Integrative Small RNA and Transcriptome Analysis Provides Insight Into Key Role of Mir408 Towards Drought Tolerance Response in Cowpea

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Research Article

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Abstract

Cowpea, a highly nutritious legume crop exhibits high resilience to heat and drought stress. Small RNA and transcriptome sequencing was performed in an elite high-yield Indian cultivar, Pusa Komal. Overall, 233 conserved and 10 novel drought-responsive miRNAs, and around 2995/497 drought-responsive genes were observed in libraries generated from leaf/root tissues of cowpea. Using 5' RLM-RACE approach, NAC29, auxin response factor 8, copper chaperone for superoxide dismutase, scarecrow-like protein 6, mitochondrial substrate carrier family protein B, and laccase 12 were identified as cleaved targets of differentially expressed miRNAs such as miR167, miR171m, miR398c, miR408, miR1514, novel_792, and novel_796 in cowpea. Moreover, the potential role of differentially up-regulated miR408 in cowpea was exploited by the generation of transgenic cowpea lines overexpressing amiR408a-3p. The transgenic OX-amiR408 cowpea lines displayed enhanced drought and salinity tolerance as compared to control plants and maintained higher relative water content, chlorophyll, and proline content, and lower cellular H2O2 content under drought stress. The SEM analysis revealed reduced stomatal aperture and higher trichome density in transgenic OX-amiR408 lines. Furthermore, genes related to trichome development, transparent testa glabra 1 (TTG1), and glabrous inflorescence stems (GIS3) showed ~1.45-fold change in transgenic lines. Selected DGE for GO enriched terms for biological processes like lipid metabolic processes, hydrogen peroxide catabolic process, and response to oxidative stress, such as LAC3, LAC12, GDSL esterase/lipase CPRD49 and peroxidase 25 were altered in transgenic OX-amiR408 lines.

Key Message

Oxidative stress response, stomatal, trichome density and alteration in lignin metabolism as preliminary drought response in transgenic OX-amiR408 cowpea lines

Introduction

Owing to the fluctuating climatic adversities, drought stress serves as a serious threat to plant growth and productivity. Physiological, biochemical, metabolic alterations like reduced plant vigor, photosynthesis, stomatal conductance, production of essential metabolites involved in cellular pathways, grain yield, and interference in the symbiotic association of rhizobium with legumes, thereby affecting root-nodule development and subsequently, nitrogen fixation, are some of the damaging aspects of drought stress in plants (Jha et al. 2020). Hence, agriculturally profitable, nutritious, and highly climatic resilient crops are need of the hour to augment agricultural production to facilitate the ever-increasing global healthy and nutritious food demand-supply. Cowpea is a highly nutritious grain legume crop and serves as the primary protein source for millions of people in tropical and sub-tropical regions of Africa, Asia, and other developing countries of the world. Due to its ability to associate with nitrogen-fixing bacteria and vesicular-arbuscular mycorrhizal fungi, cowpea can survive in low fertile soils and grown with cereals in intercropping system. It is a promising crop for cultivation under adverse climatic fluctuations, being highly resilient to heat and drought stress in comparison to other members of the Leguminosae family (Carvalho et al. 2017).

Recent advances in high throughput sequencing have paved the way for transcriptomic study in grain legumes, like chickpea (Mantri et al. 2007, Hiremath et al. 2011, Garg et al. 2016, Badhan et al. 2018), peanut (Guimarães et al. 2012, Brasileiro et al. 2015), lentil (Singh et al. 2017) and soybean (Chen et al. 2013, Van Ha et al. 2015) to facilitate identification of drought-related gene networks for understanding varied drought response mechanisms in legume crops. Again, with the advent of high throughput sequencing, microRNAs (miRNAs) belonging to small non-coding RNAs have been tagged as potential regulators of gene expression via transcriptional or post-transcriptional gene silencing. Further involvement of miRNAs in response to varied environmental cues like salinity, drought, cold, nutrient deficiency have been studied in several plants. Owing to its small genome size (~ 620 Mb), cowpea is an ideal model system for genome-wide analysis to our increased understanding towards its drought tolerance mechanisms. However, the comparative study focusing on tissue-specific miRNAs and transcriptome profiling of cowpea vis-à-vis drought stress is yet to be accomplished.

Thereby, our study focused on the drought-responsive miRNAs and transcriptome study of a high-yield Indian drought and high-temperature tolerant cowpea cultivar, Pusa Komal. In this study, we identified 233 known and 10 novel differentially regulated miRNAs in leaf and root tissues of drought-stressed cowpea plants. Again, 21 selected differentially expressed miRNAs (DE) exhibited an inverse expression correlation with 327 putative target genes. Using the 5'-RLM RACE approach, we identified 6 specific cleaved target genes for 7 drought-responsive miRNAs in cowpea. The most promising DE, miR408 up-regulated under drought stress in cowpea, was further analysed by the generation of transgenic overexpression lines of amiR408-3p in cowpea. Taken together, our work focuses on providing insight into cowpea drought tolerance through omics and translational approach, which can be further extended to an overall improvement in legume crops.

Material And Methods

Plant material and stress treatment

Healthy cowpea (*Vigna unguiculata* L. cv. Pusa Komal) seeds were planted in pots filled with soil: sand: compost (2:1:1) mixture and grown in greenhouse condition under growth condition (26 ± 2 °C/ 60-70 % RH,16h light /8 h dark photoperiod) . One month old cowpea plants were divided into two experimental sets and six replicates were kept for each set. One set comprised of control plants that were well-irrigated with leaves showing relative water content (RWC) around 85%. The second set comprised of stress-treated cowpea plants subjected to water-deficit irrigation treatment by withholding watering for 2 weeks and relative water content (RWC) of leaves ranged between 65-66 %. Thereafter, leaves and roots from six replicates of control (CK) and drought-stressed (DS) cowpea plants were frozen in liquid nitrogen and stored at -80 ºC, until further use.

RNA extraction, small RNA, transcriptome library preparation and sequencing

Total RNA was isolated from leaf and root tissues using PureLink^{TM} Plant RNA Reagent (Invitrogen, Carlsbad, California, US), RNA quantity and quality was assessed using Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA), Qubit 2.0 Fluorometer (Thermo Fisher Scientific Inc., MA, USA) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Four small RNA (sRNA) libraries were constructed using QIAseq® miRNA Library Kit (Qiagen, Maryland, U.S.A.) and sequenced using Illumina NextSeq 550 High Output sequencing platform (Genotypic Technology Pvt. Ltd., Bangalore, India). Using UEA sRNA workbench version 3.2 (http///srna-tools.cmp.uea.ac.uk/), raw data was processed to obtain clean reads (size length 16- 40 bases) after removal of 3' adapter and low quality (<q30) reads. The clean reads were mapped to the cowpea genome (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/687/525/GCA_001687525.1_Cowpea_0.03/GCA_001687525.1_Cowpea_0.03_genomic.fna.gz) for genome distribution, and Rfam database to eliminate sRNA reads mapping to non-coding RNAs (r/t/sn/snoRNAs). For the identification and expression profiling of known miRNAs, filtered sRNA sequences were subjected to BLASTN analysis against existing miRNAs in miRBase 22. Known miRNA isoforms were classified as length variants (isomiRs) and conserved miRNA variants. The remaining sRNA sequences were analysed for the identification of novel miRNAs by the mireap_0.2 program (http://sourceforge.net/projects/mireap). Briefly, the cowpea genome library was used as a reference to identify the potential precursor sequences for the novel miRNAs. Stem-loop hairpins were retained only when they complied with: 1) mature miRNAs-associated reads were mapped in the arm region of the precursors, and 2) the free energy of the secondary structure calculated by the RNAfold server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi/), was lower than -18 kcal/mol.

Differential expression profiling of drought-responsive miRNAs (DE) and target gene prediction

Expression profiling of conserved and novel miRNAs was performed using mirDeep2 software which generates normalized counts based on information obtained from mapped data. Structural stability and minimum free energy were calculated, which subsequently provided miRDeep scores where a threshold of 0 and above was used as a screening parameter. Differential analysis of miRNA abundance in CK and DS leaf and root tissues was conducted using DESeqR-package considering p-value < 0.05 as a threshold, wherein, DE with log2fold change ≥ 1.0 or ≤ -1.0 have been considered significantly up-/down-regulated, respectively. The potential targets of DE were obtained using the web-based psRNA Target program (http://plantgrn.noble.org/psRNATarget/) with default parameters of Schema V2,2017 release. The selective targets of expectancy 5.0 or less were only considered for annotation and validation.

Comparative expression analysis for selected DE and target genes

For comparative tissue-specific analysis of miRNA expression under drought stress, 40 µg of total RNA isolated from control and drought stress leaf (CL, DL) and root (CR, DR) tissues of cowpea was used for northern assay. Probes (DNA oligos) complementary to the selected DE were end-labeled with [γ-32P] dATP (Table S1). Northern blotting and analysis were performed following the method as described by Chandra et al., 2021. First-strand cDNA synthesis was performed using QuantiTect Reverse Transcription Kit (Qiagen, USA) from the total RNA of CK and DS leaf and root tissues of cowpea. Primers as listed in Table S1 were designed using Primer Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Real-time PCR was performed using QuantiNova SYBR Green RT-PCR Kit (Qiagen, USA) on LightCycler® *480* Realtime PCR System (Roche Life Sciences, Penzberg, Germany). The qRT-PCR was set using biological duplicates and technical triplicates (n = 6) with β-tubulin gene as an internal control.

Validation of miRNA guided cleavage of target genes by 5' RLM-RACE

Using FirstChoice® RLM-RACE Kit (Thermo Fisher Scientific Inc., MA, USA), four RACE libraries specific for CK and DS leaf and root tissues of cowpea were generated. Briefly, 10 µg total RNA from each tissue was ligated to a 5'-RACE adapter (5'-GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3'), incubated at 37° C for 3 h prior to cDNA synthesis, according to the manufacturer's instructions. The nested PCR products generated using RACE Inner and gene-specific (GSP) primers (Table S1) with 1/10 volume of outer race product as template, were subsequently cloned into pGEMT-easy vector, transformed into competent DH5α bacterial cells, and sequenced.

Development of transgenic cowpea lines overexpressing amiR408

The plant binary construct for vun-miR408a-3p was obtained by overexpressing *aMIR408a-3p* under *CaMV 35S* promoter in pCAMBIA 2301 (11.6 kb). Briefly, the *ath-miR319a/a** sequences in pRS300 vector were replaced with miRNA ATGCACTGCCTCTTCCCTGGC and miRNA* GCCAGGGAAGAGGCAGTGCAT sequences using oligos (Table S1) designed by WMD3 software (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi/). The *aMIR408* precursor fragment was cloned into *Eco*RI and *Bam*HI sites of pRT101 (3.3 kb). Thereafter, the *CaMV 35S promoter-aMIR408-CaMV terminator* fragment (~1.1 kb) flanked by *Hind*III sites was further, subcloned into pCAMBIA2301. The resulting pCAMBIA2301::*35S-aMIR408* plasmid (~12.7 kb) was transformed into *Agrobacterium* strain *EHA105* by freeze-thaw method. The transgenic cowpea lines were generated following *Agrobacterium-mediated* transformation protocol, previously described by Mishra et al., 2014. The independent $T_0$and T_2 *amiR408*-OE transgenic lines (1 and 5) were confirmed for the presence of the transgene by southern blotting with *nptII* and *gus-A* probe. Briefly, 50 µg of *Eco*RI digested purified genomic DNA was run on 1 % agarose gel and transferred to nylon membrane (Hybond N+, GE Healthcare) by overnight capillary transfer following depurination, denaturation, and neutralization. A 796 bp and 864 bp purified denatured gene-specific fragment for *nptII* and *gus-A*, respectively, was labeled with [32P] dCTP by using the random primer labeling method (Prime-a-Gene Labeling System Kit, Promega, Madison, WI) at 37 °C for 1 h. The membrane was hybridized with *nptII* specific probe using PerfectHyb Plus hybridization buffer (Sigma-Aldrich, Missouri, US) at 37 °C for 16 h, washed two times at 60°C for 15 min in non-stringent, 2X SSC + 0.1% SDS and stringent (1X SSC + 0.1% SDS) condition. Thereafter, the membrane was exposed to Xray-film, stored at -80°C for 1 to 2 d prior to being photographed. The blots were stripped and re-blotted using *gus-A* probe. Similarly, northern blot analysis was performed to detect the expression of amiR408-3p in southern confirmed transgenic lines. The primers used for transgenic analysis and screening of *amiR408*-OE lines were listed in Table S1.

Studying physiological changes of *amiR408*-OE transgenic lines under drought stress

Morphological and physiological changes under water deficit and salt stress

To study phenotypic response to water deficit stress, cowpea plants were taken in two growth stages. Firstly, 14 days and secondly, one month old untransformed control and transgenic *amiR408*-OE lines 1 and 5 grown in greenhouse under controlled environmental conditions, were subjected to water deficit irrigation for a period of 15 days and revival was studied after 7 days. Further, leaf samples were harvested for total chlorophyll estimation, relative water content, proline content and detection of hydrogen peroxide (H_2O_2) by 3,3' -diaminobenzidine (DAB) staining, according to Mishra et al., 2014. Further, early-seedlings (3 days old) of untransformed and T_2 generation transgenic lines *amiR408*-OE lines 1 and 5 (T_2.1 and T_2.5) were grown hydroponically in ½ MS media supplemented with/without 200 mM NaCl for 72 h and subsequently transferred to fresh media (HiMedia, Mumbai, India), photographed on 7^{th} day of revival.

SEM analysis

Leaf samples from untransformed control and *amiR408*-OE lines were used for studying stomatal changes and trichome density. Sample preparation was performed by vacuum-infiltrating leaf samples with 2.5% (w/v) glutaraldehyde in 0.1-M phosphate buffer solution (pH 7.4) buffer. After overnight fixation, samples were further dehydrated with a graded ethanol series (50,70,80,90,100 %). Samples were dried in critical-point dryer (Leica Microsystems), mounted on carbon stub and gold-coated before imaging using Scanning Electron Microscope (Zeiss EVO 18 SEM, Oberkochen, Germany).

Transcriptome sequencing and data processing

Four RNA sequencing libraries (CL, DL, CR, DR) were prepared with Illumina-compatible NEBNext® Ultra™ II Directional RNA Library Prep Kit (New England BioLabs, MA, USA) according to manufacturer's instructions (Genotypic Technology Pvt. Ltd., Bangalore, India). Similarly, six RNA sequencing libraries prepared from leaf and root of control and T_2 generation transgenic *amiR408*-OE lines 1 and 5 were analysed using Illumina HiSeq platform (Clevergene Biocorp Private Limited, Bangalore, India). Briefly, mRNA isolation was performed from 1 µg total RNA using oligo-dT magnetic beads and further, subjected to fragmentation and priming followed by cDNA synthesis. The cDNA fragments were amplified to generate transcriptome libraries and sequenced on Illumina HiSeq XTen sequencer (Illumina, San Diego, USA) for 150 bp paired-end chemistry following manufacturer's procedure. The sequencing quality was assessed using FastQC v0.11.8 software. Transcriptome analysis was performed by processing the raw data for removal of low-quality data (<q30) and adaptor sequences using TrimGalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The pre-processed high-quality data was aligned to reference *Vigna unguiculata* genome using Hisat2 with the default parameters to identify the alignment percentage. HTSeq was used to estimate and calculate transcript abundance and differentially expressed transcripts (DEG) were calculated using DESeqR package. Transcripts were categorized into up, down and neutrally regulated based on the log2fold change cutoff value of 1. Gene Ontology (GO) annotation was performed by homology search against *Viridiplantae* protein sequences from Uniprot database, with an e-value cut-off of e-5. Pathway analysis was performed using KAAS server (http://www.genome.jp/kegg/).

Results

Transcriptome sequencing and Differential Gene Expression Analysis

One month old cowpea plants were subjected to natural soil drying process for two weeks following which the leaves started wilting and displayed reduced plant growth with a 23 % decrease in RWC (Fig. 1). In this study, four libraries (Control Leaf, CL, Control Root, CR, Drought Stressed Leaf, DL, Drought Stressed Root, DR) were sequenced using the Illumina sequencing platform. A total of 151.77 million paired-end raw data were generated and nearly 96.60% of total reads were retained as high quality (>Q30) and adapter-free processed data. An average of 88.67% of the reads were aligned to the *Vigna unguiculata* reference genome with 93.35% average alignment for CL and DL libraries and 88.5 % average alignment for CR and DR libraries (Fig. S1). Further, an average of 16 590 transcripts were found to be expressed in each of four libraries. Under drought stress, the number of up-regulated differentially regulated transcripts (DGEs) ranged around 2995/497 in leaf/root tissues, similarly, the number of down-regulated DGEs ranged around 3250/700 in leaf/root tissues as shown in the volcano plots (Fig. 2, Table S2). There were nearly 101 (up-regulated) and 151 (down-regulated) DGEs common to leaf and root RNA-seq libraries under drought stress. The DGEs were functionally annotated using homology approach (blastX) against Viridiplantae from the Uniport database and gene ontology (GO) terms were assigned. GO enrichment analysis revealed that under biological process category, transcription regulation (GO: 0006355), carbohydrate metabolic process (GO:0005975), signal transduction (GO:0007165), cell wall organization (GO:0071555) and DNA integration (GO:0015074) were the most significantly enriched terms. Under cellular component category, integral component of membrane (GO:0016021), nucleus (GO:0005634), cytoplasm (GO:0005737) and cell (GO:0005623) terms occurred significantly. Under molecular function category, ATP binding (GO:0005524), DNA binding (GO:0003677), metal ion binding (GO:0046872), protein kinase activity (GO:0004672), and sequence-specific DNA binding transcription factor activity (GO:0003700) were the most significantly enriched ones. Further, the pathway analysis for DGEs was done by searching KAAS database. A total of 232 pathways were identified for DGEs and the most enriched occurrence was observed for pathways involving chromosome and associated proteins [03036], transporters [02000], transcription factors [03000], exosome [04147], membrane trafficking [04131] and chaperones and folding catalysts [03110] (Fig. 3a, b). Plants display efficient sensory correlations with outside environment to facilitate cellular homeostasis by vital reorganization of solute transport across its membrane using several active and passive transporters under abiotic stress (Conde et al. 2011). Among the drought-responsive DGEs, several members of solute carrier family proteins, glutathione-S-transferase, aquaporins, major facilitator superfamily (MFS), MATE-type proteins, heat shock proteins (HSP) and PRA-1 family proteins were significantly up-regulated. Similarly, transcription factors previously known as regulators of drought stress signal transduction pathway constituted 23% in leaf and 26% in root, with a higher occurrence of *MYB*, *NAC*, *homeobox leucine zipper, heat shock TFs*, *WRKY*, *AP2/ERF*, *EREBP* and *NFYA* in leaf and root library in cowpea (Fig. 3c). Common DGE reported in cowpea in previous reports were also detected in our leaf library. *9-cis-epoxycarotenoid dioxygenase* (*VunNCED1*) and *zeaxanthin epoxidase* (*VunABA1*) involved in ABA biosynthesis, *cowpea clones responsive to dehydrationCPRD8*, *CPRD 22* (Iuchi et al. 1996, 2000) were found to significantly up-regulated under drought stress in cowpea (Table S2).

Small RNA sequencing and analysis

For identification of drought-responsive miRNAs in cowpea, four sRNA libraries (Control Leaf, CL, Control Root, CR, Drought Stressed Leaf, DL, Drought Stressed Root, DR) were prepared and sequenced in this study. Overall, 160 981 576 total reads and 20 759 281 unique reads were obtained from all four libraries. After subsequent discarding of nearly 140.22 million reads owing to low quality and size trimming, 5.03 million high quality and non-redundant reads were retained for analysis with 7 925 343 (CL), 7 038 949 (DL), 3 139 238 (CR) and 2 040 451 (DR) reads in each library. Nearly, 90-99% of clean reads aligned to cowpea genome assembly (ASM411807v1). After removal of nearly 1.6 million reads mapping to ribosomal RNAs (rRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), and transfer RNAs (tRNAs), a total of 12.3 million clustered reads from all four libraries were further used for identification of conserved and novel miRNAs in cowpea (Table 1). The percentage of sRNA abundance across the length distribution range of 16-40 nt in all four libraries, showed 24 nt sRNAs (30.4 - 56.6 %) were more abundant than 23 nt (14.5 - 47 %), 22 nt (18.9 - 22.6 %) and 21 nt sRNAs (13.8 - 19.03 %) (Fig. S2). The presence of high endogenous small interfering RNAs (24 nt siRNAs) in both control and drought stressed libraries was consistent with previous studies in switch grass (Xie et al. 2014) and foxtail millet (Wang et al. 2016).

Identification of known and novel miRNAs

For identification of known miRNAs in cowpea, clean unique reads from all four libraries were mapped against mature Viridiplantae miRNAs at miRbase 22 database by homology-based approach, with an e-value of e-4 and non-gapped alignment. Overall, 250 known miRNAs with 240 (CL), 242 (DL), 222 (CR) and 201 (DR) miRNAs specific to each library were identified (Fig. S3a, Table S3). Out of 48 conserved miRNA families, miR159 family displayed highest members followed by miR171, miR156, and miR396 (Fig. S3b). Again, miR159a and miR1507 were observed to be most abundant in reads. For detection of putative novel miRNAs, the unaligned unique reads were mapped against cowpea genome with no mismatch and processed using Mireap_0.22b. The prediction was based on alignment, formation of stem loop structure, minimum free energy as calculated with Vienna package for the secondary structure and location of mature miRNA on the precursor arm. The secondary structure was visualized using RNAfold. A total of 837 novel miRNAs were predicted in all four libraries with 198 (CL), 353 (DL), 212 (CR) and 123 (DR) miRNAs in each library (Fig. S3a). The common occurrence of specific known and novel miRNAs in each library was observed (Fig. S3c, d). In this study, 224 high-confident novel miRNAs with (MFEI $\geq$ 0.85) and free energies of stem loop structures ranging from -20 to -112.2 kcal·mol^{-1} were

obtained (Table S3). Legume specific miRNAs such as miR4415, miR5037, miR5374, miR5770, miR6300 and miR9726 (Subramanian et al. 2008, Kulcheski et al. 2011, Zhai et al. 2011, Turner et al. 2012, Goettel et al. 2014) reported only in soybean, similarly, mir2199 (Jagadeeswaran et al. 2009) and miR2650 (Lelandais-Brière et al. 2009) so far known in barrel clover, were also detected in all four libraries in cowpea (Table S3). Owing to the splicing variation, several isoforms of miRNAs are generated and their differential accumulation in distinct tissues of plants have been evident (Srivastava et al. 2015). Similarly, several isoforms (isomiRNAs), novel variants and novel star miRNAs for known miRNAs were also observed in this study. IsomiRNAs are a result of imprecision or alternate cleavage by Dicer during pre-miRNA processing with additional nucleotide at 5'- or 3'- end of mature miRNA (Kulcheski et al. 2011). IsomiRNAs for miR1507a, miR1511, miR166, miR167 and miR399 were detected in this study. Similarly, novel isoforms for miR172c, miR2111a, miR477c, miR482g, miR4407, miR530b, miR862a, miR1510b, miR2597, miR5225, and miR5261 were identified in the four libraries of cowpea (Table S4).

Differentially expressed miRNAs (DE)

The miRNA abundance was represented as absolute read count and normalized counts (Reads per million mapped miRNA reads, RPM = (Absolute count * 1,000,000)/ library size) for known and novel miRNAs in four libraries. The differential miRNAs (DE) in control and drought stress leaf and root tissues of cowpea libraries were generated using DESeq tool considering size factor. Based on normalized read counts, miRNAs with log2fold change (≥ or ≤ 1) and p-value (≤ 0.05), were considered to be differentially expressed (Table S5). Among, 144 DE in cowpea leaves, 81 up-regulated and 63 down-regulated (Fig. 4a). It was observed that miR393a-5p, miR395i, miR396a-3p, miR5770a were highly induced, contrastingly, miR156a, miR169s, and miR2111, miR482a-3p were highly repressed under drought stress (Fig. 4b, Table S5). Similarly, among 89 DE in roots, 42 up-regulated and 47 down-regulated. Further, DE such as miR166a/b/k-3p, miR319b-3p, miR396e, and miR397a were highly induced, however, miR167d, miR171m/q, miR319d/g, miR396a-5p, miR5374-5p and miR6478 were highly repressed under drought stress (Fig. 4b, Table S5). Further, we observed that miR164e-5p, miR166b, miR169b-5p, miR171p/n, miR403 and miR408 were highly up-regulated whereas, miR164g-5p, miR166e-3p, miR319d/g, and miR396a were highly down-regulated among 54 common DE shared in leaf and root sRNA libraries (Fig. 4c, Table S5). 10 novel DE were differentially expressed in all four libraries under drought stress in cowpea. Selected conserved DE validated by northern blotting showed similar expression pattern to sequencing data except miR171m and vun_novel_27, a novel star of miR408 (Fig. 5a, c).

Target prediction, expression analysis and validation by 5' RNA ligase mediated rapid amplification of cDNA ends (5' RLM-RACE)

The target prediction for DE in all four libraries was performed using psRNAtarget (Schema V2 release) online tool (http://plantgrn.noble.org/psRNATarget/) using reference cowpea cDNA sequences with default parameters (Table S5). Further, the cleavage of putative targets of selected DE validated in northern analysis were assayed by 5' RLM-RACE method (Fig. 6). It was observed that vun-miR167b, vun-miR398c and vun-miR171m cleaved auxin response factor 8 (*ARF8*, LOC114188087), copper - chaperone for superoxide dismutase (*CCS*, LOC114178604) and scarecrow-like protein 6 (*SCL 6*, LOC114166860) exactly at 10/11th position unlike, vun_novel_792 which cleaved mitochondrial substrate carrier family protein B (*mcfB*, LOC114194150) at 12/13th position from 5'- end of miRNA. Again, 5'-cleaved PCR products (2 bands, Fig. S4) for NAC transcription factor 29 (*NAC 29*, LOC114167348) was obtained at 10/11th position from 5'- end of vun-miR1514a-5p and vun_novel_796. The above-mentioned targets were observed in CL RACE library and their expression levels were high in drought stressed as compared to control leaves (Fig. 5b). However, laccase-12 (*LAC 12*, LOC114177496) was observed to be precisely cleaved at 13 bp upstream of cleavage site of vun-*miR408* in DL RACE library.

Physiological analysis of transgenic cowpea lines overexpressing *amiR408*

Transgenic cowpea lines overexpressing *amiR408* were generated by using cotyledonary nodes as explants with a transformation efficiency of 2 % using 300 explants, following *Agrobacterium-mediated* transformation method as described in Mishra et al., 2014. Two independent OX-amiR408 lines, T_0.1 and T_0.5 were confirmed with the presence of single transgene copy, using a 0.796 kb of *nptII* probe and a 0.864 kb of *gus-A* probe. Thereafter, the expression of a*miR408*-3p was observed in T_2 generation overexpression lines of T_0.1 and T_0.5, using northern blotting (Fig. 6). Early vegetative stage, 14 days old cowpea plants were subjected to water deficit and salinity stress in soil for 2 weeks. It was observed that both control and transgenic lines displayed stunted growth under water deficit condition, however, primary leaves of control plant, wilted and yellowed as compared with transgenic lines (Fig. 7c). Moreover, the 3 days old cowpea seedlings were studied for withstanding salt stress with 200 mM NaCl, as salinity stress is known to be detrimental towards growth at early seedling stage. However, the transgenic cowpea lines survived at 200 mM NaCl/ 72 h and post-revival to ½ MS media, in contrast to control cowpea plants which displayed drastic growth defect and no survival (Fig. 7a, b). Similarly, one-month old transgenic lines displayed comparatively better survival and faster revival rate with early flowering (Fig. 8a), and also maintained a higher RWC and chlorophyll content under drought stress as compared to control plants under drought stress (Fig. 8b). The determination of osmo-protectant like proline has been used as a criterion to observe the cellular osmotic adjustment in drought adaptation response in plants (Golldack et al. 2014). Increased proline content under drought stress has been reported in some cowpea cultivars (Hamidou et al. 2007). Similarly, in this study, 21.1 % increase (4.998 µmoles/g FW) in cowpea control and an average of 13.6 % increase in transgenic lines (6.184 and 6.468 µmoles/g FW: line 1 and 5, respectively) signified the importance of proline under drought stress. Furthermore, DAB assay showed lesser occurrence of H_2O_2 under stress. The transgenic lines displayed early flowering and a higher seed yield (avg. 11-12

seeds/ pod) than control (avg. 10 seeds/ pod) under control condition (Fig. 8c) however, with no overall significant change was observed in seed weight (data not shown). The stomatal aperture with an average length of 21.53 and 14.84 µm (Mag. 3.15 K X) with 3 replicates was observed in SEM analysis and guard cells displayed higher turgidity in transgenic lines as observed under light microscope (Leica DM500, Germany) (Fig. 9).

RNA-Seq, DGE and expression analysis for transgenic cowpea lines overexpressing amiR408

RNA-seq was performed for identification of differentially expressed genes between untransformed control and T_2 transgenic cowpea lines overexpressing amiR408 and DGE were identified using DESeq2 package. Further, genes with read count less than 5 were ignored and absolute log2fold change ≥ or ≤ 1 and p-value 0.05 were considered significant. Out of 28 681 tested genes, a total of 5577/2846 and 3120/2042 common DGEs were up- and down-regulated in leaves/roots of control and OX-amiR408 lines, T_2.1 and T_2.5 (Table S6). The expression analysis for targets of a*miR408*-3p, such as *plantacyanin* and *laccase-like multicopper oxidasesLAC3* and *LAC12*, revealed down-regulation in both control untransformed plants under drought stress and transgenic lines (Fig. 10). Among the DGEs, genes known to be positively related to trichome development such as, transparent testa glabra 1 (*TTG1*), transcription factor glabra 3-like (*GL3*) and zinc finger protein *glabrous inflorescence stems* (*GIS3*) were found to be up-regulated in leaves of CK under drought and both transgenic lines (Fig. 10, Table S6. C-D). In addition, the SEM analysis for control and transgenic OX-amiR408 lines under normal conditions, also revealed an increased trichome occurrence (Fig. 9b). The KEGG pathway analysis of the most significant DGE in transgenic OX-amiR408 lines, revealed transcription factors like, WRKY, ERF, MYB, AP2, bHLH-type and homeobox leucine zipper and membrane transporters like aquaporins, solute/sugar transporters and cation/proton exchangers were potentially enriched in transgenic lines (Fig. 11a, b). The GO enrichment analysis of transgenic OX-amiR408 lines revealed regulation of transcription, hydrogen peroxide catabolic process, response to oxidative stress, cell wall organization, metal ion transport and metabolic processes were significantly enriched in biological processes category. For instance, real-time expression analysis for selected DGE such as, *PER25* (LOC114162544) peroxidase homologue of AT2G41480, involved in lignification process in *Arabidopsis* (Shigeto et al. 2014, 2015), increased drastically in leaves of control plants under drought and also in transgenic cowpea lines. *WRKY75* (LOC114164714, AT5G13080), known to be expressed under Pi deprivation and also, in development of lateral roots in *WRKY75* RNAi-lines (Devaiah et al. 2007) in Arabidopsis, and observed to be down-regulated in salt and osmotic stress in poplar (Zhao et al. 2019), was also observed to be repressed in transgenic cowpea lines. Again, DGE such as, GDSL esterase/lipase *CPRD49* (LOC114183596, AT3G11210), involved in lipid metabolism up-regulated in control plants under drought stress, however decreased in transgenic lines. *NAC62* (LOC114167089), was observed to be up-regulated in control plants under stress and also in transgenic cowpea line 5 plants (Fig. 10, Table S6).

Discussion

miRNA-mRNA network in cowpea under drought stress

Drought being a multi-dimensional stress imposes severe phenomenal changes in grain legumes with restricted growth, development, and metabolic functioning. Plant responses to varied environmental cues, instigates complex reprogramming of responsive genes at transcriptional level in cells. Further, with the advent of high-throughput sequencing techniques, genotype-based/ tissue-specific transcriptome analysis has contributed to the present understanding of molecular patterns, pathways, and processes for adaptive response against resilient environmental factors in plants. Small RNAs are known as potent regulators of plant growth and development under abiotic stress such as salinity, drought, and temperature fluctuations. Variation in tissue/genotype specific miRNA response has been observed under drought stress, owing to molecular plasticity in miRNA abundance and function in several legumes like *Phaseolus vulgaris* (Arenas-Huertero et al. 2009, Wu et al. 2017), *Medicago truncatula* (Wang et al. 2011), *Lathyrus sativus* (Bhat et al. 2020), *Glycine max* (Kulcheski et al. 2011, Zhou et al. 2020), *Cajanus cajan* (Buch et al. 2020), *Macrotyloma uniforum* (Yasin et al. 2020). In our study, among the significant DE, cowpea isoforms of gma-miR9726, gma-miR169u, decreased -3.66, and -5.87-fold and gma-miR5770a increased 11-fold, in leaf library, similar to expression in drought-sensitive and drought-tolerant common bean (Wu et al. 2017). pvu-miR482-5p decreased -8.9-fold in root library however, no expression change was observed in common bean under drought stress (Arenas-Huertero et al. 2009).

Among the validated stress-responsive miRNAs in our study, miR1514a is an important legume specific miRNA, particularly induced in roots of drought tolerant cultivar of common bean under drought stress and targeted two NAC transcription factors (TF) *NAC000* and *NAC700*, detected by degradome analysis (Sosa-Valencia et al. 2017). Further, it was detected in higher expression in leaves than roots in northern blots, under control conditions in common bean (Arenas-Huertero et al. 2009), similar to our finding in cowpea. However, miR1514a was detected in CL in northern blot and was found to cleave a NAC TF *NAC29*, a homologue of *NAC000*, as detected in 5'-RLM RACE in CL RACE library in cowpea. This was further confirmed with increased relative expression of *NAC29* (7.9-fold) in drought stressed leaves of cowpea, unlike no expression data for *NAC000* in root of common bean, indicating the potential role of *NAC29* for drought tolerance mechanism in cowpea. Similarly, miR398 is a universal stress responsive miRNA observed under oxidative stress, salinity, drought, and abscisic acid stress (Zhu et al. 2011). In cowpea, out of three isoforms of miR398a/b/c, only miR398c was down-regulated in response to drought stress. The major validated targets of miR398 in legumes are cytosolic copper/zinc superoxide dismutase *CSD1*, chloroplastic *CSD2* and copper chaperone for superoxide dismutase (*CCS*) in *Glycine max* (Zhou et al. 2020), mitochondrial cytochrome c oxidase subunit *COX5b* in *Medicago truncatula* (Trindade et al. 2010), and protein disulphide isomerase (*PDI*) in *Medicago sativa* (Pokoo et al. 2018). Under oxidative stress, SOD plays a vital role against detoxification of reactive

oxygen species (ROS) acting as a first line defence system in plants (Mittler 2002). Moreover, CCS acts as a chaperone which delivers Cu cofactor to activate CSD1 and CSD2 (Chu et al. 2005). In our case, we identified *vun-CCS* as a cleaved RACE product for miR398c in CL RACE library. miR398c was observed only in CL in northern analysis and *vun-CCS* up-regulated (8.5- fold) in DL, indicating, cowpea adapts control against oxidative stress as an immediate response mechanism to drought stress. Similarly, *gma-CCS* was also identified as a confirmed target using 5' RLM-RACE and transient-GFP dependent gene expression method in *Arabidopsis* mesophyll protoplast cells for gma-miR398c (Zhou et al. 2020). miRNA 167 and miR171 were observed to be repressed under drought stress in our study consistent with findings reported in *Lathyrus sativus* (Bhat et al. 2020) and *Medicago truncatula* (Wang et al. 2011), respectively. So far, miR171-targeted *SCL* transcription factors, *SCL6* homologues involved in nodulation in *Lotus japonicus* (De Luis et al. 2012) have been confirmed through RACE approach, moreover, SCL6 up-regulated (2.8-fold) in leaves under drought stress indicating its differential role in cowpea. miR167 is identified as a potential regulator in auxin and ABA signalling under drought stress in maize (Wei et al. 2009) and was observed to target *ARF8* in adult leaves, flowers, and inflorescences in *Arabidopsis* (Wu et al. 2006). We found in our studies, *ARF8* was cleaved in control leaves and increased upon drought stress by nearly 2-fold in leaves with decrease in miR167 as evident in northern blots. Interestingly, a *mitochondrial substrate carrier family protein B-like (mcfB)*, homologue of AT3G55640, was found to be cleaved by 22 nt novel miRNA, vun_novel_792 at 12/13th position in CL RACE library. Further, a 5.8-fold increase in leaf, and a reduction in roots was observed for *mcfB* under drought stress. MCFs are particularly transporters of wide range of substrates like nucleotides, amino acids, di- and tricarboxylates, cofactors, vitamins, phosphate and H ions (Nunes-Nesi et al. 2020). According to the expression profile data analysed for *Arabidopsis* MCFs available at "The Bio-Analytical Resource for Plant Biology database", Nunes-Nesi et al., 2020 showed AT3G55640 to be highly up-regulated under cold, oxidative and salt stress in shoot tissues, thereby suggestive of its importance in abiotic stress.

miR408 is a multistress-response miRNA, known to be differentially regulated in several plants like Arabidopsis (Song et al. 2018), wheat (Feng et al. 2013), barley (Kantar et al. 2010), barrel clover (Trindade et al. 2010). miR408a-3p was observed to be up-regulated and more abundant in leaves in our study, in contrast with no change in both drought-sensitive and tolerant cowpea cultivars (Barrera-Figueroa et al. 2011). Further, an abundant novel isoform of mir408-5p (vun_novel_27), also observed in other legumes like chickpea (Srivastava et al. 2015), sacred lotus (Zheng et al. 2013) was detected under drought stress in our study. Till now, *LAC12* have been experimentally confirmed as miR408 target in only Arabidopsis (Zhang et al. 2014) and grapevine (Leng et al. 2017). However, a cleaved product of *LAC 12*, 24-nt upstream to 10/11th cleavage site of *miR408* was obtained in our study. The overall regulatory function of *miR408* was studied by generation of *OX-miR408* transgenic lines in cowpea. The known targets, plantacyanin (LOC114176940), laccase-3 (LOC114193262) and 5' RLM-RACE identified laccase-12 (LOC114177496) were observed to be down-regulated under drought stress and also in transgenic OX-amiR408 lines. Overexpression of miR408 resulted in enhanced drought tolerance in chickpea (Hajyzadeh et al. 2015), and non-legume crops ryegrass (Hang et al. 2020), however, transgenic *OX-miR408 Arabidopsis* lines exhibited increased drought sensitivity and salinity tolerance (Ma et al. 2015). In cowpea, miR408 enhanced both drought and salinity tolerance in transgenic OX-amiR408 lines which maintained a higher water potential, proline accumulation, lesser H_2O_2 level under drought stress. miR408 has been reported to up-regulate in response to ABA and salt stress in poplar (Jia et al. 2009), rice (Mutum et al. 2013). Here, in this study, several ABA synthesis and responsive genes were altered as evident in RNA-seq of OX-amiR408 lines indicating plausibly, miR408 and ABA are interlinked. Trichomes are specialized epidermal structures known to be involved in drought tolerance in *Caragana korshinskii* by reducing water loss and thereby, transpiration rate (Ning et al. 2016). Increased trichome density was observed as an altered morphological feature in leaves of transgenic lines in cowpea. Surprisingly, ryegrass transgenic lines overexpressing *Os-miR408* (Hang et al. 2020) also displayed increased bristle like trichomes on leaf surface.

Conclusions

The study focused on the differential expression analysis of miRNAs and their target genes in leaf and root tissues of cowpea under drought stress. miR169b, miR319f, miR408, and novel_27, an isoform of miR408-5p up-regulated, in contrast to miR167, miR171m, miR398c, and miR1514a, observed to down-regulate under drought stress in leaves of cowpea, as detected by sRNA sequencing and validated by northern analysis. miR408, being a universal multi-stress-responsive miRNA, was highly abundant under drought stress, particularly in leaf tissue of cowpea. Thereby, this study focused on functional characterization of miR408a-3p, by generation of transgenic lines overexpressing amiR408a-3p. The transgenic lines were tolerant to drought and salinity stress exhibiting higher relative water content, proline content, lesser H_2O_2 production, maintaining cellular homeostasis, higher trichome density for reduced water loss, and recovery rate post drought stress. The target genes of miR408, mainly, laccases were found to be repressed and moreover, RNA-seq of transgenic lines indicated DGE involved in lipid metabolism process. Laccases have been previously reported as glycosylated multicopper oxidoreductases involved in oxidization of monolignols to facilitate lignin polymerization process. Alterations in lignin composition has been observed in response to varied environmental stimuli in plants, reportedly, a reduction in lignin biosynthesis was observed in maize under drought stress (Alvarez et al. 2008). Reduction in level of *LAC3* and *LAC12* might be contributing to reduced lignin content in cowpea and indirectly serving as an essential trait in cowpea under drought stress. Moreover, engineered *Arabidopsis* lines with low lignin and xylan have been shown to display improved drought tolerance, accumulated higher ABA content, stomatal closure and reduced water loss under drought stress (Yan et al. 2018). Again, plants with low lignin and xylan content serve as cost-effective feedstocks for biofuel production. Futuristic study focusing on genes involved in lignin metabolism in cowpea under drought stress is necessary to extend our understanding to inherent drought tolerance mechanisms in cowpea.

Declarations

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Conflicts of Interests

The authors declare that they have no known competing financial and personal interests that could have appeared to influence the work reported in this paper.

Data availability

All extracted data from sequencing of cowpea samples used for this work are available in Supplementary Data.

Author contribution

SM and BPS contributed to conceptualizing and designing of the manuscript. SM executed, contributed to the overall data generation and analysis. GS partly managed and executed expression data analysis of transgenic samples. SM wrote and reviewed the manuscript. SM is grateful to Women Scientist Scheme-A, DST, for fellowship and project grant. GS is grateful to UGC for fellowship.

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Tables

Table 1. Summary of sRNA library analysis in four libraries of cowpea.

	Control Root CR	Drought Stressed Root DR	Control Leaf CL	Drought Stressed Leaf DL
Total Reads	51640632	41405064	33548108	34387772
Total Reads after 3' adaptor removal	49983993	40021624	31397027	32630971
Total Reads after length range filtering (16-40)	24214343	18402552	26470681	27458658
Trimmed Unique Reads	3447018	2264982	7966479	7080802
Reads aligned to genome	3139238	2040451	7925343	7038949
% reads aligned to genome	91.07%	90.09%	99.48%	99.41%
rRNA	603727	505847	234150	215352
snoRNA	11195	8167	6769	5112
snRNA	8218	5595	3383	3062
tRNA	194	158	63	63
Clustered Reads	1742523	1057843	5158448	4394975
Reads aligned to mirBase	4720	3099	13861	11984
Known miRNA Unique	222	201	240	242
Reads used for Novel miRNA	17312	12123	42381	39693
Novel miRNA predicted	212	123	198	353
Putative miRNAs	337232	217705	318638	278438

Figures

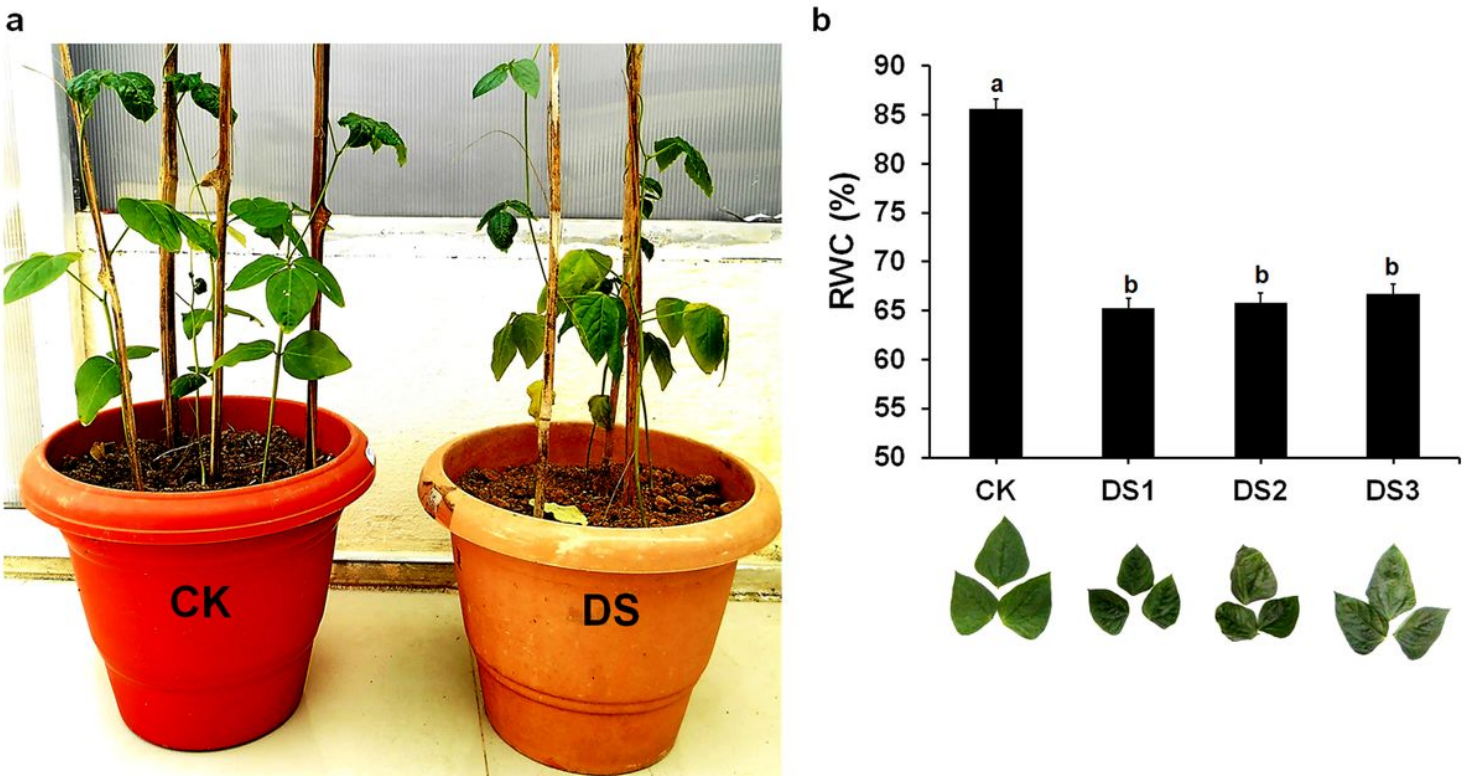

Figure 1

Drought treatment in cowpea. a One month old cowpea plants subjected to drought stress for a period of 2 weeks.b Measurement of relative water content (RWC) in leaves of CK and DS cowpea plants. Control Plant: CK, and Drought stressed plant: DS

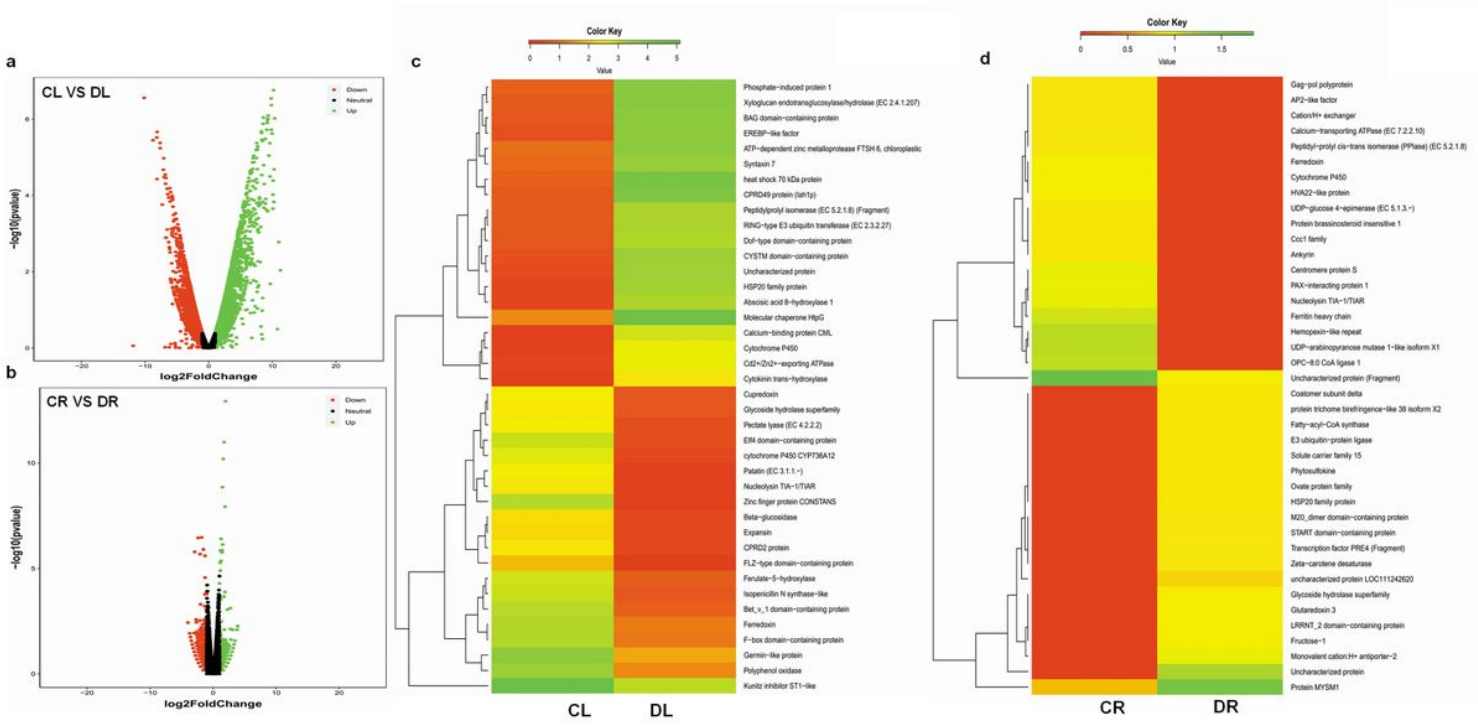

Figure 2

Transcriptome analysis of control and drought stressed libraries in cowpea. a-b Volcano plot showing the differentially expressed transcripts (DGE) up- and down- regulated in leaf (CL vs DL) and root (CR vs DR) libraries of cowpea. c-d Heat map of 20 most significant up-/down-regulated DGE in leaf and root library of cowpea observed under drought stress. Control Leaf, CL, Control Root, CR, Drought Stressed Leaf, DL, Drought Stressed Root, DR

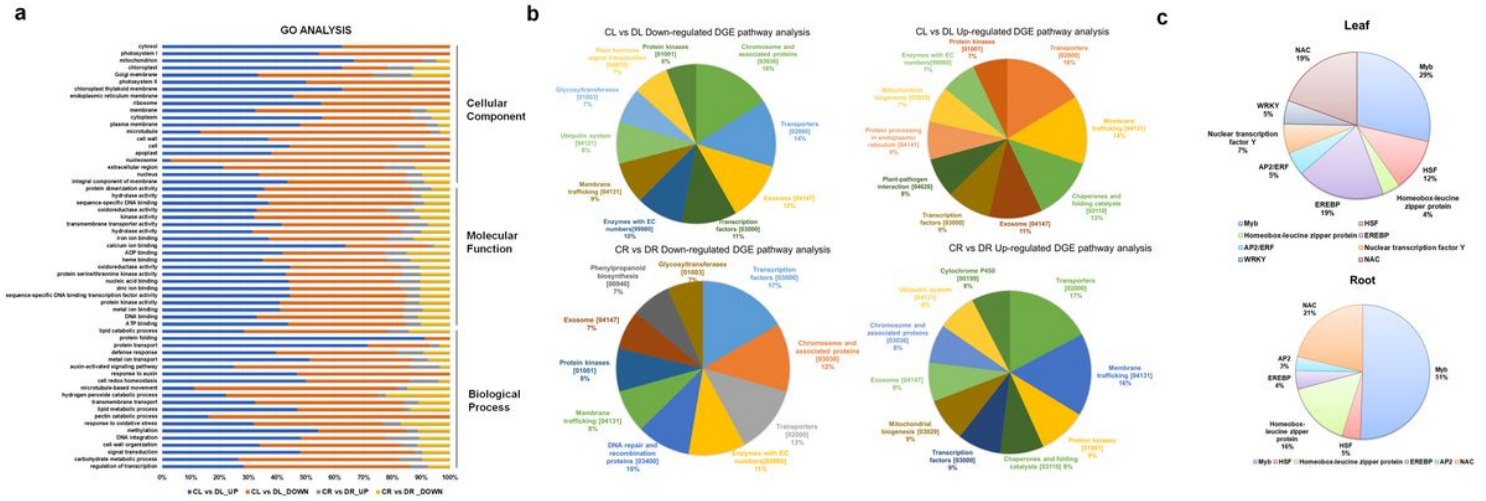

Figure 3

GO enrichment and KEGG pathway analysis of significant DGEs in control and drought stressed libraries in cowpea. Graphic representation of a GO enrichment and b pathway analysis for up- and down- regulated DGE (% of occurrence) in leaf (CL vs DL) and root (CR vs DR) libraries of cowpea, grouped under molecular function, biological processes and cellular component terms. c Transcription factors like MYB, NAC, homeobox leucine zipper, heat shock TFs, WRKY, AP2/ERF, EREBP and NFYA enriched in leaf and root libraries under drought stress. Control Leaf, CL, Control Root, CR, Drought Stressed Leaf, DL, Drought Stressed Root, DR

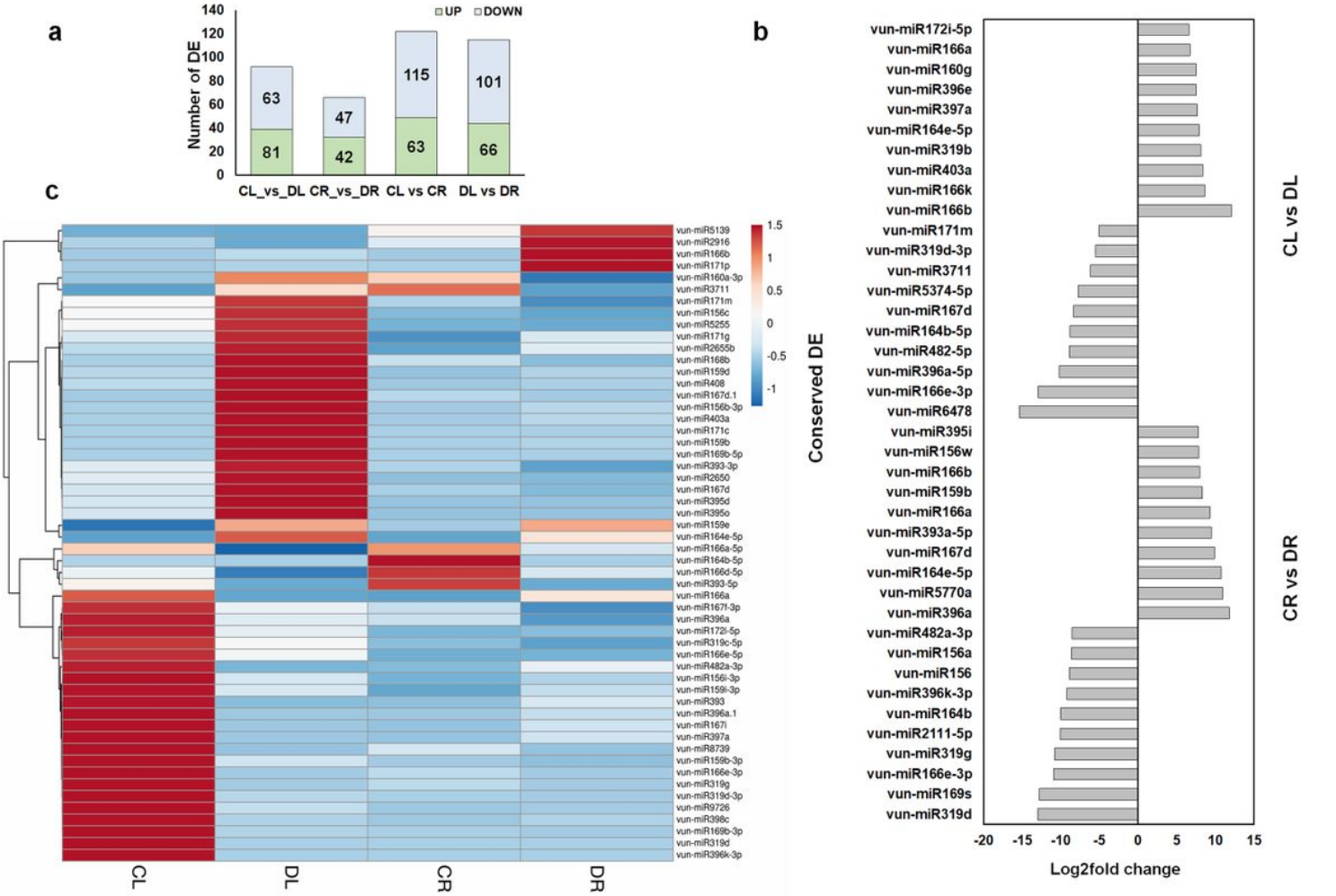

Figure 4

Differentially expressed drought responsive miRNAs (DE) in sRNA libraries of cowpea. a Total number of known DE identified in each cowpea sRNA library. b Selected 20 most significant, highly induced and repressed DE in leaf and root control vs drought stress library in cowpea. c Heat-map generated using ClustVis web tool (http://biit.cs.ut.ee/clustvis/) showing common DE identified in all four libraries (CL, DL, CR, DR). Control Leaf, CL, Control Root, CR, Drought Stressed Leaf, DL, Drought Stressed Root, DR

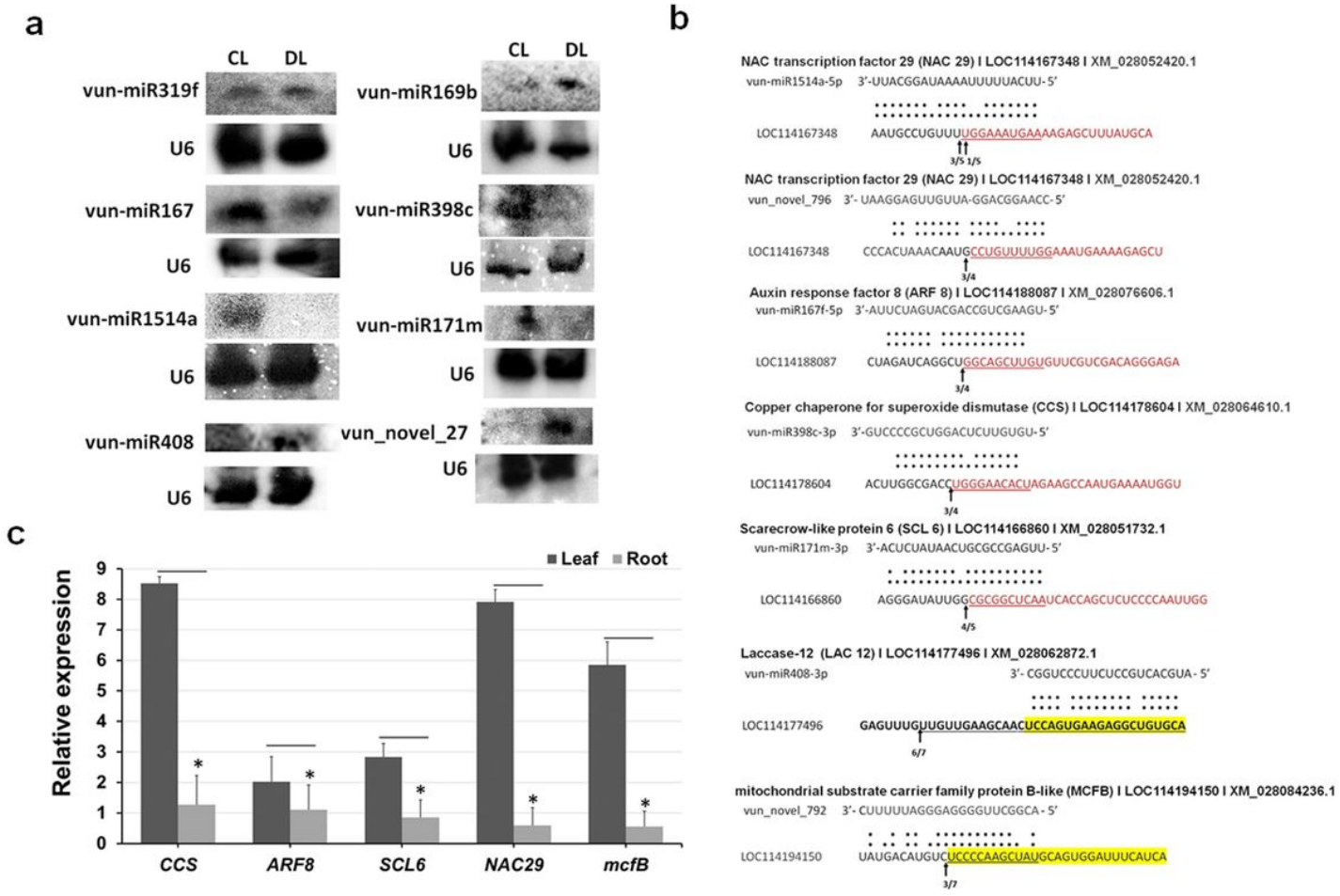

Figure 5

Expression analysis of miRNA and corresponding target genes with miRNA cleavage site identified in 5'-RLM-RACE. a Northern blot of selected DE observed in cowpea CL/DL library in cowpea. Conserved DE members of miR167, miR169, miR171, miR319, miR398, miR408, miR1514 and novel miRNA vun_miR_27 were detected in control (CL) and drought-stressed (DL) leaf library of cowpea. b Validation of candidate target genes cleaved by selected DE in cowpea by 5' RNA ligase mediated rapid amplification of cDNA ends (5' RLM-RACE). The target genes NAC 29, ARF 8, SCL 6, CCS, LAC 12, and mcfB were obtained as cleaved products of DE miR1514/ vun_novel_796, miR167, miR171, miR408, and vun_novel_792, respectively. The cleavage of the targets was observed at canonical 10/11th position in most cases, but, also at 12/13th position in mcfB. c Real-time PCR analysis of selected target genes confirmed as cleaved products for selected DE in 5' RLM-RACE approach. The target genes NAC 29, ARF 8, SCL 6, CCS, and mcfB exhibited inverse expressional co-relation with their respective miRNA counterparts, as compared to northern analysis

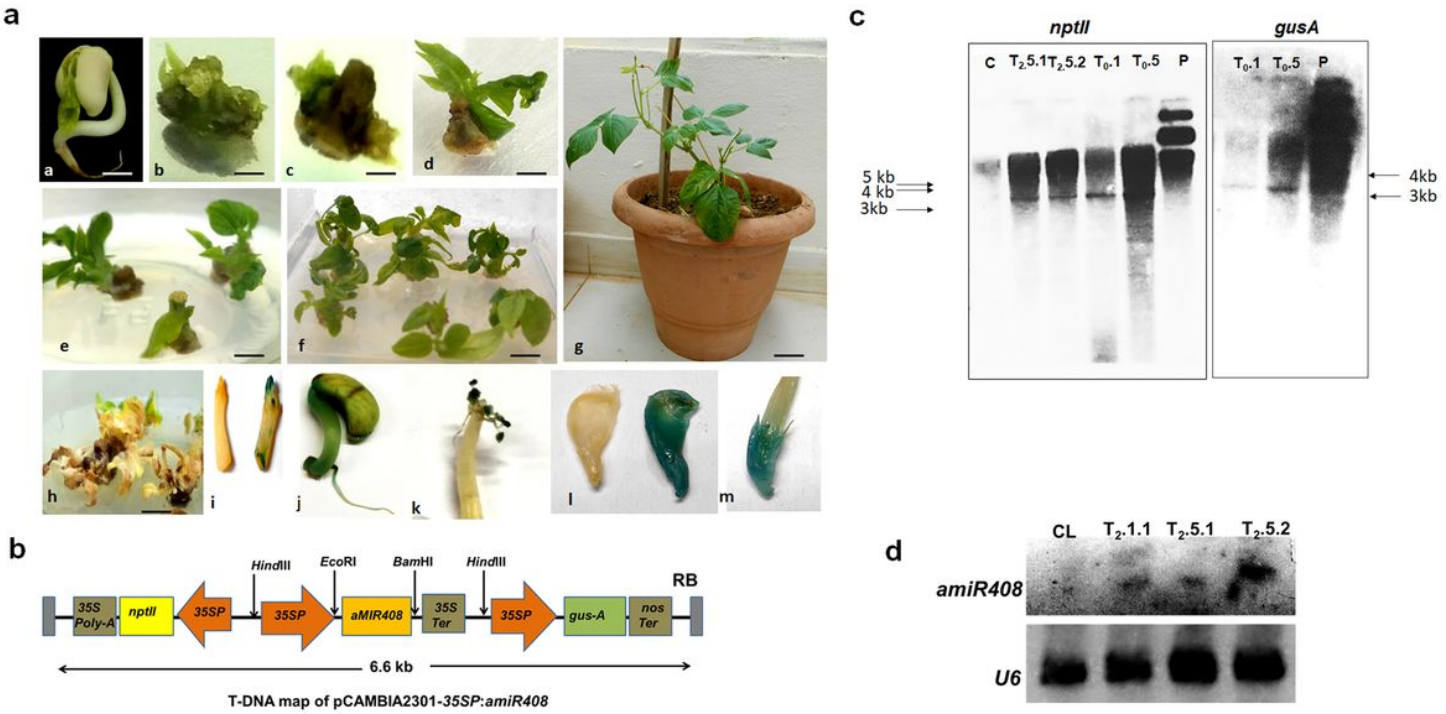

Figure 6

Generation and validation of transgenic cowpea lines overexpressing amiR408. a Agrobacterium-mediated transformation of cowpea using pCAMBIA2301::35S-aMIR408 plant binary construct, representative image of (a) 4-day old cowpea seedling, (b-f) shoot regeneration and elongation from auxiliary meristem selected on MSB5 media (pH 5.8) supplemented with different concentrations of BAP (5, 2.5, 1 µM) and 0.5 µM kinetin, 150 mg/l kanamycin and 500 mg/l cefotaxime at subsequent selection stages , (g) stable T0 transgenic cowpea line 1, (h) control untransformed plants selected on selection media supplemented with 150 mg/l kanamycin, (i) Transient GUS expression of untransformed/transformed cotyledonary explants after 3-days of co-cultivation with EHA105 harbouring recombinant plant binary construct, (j-m) Stable GUS expression of cotyledon, anthers, flower, and sepal in transgenic cowpea lines. b T-DNA map of pCAMBIA2301::35S-aMIR408 plant binary construct. c Southern blot of EcoRI digested genomic DNA of control and transgenic lines 1 and 5, using probes for nptII and gus-A d Northern blot performed with amiR408 oligo probed with P32-γ-ATP to confirm the expression of amiR408 in transgenic lines, and U6 as internal control

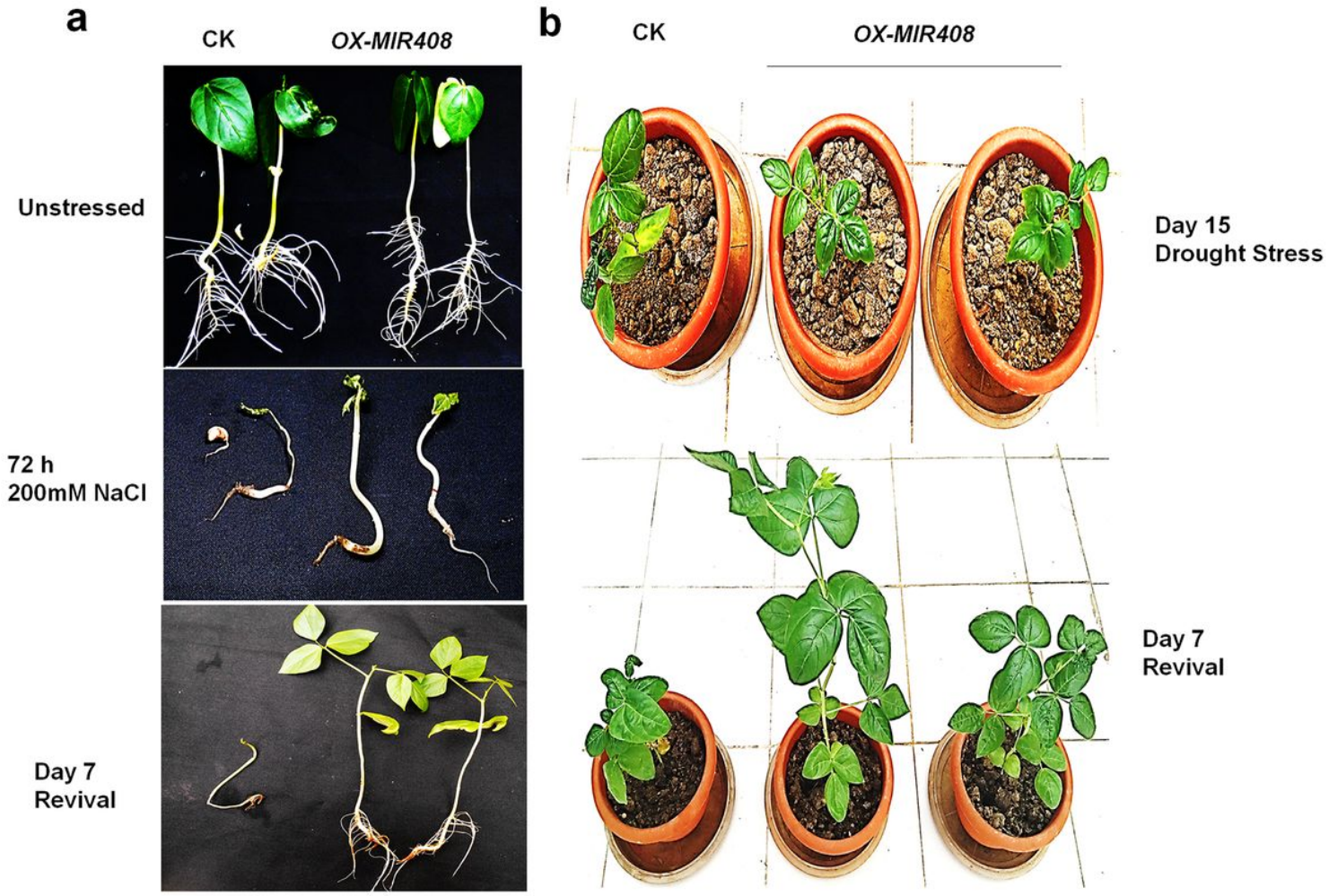

Figure 7

Phenotypic study of control and transgenic cowpea lines overexpressing amiR408 under drought and salinity stress. a-b At early seedling stage, 3-days old cowpea seedlings were exposed to 15% PEG 6000 and salt stress of 200 mM NaCl. The revival was scored after 7 days. c 14-days old cowpea plants were subjected to water deficit in soil for 2 weeks and revival observed after 7 days

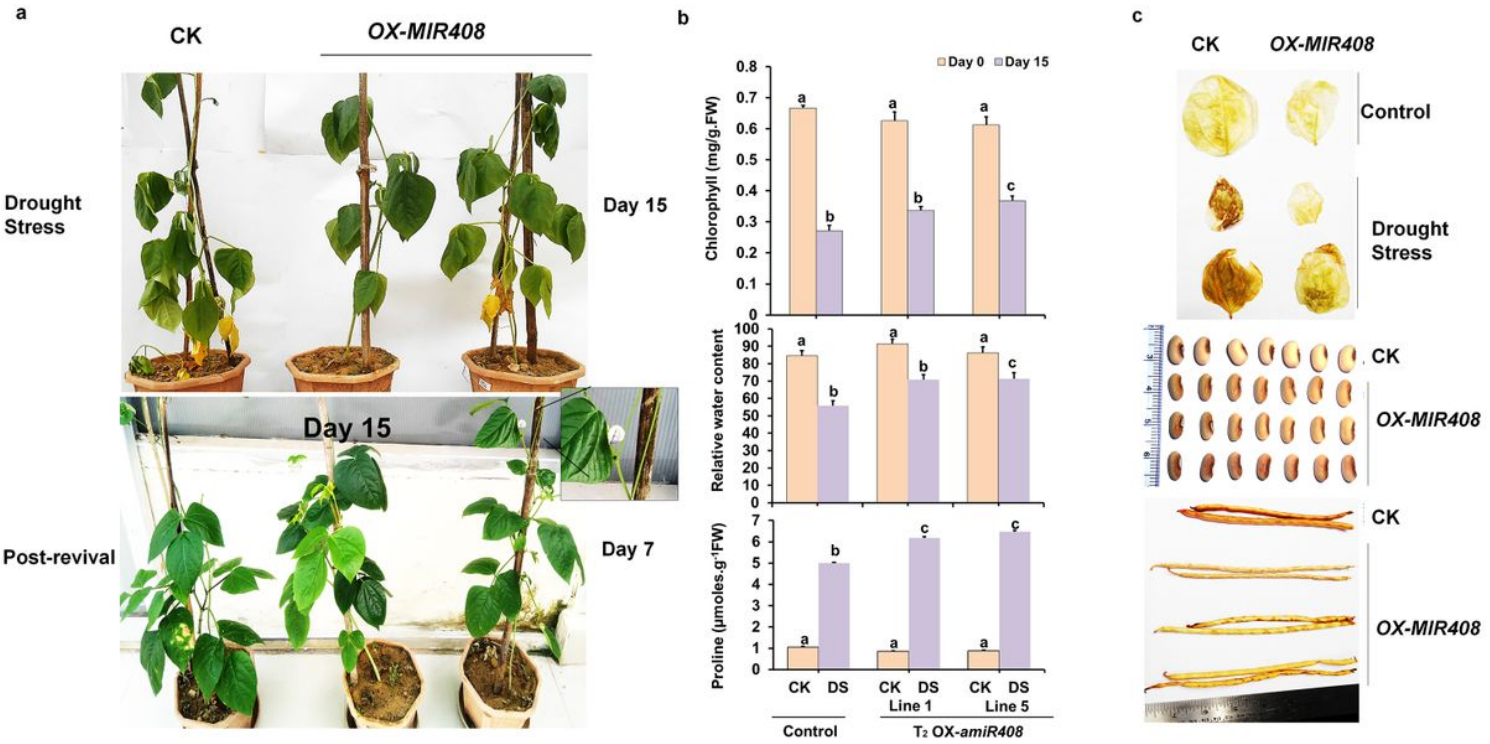

Figure 8

Response of mature control and transgenic cowpea lines overexpressing amiR408 under drought stress. a-b One month old cowpea plants were subjected to water-deficit irrigation for 14 days and revival scored after 7 days of re-watering condition. c Chlorophyll (mg/g. FW), relative water content (%), and proline content (µmoles/g. FW) estimation was performed under control (Day 0 stress) and stress treatment (Day 14 stress) in control and transgenic lines. d Detection of hydrogen peroxide (H2O2) by 3,3' -diaminobenzidine (DAB) staining in leaves of control (CK) and transgenic cowpea lines under unstressed control and drought stress. Pods and their respective seeds were observed for morphological difference in control and transgenic lines. Statistical significance was according to paired t-test with *p<0.05

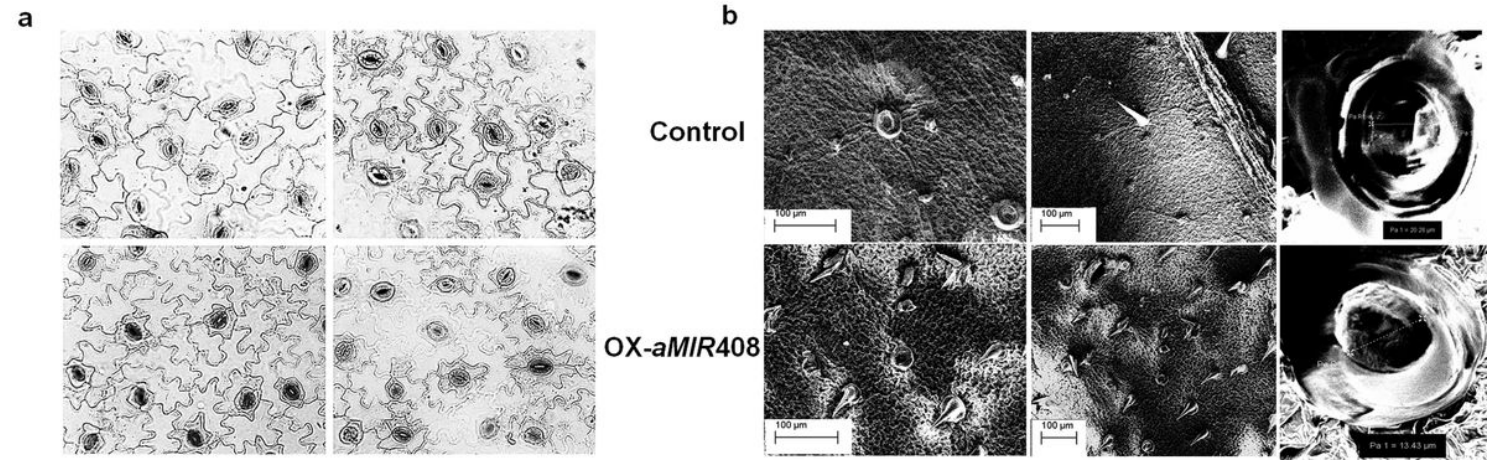

Figure 9

Study of stomata in control and transgenic cowpea lines overexpressing amiR408. a The fully expanded mature leaves were peeled, mounted on glass slide and observed at 40X, under light microscope (Leica DM500, Germany). The epidermal cells appeared reduced and elongated in size and stomata guard cells displayed higher turgidity in transgenic lines as compared to control cowpea plants. b Scanning electron microscope (SEM) analysis revealed higher occurrence of trichomes and reduced stomatal aperture (Mag. 3.15 K X).

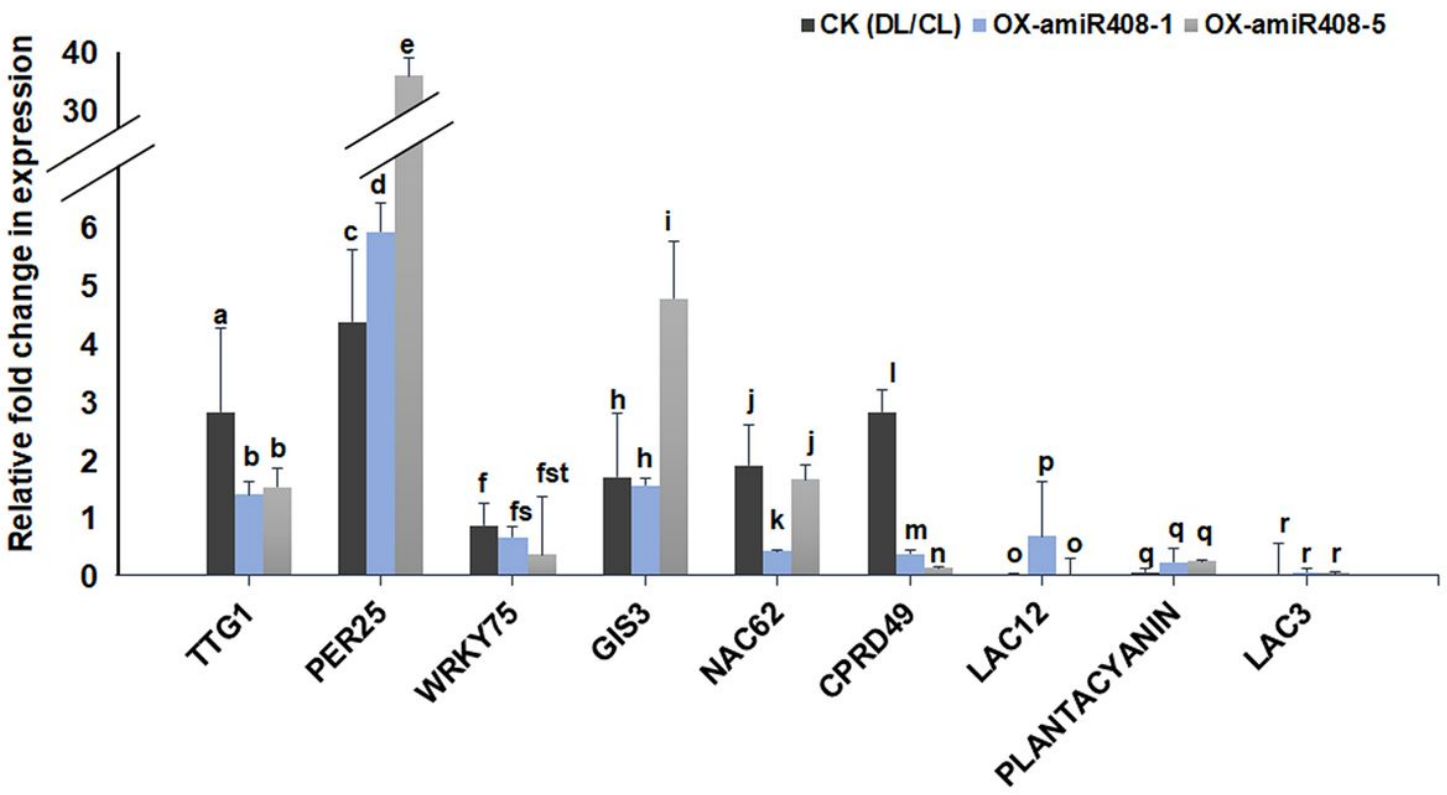

Figure 10

Real-time PCR analysis of miR408 target genes in control and transgenic cowpea lines overexpressing amiR408. The real-time PCR analysis of target genes of miR408, i.e., Plantacyanin, LAC3, LAC12 and selected DGEs like PER25, WRKY75, CPRD49, and NAC62 was performed in leaf and root of control untransformed plants under drought stress and transgenic lines under normal condition. Statistical significance was according to paired t-test with *p<0.05

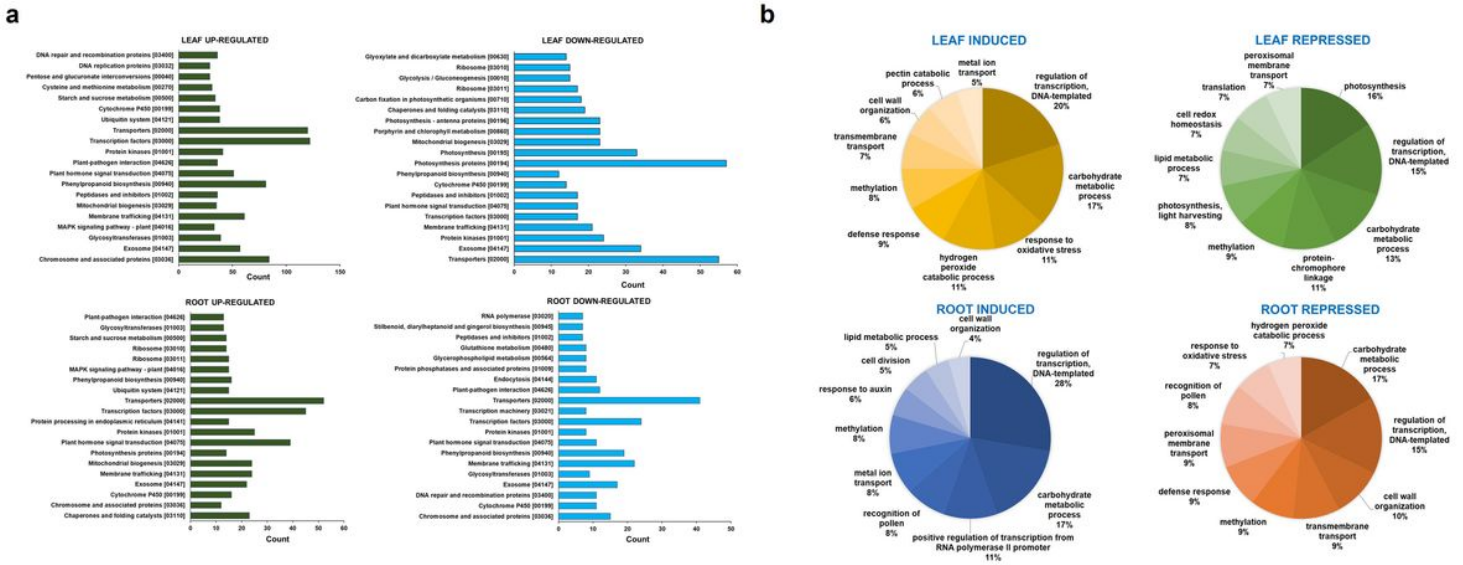

Figure 11

KEGG Pathway and GO enrichment analysis of up-/ down-regulated differentially expressed genes (DGE) in control and transgenic OX-amiR408 lines. The 20 most a enriched KEGG pathway and b GO terms for biological processes, induced and repressed, were plotted against the number of counts in each leaf and root library compared in control and transgenic OX-amiR408 lines

Supplementary Files

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

- Supplementarydata.docx
- TableS1.xlsx
- TableS2.xlsx
- TableS3.xlsx
- TableS4.xlsx
- TableS5.xlsx
- TableS6.xlsx