



Identification of micro RNAs related to drought stress in wild beet (*Beta corolliflora*) roots

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Abstract Sugar beet (*Beta vulgaris* L.) is an economically valuable industrial plant, and drought stress adversely affects sugar quality and yield. *Beta corolliflora*, a species of the wild beet, shows tolerance to drought stress. It is considered to be drought-tolerant, but the role of microribonucleic acids (miRNAs) in response to drought has not been thoroughly studied in this plant. In this study, the expression profiles of miRNAs in the roots of *Beta corolliflora* genotype (Drought) and its control (Control) were compared by small RNA sequencing after drought stress treatment. Ultimately, 83 known miRNAs and 148 miRNAs from 70 different miRNA families and their related target genes were identified. Under drought stress, nine miRNAs were upregulated and

10 downregulated in roots. The detected hairpin sequences represented three different small RNA sequences which could be potential miRNA candidates. Gene Ontology analysis (GO) of the target genes indicated that most genes were possibly associated with catalytic activity (molecular function), metabolic processes (biological process), and cellular components. Based on the Kyoto Encyclopedia of Genes and Genomes Enrichment analysis (KEGG), the target genes were highly associated with glycine, serine, threonine, and amino and nucleotide metabolism. In this study, drought stress resulted in limited changes in miRNA expression, indicating restricted regulatory plasticity rather than extensive miRNA reprogramming. These findings provide new information on the molecular mechanisms of drought responses in wild sugar beet, which could be extended to the number of plant drought tolerance genes.

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Introduction

Drought stress is a major constraint on plant growth. Yield loss occurs under drought stress, typically ranging between 30 and 90% based on the crop species (Dietz et al. 2021). To survive drought conditions, plants adapt to consume water efficiently, tend to develop drought tolerance, and improve their

physiological, morphological, and molecular adaptations at the organ and cellular levels (Naikwade 2023).

Sugar beet (*Beta vulgaris* L.), as the important industrial plant around the world, is directly affected by drought and this stress factor remarkably decreases the yield, sugar rate, and sugar quality of this crop (Choluj et al. 2004). Therefore, the cultivation of this plant is restricted to semi-humid regions. Cultural sugar beet belongs to *Amaranthaceae/Chenopodiaceae* family, Betoideae subfamily, and *Beta* genus. *Beta vulgaris*, $2n=18$, is a dicotyledonous biennial plant. Due to the remarkable resistance of *Beta corolliflora* to abiotic stress (Wu et al. 2013; Lv et al. 2018), this plant seems to be suitable as a genetic source for stress resistance studies. The monosomic M14 inter-genus hybrid line resulted from *Beta corolliflora* and *Beta vulgaris* crossing (Guo et al. 1994) has been shown to resist drought, cold, and salinity stresses (Zhang et al. 2016). In another study, the *M14 glyoxalase I* gene was cloned from the M14 monosomic line and transferred to tobacco, and it was reported that this gene provided cellular detoxification capability and tolerance to abiotic stress conditions (Wu et al. 2013). These studies have emphasized that *Beta corolliflora* is a genetic source of abiotic resistance.

miRNAs are small RNAs in the non-coding RNA class that post-transcriptionally regulate gene expression. 95% of miRNAs are comprised of 18–24 nucleotides, with an average length of 22 nucleotides (Puchta et al. 2021). miRNAs bind to the target mRNA complementarily and play a role in the regulation of gene expression by degrading the mRNA or suppressing mRNA translation (O'Brien et al. 2018; Dong et al. 2022). Many miRNAs play a role in the defense system developed by plants against biotic and abiotic stresses (Zhang et al. 2017; Shengli et al. 2017).

In this study, we aimed to reveal the miRNA profile associated with drought tolerance in the roots of the wild beet *Beta corolliflora*. However, this association with drought tolerance has not been investigated in sugar beet breeding studies. In this context, drought stress-responsive miRNAs and pathways related to the possible target genes of these miRNAs were determined.

Materials and methods

Plant growth and drought stress treatment

To select the drought stress-resistant genotypes, 12 genotypes belonging to the *Beta corolliflora* species were obtained from the gene bank of the Aegean Agricultural Research Institute of Türkiye, Ministry of Agriculture and Forestry (Table S1). Germination of *Beta corolliflora* seeds was performed according to Ergül et al. (2018). Genotypes that showed healthy rooting for 30 days in the rooting medium were transplanted into sterile soil (120 °C, 20 min.) to acclimate to external conditions.

After an acclimatization period (approximately three weeks), drought stress in the form of water deficiency was applied to 12 *Beta corolliflora* genotypes for 11 days. The “optimal drought stress time interval” was determined simultaneously for the 12 genotypes using drought tests. For this purpose, the osmotic potential (OP) was measured on day 0 (control), 1th, 5th, 7th, and 9th days (d.) in plants exposed to drought stress. From day 5, OP was assessed at 2–3 d. intervals using a Viscor (vapor pressure) osmometer. The results of *Beta corolliflora* genotypes (mmol/kg) were converted to MPa (–) according to OP (MPa) = $-c \times 2.58 \times 10^{-3}$ (Bagatta et al. 2008). Measurements were obtained from at least two plants per genotype. Although measurements could not be taken for the other 11 genotypes on day 11 (due to plant death, etc.), the *Beta corolliflora* genotype 127 (TR 51285; Bilecik-Türkiye) was determined to be the most resistant one due to its MPa value (mean MPa: – 3.22) and normal general appearance. Stress (11th day) and control (day 0) samples of genotype 127 root tissue were washed under laminar flow (for 15 min in 10% bleach, and 3–4 times rinse with sterile ddH₂O), followed by drying and storage in sterile tubes at – 80 °C.

Small RNA library constructing and small RNA sequencing

Total RNA isolation from control (Control) and drought-stressed root samples (Drought) was performed using Wizard Promega Total RNA isolation kit (Cat no: Z3100) according to the manufacturer's instructions. RNA quality was tested by agarose gel electrophoresis and using a NanoDrop (ND-1000) spectrophotometer.

Small RNA sequencing library was prepared with Illumina HiSeq technology using the TruSeq small RNA library preparation kit. Raw data were obtained by sequencing cDNAs on the Illumina HiSeq (1×50 SR) platform (Genewiz Inc. Laboratory, USA) according to the manufacturer's instructions. Raw data were acquired in fastq format and converted to fasta format for nucleotide or protein sequencing using CLC-GW version 11 program (www.qiagenbioinformatics.com).

Identification of small RNAs

Adapter sequences were extracted and filtered (length filtering, filtering sequences with at least five reads) using the CLC-GW version 11 program (www.qiagenbioinformatics.com) to detect sequences that were identical to the reads of the obtained raw data, and small RNA sequences were determined by counting clean reads. The identified small RNA sequences of each sample were mapped to the *Beta vulgaris* genome and *Beta corolliflora* RNA-sequencing contigs using the CLC-GW version 11 program. Mapping to the *Beta vulgaris* genome was performed using the reference genome obtained from "The *Beta vulgaris* Resource" (<http://bvseq.boku.ac.at/Genome/Download/RefBeet-1.2>). The reference genome of *Beta corolliflora* is not available. Therefore, mapping was performed using unpublished RNA-sequencing data of *Beta corolliflora* included in the project (TÜBITAK, project no: 115O391) performed by the corresponding author.

The miRBase V.22 database (<http://www.mirbase.org/ftp.shtml>) was downloaded using the CLC-GW version 11 program (www.qiagenbioinformatics.com) to identify small RNA sequences paired with known miRNAs in each sample, and small RNA sequences were annotated using known miRNA sequences from all plant species (Viridiplantae). miRBase annotation was performed according to the default parameters of the program (Kozomara and Griffiths-Jones 2011, 2014).

Identification and fold change based differential expression analysis of known miRNAs

As a result of annotation, miRNAs that could pair with small RNA sequences were grouped as mature, pre-mature and variants. Mature miRNAs were paired with small RNA sequences, and each sample was normalized considering the original expression values.

During adapter trimming, mismatches were allowed with a mismatch cost of 2 and a gap cost of 3, and internal and end matches were permitted. Normalized expression values (Zhou et al. 2019) were calculated for each miRNA in each sample using the transcripts per million (TPM) normalization method. Expression increases or decreases were determined by comparing the control and stress samples according to the normalized expression values. Differential expression analysis was performed based on the fold change (FC) values calculated from the normalized expression data. Fold change was determined by comparing miRNA expression levels between control and stress conditions, and miRNAs showing increased or decreased expression ($FC \geq 1.5$) were identified accordingly.

Classification of non-coding RNAs

Non-coding RNA sequences were obtained from the "rnacentral.org" web server for all plant species (Viridiplantae). First, BLAST was performed with ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA) using the CLC-GW version 11 program (www.qiagenbioinformatics.com) to detect the largest non-coding RNA group. Subsequently, a second BLAST was applied to the unmapped sequences to detect miRNA, pre-RNA, and other RNAs. BLAST was then performed with coding sequences (cds) belonging to *Beta vulgaris* to determine whether the non-annotated sequences in the second BLAST were in the coding regions or not.

Identification of other unknown small RNAs

Potential miRNA candidates were identified by determining the possible hairpin structures of the remaining (not paired) small RNA sequences as a result of miRBase annotation, non-coding RNA classification, mapping to the BNYVV genome, and BLAST with *Beta vulgaris* cds sequences. Possible hairpin structures of small RNA sequences were determined using the MiRCat program (Paicu et al. 2017) with the *Beta vulgaris* genome, DNA contigs of *Beta corolliflora*, and RNA-sequencing data. To analyze whether the sequences of the determined hairpin structures were non-coding RNA, BLASTn (word size: 11, E-value: 5) was performed by selecting all non-coding RNAs for plants in the NCBI-BLAST database. BLASTx

(word size: 11, E-value: 10) was used to determine whether it encodes a protein or not.

Target, pathway and ontology analyses of all identified known miRNAs

Target analysis for all small RNA sequences paired with known miRNAs selected six small RNA sequences with hairpin structures, and mature miRNA sequences were analyzed using the “psRNA Target” web server (Zhou et al. 2019). Since *Arabidopsis thaliana*, the model organism for plants, has the most comprehensive data set in the existing miRNA target analysis web applications, cDNA sequences of this species were used as reference considering the homology. According to the target analysis parameters, the number of targeted genes was determined to be one to select the target gene with the best match.

As a result of the target analyses (for all small RNA sequences paired with known miRNAs and small RNA sequences with hairpin structures), the cDNA sequences of the relevant genes were downloaded from the “Ensembl plant” web server (<http://plants.ensembl.org>), using the IDs of the genes determined as targets in *Arabidopsis thaliana* (Ensembl plants, biomaRt module parameters: Dataset; *Arabidopsis thaliana* genes, Filter; Transcript stable ID, Trait; cDNA sequence). Using these sequences, ontology-based pathway analyses were performed in the Kyoto Encyclopedia of Genes and Genomes (KAAS; KEGG automatic annotation server) module of the KEGG web server (<https://www.genome.jp/kegg/kaas>).

Ontology analysis was performed using the “Panther” web server (<http://pantherdb.org>) with the IDs of the genes identified as targets in *Arabidopsis thaliana* (reference sequence) for small RNA sequences paired with known miRNAs.

Validation of miRNAs and target genes by qRT-PCR

All qRT-PCR reactions were performed using a Roche Light Cycler (LC 480) Real-Time PCR instrument.

qRT-PCR analyses on miRNAs

cDNA synthesis based on RNAs was performed using the miScript II Reverse Transcription Kit (Qiagen,

Germany) according to the manufacturer’s instructions. qRT-PCR reactions were performed using the protocol of the miScript SYBR Green PCR Kit (Qiagen, Germany). According to this kit, each reaction was performed with 5 µl 2× QuantiTect SYBR Green PCR Master Mix, 1 µl 10× miScript Universal Primer (Reverse), 1 µl 10× Forward Primer, 2 µl RNase-free water and 1 µl cDNA in a total volume of 10 µl. For qRT-PCR, forward primer design was carried out as specified by Timotijević et al. (2015). For reverse primer design, the specific “universal reverse primer” included in the kit was used. The qRT-PCR program was based on the binding temperature of each primer paired with preliminary optimizations, and the program was similar to the target gene RT-PCR program (described below). The *U6* (small nuclear RNA) gene (Turner et al. 2013; Nadiya and Thomas 2019) was chosen to normalize miRNA data in the reactions.

Information on the selected miRNAs and primers used in the qRT-PCR reactions is provided in Table S2. All qRT-PCR analyses were performed in three biological and three technical replicates.

After qRT-PCR reactions, expression analyses were performed using the cycle threshold (Ct) values of the five miRNAs and the endogenous reference RNA (*U6*). Relative expression (fold change) analysis was performed according to the $2^{-\Delta\Delta Ct}$ (ddCt) method, considering the Ct values of the samples (Livak and Schmittgen 2001).

qRT-PCR analyses on target genes

Expression analyses of the *AGO1* gene, determined as the target gene of miR168, and the *HOT5* gene, determined as the target gene of potential miRNA candidate 2, were performed by qRT-PCR. Accordingly, cDNA synthesis was carried out using the First Strand cDNA Synthesis kit (Roche, Cat No: 04896866001) and the relevant protocol.

Because there is very little sequence information for *Beta corolliflora* in bioinformatics databases, the reference sequences of the target genes of the selected miRNAs in *Arabidopsis thaliana*, which were determined by BLAST at NCBI, were used for primer designation. This was carried out using the NCBI primer designing tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Histone *H3.3* (NCBI: XM_010682705.1) was chosen as the housekeeping gene for data normalization.

For each primer, standard curves were prepared (efficiency and slope values were close to 2.2 and -3.2 , respectively) from serial dilutions (i.e., 1/10–1/100,000) of a control cDNA. By means of the specified optimization conditions, qRT-PCR reactions were carried out using the SYBR Green I Master Kit (Roche) and the relevant protocols.

All qRT-PCR analyses were performed in three biological and three technical replicates. Melting curve analyses were performed after the qRT-PCR program to detect non-specific amplification products in the reactions. Data analysis was performed similarly to that of qRT-PCR for miRNAs. In addition, the Uniprot (<https://www.uniprot.org/>), TAIR (<https://www.arabidopsis.org/>), and Araport (<https://www.araport.org/>) websites were used for names and brief descriptions of the target genes included in the discussion. Bar graphs showing the relative expression of both miRNAs and their target genes, based on sequenced raw data, normalized data and qRT-PCR (fold-change) data were generated using Microsoft Excel.

Results

High-throughput sequencing of small RNA libraries

After sequencing, 146,182,479 raw sequences (readings) were obtained from the control sample, and 143,441,673 raw sequences (readings) were obtained from the stress sample. The raw data for each sample are presented in Table 1. After adapter removal, sequence length filtering, removal of repetitive nucleotides, and filtering of sequences supported by at least five reads, the analysis was continued with the remaining 12,536,752, 66,597,354, and 20,410,254 sequences,

respectively. Based on sequences supported by at least five reads, 128,334 small RNAs were identified in the Control sample and 135,841 small RNAs in the Drought sample (Table 1).

Small RNAs mapping

Mapping to the *Beta vulgaris* genome

Control sample was mapped at a rate of ~92%, and Drought sample was mapped at ~89%. The mapping data of the *Beta vulgaris* genome samples are presented in Table S3. In contrast, the small RNA sequences detected were highly mapped to *Beta corolliflora* RNA-sequencing data. The mapping results of the *Beta corolliflora* RNA-sequencing data are presented in Table S4.

Known miRNA analyses

The miRBase annotation results for the samples are presented in Table 2. According to this Table, 66,961 sequences in and 178,866 sequences in the Drought sample were paired with known miRNA sequences. These sequences with different reading counts represented 798 and 1272 small RNA sequences, respectively (5628 different miRNAs considering the common ones).

When the annotated sequences were grouped according to the mature, pre-mature and variants, 186 sequences in Control and 201 sequences in Drought sample were determined to pair with known miRNAs considering the miRBase annotation results (Table 3). In addition, when grouped as mature and variant miRNAs 83 sequences in each sample (211 different mature miRNAs when common ones were considered) paired with known miRNAs (Table 3).

Table 1 Small RNA Sequencing data overview and trim and filter results

Small RNA Sequencing data overview					
Sample	Reading count		Product	Sample	Reading count
Control	146,182,479		7455	95,39	37,97
Drought	143,441,673		7315	95,13	37,86
Trim and filter results					
Sample	Reading count	Adaptor trim, 16–24 nt	Poly ATCG trim	Count > 4	Small
Control	146,182,479	14,593,481	14,581,127	12,536,752	128,334
Drought	143,441,673	22,246,607	22,238,852	20,410,254	135,841

Table 2 miRBase annotation results

Sample	small RNAs count	Annotated	Percent (%)	Reading count	Annotated	Percent (%)
Control	128,334	798	0,60	12,536,752	66,961	0,50
Drought	135,841	1272	0,90	20,410,254	178,866	0,90

Table 3 miRBase annotation detailed results (mature, pre-mature, and variant)

Sample	miBase annotation	Mature, pre-mature and variants	Mature and variants	Mature (miRNA family)
Control	798	186	83	38
Drought	1272	201	83	40

The ungrouped miRNAs with the highest counts in all samples are listed in Table S5. Accordingly, miR2916, miR166c, and miR159 in the Control, and miR2916, miR894, and miR159 in the Drought

sample were determined to be the miRNAs with the highest counts.

Mature miRNA analyses

As a result of miRBase annotation, small RNA sequences were found to pair with 148 miRNAs from 70 miRNA families (Table S6). Among the 63 sequences of known mature miRNAs in the Control and 65 sequences in Drought, no paired or common miRNAs were detected. The mature miRNAs that paired with the small RNAs with the highest expression are given in Table 4.

Table 4 Mature miRNAs paired with small RNAs with the highest expression value in Control and Drought

Control			
miRNA sequence (miRBase)	Expression value	miRNA name	Pairing type
TCGGACCAGGCTTCATTCCC	5424	miR166b//miR166c	Mature 3' end
TTTGGATTGAAGGGAGCTCTA	3433	miR159a//miR159	Mature 3' end
TGGGGACTCGAAGACGATCATAT	1577	miR2916	Mature 3' end
TTTGGATTGAAGGGAGCTCTG	640	miR159b	Mature 3' end
TTCCACAGCTTTCTTGAAGCTG	636	miR396	Mature 3' end
GATCCCCGCAACGGCGCCA	465	miR8175	Mature 3' end
TTGTCAGGCTTGTTATCTCC	423	miR7972	Mature 3' end
ATTTCGGACCAGGCTTCATTCCCC	410	miR166	Mature 3' end
TCGGACCAGGCTTCATTCCCTC	404	miR166a//miR166d	Mature 3' end
TCGGACCAGGCTTCATTCCCC	401	miR166a//miR166e//miR166b//miR166d	Mature 3' end
Drought			
miRNA sequence (miRBase)	Expression value	miRNA name	Match type
TGGGGACTCGAAGACGATCATAT	11.871	miR2916	Mature 3' end
TTTGGATTGAAGGGAGCTCTA	6.462	miR159a//miR159b//miR159	Mature 3' end
CGTTTCACGTCGGGTTACCC	6.256	miR894	Mature 3' end
TGGCGCTAGAAGGAGGGCCT	3.238	miR3711	Mature 3' end
TCGGACCAGGCTTCATTCCC	3.146	miR166c	Mature 3' end
TTTGGATTGAAGGGAGCTCTG	3.143	miR159b	Mature 3' end
GATCCCCGCAACGGCGCCA	1862	miR8175	Mature 3' end
TCGCTTGGTGCAGGTCGGGAA	432	miR168b//miR168	Mature 3' end
AAACCTGGCTCTGATACCA	354	miR5139	Mature 3' end
TGTGTTCTCAGGTCGCCCCTG	352	miR398b	Mature 3' end

Table 5 miRNAs, miRNA-expression (fold-change) and target genes detected in the Drought sample (↑: upregulated, ↓: downregulated)

miRNA	Log2 fold-change*	Target of miRNA
miR168	↑1.94	Argonaute (<i>AGO1</i>)
miR398	↑8.60	–
miR398b	↑2.38	–
miR408	↑2.37	Plantacyanin (<i>ARP</i> N)
miR894	↑19.41	Plant ubiquitin regulatory X domain-containing protein (<i>PUX9</i>)
miR3711	↑23.13	–
miR5054	↑5.88	–
miR5139	↑2.34	B-box proteins (<i>BBX23</i>)
miR8175	↑2.46	–
miR162	↓7.96	Dicer-like proteins (<i>DCL1</i>)
miR165/166	↓3.07	<i>Phavoluta</i> (<i>PHV</i>)
miR319	↓1.88	DNA-binding domain (<i>MYB104</i>)
miR393	↓8.18	F-box proteins (<i>AFB3</i>)
miR396	↓5.72	Alcatraz 1 (<i>AC11</i>)
miR403	↓11.19	Argonaute (<i>AGO2</i>)
miR535	↓3.30	Nucleotide-binding protein (<i>NB</i>)
miR1515	↓15.78	Remorin (<i>REM</i>)
miR3627	↓1.97	F-box/LRR protein (<i>FBXL</i>)
miR7972	↓14.05	Dynein-specific chain proteins (<i>DYNI</i>)

* significant differential expression after drought stress (showing at least 1.5 fold expression change)

Control and Drought samples were compared in terms of normalized expression values, total of nine upregulated and ten downregulated miRNAs were detected (Table S7 and Table 5).

Classification of non-coding RNAs

BLAST results (based on sequencing) and small RNA BLAST results are given in Table S8. According to the reading-based BLAST results, the rRNA and tRNA ratio was lower in the Control. Considering the small RNA BLAST results, it was observed that the rRNA and tRNA ratio difference was low.

Other unknown small RNAs

As a result of the hairpin structure analysis for 262 sequences in the Control and 491 sequences in the Drought sample which were earlier showed no pairing in the BLAST result, 173 hairpin sequences belonging to a total of 74 different sequences (unpublished data) were determined. Moreover, it was determined that six small RNAs in the Control and nine in the Drought may include a hairpin structure. Three

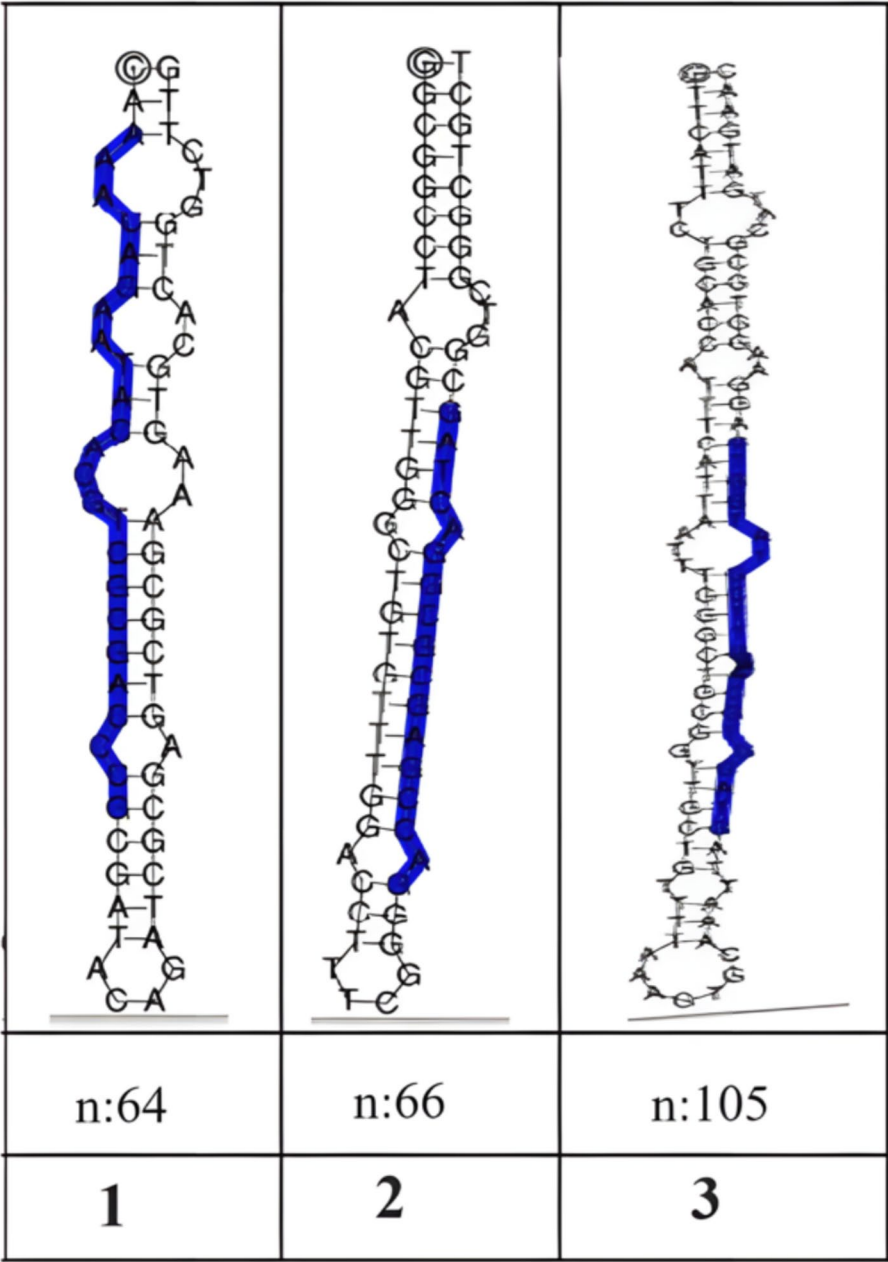
sequences (potential miRNA candidates) that were common to the Control and to Drought samples were identified (repetitive base content removed) as hairpin sequences (Fig. 1). The expression values and hairpin sequences of these potential miRNA candidates are given in Table S9.

Target, pathway, and ontology analyses in known miRNAs

According to the ontology analysis results, no significant difference was detected between the samples in terms of molecular function and biological process classification. In the molecular function group, the most significantly enriched GO terms were catalytic, binding, and transporter activities. Within the biological process group, the most significantly enriched GO terms were metabolic process, biological regulation, cellular organization, and biogenesis and localization. Under the cellular component group, the most significantly enriched GO terms included organelle and cell (Fig. 2).

We also performed KEGG pathway analysis on the predicted target genes of known miRNAs, which were

Fig. 1 Secondary hairpin structures of three potential miRNA candidates identified under drought stress in wild sugar beet (*Beta corolliflora*) (sequences which are highlighted with blue colour show mature miRNA, n: nucleotide number)



divided into different enriched categories, including glycine, serine, and threonine metabolism amino and nucleotide metabolism, oxidative phosphorylation, and the cell cycle. Number of genes targeted by small RNA sequences matching miRNAs according to pathways (KEGG), are given in Fig. 3.

Considering the affected pathways in the ontology analysis of the target genes of small RNA sequences pairing with known miRNAs, there was

no remarkable difference in terms of the pathways between the Control and Drought samples. However, among the important pathways affected by drought stress, amino sugar and nucleoid sugar metabolism, and glycine, serine, and threonine metabolism were identified (Figs. 4, 5).

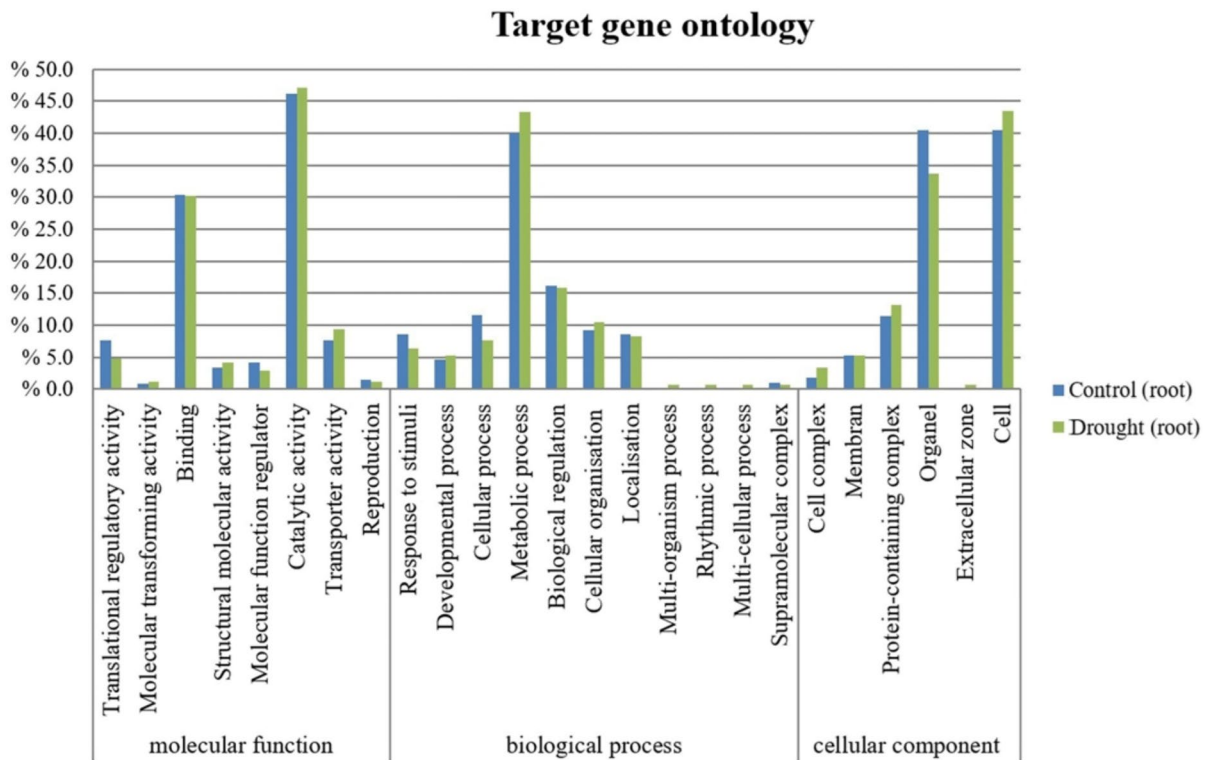


Fig. 2 GO characterization of predicted target genes of miRNAs of top GO terms in each molecular function, cellular component, and biological process categories

miRNA and target gene validation

It was concluded that in Drought, the expression levels of miRNAs selected for validation purposes obtained as a result of sequencing, and the expression

levels of these miRNAs obtained as a result of qRT-PCR, were directly proportional to the expected values. Bar graphs of miRNAs and their target genes are presented in Fig. 6a, b, respectively.

Drought sample

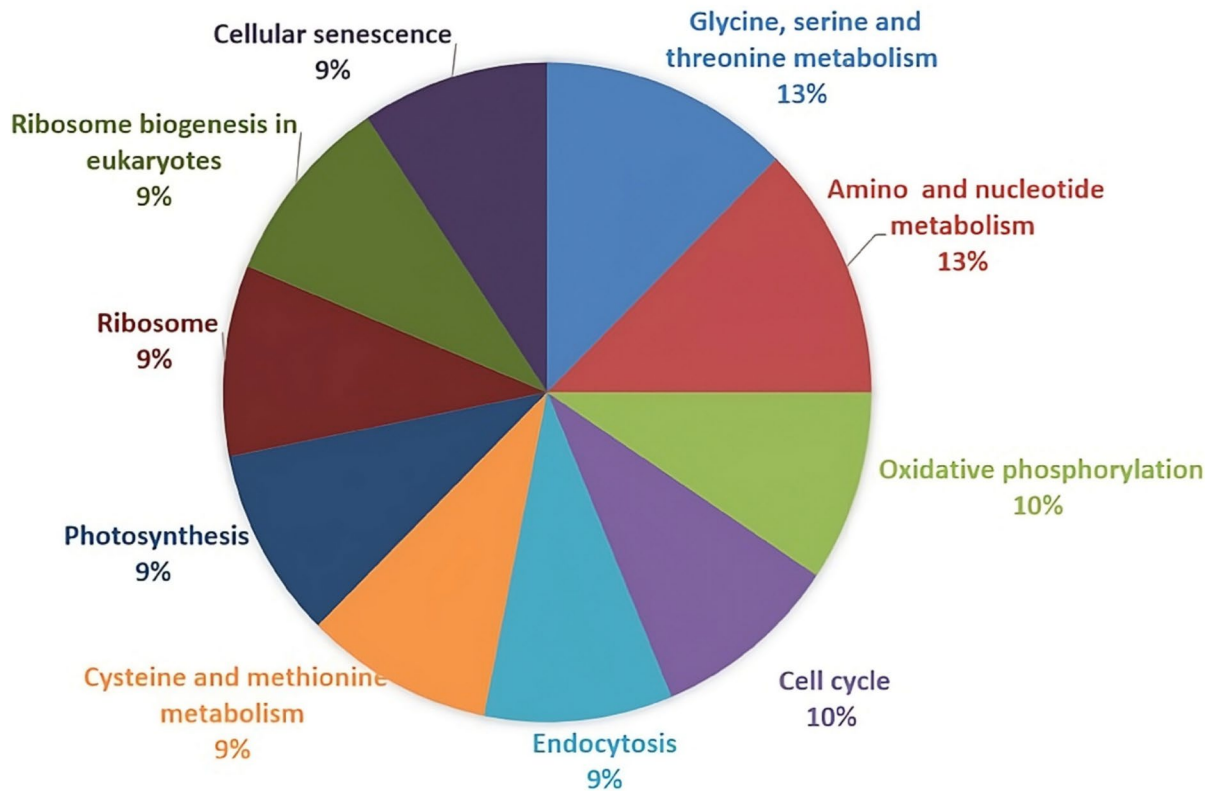
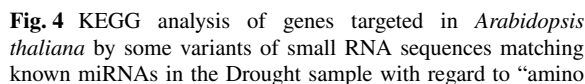


Fig. 3 The most significantly enriched KEGG pathways for drought-stress responsive target genes. %: percentage of the number of genes determined

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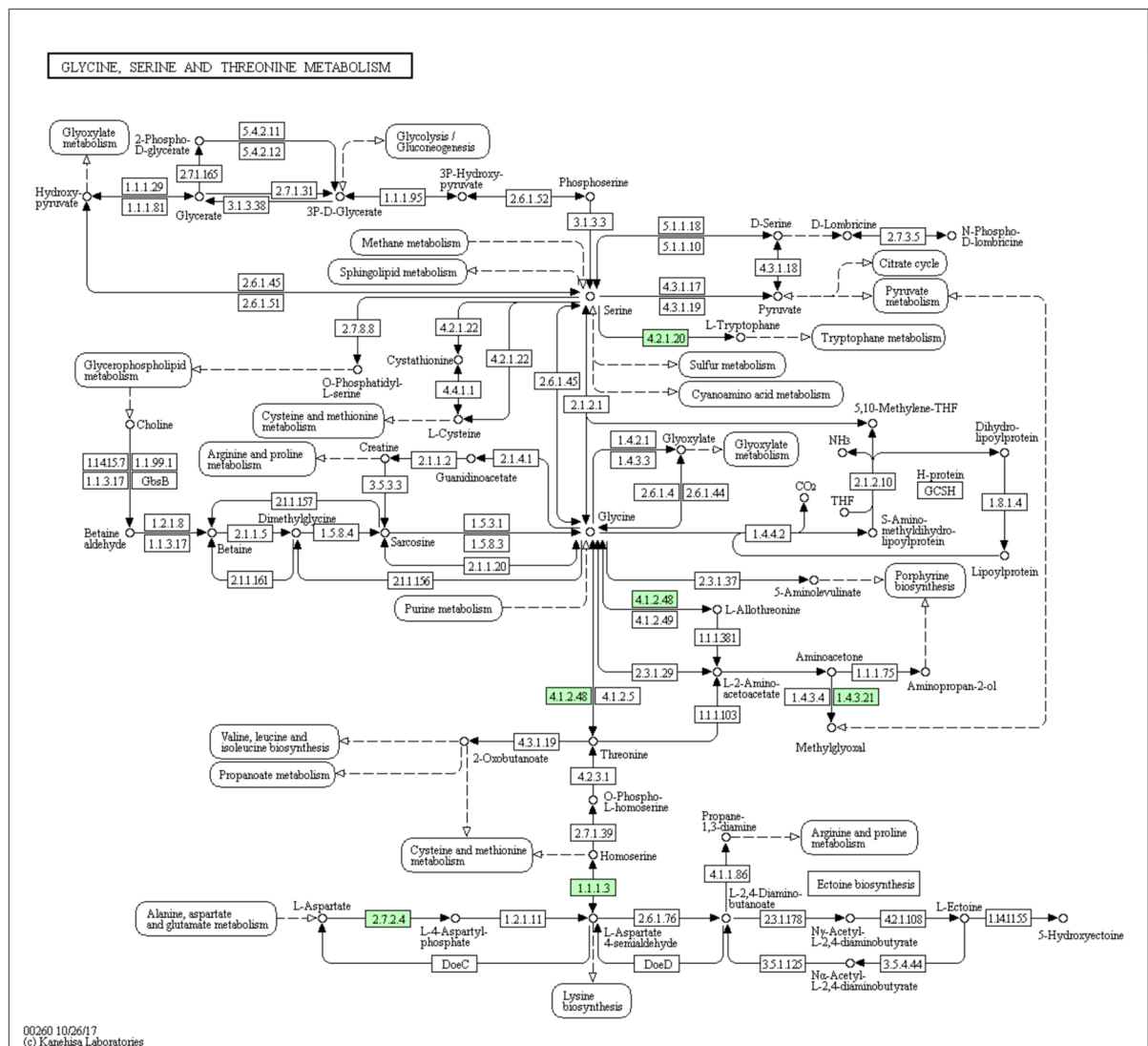
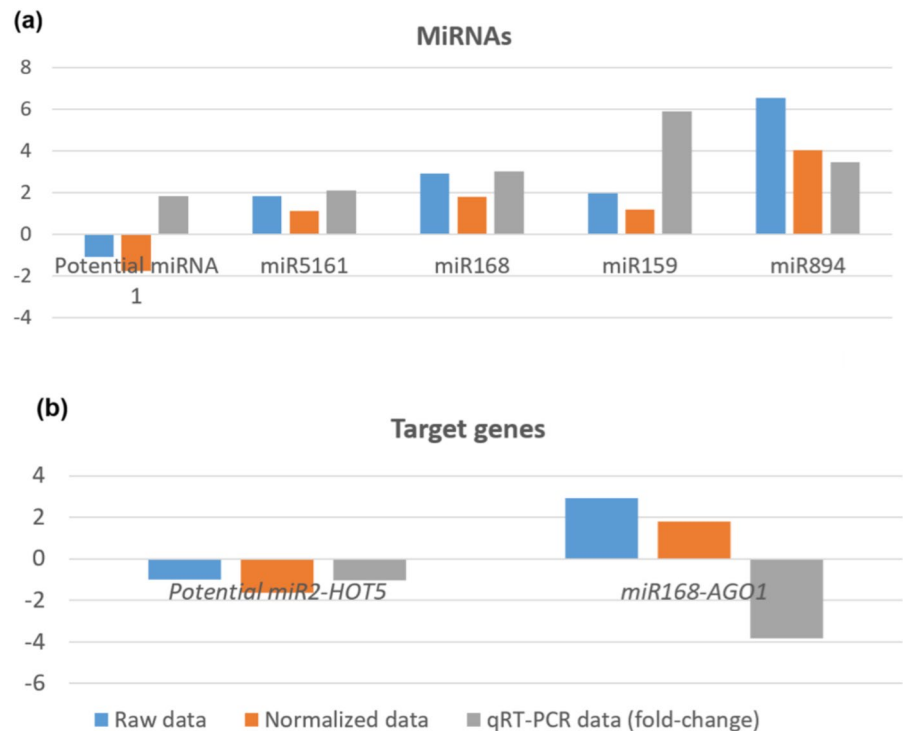


Fig. 5 KEGG analysis of genes targeted in *Arabidopsis thaliana* by some variants of small RNA sequences matching known miRNAs in the Drought sample with regard to “glycine, serine and threonine metabolism”, (green marked genes represent targeted ones)

Fig. 6 Bar graphs showing the expression levels (fold-change) of miRNAs and their target genes



Discussion

When the latest taxonomy of the genus *Beta* was examined, there were a total of 4 sections, namely *Beta* including cultivated (*Beta vulgaris* L.) and wild (*B. maritima*, *B. adanensis*, *B. macrocarpa*, *B. patula*) species and also *Corollinae*, *Nanae* and *Procumbentes* wild species (Ford-Lloyd 2005; Kadereit et al. 2006). Among these wild species, autotetraploid (4x) *Beta corolliflora* in *Corollinae* section is significantly resistant to abiotic stresses, indicating that it can be used as a genetic resource against these abiotic stress factors (Guo et al. 1994; Frese et al. 2001; Wu et al. 2013).

Nowadays, studies on non-coding RNA sequencing-based studies focus especially on plants such as cotton (Ruan et al. 2009), alfalfa (Szittyta et al. 2008), corn (Wang et al. 2011) and rice (Morin et al. 2008). Also, miRNAs associated with drought stress have been investigated in many plants (Akdogan et al. 2016; Bouzroud et al. 2018), especially in *Arabidopsis* (Ma et al. 2015) and rice (Xia et al. 2020). However, studies on the determination of non-coding RNAs and their target genes involved in drought stress response are quite limited (Yolcu et al. 2021a,

b) in sugar beet (Zou et al. 2022, 2023), especially in wild beets.

In this study, the miRNA profile of wild beet *Beta corolliflora* roots was revealed under drought stress conditions, and the miRNAs expression levels and pathways associated with genes targeted by these miRNAs were determined. To our knowledge, the current work is the first to report characterization and analysis of miRNAs isolated from wild beet *Beta corolliflora* roots using high-throughput small RNA sequencing.

When small RNA sequences matching with miRNAs were grouped as mature miRNAs, 83 miRNAs were identified in the Drought sample. It was observed that, on average, the miRNAs included ~21 long nucleotides, as also reported by Zhou et al. (2016) and Ji et al. (2018). A total of 9 upregulated and 10 downregulated miRNAs (> 1.5 fold-change) were detected in the Drought sample, and this number of miRNAs is less than that of wild rice root miRNAs (Zhang et al. 2017).

GO enrichment and KEGG pathway analyses of miRNAs target genes can help us understand the functions of miRNAs more effectively (Muhammad et al. 2022). According to molecular function, the

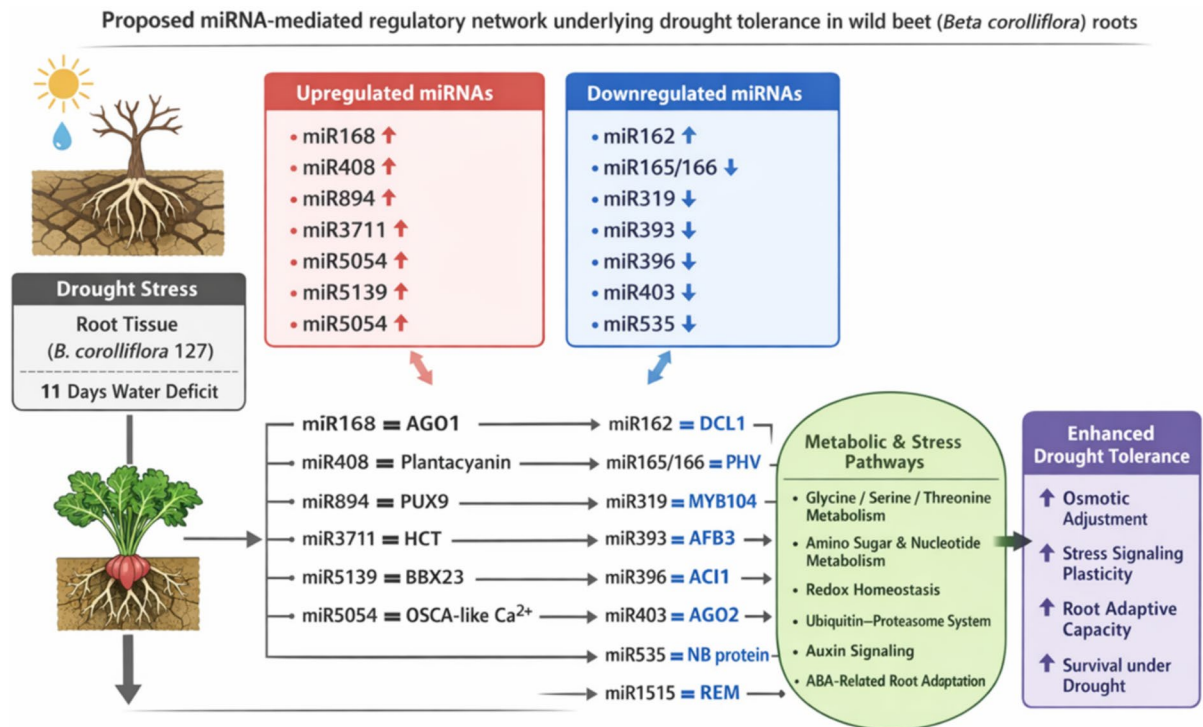


Fig. 7 Proposed miRNA-mediated regulatory mechanism underlying drought tolerance in wild sugar beet (*Beta corolliflora*) roots

target genes for drought-responsive miRNAs were significantly enriched in catalytic activity. In addition to this, within the biological process group, the most significantly enriched GO term was the metabolic process. These results indicated that miRNAs may play a role in drought resistance by modulating target genes controlling oxidoreductase activity which is in agreement with the report of Huang et al. (2014).

According to KEGG pathway analysis of the target genes for miRNAs, the most significantly enriched pathway involved glycine, serine, threonine, amino sugar, and nucleotide sugar metabolism. Glycine and threonine can act as synthetic substrates of osmotic regulatory substances, and their accumulation may play a critical role in plant responses to abiotic stress (Chang et al. 2020). On the other hand, Diniz et al. (2020) showed that drought stress greatly affects the primary metabolism of plants, including amino acid and carbohydrate metabolism. Both KEGG and GO outcomes suggested a probable regulatory role of miRNAs in wild sugar beet (Drought) response to drought. The action figure model, created to provide an integrated

summary of the main findings of this study, is presented in Fig. 7.

Evaluation of Pre-miRNAs and target genes for drought stress

In some cases, expression of the plant miRNAs in response to drought stress is not similar in the same species. Different species can show similar or opposite expression profiles, and even the same species may show different expression patterns for the same miRNA (Banerjee et al. 2017). This is attributed to the different developmental stages at which the plant is exposed to drought stress and the characteristics of the genotype (Singh et al. 2020). In this framework, miRNAs that showed significant changes in expression under different drought stress conditions were compared.

In this study, miR319 was determined to be down-regulated and associated with the DNA-binding domain (*MYB104*) as a target gene. While miR159 and miR319 regulate the expression of *MYB* transcription factors, miR319 also targets transcription

factors. Studies have shown that the miR159 and miR319 gene families are closely related and include 21 common nucleotides (Palatnik et al. 2007). In addition, recent studies have revealed connections between miR159 and miR319 with a number of target genes (Akdogan et al. 2016). According to the findings, while insignificant changes could be detected in the expression level of miR159 in Drought sample, the expression level of miR319 was downregulated slightly similar to that reported in other studies (Singh et al. 2020). In plants, miR319 has been proven to play a role in abiotic stress tolerance by targeting the transcription factor “*TEOSINTE BRANCHED/CYCLOIDEA/PCF (TCP)*” and the expression of miR319 is inversely correlated with the expression of the target gene *TCP4* transcription factor protein (Baulies et al. 2022). However, recent studies have shown that miRNAs in plants are effective in drought tolerance not only at the leaf level but also through multi-layered mechanisms such as root system formation, water transport, xylem anatomy, and ROS/ABA-based stress regulation (Balhara et al. 2024; Zhakypbek et al. 2025). Considering this, the downregulation of miR319 in the root tissue of the Drought sample in our study and the presence of the potential *MYB* transcription factor (*MYB104*) target point may indicate a root-based adaptive drought tolerance strategy, which is different from the classical TCP-based system. This situation can be considered as reflecting a potential regulatory mechanism for drought adaptation in root tissue based on the possible relationship between miR319 and *MYB104* (Balhara et al. 2024).

In our study, it was determined that miR162 was downregulated in a drought-tolerant *Beta corolliflora* genotype (Drought sample), and the target gene was revealed to be *Dicer-like 1 (DCL1)*, which plays an important role in miRNA biogenesis (Li et al. 2023a, b). In a different abiotic stress study, it was explained that miR162 downregulation under low-temperature stress, similar to this study, led to increased *DCL1* gene expression, which in turn increased the production of other miRNAs (miR160, miR164, miR171, etc.) and induced the activation of stress response genes (Li et al. 2023a, b). In this study, however, no upregulation was observed in miRNAs such as miR160, miR164, and miR171, along with miR162 downregulation. This suggests that the miR162-*DCL1* relationship is not only important for homeostasis, but also a significant component of adaptive

regulation under drought stress conditions. Based on this, it can be hypothesized that miR162 downregulation in *Beta corolliflora* genotype (Drought sample) adaptively restructures the capacity of miRNA biogenesis, enabling more effective and coordinated responses to drought stress conditions through regulatory adjustment (Xie et al. 2003; Shao et al. 2015). In this study, the target analysis determined that miR168 suppressed the *AGO1* gene and miR403 suppressed the *AGO2* gene. While the expression level of miR168 was slightly upregulated in the Drought sample, the expression level of miR403 was downregulated significantly. miR168 and miR403 target the *Argonaute (AGO)* gene, which is involved in RNA silencing. Targeting the *AGO* gene by miR168 and miR403, which together play a role in silencing the target mRNA, directly affects post-transcriptional gene regulation (Várallyay et al. 2010; Liu et al. 2020). In this context, upregulation of miR168 contributes to the preservation of essential miRNA biogenesis and function by preventing the increase in *AGO1* levels, while downregulation of miR403 may allow the activation of different RNA silencing responses under stress conditions by removing the suppression on *AGO2/AGO3* (Jagtap and Shivaprasad 2014). Current studies show that miR168-*AGO1* and miR403-*AGO2* regulations are associated with different stress tolerances not only in model plants, but also in cultivated ones (Dolata et al. 2016; Zhang et al. 2023; Manacorda et al. 2024). These findings support the idea that the combination of miR168 upregulation and miR403 downregulation found in our study may not be coincidental; it may be an adaptive and evolutionarily regulatory strategy (Vaucheret et al. 2006).

According to the analysis findings, the expression level of miR165/166 was downregulated in Drought sample. Similarly, in different plant species, miR165/166 has been described to negatively mediate different targets, including seed and root development and ion uptake, and is also involved in the modulation of various developmental processes (Singh et al. 2014, 2020; Iwamoto and Tagiri 2016). miR165/166 targets the class III homeodomain leucine zipper family of transcription factor genes, including *Phabulosa (PHB)*, *Phavoluta (PHV)*, and *Revoluta (REV)*, which are involved in meristem maintenance, leaf polarity formation, and root differentiation (Zhou et al. 2015; Yang et al. 2019). It is widely believed that downregulation of miR165/166 is associated with increased

stress tolerance, and this mechanism has been observed as a common stress response mechanism in many plants (Ding et al. 2013; Scintu et al. 2023). Accordingly, it is hypothesized that miR165/166 downregulation increases the expression of target genes and subsequently enhances ABA homeostasis and stress adaptation (Yadav et al. 2021). From this perspective, the current findings are consistent with those of other studies, and the presence of this mechanism may also be present in *Beta corolliflora* genotype in response to drought stress.

It has been shown that miR393 affects the expression levels of *TIR1*, auxin signaling *F-box* (*AFB1*, *AFB2* and *AFB3*) genes in response to abiotic stress (Wang et al. 2018). In this study, similar to some other studies, the expression level of miR393 was downregulated in the Drought sample (Jiang et al. 2022). Our results also indicated that the auxin receptor *AFB3* is targeted by miR393. It has been shown that miR393 affects the expression levels of *TIR1* and auxin signaling *F-box* (*AFB1*, *AFB2*, and *AFB3*) genes in response to abiotic stress (Wang et al. 2018). In a drought stress study on miR393 family members and their target genes *GmTIR1* in soybean root tissue, it was explained that miR393 patterns showed different expression changes under drought stress. In the same study, it was also explained that miR393a decreased drought tolerance by triggering an increase in antioxidant capacity, root vigor and relative water content (RWC) (Xing et al. 2022). In this study, the downregulation of miR393 in the Drought sample highlighted a species-specific adaptive response that allowed for the fine-tuning of auxin signaling (Chen et al. 2012) by increasing the expression of the potential auxin receptors *TIR1/AFB*. In conclusion, the fact that miR393 can show both increased and decreased expression under stress conditions in different plant species reveals that this miRNA is a highly important regulator in drought response, among other stress factors (Jiang et al. 2022), and plays a strong role in auxin-mediated stress adaptation (Ding et al. 2013).

According to our analysis, the downregulated miR396 specifically targets the *Alcatraz 1* (*AC11*) gene, a transcription factor belonging to the basic helix–loop–helix (*bHLH*) family, which is expressed in the plant vascular system. miR396 has been associated with drought stress responses in several studies, in which a complex pattern of possible functions has emerged. However, contrasting results have been

reported for some species. For example, miR396 was downregulated by drought in rice and grapevine, but in another study, miR396 was shown to be upregulated by drought in soybean leaves but downregulated in soybean roots (Liu et al. 2017; Fracasso et al. 2021). Although the classical target of miR396 is Growth-Regulating Factors (*GRFs*) (Szczygieł-Sommer and Gaj 2019), *bHLH-type factors* have also been identified in some plant species (Bao et al. 2014; Debernardi et al. 2012). These targets involved in drought stress response may act as secondary regulators. In this context, the presence of a *bHLH* gene similar to the *AC11* gene suggests the possibility of a different species- and root tissue-specific target gene association in drought stress tolerance.

In this study, downregulation of miR535 was observed in Drought sample. Although it is stated to miR535 targets the *SPL* gene (Gelaw and Sanan-Mishra 2021), our findings suggest that miR535 may potentially targets a nucleotide-binding protein (*NB*) responsible for alternative splicing. *NB* proteins play a critical role in ROS signaling, cell death regulation, and activation of defense pathways during abiotic stress (Wang et al. 2023). While studies have reported that miR535 exhibits species-specific different expression profiles under stress conditions (Fard et al. 2017; Zhang et al. 2014; Hivrale et al. 2016), miRNA and *NB* gene interactions have been reported to constitute an important regulatory mechanism for drought adaptation (Zhakypbek et al. 2025). Therefore, the miR535–*NB* gene interaction observed in *Beta corolliflora* under drought stress may represent a putative novel regulatory relationship in drought tolerance. Recently, miR535 overexpression and clustered Regularly Interspaced Palindromic Repeats (*CRISPR/Cas9*) knockout in rice enhanced stress tolerance in presence of dehydration stress (Yue et al. 2020).

Interestingly in this study, miR1515 showed a significant downregulation profile in the Drought sample (targets Remorin (*REM*)). Although the role of root miR1515 in response to drought stress is still unclear, it was also reported that miR1515 was significantly expressed in drought-sensitive cowpea leaves (Barrera-Figueroa et al. 2011) and in *Phaseolus vulgaris* (Chaudhary et al. 2021) leaves in response to drought stress and ABA treatments, respectively. On the other hand, recent studies have shown that *REM* proteins exhibit changes in gene

expression and protein levels under abiotic stress conditions (e.g., drought and salt), thus contributing to the environmental adaptation of plants (Li et al. 2023a, b; Yang et al. 2024a, b). Therefore, downregulation of miR1515 under drought stress may lead to higher expression of the target *REM* gene, which may contribute to the emergence of physiological effects such as increased plasma membrane signaling capacity and enhanced stress tolerance (Li et al. 2023a, b). Similarly, miR3711, which showed an upregulated profile, targeted the *hydroxycinnamoyl-transferase (HCT)* gene which is known to promote lignin synthesis (Cakir et al. 2016). miR3711 was upregulated in leaves under drought stress (Anjali et al. 2019), but has not yet been reported to be root-responsive to drought stress. Although there is little information about miR3711, considering its target gene, the *HCT* gene, phenylpropanoid regulation in response to drought stress comes to the fore. As is known, *HCT* is a central enzyme in the upstream process of lignin/monolignol synthesis (Sun et al. 2018). In this study, upregulation of miR3711 and suppression of *HCT* gene expression, may be associated with a decrease in lignin synthesis or a shift in efflux to other phenolic products (e.g., flavonoids, soluble phenols). This change may also affect drought adaptation in *Beta corolliflora* through cell wall modifications, osmotic balance or antioxidant profile adaptations. Such phenylpropanoid modifications in drought responses have been reported in previous studies (Yu et al. 2020). Therefore, further functional studies are needed to understand the molecular mechanisms of miR1515 and miR3711, their roles in signal transduction, and contributions to stress tolerance.

In the Drought sample, miR5054 was significantly upregulated. Recent studies have revealed that *ZmOSCA2.4* (hyperosmolality-gated calcium-permeable channel proteins) may be a target of miR5054 and plays an active role in drought tolerance in maize. However, the role of miR5054 in drought stress remains unclear and further research is needed (Cao et al. 2020). Furthermore, in our study, miR408 was significantly upregulated under drought stress. Consistent with previous studies, miR408 targets plantacyanin transcripts in various plant species and reduces plantacyanin levels under stress conditions (Trindade et al. 2010; Yang et al. 2024a, b). miR408-plantacyanin regulation has been shown to be associated with

abiotic stress response and redox homeostasis (Gao et al. 2022).

In this study, target gene identification could not be achieved in root tissues related to downregulated miR398 and miR398b and upregulated miR8175. Current studies reveal that the limited genome and transcriptome annotations, particularly in wild species, make it difficult to identify targets of low-abundance or species-specific miRNAs using computational methods. Furthermore, downregulation of the miR398 family can lead to translational regulation of target genes instead of mRNA degradation, further complicating target identification (Srivastava et al. 2014; Pandey et al. 2025). The limited characterization of miR8175 in the literature and the lack of experimentally validated targets are considered possible reasons for the inability to identify the target gene (He et al. 2014). These results indicate that miRNA-target relationships in wild species are more complex than those in cultivated plants and that, important regulatory mechanisms are still open for discovery.

Finally, in this study, miR894 was significantly upregulated in wild sugar beet under drought stress, and the plant *ubiquitin regulatory X domain-containing* protein (*PUX9*) gene was identified as the target gene. Although the functional role of miR894 in the context of drought stress has not yet been characterized in detail *PUX* genes play a central role in the regulation of the ubiquitin–proteasome system (UPS) in plants by interacting with the *CDC48/p97 AAA-ATPase* complex (Rancour et al. 2004; Li and Jarvis 2024). As is known, the ubiquitin–proteasome system is a critical regulatory mechanism in stress signaling, hormone responses, and protein quality control processes in plants under various abiotic stress conditions, including drought (Stone 2014). In this context, the increase in miR894 expression under drought stress may potentially contribute the reorganization of UPS-mediated protein degradation by suppressing *PUX9* expression levels. Similarly, it has previously been reported that many miRNAs whose expression levels change under stress conditions regulate plant stress tolerance at the post-transcriptional level by targeting stress-related genes, including protein degradation pathways (Zhakypbek et al. 2025). These results highlight the possibility that miR894 may be part of a putative genotype-specific regulatory mechanism contributing to drought tolerance in wild sugar beet. However, although the data obtained constitute a preliminary starting point in terms of wild sugar

beet miRNAs, additional functional studies are needed to confirm the relationships between miRNAs and target genes. The limited changes in miRNA expression observed in our study do not indicate an absence of stress response but rather may reflect limited plasticity, defined as the selective and the fine-tuned modulation of the miRNA regulatory network under stress conditions. This pattern suggests that the studied genotype may possess a constitutive or pre-adaptive defense capacity, in which drought responses are mediated through fine-tuning of only a few key miRNAs rather than extensive global reprogramming. Consistent with this interpretation, previous studies have reported that miRNA response patterns are highly tissue- and time-dependent, and that in some drought-tolerant genotypes, only a limited number of miRNAs show significant differential expression (Guo et al. 2017; Ferdous et al. 2017). Moreover, recent studies have demonstrated that miRNA regulation under drought stress modulates specific adaptive mechanisms, such as hormone signaling and antioxidant pathways, rather than causing broad expression shifts, supporting the view that limited miRNA changes can represent a functional and effective stress adaptation strategy (Zhakypbek et al. 2025). Overall, these observations are consistent with and support the findings of the present study.

Despite the findings obtained in this study, a limitation of this work is the lack of biological replication in the sequencing analysis. Therefore, the results should be interpreted with caution, and further studies including biological replicates are needed to validate these findings. Nevertheless, the present results provide preliminary insights into miRNA-mediated responses under drought stress in *Beta corolliflora*.

Conclusion

The fact that the miRNA profile of wild *Beta corolliflora*, which is more resistant to drought than cultivated sugar beet, is partially affected by drought stress suggests that drought tolerance may involve mechanisms other than RNA silencing in this species. To our knowledge, the current work is the first to report the systemic retrieval, characterization and analysis of miRNA isolation from wild beet (*Beta corolliflora*) root using small RNA sequencings.

The expression of a small number of conserved miRNAs was decreased in drought-stressed sample

compared to that in the control. Moreover, no event changes were detected in plant hormone pathways involved in the response to drought and oxidative stress and in the expression of genes related to anti-oxidant defence mechanisms.

Important and conserved miRNAs included in plant miRNA databases were also densely detected in *Beta corolliflora*. This is an important result in terms of detecting such conservation in wild species, considering that the majority of studies on miRNAs involve only cultivated species. However, although the obtained data provide a foundational starting point for wild sugar beet miRNAs, additional functional studies are required to validate miRNA–target gene interactions. Some of the known miRNAs detected in *Beta corolliflora*, including their target genes, have not been mapped to the *Beta vulgaris* genome and should be evaluated. In this work, findings which may enable a better understanding of the complex response mechanism developed by wild beet against stress conditions have been obtained. In conclusion, the limited miRNA expression changes observed under drought stress indicate restricted regulatory plasticity, suggesting a fine-tuned and potentially pre-adapted drought response in the studied genotype. These findings may provide a basis for new strategies to minimize the negative effects of stress factors and improve root drought stress tolerance in sugar beet production.

Author contributions UK: Methodology, conceptualization, data curation, software, formal analysis, writing—original draft, writing—review and editing, MH: Visualization, software, validation, formal analysis, AE: Methodology, project administration, supervision, conceptualization, writing—original draft, writing—review and editing, funding acquisition. All authors read and approved the manuscript.

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Data availability All the raw data sets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest

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