Indian J. Genet., 81(2): 266-276 (2021)
DOI: 10.31742/IJGPB.81.2.9

Effect of drought stress on morphological, biochemical, physiological traits and expression analysis of microRNAs in drought-tolerant and sensitive genotypes of chickpea

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(Received: December 2020; Revised: April 2021; Accepted: May 2021)

Abstract

Drought stress poses a global threat for sustainable growth and productivity of major legumes, including chickpea. There was severe reduction in biomass of the chickpea plants subjected to drought and the effect on leaves was most apparent. However, there was not much difference in the root volume(s) suggesting a drought tolerance mechanism other than the root traits in Pusa 362, a drought tolerant genotype as compared to SBD377, a drought sensitive genotype. The proline accumulation was significantly higher in Pusa 362 as compared to SBD377. Recently miRNAs (21-24 nt in length, endogenous, non-coding RNAs) have emerged as major regulatory molecules which have been shown to regulate gene expression during drought stress conditions. Among the 16 validated miRNAs, expression of miR167, miR168 and miR171 showed significant upregulation (>3-fold) in root tissue of drought tolerant genotype. These miRNAs targets auxin response factors, WD-repeat and scarecrow-like transcription factors, respectively which are known to play important role in drought stress in plants suggesting direct role of these miRNAs during drought tolerance in chickpea. miR390 and miR2118 were up-regulated in shoot samples in Pusa 362. Among the novel miRNAs, nov_miR8 in root and nov_miR2 in shoot tissue showed maximum expression in Pusa 362. Nov_miR2 targets GMP synthase and nov_miR8 targets gene encoding laccase. GMP synthase are involved in synthesis of purine nucleotides which among other functions play key role as secondary messenger in signal pathways activated during stress conditions. These drought-responsive miRNAs are likely to provide novel insights into post transcriptional gene regulation under drought stress conditions in chickpea at molecular level.

Key words: Chickpea, Drought stress, Chlorophyll, Proline, RWC, SOD, microRNAs

Introduction

Chickpea (*Cicer arietinum*) is the third most important grain legume crop in terms of total global production (12.09 Mt) after soybean and dry bean (FAOSTAT, 2019). In India, it occupies approximately 38% of the total area under pulses and contributes to about 46% of the total pulse production (Dixit et al. 2019). It is a self- pollinated, diploid annual crop grown in arid and semiarid regions worldwide. It is rich in proteins (20–25%) and essential amino-acids.Its ability to fix atmospheric nitrogen in soil, results in increased soil fertility. Owing to its high commercial value and nutritional importance, several research efforts have been carried out in the recent past to increase its production. Although the chickpea production potential is high, it has not been fully realized owing to several biotic stresses like *Fusarium* wilt, *Ascochyta* blight and abiotic stresses like drought, salinity and high temperature (Toker et al. 2007; Jha et al. 2014; Kashiwagi et al. 2015; Dasmandal et al. 2020). Among the many abiotic stresses, drought has a major negative effect on chickpea production (Boominathan et al. 2004; Deokar et al. 2011). The prevalence of drought at the pod filling stage commonly referred to as terminal drought, results in reduced flower and pod production which eventually leads to reduced production. In a recent estimate drought stress can cause up to 50% production losses in chickpea (Kaloki et al. 2019).

In order to breed for drought tolerant varieties, it

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Published by the Indian Society of Genetics & Plant Breeding, A-Block, F2, First Floor, NASC Complex, IARI P.O., Pusa Campus, New Delhi 110 012; Online management by www.isgpb.org; indianjournals.com

is important to understand the molecular mechanisms which determine the drought responsiveness of different genotypes when exposed to drought stress conditions. The drought tolerance trait is regulated by hundreds of genes and transcription factors that control various morphological and physiological responses to drought stress. The elucidation of the complex mechanisms underlying drought tolerance will accelerate the development of new drought tolerant varieties. Numerous efforts by several groups, in this regard are underway, towards generation of molecular and genomic resources which includes the transcriptome and genome sequence of chickpea (Jain et al. 2013; Varshney et al. 2013; Garg et al. 2016; Mashaki et al. 2018).These resources serve as valuable tools and provide information regarding different genes and their expression profile in different tissues as well as under different biotic and abiotic stresses. This is very important and better understanding of plants at morphological level coupled with biochemical and physiological analysis is likely to provide answers to handling complex trait like drought. The understanding of physiology of plant responses to drought has emerged as a major area and is likely to provide key solutions towards production of drought-resistant crops (Gupta et al. 2020).

MicroRNAs (miRNAs) are small regulatory RNAs of 20-24 nucleotide (nt) length derived from single-stranded stem-loop precursors (Sarkar et al. 2017; Li et al. 2020) and have profound role in gene regulation. Lot of attention has been focused on the importance of post-transcriptional gene regulation by miRNAs since their identification and involvement in plant development (Carrington et al. 2003). Increasing evidence suggests involvement of miRNAs in development as well as stress responses, both biotic and abiotic. miRNAs have emerged as important modulators in drought tolerance and avoidance mechanisms via control of the gene expression under drought stress (Sunkar 2010; Sunkar et al. 2012). In plants, miRNAs not only control the gene at post-transcriptional level but they also interact with each other in regulatory networks affecting development, responses to biotic and abiotic stresses and plant-environment interactions (Kohli et al. 2014; Song et al. 2019). Plant miRNA expression in response to abiotic and biotic stress is generally spatial (plant tissue) and temporal (developmental/growth stage) specific (Vakilian 2020).

Although miRNAs have been extensively studied in other plants, their regulatory mode of action in response to drought stress has not been studied in chickpea. The present study was therefore undertaken with the objective to understand and draw novel insights into the drought tolerance mechanisms in chickpea. For this purpose, two different genotypes, Pusa 362 (drought tolerant) and SBD 377 (drought sensitive) contrasting for drought stress tolerance were analysed (Kumar et al. 2018). Besides morphological, physiological and biochemical analysis the expression profile of drought-responsive miRNAs was also studied in these chickpea genotypes under drought stress conditions.

Materials and methods

Plant material and drought stress treatment

Two chickpea genotypes with contrasting drought stress tolerance (Pusa 362, drought-tolerant and SBD 377, drought-sensitive) were used in this study. The genotypes were grown in a net house (IARI, New Delhi, India) in rabi season (2016-17). Plants were grown in 12-inch diameter pots with normal field soil mixed with farmyard manure (FYM). Three seeds were sown in each pot and subjected to natural solar radiation. Three replicate per treatment were arranged in a completely randomized block design. All plants were grown under well-watered conditions up to 45 days following emergence. 45 days-old plants were subjected to the water deficit stress by withholding watering on the stressed pots for 45 days while controlled pots were watered in every two to three days to soil capacity. These plants were used for estimating the biochemical and physiological parameters. Root and shoot samples were frozen immediately in liquid nitrogen and stored at -80^{o}C until use. Three replicates of each sample were collected for this study.

Measurement of physiological parameters

Estimation of relative water content (RWC)

Leaf relative water content (RWC) was used as a measure to assess the water status of plant tissue. RWC was measured by method described by Turner (1981). Fully expanded third leaf from top of each plant was collected, between 11A.M.–12 noon. Samples were weighed for fresh weight and then were hydrated for 4 h by floating in de-ionized water in petriplates.These were weighed to record the turgid weight (TW). The samples were dried at 80°C for 24 h and dry weight was recorded (DW). Weight of the tissues was recorded till the weight became constant.

Leaf relative water content was calculated according to the following equation:

RWC (%) = [(Fresh weight – Dry weight) / (Turgid Weight – Dry Weight)] x 100.

Estimation of soil moisture content (SMC)

Soil moisture content was assessed as described by Black (1965). Soil sample from a depth of 15-20 cm was taken in an aluminium container (C1) from the pots (30 cm in size) by using soil augers. Fresh soil along with the container was weighed immediately (W1), oven dried at 100-110°C for 24 h and re-weighed (W2). The soil moisture content was calculated using the following formula:

Soil moisture content (%) = {[(W1-C1)-(W2-C1)]/ (W2-C1)} x 100

Where: W1-C1 is moist soil weight and W2-C1 is dry soil weight.

Measurement of biochemical parameters

Estimation of chlorophyll content

The amount of total chlorophyll was estimated according to Arnon (1949). 100 mg of fresh sample was taken, cut into small pieces and suspended in test tubes containing 10 ml of dimethyl sulphoxide (DMSO). Test tubes were incubated at 60°C for 3 to 4 h in incubator. Then the extract was filtered to remove the pieces of leaves. The extract was transferred to a cuvette and the absorbance was read in a spectrophotometer at 645 and 663 nm against DMSO blank. The total chlorophyll content was calculated by using the following formula-Total chlorophyll (µg/ml) = 20.2 (A645) + 8.02 (A663).

Estimation of proline content

Assessment of proline content was performed in control and drought stress samples by following the method of Bates et al. (1973). Proline was extracted from 0.2 g fresh leaf samples that were homogenized in 3% (w/v) aqueous sulphosalicylic acid. These homogenized samples were filtered out using Whatman filter paper through funnel. 1ml of filtrate was mixed with 1ml of glacial acetic acid and 1 ml of acid ninhydrin and kept at 98 ° C for 1 h in water bath. The reaction was terminated by placing it on ice for 5 min. After adding 3 ml of toluene the fraction with chromophore containing toluene was separated from aqueous phase and absorbance was read at a wavelength of 520 nm. Proline concentration was determined using a calibration curve and expressed as µ mol proline g^{-1} FW.

Estimation of superoxide dismutase enzyme

Superoxide dismutase assay was performed as per the method of Dhindsa et al. (1981). Leaf samples (1 g) were homogenized in 10 ml extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA). Then, the homogenized extract was centrifuge at 13000xg for 20 min at 4°C. After centrifugation, supernatant was collected and used as enzyme source. Total 3 ml of reaction mixture consisting of 0.2 ml methionine (200 mM), 0.1 ml nitroblue tetrazolium chloride (NBT) (2.25 mM), 0.1 ml EDTA (3 mM), 1.5 ml phosphate buffer (pH 7.8) (100 mM), 0.1 ml of sodium carbonate (1.5 M), 0.2 ml enzyme extract and distilled water (to make a final volume of 3.0 ml) was prepared. Then, 2 mM riboflavin (0.1 ml) was added to 3 ml reaction mixture to start reaction and kept under two 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave maximal colour, served as control. Switching off the light and putting the tubes into dark stopped the reaction. A non-irradiated complete reaction mixture served as a blank. Absorbance was recorded at 560 nm in spectrophotometer.One unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50 % in comparison with tubes lacking enzyme.

$$\text{Unit of enzyme} = \frac{\text{Control - Sample}}{\text{Control /2}}$$

Isolation of RNA and expression analysis of drought-responsive miRNAs

Drought-responsive miRNAs were obtained from high-throughput deep sequencing of small RNA libraries (data unpublished). Poly(A)-tailed based quantitative real-time PCR (qRT-PCR) of selected miRNAs was performed using method as described by Kohli et al. 2014. Sixteen miRNAs, including 10 conserved and 6 novel, were used for validation in the root and shoot tissues of both the genotypes. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA was quantified by NanoDrop 1000 (Thermo Fisher Scientific, MA, USA). RNA integrity was checked by running samples in 1.2 % denaturing agarose gel. Total RNA was poly (A)-tailed using the Poly(A) Tailing Kit (Ambion, USA) according to the manufacturer's instructions and was reverse transcribed into cDNA.

For qRT-PCR, the sequences of the specific mature miRNAs served as the forward primer and RTQ uni-primer, having an adaptor sequence served as the reverse primer (Table 3). The 5S rRNA was used as an internal control for normalization. Three biological replicates were used per sample in addition to three technical replicates, along with a no template control (NTC). The data was analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) and reported as the means of standard errors (SE) of three biological replicates.

Target gene identification

miRNA target mRNA genes were identified using miRanda(http://www.microrna.org) and psRNAtarget (http://plantgrn.noble.org/psRNATarget/) software using default parameters.

Statistical analysis

All the experimental data recorded were average mean of at least three independent biological replicates. SE was calculated as the means of standard deviation of three biological replicates.The significance of differences was determined using the Student's t-test.

Result

Effect of drought stress on morphological characters in contrasting genotypes

When 45-day old chickpea plants were subjected to drought stress of 45 days, there was reduction in height of plants of both the tolerant and sensitive genotype. However, the percent reduction in height/ biomass volume was more in SBD 377, a drought sensitive genotype as compared to Pusa362, a drought tolerant genotype (Fig. 1A). The root tissue was carefully retrieved from the plants grown under control and stress conditions. As is evident from the Fig. 1B there is not much change in the root length/volume of both the genotypes under control and stress conditions. However, there was drastic reduction in the leaf size/ volume of the plants subjected to drought stress (Fig. 1C). Here, we would like to point out that Pusa362 is a genotype with uni-imparipinnate leaf having 9-15 leaflets; one normally comes across in chickpea. However, SBD 377 is a genotype with simple leaf.

Effect of drought stress on physiological parameters of genotypes with contrasting tolerance

The relative water content is used as a measure of water potential of the plant. Under well-watered condition Pusa362 and SBD 377 had high RWC of

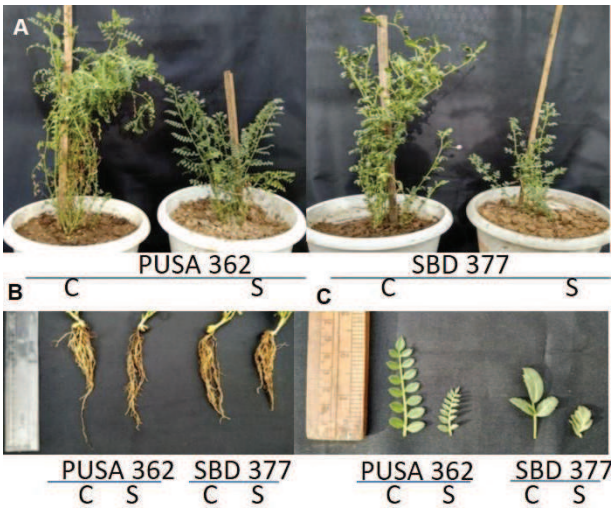

Fig. 1. Effect of drought stress on morphological characters of two chickpea genotypes: Pusa362, drought tolerant and SBD377, drought susceptible. 45-day old plants were subjected to drought stress of 45 days: A. Variation in plant height B. Variation in root length and C. Variation in leaf morphology (C: Control; S: Drought stress). Please note that SBD377 is a genotype with simple leaf

72% and 70%, respectively. However, with the withholding of water the RWC dropped to as low as 55 % and 51 % in Pusa362 and SBD 377, respectively (Table 1). The soil moisture content was comparable

Table 1. Analysis of the physiological parameters in two chickpea genotypes: Pusa362, drought tolerant and SBD377, drought susceptible under control and drought stress conditions. The values are expressed as mean ± S.E. for triplicates

Physiological parameter		Drought tolerant Pusa362	Drought susceptible SBD377
Relative Water Content (%)	Control	72.18 ± 1.69	70.166 ± 1.32
	Stress	55.96 ± 0.60	51.82 ± 0.75
Soil Moisture Content (%)	Control	28.59±1.69	27.29 ± 1.32
	Stress	12.48 ± 0.60	9.733 ± 0.75

in both the genotypes (28 % and 27 % for Pusa362 and SBD 377, respectively) under control condition. With imposition of drought stress the soil moisture content decreased sharply and was12 % in Pusa362 as against 9 % in SBD 377.

Effect of drought stress on biochemical traits in contrasting genotypes

Drought stress is known to alter the amount of chlorophylls and carotenoids in plant tissues (Hussein et al. 2008). Likewise, drought stress imposed on chickpea plants led to significant decrease in the total chlorophyll content in both genotypes (Fig. 2A). However, the percent reduction in chlorophyll content was more in SBD 377, a drought sensitive genotype as compared to the resistant genotype.

Drought tolerance is generally associated with the accumulation of osmoprotectants such as proline. Towards that end, the proline content was estimated in both resistant and sensitive genotypes under control and stress conditions. There was a considerable increase in proline content in both tolerant and sensitive genotypes under stress condition and moreover, proline content in the tolerant genotype (0.103 μmol g-1 FW) was more as compared to the sensitive one (0.089 μmol g^{-1} FW) (Fig. 2B). This implies that the tolerant genotype has an intrinsic mechanism to resist the changes in water status in its environment by regulating its proline concentration.

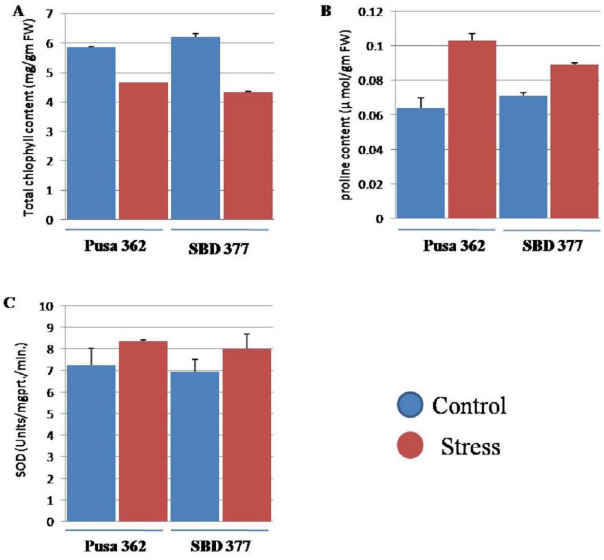

Fig. 2. Effect of drought stress on biochemical characteristics of chickpea genotypes: Pusa362, drought tolerant and SBD377, drought susceptible. a. Chlorophyll content b. Proline content c. Superoxide dismutase. For statistical analysis, Students t-test was performed and significant differences with a p value < 0.05 are depicted with *. All observations were taken for three biological replicates (n = 3)

Generally genotypic differences in drought tolerance are attributed to increase in antioxidant enzymes like Superoxide dismutase. Superoxide dismutase activity was found to be increased in both tolerant and sensitive chickpea genotype under drought stress condition (Fig. 2C). However, the significantly increased SOD activity was observed in drought tolerant genotype as compared to the sensitive one.

Expression analyses of miRNAs in shoot and root tissues under drought stress

The drought-responsive miRNAs were selected on the basis of literature and data available with us after carrying out the preparation of small RNA libraries for identification of miRNAs (unpublished data). Expression analysis of 16 miRNAs, including 10 conserved and 6 novel, was evaluated in root and shoot tissues of both tolerant and sensitive genotype subjected to drought stress. miR390 and miR2118 showed significantly higher expression in shoot tissue of drought tolerant genotype (Pusa362) in contrast to down regulation in sensitive genotype (SBD 377) under stress (Fig. 3A). Similarly, nov_miR2 was up-regulated in tolerant and down-regulated in sensitive genotype under drought stress in shoot tissue (Fig. 3B), indicating their probable role in drought tolerance. In root, 3 miRNAs (miR167, miR168 and miR171) were up-regulated in tolerant genotype (Pusa362) and down-

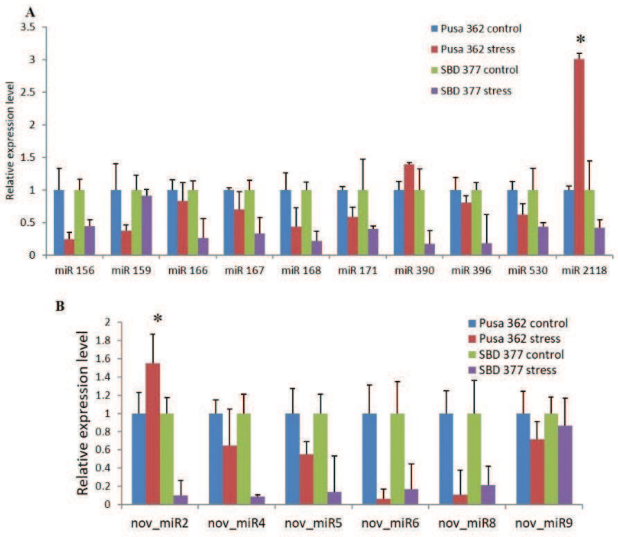

Fig. 3. Expression analysis, using qRT-PCR, of selected miRNAs in shoot tissues of chickpea genotype - Pusa362, drought tolerant and SBD377, drought susceptible: A. Conserved and B. Novel miRNAs. * indicates statistically significant change (p < 0.05) in expression

Table 2A. Conserved miRNAs and target genes in plant drought stress.

miRNAs	Target genes	References
miR156	Squamosa promoter-binding-like protein	Li et al. 2020, Song et al. 2019
miR159	Transcription factor GAMYB	Li et al. 2020, Song et al. 2019
miR166	Homeobox-leucine zipper protein	Li et al. 2020, Song et al. 2019
miR167	Auxin response factor (ARF), *Indole-3-acetic acid–ala resistant* (IAR3)	Li et al. 2020, Song et al. 2019
miR168	AGO1, WD repeat and HMG-box DNA-binding protein	Li et al. 2020, Song et al. 2019
miR171	Scarecrow-like protein (SCL)	Li et al. 2020, Song et al. 2019
miR319	Transcription factor TCP	Li et al. 2020, Song et al. 2019
miR390	*TAS3* (TRANS-ACTING siRNA3)	Li et al. 2020, Song et al. 2019
miR396	Growth-regulating factor (GRF)	Li et al. 2020, Song et al. 2019
miR530	Zinc knuckle protein	Kohli et al. 2014
miR2118	Disease resistance protein (TIR-NBS-LRR class)	Kohli et al. 2014, Song et al. 2019

Table 2B. Novel miRNAs and target genes in plant drought stress.

miRNAs	Target genes	References/tools used
nov_miR2	GMP synthase	miRanda and psRNA target
nov_miR4	Protein FAM135B, E3 ubiquitin-protein ligase BAH1	miRanda and psRNA target
nov_miR5	TMV resistance protein N, methionine gamma-lyase	miRanda and psRNA target
nov_miR6	protein TAR1, probable xyloglucan galactosyl transferase GT19	miRanda and psRNA target
nov_miR8	laccase-4, cation/H(+) antiporter 15	miRanda and psRNA target
nov_miR9, WD repeat-containing protein, peroxisomal and mitochondrial division factor		miRanda and psRNA target

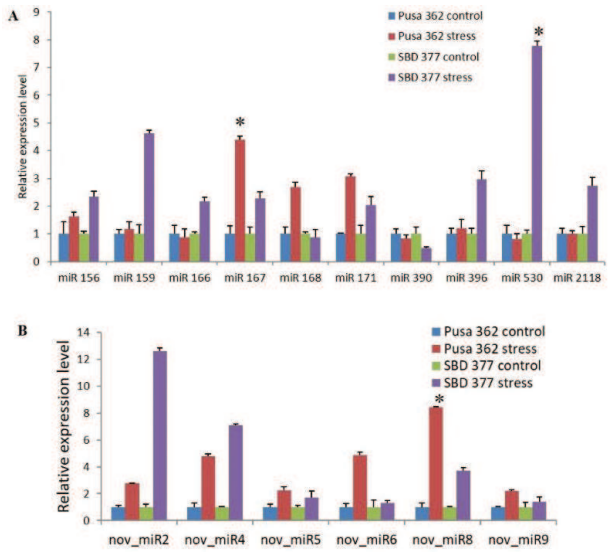

Fig. 4. Expression analysis of selected miRNAs in root tissues of chickpea genotypes - Pusa362, drought tolerant and SBD377, drought susceptible: A.Conserved and B. Novel miRNAs. * indicates statistically significant change ($p < 0.05$) in expression

regulated in sensitive genotype (SBD377) under drought stress (Fig.4A). Two novel miRNAs (nov_miR6 and nov_miR8) were also up-regulated in tolerant genotype and down-regulated in sensitive genotype under stress (Fig. 4B). Interestingly, nov_miR2 which showed downregulation in shoot tissue exhibited more than 10-fold up-regulation in root tissue in the sensitive genotype.

Discussion

Drought stress alters many physiological, biochemical and metabolic processes in plants (Gunes et al. 2006). Effect on plant morphology like reduction in shoot biomass, development of a deeper root system and reduction in the number lateral roots are the earliest plant responses to drought. Morphological traits like yield component traits, root system architecture etc. have been widely studied and have been shown to contribute to drought stress tolerance to various extent in different crop species (Bartels and Sunkar 2005). Thus it becomes extremely important to evaluate the drought responsiveness of different genotypes under drought stress conditions in terms of these traits. The

Table 3. Sequences of primer pairs used for qRT-PCR validation reaction of miRNAs

S.No.	Primer name	Primer Sequence (5' - 3')
1	RTQ-RP	CGAATTCTAGAGCTCGA GGCAGG
2	5S RNA-F	ATCAGAACTCCGCAGTTAA GCGTG
3	miR156-F	TTGACAGAAGAGAGAGAGCAC
4	miR 159-F	TTTGGATTGAAGGGAGCTCTA
5	miR166-F	TCGGACCAGGCTTCATTCCCC
6	miR167-F	TGAAGCTGCCAGCATGATCTG
7	miR168-F	TCGCTTGGTGCAGGTCGGGAA
8	miR 171-F	TTGAGCCGCGCCAATATCACT
9	miR 390-F	AAGCTCAGGAGGGATAGCGCC
10	miR 396-F	CTCAAGAAAGCTGTGGGAGA
11	miR530-F	TGCATTTGCACCTGCACTTTA
12	miR 2118-F	TTACCGATTCCACCCATTCCTA
13	nov_miR 2-F	GTGATATTGTTTCTGCTCATTT
14	nov_miR 4-F	TTTTACTGGACTGAGATGATTT
15	nov_miR 5-F	TAGCGAGTATCTGTGCCTCTG
16	nov_miR 6-F	GTTCGAATTGTAGTCTGGAGA
17	nov_miR 8-F	CAGGTGCGATCATACCAGCA
18	nov_miR 9-F	GTGTGGGTGCGTGTGGATGT

growth of a plant is accomplished through integration of different processes like cell division, enlargement and differentiation. This involves genetic, physiological, biochemical, ecological and morphological events and their interactions. The quality and quantity of plant growth thus, depends on these events, which are in turn affected by drought stress during the early and late development stage.

In this study, drought stress given to chickpea plants, at late developmental stage, decreased the plant height which was observed in both tolerant and sensitive genotypes. However, only minor differences in root length were noted but there was a drastic reduction in leaf size in both genotypes due to drought stress, also observed in faba beans (Abid et al. 2017). The plants subjected to drought stress had significantly reduced RWC as compared to control plants (Pandey et al. 2013). The plants were subjected to drought stress in large pots. Although pot studies cannot recreate the complex heterogeneous factors present in field, it is good practice to set gradual intensification of water deficit over at least several weeks (Snow and Tingey 1985) and this is what was precisely done in this study. The soil of pots subjected to drought stress by withholding water had soil moisture content of 9-12 % as compared to the 27-28 % for control plants indicating successful establishment of drought conditions.

Drought stress can also alter the amount of chlorophyll and carotenoids (Hussein et al. 2008) in plant tissues. Significant differences in total chlorophyll content were observed between tolerant and sensitive genotype grown under drought stress in present study. The reduction in chlorophyll was more in sensitive genotype than the tolerant one. Decreased or unchanged total chlorophyll level during drought stress has been reported in other species, depending on the severity and duration of drought (Kpyoarissis et al. 1995). A decrease of total chlorophyll content under drought stress implies a lowered capacity for light harvesting capacity of chloroplast. Since the production of reactive oxygen species (ROS) is mainly driven by excess energy absorption in the photosynthetic apparatus, the degradation of light absorbing pigments (chlorophyll and carotenoids) might be an adaptive, catabolic mechanism to avoid an excessive increase in ROS, are observed in wheat cultivars under drought stress (Herbinger et al. 2002).

The drought tolerance mechanism in plant is usually associated with accumulation of osmoprotectants such as proline (Abid et al. 2017). Many reports suggest accumulation of proline under drought stress in different crops including chickpea (Khanna et al. 2016). Under drought stress conditions, the production of proline increases, which allow the plant to adjust its osmotic cell potential so as to maintain cell turgor, which contributes towards drought tolerance. Proline accumulation is believed to play adaptive roles in plant stress tolerance (Verbruggen and Hermans 2008). Accumulation of proline has been advocated as a parameter of selection for drought stress tolerance in *Catharanthus roseus* and other plants (Yancy et al. 1982; Jaleel et al. 2007). In our study significant increase in proline content, under drought stress, was also observed for both tolerant and sensitive genotypes. However, the accumulation of proline was more in Pusa362, drought tolerant genotype as compared to SBD 377, drought sensitive genotype. Thus, increase in proline content in both genotypes implies that it is an adaptive strategy to withstand drought stress.

Antioxidant defence system plays an important role in plant under stress conditions. It is obvious from result that chickpea plants produce more amounts of Superoxide dismutase enzyme under stress, as compared to control condition. This suggests that increase in SOD activity in chickpea may be related to induction of antioxidant responses that protect the plants from oxidative damage. Superoxide dismutase activity increased in both the genotype under drought stress condition compared to control condition. As far as genotype is concerned, Pusa362, tolerant genotype had marginally more amounts of SOD enzyme than sensitive genotype. SOD constitutes the first line of defence via detoxification of superoxide radical, there by maintaining the plant tissue as reported in wheat (Sairam and Saxena 2000). The increased activity of SOD in Pusa362 agrees well with its ability to tolerate drought stress better than SBD377, a drought sensitive genotype.

In this study, the opposite correlation of miRNA expression between tolerant and sensitive genotype was observed for miR2118 and miR390 under stress in shoot tissue. The expression level of miR2118 was elevated by 3-fold in tolerant genotype. miR2118 is a legume specific miRNA and targets gene encoding TIR-NBS-LRR domain protein, as reported in chickpea and other crops (Kohli et al. 2014). The role of miR2118 has been earlier reported in response to drought, cold, salinity and ABA in *Medicago truncatula* (Jagadeeswaran et al. 2009) and *Phaseolus vulgaris* (Arenas-Huertero et al. 2009). Till now, the upregulation of miR2118 under drought stress in chickpea has not been reported in literature. Thus, higher expression of miR2118 has also been observed in our data and together with its predicted target gene (TIR-NBS-LRR) we can infer that miR2118 might be regulating the drought tolerance mechanism in a species-specific manner under drought stress in chickpea. However, a detailed study involving over-expression studies of miR2118 to prove this contention is required. Another miRNA, miR390, is reported to target *TAS3* siRNA which in turn regulates ARF genes expression (Haiping et al. 2018). The ARF genes have been shown to be involved in leaf, root and flowering time alteration in *Arabidopsis* and soyabean (Haiping et al. 2018). Although the role of miR390 in shoot development through miR390-TAS3-ARF pathway has been well documented in land plants, there are few studies documenting its role in drought stress. miR390 was up-regulated in our data in drought stress and similar observation of elevated expression of miR390 under drought stress was reported in *Vigna unguiculata* (Ding et al. 2013).

In root tissue, 5 miRNAs were identified to be differentially regulated in tolerant and sensitive genotype. The expression of miR167, miR168 and miR171 was up-regulated in Pusa362, drought tolerant genotype under stress. It has been reported that miR167 targets *ARF6*/*8,* which in turn are involved in floral development in crop (Haiping et al*.* 2018). Based on the inverse expression pattern of miRNA and target gene it can be inferred that the upregulation of miR167 may reduce the target accumulation in stress conditions in the current study. Role of miR168 has been previously reported in rice and *Arabidopsis* in response to drought stress.miR168 targets AGO1 which is involved in miRNA biogenesis (Bakhshi et al. 2016). Thus, mutating AGO1 or overexpressing miR168 results in ABA hypersensitivity and enhanced drought tolerance, suggesting that the miR168-AGO1 module is involved in the ABA dependent drought tolerance in *Arabidopsis* (Li et al. 2020). miR171 targets Scarecrow-like transcription factor (Jagadeeswaran et al*.* 2009), which is involved in root development. Thus, an up-regulation of miR171 eventually decreases the transcript accumulation of scarecrow-like transcription factor as previously reported (Jain et al. 2014). It has been previously established that transcription factors encoding mRNAs constitute the major fraction of the miRNA targets in chickpea (Jain et al. 2014). The scarecrow like TF belongs to the GRAS family of TFs and has been shown to be involved in altering the root growth and development. The inverse relationship between miR171 and Scarecrow-like transcription factor might be indicative of differential expression of these TFs under drought stress conditions.

Among the six validated novel miRNAs, nov_miR2 showed contrasting expression pattern in both the genotypes in root and shoot tissues. The expression of nov_miR2 was elevated 12-fold in sensitive genotype (SBD377) in root tissue but showed down-regulation in shoot tissue. While in case of tolerant genotype, Pusa362, it exhibited opposite expression pattern. Another novel miRNA, nov_miR8 showed upregulation in both the genotypes in root tissue with higher fold (about 10-fold) up-regulation in tolerant genotype. However, in shoot tissue it was down-regulated in tolerant genotype. Nov_miR2 and nov_miR8 targets genes encoding GMP synthase and laccase, respectively. GMP synthase is involved in synthesis of purine nucleotides and involvement of

cyclic nucleotides (cGMP) as secondary messengers is one of the key steps in perceiving any signal due to abiotic or biotic stress in plants (Joshi et al. 2016).

The present study has led to identification of novel miRNAs and their potential target genes. These miRNAs can serve as potential candidate miRNAs which can be utilized for further understanding of miRNAs mediated regulation of gene expression under drought stress conditions in chickpea. In addition, the manifestation of drought stress as physiological and biochemical changes in plants demonstrate the importance of these traits in order to quantify the stress and devise suitable selection strategies to select for drought tolerance trait in crop germplasm.

In this study, we observed that chickpea plants show various adaptive strategies to drought stress, which range from simple morphological or physiological or biochemical traits that serve as important stress tolerance markers to striking differences in expression of genes and miRNAs with far-reaching effects on drought stress tolerance. The better understanding of all these intricate mechanisms will pave way for the development of drought stress tolerant chickpea.

Authors' Contributions

Conceptualization of research (PKJ); Designing of the experiments (LS); Contribution of experimental materials (CB, KG, RK, AD, VP, PKJ); Execution of field/ lab experiments and data collection (LS, DK, PKJ); Analysis of data and interpretation (PKJ, KG, LS); Preparation of the manuscript (LS, DK, PKJ).

Declaration

The authors declare no conflict of interest.

Acknowledgment

The authors thankfully acknowledge the support from NPFGGM project (2001-3003). The first author also acknowledges the support of National Agricultural Higher Education Project- Centre for Advanced Agricultural Science and Technology (NHEP-CAAST) funded by World Bank and ICAR, New Delhi, India. He is also thankful to DBT, Govt. of India for providing JRF fellowship for conducting the research work.

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