In silico identification and prediction of *Coffea arabica* L. microRNA and their potential role in drought stress regulation

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Abstract
MicroRNA is a small endogenous RNA molecule that inhibits gene expression in eukaryotes. Identifying such small RNA entities is critical for understanding the regulation of metabolic pathways in plant systems. In this study, a computational technique was employed to identify the miRNAs in Coffea arabica L. that regulate key metabolic pathways by targeting the expression of specific proteins and transcription factors. A total of 10 potential conserved miRNA sequences were predicted to regulate 9 transcription factors belonging to the ethylene response transcription factor (ERF) family, which are expressed under drought conditions.

The study also discusses the relevance of such putative target genes/transcription factors in regulating plant growth and development under water scarcity conditions. The present study will offer a novel perspective for understanding the surveillance and tolerance mechanism of miRNA under drought and a better scope in generating high-yielding coffee varieties using transgenics.

Keywords: *Coffee arabica*, miRNA, drought, target annotation.

Introduction

With the increasing growth of the human population, the need for fulfilling the ever-increasing demand of the population has turned out to be an unavoidable challenge, especially in the case of crop production. Apart from human demand, the changes in climatic conditions and hydrological factors such as depletion of moisture and humidity have been reported to negatively regulate the overall gross productivity of food[6].

Thus, priorities need to be set to mitigate issues like soil fertility, moisture retaining capacity, insignificant regeneration capacity and altered photosynthetic activity related to crop failure[42]. Even sustainable approaches in the agronomic sector need to be prioritized including crop improvement, development of resilient varieties and well-equipped sustainable farming approaches. Such approaches should not be restricted to developing crops to overcome food scarcity; rather, they should include cash crops like tea, coffee, cotton, sugarcane. Among various abiotic stress factors, drought is the most common and a frequent one. In some areas its annual scenario has a detrimental effect on plant productivity, causing substantial loss. It is associated with insufficient water availability for normal life cycle and metabolism, thus, resulting in reduced stomatal conductance, CO_2 assimilation, photosynthetic rate and wilting[25].

Drought negatively regulates cellular homeostasis and membrane protein-lipid integrity that are known to be associated with withstanding the harsh environmental conditions[27]. The foremost prevailing outcome of drought is enhanced reactive oxygen species (ROS) generation in the subcellular components of cells like mitochondria, peroxisomes and chloroplasts[11]. Reduced water content of soil is also associated with poor mineral absorption, more particularly for the uptake and translocation of potassium ions responsible for plant endurance[3]. However, such stress can be mitigated through antioxidant enzymes and osmolytes by up-regulating the genes responsible for the ROS scavenging mechanism. These genes could also be regulated by several non-coding small RNA transcripts.

One such molecule is microRNA (miRNA), which is nearly 18-22 nucleotides (nt) long and targets specific RNA transcripts or transcription factors that are associated with the gene expression pattern. Unlike siRNAs, the transcription of miRNAs is facilitated by the DNA dependent RNA polymerase II enzyme from their respective MIR genes and these transcripts undergo modification to form the primary transcript, pri-miRNA. Further, these primary transcripts from the hairpin loop structure are detected, cleaved and processed (3/ methylation) to form the double stranded precursor miRNA (pre-miRNA) with the help of proteins like Dicer-like 1(DCL-1), HYPONASTIC LEAVES (HYL1) and HUA Enhancer 1 (HEN1) in plants[43].

The double-stranded miRNA-miRNA* duplexes are transported to the cytoplasm and bind with the AGO protein, AGO1, to form a miRISC complex that regulates the gene expression at the translational level. At this level ALTERED MERISTEM PROGRAM1 (AMP1) together with the RISC complex resists target mRNA binding on the polyribosome of the rough endoplasmic reticulum whereas, at the transcriptional level, the cleavage of target mRNA takes place by AGO facilitated by high mobility group 1 (HMG1) and HYDRA protein 1 (HYDRA1).

Plant miRNAs are found to take part in the different biochemical and molecular mechanisms that facilitate

growth, development and numerous biological processes by their action of cleavage on the coding region of the target gene. Whereas in the case of animals, the miRNAs usually repress gene expression by the process of translational attenuation[33]. Many miRNAs have been found to play a significant role against biotic and abiotic stress conditions. Several previous studies reported the role of miRNAs in plant nutrient deprivation like nitrogen[53], phosphate[5,9], sulfate[22] and copper[1]. Earlier studies also suggest miRNA-associated regulation of genes under stress conditions like drought[17], heat[51], salinity[18], heavy metal[32], oxidative stress[45] and also in biotic stress[24].

Evidence has been reported describing the up- or down-regulation of the specific MIR genes under drought stress in different plant species such as tobacco[18], rice[54], wheat[7], Chinese white poplar[41], tea[30] and mandarin[10]. The livelihood of more than 25 million farmers is dependent on coffee production in over 60 countries in tropical regions; some are in a vulnerable state due to the effects of climate change[38]. As of 2024/2025, the total production of Arabica and Robusta coffee beans reached 174.8 million bags (https://www.fas.usda.gov/). However, its production is limited by constitutional reforms, market price fluctuations, climate changes, rising temperatures, unpredictable weather patterns and increased pest pressure[4].

Under drought conditions, *Coffea arabica* is more economical in terms of water retention and transpiration than *C. canephora*[12,35]. *Arabica* coffee exhibits drought tolerance through intact stomatal conductance, transpiration rate, photo-inhibition, recovery of photosynthetic mechanisms, osmotic adjustment, relative water content and maximum bulk modulus of elasticity[13,14]. However, the climatic variations, especially increased temperature and decreased rainfall, affect the bean weight and yield. To date, a few scientific reports have addressed the role of miRNA in *Coffea arabica*[2,8,16].

Systematic research on *C. arabica* miRNA and its role in mitigating drought stress is still unaddressed due to a lack of sufficient data. Therefore, the present study was undertaken with the hypothesis of identifying miRNAs in *Coffea arabica,* exploring their interaction with drought-responsive genes and establishing their corresponding regulatory networks.

Material and Methods

Isolation of miRNA and Drought-responsive gene: A total of 60 (as of January 2025) *C. arabica* miRNAs and their isoforms have been retrieved from research articles by exploring journal archives, namely Google scholar (https://scholar.google.com/) and PubMed Central (https://pmc.ncbi.nlm.nih.gov/)[2,8,16,40] which were not available at the miRBase database[19]. Following the elimination of redundant miRNA sequences, the residual miRNA sequences were further examined for possible targets within drought-responsive genes of *C. arabica*. To do so, cDNAs of 24 drought-responsive genes were retrieved, which are available at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/).

All extracted miRNAs were subsequently subjected to homology analysis against drought-responsive cDNA sequences of *Coffea arabica* to identify potential precursor sequences for the 54 putative miRNAs. To perform BLASTn, the BioEdit Sequence Alignment Editor[20] tool was utilized. The sequences with a minimum 18-nucleotide match region with default parameters such as low complexity, 0-2 mismatches, an e value of 0.01 and BLOSUM62 matrix were considered to detect potent miRNA precursor candidates[10].

The cDNA sequences exhibiting homology with the extracted mature miRNAs were further analyzed to identify their precursor sequences. The secondary structures, particularly the stem-loop configurations, were predicted using the publicly available web-based server Mfold[55], employing default parameters: folding temperature at 37°C, 1 M NaCl ionic conditions without divalent ions, percent suboptimality set at 5%; maximum interior/bulge loop size of 30 nucleotides and maximum asymmetry for interior/bulge loops of 30.

Potential miRNA candidates were identified based on the following criteria: presence of at least 18–22 nucleotide matches at a single arm of the stem loop, no more than six mismatches between the miRNA and miRNA* sequences, an intact double-stranded region without breaks, A+U content between 30–70%, a highly negative minimal free energy (MFE) and a minimal free energy index (MFEI) as described by Zhang et al.[52]

Identification and functional annotation of the potential miRNA target genes: To predict the putative targets of the identified conserved miRNAs, a homology algorithm was employed using the publicly available online tool psRNATarget (2017 update)[15]. In the present study, the extracted 24 drought-responsive cDNA sequences were used as reference targets with the criteria such as a maximum e-value kept at 6; a complementary scoring length of 19; a maximum UPE of 25; 17 and 13 nucleotide flanking regions around the target upstream and downstream respectively; a 9-10 nt translational inhibition range and <4 permitted mismatches in the seed region.

The Gene Ontology Resource (https://geneontology.org/) tool was subsequently utilized for the analysis of the regulatory role of identified target proteins and transcription factors, facilitating annotation within ontology categories such as biological process, molecular function and cellular component. Additionally, the annotated targets were further curated for associated metabolic pathways using KASS-KEGG Automatic Annotation Server (KASS) (https://www.genome.jp/kegg/kaas/) which compares target sequences against the KEGG GENE database via local

alignment and annotates KEGG Orthology (KO) terms to the target genes along with KEGG pathway.

Phylogenetic analysis of predicted miRNA: To elucidate the expression patterns and regulatory mechanisms of miRNAs under drought, a phylogenetic analysis was conducted for potential miRNAs involved in the drought stress response. To perform this, homologous sequences of all the predicted drought-responsive mature conserved miRNAs were extracted from miRBase database. Multiple sequence alignments of these sequences and their homologs were performed using ClustalW with the following parameters: an e-value threshold of 0.001, gap opening penalty of 15.00, gap extension penalty of 6.66 and a maximum mismatch allowance of 3 nucleotides.

The aligned mature miRNA sequences were subsequently subjected to phylogenetic tree construction, employing neighbor-joining method, with bootstrap analysis set to 1,000 replicates to assess the robustness of the tree topology. Evolutionary distances were computed using the maximum composite likelihood method as described by Trees[47] and Tamura et al.[46] All these analyses were carried out using locally installed MEGAX platform[26].

Results and Discussion

***Coffea arabica* miRNA and their characteristics**: Based on comprehensive literature survey, a total of 90 distinct miRNAs have been reported in *C. arabica* to date. The majority of these miRNAs were identified through *in silico* prediction method. Among them, 18 were predicted by Rebijith and his coworkers[40]. The prediction process was contingent upon the availability of expressed sequence tags (ESTs) and genome survey sequences (GSS).

Consequently, the identification of miRNAs in *C. arabica* has been influenced by the accessibility of such nucleotide sequences. Akter and co-workers[2] reported the presence of miR393 in the *C. arabica* genome[2] whereas Chaves et al reported 24 including two sequences each for Car-miR159a, Car-miR390a and Car-miR393a and Devi et al identified 20. These miRNAs are believed to play regulatory roles in various metabolic pathways that facilitate plant growth and development (Table 1).

The analysis of mature conserved miRNAs revealed that the majority of these miRNAs ranged from 18 to 24 nucleotides in length, consistent with miRNAs reported in other plant species. Notably, a predominant proportion of the miRNAs were 21 nucleotides long (Figure 1). A+U content of these miRNAs resided within the range, aligning with the findings by Zhang et al[52]. Sequence alignments indicated that 60 of these miRNAs were unique and non-redundant. These miRNAs were subsequently subjected to target prediction analyses to identify potential gene targets, particularly among drought-responsive genes.

Among the sequences retrieved from the NCBI database, several remained uncharacterized with accession numbers such as GW436790.1, GW469985.1, GW469938.1, GW436120.1, GW435975.1, GW465238.1, GW459496.1 and GT682773.1. To investigate whether these sequences encode MIR gene transcripts, a local database was constructed using corresponding cDNA sequences of *C. arabica*.

Table 1
List of extracted miRNAs of *C. arabica* and their nucleotide composition[2,8,16,40]

miRNA	Reference EST/GSS/ cDNA ID	Sequence	NT length	A	C	G	U/T	(A+U) %	(G+C) %
Car-miR172c[40]	GW446798	GUAGCAUCAUCAAGAUUCACA	21	8	5	3	5	61.90	38.10
Car-miR5658a	GR996153	AUGAUGAUGAUGAUGAUGACA	21	8	1	6	6	66.67	33.33
Car-miR5658b	GT020019	AUGAUGAUGAUGAUGAUGAAU	21	8	0	6	7	71.43	28.57
Car-miR1171a	GW473864	AGGAGUGGAGUGGAGUGGAGUGG	23	5	0	14	4	39.13	60.87
Car-miR1171b-1	GT680258	UGGAGUGGAGUGGAGUGGAGUGG	23	4	0	14	5	39.13	60.87
Car-miR1171b-2	GT681553	UGGAGUGGAGUGGAGUGGAGUGG	23	4	0	14	5	39.13	60.87
Car-miR156f	GW427474	UUGACAGAAGAGAGAGAGCAUA	22	10	2	7	3	59.09	40.91
Car-miR167g	GT671631	UGAAGCUGCCAGCAUGAUCUGG	22	5	5	7	5	45.45	54.55
Car-miR4414	GW458140	UGCUGCUGACUCGUUGGCUC	20	1	6	6	7	40.00	60.00
Car-miR5368	GR988681	GGACAGUCUCAGGUAGACA	19	6	4	6	3	47.37	52.63
Car-miR2673a	GW478692	CAUCUUCCUCUUCCUCUUCCUC	22	1	11	0	10	50.00	50.00
Car-miR5532	GT001031	AUGGAAUAUAUGACAAAGGUGG	22	9	1	7	5	63.64	36.36
Car-miR390b	GW445903	AAGCUCAGGAGGGAUAGCGCC	21	6	5	8	2	38.10	61.90
Car-miR166c	GT690348	CCGGACCAGGCUUCAUCCCAG	21	4	9	5	3	33.33	66.67
Car-miR396e	GW491635	UUCCACAGGCUUUCUUGAACGA	22	5	6	4	7	54.55	45.45
Car-miR398[2]	GW479264	UGUGUUCUCAGGUCACCCCUU	21	2	7	4	8	47.62	52.38
Car-miR319[8]	GW458822	AUUGGACUGAAGGGAGCUCCC	21	5	5	7	4	42.86	57.14
Car-miR828a	GW491209	UCUUGCUCAAAUGAGAAUUCCA	22	7	5	3	7	63.64	36.36
car- miR393	GW432396.1	TCCAAAGGGATCGCATTGATCC	22	6	6	5	5	50.00	50.00

car- miR159a	GW458140.1	UUUGGAUUGAAAGGAGCUCU	20	5	2	6	7	60.00	40.00
		GAGCUCCUUGAAGUCCAAUAG	21	6	5	5	5	52.38	47.62
Car-miR166h1	HQ696508.1	UCGAACCAGACAGCAUUCCCC	21	6	9	3	3	42.86	57.14
Car-miR166h2	GU123896.1	UCGAACCAGACAGCAUUCCCC	21	6	9	3	3	42.86	57.14
Car-miR166h2	GU123896.1	UCGAACCAGACAGCAUUCCCC	21	6	9	3	3	42.86	57.14
Car-miR167a1	GU123897.1	AUCAUGCUGGCAGCUUCAACUGAU	24	6	6	5	7	54.17	45.83
Car-miR167a1	GU123898.1	UGAAGCUACCACAUGAUCUGA	21	7	5	4	5	57.14	42.86
Car-miR167a2	GU123897.1	UGAAGCUGCCAGCAUGAUCUAA	22	7	5	5	5	54.55	45.45
	GU123898.1	AUGAGCCGAACCAAUAUCACU	21	8	6	3	4	57.14	42.86
Car-miR171f	ED796419.1	UGAGAAUCUUGAUGAUGCUGCAU	23	6	3	6	8	60.87	39.13
Car-miR172d	GW446798.1	GUGUAGCAUCAUCAAGAUUCACA	23	8	5	4	6	60.87	39.13
Car-miR319	GW427525.1	UUGGACUGAAGGGUUUCCUUC	21	3	4	6	8	52.38	47.62
	GW427827.1								
	GW431253.1								
Car-miR319b	GW445971.1	UAGCUGCCGACUCAUUCAUUCA	22	5	7	3	7	54.55	45.45
Car-miR319b	GW450284.1								
Car-miR390a1	GW445903.1	CGCUAUCCCUCCUGAGCUUUA	21	3	8	3	7	47.62	52.38
		AAACUCAGGAUGGAUAGCGCC	21	7	5	6	3	47.62	52.38
Car-miR390a2	GW445903.1	CGCUAUCCCUCCUGAGCUUUA	21	3	8	3	7	47.62	52.38
		AAACUCAGGAUGGAUAGCGCC	21	7	5	6	3	47.62	52.38
Car-miR393a	GW432396.1	AUCAUGCUAUCCCUUUGGAU	20	4	5	3	8	60.00	40.00
		UCCAAAGGGAUCGCAUUGAUCC	22	6	6	5	5	50.00	50.00
Car-miR398a	GW479264.1	UGUGUUCUCAGGUCACCCCUU	21	2	7	4	8	47.62	52.38
Car-miR398a	GW471972.1								
Car-miR398a	GW444864.1								
Car-miR5368	NC_008535.1	GGACAGUCUCAGGUAGACA	19	6	4	6	3	47.37	52.63
	NC_008535.1								
	EF044213.1								
	EF044213.1								
	ED796313.1								
	ED796968.1								
	GW465047.1								
	GW465437.1								
	GW473119.1								
	GT020948.1								
	GT020845.1								
Car-miR7122a[16]	HQ696508.1	ACCGCGUUUCUUUGUAUAAAG	21	5	4	4	8	61.90	38.10
		UUAUACAGAGAAACCGCGGUUG	22	7	4	6	5	54.55	45.45
Car-miR835a	ED795605.1	UGAAGAAGGUAAGGAAGAAAG	21	11	0	8	2	61.90	38.10
Car-miR393	GW432396	AUCCAAAGGGAUCGCAUUGAUC	22	7	5	5	5	54.55	45.45
Car-miR393b	GW432396	UCCAAAGGGAUCGCAUUGAUC	21	6	5	5	5	52.38	47.62
Car-miR393b-3p	GW432396	AUCAUGCUAUCCCUUUGGAU	20	6	6	5	5	55.00	55.00
Car-miR393d	GW432396	UCCAAAGGGAUCGCAUUGAUCC	22	6	6	5	5	50.00	50.00
Car-miR854d	GW438532	GAGGAGGGGGAGGAGGAG	18	5	0	13	0	27.78	72.22
Car-miR1134	EG702329	AAGAAGAAGAAGAAGAAGAAG	21	14	0	7	0	66.67	33.33
Car-miR5809	GW454016	UCGUCGCCGGCGACCACAGC	20	3	9	6	2	25.00	75.00
Car-miR482d	GW443948	CCUUUCCCAGGCCUCCCAUGCC	22	2	12	3	5	31.82	68.18
Car-miR397b	GT716079	UGGGUGCAGCGUUGAUGU	18	2	2	8	6	44.44	55.56
Car-miR426	GW459110	UUUUGGAAGUUGGUCCUU	18	2	2	5	9	61.11	38.89
Car-miR1879	GW466885	GUAUGGUUUAGGGAUGAUGU	20	4	0	8	8	60.00	40.00
Car-miR414	GT677142	UCAUCCUCAUCAUCCUCAUC	20	4	9	0	7	55.00	45.00
Car-miR390a-3p	GW445903	CGCUAUCCAUCCUGAGUUU	19	3	6	3	7	52.63	47.37
Car-miR390b	GW445903	AAGCUCAGGAGGGAUAGCGCC	21	6	5	8	2	38.10	61.90
Car-miR390d-3p	GW445903	CGCUAUCCAUCCUGAGUUUUA	21	4	6	3	8	57.14	42.86
Car-miR390e	GW445903	AGCUCAGGAGGGAUAGCGCC	20	5	5	8	2	35.00	65.00
Car-miR1110	GT693786	AGGGGCAGUGGGCAAGGA	18	5	2	10	1	33.33	66.67
Car-miR533a-5p	GW475066	GCUGGCCAGGCUCUGAAGG	19	3	5	8	3	31.58	68.42
Car-miR2118e	GW443948	UUCCCAGGCCUCCCAUGCC	19	2	10	3	4	31.58	68.42
Car-miR5212*	ED796316	UGGAUUUCGUAUUUCUUUGG	20	2	2	5	11	65.00	35.00

Table 2
Potential conserved miRNAs and their target genes/ transcription factors responsible for drought tolerance in *C. 59rabica*.

miRNA	Target Acc.	miRNA start position	miRNA end position	Target start position	Target end position	Alignment	Inhibition	Target Description
Car-miR1134	KF743541.1	1	21	74	94	.:::::::::::::::::	Cleavage	ERF transcription factor 02 mRNA, complete cds
Car-miR156f	KF743541.1	1	22	88	109	:::::::.::::::::	Cleavage	ERF transcription factor 02 mRNA, complete cds
Car-miR167a1	KF743552.1	1	24	3	26	.::::::::::::::..:::	Cleavage	ERF transcription factor 13 mRNA, complete cds
Car-miR2673a	KF743545.1	1	22	348	369	::::::::::::::	Cleavage	*Coffea arabica* ERF transcription factor 06 mRNA, complete cds
Car-miR5532	KF743549.1	1	22	604	625	.::.:::::::::.:::.:::	Cleavage	*Coffea arabica* ERF transcription factor 10 mRNA, complete cds
	KF743548.1	1	22	604	625	.::.:::::::::.:::.:::	Cleavage	*Coffea arabica* ERF transcription factor 09 mRNA, complete cds
Car-miR5564b	GW435975.1	1	20	236	255	:.::::::::::::	Cleavage	CA00-XX-BP1-005-D06-JE.F *Coffea arabica* BP1 *Coffea arabica* cDNA clone CA00-XX-BP1-005-D06-JE, mRNA sequence
Car-miR5658a	KF743541.1	1	21	911	931	::::::::::::.:::	Cleavage	ERF transcription factor 02 mRNA, complete cds
	KF743549.1	1	21	599	619	:::::::::.:::::	Cleavage	ERF transcription factor 10 mRNA, complete cds
	KF743548.1	1	21	599	619	:::::::::.:::::	Cleavage	ERF transcription factor 09 mRNA, complete cds
Car-miR5658b	KF743541.1	1	21	914	934	::::::::::.:::::	Cleavage	ERF transcription factor 02 mRNA, complete cds
	KF743549.1	1	21	599	619	::::::::::.:::::	Cleavage	ERF transcription factor 10 mRNA, complete cds
	KF743548.1	1	21	599	619	::::::::::.:::::	Cleavage	ERF transcription factor 09 mRNA, complete cds
Car-miR7122a	GW436790.1	1	21	287	307	::::::::::.::.::	Cleavage	CA00-XX-BP1-001-D07-JE.F *Coffea arabica* BP1 *Coffea arabica* cDNA clone CA00-XX-BP1-001-D07-JE, mRNA sequence
	KF743542.1	1	21	53	73	::::::::::.::.::	Cleavage	ERF transcription factor 03 mRNA, complete cds
	GW436120.1	1	21	287	307	::::::::::.::.::	Cleavage	CA00-XX-BP1-005-D07-JE.F *Coffea arabica* BP1 *Coffea arabica* cDNA clone CA00-XX-BP1-005-D07-JE, mRNA sequence
	GW435975.1	1	21	285	305	::::::::::.::.::	Cleavage	CA00-XX-BP1-005-D07-JE.F *Coffea arabica* BP1 *Coffea arabica* cDNA clone CA00-XX-BP1-005-D07-JE, mRNA sequence

Car-miR835a	KF743541.1	1	21	31	51	::::::.::.::::: ::	Cleavage	*Coffea arabica* ERF transcription factor 02 mRNA, complete cds

Utilizing BLASTn and the Mfold program, it was determined that none of these transcripts possessed the characteristic hairpin loop structures necessary for forming putative *C. arabica* miRNAs during post-transcriptional processing. Consequently, these transcripts were further analyzed for target prediction to elucidate their potential regulatory roles.

Potential targets of *C. arabica* miRNAs among drought-responsive genes: The miRNAs are critical regulators of gene expression, influencing various metabolic processes. Prior research has established the association of miRNAs with stress response mechanisms across multiple species[39]. Differential expression of specific miRNAs such as miR156, miR166, miR171, miR398, miR896, miR1867, miR528, miR474, miR396, miR1881, miR894 and miR1432 has been implicated in conferring drought tolerance in *Triticum dicoccoides*[21]. Additionally, studies have demonstrated the regulatory roles of miR169, miR319 and miR167 in abscisic acid (ABA) signaling pathways during drought stress[24]. However, the lack of comprehensive RNA sequencing data has limited the investigation of these miRNAs and their expression profiles in *Coffea arabica*'s drought response.

In the current study, we predicted the potential regulatory relationships between miRNAs and drought-induced transcripts in *C. arabica*. Using psRNATarget software, ten target genes/transcription factors were identified for ten Car-miRNAs (Table 2). Further analysis of EST and GSS accession data indicated that most of these miRNAs are likely to be expressed in leaf tissues. In addition, miR5658a, miR5658b and miR5564b, identified from EST/GSS sequences were associated with tissues such as leaf, fruit, flower, root and germinating seed, suggesting their broader expression profile in *C. arabica*. Notably, the predominant expression of the predicted target proteins was observed in leaf tissue, with many belonging to the transcription factor family involved in ethylene signaling, particularly the Ethylene Response Factor (ERF) family including ERF2, ERF3, ERF6, ERF9, ERF10 and ERF13.

ERFs, as members of the APETALA2/ethylene-responsive element-binding protein family, play vital roles in stress tolerance by activating the transcription of stress-related genes[28]. Some ERFs, such as ERF3 and ERF5, are also reported as negative regulators of drought-responsive genes, with ERF3 repressing defense-related gene expression and ERF5 negatively regulating chitin-mediated defense responses[31,44]. These findings suggest a high likelihood that miRNAs modulate the expression of these target genes under drought conditions in *C. arabica*, influencing the plant's adaptive stress responses. Among the predicted target members of the ERF transcription factor family, ERF2 was identified as a primary target of multiple Car-miRNAs, including Car-miR156f, Car-miR835a, Car-miR1134, Car-miR5658a and Car-miR5658b. Functional annotation of ERF2 indicates its involvement in several key biological processes such as positive regulation of cell division (GO:0051301), ethylene-activated signaling pathway (GO:0009873), induced systemic resistance, jasmonic acid-mediated signaling pathway (GO:0009864), phloem or xylem histogenesis (GO:0010087) and positive regulation of DNA-templated transcription (GO:0045893), collectively contributing to the modulation of hormone signaling pathways (Figure 2).

Additionally, Car-miR7122a was predicted to target ERF3, which is associated with biological processes including defense response (GO:0006952), negative regulation of ethylene-activated signaling pathway (GO:0010105) and molecular functions such as DNA-binding transcription factor activity (GO:0003700) and transcription cis-regulatory region binding (GO:0000976). The upregulation of Car-miR7122a under drought conditions could thus negatively regulate ERF3 expression, potentially enhancing cellular defense mechanisms against drought stress, as observed in *Vitis vinifera*[36]. Most of the ERFs targeted by Car-miRNAs were involved in molecular functions related to DNA-binding, DNA-binding transcription factor activity and cis-regulatory region binding (GO:0003677, GO:0003700, GO:0000976).

Furthermore, targets of Car-miR5632, Car-miR5658a and Car-miR5658b were associated with ethylene-activated signaling pathways and cellular defense responses. Previous studies have highlighted the role of ERFs in drought stress, particularly in regulating enzymes such as 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and oxidase, which facilitate ethylene biosynthesis under drought conditions[23].

This process leads to leaf senescence, restricted root elongation and stomatal closure, collectively contributing to drought adaptation[50]. Additionally, ERFs contribute to the acceleration of drought-responsive gene expression by binding to dehydration-responsive element/C-repeat (DRE/CRT) motifs, thereby enhancing stress tolerance[37]. Pathway analysis further suggests that these ERF-associated processes are involved in ATP-dependent chromatin remodeling, ubiquitin-mediated proteolysis, plant hormone signal transduction and mitogen-activated protein kinase (MAPK) signaling pathways. In the MAPK pathway, MAP kinases phosphorylate ERFs, modulating their DNA binding capacity and stress-related signaling responses[49] (Table 3). This study investigated the role of drought-induced gene expression in *Coffea arabica*, with a focus on genes primarily involved in DNA binding, defense responses and ethylene signaling pathways (Figure 3).

Table 3
Potential drought-responsive *C. arabica* miRNA target proteins/transcription factors and their involvement in metabolic pathways

Target	miRNA	Metabolic pathways
ERF transcription factor 02	Car-miR1134 Car-miR5658a Car-miR5658b Car-miR156f Car-miR835a	Plant hormone signal transduction (ath04075)
ERF transcription factor 03	Car-miR7122a	mRNA surveillance pathway (ath03015)
ERF transcription factor 06	Car-miR2673a	Carbon metabolism (ath01200), Ubiquitin mediated proteolysis (ath04120), Proteasome (ath03050), mRNA surveillance pathway (ath03015) , Nitrogen metabolism (ath00910), Monoterpenoid biosynthesis (ath00902), Plant hormone signal transduction (ath04075), MAPK signaling pathway – plant (ath04016), ATP-dependent chromatin remodeling (ath03082)
ERF transcription factor 09	Car-miR5532 Car-miR5658a Car-miR5658b	Carbon metabolism (ath01200), Ubiquitin mediated proteolysis (ath04120), Proteasome (ath03050), mRNA surveillance pathway (ath03015) , Nitrogen metabolism (ath00910), Monoterpenoid biosynthesis (ath00902), Plant hormone signal transduction (ath04075), MAPK signaling pathway – plant (ath04016), ATP-dependent chromatin remodeling (ath03082)
ERF transcription factor 10	Car-miR5532 Car-miR5658a Car-miR5658b	Carbon metabolism (ath01200), Ubiquitin mediated proteolysis (ath04120), Proteasome (ath03050), mRNA surveillance pathway (ath03015) , Nitrogen metabolism (ath00910), Monoterpenoid biosynthesis (ath00902), Plant hormone signal transduction (ath04075), MAPK signaling pathway – plant (ath04016), ATP-dependent chromatin remodeling (ath03082)
ERF transcription factor 13	Car-miR167a1	Ubiquitin mediated proteolysis (ath04120), Proteasome (ath03050), mRNA surveillance pathway (ath03015), Nitrogen metabolism (ath00910), Monoterpenoid biosynthesis (ath00902), Plant hormone signal transduction (ath04075), MAPK signaling pathway – plant (ath04016), ATP-dependent chromatin remodeling (ath03082)

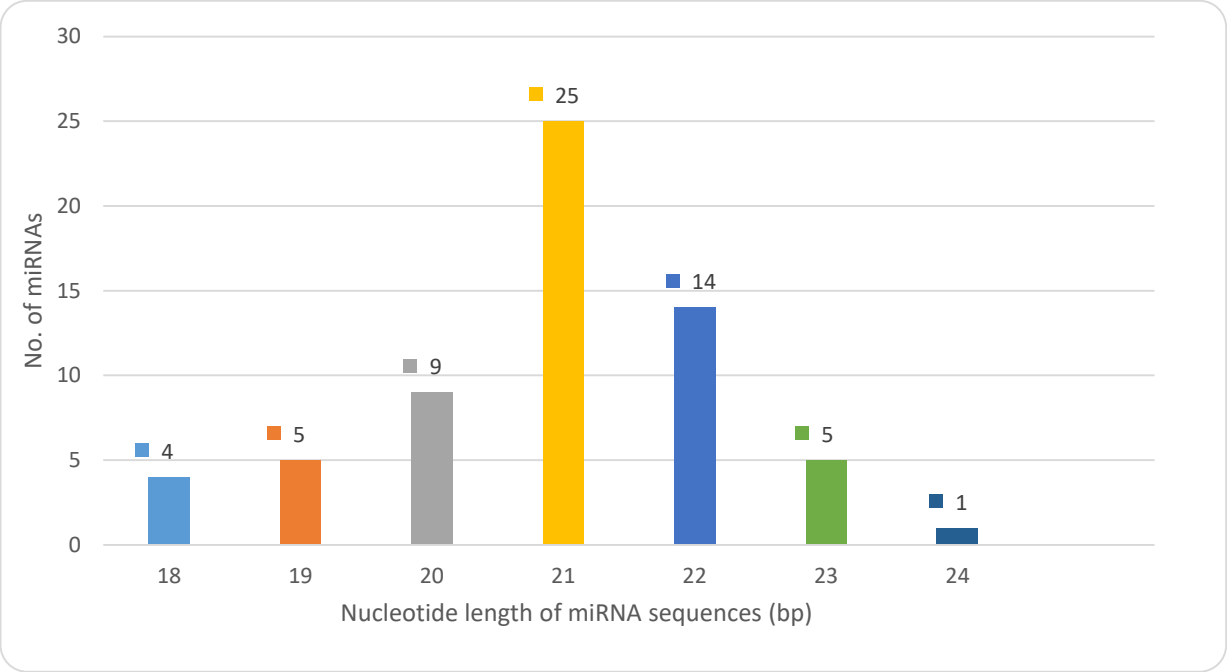

Figure 1: Nucleotide length of *C. arabica* miRNAs and their frequency of occurrence (X-axis represents length of miRNA and Y-axis represents the frequency of miRNAs)

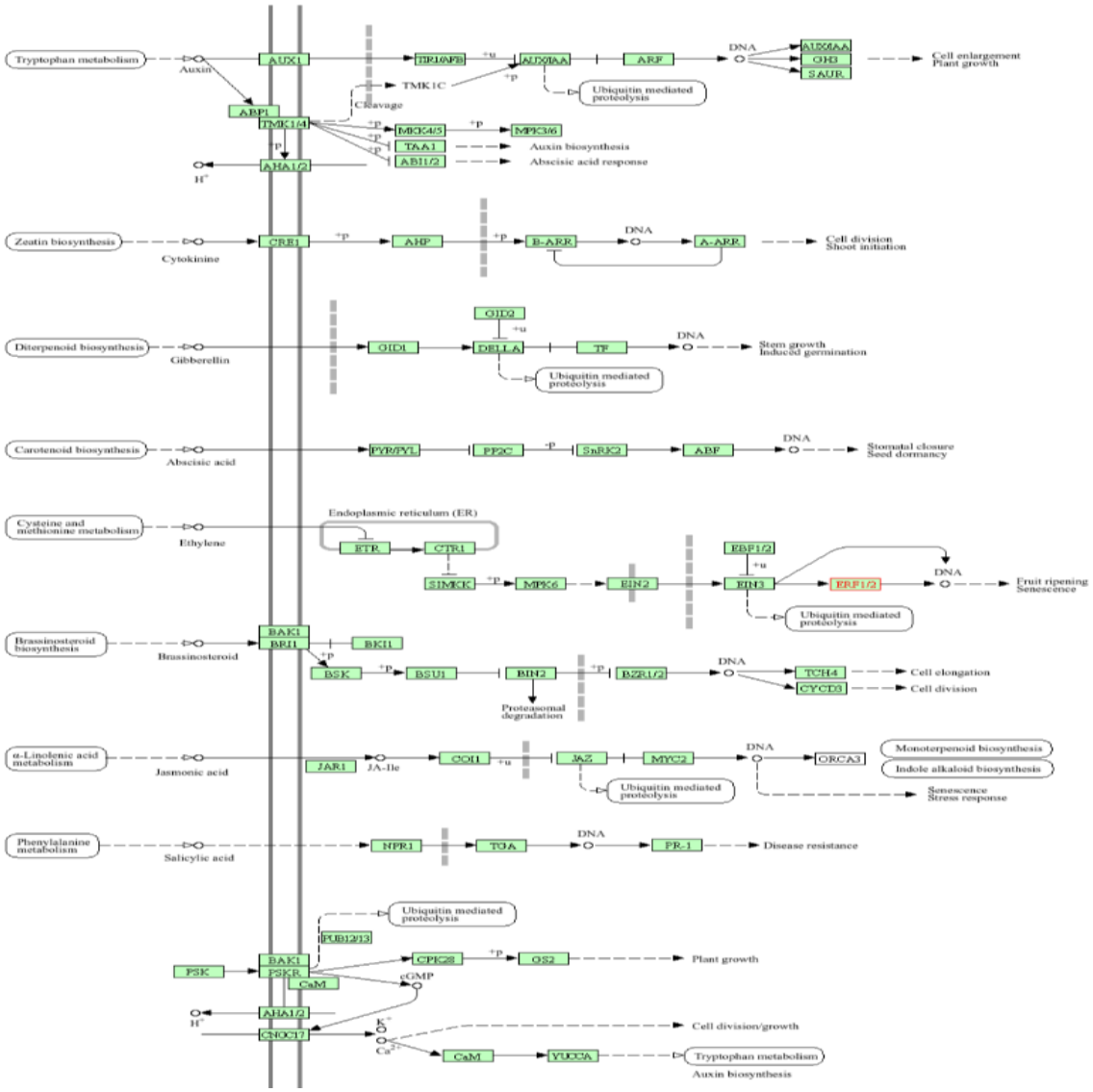

Figure 2: Plant hormone signal transduction pathway (ath04075) associated with ERF2 generated in KEGG Pathway tool

These genes are predicted to be potential targets of Car-miRNAs including Car-miR1134, Car-miR5658a, Car-miR5658b, Car-miR156f, Car-miR167a1, Car-miR7122a, Car-miR835a, Car-miR2673a, Car-miR5532 and Car-miR5564b. Notably, several target genes such as ERF-6, ERF-9, ERF-10 and ERF-13 are associated with monoterpenoid biosynthesis which functions as an osmoprotectant under water deficit conditions.

Additionally, ERF transcription factors play a crucial role in mitigating oxidative stress by regulating the synthesis of reactive oxygen species (ROS) scavengers including proline, malondialdehyde and flavonoids[49]. Evidence from drought stress studies indicates that ERF-mediated regulation can lead to the up-regulation of nuclear factor-Y (NF-Y) transcription factors, which are involved in stress response pathways[28,48]. In our analysis, members of the ERF family were predicted as potential targets of Car-miR156f, miR167a and miR835a, suggesting they may influence ABA-dependent stress response mechanisms by binding to the upstream C-repeat/dehydration-responsive element (DRE) within NFYA promoters[34].

Consequently, the modulation of target gene expression through the regulation of these miRNAs either by down-regulation or up-regulation could be leveraged for transgenic development aimed at enhancing drought tolerance. However, validation of these interactions through experimental approaches such as RT-PCR and RLM-RACE is essential to substantiate these predictions.

Convergence and phylogenetic characteristics of potential drought-responsive miRNAs: In order to understand the evolutionary history and relationship among drought-responsive miRNAs in relation to their origin, function and expression patterns, a phylogenetic analysis was conducted. Prior studies on miRNAs have revealed that miRNAs are highly conserved regulatory elements that perform similar roles across diverse species; however, their expression profiles often vary depending on their degree of

conservation, functional divergence and environmental adaptation. In this study, we analyzed the evolutionary relationships among selected drought-responsive miRNAs, including Car-miR156f, Car-miR835a, Car-miR7122a and Car-miR167a1 and their homologs reported in other species (Figure 4). Using the default parameters in MEGA X software for the construction of a phylogenetic tree via the neighbor-joining method, it was observed that Car-miR156f and Car-miR835a cluster closely with their respective homologs in *Glycine max* and *Arabidopsis lyrata* [Figure 4(A) and 4(B)].

Additionally, car-7122a clustered proximally with *Prunus persica*, while Car-miR167a1 grouped with orthologs from *Oryza sativa* and *Zea mays* [Figure 4(C) and 4(D)]. These phylogenetic affiliations suggest a shared evolutionary origin with these plant species, underscoring the potential functional relevance of the identified drought-responsive miRNAs in *C. arabica*'s stress resilience mechanisms. However, due to limited availability of mature miRNA sequences in the miRBase database, the phylogenetic analysis was restricted to these four miRNAs, with the remaining seven putative drought-responsive miRNAs lacking sufficient orthologous data for comprehensive phylogenetic reconstruction.

Conclusion

The present research aimed to identify drought-responsive miRNAs in *Coffea arabica* L. The novel findings also included the identification and characterization of miRNA-target proteins expressed under water deficit conditions in *C. arabica*. Computational analysis revealed a total of 10 conserved miRNAs in *C. arabica*, involved in various aspects of plant development. These miRNAs were found to regulate their respective drought-responsive target gene expressions via post-transcriptional cleavage. Notably, the same target proteins or transcription factors appeared to be regulated by multiple miRNAs.

However, the number of such target genes was limited, primarily due to a lack of a comprehensive miRNA profile for *C. arabica*. This analysis provides a foundation for future research aimed at identifying the novel miRNAs that contribute to drought tolerance in coffee and offers a model for understanding the molecular mechanisms underlying drought response in plants.

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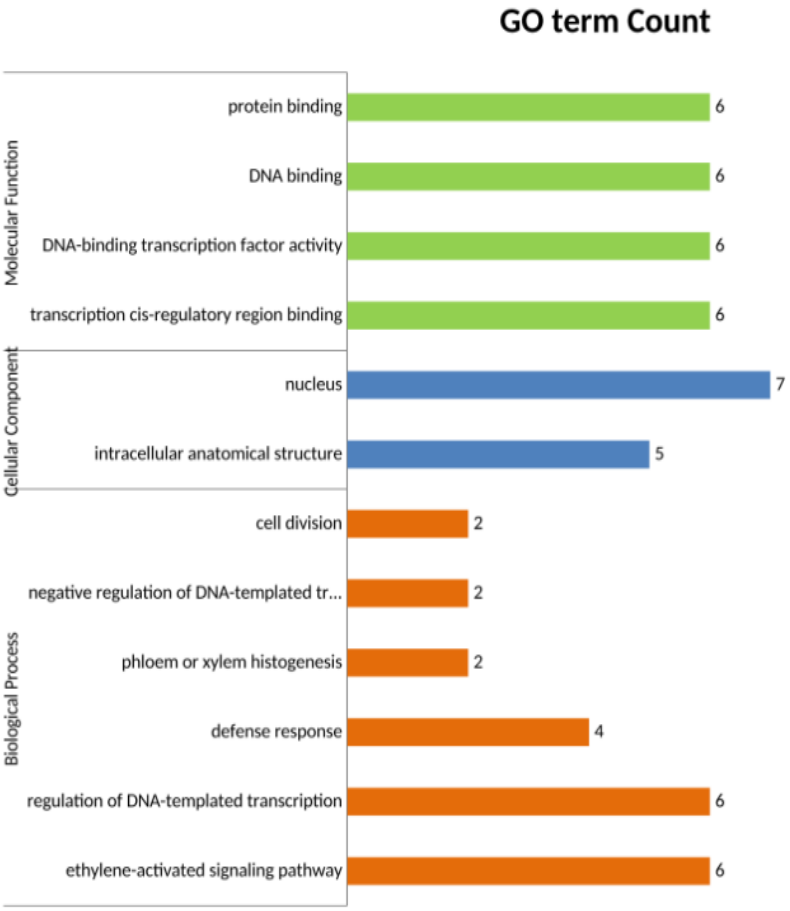

Figure 3: GO annotations of identified target genes of *Coffea arabica* miRNAs

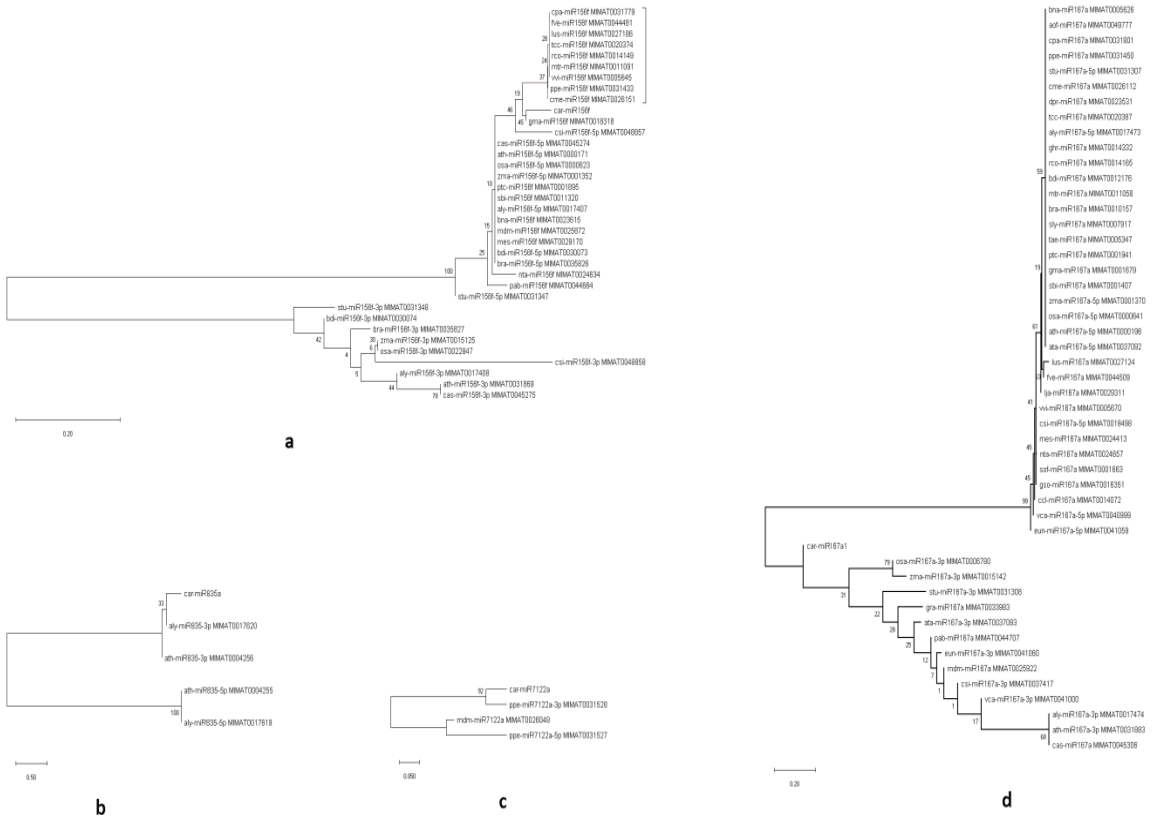

Figure 4: Phylogenetic analysis of *Coffea arabica* miRNAs (A) Car-miR156f, (B) Car-miR835a, (C) Car-miR7122a and (D) Car-miR167a1

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