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Full Length Research Paper

Differential expression of microRNAs in roots and leaves of Hyacinth bean (*Lablab purpureus*) under drought stress

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Post-transcriptional regulation of gene expression via microRNAs (miRNAs) plays crucial role in plant responses to environmental changes. Hyacinth bean, a versatile adapter to drought conditions, exhibited miRNA mediated regulation of gene expression in tissue-specific manner as revealed by Reverse Transcription quantitative PCR (RT-qPCR). Contrasting expression pattern of two miRNAs; miRNA167 and miRNA393 in root and leaf tissues suggested different targets and functional divergence of miRNAs within the same species. Target prediction and gene annotation showed that majority of the target genes included transcription factors involved in plant growth, development and stress responses. Membrane transporters, metabolic enzymes, antioxidant enzymes and components of miRNA biogenesis were other predicted targets, suggesting the crucial roles of these miRNAs in diverse physiological functions under drought stress. Considering the expression pattern of drought responsive miRNAs and functional annotation of their predicted targets a possible regulatory network operating in roots and leaves of hyacinth bean, contributing to signal transduction pathways for osmotic homeostasis, and antioxidant defense is proposed.

Key words: Drought, *Lablab purpureus*, Hyacinth bean, Differential expression, microRNA.

Abbreviations: RT-qPCR, Reverse Transcription quantitative PCR; miR, microRNA; ABA, Abscisic acid; CSD, Cu/Zn-superoxide dismutase; GST, Glutathione Transferase; BADH, Betaine Aldehyde Dehydrogenase; NFYA, Nuclear Factor Y Subunit A; DREB, Dehydration-Responsive Element Binding protein; ARF, auxin response factor; TIR, transport inhibitor response; CUC2, Cup shaped cotyledon 2; TF, Transcription Factor.

INTRODUCTION

Water accounts for 80-90% of the fresh weight of most herbaceous plant and over 50% of the fresh weight of woody plants, hence availability of water plays an imperative role in plant growth and development (Kramer and Boyer, 1995). Drought is a widespread adverse environmental condition which is likely to increase in upcoming years. Climate change with 4°C warming is projected to reduce renewable surface water and groundwater resources significantly in most dry subtropical regions. Crop productivity in these environments can only be increased by the development of plants that are well adapted to dry conditions, which requires complete understanding of the drought responsive mechanisms in plants.

In plants, mild drought conditions induce regulatory mechanisms for uptake and water loss allowing maintenance of relative water content of tissues within the limits and the change in photosynthetic process at

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negligible levels. Whereas severe drought induces adverse changes in plant metabolic processes, stomatal movement, nutrient uptake, and production of photosynthetic assimilates, which ultimately cause reduced growth and crop losses (Neumann 2008). Plants have evolved series of mechanisms to circumvent the detrimental effects of drought stress. Changes in gene expression play an important role in plant drought stress response. Genes induced during drought stress are classified into two groups. The first group comprises of components that protect the cell against drought stress directly, such as osmolytes, antioxidants, antioxidant enzymes, chaperones, LEA (late embryogenesis abundant) proteins, water channel proteins and transporters. The second group comprises of protein components involved in the signal transduction such as protein kinases and transcription factors that regulate gene expression in drought stress response (Shinozaki and Yamaguchi-Shinozaki 2006).

By far, the most frequently invoked regulation of gene expression is at transcriptional level. Complementing transcriptional regulation, post transcriptional regulation has contributed to an additional layer of regulation of gene expression. Increasing evidences in recent times suggest a pivotal role of miRNAs in regulation of gene expression post-transcriptionally. While they mediate cleavages of target mRNAs when there is perfect complementary binding, and inhibit translation when binding is partially complementary. miRNAs have been characterized from drought stressed plant species such as *Arabidopsis* (Sunkar and Zhu, 2004), *Phaseolus vulgaris* (Arenas-Huertero et al., 2009), rice (Zhou et al., 2010), cowpea (Barrera-Figueroa et al., 2011), tobacco (Frazier et al., 2011), soya bean (Kulcheski et al., 2011) suggesting their inevitable role in drought stress tolerance mechanisms, and have paved way for better understanding of the regulatory process mediated by miRNAs.

Lablab purpureus (Hyacinth bean) is a drought tolerant crop and grows well in dry lands with limited rainfall. Adaptive strategies of Hyacinth bean to drought with respect to physiological and biochemical responses (D'Souza and Devaraj 2011), polyamine synthesis (D'Souza et al. 2014) are well documented. Also, at molecular level, numerous ESTs are characterized (Yao et al. 2013; Kokila and Devaraj 2015). Understanding the regulation of gene expression by miRNAs would lead to better understanding of the drought responsive mechanisms in Hyacinth bean. Differential expression pattern of miRNAs in roots and leaves of Hyacinth bean and functions of their predicted target genes during drought stress are described hereunder.

METHODOLOGY

Plant materials and stress conditions

The seeds of Hyacinth bean (cv. HA-4) were obtained from National Seed Project, University of Agricultural Sciences, GKVK, and Bangalore, India. Seeds were surface sterilized with 0.1 % (w/v) mercuric chloride for 1 min, rinsed immediately with large volume of distilled water, and soaked overnight in distilled water. Soaked seeds were sown in trays containing vermiculite and acid-washed sand (1:1 w/w) and watered daily, once. The germination was carried out under natural greenhouse conditions; day/night temperature and relative humidity were 30/25 °C, and 75/70 %, respectively. The average photoperiod was 12 h light/12 h dark. The seedlings were grown in green house conditions for 10 days. Drought stress was induced in 10 days old seedlings by withholding water. Root and leaf samples were collected 6 days after drought stress for isolation of total RNA. Plants that were watered regularly served as control.

Total RNA isolation

Total RNA was extracted by homogenizing leaf tissue in liquid nitrogen and pre-chilled Tri-Reagent (Sigma–Aldrich) at room temperature. The homogenate was centrifuged at 8,000×*g* for 10 min at 4°C and the supernatant was transferred to a fresh centrifuge tube. The sample was left to settle for 5 min at RT, 8 mL of chloroform was then added and the mixture was shaken vigorously for 15 s. The mixture was left to stand for 15 min at RT and centrifuged at 8,000×*g* for 15 min at 4°C. The colourless upper aqueous phase was transferred to a fresh centrifuge tube and mixed with 20 mL isopropanol. The tube was briefly vortexed, then allowed to stand for 10 min at RT and centrifuged at 8,000×*g* for 10 min at 4°C. After removal of the supernatant, the RNA precipitate was immediately dissolved RNase-free water. The concentration and purity of isolated total RNA sample was assessed by A260/ A280 ratios and A260/A230 ratios using Biomate 3S UV-Visible spectrophotometer (Thermo Scientific).

Expression profile of miRNA via stem–loop RT-PCR

The miRNA primers and stem-loop RT-qPCR experiment indicated in table 1 were designed according to Chen et al. (2005) and Varkonyi-Gasic et al. (2007). Briefly, 1µg total RNA and 50 nM stem–loop primers were mixed with RNase-free water to a total volume of 12.5 µL and incubated at 65°C for 5 min followed by ice-cooling. Then, 4 µL RT-Buffer, 2 µL of dNTP mix (Thermo Scientific), 0.5 µL Ribolock RNase inhibitor (Thermo Scientific) and 1 µL RevertAid Reverse transcriptase 200U (Thermo Scientific) were added to a final volume of 20 µL. Reverse transcription reaction was performed on Eppendorf master cycler by incubating at 42 °C for 60 min, followed by 70°C for 10 min to inactivate the enzyme.

SLP- Stem loop primer, FP- Forward primer

The qPCR reactions were carried out with Biorad iQ5 Multicolor Real Time PCR Detection System, using Biorad SYBR green Supermix in a 20 µl reaction volume

Table 1. List of primer sequences used for RT-qPCR analysis.

miRNAs	Primer Sequence 5' to 3'
miR160	SLP: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGCAT
	FP: TTCCTTGCCTGGCTCCCTGT
miR162	SLP: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGATG
	FP: CGCGCCTCGATAAACCTCTG
miR164	SLP: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCACG
	FP: TTCCTTGGAGAAGCAGGGCA
miR167	SLP: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGATC
	FP: TTGCTTGAAGCTGCCAGCAT
miR168	SLP: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCCCG
	FP: TTCCTTCGCTTGGTGCAGGT
miR169	SLP: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCGGCA
	FP: TTGCTCAGCCAAGGATGACT
miR319	SLP: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGGAG
	FP: TCGCGTTGGACTGAAGGGAG
miR393	SLP: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGATCA
	FP: CGGCTTCCAAAGGGATCGCA
miR395	SLP: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAGTTC
	FP: CGGCCATGAAGTGTTTGGGG
miR398	SLP: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGGGG
	FP: CGGCCTGTGTTCTCAGGTCA
miR156	SLP: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC
	FP: CGCGCCTGACAGAAGAGAGT
Universal Reverse primer: GTGCAGGGTCCGAGGT	

containing 0.5µL cDNA, 10 µL 2X SYBR Green mix, 0.5 µl each of 1 µM forward and reverse primer. The reaction conditions were; an initial denaturation step of 95°C/4 min, followed by 40 cycles of 95°C/30 s for denaturation, 58°C/30 s for annealing and 72°C/20 s for extension. Each reaction was run in triplicate. A non-template control was also included in each run. The real time data was normalized by relative quantification method (Livak and Schmittgen 2001), using miRNA 156 as reference gene which was experimentally proved to be stable in *Lablab purpureus* (Thilagavathy and Devaraj 2016).

Target prediction and annotations

Target prediction was performed using online prediction algorithm psRNA Target program (http://plantgrn.noble.org/psRNATarget) against the *Arabidopsis thaliana* Unigene DFCI gene index (AGI) Version 15. The target genes predicted by psRNATarget followed the criteria proposed by Schwab et al., (2005); allowing one mismatch in the region complementary to nucleotide positions 2-12 of the miRNA, and three additional mismatches between positions 12-22, but with no more than two continuous mismatches. To better understand the functions of target genes and their metabolic regulatory network during drought stress in Hyacinth bean, annotation of target function was performed using web-based Gene Ontology searching tools AmiGO (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) and QuickGO (https://www.ebi.ac.uk/QuickGO).

RESULTS AND DISCUSSION

Changes in soil water levels are first perceived by roots, the primary organs in which drought stress responses are initiated. Later, due to alteration in metabolic and photosynthetic processes, major drought stress responses are elicited in leaf tissues. Hence, both in root and leaf tissues, expression of various miRNAs have been found to be altered during drought stress. Although conserved miRNAs have identical sequences and often share common targets, their expression pattern can be species specific, and also, tissue-specific within a given species which became evident in our study, wherein all the 10 miRNAs showed different levels of expression in drought stressed root and leaf tissues (Figure 1). Interestingly, two miRNAs; miR167 and miR393 showed contrasting expression pattern in root and leaf tissues, suggesting their functional divergence within the same species.

Identification of miRNA target transcripts is necessary to obtain an insight into the biological role of miRNA in drought stress adaptation. The presence of near-perfect complementarities between miRNA and their target

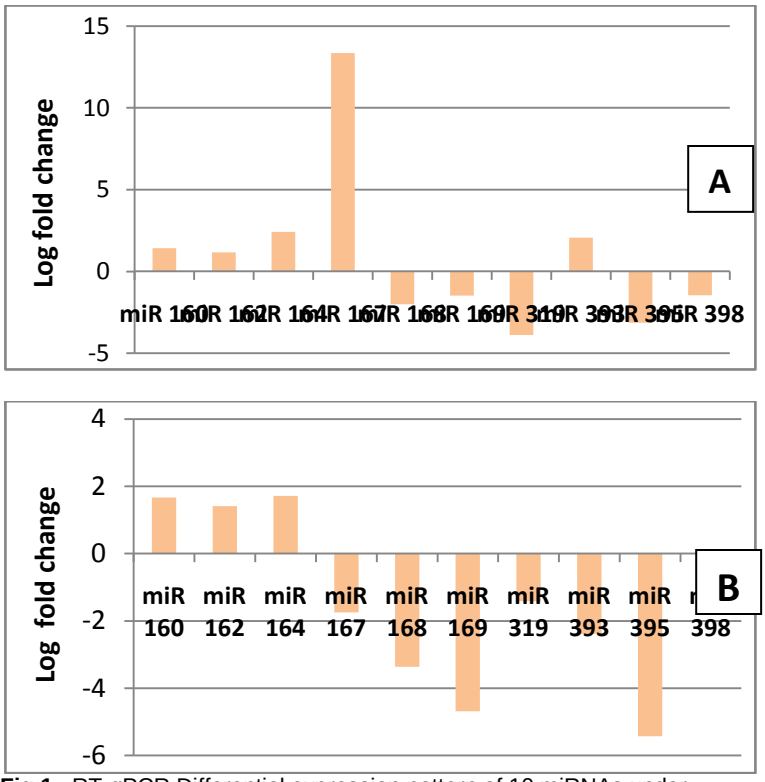

Fig 1. RT-qPCR Differential expression pattern of 10 miRNAs under drought stress in root (A) and leaf (B) samples of Hyacinth bean.

transcripts has been successfully exploited in plants for computational analysis. The details of target transcripts with significant functions, Gene Ontology (GO) term and target inhibition mode for down-regulated and up-regulated miRNAs in both root and leaf tissues are provided in table 2 and 3, respectively. Table 4 shows the details for miRNA up-regulated in roots, but down-regulated in leaf tissues of Hyacinth bean.

Gene ontology analysis showed that targets of characterized miRNAs encode an array of gene products with wide-range of biological functions. Of the predicted targets, transcription factors (TFs) that regulate gene expression accounted for 34%. As transcription factors are major regulators of gene expression under abiotic stress, by targeting genes coding for TFs, miRNAs seem to act as super regulators. Enzymes involved in metabolic processes accounted for 23%, indicating significant metabolic changes during stress response. Components of stress response mechanisms and auxin activated signaling pathway accounted for 14% each of the targets predicted for miRNAs, suggesting role of miRNAs in regulation of stress response and developmental events of the plant system. Interestingly, 4% of the predicted targets are known to play a role in biogenesis of miRNA and their functioning, indicating the auto-regulation of miRNA expression. Membrane transporters, membrane components, components of chromatin remodeling complexes and molecules involved in translation formed 4%, 3%, 2%, and 2% of the target genes, respectively, as shown in Fig. 2. From these results, it appears that miRNAs in hyacinth bean influence drought response primarily via TFs, metabolic pathways and auxin mediated signaling pathways. This observation is in concurrence with the reports of drought induced miRNA as regulators in rice which had the TFs as the major targets (Li et al., 2010).

Drought stress tolerance in plants is established by physiological and metabolic changes that are controlled by changes in gene expression, which is further known to be regulated by the action of miRNAs. This necessitates regulation of miRNA expression during drought. Two miRNAs characterized in hyacinth bean were predicted to target genes central to miRNA biogenesis and function; while miR162 targets DCL1, miR168 targets AGO1. DCL1 functions in the miRNA biogenesis pathway by cleaving primary miRNAs (pri-miRNAs) and precursor miRNAs (pre-miRNAs) to form mature miRNA. AGO1, the catalytic subunit of the RNA-Induced Silencing Complex (RISC) is responsible for post-transcriptional gene silencing. Expression of AGO1 is controlled through a feedback loop involving the miR168, and this is shown to ensure the maintenance of AGO1 homeostasis that is pivotal for the correct functioning of the miRNA pathway (Vaucheret et al. 2004). The fact that DCL1 and AGO1 are also miRNA

Table 2. Potential target genes of down-regulated miRNAs predicted through psRNA target

miRNA	Target gene	Functional process (Gene Ontology)	Target inhibition
miR168	AGO 1 (Argonaute 1)	Gene silencing by miRNA (GO:0035195)	Cleavage
	SUVR 4- Histone-lysine N-methyl transferase	Histone –lysine N- methyl transferase activity (GO:0018024)	Cleavage
	Glutathione transferase	Defense response (GO:0006952) Glutathione metabolic process (GO:0006749) Oxidation reduction process (GO:0055114)	Cleavage
	Glycosyl transferase family 8 protein-like	Transferase activity (GO:0016757)	Cleavage
	Allantoate amidohydrolase	Purine nucleobase catabolic process (GO:0006145)	Cleavage
	hyuC-like protein	Cellular amino acid biosynthetic process (GO:0008652)	Cleavage
miR169	CCAAT-binding transcription factor (CBF-B/NF-YA) family protein;	Transcription factor activity (GO:0003700)	Cleavage
	Nuclear transcription factor Y subunit A-3	Transcription factor activity (GO:0003700)	Cleavage
	Nuclear transcription factor Y subunit A-1	Transcription factor activity (GO:0003700)	Cleavage
	Nuclear transcription factor Y subunit A-2	Transcription factor activity (GO:0003700)	Cleavage
	Nuclear transcription factor Y subunit A-9	Transcription factor activity (GO:0003700)	Cleavage
	Nuclear transcription factor Y subunit A-10	Transcription factor activity (GO:0003700)	Cleavage
	lectin protein kinase family protein	Protein phosphorylation (GO: 0006468)	Cleavage
miR319	Ankyrin like protein	Integral component of membrane (GO:0016021)	Translation
	R2R3-MYB transcription factor	Transcription factor activity (GO:0003700)	Cleavage
	MYB transcription factor	Transcription factor activity (GO:0003700) Response to Abscisic acid (GO:0009737)	Cleavage
	Aldehyde dehydrogenase 22A1 precursor	Oxidation reduction process (GO:0055114)	Cleavage
	Betaine aldehyde dehydrogenase	Oxidation reduction process (GO:0055114) Response to Abscisic acid (GO:0009737) Response to water deprivation (GO:0009414)	Cleavage
	Teosinte branched1 - like protein	Transcription factor activity (GO:0003700)	Cleavage
miR395	Dehydration-responsive element binding protein 6	Transcription factor activity (GO:0003700)	Translation
	Glycoside hydrolase	Carbohydrate metabolic process (GO:0005975) Beta mannosidase activity (GO: 0004567)	Cleavage
	ATP sulfurylase	Hydrogen sulfide biosynthetic process (GO:0070814)	Cleavage
	Amino acid permease	Amino acid transmembrane transporter activity (GO:0015171)	Cleavage
	Cellulose synthase catalytic subunit-like protein	Cellulose biosynthetic process (GO:0030244) Plant-type cell wall biogenesis (GO: 0009832)	Cleavage
	cellulose synthase isolog	Cellulose biosynthetic process (GO:0030244) Plant-type cell wall biogenesis (GO: 0009832)	Cleavage
	U2 auxiliary factor small subunit	mRNA splicing via spliceosome (GO:0000398)	Cleavage
miR398	CSD(Cu-Zn Superoxide Dismutase)	Cellular response to oxidative stress (GO:0034599) Removal of superoxide radicals (GO:0019430)	Cleavage
	n-acetylglucosaminyl-phosphatidylinositol-like protein	GPI anchor biosynthetic process (GO:0006506)	Cleavage
	Endoglucanase 16 precursor	Cellulose catabolic process (GO:0030245)	Cleavage
	30S ribosomal protein S10-like	Translation (GO:0006412)	Cleavage
	Protochlorophyllide oxidoreductase	Oxidation reduction process (GO:0055114) Chlorophyll biosynthetic process (GO:0015995) Respone to ethylene (GO:0009723)	Translation
	Non-specific lipid-transfer protein 1 precursor	Lipid transport (GO:0006869)	Cleavage

Table 3. Potential target genes of up- regulated miRNAs predicted through psRNA target.

miRNA	Target gene	Functional process (Gene Ontology)	Target inhibition
miR162	Cytochrome P450-like protein	Electron transport chain (GO:0022900)	Cleavage
	Receptor kinase-like protein	Cellular Response To Abscisic Acid Stimulus (GO:0071215) Cellular response to water deprivation (GO:0042631)	Cleavage
	Endoribonuclease Dicer homolog	Production of miRNAs (GO: 0035196)	Cleavage
	Transcription factor E2Ff	Transcription factor activity (GO:0003700)	Cleavage
	Hydrolase-like protein	Hydrolase activity (GO:0016787)	Translation
miR160	Putative auxin response factor	Transcription factor activity (GO:0003700) Auxin activated signaling pathway (GO:0009734)	Cleavage
	Auxin response factor 10	Transcription factor activity (GO:0003700) Auxin activated signaling pathway (GO:0009734) Root cap development (GO:0048829)	Cleavage
	Auxin response factor 16	Transcription factor activity (GO:0003700) Auxin activated signaling pathway (GO:0009734) Root cap development (GO:0048829)	Cleavage
	Auxin response factor 17	Transcription factor activity (GO:0003700) Auxin activated signaling pathway (GO:0009734)	Cleavage
miR164	Cup-shaped cotyledon 1	Transcription factor activity (GO:0003700) Lateral root development (GO:0048527) Negative regulation of cell division (GO:0051782)	Cleavage
	CUC 2	Transcription factor activity (GO:0003700) Leaf development (GO:0048366)	Cleavage
	NAM (no apical meristem)-like protein	Transcription factor activity (GO:0003700)	Cleavage
	NAC domain-containing protein 21/22	Transcription factor activity (GO:0003700) Auxin activated signaling pathway (GO:0009734)	Cleavage
	NAC5 protein	Transcription factor activity (GO:0003700)	Cleavage
	DEAD-box ATP-dependent RNA helicase 13	Helicase activity (GO:0004386)	Cleavage

targets suggests that plant miRNAs play a role in tuning their own biogenesis and turnover. Up-regulation of miR162 and down-regulation miR168 in hyacinth bean suggested the existence of such a feedback system as a possible mechanism to regulate the levels of miRNAs, which in turn regulates various processes during drought tolerance.

Considering the pattern of expression of miRNAs, and their predicted targets, a possible regulatory cascade is proposed for hyacinth bean roots (Figure 3) and leaves (Figure 4). Plant growth and development is one of the vital processes that are regulated by miRNAs during drought. Growth is accomplished by cell division, cell elongation and differentiation. These cellular processes are directly affected by the reduced cellular turgor pressure caused by water deficit. Since roots are the tissues through which plants first encounter drought stress, they respond to drought by reduced root growth and development. Apart from this, phytohormone auxin plays a central role in plant growth signaling pathway, which is predominantly regulated by miRNAs. miR393, predicted to target mRNAs encoding TIR1 and GRR1 is closely related F-box proteins, which target auxin repressors AUX/IAA proteins for proteolysis and promote auxin mediated growth processes. As, miR160 and miR167 are the most important regulators of root growth and development, up-regulation of these miRNAs could lead to down regulation of ARF10, ARF16 and ARF17 targeted by miR160, ARF6 and ARF8 targeted by miR167, resulting in reduced auxin signaling and reduced root growth. Apart from inhibiting auxin signaling and reduced root growth via miR160 and miR167, lateral root growth was also negatively affected by down regulation of NAC TFs under the control of up regulated miR164. Inhibition of lateral growth development regulated by NAC TFs targeted by miR164 was also reported in rice (Fang et al., 2014) Over expressed miR164 is also known to negatively affect leaf growth and development via CUC2 TF as has been reported for *Arabidopsis thaliana* (Raman et al., 2008).

ABRE (Abscisic acid Responsive Element) and DRE

Table 4. Potential target genes predicted through psRNA target for miRNAs up- regulated in root and down regulated in leaf tissues.

miRNA	Target gene	Functional process (Gene Ontology)	Target inhibition
miR167	Glycine-rich protein precursor	Defense response (GO:0006952)	Cleavage
	Cysteine-rich repeat secretory protein 57 precursor	Anchored component of membrane (GO:0031225)	Cleavage
	Auxin responsive transcription factor	Transcription factor activity (GO:0003700) Auxin activated signaling pathway (GO:0009734)	Cleavage
	Chloroplast chaperonin 10	Protein folding (GO:0006457) Positive regulation of superoxide dismutase activity (GO:1901671)	Translation
	Extracellular calcium sensing receptor	Regulation of stomatal closure (GO: 009033) Cellular response to calcium (GO: 0071277)	Translation
	Auxin response factor 2	Transcription factor activity (GO:0003700) Auxin activated signaling pathway (GO:0009734)	Cleavage
	Auxin response factor 8	Transcription factor activity (GO:0003700) Auxin activated signaling pathway (GO:0009734)	Cleavage
miR393	GRR1-like protein	Auxin activated signaling pathway (GO:0009734)	Cleavage
	TIR1 (Transport Inhibitor Response 1)	Auxin activated signaling pathway (GO:0009734) Protein ubiquitination (GO:0016567) Lateral root formation (GO:0010311)	Cleavage
	Probable WRKY transcription factor 33	Transcription factor activity (GO:0003700) Response to osmotic stress (GO:0006970) Response to water deprivation (GO:0009414)	Cleavage
	Mn-specific cation diffusion facilitator transporter	Cation transmembrane transporter activity (GO:0008324)	Cleavage
	Myrosinase-binding protein homolog	Carbohydrate binding (GO:0030246)	Cleavage
	1-D-deoxyxylulose 5-phosphate synthase-like protein	Metabolic process (GO:0008152)	Cleavage
	CPL3 (C-Terminal Domain Phosphatase-Like 3	CTD phosphatase activity (GO:0008420)	Cleavage

(Dehydration Responsive Element) which function in ABA-dependent and ABA-independent gene expression, respectively, are two major *cis*-acting elements under osmotic stress responses in most of the stress-inducible genes (Zhu 2002; Yamaguchi-Shinozaki and Shinozaki 2005). ABA-independent pathway is controlled by miR395 that is predicted to target DREB TFs which bind to DRE. Although DREB-mediated drought response is not fully elucidated yet, enhanced drought tolerance through DREB mediated pathways is considered to involve transcriptional activation of genes such as LEA proteins which are thought to protect macromolecules, such as; enzymes, lipids, from dehydration, and dehydrins that help in maintaining cell volume, thus preventing cellular collapse. Some dehydrins which contain relatively large amounts of Hydrogen ions and other reactive amino acid residues on their surface, also exhibit reactive oxygen species (ROS) scavenging and metal ion binding properties (Hanin et al. 2011). Thus, down-regulation of miR395 in hyacinth bean root and leaf tissues can lead to drought tolerance by increased expression of DREB TFs.

Khraiwesh and others have reported that ABA-dependent stress responses is regulated by two miRNAs miR319 and miR169. miRNA 319 that is predicted to target MYB TFs and miR169, predicted to target NFYA TFs are down-regulated in roots and leaves of hyacinth bean. Down-regulation of miR319 leads to increased accumulation of MYB TFs, one of the major classes of TFs in plants that are known to enhance drought tolerance through regulation of stomatal movement, ROS detoxification, and maintenance of osmotic balance. Similarly, down-regulated miR169 under drought stress may contribute to the induction of the *NFYA* TFs (Khraiwesh et al. 2012).

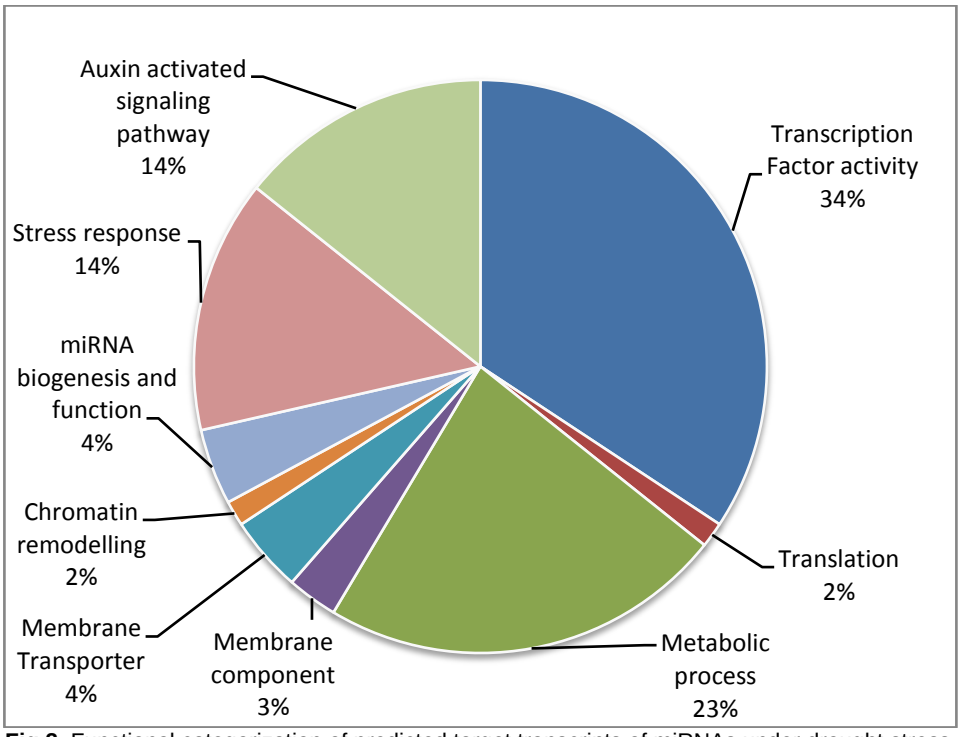

Fig 2. Functional categorization of predicted target transcripts of miRNAs under drought stress in Hyacinth bean.

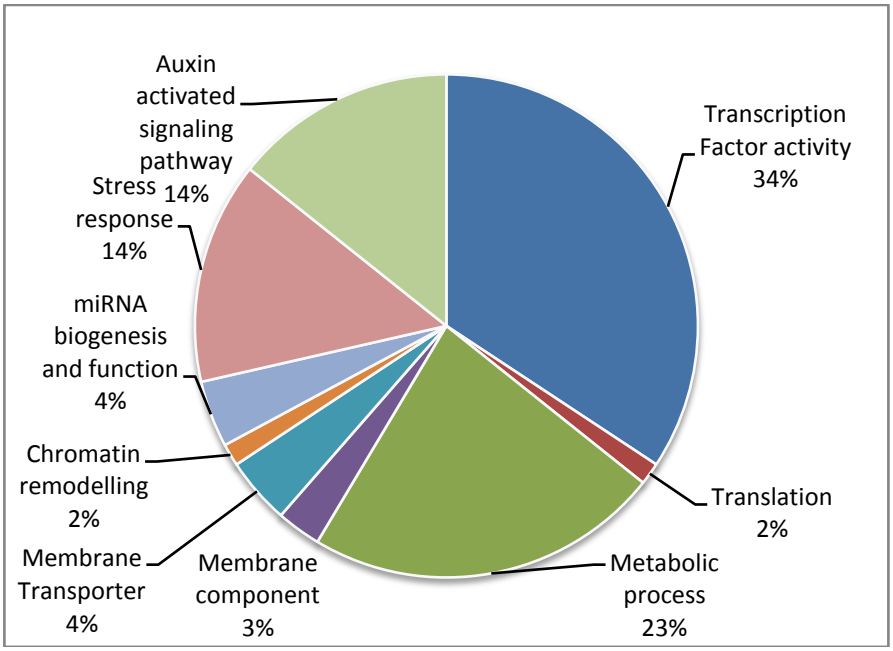

Fig 2. Functional categorization of predicted target transcripts of miRNAs under drought stress in Hyacinth bean.

NFYA expressed in guard cells is known to control stomatal aperture, while *NFYA* expressed in other cells are crucial for the expression of a number of drought stress-responsive genes, such as glutathione transferase or peroxidase based on microarray analysis (Li et al. 2008). Thus, elevated levels of MYB and NFYA due to down-regulation of miR319 and miR169 respectively, is expected to reduce water loss in hyacinth bean.

Besides regulating the level of TFs involved in activation

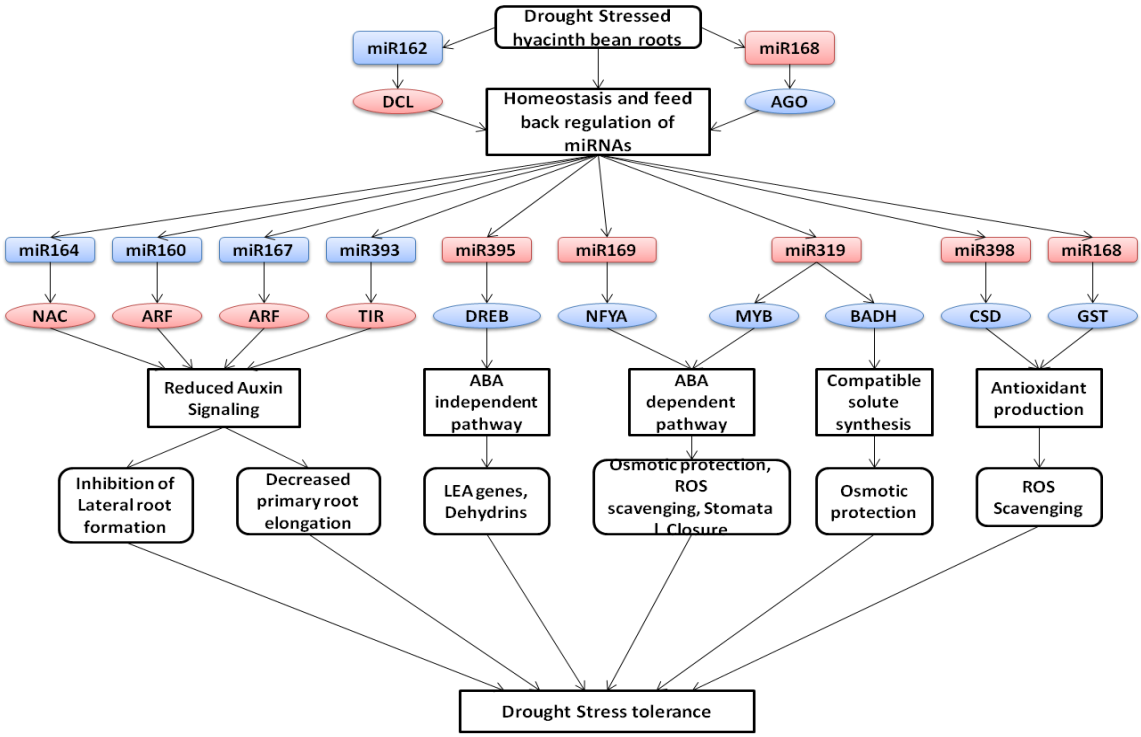

Fig 3 Potential regulatory network of drought-responsive miRNAs drawn based on predicted targets, and their roles in Hyacinth bean roots. miRNAs and targets in red indicates down regulation and blue indicates up regulation.

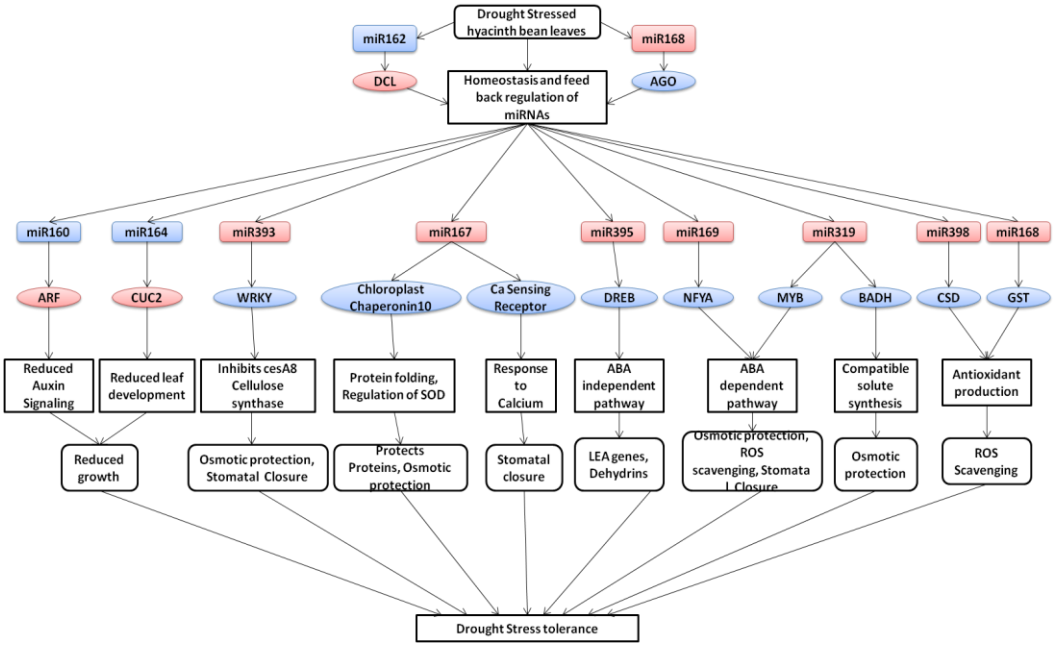

Fig. 4. Potential regulatory network of drought-responsive miRNAs drawn based on predicted targets, and their roles in Hyacinth bean leaves. miRNAs and targets in red indicates down regulation and blue indicates up regulation.

of drought responsive genes, miRNAs can directly control the levels of antioxidants and osmoprotectants. Transcript encoding antioxidant enzyme, Cu-Zn Superoxide dismutase (CSD) that scavenges superoxide radicals produced during stress is the potential target for miR398 (Sunkar et al. 2006). Down-regulation of miR398 thus can contribute to robust antioxidant system involving antioxidant enzyme CSD in

hyacinth bean. Another antioxidant enzyme Glutathione transferase (GST) mRNA was predicted to be the target of miR168. Plant GSTs conjugate Glutathione (GSH) with endogenous products of oxidative damage initiated by hydroxyl radicals and lead to their detoxification. Betaine aldehyde dehydrogenase, an enzyme involved in the synthesis of the osmoprotectant Glycine betaine is predicted to be the target of down-regulated miR319 in hyacinth bean. In hyacinth bean miR 398, miR 168 and miR 319 are down regulated in root and leaf tissues under drought, thus can lead to increased accumulation of CSD, GST's, and glycine betaine to ensure removal of ROS and maintain osmotic balance, substantiating the biochemical responses of hyacinth bean during drought (D'Souza & Devaraj 2011).

Two miRNAs; miR167 and 393 showed contrasting expression profile in root and leaf tissues of hyacinth bean. While they were up-regulated in roots probably causing decreased root growth by regulating auxin signaling, they were down-regulated in leaves indicating different functions in leaf by controlling different targets (Fig 4). Chloroplast chaperonin 10 that aids in protein folding and Calcium sensing receptors that control stomatal movement are also the predicted targets of miR 167. Therefore, down-regulation of miR 167 in leaves can lead to increased accumulation of Chloroplast chaperonin 10 and Calcium sensing receptors, necessary for drought tolerance. Down-regulated miR 393, is predicted to target WRKY33 TFs, known to inhibit CesA8 (Cellulose synthase sub unit), which may enhance drought and osmotic tolerance in hyacinth bean as shown in *Arabidopsis thaliana,* wherein mutation in the cellulose synthase gene led to the accumulation of soluble sugars to a higher level in cells, and subsequently increased drought and osmotic tolerance. (Chen et al. 2005).

To conclude, tissue-specific expression of miRNAs characterized from leaves and root tissue of hyacinth bean, and wide range of targets predicted indicated that drought stress responses mechanisms and their regulation in hyacinth is highly complex. The complexity is also exemplified by contrasting expression pattern of miR167 and miR393, indicating the possibility of diverse targets for the same miRNA in different tissues of same plant. These finding provide insights to the miRNA controlled post-transcriptional regulation during drought stress responses, which can be potentially exploited in development of new drought tolerant crops.

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