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miR166h Directed Cleavage of Target Upon PGPR Inoculation Under Drought Stress and Tissue-Specific Expression Analysis Under Abiotic Stresses in Chickpea.

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Abstract

Legumes are an indispensable food after cereals with extensive production across the world. The legume production is imposed with limitations and has been augmented by various environmental stresses. The symbiotic relations between legumes and rhizobacteria have been an intriguing topic of research in view of their roles in plant growth, development and various stress responses. Recent advances on gene networks involving plethora of evolutionarily conserved miRNAs have been investigated pertaining to their roles in plant stress responses. The interaction between plant growth promoting rhizobacteria (PGPR) strain *Pseudomonas putida* RA, MTCC5279 and abiotic stress responsive miRNAs have previously been studied with roles in abiotic stress mitigation by modulating stress responsive miRNAs and their target genes. The present studyis an investigation involving the role of RA in abiotic stress responsive miR166h for drought mitigation in tolerant *desi* chickpea genotype. miRNA166 directed cleavage of its target, *ATHB15* has been drifted of drought treated plantlets upon RA inoculation using 5´RLM-RACE analysis. Drought stressed chickpea plants when inoculated with growth promoting rhizobacteria, RA, the inverse correlation in expression patterns were noticed in miR166h and its validated target, *ATHB15*. Tissue-specific expression patterns in 15 days old chickpea seedlings including leaves, shoot and roots when exposed to salinity, drought and abscisic acid at different time points indicated the role of miR166 in different abiotic stress response. In view of the results, validation and functional characterization of such interactions involving stress responsive miRNAs along with microbial stress management techniques could be an important technique for crop improvement.

Key Message

The present study includes target validation of miR166hin *desi* chickpea using 5´RLM-RACE analysis as well as expression analysis of miR166h and validated target under different abiotic stresses.

1. Introduction

Legumes are important crops known for their nitrogen supplementation in soil as well as nitrogen-fixing capacity in association with soil bacteria. Chickpea (*Cicer arietinum* L.) is one of the food legumes and an imperative diet portion rich in proteins with essential amino acids. It lines second amongst all leguminous crops and is grown in dry/semi-dry regions of tropics as a rain fed crop (Jatan et al. 2019; Shah et al. 2020). It is cultivated with a coverage of 13718980 ha with a total production of 14246295 tonnes along with productivity of 10384 hg/ha globally **(FAOSTAT, 2019).** The foremost producer of chickpeais India with a total production of 9937990 tonnes and productivity of 10410 hg/ha from an area 9547030 ha **(FAOSTAT, 2019).** However, global climate change including drought, temperature stress and excessive moisture has imposed restrictions on its annual production. These climatic factors affect the crop yield globally (Devasirvatham and Tan 2018**).** Among major abiotic stresses, drought is most detrimental for plants **(Khan et al. 2018).** Drought stress accounts for approximately 7-51% yield loss in chickpea worldwide (Khodadadi 2013; Hajyzadeh et al. 2015; Jatan et al. 2019). Drought stress hampers the overall physiology of crops and hence leads to severe multi-dimensional effects. However, crop plants

respond to drought stress by implementing certain morphological, physiological, biochemical and molecular mechanisms. These mechanisms lead to gene reprogramming and altered expression of various stress modulating pathways including various genes, transcription factors (TFs), metabolites and small non-coding regulatory miRNAs resulting in drought stress mitigation in crop plants (Lata et al. 2015; Tiwari et al. 2017; Jatan et al. 2019).

Small RNAs including microRNAs work with stress adaptive machinery in plants involving suppression of various genes and target mRNAs involved in stress response mechanisms (Sunkar et al. 2012). miRNAs are 20-24nt small RNAs with their high complementarity binding to the target messenger RNAs (mRNAs),thus modulating gene expression events and in turn regulating plant development and abiotic stress responses (Trindade et al. 2011; Sunkar et al. 2012; Millar 2020). miRNAs undergo gene silencing and bind to their specific targets on the basis of structural complementary. This silencing of genes could be either repression at translational levels or cleaving them(Voinnet 2009; Sunkar et al. 2012). Along with their roles in plant development at cellular, biological and molecular levels, the miRNAs have also been functionally analysed and critically investigated for their roles in environmental stress signalling (Zhang 2015; Xia et al. 2018). Various crop plants have been studied for the variation in miRNAs expression patterns during extreme water deficit and drought stress **(Liu et al. 2017**, Guo et al. 2017, Jatan et al. 2019, Millar 2020**).**

Evolutionarily conserved and known miRNAs including miR166 have been identified via deep-sequencing in chickpea (Kohli et al. 2014; Srivastava et al. 2015; Jatan et al. 2019). miR166 has both developmental and stress responsive roles along with its target HD-ZIP III, the leucine zipper family protein that exhibits inverse expression patterns with miR166. The developmental roles functionally controlled by miR166 in correlation with HD-ZIP III family genes involve shoot apical meristem development (SAM), shoot vasculature development (Mandel et al. 2016), root (Singh et al. 2017), seed (Li et al. 2017), and organ polarity (Husbands et al., 2009) along with nutrient uptake (Iwamoto and Tagiri 2016) and vegetative dormancy (Kitazumi et al. 2015) in various plants. HD-ZIP III proteins are conserved in nature and are induced when drought and salinity stress are imposed on plants (Chen et al. 2014). The homeobox-leucine zipper protein-15 (*ATHB15*) placed under HD-ZIP III class has been identified in various plants with roles like procambium and xylem and vascular differentiation, plastid development and secondary cell wall development (**Ohashi and Fukuda 2003;** Ochando et al. 2006; Du and Wang 2015**).** *ATHB15* also known as *CORONA* (*CAN*) has drought responsive roles with indications of regulatory control of miR166 on the target expression validated in plants like *Triticum dicoccoides, Sorghum bicolor, M. truncatula, Camellia sinensis* and chickpea (Jia et al. 2009; Du and Wang 2015; Li et al. 2017; Ding et al. 2018; Jatan et al. 2019). The target mapping by miR166 directed cleavage of ATHB15 has only been reported in *Arabidopsis* until now (Kim et al. 2005). Hence, the present study is an attempt to delineate *ATHB15* as the target of car-miR166 in chickpea using 5´RLM-RACE.

Further, PGPR's are investigated to have roles in host plant growth promotion by attuning morpho-physiological, biochemical and molecular responses, increasing availability of soil micronutrients and production of phytohormones. Plants inoculated with PGPR have shown to be better survivors under

drought stress in arid and semi-arid regions (Tiwari et al. 2016; **Khan et al. 2018). ***Pseudomonas putida*, a PGPR strain MTCC5279 (RA) has been reported to have miRNA-mediated drought stress mitigating roles revealed by a genome-wide analysis of stress responsive miRNAs upon inoculation with or without RA (Jatan et al. 2019). The study also highlighted the role of PGPR in modulating the expression of conserved miR166h and its potential target *ATHB15.*Hence, mapping of selected target cleavage by miR166h in chickpea through 5´RLM-RACE would provide better analysis of its role in chickpea and its relationship with its cognate target *ATHB15.* The tissue-specific expression patterns of both miR166 and *ATHB15* would also help to better understand the mechanism of action which is yet not fully deciphered.

2. Materials And Methods

2.1 Plant material, stress treatment and library construction

The tolerant *desi* chickpea cultivar BG-362 was selected for the experiment. The seeds were initially washed with RO water and further with 0.1% (w/v) mercuric chloride solution for surface sterilization and after washing three to four times with sterile Milli-Q water, the seeds were washed with 70% ethanol and repeatedly washed with sterile Milli-Q and soaked overnight. The overnight water imbibed seeds were placed in Petridishes on wet autoclaved Whatman No. 1 filter paper and incubated in growth chamber for 3-4 days. The germinated seeds were transferred to Hewitt media containing hydroponic trays under standard growth conditions for two weeks. After two weeks the experiment was designed for library construction involving 1% RA suspension inoculation in two trays for 24h and the rest two trays were kept uninoculated. The bacterial culture of RA available in our institute was grown with conditions at 250 rpm and 28°C. After 24h, the uninoculated and inoculated seedlings were subjected to drought stress using 20% (w/v) polyethylene glycol (PEG) 6000. The untreated non-stressed condition was maintained for control. The treated and untreated plants were harvested at 0, 72 and 168h time intervals. The time intervals for physiological and biochemical studies involve 0, 24, 72 and 168h. Hence, four set of plants included control, drought, drought+RA and RA. All the tissues were harvested in three independent biological replicates and were immediately in liquid nitrogen and preserved carefully after labelling in −80°C for further experimental analysis.

2.2 Morphological analysis

All four set of plants were evaluated for various morphological parameters in terms of drought stress response evaluation. The number of biological replicates of plants used for experimental analysis at each time interval was five. The samples were wiped carefully after removing from hydroponics medium and different parameters were recorded thereafter. After the fresh weight analysis, the samples were kept in blotting sheets and were dried in hot air oven at 60°C for 4 days and then dry weight was recorded. The morphological parameters were studied and noted as described elsewhere (Tiwari et al. 2016).

2.3 Electrolyte Leakage (EL)

The electrolyte leakage (EL) of treated and untreated roots of chickpea plants was measured according to method explained by **Lata et al (2011) and** Tiwari et al (2016) with minor modifications. About 100 mg of fresh root samples were taken from all sets and further incubated in 20 ml sterile deionized Milli-Q water in 50 ml sterile Falcon tubes for 40 mins at 120rpm at room temperature. After 1h, the initial electrolyte conductivity (E1) was measured using Orion 5star conductivity meter (Thermo Scientific, USA). The tubes were further kept in boiling water with temperature 90°C in a water bath for 40 min and after cooling for some time, the final electrolyte conductivity (E2) was recorded. EL was calculated as by the formula mentioned in previous reports **(**Tiwari et al. 2016).

2.4 Relative water content (RWC)

For the assessment of relative water content in both control and treated samples, the leaf samples were taken in triplicate **(Lata et al. 2011;** Tiwari et al. 2016). The leaf samples of similar size were collected and their fresh weight (FW) was recorded immediately. Afterwards, these samples were soaked in 30 ml sterile Milli-Q water for 4h in Petridish after which their turgid weight (TW) was recorded. These samples were then kept for drying in hot air oven at 60°C for 48h after which their dry weight (DW) was recorded. The readings were then subjected to calculation of RWC using formula: RWC% = (FW-DW)/(TW-DW)*100 **(**Barrs and Weatherley 1962**).**

2.5 Lipid peroxidation assay

The modified protocol of Heath and Packer (1968**)** was used for estimation of lipid peroxidation. The aldehyde product, malondialdehyde (MDA) was measured using 2-thiobarbituric acid (TBA) reaction. 0.1% (w/v) TCA solution was prepared from which 500 µl was used for homogenization of ~100 mg leaf tissue samples from all sample sets in triplicates. The mixture was centrifuged at 13,000 g at 4°C for 10 min. The 500 µl supernatant was mixed with 1.5 ml of 0.5% TBA and incubated for 25 min at 95°C. The reaction was inhibited after 5 min incubation on ice and the absorbance was measured at 532 nm and 600 nm in a microplate reader.

2.6 Proline estimation assay

The amino acid proline estimation was measured using the protocol of **Carillo and Gibbon (2011).** 1ml of 70% ethanol was used to homogenize ~100 mg of leaves. 50 µl ethanolic extract was mixed with reaction mixture prepared by mixing 1% w/v ninhydrin in 60% (v/ v) acetic acid and 20% (v/v) ethanol. The reaction was incubated at 95°C for 20 min followed by 5 min on ice and then absorbance was recorded in microplate recorder at 520 nm.

2.7 Total RNA isolation for control and treated samples

Total RNA was isolated using mirPremier® microRNA Isolation Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. The root samples were taken for quantitative real-time PCR analysis of all four sample sets for miR166h and its target *ATHB15*expression analysis. For tissue-specific expression analysis, total RNA was isolated from different tissues as well as root, shoot and leaves with 15 days old seedlings harvested at time intervals 0 h, 1 h, 24 h and 72 h with different abiotic stress treatments like

20% polyethylene glycol (PEG) (6000), 100mM NaCl and 10μM ABA. Purity and quantity of isolated RNA samples was checked using Nano-Drop (Nanodrop 1000, Thermo Scientific, USA) and 1.2% formaldehyde-agarose gel electrophoresis. Turbo DNA-free™ kit (Ambion, Life Technology) was then used for DNase treatment of 5 μg RNA.

2.8 cDNA synthesis of miR166h and *ATHB15*

The purified RNA samples were used for cDNA preparation using Taqman® MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's protocol. Stem-loop reverse transcription primer of miR166h and mature miR166h forward primer were designed according to the previous study (Jatanet al. 2019). Universal reverse primer was designed as described **(Kramer et al. 2011).** The target cDNA was prepared using Verso cDNA synthesis kit (Thermo Scientific, USA) according to the manufacturer’s protocol. The information regarding target gene prediction and its functions was gathered as per Jatan et al (2019).

2.9 Stem-loop quantitative real-time PCR (SL-qRT) for car-miR166 expression analysis

The SL-qRT-PCR analysis was performed to validate the expression levels of selected conserved car-miR166h in control and treated samples. Real-time PCR was done using Applied Biosystems^{TM} SYBR^{TM} Green PCR Master mix on a 7500 fast real-time PCR machine (Applied Biosystems, USA). Real time primers were designed as described by Jatan et al (2019). *U6* snRNA was used as an internal control gene for miR166h expression data normalization **(Jain et al. 2014).** The relative expression of the miR166h in different treated samples vs. control was calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

2.10 qRT-PCR expression analysis of predicted target *ATHB15*

To analyse the relative expression of respective target gene *ATHB15*in control and treated samples, chickpea glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as a reference gene for transcript normalization (Garg et al. 2010; Jatan et al. 2019). qRT-PCR analysis was performed on 7500 fast real-time PCR machine (Applied Biosystems, USA). The relative expression of the target genes was determined using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

2.11 Phylogenetic analysis of target

The target was subjected to evolutionary phylogenetic analysis in different leguminous crops in comparison to chickpea using Molecular Evolutionary Genetics Analysis (MEGA X) (Kumar et al. 2018). The sequences were retrieved from NCBI using BLAST and were aligned further using CLUSTAL W and sequentially the data was analysed by neighbour-joining tree method using bootstrap test.

2.12 Phylogenetic analysis of pre-miR166h

Sequences of miR166 precursors (pre-miR166h) of different crops plants were collected from NCBI using BLAST application and then aligned using Clustal W. Subsequently, phylogenetic tree was constructed with the bootstrap value calculated with 1000 replicates using maximum likelihood method on MEGA-X.

2.13 Promoter analysis of mir166

For prediction of transcription start site (TSS) in miR166 gene, TSSP software from Softberry(http://linux1.softberry.com/berry.phtml?topic=tssp&group=programs&subgroup=promoter)was used.A1500 bps upstream of precursor sequence was selected for promoter analysis and the *cis* regulatory motifs were identified using Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) as described by Li et al (2017).

2.14 Detection of miR166 cleaved target ATHB15 mRNA by 5´RLM RACE

For the validation of predicted target of miR166h, modified 5´ RACE was performed. For this validation, the FirstChoice RLM-RACE Kit (Ambion, USA) was used with slight modifications. 1 µg of total RNA from RA inoculated drought treated root samples were isolated, checked for purity and quantity and further subjected to adapter ligation. This was followed by cDNA synthesis in which ligated product was used as a template. Specific gene reverse primers were designed for 5' RLM-RACE and checked by OligoEvaluator™ (Sigma) **(Supplementary Table 1).** PCR reactions for cDNA amplifications using primer combinations with PCR cycling conditions set according to manufacturer's instructions were performed. The annealing temperature was optimized and the single PCR fragments were cloned into the pGEM-T Easy Vector (Promega, USA) and sequenced to identify the 5´end of the amplified target gene.

2.15 Relative expression analysis of miR166 in different chickpea tissues

cDNA for miR166h, *U6* and target were synthesised from different tissue samples of chickpea and further studied for relative expression analysis. Stem-loop primers for miR166h and U6 were used for reverse transcription. For transcript normalization, *GAPDH* was used (Garg et al. 2010). Expression analysis of miR166h and its target was performed in triplicates. The relative expression of the miR166h and target gene in different samples was calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

2.16 Tissue-specific expression analysis in abiotic stresses

The leaves, shoot and root samples were harvested from control and different abiotic stress treatments including drought, salinity and ABA treatments according to the manufacturer's instructions. The relative

expression of the miR166h and target gene in different treated samples compared to the control was calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

2.17 Statistical analysis

Relative expression data for miR166 and its target gene, *ATHB15* from three independent biological replicates were calculated as the mean with standard error (mean ± SEM). One-way analysis of variance (ANOVA) using Duncan's multiple range tests (DMRT) was used for significant differences in variance between average values of control and treated plants with the analysis of significant difference between the means ($p < 0.05$). Standard deviation (SD) values were calculated using the mean of the replicates.

3. Results

The expression patterns of miR166h and *ATHB15* were studied in the present study upon RA inoculation in chickpea tolerant cultivar subjected to drought stress. Control samples with standard conditions and RA inoculated and uninoculated samples with drought stress treatment were brought under study where differential expression patterns were observed. This miRNA and its target have been found to have inverse correlation expression patterns in chickpea. When comparing all four libraries as explained by **(Jatan et al. 2018;** Jatan et al. 2019) and we also researched that RA in relation to miR166h has roles in drought tolerance and response. Transcripts with ≥ 1.0 were considered as upregulated and that of ≤ −1.0 fold-change values as down-regulated.

3.1 Effect of drought stress on morphological parameters with and without RA- inoculation

To determine the effect of RA on drought stress treated plants, the morphological study was done. The RA-inoculated samples showed better growth with and without drought stress **(Figure. 1a and 1b).** The primary root length was found to be increased by 42% and 50% in drought treated RA-inoculated plants as compared to drought treated uninoculated plants at late time intervals (72h and 168h) respectively. The shoot length was also comparatively higher with 14% increase in drought treated RA inoculated plants at 168h in comparison to drought treated control plants. The fresh weight and dry weight also indicated towards stress adaptive efficacy. Huge differences were observed in the numbers of lateral branches with almost (~3 fold) increase in number of lateral branches in RA-inoculated drought stressed plants after 168h of stress treatment. Significant difference in lateral roots number was observed in drought induced RA inoculated plants at 168h with 66% increase as compared to drought treated plants indicating the role of RA in drought stress endurance in chickpea.

3.2 Effect of drought stress on physiological parameters with or without RA inoculation

Electrolyte leakage was progressively increased in drought treated plants as compared to control whereas RA inoculated drought stressed plants showed comparative decline in EL at 72h (50%) and 168h (80%). The RA inoculated plants showed better results as compared to drought at 72h (72%) and 168h (75%) (Fig. 2a). The RWC was progressively higher with increase in time to about 63% and 115% at 72 and 168h in drought treated RA inoculated samples respectively as compared to drought treated samples. The RA inoculated plants had relatively higher water content (69%) at 72h as compared to drought (Fig. 2b).

3.3 Effect of drought stress on biochemical aspects with or without RA inoculation

The MDA content was highest in 72h of drought treated plants and decreased with increase in stress exposure with lowest MDA content recorded in RA inoculated non stressed plants at 168h (0.04 nmols MDA mg^{-1} FW). The lipid peroxidation levels were less in RA inoculated drought treated plants at all time intervals involving 75% and 54% at 72h and 168h time intervals as compared to drought treated plants at the same time points (Fig. 2c). Further accumulation of compatible osmolyte, proline showed significant increase with progression of drought stress whereas significant decrease in its content was observed in RA inoculated plants in comparison to stress treatment. The RA inoculated drought treated plants showed comparatively less proline content as compared to drought treated plants by 20% (72h) and 40% (168h) (Fig. 2d).

3.4 Expression analysis of miR166h and *ATHB15* under drought stress in different samples

The expression analysis of chickpea root samples for miR166h and *ATHB15* in all four sample sets at two time points of drought stress (72h and 168h) was studied. The quantitative expression analysis indicated inverse correlation between the selected miRNA and its target. MiR166 was highly upregulated under drought with (~2.8 fold expression) at 7h and 168h with its target was found to be downregulated (-1.3 fold). In case of drought stressed RA-inoculated plants, the fold change in expression levels of miR166h was found to be increased as compared to drought stress at 72h (3.3fold) and 168h (3.2 fold) and the target was downregulated by -1.6 and -1.5 fold, respectively at respective stress durations. Further, RA inoculated non stressed plants resulted in downregulation of miR166h with upregulation of its target. MiR166h expression levels were found to be -1.1 and -1.5 fold whereas *ATHB15* showed an upregulation with 1.9 and 1.6 fold change in expression at 72h and 168 h, respectively (Fig. 3). Hence, these quantitative expression levels indicate the role of miR166 in RA-mediated mechanism of mitigating drought stress.

3.5 Phylogenetic status of *ATHB15* and pre-miR166h

We examined the phylogenetic status of miR166 target *ATHB15* of chickpea with other leguminous plant species. A simple nucleotide BLAST of *ATHB15* in NCBI was performed that identified sequence similarity ranging from 77 to 100% and query coverage of 81 to 100 %. Phylogenetic analysis revealed that the target *ATHB15* of miR166in chickpea was closely related to *ATHB15*of *Medicago truncatula*(Fig. 4a)

regulating vascular development and cambium formation (Li et al. 2020). Whereas, reduces the formation of lateral roots and nodulation when suppressed by its miRNA (Boualem et al. 2008). This target has not yet been characterized in many crop plants and hence need more interest. Similarly pre-miR166h was directed for phylogenetic relationship using same procedure. The sequence similarity was ranging from 36 to 100% and query coverage of 97 to 100 %. MiR166h of chickpea is most similar in *Hevea brasiliensis* with 98% query cover (Fig. 4b). Kuruvilla et al. (2016) also studied the roles of pre-miR166 in *Hevea brasiliensis*and hence more plants should be studied for roles in pre-miR166 in different plants.

3.6 Target validation for conserved car-miR166h

For experimental validation of regulatory target of car-miR166h was done using modified 5´end rapid amplification of cDNA ends (5' RLM-RACE) approach. Based on the sequencing results of mRNA cleavage of predicted target gene, *ATHB15*cleavage site was found opposite to the 10th position from the 5'end of the miRNA. Based on the predicted target for the conserved miR166h,11 RACE experiments were done using root samples of RA inoculated drought stress treated chickpea. The results presented in Fig. 5 indicate that our prediction approach correctly identified target *ATHB15* for conserved miR166h in chickpea.

3.7 Analysis of cis-regulatory motifs in promoters of miR166

The promoter analysis was carried out for better understanding of miR166 regulation in chickpea in terms of abiotic stress response. To identify and study predicted putative *cis*-regulatory elements localised in the promoter region that was located according to the analysis based on the assumed position of transcription start site (TSS) was done using *in silico* approach. The observed promoter elements included light response, hormone response, plant development, metabolism, defence and stress responses and others. The different cis-regulatory elements analysed in promoter of pre-miR166h in chickpea with their putative positions have been annotated **(Table.1).** The *cis*-regulatory elements were not distributed uniformly in miR166 promoters. The light-responsive elements were most abundantly found in the promoters. Preferably five types of stress-responsive elements were observed namely, ABRE (ACGTG, the abscisic acid (ABA) response element), CGTCA-motif (methyl–jasmonate–responsive *cis*-regulatory element), TC-rich repeats (GTTTTCTTAC, defence and stress responsive *cis*-regulatory) elements and TCA-elements (CCATCTTTTT, salicylic acid responsive *cis*-regulatory elements) (Fig. 6**).** For better understanding of the roles of these *cis*-regulatory motifs, quantitative real-time PCR analysis of different tissues harvested from drought, salinity and ABA treatments was performed.

3.8 Tissue-specific expression of miR166h and ATHB15

The relatively highest expression levels of miR166h was observed in pods (1.9 fold) followed by flowers (1.8 fold), whereas the expression of *ATHB15* was found to be highest in mature shoot (1.7 fold) followed

by immature leaf (1.2 fold) (Fig. 7a). Further expression analysis of miR166 and its target was done in different tissues harvested after drought, salinity and ABA stress treatments. Expression patterns in different tissues and different stresses suggested inverse correlation between miR166h and its target. On exposure to drought stress, the expression of miR166h in leaf tissue was found to be downregulated under control conditions as well as during 20% PEG6000 treatment with -0.5 and -1.3 fold relative expression levels at 24h and 72h of stress exposure, respectively. The drought stress treated shoot at 24h showed 2.6 fold whereas at 72h the expression levels were declined to basal levels with 1.2 fold change. Further, root tissue analysis revealed high upregulation on drought exposure with 2.4 (1h), 6.4 (24h) and 6.6 fold expression at 72h of stress. In accordance to miR166h expression, *ATHB15* showed decline in expression in leaf tissue at 24h and 72h with 1.6 and 2.0 fold expression, respectively while drought treated shoots showed -0.3 fold-change expression at other time intervals. Relative expression levels were found to be comparatively declined (-3.6 and -4.4 fold) in roots at later time points (Fig. 7b).

The expression analysis of conserved miR166h and *ATHB15* was also studied under100mM NaCl treatment that revealed differential expression patterns at different time intervals. *ATHB15* was found to be increasingly upregulated by the increase in time interval with 2.1-foldat 24h and 1.9 fold at 72h fold expression in control along with significant upregulation (3.4 and 5.3 fold) values when treated samples were subjected to relative fold expression study. The inverse expression patterns of miR166h were observed with that of *ATHB15*that showed highly downregulated expression with -0.4, -1.7 and -2.4 fold expression at 1h, 24h and at 72h. The salinity stress treated shoot samples showed downregulation of miR166h with significant fold change (-1.6 and -1.8) at 24h and 72h, respectively whereas *ATHB15* showed highest expression at 72h with 2.8 fold expression. The target gene showed upregulation (0.6, 1.7 and 5.7 fold) in roots with increasing stress duration (1h, 24h and 72h). The miR166h showed a significant decline (0.8, -1.3 and -3.2 fold) with increasing time intervals indicating the important roles of miR166h in salinity endurance (Fig. 7c).

Variations in expression patterns were also observed upon treatment with 10μM ABA observed. MiR166h exhibited upregulation (2.1 fold) at 1 h in leaves but showed a decline (-3.2 fold) at 24 h and again an increase at 72h (2.9 fold) whereas *ATHB15*showed an upregulation (1.6 fold) at 1h but downregulation (-1.0 and -3.0 fold) at 24h and 72h, respectively. ABA treated shoots showed high upregulation of miR166h only at 24h (4.7fold) and no significant expression at 1h and 72h whereas basal expression levels were noted in *ATHB15* upon ABA stress treatment at all time intervals. Roots treated with ABA showed highly downregulated expression (-4.0 and -3.5 fold) of *ATHB15* at 24h and 72h, respectively with gradual increase in regulation in control samples whereas the miR166h expression was higher at 1h but declined with increase in time interval resulting in expression levels as 4.8 fold at 1h, 3.2 fold at 24h and 2.3 fold at 72h of stress (Fig. 7d).

4. Discussion

With the discovery of plethora of miRNAs in response to drought stress has led to initiation of in-depth research to decipher their mechanism of action **(Ding et al. 2013;** Shuai et al. 2013; Zhang 2015; Shriram

et al. 2016; Jatan et al. 2019). Drought stress is the most common and detrimental environmental stress factor that reduces crop production and yield throughout the world. Drought stress adaptation of crop plants can be achieved through breeding, genetic engineering or gene editing for enhanced agricultural productivity. However, microbial techniques involving microbes for improving stress tolerance of crop plants is gaining importance due to the constraints of labour, cost, time and ethical issues (Nautiyal et al. 2013). The present study discusses about the positive modulatory aspects of RA as well as miR166h-mediated response to drought stress in *desi* chickpea genotypein stress alleviation. Plants subjected to drought stress perceive the simulations primarily through roots. Hence, root growth is one of the major indicators of stress management. Longer root system helps the plants under stress to extract soil water and nutrients from deep soil, thus helping to sustain better growth and survival under drought stress conditions (Lopes et al. 2011; Tiwari et al. 2016). RA-inoculation increased shoot length under drought conditions as compared to the drought but the difference was not significant. Increase in shoot length upon PGPR inoculation under drought stress has been reported for improved stress tolerance by Bresson et al (2013) whereas no significant differences were observed by Tiwari et al (2016). The increase in fresh weight and dry weight of RA-inoculated plants and drought stressed plants with RA inoculation as compared to drought stress uninoculated plants could be correlated with the increase in the number of shoot lateral branches, internodes with the increase in the primary root length and number of lateral roots at different time points. Several previous studies account such results (Yang et al. 2009; Grover et al. 2014; Tiwari et al. 2016).

EL and RWC are imperative topics in terms of abiotic stress tolerance used as biochemical stress markers. RWC is the indicator of the balance between absorbed water and consumed water during transpiration process followed in plants under stress **(Lata et al. 2011;** Kang et al. 2014; Tiwari et al. 2016). The RWC of RA-inoculated drought stress plants was higher as compared to drought stress uninoculated plants suggesting the beneficial roles of RA in improving relative water levels that help plant cope water stress in confirmation with previous studies (Kang et al. 2014; Tiwari et al. 2016). The cell membrane integrity in plants could be assessed by estimating electrolyte leakage (Cha-um et al. 2013; Ullah et al. 2018). A comparative reduction in EL upon RA inoculation in drought stressed plants suggested the role of RA in maintaining cell membrane integrity in chickpea in accordance with previous studies (Kang et al. 2014; Tiwari et al. 2016). Further, analysis of recovered plants from drought stress at 24, 72 and 168 h suggested that degree of severity of stress increases with the time interval indicating severe drought during 168h. Further, it was observed that the degree of stress severity increased with drought stress progression with maximum affect at 168 h of stress confirming the results of previous study on chickpea (Tiwari et al. 2016).

Lipid peroxidation in plants result in the production of one of the aldehyde products known as malonaldehyde (MDA) which is often used as a biomarker for oxidative stress estimation **(Lata et al. 2011).** Our findings are in accordance to earlier studies where MDA levels indicated the membrane integrity functions in drought treated chickpea seedlings (Bhushan et al. 2007; Tiwari et al. 2016). Membrane damage could overcome to acceptable levels due to low MDA accumulation in RA inoculated as compared to uninoculated plants. Proline being a compatible solute maintains osmotic turgor and

help plants to overcome drought stress (Grover et al. 2014). Manifold accumulation in proline content has been reported with increasing drought stress (Lata et al. 2015). The proline levels highly increased during drought stress uninoculated conditions whereas significant reduction in osmolyte levels under RA inoculation with subsequent restoration of proline content in drought treated RA inoculated plants at all intervals. The role of RA as explained by Tiwari et al. (2016), could be due to possibly induced systemic response (IST) on chickpea leading to enhanced soil moisture content for improved nutrient uptake, biofilm formation and root exudations for enhanced root growth thereby improving plant health status under drought stress condition.

A recent study on miRNA profiling using high throughput sequencing in chickpea led to the discovery of RA-responsive miRNAs involved in regulating drought stress response by modulating the expression of stress responsive genes (Jatan et al. 2019). The same study reported that miRNAs expression patterns were modulated in the presence of RAin *desi* chickpea genotype. It was observed that RA inoculation modulated the expression of miR166h in response to drought stress resulting in the upregulation of miR166h in comparison to drought uninoculated plants. The study reported a downregulation of *ATHB15*which belongs to the family of HD-ZIP III TFs andthe target of miR166h in RA inoculated drought stress treated plants indicating the crucial role of RA in the adaptation and restoration of cellular homeostasis under drought stress by the regulation of miR166h and its target expression resulting in enhanced growth and development of plants (Jatan et al. 2019).

In this study, the phylogenetic analyses of pre-miR166 and *ATHB15* have been performed to understand their evolutionarily conserved nature. *ATHB15* has been previously characterized to a lesser extent as the target of miR166 in *Zinnia* (**Ohashi and Fukuda 2003)** and *Arabidopsis* (Kim et al. 2005). Further, RLM-RACE is used for analysing miRNA-guided sequence-specific mRNA target endo-nucleolytic cleavage (Llave et al. 2002; Donaire et al. 2011). This procedure has been successfully utilized for validation of miRNA targets (Llave et al. 2002; Jones-Rhoades and Bartel 2004). In this study, the RLM-RACE confirmed that *ATHB15* is indeed the target of miR166hin *desi* chickpea genotype. The validated target mRNA transcript showed perfect cleavage site that mapped at the 10th from the 5'-end of the binding site for miR166hjustifying the previous report in *A.thaliana*(Kim et al. 2005). The earlier study reported the role of *ATHB15* in vascular development while no such validation has been reported till date suggesting *ATHB15* to be the mRNA target of miR166 in chickpea under drought stress or PGPR inoculation.

The *cis*-regulatory ACGT-containing ABREs have been functionally demonstrated in ABA-modulated transcription roles in various stress responsive genes subjected to ABA and water stress in maize by Pla et al. (1993). It has been demonstrated that bZIP proteins bind with more affinity to these elements and further regulate ABA signalling and abiotic stress responses (Liao et al. 2008). Such bZIP proteins bind to the *cis*-elements in the promoter region and modulate the stress responses by either directly binding or indirectly by activating various other stress responsive genes. Liao et al. (2008) reported negative regulatory ABA signalling involving ACGT elements leading to salinity and freezing tolerance in transgenic *Arabidopsis.* Promoter analysis of *Oryza sativa* lectin receptor-like kinases (*LecRLKs*) indicated the involvement of various *cis*-regulatory genes including TC-rich repeats under biotic stress

(Passricha et al. 2017). *ZmMYB30* of *Zea mays* is also enriched with TC-rich repeats in its promoter and has roles in terms of salinity stress (Luo et al. 2020). *PDI* gene family in *Solanum lycopersicum*also has TC-rich repeats in promoter pointing their role towards the abiotic stress mitigation (Wai et al. 2021). The ESTs under salinity stress in *Artemesia annua* were also studied for the presence of *cis*-regulatory elements and showed the role of TCA-elements in salicylic acid response and stress modulation **(Alam and Balawi 2020).**

Tissue-specific expression analysis is a useful tool to examine the transcriptional networks involving miRNAs and their targets in various plant tissues **(Celik and Akdas 2019).** miR166 has been studied in different plants for its relative expression and abundance in different plant tissues. In this study, contrasting expression patterns are noted between miR166 and its target gene in *desi* chickpea genotype upon different abiotic stress treatments including salinity, drought and ABA on different plant tissues. NaCl treated leaves showed significant downregulation of miR166h with gradual increase in the expression of its target as discussed in previous reports on maize, chickpea and guava (Ding et al. 2009; Kohli et al. 2014; Sharma et al. 2020). The tissue-specific expression in shoot followed the same expression patterns of miR166h and its target as observed in leaves. The drought treated root samples showed high upregulation at 72h of salinity stress for the target whereas miR166h exhibited increasingly declined expression (Xie et al. 2014; **Jatan et al. 2018).**The inverse expression patterns of miR166h with its target were also studied in drought treated leaf samples in various crops (Akdogan et al. 2016; Hamza et al. 2016).The differential expression was observed in previous reports in drought treated shoot with upregulation of miR166h at 24h but down regulation or basal expression at 72h while*ATHB15*showed basal expression at 1h and was found to be downregulated with stress progression in wheat (Akdogan et al. 2016). On the other hand, drought treated root samples showed higher fold expressions of miR166h as compared to its target which showed decline in expression at later time points of stress treatment in confirmation to previous reports (Trindade et al. 2011; Kantar et al. 2011; Gentile et al. 2015; Jatan et al. 2019).Phytohormonal treatment resulted in variation in expression patterns of both miR166h and *ATHB15* where ABA stressed leaf showed miR166h upregulation at 1h and 72h and basal expression at 24h while *ATHB15* was found to be highly downregulated at 72h. The ABA treated shoots however, drew no such significant expression relations for both miR166h and *ATHB15*except high expression levels in shoot samples at 24h for miR166h and basal expression of *ATHB15*in response to ABA. The stressed root tissues showed miR166h upregulation at 1h but gradual decline with increasing time interval while the target was also downregulated at 24h and 72h. Similar observation was reported earlier in *Arabidopsis thaliana* (Singh et al. 2017). However, no significant difference in expression patterns of miR166h and its target was observed upon ABA treatment in *Arabidopsis* and rice **(Liu et al. 2009).**

Conclusion And Future Perspectives

The tripartite plant-soil-microbe interaction is becoming an important area of research for crop improvement in terms of abiotic stress including drought. *P. putida* RA has been found to be beneficial in ameliorating drought stress in *desi* chickpea cultivar. Drought stress negatively regulated the crop by affecting the growth and development, including root length, shoot length, number of lateral roots,

branches and internodes, increased EL and MDA content with reduced RWC, enhanced compatible solutes. While RA played an important role in positively modulating the stress response with improved plant growth, membrane integrity, water status with restored antioxidants and osmolytes. At molecular levels the regulation of various stress responsive genes with inverse correlation to miRNAs have also been found to be modulated by RA. A working hypothesis for the mechanism of RA-mediated drought stress mitigation in *desi* chickpea cultivar based on the plant responses to RA from this study and literature survey in different crops has been illustrated (Fig. 8).

miR166 known to be an important drought stress-responsive miRNA targeting the HD-ZIP III TF family. In our study, miR166h was found to have positive roles under drought stress. In RA-inoculated plants, both miR166h and *ATHB15* showed inverse expression patterns suggesting the role of RA in drought stress alleviation and in growth promotion in RA inoculated plants. Based on our results as well as previous literature, we hypothesised the mechanism of RA and miR166h-mediated regulation of drought stress by targeting *ATHB15* for enhanced drought endurance in chickpea (Fig. 9). RLM-RACE confirmed that *ATHB15* is the target of miR166h in chickpea. This is the first report on the validation of*ATHB15* as the target of miR166hin chickpea upon PGPR inoculation under drought stress. The inverse correlation between miR166h and its target *ATHB15* in different tissues of chickpea under different abiotic stresses indicated the crucial role of miR166h in stress mitigation by expression modulation of its target.

Hence, this study highlighted the beneficial roles of RA-inoculation in the modulation of miR166h and its target gene expression in drought stress mitigation and its utilization in various crop improvement strategies. Further,differential expression and post-transcriptional gene regulation by miR166h under different abiotic stresses could be an interesting topic of research in different crop plants for augmentation of growth, yield and other desirable agronomic attributes under stress conditions. Various recently emerged techniques like CRISPR-Cas9 genome editing and miPEPscan be effectively employed to further investigate the biological functions of miR166 and can be successfully exploited for the improvement programmes of various crop plants.

Declarations

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Author contribution statement

CL conceived and designed the research. CL and IS provided facilities and funding for the research. AY conducted the experiments and wrote the manuscript. AY, SK and RV analysed the data. CL, SPR and IS

critically reviewed the manuscript. All authors read and approved the manuscript. Authors also pay their gratitude to Dr. Puneet S. Chauhan for providing the *Pseudomonas putida* bacterial strain (RA).

Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table. 1 Cis-acting elements of *Cicer arietinum* pre-miR166h promoter from PlantCare database

cis-element	Position	Strand	Organism	Sequence	Function
ABRE	1344 1358 1484	- - -	*Arabidopsis thaliana*	ACGTG	*cis*-acting element involved in the abscisic acid responsiveness
ARE	1136	+	*Zea mays*	AAACCA	*cis*-acting regulatory element essential for the anaerobic induction
ATCT-motif	495	-	*Pisum sativum*	AATCTAATCC	part of a conserved DNA module involved in light responsiveness
AuxRR-core	1161	-	*Nicotiana tabacum*	GGTCCAT	*cis*-acting regulatory element involved in auxin responsiveness
Box 4	1217	-	*Petroselinum crispum*	ATTAAT	part of a conserved DNA module involved in light responsiveness
CAT-box	748	-	*Arabidopsis thaliana*	GCCACT	*cis*-acting regulatory element related to meristem expression
CCAAT-box	1473	+	*Hordeum vulgare*	CAACGG	MYBHv1 binding site
CGTCA-motif	132	-	*Hordeum vulgare*	CGTCA	*cis*-acting regulatory element involved in the MeJA-responsiveness
G-Box	1344	+	*Pisum sativum*	CACGTT	*cis*-acting regulatory element involved in light responsiveness
G-Box	1358 1484	- -	*Arabidopsis thaliana*	TACGTG TACGTG	*cis*-acting regulatory element involved in light responsiveness
GA-motif	170	-	*Arabidopsis thaliana*	ATAGATAA	part of a light responsive element
GATA-motif	1119	-	*Arabidopsis thaliana*	AAGATAAGATT	part of a light responsive element
GT1-motif	583	-	*Arabidopsis thaliana*	GGTTAA	light responsive element
P-box	991	-	*Oryza sativa*	CCTTTTG	gibberellin-responsive element
TC-rich repeats	467 1331	- +	*Nicotiana tabacum*	GTTTTCTTAC GTTTTCTTAC	cis-acting element involved in defence and stress responsiveness

	1239	-		ATTCTCTAAC	
TCA-element	736	+	*Nicotiana tabacum*	CCATCTTTTT	cis-acting element involved in salicylic acid responsiveness
TGACG-motif	132	+	*Hordeum vulgare*	TGACG	cis-acting regulatory element involved in the MeJA-responsiveness
chs-CMA2c	1354	-	*Daucus carota*	ATATACGTGAAGG	part of a light responsive element

Figures

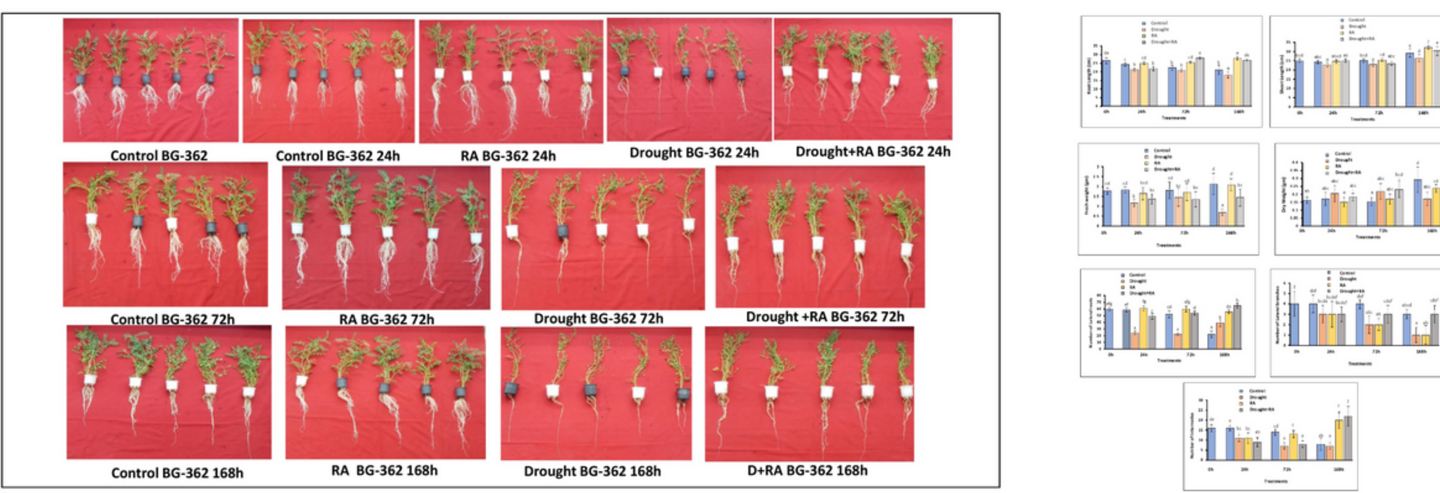


Figure 1

a. Library preparation of chickpea grown in hydroponics including Control, RA, Drought and Drought+RA at different time intervals (0h, 24h, 72h and 168h). b. Different morphological analysis of control and treated chickpea plants at different time intervals with and without RA inoculation. Duncan multiple range test has been used for significant differences from the mean of five biological replicates. The letters C represent control, RA represent the PGPR inoculation in control, D represent drought and D+RA represents drought+PGPR inoculation respectively.

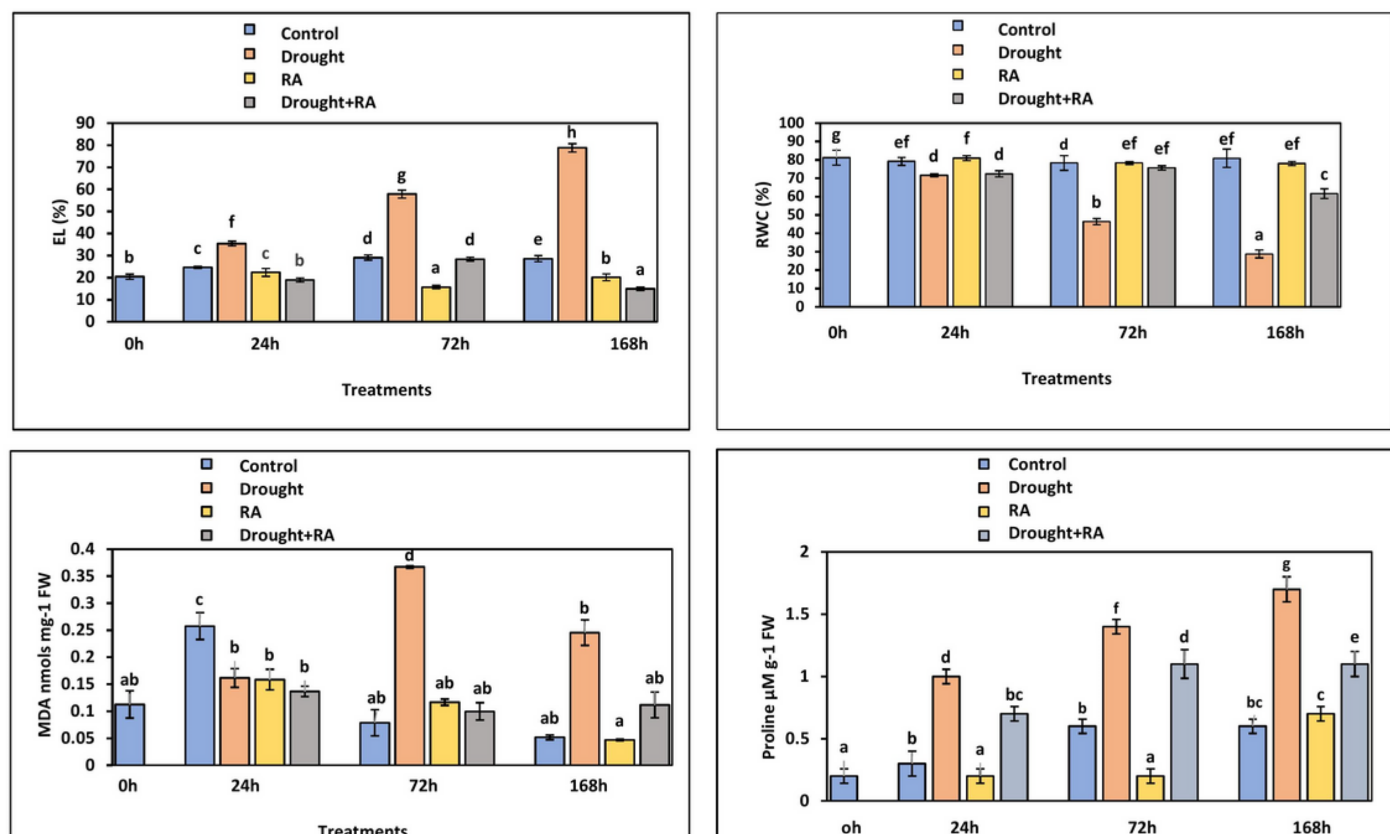
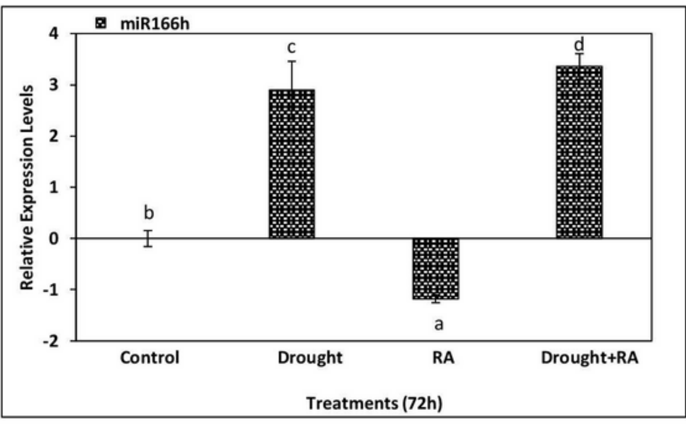
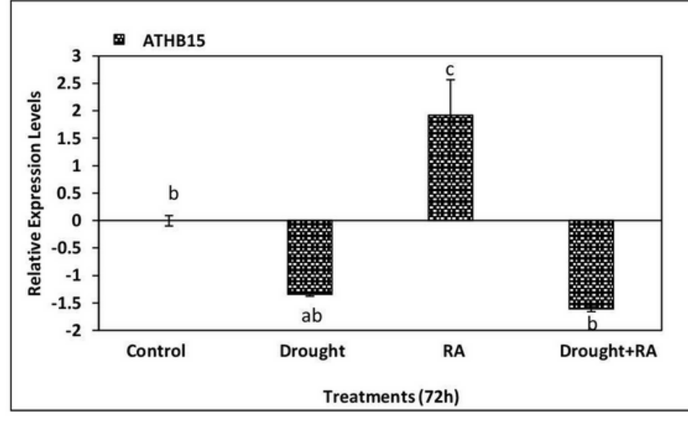
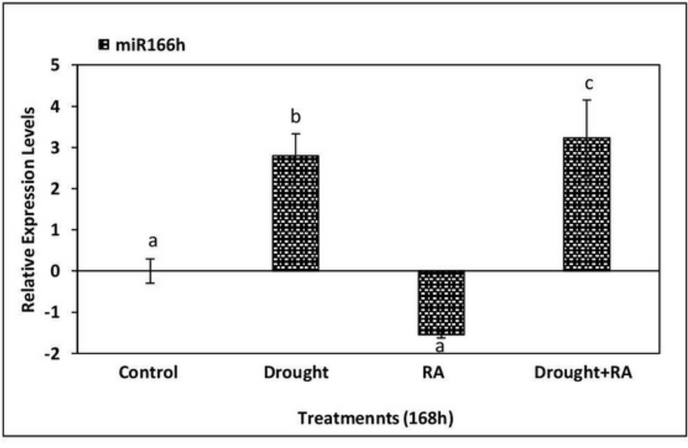


Figure 2

(A). Determination of EL at 0, 24, 72 and 168h with and without RA inoculation. (B).Determination of RWC (%) at 0h, 24h, 72 and 168h with and without RA inoculation. (C). Determination of MDA levels at 0h, 24h, 72 and 168h with and without RA inoculation. (D). Proline estimation in control and drought treated plants with and without RA inoculation. The letters indicate significant differences from three biological replicates mean obtained from Duncan multiple range test.







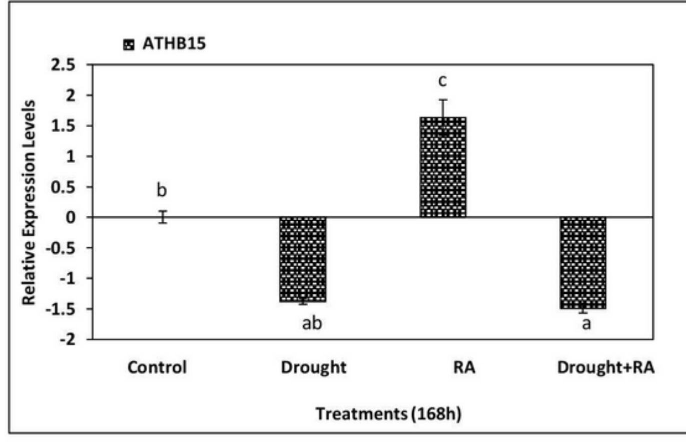
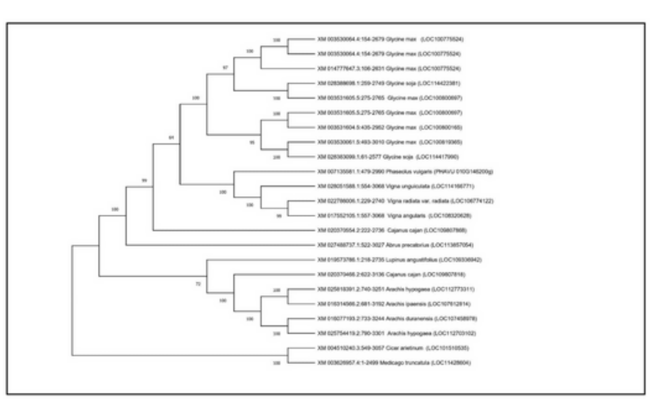


Figure 3

Expression analysis of conserved miR166h and its predicted target ATHB15 by qRT-PCR in chickpea, BG-362 on drought exposure at 72h and 168h in the presence or absence of RA. The letters in the graph indicate towards the significant differences determined by Duncan's multiple range test with p-value ≤ .05.



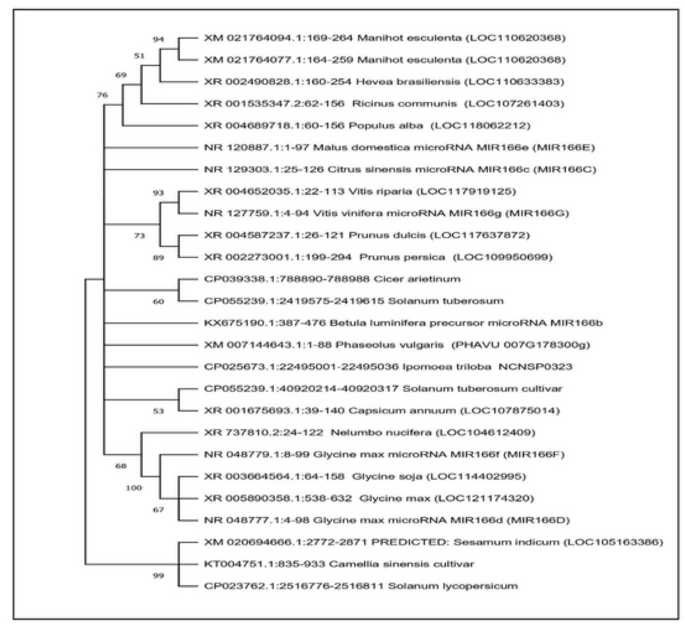


Figure 4

a. Evolutionary relationships of ATHB15: The evolutionary analyses were conducted in MEGA X and the evolutionary history was inferred using the Neighbor-Joining method. The associated taxa were clustered together in the bootstrap test (1000 replicates) with evolutionary distances computed using the p-distance method. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 2536 positions in the final dataset. b. Evolutionary analysis by Maximum Likelihood method of pre-miR166h: The evolutionary analysis was conducted using MEGA X including Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-985.96) is shown. The initial tree(s) for the heuristic search were obtained automatically using Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value.

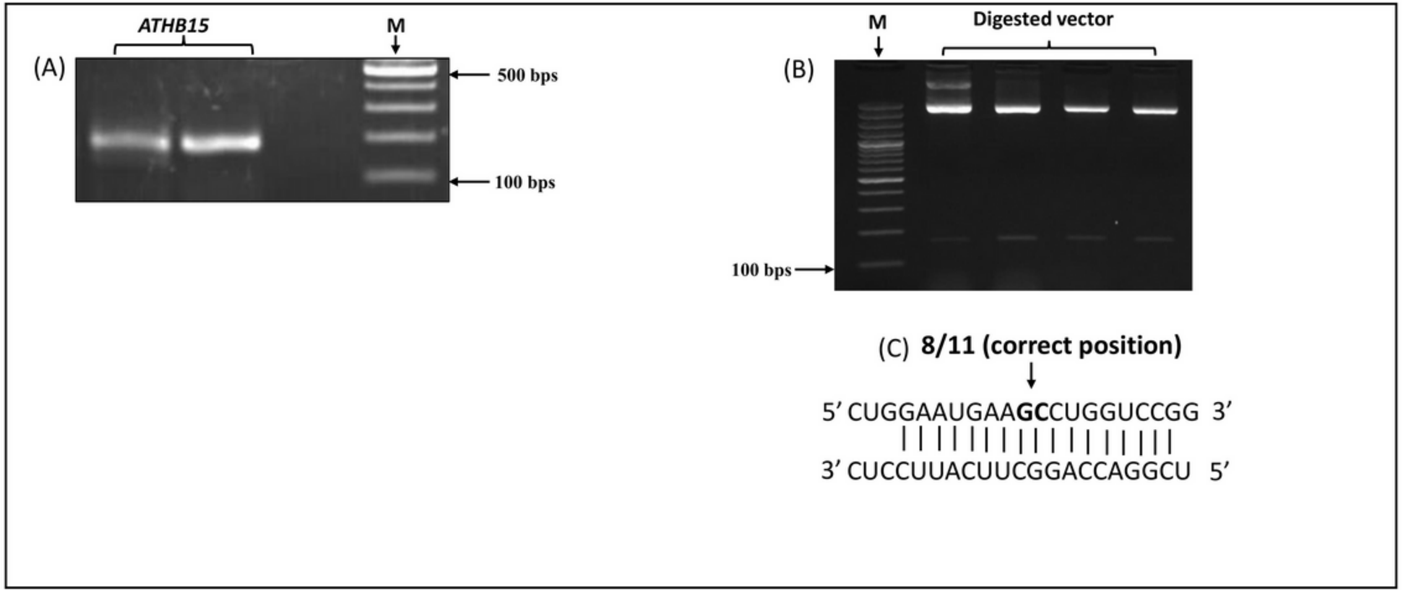


Figure 5

(A) Cleavage product of ATHB15 after nested PCR with inner forward adaptor primer and inner reverse gene specific primer combinations. (B) Digestion of cloned pGEMT vector using EcoRI resulting in cleavage product of desired size. (C) Cleavage of ATHB15 mRNA by miR166h as validated by modified 5’RLM-RACE is shown. The fraction of clones obtained with results were 8 out of 11 sequenced clones and cleavage was at expected 5’end and is also indicated in the figure.

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>PlantCARE_pre-miR166 (Cicer arietinum)
+ TTTTTTTTTG TAAAATAAGT TTACATGAAC TGAATTATCA TAGTCCATTT TGTTCACATA TGTAGTAAAT
- AAAAAAAAAC ATTTTATTCA AATGTACTTG ACTTAATAGT ATCAGGTAAA ACAAGTGTAT ACATCATTTA

+ TTAATAAACT AAGTTTACGT ATGTTATATG TGAATTAAAA TGTTGTAACT AAAAAGAAAA ATGACGTAAC
- AATTATTTGA TTCAAATGCA TACAATATAC ACTTAATTTT ACAACATTGA TTTTTCTTTT TACTGCATTG

+ CAATAAAATG TTTAATGAAT GGAAAATTCT TATCTATCAG GCATTGAATT CAATTCTTTC AATGTTAAAA
- GTTATTTTAC AAATTACTTA CCTTTTAAGA ATAGATAGTC CGTAACTTAA GTTAAGAAAG TTACAATTTT

+ AGTAAGTTGA GTTTTTTCTT TTTTAGGTAA ATTGAAATTA TTTCCACCAT ATGCATATAT GTGTTGAACT
- TCATTCAACT CAAAAAAGAA AAAATCCATT TAACTTTAAT AAAGGTGGTA TACGTATATA CACAACTTGA

+ CTAAAATTCA ATCCATCAAG TTGTAGTTAA CTGCTGATGA ATTGAATTTT GTGTAACCTG ATAAATTTTT
- GATTTTAAGT TAGGTAGTTC AACATCAATT GACGACTACT TAACTTAAAA CACATTGGAC TATTTAAAAA

+ TATAGGACTA ATGATTTTTT TAGGACTATA TGATAACATA CTATATGGAT TTCAAGTAAA ATTAGTCCAA
- ATATCCTGAT TACTAAAAAA ATCCTGATAT ACTATTGTAT GATATACCTA AAGTTCATTT TAATCAGGTT

+ GATTTGAAAA GTGAAAACGT ATAAGCACAG TTTACAATTA CCTAAGGTAA AAAAACAGGC GTGCAAATGA
- CTAAACTTTT CACTTTTGCA TATTCGTGTC AAATGTTAAT GGATTCCATT TTTTTGTCCG CACGTTTACT

+ AAGAGGATTT GATTTGGATA TAAAAATTTA TAAAACTCTA TTTTTCTGTT TCCCTTCGTT TTCTCTTGTT
- TTCTCCTAAA CTAAACCTAT ATTTTTAAAT ATTTTGAGAT AAAAAGACAA AGGGAAGCAA AAGAGAACAA

+ TTAAGCTTAA CATTATGGTA ACTTAACCAA ACTGATTTTT ACTTGTGATG AAGGGATGCT TCTTGCACAT
- AATTCGAATT GTAATACCAT TGAATTGGTT TGACTAAAAA TGAACACTAC TTCCCTACGA AGAACGTGTA

+ GTCAAACAGA TTTTCAAGTC TGTAACATAA GCCAATTATA TATATTTTTT GATGAAATCC AATTATATAT
- CAGTTTGTCT AAAAGTTCAG ACATTGTATT CGGTTAATAT ATATAAAAAA CTACTTTAGG TTAATATATA

+ TTTTACTTTA GAATCAACGA ACACTCTCAT CAAATGCATC TTTTTAAAGT GGCAAGCAAA AGAGAACTAC
- AAAATGAAAT CTTAGTTGCT TGTGAGAGTA GTTTACGTAG AAAAATTTCA CCGTTCGTTT TCTCTTGATG

+ TTTAAATACA CACTTTAATT CACTATACAA GTCCCTTTGA CAACACCAAT ATCCTATAAG CAATAAACTT
- AAATTTATGT GTGAAATTAA GTGATATGTT CAGGGAAACT GTTGTGGTTA TAGGATATTC GTTATTTGAA

+ CATCGTGTTT TTCTCAACAA TAAAATAAGT ACTAGTACCC CAAAAAAACA AGGAATGAAT TATAACATTG
- GTAGCACAAA AAGAGTTGTT ATTTTATTCA TGATCATGGG GTTTTTTTGT TCCTTACTTA ATATTGTAAC

+ TAGTCTCAAA AAATTTACAC TAAATATATG TTTAATTGCC CAAATCAAAA TGTTTCTCAC CAATTCATGA
- ATCAGAGTTT TTTAAATGTG ATTTATATAC AAATTAACGG GTTTAGTTTT ACAAAGAGTG GTTAAGTACT

+ AGCAATTAGG CAAAAGGGAA TAAGCAATGG CGTTTGTGTT TGGAATTGTG GCCATGTTTG GTAGACTTAA
- TCGTTAATCC GTTTTCCCTT ATTCGTTACC GCAAACACAA ACCTTAACAC CGGTACAAAC CATCTGAATT

+ AGGAAGCTTT GTAAGTCTTT TTAATTTATG CTACAAAAAG GCTTTTACTT TTGCATCACT AATGCACCAA
- TCCTTCGAAA CATTCAGAAA AATTAAATAC GATGTTTTTC CGAAAATGAA AACGTAGTGA TTACGTGGTT

+ TCTTTTCTTG ATTTGAAACC ATTTGCCATG AATGAGGCAT ATGGACCCTA TTTACACTAT GAATTGTCTT
- AGAAAAGAAC TAAACTTTGG TAAACGGTAC TTACTCCGTA TACCTGGGAT AAATGTGATA CTTAACAGAA

+ TCCTTTTCAT TTTTCCAAAG ACCATAATTA ATTTGTGTTC AGTTTGAGTT TAGAGAATTA ATTAACAATA
- AGGAAAAGTA AAAAGGTTTC TGGTATTAAT TAAACACAAG TCAAACTCAA ATCTCTTAAT TAATTGTTAT

+ CTAAAATTTG TTATCACAAG TTAATAATTA TACTAATTAA TGGGTGACAT AGGCACTTGG AAAATTGTAC
- GATTTTAAAC AATAGTGTTC AATTATTAAT ATGATTAATT ACCCACTGTA TCCGTGAACC TTTTAACATG

+ GTTTTCCTAC ACACACGTTC CCACCTTCAC GTATATAAGG GATAATTAGG GCCCATAAGA AAAACATTGC
- CAAAAGGATG TGTGTGCAAG GGTGGAAGTG CATATATTCC CTATTAATCC CGGGTATTCT TTTTGTAACG

1/3

+ ATGGCCAAAT CAAAATCAAA CAGTGCTCTT CCATAATATC AATACCTTCT CTCCCAATTT TCTCATAACA
- TACCGGTTTA GTTTTAGTTT GTCACGAGAA GGTATTATAG TTATGGAAGA GAGGGTTAAA AGAGTATTGT

+ ACCAACGGAA ACACACGTAC TTACGAAA
- TGGTTGCCTT TGTGTGCATG AATGCTTT
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Figure 6

The sequence of miR166h promoter indicated different cis-acting elements that regulate genes. The elements involved in different stress responses are marked with specific color. Grey: cis-acting regulatory element involved in the MeJA-responsiveness (TGACG-motif), pink: cis-acting regulatory element involved in the MeJA-responsiveness (CGTCA motif), blue: cis-acting element involved in defense and stress

responsiveness (TC-rich repeats), green: cis-acting element involved in salicylic acid responsiveness (TCA element) and yellow: cis-acting element involved in the abscisic acid responsiveness (ABRE).

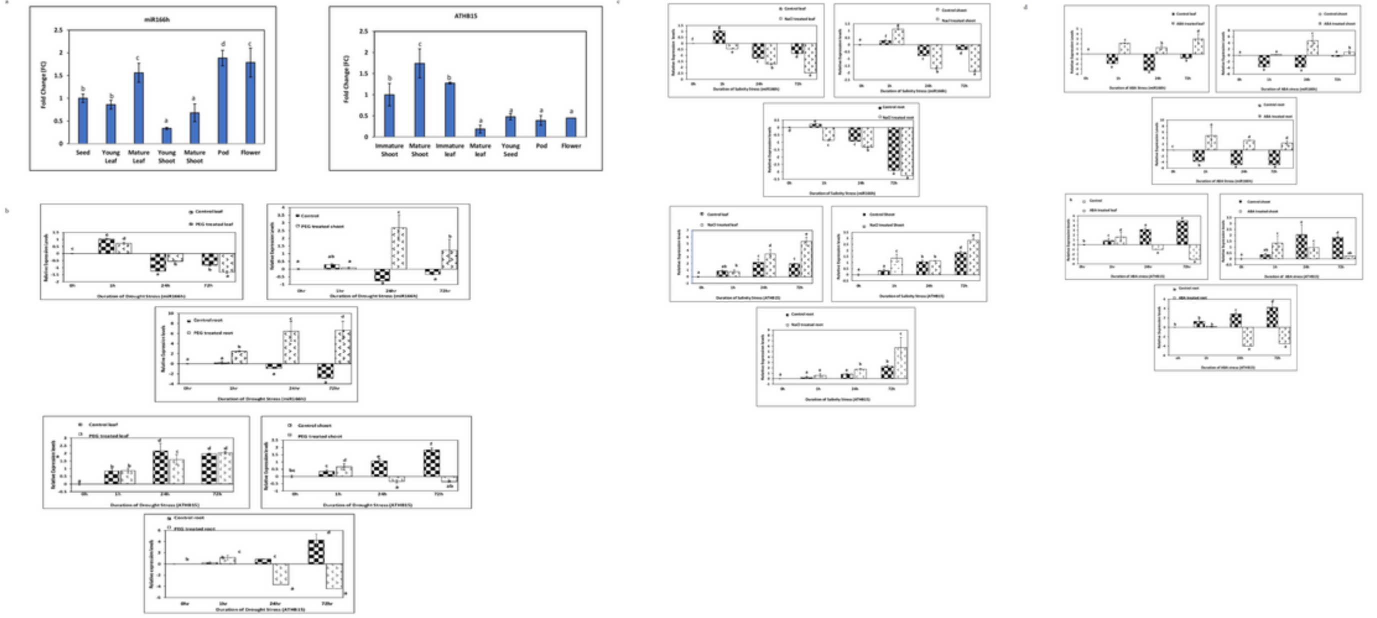

Figure 7

a. Expression analysis of miR166h and ATHB15 in different tissues of chickpea including shoot, leaf, flower, pod and seed. The letters in the graph indicate towards the significant differences determined by Duncan's multiple range test with p-value ≤ .05. b. Expression analysis of miR166h and ATHB15 of chickpea in drought treated tissues (leaf, shoot and root) harvested at different time intervals 0h, 1h, 24h and 72h. The letters in the graph indicate towards the significant differences determined by Duncan's multiple range test with p-value ≤ .05. c. Expression analysis of miR166h and ATHB15 of chickpea in salinity stress treated tissues (leaf, shoot and root) harvested at different time intervals 0h, 1h, 24h and 72h. The letters in the graph indicate towards the significant differences determined by Duncan's multiple range test with p-value ≤ .05. d. Expression analysis of miR166h and ATHB15 of chickpea in ABA treated tissues (leaf, shoot and root) harvested at different time intervals 0h, 1h, 24h and 72h. The letters in the graph indicate towards the significant differences determined by Duncan's multiple range test with p-value ≤ .05.

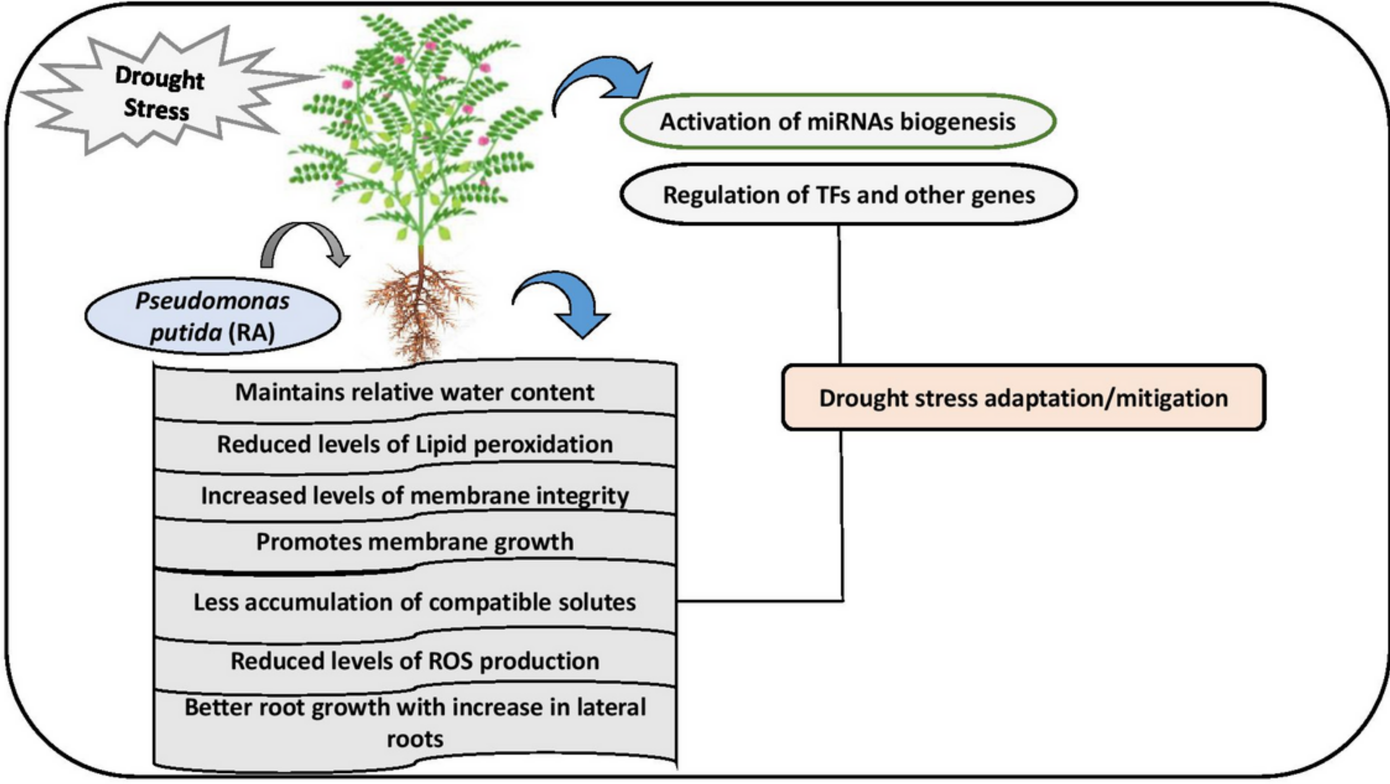

Figure 8

A representative model of the molecular basis ofdrought stress mitigation in chickpea inoculated with PGPR (RA)

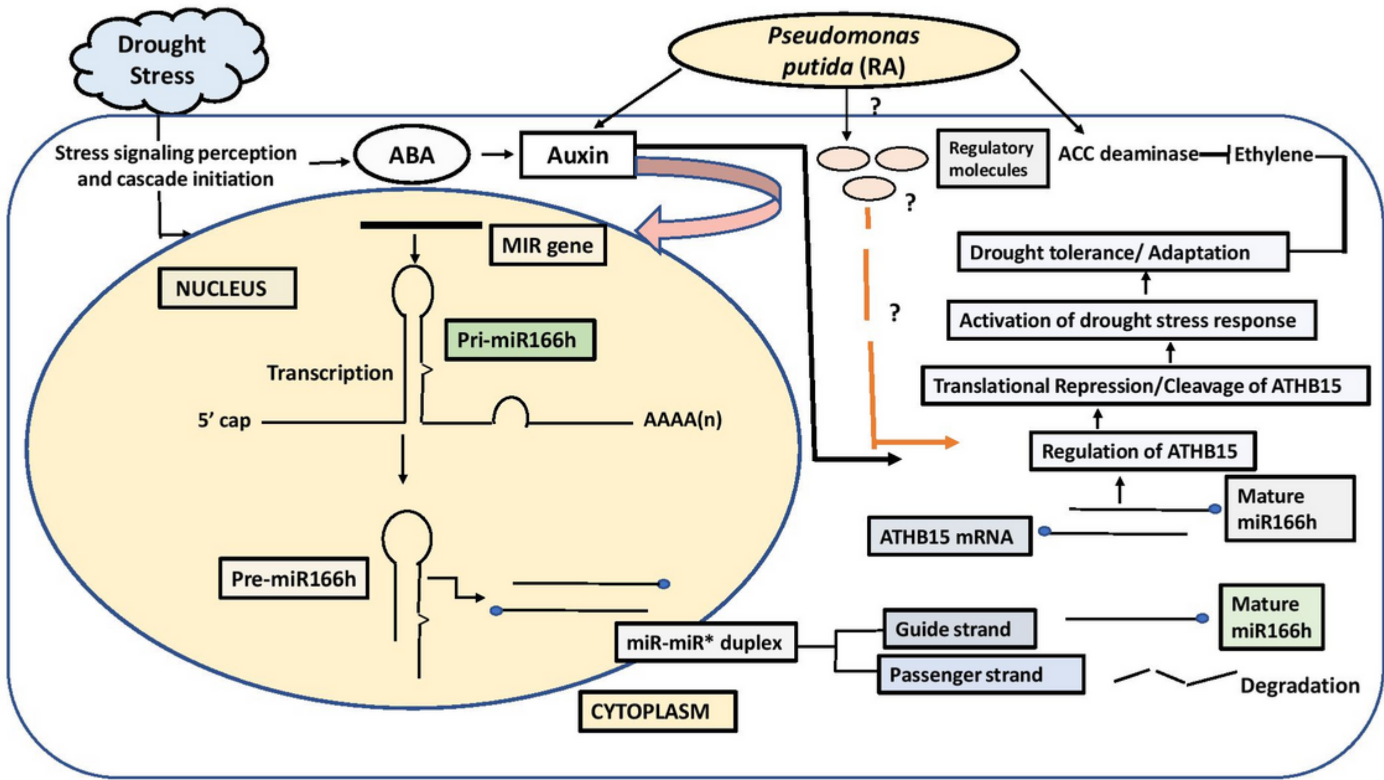

Figure 9

A schematic representation of the role of miR166h in response to drought stress in chickpea inoculated with plant growth promoting rhizobacteria, RA strain.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.docx