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Original Article

Small RNA sequencing reveals important miRNA function in *Arabidopsis thaliana* calli in response to combined drought and nitrogen deficiency

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ABSTRACT

MicroRNAs and transcription factors are crucial components of the stress response network in plants. *Arabidopsis* callus tissues were exposed to drought and nitrogen deficiency conditions using the Murashige and Skoog culture medium. Sequencing identified 117 differentially expressed miRNAs within comparisons. mir156j, mir157b, mir166b, mir164a, mir160c, mir158b, mir156b, mir159b were among those detected. Upon combination stress, mir160c was upregulated (7.49) whilst downregulated (-6.87) in the drought-only treatment. Some of the known miRNAs identified included mir390, mir781, mir1543, mir829, mir8155, mir5643, and mir403. A total of 17,003 potential target genes were predicted for the identified *Arabidopsis* miRNAs. The target genes identified by psRNATarget were enriched in gene ontology categories and included processes such as Transcription regulator activity (GO:0140110), DNA binding transcription factor activity (GO:0003700), RNA helicase activity (GO:0003724), Gravitropism (GO:0048653), and Signal transduction (GO:0007165). Furthermore, among the transcription factor genes studied, HD-Zip protein family genes were detected to have varying expressions. UBC24 was upregulated by nitrogen deficiency, while REVOLUTA (controlled by mir165 and mir166) along with RH39 were downregulated after the nitrogen deficiency treatment. Our findings highlight varied expressions within miRNA families depending on the stress condition, along with strand-specific regulation and functions of miRNA target genes which can be leveraged to enhance agricultural productivity.

Keywords: abiotic stress, climate change, drought, miRNA profiling, nitrogen deficiency, plants, transcriptomics.

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Introduction

Abiotic stress is a common phenomenon encountered by plants in the wild, affecting metabolism, growth, and development and in most cases, leading to a decrease in crop yield. Adverse climatic conditions together with human activities like pollution are the main reason for the subsequent increase in abiotic stress (Oshunsanya *et al.*, 2019; Kumar, 2020). Drought and nitrogen deficiency are among the predominant abiotic stresses plants face (Song *et al.*, 2019). Drought stress is caused by water insufficiency due to continuous loss of water through transpiration and evaporation, causing extensive cellular damage to the plant by the formation of reactive oxygen species (ROS) (Seleiman *et al.*, 2021; Zhang *et al.*, 2021). Nitrogen deficiency, on the other hand, refers to the inadequate supply of nitrogen. Nitrogen (N), one of the major components that form chlorophyll and amino acids, is a crucial element for plant viability (Leghari *et al.*, 2016). However, nitrogen is not directly available to plants in the soil as atmospheric N needs to be converted to be used. The long steps in the nitrogen conversion cycle lead to decreased nitrogen availability, causing deficiency in plants. Moreover, water availability affects nitrogen uptake because nitrogen must be dissolved in water for plant roots to absorb it (Buljovcic & Engels, 2001: Gloser *et al.*, 2020). Therefore, changes in water availability can alter the concentration of nitrogen in the soil, impacting its uptake by plants (Sajjad *et al.*, 2021).

The combined effect of nitrogen deficiency and drought has been observed to greatly impact plants (Song *et al.*, 2019). Leaf chlorosis due to reduced chlorophyll production in potato plants was attributed to nitrogen deficiency and drought stress (Wellpott *et al.*, 2023). Drought stress can additionally cause wilting, leaf curling, and stomatal closure to minimize water loss (Ali *et al.*, 2022).

Considering the detrimental effects of such conditions, plants have developed intricate mechanisms to adapt and respond to stress, involving complex changes at the molecular, physiological, and biochemical levels, controlled by a network of genes. One of the most prominent regulators of these pathways are microRNAs (miRNAs) (Wang *et al.*, 2004). MicroRNAs are small, non-coding RNA molecules that play important regulatory roles in gene expression (Wang *et al.*, 2004; Zhang, 2015; Luo *et al.*, 2022). In plants subjected to abiotic stress, they can either enhance stress tolerance or promote stress sensitivity depending on the specific miRNA and its target gene (Hajdarpašić & Ruggenthaler, 2012; Sunkar *et al.*, 2012; Shah & Ullah, 2023; Pandita & Pandita, 2023). Cui *et al* (2006) suggested that miRNAs constitute approximately 1% of the anticipated genes in higher eukaryotic genomes and that miRNAs could potentially regulate a substantial portion, ranging from 10-30% of all genes. The stress responses mediated by microRNAs often display a strong correlation with target transcription factor genes that function in the transcriptional regulation of downstream stress-responsive genes (Palatnik *et al.*, 2003; Sunkar *et al.*, 2012).

Transcription factors (TFs) also play crucial roles in coordinating stress responses by gene expression regulation (Lindemose *et al.*, 2013; Mitsis *et al.*, 2020; Zhang *et al.*, 2023). They do this by binding to specific DNA sequences and modulating the recruitment of RNA polymerase thus activating or deactivating target genes responsible for stress signaling. Previous studies have provided an outline of the following transcriptional factors: basic leucine zipper (Liu *et al.*, 2023), WRKY (Erpen *et al.*, 2018), homeodomain-leucine zipper (Mao *et al.*, 2016), myeloblastoma (Ren *et al.*, 2023), drought-response elements binding proteins/C-repeat binding factor (Vazquez-Hernandez *et al.*, 2017), shine (Shi *et al.*, 2011), wax production-like (Hrmova & Hussain, 2021), and TATA-binding protein (Bertrand *et al.*, 2005). The Teosinte branched1/Cincinnata/proliferating cell factor (TCP) family consists of genes that encode transcription factors specific to plants. This domain in plants consists of a primary section and two alpha helices connected by a loop. The

characteristic presence of amino acids in the TCP domain makes it a unique DNA-binding domain in plants (Giraud *et al.*, 2010; Viola *et al.*, 2023).

As potential miRNA target genes, we considered certain Homeodomain leucine (HD-Zip) factors (PHAVOLUTA, TCP24, PHABULOSA, REVOLUTA, CORONA) along with Ubiquitin-Conjugating Enzyme 24 (UBC24), Teosinte Cycloidea Proliferating Cell Factor (TCP24) and a DEAD-box protein-coding gene to assess specific responses of these genes under combined stressors. These target genes were chosen based on their diversity in terms of functions and miRNA-targeted transcription factor activity. They are also known to influence plant's response to abiotic stress either directly or indirectly through their roles in growth and development (Mukhopadhyay & Tyagi, 2015; Sornaraj *et al.*, 2016; Gong *et al.*, 2019; Li *et al.*, 2022; Zhang *et al.*, 2023). In addition, we performed small RNA sequencing to better understand the mechanism underlying the combined drought and nitrogen deficiency stresses. Profiling miRNAs under stress conditions offers promising avenues to enhancing breeding programs and genetic engineering efforts in agriculture. This can permit researchers to identify and manipulate key pathways to improve crop resilience and productivity. miRNA-based technologies have been successfully applied to improve resistance to abiotic and biotic stresses in crops such as rice and wheat, demonstrating their potential to reprogram plant responses and enhance resilience (Nogoy *et al.*, 2018). Additionally, the use of miRNAs in genome editing has shown success in improving traits in rice, highlighting their broader applicability across various plant species (Mangrauthia *et al.*, 2017).

Materials and Methods

Plant growth, tissue culture, and stress treatments

Arabidopsis thaliana Col-0 (Columbia-0) seeds used in this study were originally obtained from The Center for Biotechnology (CeBiTec), Bielefeld University. Seeds were surface sterilized by 70% ethanol wash, three times, and 100% ethanol wash once. Sterile seeds were dried and sown on plates containing Murashige and Skoog (MS) (Murashige & Skoog, 1962) medium supplemented with 3% sucrose and 0.9% agar. Plates were sealed, and seeds were allowed to germinate in the plant growth chamber at 25±2 °C, 16h light/8h dark conditions (Nüve, TK 252). Callus induction media was additionally supplemented with 0.5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) (Sigma, D7299). Plant roots were selected as explants for callus culture due to their high regenerative property, high responsiveness to plant growth regulators, and adaptability to environmental stresses (Fehér, 2019).

Nitrogen deficiency was created by using modified MS basal salts without NH_4NO_3 (Sigma M2909). To prepare this stress medium, 3% sucrose and 2.7 g/L of modified MS were dissolved in distilled water and 0.9% agar was added. 0.5 mg/L 2,4-D was added after sterilization by autoclave.

Polyethylene glycol 8000 (PEG 8000) was used to simulate drought conditions *in vitro*. Low water potential treatment was applied according to the protocol modified by Verslues *et al.* (2006) at an osmotic potential of -0.7Mpa. To prepare the solid phase (½ strength MS), 2.2 g/L MS salts, 1.2 g/L MES (2-(N-morpholino) ethanesulfonic acid) (6mM) and 3% sucrose were dissolved in distilled water. The pH of the solution was adjusted to 5.7. After adding 0.9% agar, the media solution was sterilized by autoclaving, and then 0.5 mg/L 2,4-D was added. To prepare the PEG overlay solution, ½ strength MS media was prepared without agar, and an appropriate amount of PEG 8000 was added gradually to reach -0.7 MPa and stirred until completely dissolved. PEG overlay solution was poured on solidified agar plates in equal volumes. Petri dishes were left at room temperature for at least 12-15 hours to allow the PEG to diffuse into agar plates. The overlay solution was carefully removed, and plates were stored at 4 °C for further use.

To prepare the combined stress medium, a modified MS salt mixture (without NH_4NO_3) was used along with PEG 8000, following protocols for both drought stress and nitrogen deficiency conditions as outlined above. The PEG overlay was added to the plates, simulating drought stress while the nitrogen-free MS medium induced nitrogen deficiency. This setup ensured that calluses were subjected to both stresses simultaneously. The prepared medium plates were stored at 4°C. 1-month-old calluses were transferred to control, nitrogen deficiency, drought, and combined stress MS medium containing 1 mg/L 2,4-D. This was done three separate times for the different experimental groups. Callus tissues were collected after 7 days in the growth chamber (75% humidity, 16/8-hr photoperiod (Panasonic MLR-352H-PE). All samples were snap-frozen by liquid nitrogen and stored at -80°C.

RNA Isolation, cDNA Synthesis, and Sequencing Workflow

Total RNA was isolated from *Arabidopsis thaliana* callus tissues using Hibrizol (HibriGen, Turkey) and phenol:chloroform extraction as recommended by the manufacturer. After extraction, the RNA samples were dissolved in DEPC (Diethyl Pyrocarbonate)-treated water. The purity and concentration of the extracted RNA were measured using the spectrophotometer. Extracted RNAs were visualized using agarose gel electrophoresis to determine their integrity (the presence of three RNA-specific bands). RNA samples with an A260/A280 ratio between 1.8-2.0 and which showed the 3 bands (28S, 18S, and 5S) during electrophoresis were sent to a local company (Genoks, Turkey) for sequencing by service procurement. Both the quantity and quality of the RNA were verified using Agilent 2100 Bioanalyzer (Agilent Technologies, USA).

cDNA library preparation and sequencing reactions were conducted by the Genoks, Turkey. After the size selection procedure to select RNA molecules in the 18-40 nucleotide range, short adapters were ligated to the 3' and 5' ends of the small RNA to prevent degradation. These adapters also served as primer binding sites, enabling the amplification of small RNA molecules to generate sufficient material necessary for quality sequencing. The size distribution was verified using the Agilent 2100 Bioanalyzer and the libraries were quantified with the Qubit fluorometer (Thermo Fisher, USA). The small RNA libraries were sequenced on an Illumina NextSeq 2000 using 50bp long single-end (SE) sequencing. The sequencing data has been deposited in NCBI GEO with accession number GSE268439.

Sequence quality was assessed using Mirtrace software, v1.0.1 (Kang *et al.*, 2018). Fastp v0.23.2 (Chen *et al.*, 2018) software was used to filter reads by length. Fastp effectively removed low-quality bases, adapter sequences, and excessively short or overly long reads, thereby improving the overall quality of the dataset. Only high-quality reads of the desired size range were retained for downstream analysis. Filtered reads were then aligned to the reference genome using Bowtie v1.3.1 (Langmead *et al.*, 2009) software. All steps of data analysis were provided by service procurement.

Detection of known small RNAs and identification of miRNA target genes

Preprocessed reads were aligned using miRDeep-P2 v1.1.4 (Kuang *et al.*, 2019) to the *Arabidopsis thaliana* reference genome (TAIR10) using a short read aligner such as Bowtie software to detect known small RNAs. For this process seqkit v2.4.0 (Shen *et al.*, 2016) was used for file conversion then using the fastx_toolkit v0.0.14 (Gordon & Hannon, 2017), the non- miRNA files

were excluded. The above procedure was repeated for each sample, and known miRNAs for each sample were obtained. The abundance analysis was performed using the featureCounts command of the software subread v2.0.3 (Liao *et al.*, 2019). The target genes of known miRNAs were detected using the psRNATarget (Dai & Zhao, 2011) web tool. psRNATarget uses the Smith-Waterman algorithm to perform sequence alignment considering sequence complementarity and target site accessibility. *Arabidopsis thaliana* was specified as a reference, and known miRNA sequences were used as inputs. The maximum target parameter was determined as 200, and the maximum false base pairing parameter was determined as 2.

Differential expression analysis between groups

DESeq2 v1.38.3, an R package (Love *et al.*, 2014), was used to perform differential expression analysis. The results of 6 different comparisons (A *vs.* B, A *vs.* C, A *vs.* D, B *vs.* C, B *vs.* D, C *vs.* D) for four different groups (Control (A), Nitrogen deficiency (B), Drought (C), Combination (D)) were examined. For this purpose, a "meta" file describing the samples and conditions was also used. The filtering parameters used for differentially expressed genes were adjusted as p-value (padj) < 0.05 and log2foldchange > 1. These differentially expressed genes were then compared for Gene Ontology enrichment.

Target gene enrichment and Gene Ontology analysis

Target gene enrichment analysis to assess whether a specific set of genes is overrepresented or enriched in predefined gene sets or functional categories compared to what would be expected by chance was done using ShinyGO (v0.77). The list of differentially expressed genes (DEG) was analyzed for enrichment in Gene ontology (GO) classification and functional enrichment (Ge *et al.*, 2020). The main role of Gene ontology was to classify the target genes into molecular function (mf), biological process (bp), and cellular component (cc). The FDR parameter was set to 0.05, and the maximum pathway parameter to 20. The enrichment analysis results were visualized using the *ggplot* R package, v3.4.2 (Villanueva & Chen, 2019).

Analysis of target gene expression levels

Previously designed primers (Tab. 1) for some target genes were assessed by PCR. For this, 2 µl of synthesized cDNA samples were used as a template in a final volume of 20 µl containing 1x SYBR Green Mix (Hibrigen, Turkey) and gene-specific primers. The primers were designed using NCBI Primer BLAST and the list of primers is given in Table 1. qPCR was performed on a CFX 96™ Real-Time System (BIORAD, USA). The reaction conditions were as follows: 95 °C for 1 minute, 40 cycles of 95 °C for 15 s and at the following annealing temperatures for the different genes (PHABULOSA, REVOLUTA, RH39 at 64°C; PHAVOLUTA, TCP24 at 63.5°C; CORONA, UBC24 at 62°C) for 15 s and final extension at 72°C for 45 s. Melting curve analysis was performed for all reactions. Following qRT-PCR, differences in gene expression levels were assessed by analyzing the mean C_T values with the positive control housekeeping gene *Actin*. *Actin* was chosen due to its consistent and stable expression in various tissues and developmental stages across different experimental conditions (Czechowski *et al.*, 2005; Kozera & Rapacz, 2013; Chen *et al.*, 2019). The relative gene expression was quantified using the comparative ΔΔCT method (Livak & Schmittgen, 2001).

Table 1. Primer sequences used for gene expression analysis by qPCR

Gene Name	Gene ID		Primer Sequences (5'-3')
REVOLUTA	AT5G60690	Forward Reverse	CGACGGGTGGTCTACGATGC AGAGCACGCCTCCAAGGAAC
CORONA	AT1G52150	Forward Reverse	AAACCAGGCGGGACTTGACA GCGAAGCCCTGTTGCATGAT
PHAVOLUTA	AT1G30490	Forward Reverse	GGAAGTGGTGTCCTCTGTGCC CTGCCCATTCAGCTCGGTGT
PHABULOSA	AT2G34710	Forward Reverse	CGACGGGTGGTCTACGATGC AGAGCACGCCTCCAAGGAAC
TCP24	AT1G30210	Forward Reverse	CGGAGACTTTTGATGGTCGGC ATGCTCAGGGCTGCACAAAC
UBC24	AT2G33770	Forward Reverse	AAAGGGTTGGCTCAGTCCCA AGCCCATCGTGATATGGCGT
RH39	AT4G09730	Forward Reverse	ACCGTGTGGCGAAGTCCATA ACCAGGGGTTCCAACAACCA
ACTIN	AT1G49240	Forward Reverse	GCCAGTGGTCGTACAACCG TCCATGAGGTAATCAGTAAGGTAC

Results

Analysis of Sequencing data

After trimming adapters, low-quality reads were filtered based on sequence quality, read length, and abundance of non-miRNA sequences. Checks performed based on read length ensured all reads were between 18–26 bases. In total, 51 million clean reads were obtained (Table 2).

Alignment of Reads to the *Arabidopsis thaliana* Reference Genome

Clean reads were aligned to the *Arabidopsis thaliana* reference genome using the Bowtie software (v 1.3.1) for mapping (Langmead *et al.*, 2009). The *Arabidopsis thaliana* reference genome (TAIR10) was used, and its annotations were obtained from the Ensembl (Cunningham *et al.*, 2022). Firstly, an index was created for the reference genome alignment. After indexing with Bowtie, index files with "ebwt" extension were created in various sizes and numbers, and alignment was performed. Alignment files in SAM (sequence alignment map) format of each sample were obtained. The resulting SAM files were used as input in the abundance analysis.

Determining Abundance Levels of Small RNAs

This was performed using the 'featureCounts' command of the sub-read (v2.0.3) software.

The control (A), nitrogen deficiency (B), drought (C), and combined (D) stress conditions were aligned to the reference genome at rates of 93.20%, 94.15%, 93.13%, and 93.21% respectively, as seen in Table 3.

Sequencing analysis detected 247 known miRNAs for the control sample, 322 for the nitrogen deficiency, 329 for the drought, and 324 for the combined stress condition. An average of

Table 2. Sequencing statistics for the *Arabidopsis* transcriptome.

Sample	Raw reads	Clean reads	Clean reads Q20 (%)	Clean reads Q30 (%)	Clean reads ratio (%)
Control (A)	12,606,109	12,408,557	99.23	97.20	98.43%
Nitrogen deficiency (B)	12,863,663	12,661,613	99.34	97.42	98.43%
Drought (C)	12,381,853	12,047,539	98.82	96.30	97.30%
Combination (D)	15,118,462	14,628,023	99.27	97.28	96.76%

Table 3. Alignment ratios of reads to the reference genome.

Treatment	A	B	C	D
Read number	708,936	737,950	588,979	836,976
Aligned read number	660,705	694,800	548,493	780,133
Alignment (%)	93,20	94,15	93,13	93,21

178 miRNAs were identified in all stress conditions (Fig. 1). Nineteen known miRNAs were common in the B & C sample, 9 in A & B, 24 in C & D, and 4 in A & D (Fig. 1).

Combined drought and nitrogen deficiency-responsive miRNAs

The DESeq2 software identified 117 differentially expressed miRNAs belonging to 12 families (mir156, mir157, mir158, mir159, mir160, mir161, mir162, mir163, mir164, mir165, mir166, and mir167) between the comparisons (Fig. 2). Differentially expressed miRNAs for each comparison were also obtained. The resulting p-values for each comparison were adjusted to control the false discovery rate (FDR), and miRNAs that met the criteria of adjusted p-value (padj) < 0.05 and log2 fold change > 1 were identified as upregulated miRNAs, while miRNAs that satisfied the criteria of padj < 0.05 and log2 fold change < -1 were identified as downregulated miRNAs.

Gene families mir156, mir157, and mir158 were consistently expressed across all conditions. All 12 families were exclusively expressed in the nitrogen deficiency and combined comparison (Fig. 4). The mir156 family had the most significantly upregulated miRNAs, with mir156j having an 8.12 log2foldchange, mir156d a 7.49 log2foldchange, while mir159b and mir158b had similar log2foldchange of 7.26. Generally, mir157b, mir157c, mir158a, mir158b, mir159a, mir159b, mir159c, mir160b, mir160c, mir162a, mir163, mir164a, mir164b, and mir166b were upregulated just for the nitrogen deficiency treatment whilst the downregulated miRNAs for this condition included

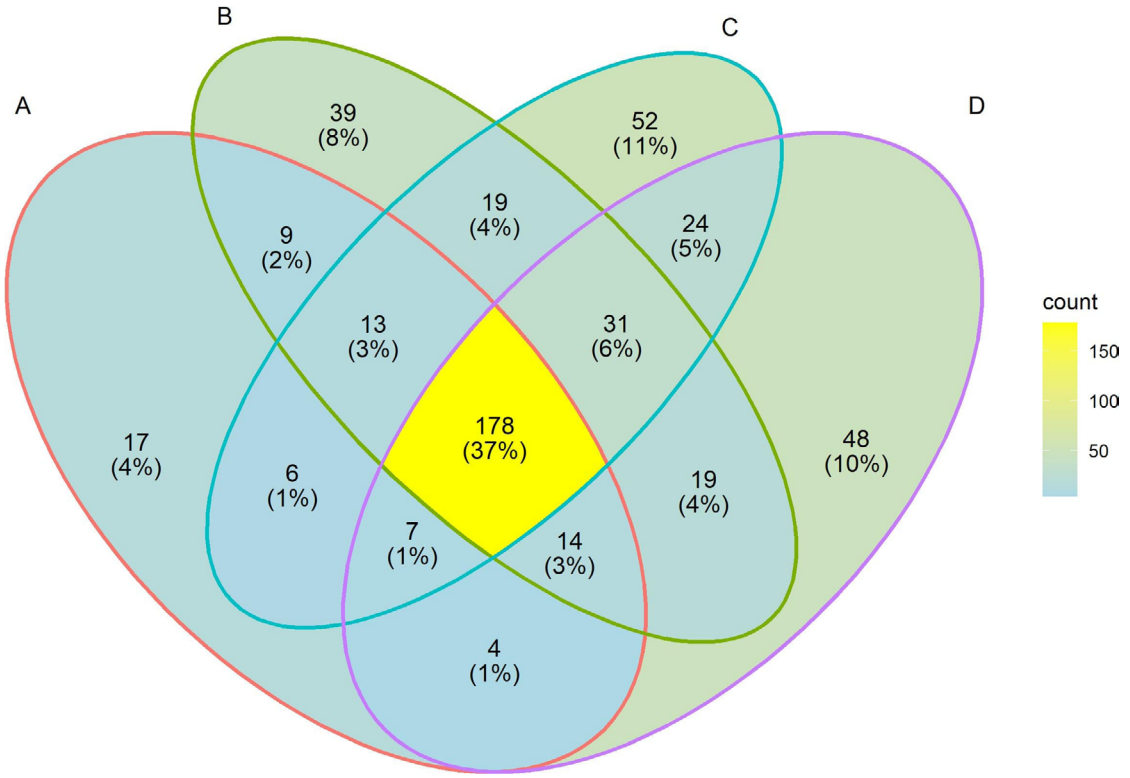

Figure 1. Venn diagram showing the intersection of miRNA found. A (control), B (nitrogen deficiency), C (drought), D (combination).

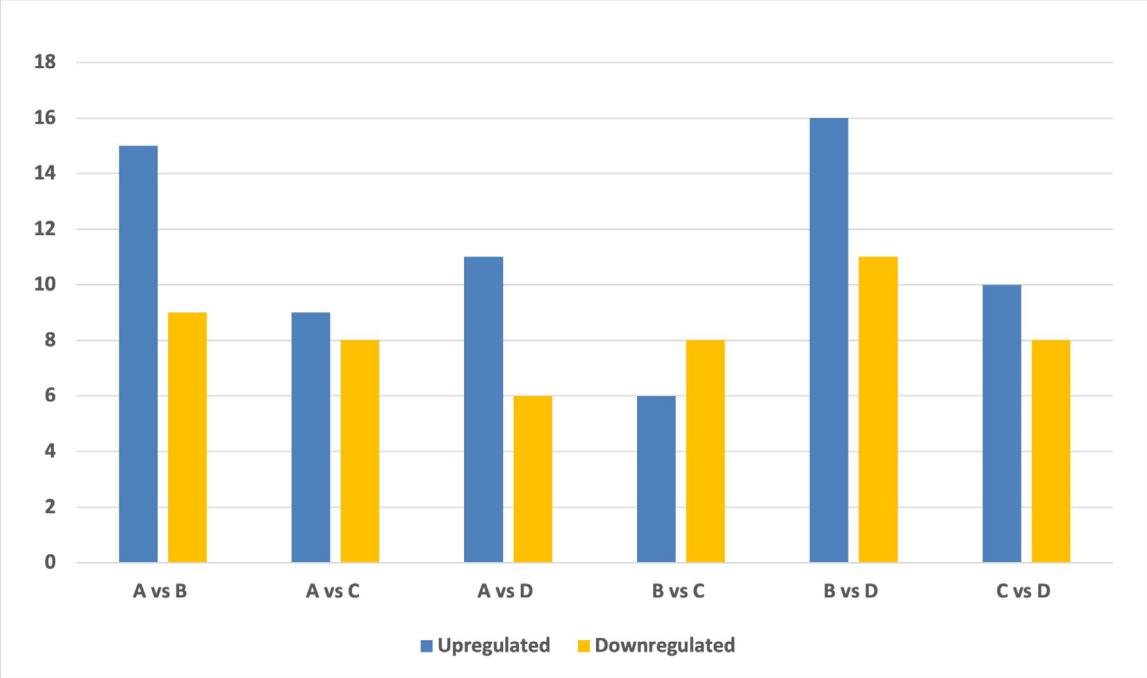

Figure 2. Statistics of differentially expressed miRNAs between comparisons. The X-axis represents comparisons between sample groups. The Y-axis represents the number of differentially expressed miRNAs. Blue: upregulated, red: downregulated.

mir157a, mir165a, mir165b, mir156a, mir156f, mir156d, mir156c, mir156j, mir156b (Fig. 4A).

Apart from mir156f, mir160a, and mir160b, the miRNA upregulated during the nitrogen deficiency treatment (mir157b, mir158b, mir159a, mir159c, mir161, mir162a) were also upregulated for the drought treatment. However, mir156d, mir 156j, mir156a, mir156b, and mir156c, in addition with mir157c, mir158a, and mir160c showed a downregulation in drought treatment. Table 4 gives a detailed comparison and expression changes of these miRNAs across conditions.

Table 5 summarizes the target genes of these differentially expressed miRNAs. High numbers of differentially expressed target genes for the miRNAs were obtained for each comparison as the maximum parameter for the target genes was set at 200 (Fig. 3). A detailed list of these miRNAs and predicted genes is available at Table S3.

Target gene enrichment analysis and Gene Ontology (GO)

The analysis in this study showed a statistically significant enrichment of genes in the biological process and molecular function categories (Table S4). The most common subcategories under molecular function were the transcription regulator activity (GO:0140110), DNA binding transcription factor activity (GO:0003700), RNA helicase activity (GO:0003724), and ATP dependent activity (GO:0140657). The most enriched GO genes are shown in Fig. 5. Among the top 19 DEGs between A *vs.* B in the molecular function category, polynucleotide phosphatase (GO:0098518), lipid transporter activity (GO:0005319) and RNA helicase (GO:0003724) activity were the top 3 categories with 4, 15, and 18 unigenes respectively; RNA helicase activity (GO:0003724), ATP-dependent activity on RNA (GO:0008186) as well as Helicase activity (GO:0004386) for A *vs.* C comparison (Fig 5C). While for the A *vs.* D comparison, miRNA binding (GO:0035198), regulatory RNA binding (GO:0061980), and lipid transporter activity (GO:0005319) were significantly enriched.

In the biological process, the most common and most enriched terms were the stamen development (GO:0048443), positive regulation of development (GO:0045962), androecium development (GO:0048466), floral whorl development (GO:0048438), floral organ development

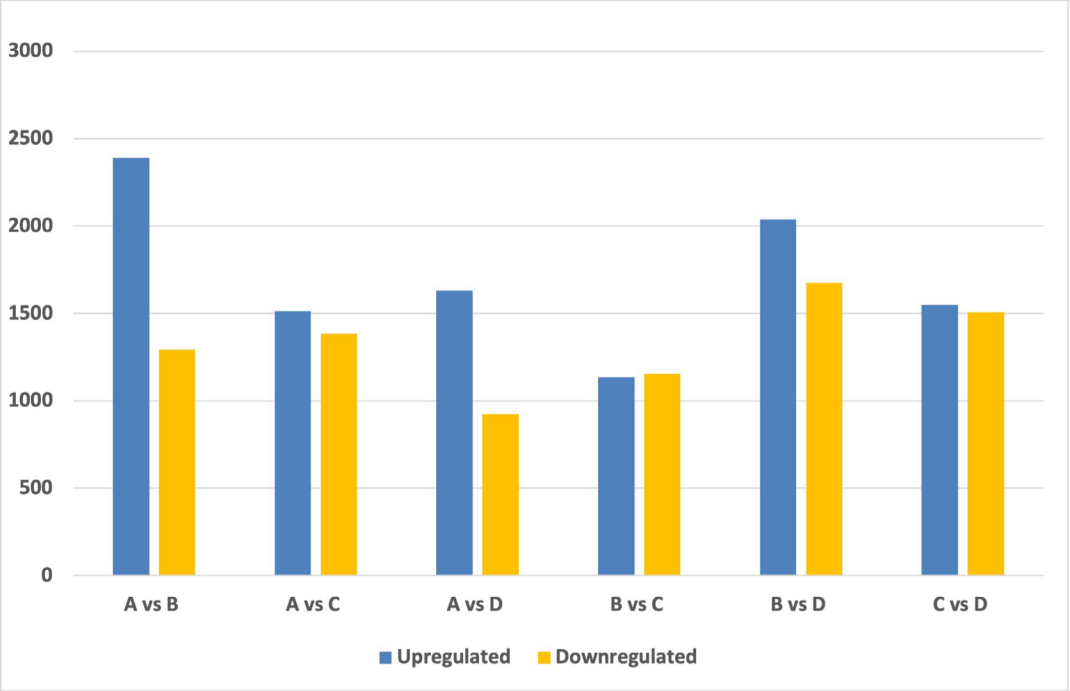

Figure 3. Statistics of differentially expressed miRNA target genes. Red columns represent downregulated miRNA target genes, and blue represent upregulated miRNA target genes.

(GO:0048437), and pollen development (GO:0009555). However, these subcategories varied within the comparison. Differentially expressed genes in the combined stress condition were generally under the processes of growth regulation, gene silencing pathways, and signal transduction pathways. Interestingly, gravitropism (GO:0048653), response to gravity (GO:0009629), and tropism (GO:0009606) processes were specific to drought stress treatment only. These, however, target a wide range of genes, some of which were identified as *A. thaliana* Peroxidase 34 (Prx34), Syntaxin of plants 41, Pip2;2 (Plasma Membrane Intrinsic Protein 2;2), and Receptor-Like Protein 23 amongst others.

Expression of target genes

The comprehensive analysis of gene expression patterns under various stress conditions in *Arabidopsis* yielded insightful findings about the regulatory responses of several genes in this study (PHAVOLUTA, TCP24, PHABULOSA, REVOLUTA, RH39, CORONA, and UBC24). All four genes encoded by the basic leucine zipper transcription domain (HD-Zip III), PHAVOLUTA, PHABULOSA, REVOLUTA, and CORONA, were implicated in all the stress conditions (Fig. 6). Results demonstrated a statistically significant downregulation in the nitrogen deficiency, drought and combined stress conditions for the PHABULOSA and the REVOLUTA genes, while the PHAVOLUTA and CORONA showed a significant upregulation for the nitrogen deficient condition and PHAVOLUTA gene demonstrated a downregulation in the drought and combined stress conditions.

Discussion

Drought and nitrogen deficiency significantly affect plant growth and development. The combined occurrence of these often results in a mutual interaction leading to several pathways and expression of certain genes (Li & Wang, 2023; Cerda & Alvarez, 2024). Plants possess the ability to synthesize or modulate the expressions of specific miRNAs in direct response to stress (Valinezhad-Orang *et al.*, 2014; Dong *et al.*, 2022). This study identified 1,222 known miRNAs in all conditions, 117 differentially expressed miRNAs, and 1,7003 corresponding target genes between comparisons.

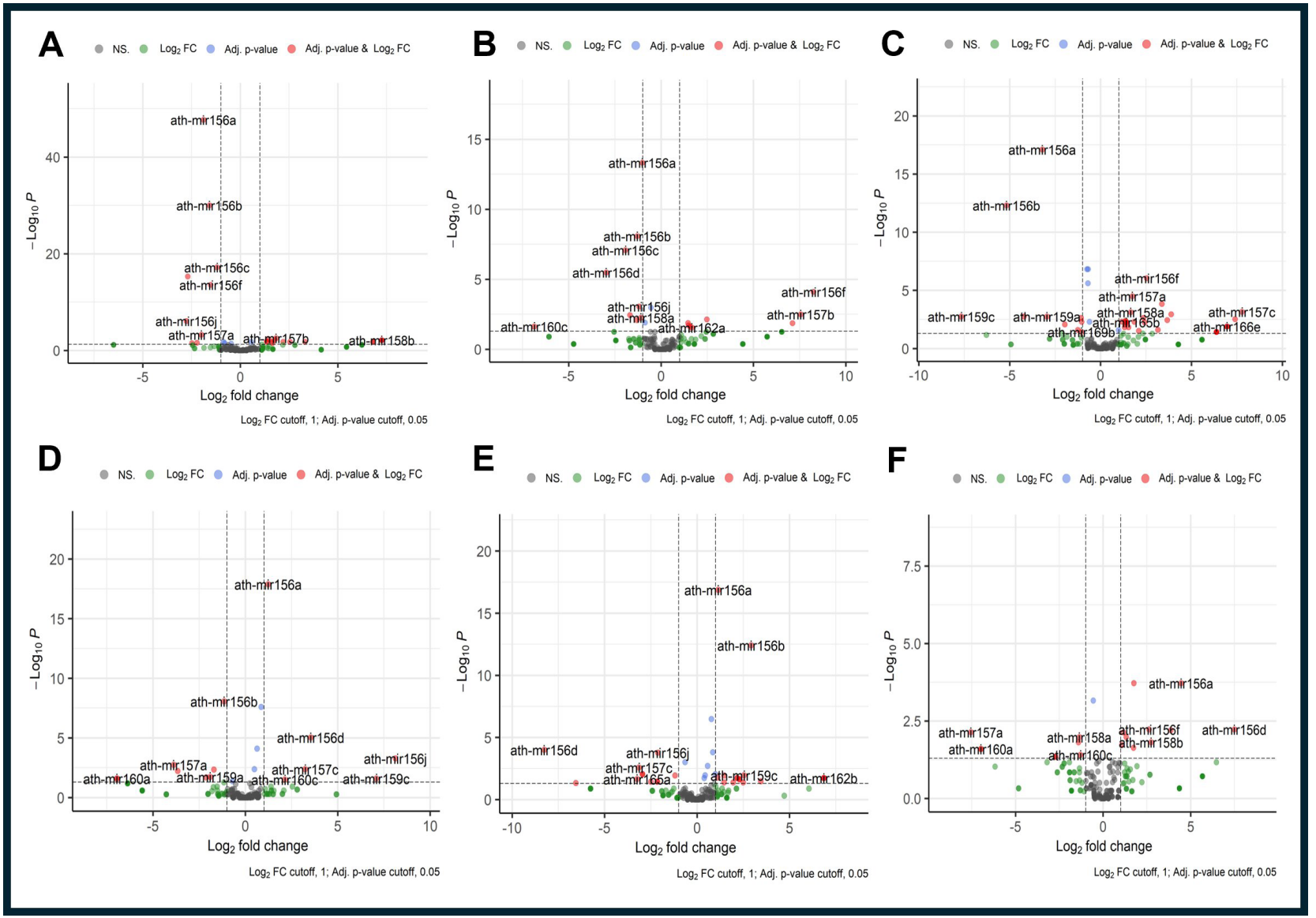

Figure 4. Volcano plots of DEGs. The y-axis shows the statistical significance of changes in miRNA expression. Higher -log10p suggests greater statistical significance. The x-axis shows fold change expressions. A: control vs nitrogen deficiency; B: control vs drought; C: control vs combination; D: nitrogen deficiency vs drought; E: nitrogen deficiency vs combination; F: drought vs combination.

Table 4. Expression changes of miRNAs across nitrogen deficiency, drought and combined stress conditions.

miRNA	B fold change	B p-value	B- FDR	C fold change	C p-value	C-FDR	D fold change	D p-value	D – FDR
mir156a	-1.8901	$9.822e^{-51}$	$2.062e^{-48}$	-1.0185	$2.252e^{-16}$	$4.731e^{-14}$	-3.2048	3.7570	$7.889e^{-18}$
mir156b	-1.5807	$9.326e^{-33}$	$9.792\ e^{-31}$	-1.2840	$7.864e^{-11}$	$8.261e^{-9}$	-5.1710	4.8722	5.1158
mir156c	-1.2013	$9.238\ e^{-20}$	$6.466\ e^{-18}$	-1.9364	$1.214e^{-9}$	$8.503\ e^{-8}$			
mir156d	-2.6878	$9.722\ e^{-18}$	$5.104\ e^{-16}$	-2.9753	$6.372e^{-8}$	0.0000			
mir156f	-1.5195	$6.682\ e^{-16}$	$2.785e^{-14}$	8.2497	0.0001	0.0001	2.534	2.1501	$9.030e^{-7}$
mir156j	-2.7644	$2.2978\ e^{-7}$	$8.042\ e^{-7}$	-1.1915	0.0000	0.0001			
mir157a	-1.9942	0.0000	0.0005				1.7471	0.0000	0.0000
mir157b	1.8358	0.0001	0.0029	7.5651	0.0001	0.0032	3.3759	0.0000	0.0001
mir157c				-1.6834	0.0001	0.0036	7.7731	0.0000	0.0007
mir158a	1.7632	0.0003	0.0064	-1.0900	0.0002	0.0056	1.6649	0.0000	0.0007
mir158b				2.4790	0.0003	0.0074	3.8850	0.0000	0.0011
mir159a	7.2618	0.0004	0.0078	-1.3069	0.0004	0.0084	-2.9442	0.0001	0.0018
mir159b							-4.2130	0.0001	0.0018
mir159c				1.4584	0.0008	0.0132	-7.6653	0.0001	0.0018
mir160a				7.1143	0.0009	0.0136	-1.0680	0.0001	0.0023
mir160b	1.3692	0.0013	0.1663	1.4950	0.0015	0.0200	2.3610	0.0001	0.0023
mir160c				-6.875	0.0019	0.0241	7.4035	0.0002	0.0030
mir161	3.3186	0.0015	0.0166	1.5958	0.0021	0.0248	3.6691	0.0003	0.0036
mir162a	1.6092	0.0015	0.0166	1.6679	0.0026	0.0291			
mir162b							1.1686	0.0004	0.0049
mir163	2.5557	0.0016	0.0166						
mir164	6.8132	0.0019	0.0178						
mir165a	-2.2182	0.0029	0.0257						
mir165b	-2.4745	0.0037	0.0313						
mir166	1.6511	0.0042	0.3333						

B: Nitrogen deficiency, C: Drought, D: Combine, FDR: False Discovery Rate

Table 5. Target gene numbers of differentially expressed miRNAs belonging to the comparison groups.

Comparison	Upregulated Target Gene Number	Downregulated Target Gene Number
A *vs.* **B**	2,390	1,292
A *vs.* **C**	1,512	1,384
A *vs.* **D**	1,631	923
B vs.C	1,136	1,354
B *vs.* **D**	2,039	1,676
C *vs.* **D**	1,549	1,507

Most of these target genes were associated with the biological process and molecular function categories of GO terms.

miRNAs were grouped into different miRNA families and mature strands (Table S1). Among the differentially expressed miRNAs in this study, mir156a, mir156b, mir156f, mir157a, mir157c, mir158a, mir159a, mir159c, mir165b, mir166e were commonly expressed in the combined stress condition. The majority of them had previously been reported as conserved between *Arabidopsis* and *Oryza sativa* plant species (Wang *et al.*, 2004;

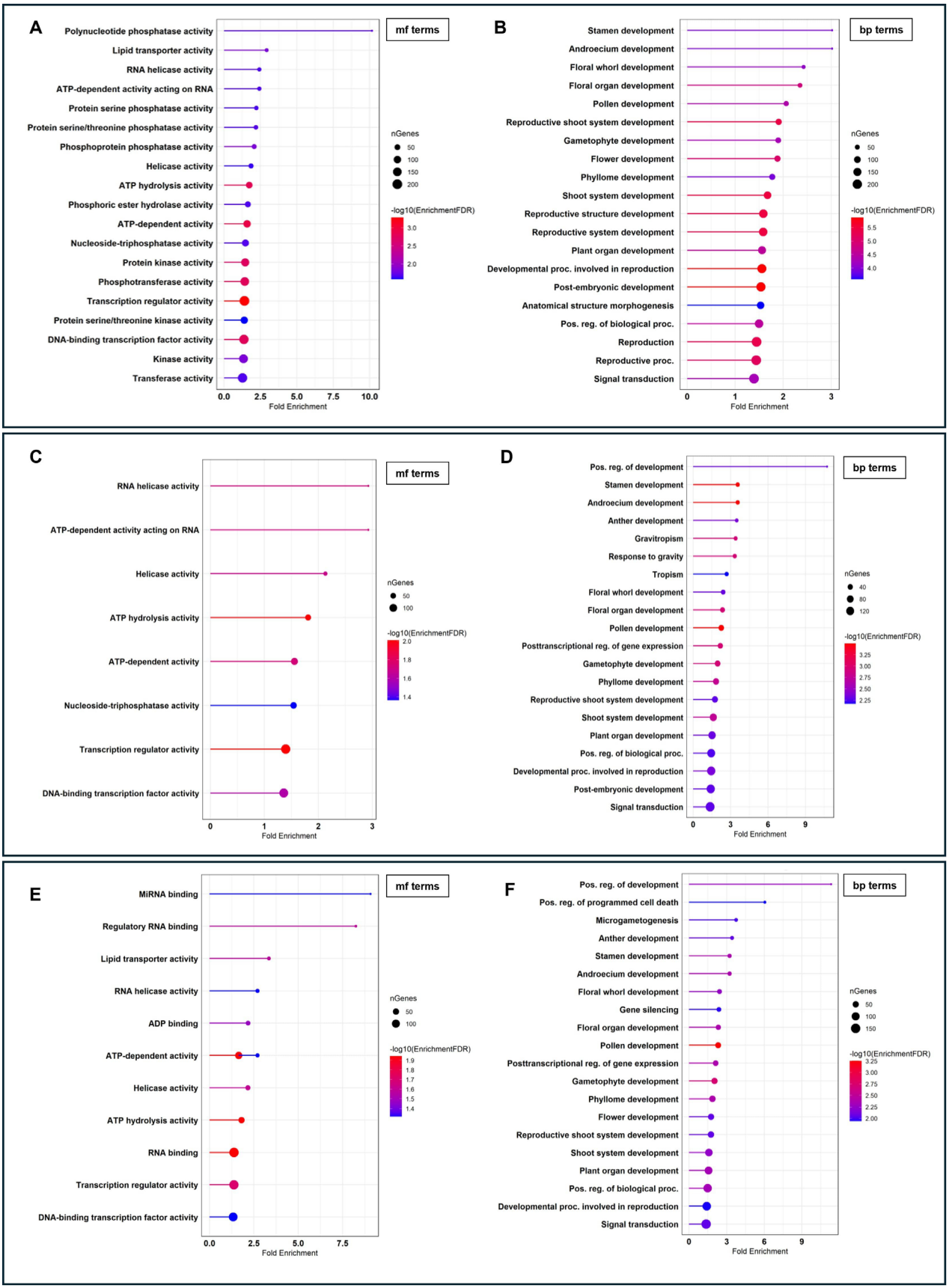

Figure 5. Target gene enrichment analysis of **A**: control vs nitrogen deficiency (MF), **B**; control vs nitrogen deficiency (BP) **C**; control vs drought (MF), **D**; control vs drought (BP), **E**; control vs combine (MF), **F**; Control vs combine (BP),

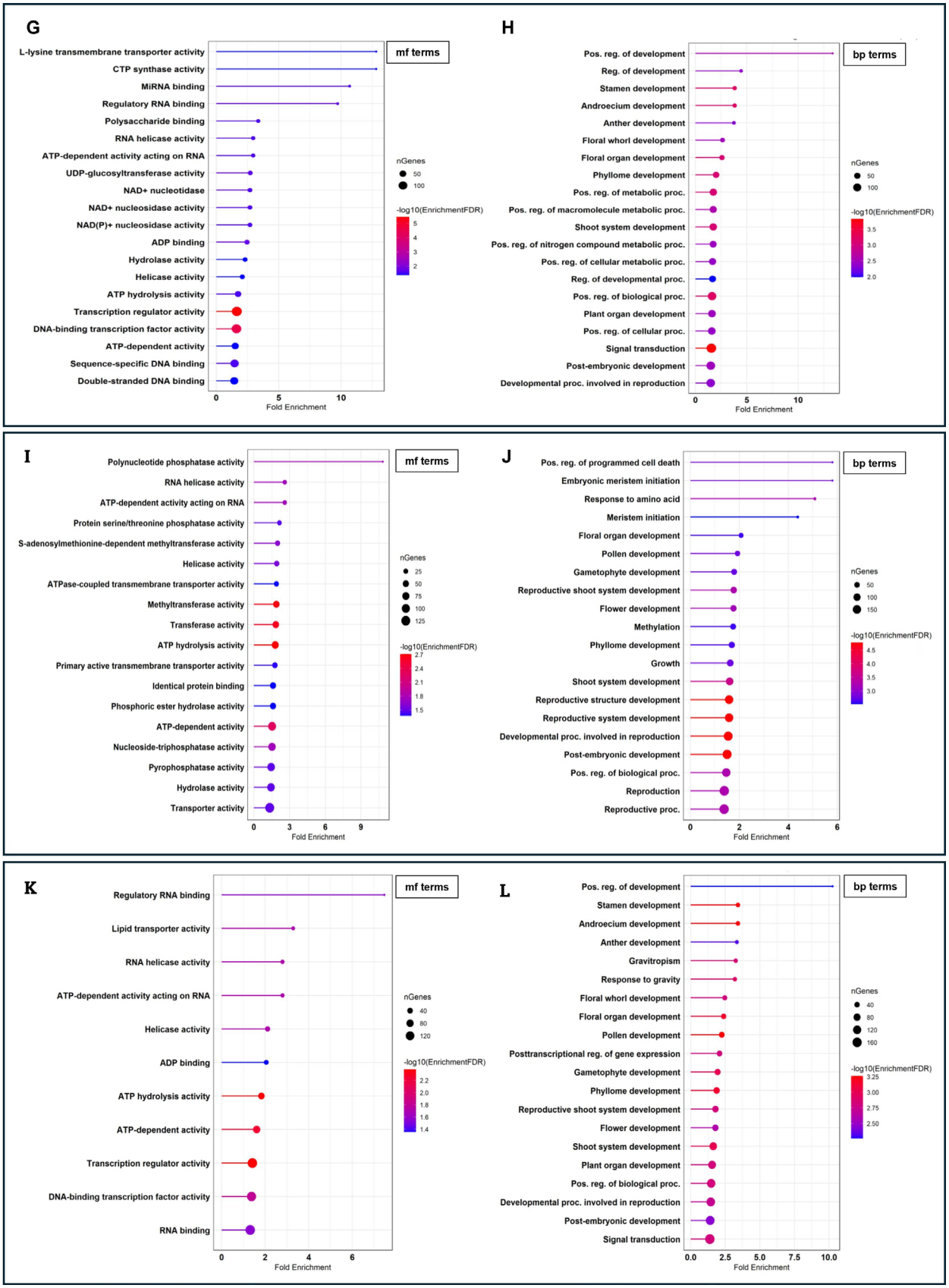

Figure 5. Cont. **G**; nitrogen deficiency vs drought (MF), **H**; nitrogen deficiency vs drought (BP) **I**; nitrogen deficiency vs combine (MF), **J**; nitrogen deficiency vs combine (BP), **K**; drought vs combine (MF), **L**; drought vs combine BP). The FDR parameter was set to 0.05 and the maximum pathway parameter to 20.

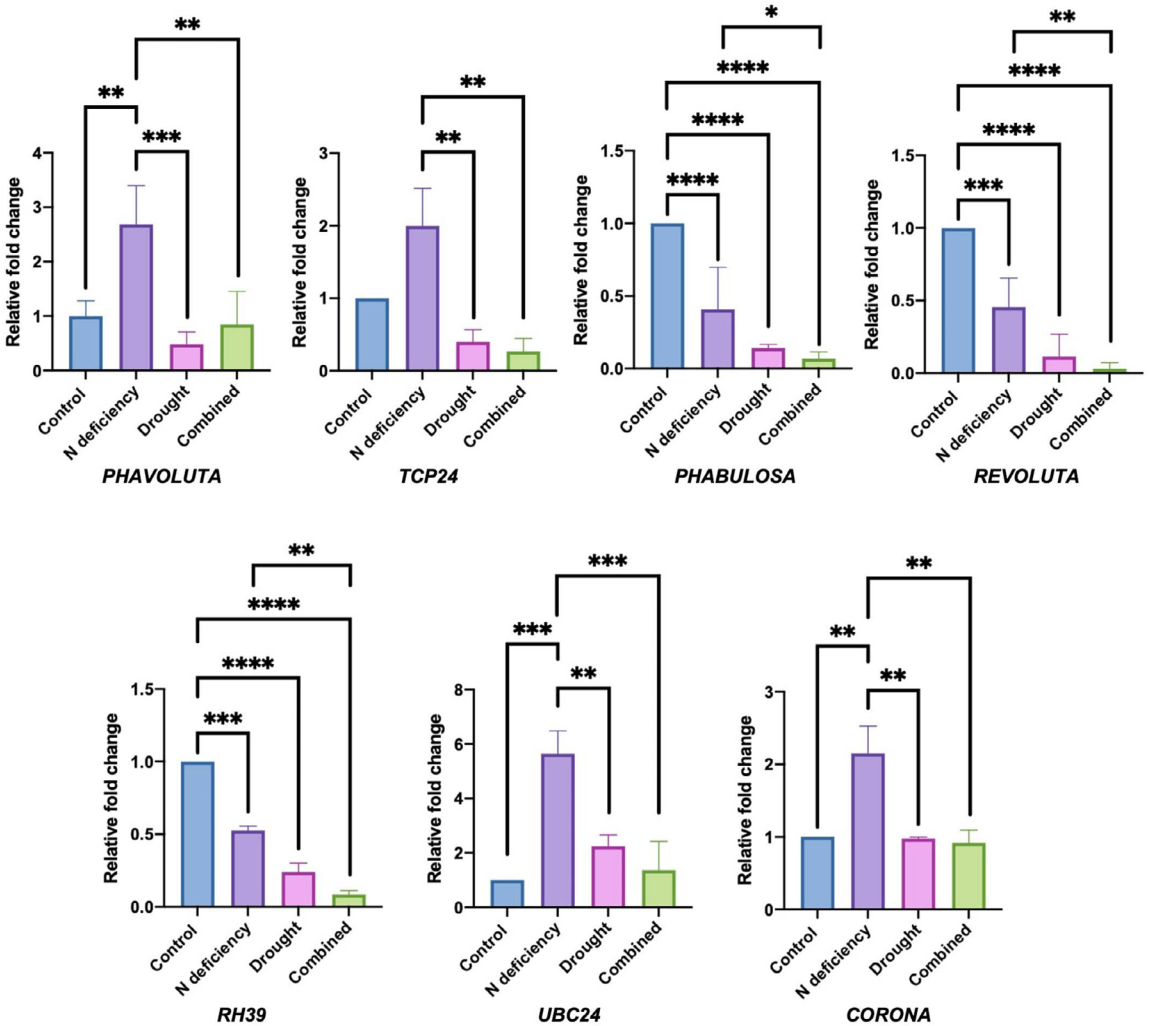

Figure 6. The relative expression levels of *A. thaliana* genes under Control, N deficiency, Drought, and Combined conditions.

Shah & Ullah, 2023). The ability of these miRNAs to mediate combined complex responses highlights their potential as targets for bioengineering crops with improved resilience to multiple stressors (Patil *et al.*, 2021). Under combined stress conditions, mir156f, mir157a, mir158a, mir157c, and mir166e were significantly upregulated as opposed to lower expression when one stress condition was taken into consideration (mir157b and mir158b in nitrogen deficiency and mir162a, mir157b, and mir156f in drought treatment). This reveals the unique regulatory dynamics of miRNAs under combined stress conditions. The downregulation of mir156a and mir156b under nitrogen deficiency and drought stress could indicate a potential shift in resource allocation, prioritizing survival over growth by altering developmental processes (Singh *et al.*, 2020; Yaşar *et al.*, 2024). mir156, previously characterized as a prominent microRNA (Wang *et al.,*2023) in stress responses and plant biological processes, was also found to be the most prevalent in this study. Past studies in *Arabidopsis* have demonstrated the involvement of members of this family in regulating drought stress responses. Zheng *et al.* (2016) reported an upregulation of mir156 under drought stress. According to Chen *et al.* (2023), an overexpression of mir156 in *Arabidopsis* resulted in increased drought tolerance, suggesting a positive role for mir156 in drought stress adaptation. Similarly, Ding *et al.* (2013) and Wen *et al.* (2024) identified drought-responsive genes targeted by mir156 in *Arabidopsis* and *Camellia*, respectively, indicating their regulatory role in drought stress signaling pathways. Despite limited research specifically linking mir156f and mir156j to nitrogen deficiency in *Arabidopsis,* studies have

revealed the responsiveness of mir156 to nitrogen availability in maize, suggesting a potential role in nitrogen metabolism (Xu *et al.*, 2011; Zhao *et al.*, 2012). This study reported a downregulation for mir156f in nitrogen deficiency and an upregulation in drought and combined stress conditions. These findings underscore mir156's central role in coordinating stress-responsive regulatory networks, particularly through its SPL3 (Squamosa promoter binding protein-like) target, and position it as a key candidate for enhancing crop resilience to multiple stresses.

The expression of mir159 is induced under drought conditions, thus negatively regulating its target gene- MYB33 (Reyes & Chua, 2007). In a study by Liang *et al.* (2015), the expression of mir165 was found to be responsive to nitrogen availability in *Arabidopsis* roots. They observed increased levels of mir165 under nitrogen deficiency conditions. This pattern was similar for mir160 (Liang *et al.*, 2012), suggesting a potential role in nutrient stress responses. Moreover, mir166 was found to be differentially expressed in response to drought stress in rice (Zhang *et al.*, 2018) and maize (Li *et al.*, 2020). Yaşar *et al.* (2024) reported a consistent significant downregulation of mir165a-3p after 7- and 14-day treatment of callus tissues under combined nitrogen deficiency and drought stress conditions, suggesting long-term adaptation to stress by modulating the expression of its target.

We observed that individual miRNAs displayed differential expression when subjected to different stress treatments; mir160c which was significantly upregulated (7.49 log2 fold change) in combined stress conditions was significantly downregulated (-6.87 log2 fold change) in drought treatment. Likewise, the upregulation of mir160a in drought and downregulation in combined stress. This was the same with mir156d upregulated (7.49 log2 fold change) in combination stress but downregulated (-2.68 log2 fold change) in nitrogen deficiency. This observation highlights the specificity of miRNA responses to distinct stress treatments. Interestingly, even members from the same miRNA family exhibited varying expression patterns within treatments. According to Gurtan & Sharp (2013), this is because they are regulated by different transcription factors or signaling pathways. An example is mir157a with target genes such as SPL13, NAC, ATHB-5, TCP2, MYB57, ATRH25, RLP9, PRR8, and bZIP transcription factor family protein that was upregulated in combined conditions, while its isoform mir157b was upregulated for the drought treatment only. All identified miRNAs had at least 50 target genes (Table S1). Unlike the other miRNAs encoding different protein domains, mir156j was specific to SPL. mir156 has been recorded to target SPL genes and regulate their expression levels, thereby influencing plant developmental processes (Li *et al.*, 2023). mir156j is known to target genes involved in developmental processes, such as the regulation of flowering time and phase transition from vegetative to reproductive growth (Wang *et al.*, 2023). The upregulation of mir156j may indicate a reprogramming of developmental pathways to prioritize stress adaptation and survival.

Wang *et al* (2012), reported sensitivity of mir159 to heat stress in *Oryza sativa*, therefore, their downregulation seen in this study could be part of the plant's adaptation to the stress conditions. Our analysis revealed that the mir159 family encodes targets for the MYB domains 33, 65,81,97,101,104 and 120, TCP transcription factor 4, TCP2, PCF 24, k-box and MADS-box transcription factor family, protein kinase C-like zinc finger protein, homeodomain-like superfamily protein, mitochondrial inner membrane translocase complex, actin-related protein C3 plant regulator RWP-RK family protein and several other proteins (Table S1). Meanwhile, mir158b targets the MYB domain protein 16, Phosphate-responsive 1 family protein, remorin family protein, histone acetyltransferase of the MYST family 1, flavin-monooxygenase glucosinolate S-oxygenase 2, regulator of chromosome condensation (RCC1) family with FYVE zinc finger domain.

Contrary to our study, Fischer *et al.* (2013) recorded an upregulation of mir160 targets under nitrogen starvation. The significant downregulation of mir160a and mir160b under nitrogen deficiency and drought stress could imply suppression of their regulatory activity in response to these stresses. The subfamilies mir160a and mir160b are

involved in auxin signaling response, which plays essential roles in plant growth, development, and stress adaptation (J Yang *et al.*, 2021; Luo *et al.*, 2022). The decrease in expression of these miRNAs might indicate a reorganization of auxin-related pathways, prioritizing stress resilience over growth stimulation. ARF10 and ARF17 are the specific targets of mir160 in *Arabidopsis* (Liu *et al.*, 2007), which likely function in transcriptional repression and hormone signaling (Mallory *et al.*, 2005). Together with ARF10 and ARF17, we identified another -ARF16 previously not reported. Our data revealed the ARF genes were targeted by the 5p-derived miRNAs, notably mir160c-5p, suggesting strand-specific targeting. mir157b was upregulated in response to nitrogen deficiency and drought individually; its expression was modulated differently in the context of combined stress. Crosstalk between stress signaling pathways may result in synergistic or antagonistic interactions that influence the expression patterns of miRNAs (Shriram *et al.*, 2016). Crosstalk is the interaction of multiple signaling pathways in plants involved in simultaneous developmental processes and stress responses (both abiotic and biotic) in plants (Sunkar & Zhu, 2007; Luo *et al.*, 2022; Samynathan *et al.*, 2023). Hormonal signaling involving abscisic acid, jasmonic acid, salicylic acid, and ethylene plays a central role in this process (Singh *et al.*, 2017) as miRNAs interact with key components of hormonal signaling pathways, allowing plants to balance responses to both form of stress. The mir165/166 family regulates root growth by targeting HD-ZIP III genes, which are also modulated by various phytohormones (Singh *et al.*, 2017). mir398 regulates responses to both oxidative stress and pathogen infection, modulating levels of reactive oxygen species, a common signaling molecule in various stress pathways (Zhang *et al.*, 2022). mir159 influences ABA signaling (involved in drought stress) and can also affect pathogen defense, demonstrating its role in balancing responses between conflicting stresses (Kumar *et al.*, 2021). Similarly, mir156 and mir160 target transcription factors involved in auxin and jasmonic acid signaling, allowing plants to adjust their growth and stress responses under combined stresses (Luo *et al.*, 2022). mir5658 and mir172 interact with transcription factors like the ERF/AP2 family, influencing transcriptome changes in response to stress (Izadi *et al.*, 2017). Wu *et al.* (2022), examined the role of miRNA-miRNA crosstalk in adaptation to environmental stress of plant populations of *Arabidopsis thaliania* through genetic variation and phenotypic plasticity.

Gene Ontology analysis revealed the following enriched processes: Transcription regulator activity (GO:0140110), DNA binding transcription factor activity (GO:0003700), RNA helicase activity (GO:0003724), and ATP-dependent activity (GO:0140657). The significant enrichment of both the DNA binding transcription factor activity and transcription regulator activity suggests that the identified DEGs include transcription factors and other regulatory proteins that directly bind to DNA and modulate the transcription of target genes. These factors and other DNA-binding proteins function as key nodes in regulatory networks, orchestrating the expression of stress-responsive genes and coordinating adaptive responses to stress (Suter, 2020). Based on enrichment analysis, 138 genes were associated with the transcription regulatory activity in the combined stress treatment. Some of the transcription factor genes included SPL (Squamosa Promoter Binding Protein-Like), MYB (Myeloblastosis) family, Homeodomain-like superfamily protein, Zinc Finger family, and AP2/ERF (APETALA2/Ethylene Response Factor) family with DREB/CBF (Dehydration-Responsive Element-Binding/C-repeat Binding Factor) family, specific to drought treatment (Sunkar *et al.*, 2012). Transcription factors involved in abscisic acid signaling influence nitrogen metabolism and responses to nitrate signals (Cerda & Alvarez, 2024). In the combined drought and nitrogen stress conditions, polynucleotide phosphatase activity, ATP-dependent activity acting on RNA, and RNA helicase activity were most enriched. Polynucleotide phosphatases can modulate gene expression by affecting the stability or processing of specific RNA molecules such as non-coding RNAs, through targeted dephosphorylation events (Bandyra *et al.*, 2016). The Phosphoesterase, APE2, and mRNA capping enzyme family proteins were

the target proteins identified in this study. ATPases function in osmotic regulation in drought conditions (Li *et al.,* 2022). RNA helicase regulates DNA/RNA metabolism under stress conditions (Singha *et al.*, 2017). The biological process-positive regulation of development (fold enrichment -11.33) and positive regulation of programmed cell death (fold enrichment -6.04) were enriched in the combined stress treatment. The high enrichment of the former may be a mechanism to optimize resource utilization during stress conditions by enhancing root growth and leaf expansion, which are crucial for accessing water and nutrients in the soil (Krouk *et al.*, 2010). Programmed cell death (PCD) is particularly relevant under drought conditions, where plants may selectively eliminate cells to conserve resources. Research indicates that miRNAs play roles in PCD by regulating genes involved in oxidative stress responses, such as the superoxide dismutase and catalase genes (Alonso-Peral *et al.*, 2010; Petrov *et al.*, 2015). miRNA-directed gene silencing is particularly relevant under nutrient deprivation. Under nitrogen stress, mir169 modulates nitrogen-responsive pathways by targeting NF-YA transcription factors. By downregulating NF-YA, mir169 contributes to resource conservation and metabolic adjustment during nutrient stress (Zhao *et al.*, 2011; Liang *et al.*, 2012). mir160 and mir167, are involved in root architecture changes, enhancing root depth and density under drought, which is beneficial when nitrogen is scarce (Gautam *et al.*, 2017; Hao *et al.*, 2022).

Transcription factor genes and associated miRNAs under drought and nitrogen deficiency stress treatments in *Arabidopsis* calli showed varying expression results. mir165 and mir166 control the activity of PHABULOSA, PHAVOLUTA, REVOLUTA, CORONA, and ATHB-8 (Rhoades *et al.*, 2002) while UCB24 and TCP24 are mir319-dependent (Fujii *et al.*, 2005; Kuo & Chio, 2011; Fang *et al.*, 2021). Our results confirmed the negative regulatory role of miRNAs and their associated target genes as the miRNAs downregulated the expression of their target genes (Fig. 6) This enhances the understanding of how miRNAs fine-tune gene expression by downregulating specific targets, enabling plants to conserve energy and optimize metabolic responses. The observed expression patterns of PHAVOLUTA and CORONA genes in all stress conditions suggest their involvement in *Arabidopsis thaliana* stress regulatory pathways (Prigge *et al.*, 2005; Mao *et al.*, 2016). Together with REVOLUTA and PHABULOSA, they regulate meristem initiation and vascular development, with different subsets of the genes being involved in each process (Prigge *et al.*, 2005; Hrmova & Hussain, 2021). However, the CORONA gene showed higher expression levels under nitrogen deficiency. Prigge *et al.* (2005) demonstrated an antagonistic role with the REVOLUTA gene function in the lateral shoot meristem. Consequently, the parallel expression dynamics shared with other genes highlight its potential regulatory relationship and interaction with other transcription factors (Gong *et al.*, 2019; Qiu *et al.*, 2022).

In response to nitrogen deficiency and drought, PHAVOLUTA was upregulated and downregulated respectively. The PHAVOLUTA with its high binding affinity to the pseudo palindromic sequence forms dimers with the HD-Zip region and the leucine zipper domain (Sessa *et al.*, 1998). As the pseudo palindromic sequence is often found in combination with other regulatory elements in the promoters of drought-responsive genes, under such conditions, activated leucine zipper transcription factors recognize and bind to specific DNA sequences (C-box elements) present in the promoter regions of drought-responsive genes (Sornaraj *et al.*, 2016).

Similarly, under combined stress conditions, different signaling pathways activate in response to individual stressors. The crosstalk between the pathways can influence the activation of basic leucine zipper transcription factors, which may be common or distinct depending on the specific combination of stresses (Viswanath *et al.*, 2023). This could explain the effect observed in all stress conditions. There is the possibility for a synergistic activation of stress-responsive genes in combined stress conditions of basic leucine transcription factors leading to a more coordinated response as some genes may have binding sites

for multiple transcription factors. According to Prigge *et al* (2005), these genes are known to play cooperative, opposite, and sometimes independent roles in *Arabidopsis* development. Overlapping transcription factor binding sites may enable plants to integrate multiple stress signals, resulting in a more resilient and adaptive response. A shared expression pattern was observed for REVOLUTA and RH39. The RH39 encodes photosynthetic proteins with the help of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Asakura *et al.*, 2012; X Yang *et al.*, 2021) which are important components in mediating signaling networks between diverse metabolic pathways to withstand various forms of stress. Consequently, these processes facilitate water conservation, regulate stomata closure, and promote nutrient uptake. Based on our findings, we suggest that RH39 plays an important role in crosstalk signaling between diverse metabolic pathways under multiple stress conditions and could be a potential target for crop improvement.

Fang *et al.* (2021) discussed the possibilities of mir319 regulating the expression of TCP genes in drought and salt stress in rapeseed plants. During periods of nitrogen deficiency, TCP24 modulates secondary cell wall thickening (Wang *et al.*, 2015; Rivai *et al.*, 2021). The transactivation domain of TCP24 recognizes and binds to certain DNA sequences, promoting interaction with the transcriptional machinery in *Arabidopsis*. The differences observed between drought and nitrogen deficiency conditions could reveal the distinctive transcriptional adjustments to these stressors. Notably, the higher expression levels of these genes under nitrogen deficiency compared to combined stress suggest a regulatory priority under the latter condition. The contrasting response of the UBC24 gene highlights its sensitivity to nitrogen deficiency. The UBC24 gene is involved in protein-protein interactions and the regulation of various biological processes, such as plant leaf senescence during conditions of nitrogen deficiency (Park *et al.*, 2018). Its phosphate homeostasis role is primarily mediated by mir399 with a downregulated expression when the plant miRNAs are overexpressed (Fujii *et al.*, 2005). These findings not only advance our understanding of miRNA-mediated regulation in plants but also provide insights into developing crop varieties better equipped to withstand the challenges posed by climate change and nutrient deficiency.

This study identified miRNAs differentially expressed and their corresponding target genes in combined drought and nitrogen deficiency stress treatments. We observed that the modulation of miRNA expression was determined by the stress treatment conditions. Combined stress treatments result in fewer downregulated miRNAs compared to upregulated ones. Bioinformatic analysis revealed enrichment of key GO terms related to transcriptional regulation, metabolic processes, developmental processes, enzyme activity, response to stimulus, and nutrient homeostasis, portraying their relation to stress responses in plants. Furthermore, we observed differential expression among the transcription factor genes assessed, confirmed the negative regulatory roles with their corresponding miRNA, and identified other transcription factor targets such as PCF24, TCP2, and TCP4 for the mir159 family and ARF16 for the mir160 family, thus suggesting miRNA targeted transcription activity. Upregulated and downregulated miRNA revealed important protein targets in combined drought and nitrogen starvation stress with strand-specific regulation of mir160c for the ARF genes. miRNA- transcription factor crosstalk unraveled the complex networks and pathways of gene regulation in cells. Strand-specific regulation could likely be a key component in environmental stress regulation.

The identified miRNA profiles from this article can serve as functional biomarkers for selecting drought and nitrogen-deficiency-tolerant crops. We propose using information about these miRNAs in genetic engineering to enhance stress tolerance, especially targeting those involved in root development and stomatal regulation. These results can be combined with traditional and genomic selection methods to facilitate the development of multi-trait crops that maintain yield and quality under stress. We recognize the importance of validating these results with functional assays and

also further research on the specific mechanisms by which miRNA regulatory pathways operate and interact with other components of the stress response network. We also recommend examining responses to a broader range of combined stresses to provide a more comprehensive understanding of plant stress adaptation. Furthermore, the use of field trials to ensure the practicality and sustainability of the engineered crops are also needed.

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Author Contributions

Emmanuela Lemnyuy Wirsiy: Conceptualization (Equal), Data curation (Equal), Formal analysis (Equal), Investigation (Equal), Methodology (Equal), Validation (Equal), Writing – original draft (Equal) . **Elif Pulat:** Conceptualization (Equal), Data curation (Equal), Formal analysis (Equal), Investigation (Equal), Validation (Equal), Writing – original draft (Equal), Writing – review & editing (Equal). **Ali Harun Arslantürk:** Data curation (Equal), Formal analysis (Equal), Visualization (Equal). **Seda Yaşar:** Conceptualization (Equal), Investigation (Equal), Methodology (Equal). Özgür Çakır (Corresponding Author): Conceptualization (Equal), Data curation (Equal), Funding acquisition (Equal), Investigation (Equal), Supervision (Equal), Writing – review & editing (Equal).

Conflict of Interest

The authors declare that there is no conflict of interest.

Supplementary Material

The following online material is available for this article:

Table S1. Identified miRNA families, sequences, and predicted roles in the stress response of target genes.

Table S2. miRNA expression changes across different stress conditions.

Table S3. miRNAs and their predicted target gene ID.

Table S4. Enriched GO categories with enrichment scores and enrichment FDR values.

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