in addition to its well-known co-chaperonin function (Weiss *et al*., 2009). The role of 20 kDa chaperonin (target of miR-971-5p) in water stress was analysed in the study.

2.6.1.5 ABC transporter C family member 13

ATP-binding cassette (ABC) transporters are a highly conserved family of ATP-binding proteins found in both prokaryotes and eukaryotes with a diversity of physiological functions. (Higgins. 1992). Although, in general, each ABC transporter is relatively specific for its own particular substrate(s), it is remarkable that there is an ABC transporter for essentially every type of molecule that must cross a cellular membrane. ABC transporters have been characterized with specificity for small molecules, large molecules, highly charged molecules and highly hydrophobic molecules–systems are known with specificity for inorganic ions, sugars, amino acids, proteins, and complex polysaccharides. Although most exhibit relatively tight substrate specificity, some are multi-specific such as the oligopeptide transporter which handles essentially all di- and tripeptides (Christopher, 2001). The role of ABC transporter C family member 13 (target of miR-449c-3p) in water stress was analysed in the study.

Materials and methods

3. MATERIALS AND METHODS

The study entitled “Expression profiling of microRNAs associated with water stress in banana (*Musa* spp.)” was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2018-2020. The detailed description of the methodologies followed for this research is mentioned below:

3.1 MAINTENANCE OF TISSUE CULTURE PLANTS

In vitro raised banana plants var. Nendran were collected from Kuzhippallam botanical garden and maintained in a glasshouse for three months by watering regularly. Two sets of plants were maintained in a glasshouse with three replications, in pots of 25 cm diameter and 30 cm height with a potting mixture consisting of soil, sand and cow dung in 1:1:1 ratio. One set served as control and the other set was used for imposing water stress (Plate 1).

Plate 1: Healthy tissue culture plants

3.2 IMPOSITION OF WATER STRESS

Water stress was imposed on three months old tissue culture raised plants (Three replication) by withholding regular irrigation for seven days when the plants are of three months old. Leaf samples were collected from stress imposed and control banana plants on the eighth day of stress imposition. After collection, all the samples were immediately frozen in liquid nitrogen and stored at -80°C until it was used for RNA extraction.

3.3 CONFIRMATION OF WATER STRESS BY RELATIVE WATER CONTENT METHOD

Water stress was confirmed in banana plants by measuring the relative water content (Mullan and Pietragalla 2012). RWC of both control and stressed plants were taken to confirm water stress. Top-most fully expanded leaves were sampled. The samples were placed in a pre-weighed airtight plastic zip cover. Samples in the cover were weighed to obtain leaf sample weight (W), after which the samples were immediately hydrated to full turgidity for 3-4 h under normal room light and temperature. Leaf discs and small leaflets were hydrated by floating on de-ionized water in a closed petri dish. After hydration, the samples were taken out of the water and were well dried of any surface moisture quickly and lightly with filter/tissue paper and immediately weighed to obtain fully turgid weight (TW). Samples were then oven-dried at 80^0C for 24h and weighed to determine the dry weight (DW). Relative water content was measured using the formulae:

$$\text{RWC (percent)} = [(W-DW) / (TW-DW)] \times 100$$

Where,

W = Sample fresh weight

TW = Sample turgid weight

DW = Sample dry weight.

The RWC of control plants and stressed plants were compared to confirm the occurrence of water stress

3.4 EXPRESSION PROFILING OF miRNAs AND THEIR TARGET GENES DURING WATER STRESS

3.4.1 RNA isolation

RNA was isolated from the leaf samples of both healthy and stress-imposed plants using the method of Rodrigues-Garcia *et al.* modified by Ekatpure *et al.* (2019). In this protocol the incubation time was increased from 10 min to 30 min at 65 ºC and chloroform: Isoamyl treatment was given twice to completely remove the cell debris, protein and phenolic compounds. RNA was precipitated with 3M LiCl by incubating overnight at 4 ºC instead of -20.

Samples (0.5 gm) were taken in a pre-chilled mortar and ground to a fine powder using liquid nitrogen. To this, 3 ml of extraction buffer (Appendix I) was added to make a homogenate and incubated in a water bath at 65 ºC for 30 min. The homogenate (approx. 750µl) was transferred to a sterile 2 ml microfuge tube using the cut tip to avoid the RNA damage and was precipitated using 5 mM potassium acetate (66µl) and absolute ethanol (150µl). The tube was vortexed briefly for 1 min. An equal volume of chloroform-isoamyl alcohol (49:1) was added, vortexed briefly, and centrifuged at16000 g for 20 min at room temperature. The supernatant was collected in a fresh tube and repeated this process to eliminate any cell debris that was suspended. The supernatant was collected in a fresh tube and 850 µl of fresh phenol chloroform-isoamyl alcohol (25:24:1) was added. It was then vortexed for 10 sec and centrifuged at 16000 g for 15 min at room temperature. The supernatant was collected in a fresh sterile tube and 850 µl of chloroform-isoamyl alcohol (49:1) was added and the tube was briefly vortexed for 10 sec and centrifuged at 16000 g for 15 min at 4ºC. To the supernatant, 3M LiCl was added and kept overnight at 4ºC. The content was then spun at 16000g for 20 min at 4ºC. The supernatant was discarded and the pellet recovered was washed twice with 70 percent ethanol. Pellet was air-dried at room temperature under the Laminar Air Flow (LAF). It was dissolved in 30 µl DEPC 0.1 % treated sterile distilled water. RNA was quantified in a NanoDrop spectrophotometer and stored at -80 ºC until use.

3.4.2 Agarose gel electrophoresis

The quality of RNA was analysed on 2 percent agarose gel in a horizontal gel electrophoresis unit. Agarose was weighed out and melted in 1X TBE (Tris base 10.8g, Boric acid 5.5g, 0.5M EDTA 4ml). Ethidium bromide 0.5 $\mu g ml^{-1}$ was added to the agarose after cooling it to about 50 ºC. The mixture was then poured into a casting tray fitted with the appropriate comb. After solidification, the comb was removed and the gel was placed in an electrophoresis tank containing 1X TBE buffer filled to around 1mm above the gel. RNA samples (5µl) were mixed with gel loading dye (1µl) (6X Bromophenol blue (0.25 percent) and Glycerol (30 percent)) and loaded into the wells. Electrophoresis was carried out at 75 V until the loading dye reached the three-fourth length of the gel. The gel was documented using the Gel Doc^{TM} XR+ documentation system (BIO-RAD).

3.4.3 Quantification of RNA

The quantification of RNA was carried using a NanoDrop spectrophotometer. The optical density of the samples was recorded at both 260 and 280 nm. The absorbance value of 1.0 at 260 nm indicates the presence of 40µg ml^{-1} of RNA. The concentration of RNA was calculated by using the formulae:

Conc. of RNA ($\mu g\ ml^{-1}$) = A_{260} x 40 x dilution factor

A_{260}/A_{280} ratio indicates the quality of RNA. Ratio ranging from 1.8 - 2, indicates good quality RNA.

3.4.4 cDNA synthesis

The RNA isolated from both control and stressed plants were converted to their complementary DNA using Verso cDNA synthesis kit (Thermo Scientific) according to the manufacturer's protocol. The kit contained reverse transcriptase enzyme and oligodT primers and an RNase inhibitor which prevents the degradation of isolated

RNA. It also contains an RT enhancer for the removal of DNA contamination. A 20µl reaction mix was prepared using the following components.

5X cDNA synthesis buffer	2 µl
dNTP mix (500 µM)	1 µl
RNA primer (2 µM)	0.5 µl
RT enhancer	0.5 µl
RNA (1µg/µl)	3 µl
Reverse transcriptase (1unit/µl)	0.5 µl
Nuclease free Water	2.5 µl
Total volume	10 µl

The contents were mixed well after giving a flash spin, PCR was carried out in a thermal cycler T100^{TM} (BIO-RAD). The thermal profile for the reverse transcription reaction of the target gene was:

42 $^{\circ}$C for 30 min

95 ^{o}C for 2 min

3.4.5 Confirmation of cDNA synthesis

The cDNA prepared was checked for genomic DNA contamination as well as successful conversion to cDNA by RT-qPCR with β-actin gene specific primers. The primers were designed spanning an intron. Genomic DNA contamination will produce a ~700 bp larger amplicon whereas cDNA produces ~100 bp amplicon. A standard PCR mix was prepared for 20 µl total volume containing:

Components	**Volume (µl)**
SYBR Green Master Mix (2X)	10 µl
Forward primer (10 µM)	0.6 µl
Reverse primer (10 µM)	0.6µl
cDNA (500ng/ µl)	2 µl
Nuclease free water	6.8 µl
Total volume	**20 µl**

The thermal profile used was as follows

Step 1: 95 ^{O}C for 3 min

Step 2: 95 ^{O}C for 15sec

Step 3: 55 ^{O}C for 15 sec

Step 4: 60 ^{O}C for 45sec. The steps from 2 to 4 were repeated for 30 cycles

Step 5: 60 ^{O}C for 5 min

After completion, the PCR products were separated on 1.5 percent agarose gel in a horizontal gel electrophoresis unit along with a 100 bp ladder. The electrophoresis was conducted at 75 V till the gel loading dye reached three-fourth of the gel and the final results were viewed using Gel Doc ^{TM} XR+ documentation system (BIO-RAD).

3.5 ISOLATION OF miRNAS

3.5.1 The miRNAs used for validation

In a previous study conducted in the Department of Plant Biotechnology, 52 mature miRNAs were computationally predicted using NovoMIR software and over 142 targets were identified for these miRNAs using psRNATarget in banana (*Musa*

acuminata) genome. From these computationally predicted miRNAs, five miRNAs were selected for this study, based on the role of their target genes in abiotic stress condition. The miRNAs and their targets selected in the present study are shown in Table 1.

Table 1 List of miRNAs selected for the study and their targets

Sl No	miRNA	Target aligned fragment	miRNA Aligned Fragment	Target description
1	miR-3900-5p	CGCGGUGAUGUCUUCUUG	GGGGAUUUUCAAGUACUGCA	Nitrate transporter 1.1
2	miR-67	CAAUCUCCCAGUUAGAGAGG	CCUCUCUGUCUGGGAGGUUG	Probable xyloglucan endotransglucosylase/hydrolase protein 33
3	miR-971-5p	UAAGAUCAAGUCGUCAGA	UUUGAUGAUUUGAAUUUA	20 kDa chaperonin
4	miR-2293	AAGUCAGAUUAUCAGAGUCA	UGAUUUUGAUGAUUUGAAUU	Putative Zinc finger protein VAR3 chloroplastic
5	miR-449c-3p	UAGAAAACUGCAGCUAUCAA	UUGAUAGUUGCACUUUUCUC	ABC transporter C family member 13

3.5.2 Amplification of miRNAs

RNA was isolated from the leaf samples of both control and stress-imposed plants using the method of Rodrigues-Garcia *et al.* modified by Ekatpure *et al.* (2019) as described in section 3.4.1.

It is difficult to extract microRNAs from the total RNA because of their small size (18-22nt). So, the amplification was carried out in a two-step process. A stem-loop RT primer was used in the first step to hybridize with the miRNAs in the total RNA and cDNA was synthesized in an RT reaction. In the second step, this cDNA was amplified using miRNA specific forward primer and universal reverse primer in a real-time PCR reaction using SYBR Green assay.

In the present study, a stable stem-loop primer sequence of 44nt (5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC -3′) reported by Chen *et al*. (2005) was used to lengthen the target cDNA. The miRNA-specific stem-loop primer used in this work was designed (Subramanian, 2017) by combining the stem-loop sequence with the reverse complement of the six 3′ nt of the mature miRNA sequence.

For the reverse transcription of miRNAs using stem-loop primers, a 10 µl reaction mix was concocted using the following components:

Components	**Volume (µl)**
cDNA synthesis buffer	2 µl
dNTP (500 µM)	1 µl
Stem-loop primers (10 µM)	0.5 µl
RNA samples (1ng/ µl)	2.5 µl
Reverse transcriptase enzyme	0.5 µl
Water	3.5 µl
Total volume	**10 µl**

The contents were mixed well and after giving a flash spin, the reaction was carried out in BIORAD's thermal cycler T100^{TM}. The following thermal profile was used for this reaction:

16 ºC incubation for 30 min, followed by

Step 1: 30 ºC for 30 sec

Step 2: 42 °C for 30 sec

Step 3: 50 °C for 1sec

Steps 1 to 3 were repeated for 60 cycles.

Step 4: 85 °C for 5 min

The cDNA samples were stored at -20 ^{O}C

3.5.3 Primer used for the amplification of miRNAs

Forward primers specific to miRNA were designed by taking the first 12 to 17 nts of the 5' end of the mature miRNA and adding 5 to 7 additional nucleotides at 5'nt for adjusting the Tm to 60^{O}C. The reverse primer was designed with the 44nt stem-loop sequence proposed by Chen *et al*. (2005). The reverse primer was universal for all miRNAs (5' CCAGTGCAGGGTCCGAGGTA-3') (Kramer, 2011). The compatibility and secondary structure formation of the forward primers were verified by using Eurofins genomics oligo analysis tool (Fig. 1).

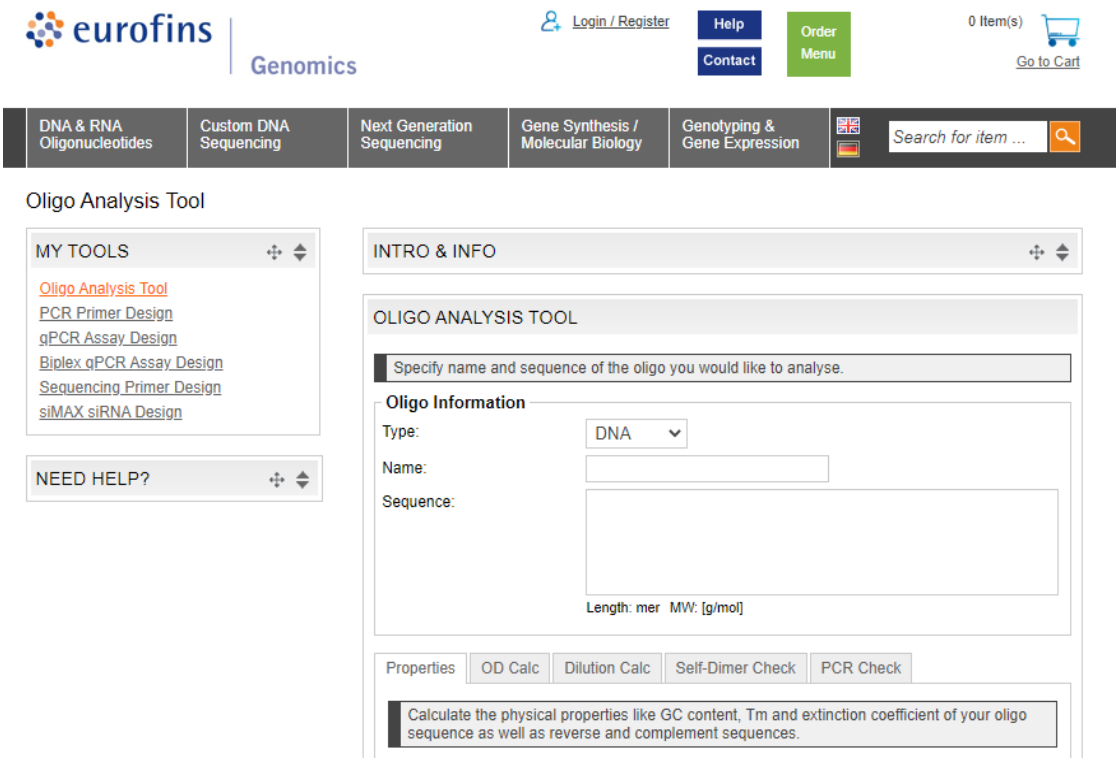


Fig. 1. Home page of eurofin's genomics oligo analysis tool software

3.5.4 Primer for amplifying target genes

Target gene-specific primers were designed using primer3plus software (Untergasser *et al.*, 2007). The software comprises an input box for sequence information and they are pasted in FASTA or EMBL format in the space provided. The region required for amplification was selected using '[ ]' indicated below the dialogue box. Default parameters like length: 20-22nt, GC percent: 50-60 percent, melting temperature: 55-56 ^{O}C were adjusted and primers were designed (Fig. 2).

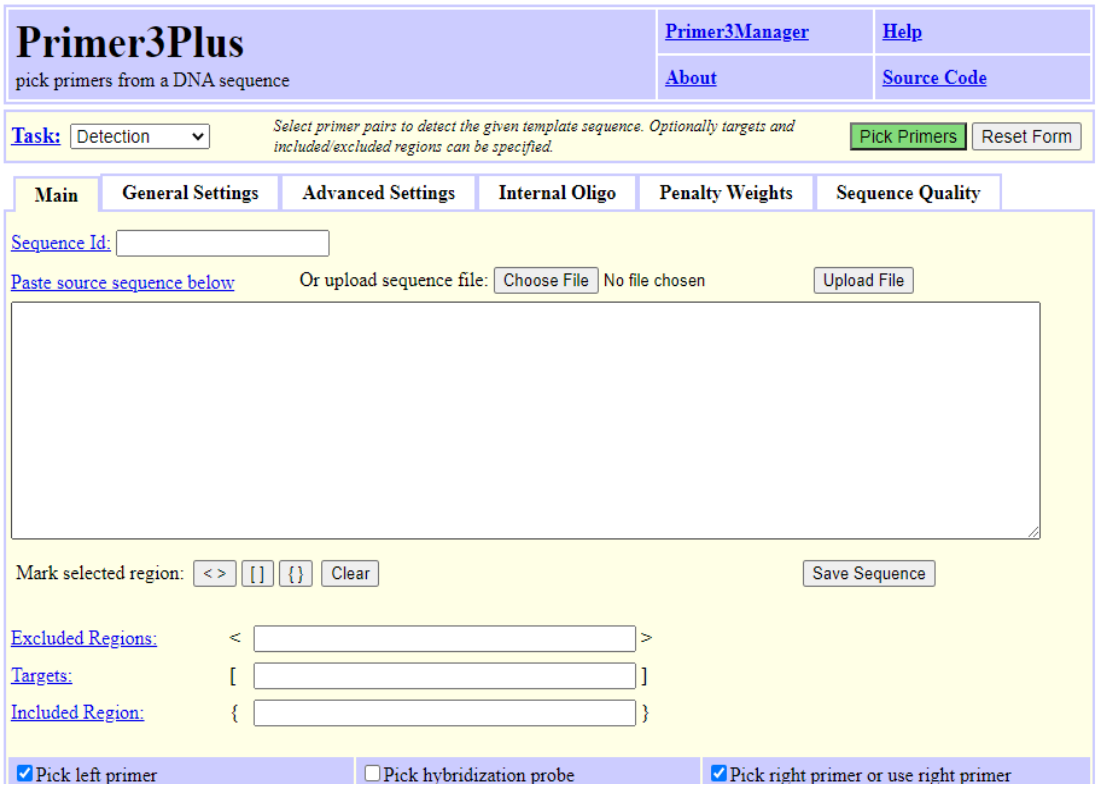


Fig. 2 Home page of Primer3Plus

The primer specificity was checked through NCBI Primer-BLAST software (Fig. 3). The software was specifically designed by NCBI which utilizes the BLAST program and global alignment algorithm to pick primers against the target gene sequence. It also checks the compatibility of the primers during PCR i.e., to find out non-specific amplification causing primer pairs (Ye *et al.*, 2012).

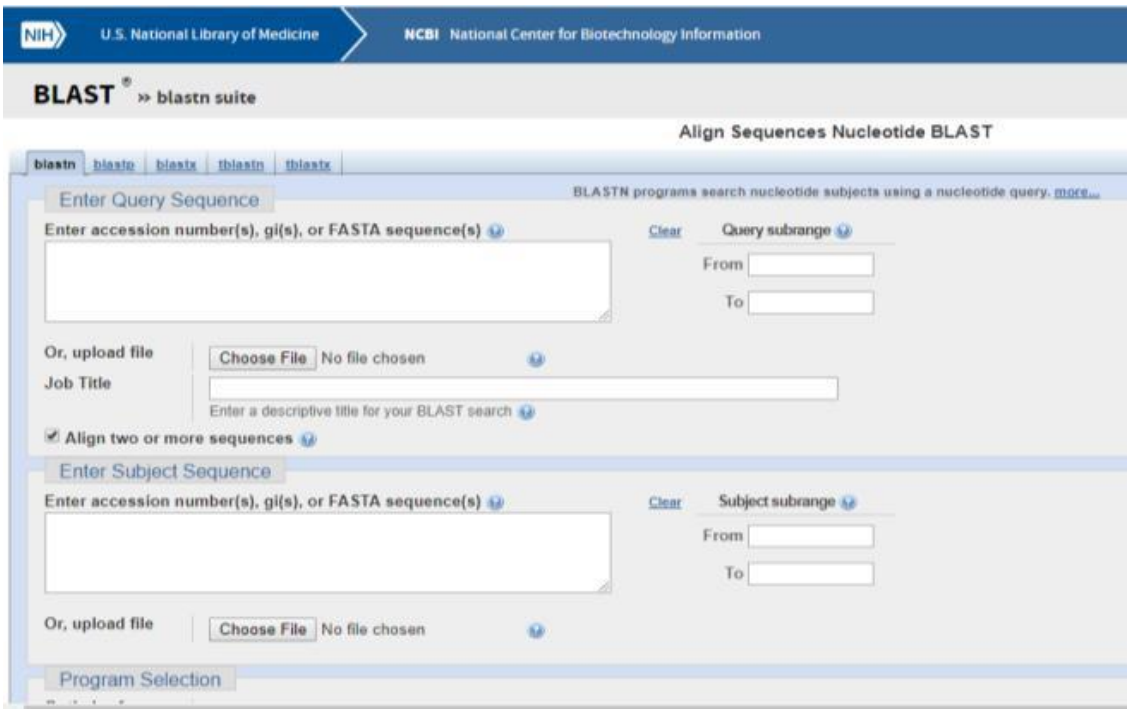


Fig. 3 Home page of NCBI Primer BLAST software

3.6 CHECKING miRNA ASSAY BY PCR

The miRNA expression in control and stress-imposed plants is checked by PCR. miRNA specific, as well as universal primers specific to stem-loop sequence, were used to amplify miRNA specific cDNA. Components for PCR were added as follows:

Components	**Volume (µl)**
10X reaction buffer (1X)	2 µl
dNTP mix (100 µM each)	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq DNA polymerase (1U/ µl)	0.5 µl
cDNA (300ng/ µl)	1 µl
Nuclease free water	13.5 µl
Total volume	**20 µl**

The thermal profile followed was:

Step 1: 95 °C for 5 min

Step 2: 95 °C for 5sec

Step 3: 60 °C for 10 sec

Step 4: 72 °C for 8 sec. Steps 2 to 4 were repeated for 30 cycles

Step 5: 72 °C for 5 min

After completion, the PCR products were separated on 1.5 percent agarose gel. The gel electrophoresis was conducted at 75 V till the gel loading dye reached three fourth of the gel. The final results were viewed in Gel Doc ™ XR+ documentation system (BIO-RAD).

3.7 CHECKING TARGET AND REFERENCE GENE ASSAYS BY PCR

The cDNA of the stress-imposed and the control banana plants were checked for the amplification of target genes using the target gene-specific primers. The PCR components were added as follows:

Components	**Volume (µl)**
10X reaction buffer (1X)	2 µl
dNTP mix (100 µM each)	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq DNA polymerase (1U/ µl)	0.5 µl
cDNA (500ng/ µl)	1 µl
Nuclease free water	13.5 µl
Total volume	**20 µl**

The thermal profile followed was:

Step 1: 95 °C for 3 min

Step 2:95 °C for 15sec

Step 3: 55 °C for 15 sec

Step 4: 72 °C for 45sec. Steps 2 to 4 were repeated for 30 cycles

Step 5:72 °C for 5 min

After completion, the PCR products were separated on 1.5percent agarose gel along with a 50 kb ladder. The gel electrophoresis was conducted at 75 V till the gel loading dye reached three-fourth of the gel. The final results were viewed in the gel documentation system (BIO-RAD).

3.8 RELATIVE EXPRESSION ANALYSIS USING REAL-TIME PCR

RT-qPCR technique was used to analyse the expression levels of miRNAs and their corresponding target genes. The analysis was done in CFX96 real-time machine (BIO-RAD). RT-qPCR reactions for all the samples belonging to the same gene/ miRNA were run on the same plate. Two technical replicates were kept for each reaction. Non-template controls were included for each assay. A reaction mixture of 20 µl was prepared using the following components:

Components	**Volume (µl)**
cDNA (50ng/ µl)	5 µl
Forward primer (10 µM)	0.6 µl
Reverse primer (10 µM)	0.6 µl
SYBR Green Master Mix (2X)	10 µl
Nuclease free water	3.8 µl
Total volume	**20 µl**

cDNA from both healthy and stress-imposed plants were amplified by initial denaturation for 5 min at 95^{O}C, followed by 45 cycles of denaturation at 95^{O}C for 5sec, annealing at 60^{O}C for 10sec and extension at 72 ^{O}C for 8sec.

Target and reference genes were amplified from the cDNA of both control and stress-imposed plants by initial denaturation at 95 ^{O}C for 2 min, followed by 40 cycles of denaturation at 95 ^{O}C for 15 sec, annealing at 55 ^{O}C for is 15 sec, and extension at 72^{O}C for 30 sec. The final extension was done at 72 ^{O}C for 5 min. PCR was followed by melt curve analysis i.e. fluorescence signals at 530nm wavelength were monitored from 65 ^{O}C to 95 ^{O}C at every 0.2 ^{O}C to determine the product specificity.

Raw fluorescence data (RFU) was generated by CFX96 software. A threshold fluorescence passing through the exponential phase of the log RFU plot of all the reactions in an assay was set manually to generate the threshold cycle (Cq) value. Cq value is the fractional number of cycles taken by each reaction to reach threshold fluorescence. Normalized relative quantities for each target gene and miRNA were generated by ΔΔCq method in excel using actin as the reference gene.

Result

4. RESULT

The study entitled “Expression profiling of microRNAs associated with water stress in banana (*Musa* spp.)**”** was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2018-20. The result of the study is presented in this chapter.

4.1 IMPOSITION OF WATER STRESS AND STRESS CONFIRMATION USING RWC METHOD

Three months old *in vitro* raised banana plants of cv. Nendran were given water stress by withholding irrigation for a period of seven days (Plate 2). The leaf samples were taken for analysis.

Plate 2. Water stress-imposed tissue culture raised banana cv. Nendran plants a) Control plants b) Plants after withholding irrigation for 7 days

4.1.1 Confirmation of water stress using relative water content (RWC) method

The leaves of banana plants (both control and water stress imposed) were collected and the RWC was calculated by taking fresh weight, turgid weight and dry weight. Water stress-imposed plants were found to have a mean RWC of 60 percent compared to the control plants which are found to have RWC of 94.06 percent (Table 2), which indicated the occurrence of water deficiency stress in the stress-imposed plants.

Table 2 Relative water content (RWC) in banana plants 7 days after withholding irrigation

Sample	RWC
Water stressed	60.003
Control	94.067
SE ± 0.839	
CD (0.001)	5.464

Treatments were found significant at 1 percent and 5 percent level of significance CD (0.01) = 5.464 CD (0.05) = 3.294

4.2 DESIGNING AND SYNTHESIS OF PRIMERS FOR MIRNAS AND THEIR TARGET GENES

4.2.1 miRNAs and their targets selected

List of miRNAs selected for the study and their corresponding targets are given in the Table 1 (Section 3.5.1)

4.2.2 Primers designed

Primers were designed for the selected miRNAs according to the report of Chen *et al*. (2005). The details of stem loop primers designed for amplification of cDNA from miRNA are given in Table 3.

Table 3: Sequence of stem-loop primers

Sl. No.	miRNA	miRNA sequence	miRNA specific stem-loop sequence
1	miR-3900-5p	GGGGATTTTC AAGTACTGCA	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACTGCAGT
2	miR-67	CCTCTCTGTCT GGGAGGTTG	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACCAACCT
3	miR-971-5p	TTTGATGATTT GAATTTA	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACTAAATT
4	miR-2293	TGATTTTGATG ATTTGAATT	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACAATTCA
5	miR-449c-3p	TTGATAGTTGC ACTTTTCTC	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACGAGAAA

Target gene specific primers were designed using primer3plus software. The list of target gene specific primers is shown in Table 4.

Table 4: Sequence of primers designed for target

Sl No	Target gene	Target sequence	Forward primer 5' to 3'	Reverse primer 5' to 3'
1	Nitrate transporter 1.1	CGCGGTGATGT CTTCTTG	CGGGAGGTT GGATTGCTTC T	CAGCTGGTGG ATCTGAGATT GA
2	Probable xyloglucan endotransglucosylase/hydrolase protein 33	CAATCTCCCAG TTAGAGAGG	TCGACCCCAC TGCAGATTTC	GGGCTTGGAG GGATAAGCTC
3	20 kDa chaperonin	TAAGATCAAGT CGTCAGA	CTCAAGCGG AGGAGCCTTC	GTTTTGCCTTC TCCAACGGC
4	Putative Zinc finger protein VAR3 chloroplastic	AAGTCAGATTA TCAGAGTCA	TCAATCCGCA GTCTGTTGGC	TGGGCAAATC CAGTCACCTC
5	ABC transporter C family member 13	TAGAAAACTGC AGCTATCAA	TAGCTTGCTG GGTATCGCAG	GGAGGGGAGA GAAAGAGTGC

The miRNA specific primers designed for amplification are shown in Table 5.

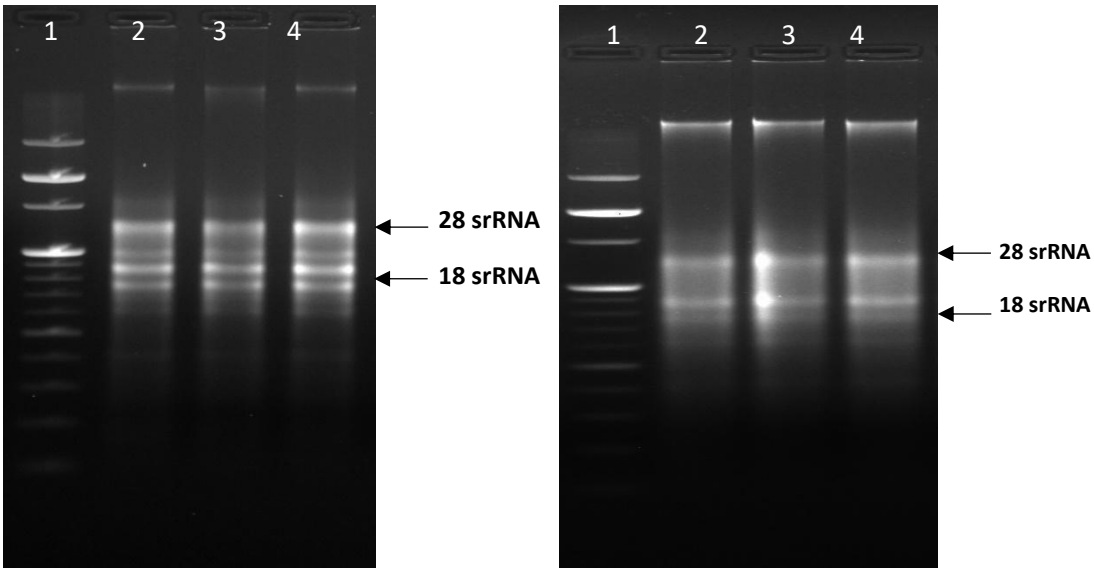
Table 5: Sequence of the forward and reverse primers designed for miRNAs

Sl. No.	miRNA	miRNA sequence	Forward primer	Universal reverse primer
1	miR-3900-5p	GGGGATTTTC AAGTACTGCA	CGGCGCGGGGAT TTTCAAGT	CCAGTGCAGG GTCCGAGGTA
2	miR-67	CCTCTCTGTCT GGGAGGTTG	CGTCGCCCTCTCT GTCTGGG	
3	miR-971-5p	TTTGATGATTT GAATTTA	GCGGCGCCTTTG ATGATTTG	
4	miR-2293	TGATTTTGATG ATTTGAATT	GCCCGCGTGATT TTGATGATT	
5	miR-449c-3p	TTGATAGTTGC ACTTTTCTC	GCCGCCTTGATA GTTGCACT	

4.3 EXPRESSION PROFILING OF miRNAs AND TARGETS DURING WATER STRESS

4.3.1 RNA isolation

RNA was isolated from leaf samples of both control and water stress-imposed plants after a period of seven days of stress, following the method of Rodrigues-Garcia *et al.* modified by Ekatpure *et al.* (2019). RNA profile on 1.5percent agarose gel (Plate 3) showed bands corresponding to 28srRNA and 18srRNA. The purity (A_{260}/A_{280}) assessed using NanoDrop microvolume Spectrophotometer ranged from 1.85 to 2.0 (Table 6).



(a) **(b)**

Plate 3: Gel profile of RNA isolated from the leaves of banana plants (a) Control (b) Water stress-imposed.

Lane 1: 100bp ladder, Lane 2 to 4: Biological replicates

Table 6: Quantity of RNA measured using NanoDrop spectrophotometer

	SAMPLE	A_{260} /A_{280}	A_{260} /A_{280}	CONCENTRATION (ng/ul)
Water stressed	**S1**	1.96	1.57	350.84
	S2	1.83	1.06	329.22
	S3	1.96	1.97	604.03
Control	**C1**	1.73	1.71	420.77
	C2	2.08	2.01	406.07
	C3	2.09	2.09	337.61

4.3.2 cDNA synthesis

cDNA synthesis of both control and water stressed samples was done using verso cDNA synthesis kit. The quality of cDNA was assessed by doing PCR with β-actin specific primers. Amplicon of expected size (106 bp) was produced in all the samples (Plate 4).

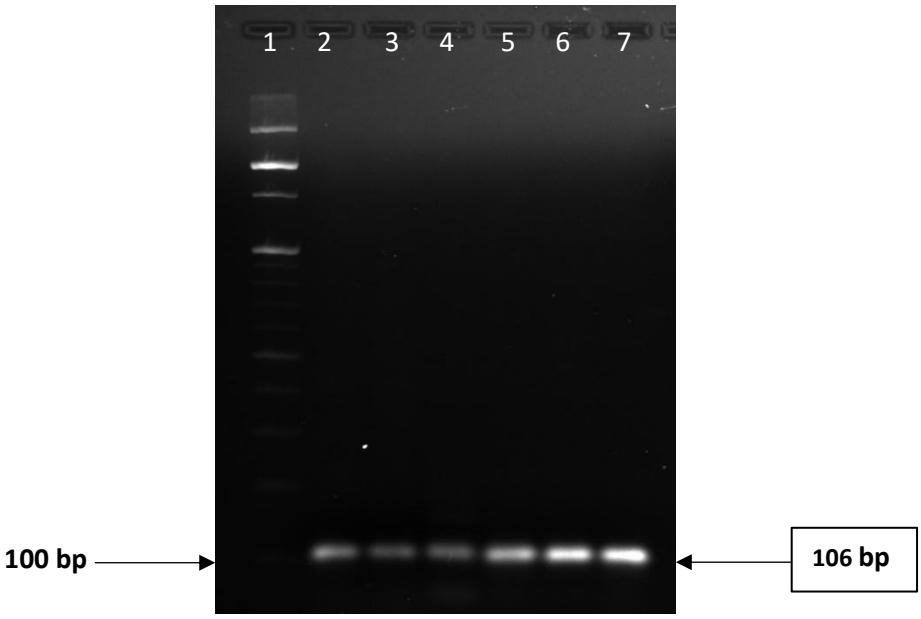


Plate 4: β-actin gene specific fragments amplified from cDNA of both control and water stress-imposed plants.

Lane 1: 100bp ladder, Lane 2 to 4: Control, Lane 5 to 7: Water stress imposed

4.3.3 Assay of primers designed for miRNAs and target genes

The details of the primers designed and synthesized are shown in Tables 4 and 5. The specificity of these primers was checked by PCR. The PCR products were separated on a 2 percent agarose gels. The primers designed for mir-67 and miR-2293 showed an amplicon of size 64 bp (expected size) in both control and water stressed plants (Plate 5A) and (Plate 5B), at the same time primers designed for miR-971-5p showed multiple bands even after doing gradient PCR (Plate 5C), whereas miR-3900 and 449 did not show amplification even after repeated assays.

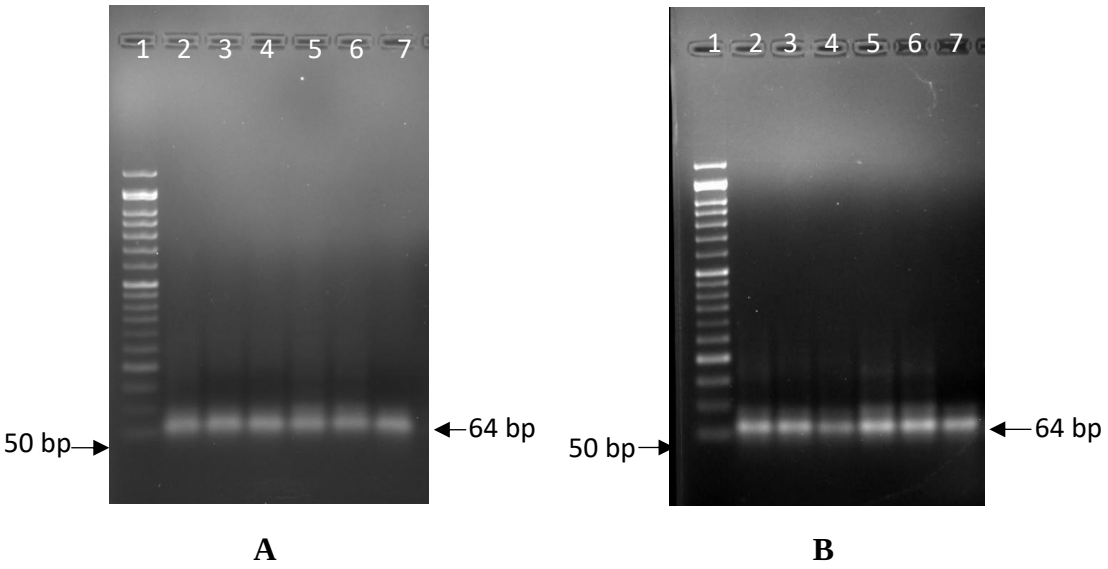


Plate 5: Amplicons of miRNAs in banana plants after 7 days of water withholding. A) miR-67 B) miR2293

Lane 1: 50bp ladder, Lane 2 to 4: Control, Lane 5 to 7: Water stressed plants

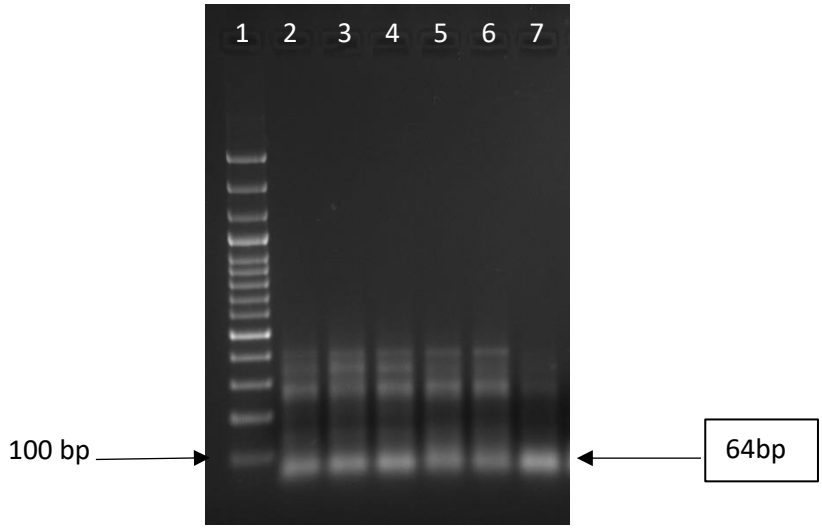


Plate 5C: Amplicons of miR-971-5p.

Lane 1: 100bp ladder, Lane 2 to 4: Control, Lane 5 to 7: Water stressed plants

PCR was performed for target gene-specific primers also and the PCR products were separated on 2 percent agarose gel. The target genes namely Nitrate transporter 1.1 gene (target of miR-3900-5p), Probable xyloglucan endotransglucosylase/hydrolase protein 33 gene (target of miR-67), 20 kDa chaperonin

protein gene (target of miR-971-5p), and Putative Zinc finger protein VAR3 chloroplastic gene (target of miR-2293) showed expression in all samples and their amplicon size were 322 bp (plate 6A), 216 bp (plate 6B), 206 bp (plate 6C) and 159 bp (plate 6D) respectively, however primers designed for target gene of ABC transporter gene for miR-449c-3p did not produce any amplicon

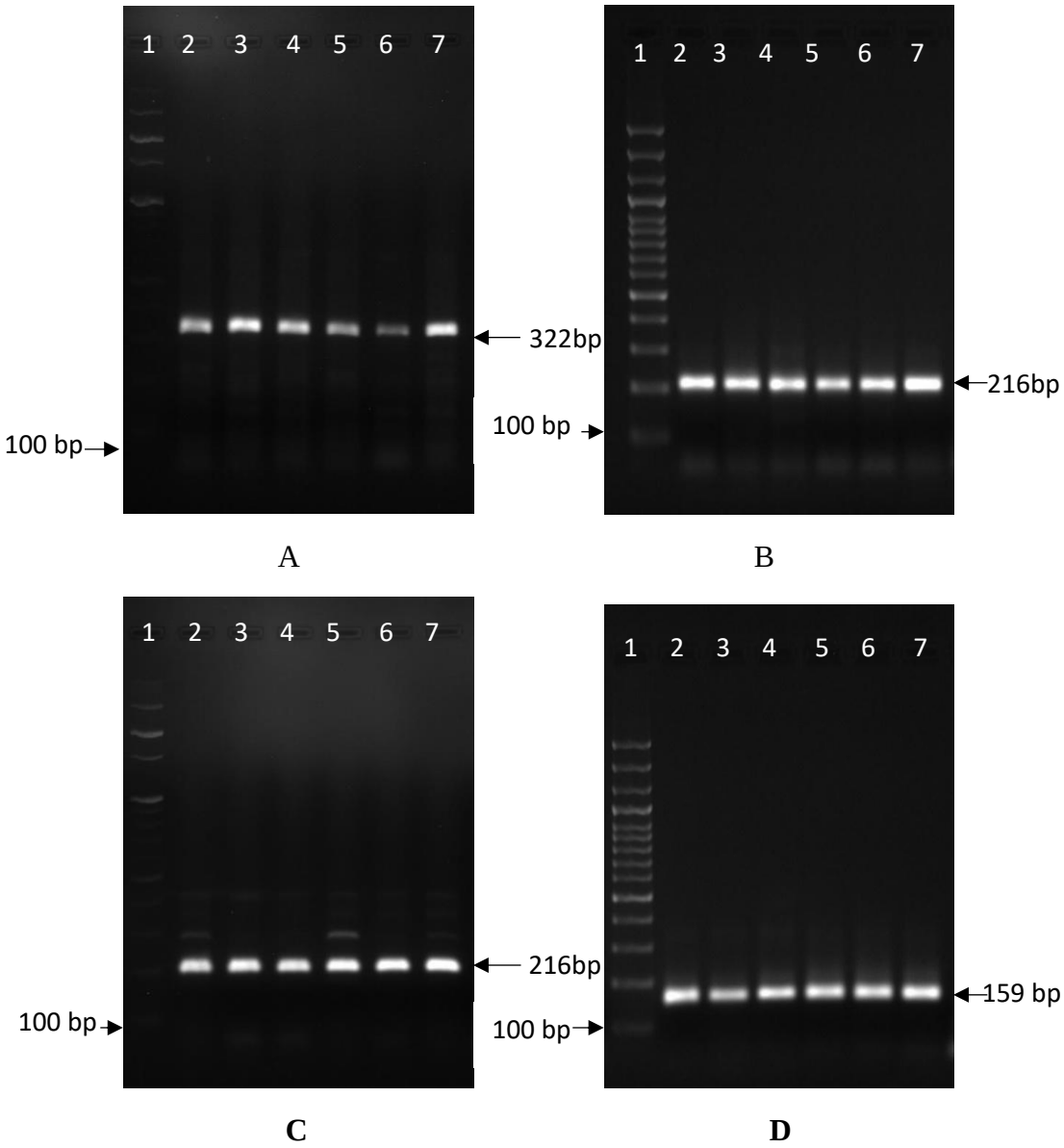


Plate 6: Amplicons of target gebes A) Nitrate transporter 1.1 (target of miR-3900-5p), B) Probable xyloglucan endotransglucosylase/hydrolase protein 33 (target of miR-67), C) 20 kDa chaperonin protein (target of miR-971-5p), D) Putative Zinc finger protein VAR3 chloroplastic (target of miR-2293).

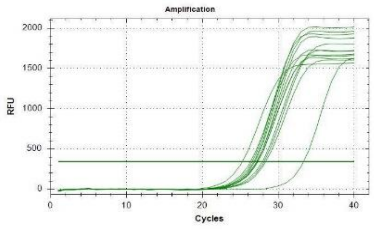
Lane 1: 100bp ladder, Lane 2 to 4: Control, Lane 5 to 7: Water stressed plants

4.3.4 Real time qPCR analysis of miRNAs and target genes in water stressed plants

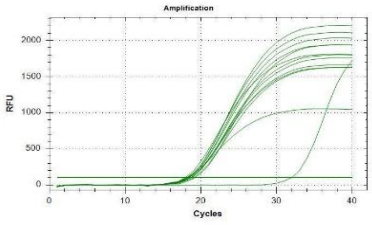
Expression of miRNA and their target genes in water stress-imposed plants was analysed on the eighth day of water stress imposition using RT-qPCR and compared with that of normal irrigated control plants. For each set three biological replicates were used and for each sample.

4.3.4.1 Raw expression data and Cq values of miRNAs and target genes

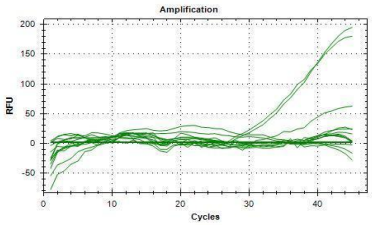
The RT-qPCR was conducted with two technical replicates for each sample of miRNA and its target gene. The amplification plot for each miRNA and their target genes were generated by RT-qPCR. Fig.4 and Fig. 5 show the raw expression data of each miRNA and their target gene respectively. The amplification plot of miR3900-5p was not good for many samples and was removed from further analysis. Cq values were generated for each assay by keeping a common threshold passing through the exponential phase of all samples. For miRNAs, the Cq value ranged from 19 to 28 (Fig. 4). For target genes, it ranged from 20 to 30 (Fig. 5). Cq values for the reference gene ranged from 20 to 25. The Cq values of miRNAs and their target genes as well as reference genes are represented in Table 7 and Table 8.



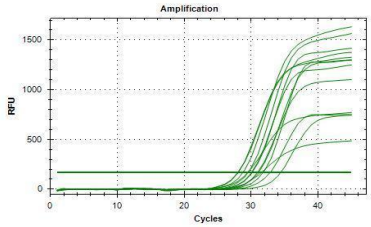
miR-67



miR-2293

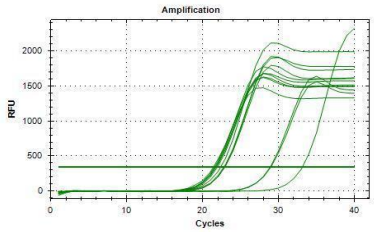


miR-3900-5p

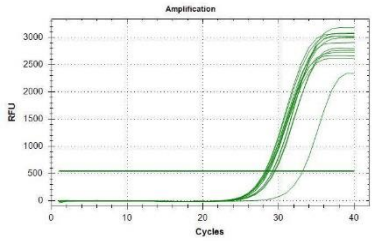


miR-971-5p

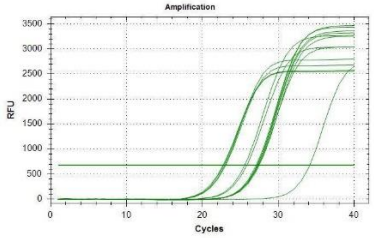
Fig. 4 Raw expression data of miRNAs



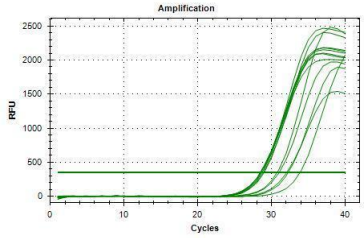
B-actin



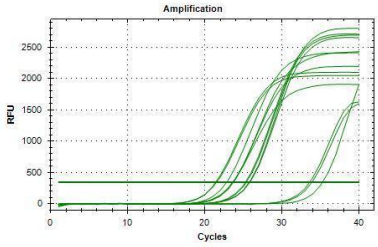
Probable xyloglucan endotransglucosylase/hydrolase protein 33



Putative Zinc finger protein VAR3 Chloroplastic



Nitrate transporter 1.1



20 kDa chaperonin

Fig. 5 Raw expression data of target genes

Table 7: The Cq values of miRNAs

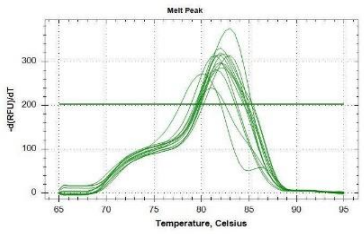
SAMPLE	miR-2293	miR-67
C1	20.04	26.52
C2	19.44	27.03
C3	19.84	26.75
S1	19.89	26.91
S2	19.74	26.79
S3	19.36	28.18

Table 8: The Cq values of target genes

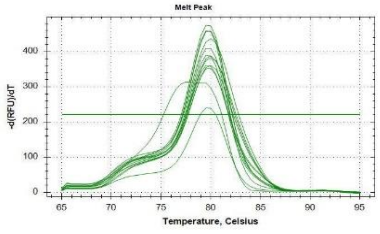
Samples	β-actin	HYD P	ZINC F	CHAP	NIT
C1	22.38	28.22	24.76	23.39	28.54
C2	20.95	28.50	21.79	21.23	28.52
C3	20.95	28.42	22.07	33.51	32.20
S1	24.85	29.35	26.36	25.12	28.72
S2	24.84	29.32	26.45	25.56	30.92
S3	25.07	28.72	26.23	23.04	28.10

4.3.4.2 Melt curve analysis

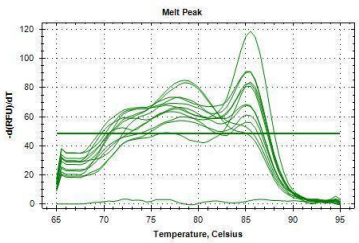
The specificity of RT-qPCR was determined by doing melt curve analysis. All miRNAs except miR-3900-5p showed single prominent peaks (Fig. 6). However, amplicons obtained for miR971-5p showed non-specific bands on agarose gel. All the target genes studied showed specific amplification (Fig. 7) using the primers designed.



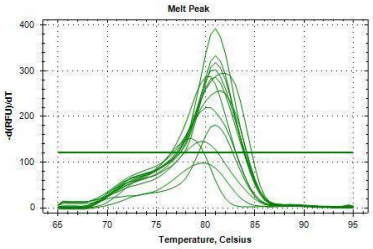
miR-67



miR-2293

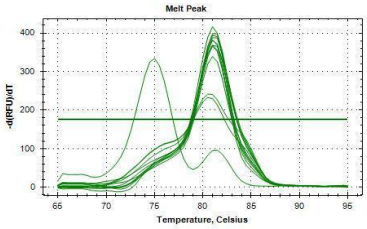


miR-3900-5p

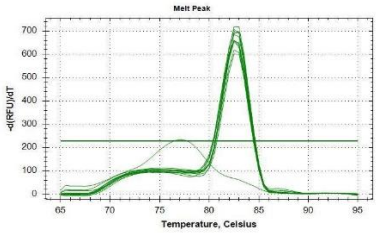


miR-971-5p

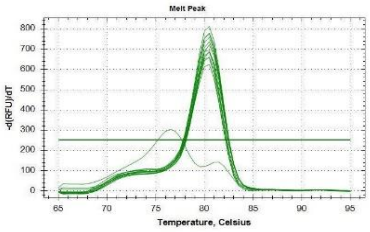
Fig. 6: Melt curve analysis of miRNAs



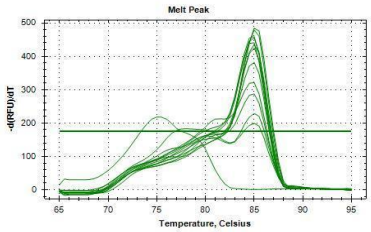
B-actin



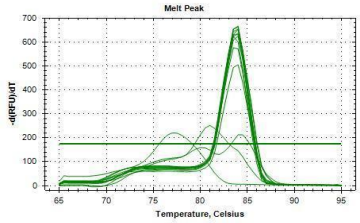
Probable xyloglucan endotransglucosylase/hydrolase protein 33



Putative Zinc finger protein VAR3 Chloroplastic



Nitrate transporter 1.1



20 kDa chaperonin

Fig. 7: Melt curve analysis of target genes

4.3.4.3 Relative expression values of miRNAs and target genes

The Cq values of control samples and water stress-imposed samples on 8th day were analysed by qBase plus software. The software generated a relative expression value of miRNAs and target genes with respect to the control samples. For the target gene, the Cq values were normalized with Cq values of β-actin (house-keeping gene). The normalised relative expression values (NRQ) are shown in Tables 9 and 10 and are represented by bar diagrams (Fig. 8, 9, 10 and 11).

Table 9: NRQ values of miRNAs

Samples	miR-2293	miR-67
C1	1	1
C2	0.56	0.25
C3	0.42	0.31
S1	6.16	4.24
S2	6.77	4.56
S3	10.34	2.04

Table 10: NRQ values of target genes

	β-actin	HYD P	ZINC F	CHAP	NIT T
C1	1.00	1.00	1.00	1.00	1.00
C2	1.00	0.30	2.89	3.43	0.78
C3	1.00	0.32	2.38	0.12	10.73
S1	1.00	2.52	1.82	0.62	1.83
S2	1.00	2.56	1.69	0.37	0.32
S3	1.00	4.57	2.32	1.15	0.66

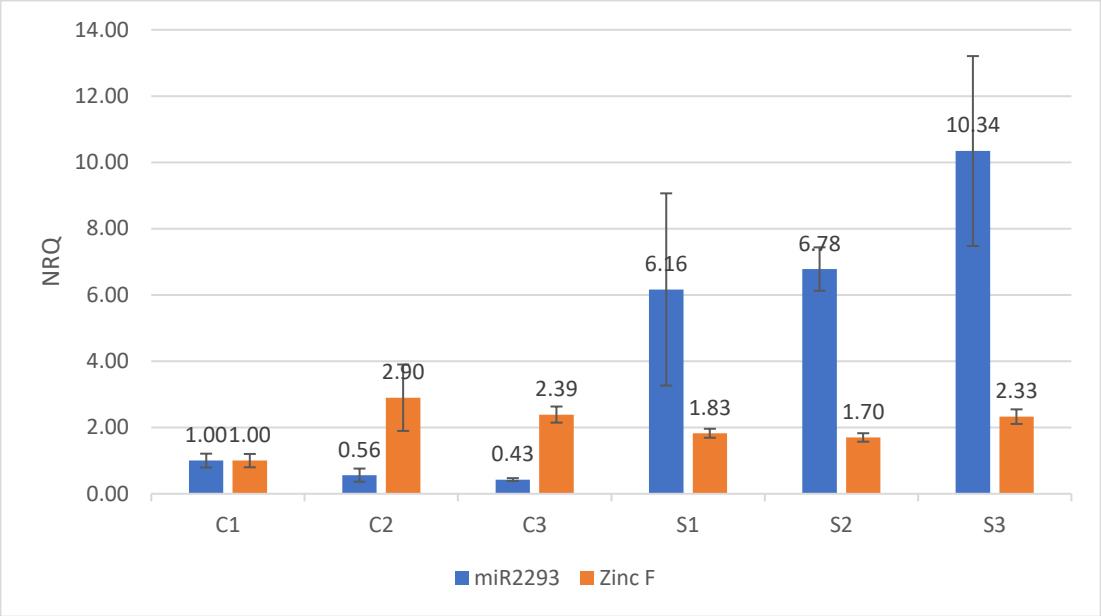


Fig. 8 Relative expression of miR-2293 and its target Putative Zinc finger protein VAR3 chloroplastic gene

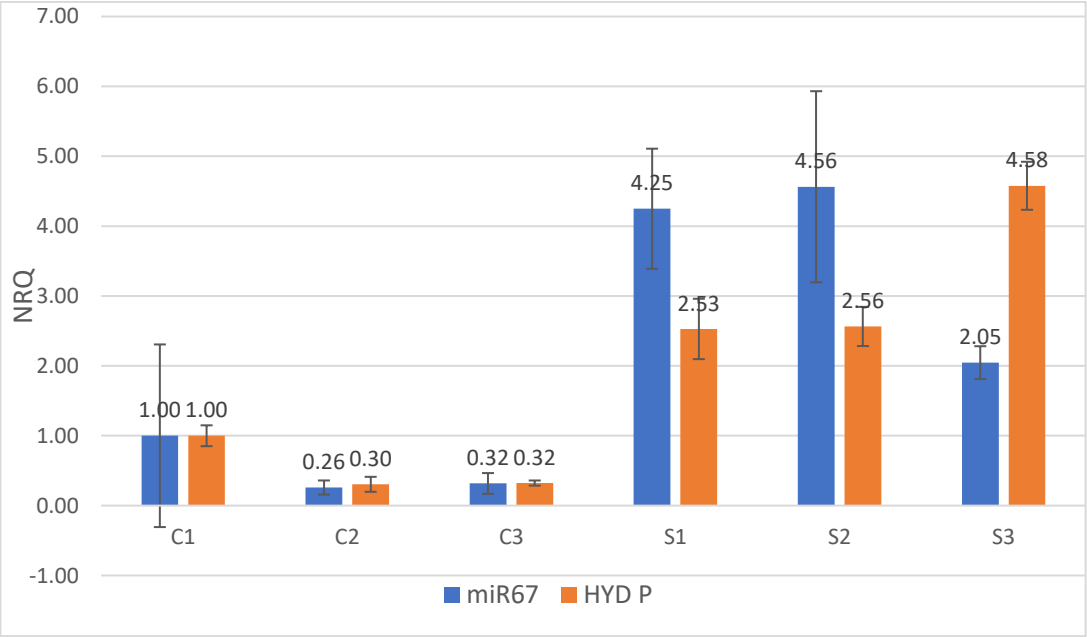


Fig. 9 Relative expression of miR-67 and its target Probable xyloglucan endotransglucosylase/hydrolase protein 33 gene

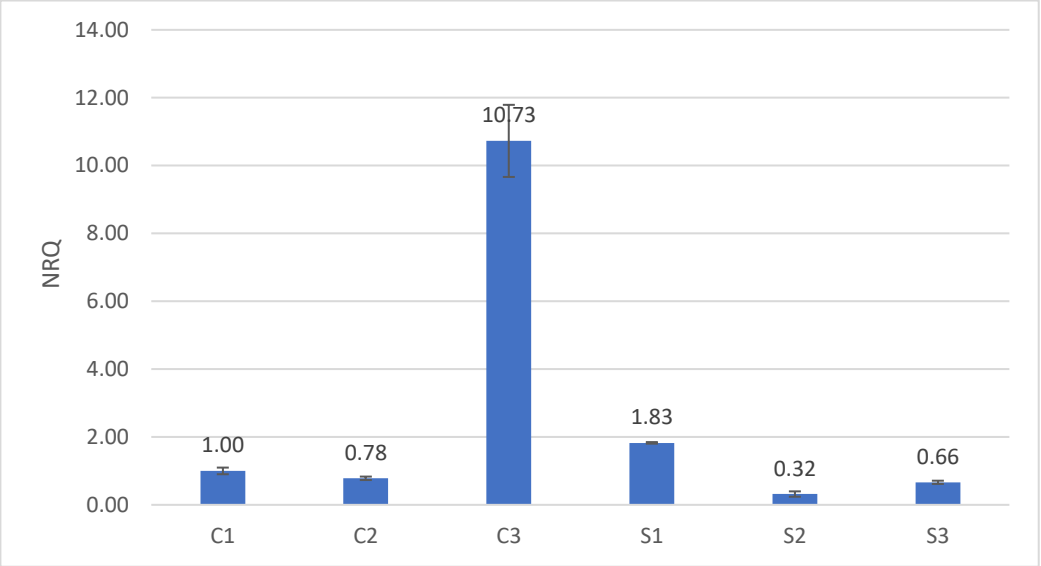


Fig. 10 Relative expression of Nitrate transporter 1.1 gene

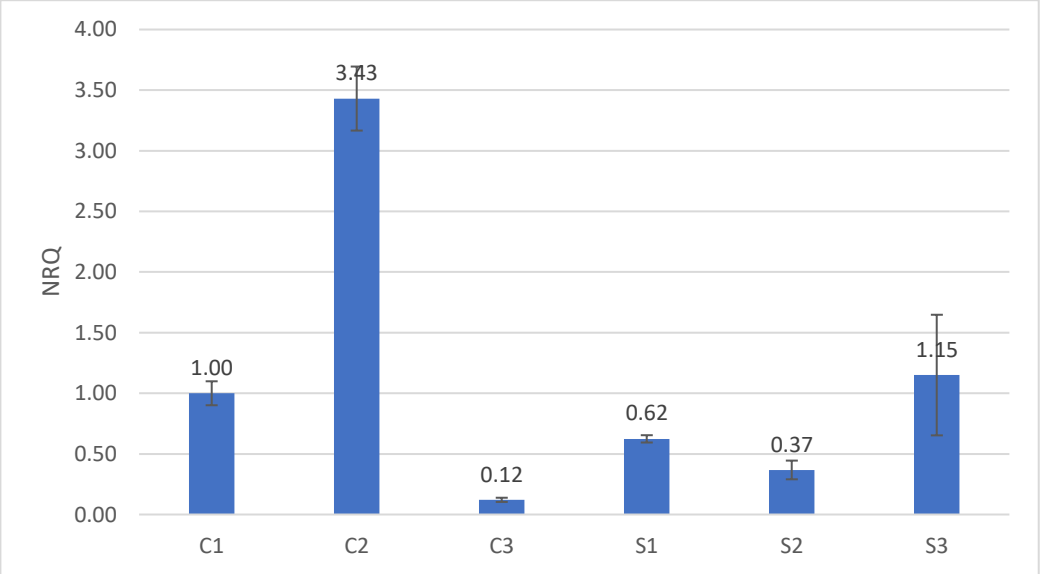


Fig. 11 Relative expression of 20 kDa chaperonin protein gene

Out of the four miRNAs studied, two miRNAs namely, miR-67, and miR-2293 showed expression in all the control and water stressed plant samples whereas miR-3900-5p miR-971 did not show expression in any of these samples. Target genes of the selected miRNAs, nitrate transporter 1.1, probable xyloglucan endotransglucosylase/hydrolase protein 33, chaperonin (20 kDa) and putative Zinc finger protein VAR3 chloroplastic showed expression in both the water stress imposed as well as control plant samples while ABC transporter C family member 13 gene didn't show expression in any of the samples.

All the water stressed plant showed an increased expression of miR-2293 and miR-67. The expression of miR-2293 was upregulated (6-10 fold) in water stressed plants compared to normal irrigated control plants. But its target gene encoding the Putative chloroplastic Zinc finger protein VAR3 did not show much variation in the expression level in water stressed plants (Fig. 8). Here the expression of miRNA and its target showed an inverse correlation.

A drastic increase in the level of miR-67 and its target was observed in the plants undergone water deficit stress. The expression of miR-67 was upregulated to a maximum of 4.6 fold in all the three water-stressed samples compared to normal irrigated control. The target gene of this miRNA, encoding Probable xyloglucan endotransglucosylase/hydrolase protein 33, also showed 2.5 to 4.5-fold increase in its expression (Fig. 9).

miR-3900-5p didn't show amplification in any of the samples, whereas its target gene encoding Nitrate transporter 1.1 showed no a variation in the water stress-imposed plants compared to control (Fig. 10).

A nonspecific amplification was observed in the case of miR-971-5p in real-time PCR whereas its target gene encoding 20 kDa chaperonin was found to be downregulated (up to 2.7fold) in water stress-imposed plants compared to that of normal irrigated control.

Discussion

5. DISCUSSION

Banana is an important commercial fruit crop in the tropic and subtropical regions of the world which is cultivated throughout the year. In India, it is the second most important crop next to mango having a gross production of 115.73 million metric tonnes (FAOSTAT, 2018). It is the most preferred crop for cultivation by farmers due to its cheap cost of cultivation and high nutritive value.

The most important constraint in banana cultivation is poised by biotic and abiotic stresses. Water deficit is one of the main limiting factors for the cultivation of *Musa* spp. The weight of the bunch is affected due to the effect of water deficit during the flowering period, which reduces the number of fingers produced.

Plants have evolved several tolerance mechanisms to overcome the abiotic stress condition. Several non-conventional approaches including the identification of drought-responsive genes and their regulation and development of transgenics are being tried to overcome water stress. The miRNA mediated gene regulation is an emerging strategy to combat various plant stresses. Among the small noncoding RNAs, microRNAs (miRNAs) constitute the most studied group of endogenous posttranscriptional silencing effectors, which serve as guides for either the cleavage or translational inhibition of complementary target transcripts (Chen, 2013), thus playing an important role in adaptation to stress. Stress-induced miRNAs generally target negative regulators of stress responses or positive regulators of processes that are inhibited by stresses and most of the newly identified miRNAs exhibit tissue- or developmental stage-specific expression patterns.

In banana, studies related to identification of miRNAs associated with abiotic stress are very few. Identification of differentially expressed miRNAs during abiotic stress can help the development of a more targeted genetic transformation or gene editing approach for imparting abiotic stress tolerance in banana.

In a previous study from our department, 52 miRNAs have been predicted in banana genome using a computational approach using the NOVOMIR tool and their target genes were identified using psRNATarget software (Mathew, 2018). The present

study was an attempt to validate some of these miRNAs for identifying water stress-responsive ones. As a preliminary study, five miRNAs were selected based on their identified target genes having possible roles in stress adaptation. The miRNAs selected for the study were miR-3900-5p (target: Nitrate transporter 1.1), miR-67 (target: Probable xyloglucan endotransglucosylase/hydrolase protein 33), miR-971-5p (target: of 20 kDa chaperonin protein), miR-2293 (target: Putative Zinc finger protein VAR3 chloroplastic), miR-449c-3p (target: ABC transporter C family member 13).

The experiment was carried out in three months old *in vitro* raised banana cv. Nendran plants to study their response towards water deficiency stress. The plants were given water stress by withholding regular irrigation for a period of seven days. The control plants received normal irrigation. After seven days, the relative water content of water stress-imposed plants was reduced to 60 percent compared to the control plants (94.06 percent). The values indicated the occurrence of water deficiency stress after 7 days of water withholding.

Among the five miRNAs studied, three have shown expression in all the samples and two of them has been found to show differential expression in water stress-imposed plants. miR-2293 and miR6-7 showed expression in in all samples whereas miR-971 showed nonspecific bands on confirmation by gel electrophoresis. miR-3900-5p and miR-449c-3p did not shown amplification in any samples. Hence for further analysis on the relative expression of miRNAs during water stress, only miR-2293 and miR67 were selected

In this study miR-2293 and miR-67 were found to be over expressed in all the water stressed plants. The expression of miR-2293 was increased 6 to 10 fold the water stressed plants. The target of miR-2293, the Putative Zinc finger protein VAR3 chloroplastic gene was found to be down regulated in all the water stress induced plants, indicating an inverse correlation between miR-2293 and its target.

The *VAR3* gene, encodes 85.9 kDa VAR3 protein containing novel repeats and zinc fingers described as protein interaction domains. VAR3 is a part of the protein complex which is required for normal chloroplast and palisade cell development. Palisade cells from water stressed plants had thinner cell walls, larger central vacuoles

and approximately the same amount of cytoplasm compared to cells from non-stressed plants. Within the cytoplasm, stressed plants had more but smaller chloroplasts with increased grana and stroma lamellae surfaces (Berlin *et al.,* 1982). VAR3 also interacts with many of the chloroplast localized enzymes and thereby affect their activity (Næsted *et al*., 2004).

Zinc finger protein family collectively called as Stress Associated Proteins (SAP), containing an N-terminal A20 and a C-terminal AN1 zinc finger domain, is a vital gene family involved in functions related to stress response and its management (Mukhopadhyay *et al*. 2004). So, in this study the higher expression of miR-2293 indicated that it is water stress responsive.

miR-67, which targets probable xyloglucan endotransglucosylase/hydrolase protein 33 gene, showed higher levels of expression (4-6 fold) in all the water stress-imposed plants. Its target gene codes for the key enzymes xyloglucan endotransglucosylase(XET) /hydrolase protein (XTHs) which are involved in metabolism of xyloglucan which is a major structural component of the cell wall matrix in several cell types at different developmental stages, including newly formed cell plates of dividing cells (Moore and Staehelin, 1988). These enzymes mediate splitting and reconnection of the xyloglucan crosslinks in the cell wall. XTHs are thought to play a pivotal role in the construction, remodelling and disassembly of the xyloglucan/ cellulose framework in type I cell walls during cell growth and differentiation due to their potential enzymatic actions (Nishitani and Vissenberg, 2007). In the present study, the expression of the target of miR-67 was also found to increase (2.5 - 4.5-fold) indicating a positive correlation between the expression of miR-67 and its target.

XTH activity is also found to be associated with wall strengthening (Maris *et al.*, 2009) since the transglucosylase activity can potentially allow integration of newly secreted xyloglucans into the cell wall thereby strengthening it. In a study, XTH and xylose isomerase genes encoding two xyloglucan-modifying enzymes were found to be up-regulated in the early stages in the elongation zone of rice roots under water deficit following stress application, indicating the role of xyloglucan in the maintenance of root growth (Yang *et al*., 2006). The overexpression of an XTH gene from pepper in transgenic plants confirmed the role of XTH in enabling better drought tolerance (Yang

et al., 2006). In another study, a transgenic Arabidopsis plants overexpressing a hot pepper (*Capsicum annuum*, cv. Pukang) XTH (CaXTH3) gene exhibited abnormal leaf morphology resulting in a severe wrinkled leaf shape, increased number of small-sized cells in the leaf mesophyll cells and, ultimately, an increased tolerance to severe water deficient stress. This suggests that CaXTH3 could be involved in remodelling of cell wall to strengthen the wall layers, and thereby it could help in the protection of mesophyll cells against water deficient stress (Cho *et al*., 2006). In another study conducted on transgenic tomato plants overexpressing CaXTH3, similar results were shown, emphasizing the key role of XTH in water-deficit stress tolerance (Choi *et al*., 2011). Increased cellulose synthesis is a way by which cell wall integrity and cell turgor pressure are maintained, and thereby allowing continuous cell growth under low water potential (Ricardi *et al*., 2014). The result of present study is also in confirmation with these studies.

The miR-971-5p that targets 20 kDa chaperonin protein showed non-specific amplification in real-time PCR. The target gene of 20 kDa chaperonin protein has shown decreased expression in stress-imposed plant samples compared to control samples. Chaperones are a group of functional proteins involved in protein folding, assembly, degradation, and protection of nascent proteins during their transport into specific organelles, in both optimal and adverse conditions (Wang *et al*. 2004; Cheng *et al*. 2015). Chaperonin 20 kDa (CPN20) is a well-known chloroplast-localized co-chaperonin that help chaperonin CPN60s in protein folding in an ATP-dependent reaction (Horwich *et al*. 2007). Several studies have shown that CPN20 mediates the antioxidant enzyme (FeSOD) activation in Arabidopsis chloroplasts (Kuo *et al*. 2013), and negatively regulates abscisic acid signalling in Arabidopsis (Zhang *et al*. 2013). Antioxidant enzyme, Superoxide dismutase (SOD) acts as a primary defence against ROS by converting O_2^- to O2 and H_2O_2, which requires a specific metal cofactor (Bowler *et al*., 1992). In this study also the stress condition showed that the reduction may be an indication of water stress.

Similarly, from a group of chaperone proteins chloroplastic 20 kDa chaperonin which was downregulated (2.83-fold) in wheat, by imposing moisture stress (Nazari *et al*., 2020). A reduction of CPN20 was first observed in wheat in response to moisture

deficient stress. Less-abundance of this protein was also reported in maize leaves under drought stress (Zhao *et al*. 2016).

miR-3900-5p that targets nitrate transporter 1.1 didn't show proper amplification in the real time PCR, NRT1.1 is a dual-affinity NO_3^- transporter which is regulated by phosphorylation and it's also able to transport auxin (Krouk *et al*., 2010). Initially characterized as an influx transporter which participate in the uptake of NO_3^- from the external medium, NRT1.1 has also been shown to display a NO3 – sensing function governing expression of NO_3^- responsive genes and NO_3^- induced changes in root development. The NRT1.1 gene is expressed in both roots and shoots. In more immature parts of the roots, NRT1.1 may also participate in xylem NO_3^- loading/ unloading in the root stele. In a study by Zhong *et al* (2015), the total nitrogen content was found to be increased in transgenic *Arabidopsis thaliana* (AtTGA4) plants, and expression of NRT2.1 and NRT2.2, that function as transporters of nitrogen uptake from soil to roots, also increased under drought stress compared to control plants. The regulatory function of N in water stress tolerance of plants depends upon the intensity of stress and N level. Proper N level supports regular plant growth and helps plants to defend against stress (Chang *et al*., 2016). It has been proposed that crops supplied with relatively higher N had better growth performance than that supplied with low N under drought stress (Haefele *et al*., 2008). Higher N mitigates the adverse effects of water stress on photosynthesis as well. Plants decrease nitrogen uptake with declining soil moisture, due to reduced nutrients coming from decomposition and mineralization (Sanaullah *et al*., 2012; Borken *et al*., 2009). Expression of the nitrate transporter genes NRT1.1, was similar in transgenic and wild type plants, indicating that NRT1.1 is a sensor in nitrogen signalling responsible for the repression of NRT2.1, and suggesting that NRT1.1 function independently (Ho *et al.,* 2009). In this study, the target gene nitrate transporter 1.1, showed reduction in its expression in the stress-imposed plants compared to the control plants.

miR-449c-3p and its target gene ABC transporter C family member did not show expression in any of the samples. ABC transporters are involved in the transport of the most important carotenoid-derived stress hormone ABA. Besides being a germination inhibitor present in dormant seeds, ABA is mainly produced in the vascular

tissues of shoots and roots as a response to water stress (Schachtman *et al*., 2008). From there it is translocated to foliar tissues, where it induces stomatal closure to minimize water loss. Consequently, ABA has to be exported from xylem parenchyma cells, transferred to leaves and imported into guard cells and other cell types in order to trigger the signalling pathways required for coping with water stress. An upregulation of ABC transporter gene is expected during water stress. In the present study, ABC transporter gene didn't show expression.

The study could demonstrate the biological validation of five computationally predicted miRNAs in banana. Out of them miR-2293 and miR-67showed differential expression in water deficit stress imposed plants and the target of miR-2293, Putative Zinc finger protein VAR3 chloroplastic, showed an inverse expression pattern, indicating the regulation of target gene by the miRNA.

The differentially expressed miRNAs might have a role in water stress responses in banana and understanding the exact mechanism of these miRNAs can aid in the development of a miRNA targeted approach for water stress tolerance in banana.

Summary

6. SUMMARY

The study entitled “Expression profiling of microRNAs associated with water stress in banana (*Musa* spp.)” was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2018-2020. The objective of the study was to analyse the expression of selected computationally predicted miRNAs in banana cultivar Nendran (*Musa* AAB) under water deficit stress conditions.

Banana is an important commercial fruit crop in the tropic and subtropical regions of the world. The average production of banana is estimated to be 115.73 million tonnes in over 120 countries and India ranks first with 30.8 million tonnes.

Soil moisture is the most dangerous abiotic stress that limits the production and productivity of banana (*Musa* spp.) Knowledge of genetic and molecular changes in the banana plant is useful to formulate programs for crop genetic improvement and developing particular desirable characteristics such as resistance and tolerance to abiotic and biotic stresses. Plant’s responses to water stress are regulated at both transcriptional and posttranscriptional levels. MicroRNAs (miRNAs) have been found to play roles in stress responses in plants. They regulate gene expression during drought stress as post-transcriptional gene regulators, by the silencing of endogenous messenger RNAs. RNA interference technology (RNAi) is found to be a promising approach for developing drought stress-tolerant crops. The present work was aimed to study the expression of drought-responsive miRNAs and their targets in banana cv. Nendran subjected to soil moisture deficit stress using real-time quantitative PCR (RT-qPCR). Salient findings from the study are summarized below.

Five microRNAs were selected from computationally predicted microRNAs based on the function of their target genes and their possible role in abiotic stress responses. The miRNAs selected were miR-3900-5p (target; Nitrate transporter 1.1), miR-67 (target: Probable xyloglucan endotransglucosylase /hydrolase protein 33), miR-971-5p (target: 20 kDa chaperonin), miR-2293 (target: Putative Zinc finger protein VAR3 chloroplastic), and miR-449c-3p (target: ABC transporter C family member 13).

The study was conducted in three months old *in-vitro* raised banana plants cv. Nendran. Soil moisture deficit stress was imposed by withholding irrigation for seven days. *In vitro* raised plants of the same developmental stage provided with normal irrigation served as control. Leaf samples were collected from both the stress imposed as well as control plants after seven days. Water stress was confirmed in banana plants by measuring the relative water content (RWC). Water stress-imposed plants were found to have a mean RWC of 60 percent compared to the control plants which are found to have RWC of 94.06 percent

RNA was isolated from the leaf samples of the control and water stress-imposed plants using the modified Rodrigues-Garcia method (Ekatpure *et al.*, 2019). Primers were designed and synthesized for the stem-loop synthesis and amplification of miRNAs and their target genes. The RNA was reverse transcribed using Verso cDNA synthesis kit and cDNA conversion was confirmed by PCR using β-actin gene primers.

Gene expression was studied by conducting RT-qPCR. Melting curve analysis was done to check the specificity of the primers and the appearance of a single peak confirmed specific amplicons. The raw Cq values were converted to relative expression values with help of qBase plus software. The values were normalized using the reference gene, β-actin. The expression of miRNAs and their target genes was analysed with reference to the control plant. Expression analysis with RT-qPCR confirmed the presence of all miRNAs except miR-3900-5p in all the samples.

Out of the five miRNAs studied, miR-2293 and miR-67 were found to be overexpressed (6-10 fold) in all the water-stressed plants. At the same time, the target of miR-2293, the Putative Zinc finger protein VAR3 chloroplastic gene was found to be down-regulated in all the water stress-induced plants, indicating an inverse correlation between miR-2293 and its target.

miR-67, which targets probable xyloglucan endotransglucosylase/hydrolase protein 33 gene was found upregulated (4-6 fold) in all the water-stress imposed plants. Here the expression of the target gene was also increased (2.5 and 4.5-fold), indicating no miRNA- target correlation between miR-67 and its target.

miR-971-5p showed nonspecific amplification in real-time PCR whereas its predicted target gene, 20 KDa chaperonin was found to have a lower expression in stress-imposed plants compared to that of control plants. miR-3900-5p didn't show amplification in any of the samples, whereas the mRNA transcript encoding Nitrate transporter 1.1 showed a reduction in its expression in stress-imposed samples.

The study suggests that the miR-2293 and miR-67 are associated with water deficit induced stress response in banana cv. Nendran. miR-2293 and its target, the Putative Zinc finger protein VAR3 chloroplastic gene showed an appropriate negative correlation. Further studies can be conducted to find out the exact role of these differentially expressed miRNAs in water stress responses.

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Appendices

APPENDIX 1

RNA extraction buffer (Ekatpure *et al.,* 2019).

Tris HCl	150Mm
SDS	4 percent (W/v)
EDTA (Ph 7.5)	100 Mm
β- mercaptoethanol	2 percent (v/v)
polyvinyl pyrrolidone (PVP)	4 percent (W/V)
RNaser free water	Treated with diethyl pyrocarbonate (DEPC) 0.1per cent

Abstract

EXPRESSION PROFILING OF microRNAs ASSOCIATED WITH WATER STRESS IN BANANA (*Musa* spp.)

By

SAYOOJ P.

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Abstract of the thesis

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2020

ABSTRACT

The study entitled “Expression profiling of microRNAs associated with water stress in banana (*Musa* spp.)” was carried out during 2018-2020, in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objective was to study the expression of computationally predicted miRNAs in banana cultivar Nendran (*Musa* AAB) under water stress conditions.

Five computationally predicted miRNAs were selected from a study previously conducted in the Department of Plant Biotechnology. The miRNAs were selected based on the function of their target genes that have a possible role in stress responses. The miRNAs selected were miR-3900-5p (target: Nitrate transporter 1.1), miR-67 (target: Probable xyloglucan endotransglucosylase/hydrolase protein 33), miR-971-5p (target: 20 kDa chaperonin protein), miR-2293 (target: Putative Zinc finger protein VAR3 chloroplastic), miR-449c-3p (target: ABC transporter C family member 13).

Three-months-old *in vitro* raised banana cv. Nendran plants were subjected to water stress by withdrawing irrigation for one week. Water stress was confirmed by measuring the relative water content in the leaf samples after 7 days. For expression analysis of miRNAs, total RNA was isolated from the leaf samples using the modified Rodrigues-Garcia method and reverse transcribed to cDNA. Stem loop primers were used for miRNAs and PCR was carried out using a specific forward and a universal reverse primer. The expression profile of the miRNAs and their target genes were studied by RT-qPCR. A melt curve analysis was performed to determine the specificity of the products. Reactions were done with three biological replicates and two technical replicates.

The results showed an upregulation of miR-2293 (6 to 10-fold) in all the plants. But the expression pattern of its target gene (Putative Zinc finger protein VAR3 chloroplastic gene) showed a reduction in its expression by half, indicating an inverse correlation between the miR-2293 and its predicted target, which in turn indicate a miRNA-target interaction in response to water stress.

The miR-67 expression showed a 2 to 4 fold increase in the water stress-imposed plant samples compared to control. Its target, Probable xyloglucan endotransglucosylase/hydrolase protein 33, also showed an increase in its expression (2.5 and 4.5 fold). However, there was no inverse correlation between the miRNA and its target.

In RT-qPCR, miR-3900-5p and miR-971-5p did not produce expected amplicons and their target genes did not show any specific pattern of expression in water stress-imposed plants. miR-449c-3p and its target ABC transporter C family member 13 gene did not show expression in any of the samples.

The study showed that miR-2293 and miR-67 are water stress responsive in banana cv. Nendran. Further studies on other identified targets may help in understanding the role of these miRNA in water deficient stress conditions.

സംഗ്രഹം

വാഴയിലെ ജല സമ്മർദ്ദവുമായി ബന്ധപ്പെട്ട മൈക്രോ ആർഎൻഎകളുടെ പ്രകടന രൂപരേഖ പഠനം (മൂസ സ്പീഷിസ്)

"വാഴയിലെ (മൂസ സ്പീഷിസ്) ജലസമ്മർദ്ദവുമായി ബന്ധപ്പെട്ട മൈക്രോ ആർഎൻഎകളുടെ പ്രകടന രൂപരേഖ പഠനം" 2018-2020 കാലയളവിൽ വെള്ളായണി കാർഷിക കോളേജ്, സസ്യ ജൈവസാങ്കേതിക വിദ്യ വിഭാഗത്തിൽ നടത്തുകയുണ്ടായി. ജല സമ്മർദ്ദ സാഹചര്യങ്ങളിൽ നേന്ത്രവാഴ ഇനത്തിൽ (മൂസ എഎബി) കമ്പ്യൂട്ടേഷണൽ രീതി പ്രകാരം പ്രവചിച്ച മൈക്രോ ആർഎൻഎകളുടെ പ്രകടന രീതി പഠിക്കുകയായിരുന്നു ലക്ഷ്യം.

സസ്യ ജൈവസാങ്കേതിക വിദ്യ വിഭാഗത്തിൽ മുമ്പ് നടത്തിയ പഠനത്തിൽ പ്രവചിച്ച അഞ്ച് മൈക്രോ ആർഎൻഎകളെ തിരഞ്ഞെടുത്തു. ജലസമ്മർദ്ദ പ്രതികരണങ്ങളിൽ ടാർഗെറ്റ് ജീനുകളുടെ സാധ്യമായ പങ്കിനെ അടിസ്ഥാനമാക്കിയാണ് മൈക്രോ ആർഎൻഎകളെ തിരഞ്ഞെടുത്തത്. മിർ-3900-5-പി (ടാർഗെറ്റ്: നൈട്രേറ്റ് ട്രാൻസ്പോർട്ടർ 1.1), മിർ-67 (ടാർഗെറ്റ്: പ്രോബബിൾ സൈലോഗ്ലുകാൻ എൻഡോട്രാൻസ്ഗ്ലൂക്കോസൈലേസ് / ഹൈഡ്രോലേസ് പ്രോട്ടീൻ 33), മിർ-971-5 പി (ടാർഗെറ്റ്: 20 കെഡിഎ ചാപെറോണിൻ പ്രോട്ടീൻ), മിർ-2293 (ടാർഗെറ്റ്: പുട്ടേറ്റീവ് സിങ്ക് ഫിംഗർ പ്രോട്ടീൻ VAR3 ക്ലോറോപ്ലാസ്റ്റിക്), മിർ-449c-3പി (ടാർഗെറ്റ്: എബിസി ട്രാൻസ്പോർട്ടർ സി കുടുംബാംഗം 13) എന്നിവയാണ് തിരഞ്ഞെടുക്കപ്പെട്ട മൈക്രോ ആർ എൻ എ കൾ.

മൂന്ന് മാസം പ്രായമുള്ള, ടിഷ്യുകൾച്ചർ വഴി വികസിപ്പിച്ചെടുത്ത നേന്ത്രവാഴത്തൈകളെ ഒരാഴ്ച ജലസേചനം പിൻവലിച്ചുകൊണ്ട് ജല സമ്മർദ്ദത്തിന് വിധേയമാക്കി. ഏഴു ദിവസത്തിനുശേഷം ഇലകളിലെ ജലത്തിന്റെ ആപേക്ഷിക അളവ് കണക്കാക്കിയാണ് ജല സമ്മർദ്ദം സ്ഥിരീകരിച്ചത്. മൈക്രോ ആർഎൻഎകളുടെ പ്രകടന വിശകലനത്തിനായി ഇലകളിൽ നിന്ന് മൊത്തം ആർഎൻഎ വേർതിരിച്ച് സിഡിഎൻഎയായി മാറ്റി. മൈക്രോ ആർഎൻഎകൾക്കായി സ്റ്റെം ലൂപ്പ് പ്രൈമറുകൾ ഉപയോഗിക്കുകയും ഒരു പ്രത്യേക ഫോർവേർഡും സാർവ്വത്രിക റിവേഴ്സ് പ്രൈമറും ഉപയോഗിച്ച് പിസിആർ നടത്തുകയും ചെയ്തു. ആർടി-ക്യുപിസിആർ വഴി മൈക്രോ ആർഎൻഎകളുടെയും അവയുടെ ടാർഗെറ്റ് ജീനുകളും പ്രകടന രൂപരേഖ പഠനവിധേയമാക്കി. മൈക്രോ ആർഎൻഎകളുടെ സുവ്യക്തത നിർണ്ണയിക്കാൻ ഒരു മെൽറ്റ് കർവ് വിശകലനം നടത്തുകയുണ്ടായി. മൂന്ന് ജീവശാസ്ത്രപരമായ പകർപ്പുകളും രണ്ട് സാങ്കേതിക പകർപ്പുകളും ഉപയോഗിച്ചാണ് പഠനം നടത്തിയത്.

എല്ലാ സസ്യസാമ്പിളുകളിലും മിർ-2293 യുടെ പ്രകടനത്തിൽ 6 മുതൽ 10 മടങ്ങ് വരെ വർദ്ധനവ് കാണിക്കുന്നതായി കണ്ടു. എന്നാൽ

അതിന്റെ ടാർഗെറ്റ് ജീനിന്റെ (പുട്ടേറ്റീവ് സിങ്ക് ഫിംഗർ പ്രോട്ടീൻ വിഎആർ3 ക്ലോറോപ്ലാസ്റ്റിക് ജീൻ) പ്രകടനം അതിന്റെ ആവിഷ്കാരത്തിൽ പകുതിയായി കുറയുന്നതായും കണ്ടു, മിർ-2293 ഉം അതിന്റെ ടാർഗെറ്റും തമ്മിലുള്ള ഈ വിപരീത ബന്ധം ജല സമ്മർദ്ദത്തോടുള്ള ഒരു മൈക്രോ ആർഎൻഎ-ടാർഗെറ്റ് പ്രതിപ്രവർത്തനത്തെ സൂചിപ്പിക്കുന്നു

നിയന്ത്രിതസസ്യവുമായി താരതമ്യപ്പെടുത്തുമ്പോൾ ജല സമ്മർദ്ദം ചെലുത്തിയ സസ്യസാമ്പിളുകളിൽ മിർ-67, 2 മുതൽ 4 മടങ്ങ് വരെ വർദ്ധനവ് പ്രകടനത്തിൽ കാണിക്കുന്നതായി കണ്ടു. അതിന്റെ ടാർഗെറ്റ്, പ്രോബബിൾ സൈലോഗ്ലുകൻ എൻഡോട്രാൻസ്ഗ്ലൂക്കോസൈലേസ് / ഹൈഡ്രോലേസ് പ്രോട്ടീൻ 33 എന്നിവയും അതിന്റെ പ്രകടനത്തിൽ വർദ്ധനവ് കാണിക്കുന്നതായും കാണാൻ കഴിഞ്ഞു (2.5-4.5 മടങ്ങ്).

ആർടി-ക്യുപിസിആർ ഇൽ, മിർ-3900-5പി, മിർ-971-5പി എന്നീ മൈക്രോ ആർഎൻഎകൾ പ്രതീക്ഷിച്ച ആംപ്ലിക്കോണുകൾ ഉൽപാദിപ്പിച്ചില്ല, മാത്രമല്ല അവയുടെ ടാർഗെറ്റ് ജീനുകൾ ജലസമ്മർദ്ദം ചെലുത്തുന്ന സസ്യങ്ങളിൽ ഏതെങ്കിലും പ്രത്യേക രീതി പ്രകടിപ്പിക്കുന്നതായി കാണാൻ കഴിഞ്ഞില്ല. മിർ-449c-3 പി, അതിന്റെ ടാർഗെറ്റ് എബിസി ട്രാൻസ്പോർട്ടർ സി കുടുംബാംഗം 13 ജീൻ എന്നിവയുടെ സാന്നിദ്ധ്യം ഒരു സാമ്പിളിലും പ്രകടിപ്പിക്കുന്നതായി കാണാൻ കഴിഞ്ഞില്ല.

നേന്ത്രവാഴയിൽ മിർ-2293, മിർ-67 എന്നീ മൈക്രോ ആർഎൻഎകൾ ജല സമ്മർദ്ദത്തോട് പ്രതികരിക്കുന്നതായി പഠനം തെളിയിച്ചു.