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Function and Gene Network Mapping for Target Genes of *miR414* and *miR2102* under Drought Stress in Barley

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Abstract

Barley (*Hordeum vulgare* L.), an important crop plant and an annual grain cereal, is widely cultivated in dry areas globally. Barley's drought tolerance is multi-genic. There is little information on the candidate genes linked to drought tolerance. This study aimed to evaluate the function and gene network mapping for *miR414* and *miR2102* target genes under drought stress in barley. psRNATargets served were used to find target sequences of desired microRNAs. The results of this study revealed that NUC-L1, At5g14050 (GRF1), YAO, and RPL5A genes exhibited the highest contrast with other genes in the network. The expression of identified target genes was evaluated in two barley genotypes (Nimruz as a drought-tolerant cultivar and Spontaneum as the drought-sensitive species) under drought stress. The expression analysis results showed that each genotype exhibited a distinct expression pattern for the target genes. Nimruz cultivar had a higher expression for all target genes than Spontaneum. The transcription level of *NUCL1* increased by about 56-fold under drought stress in Nimruz after 72 hrs, whereas only slight or no significant elevations were observed in Spontaneum. Based on the results, the expression of the *GRF1* gene was significantly ($P < 0.05$) influenced by genotype and drought stress. The Nimruz cultivar generally exhibited higher expression than the Spontaneum genotype, with both genotypes showing increased expression under stress conditions. Under drought stress in Nimruz, the transcription levels of the *GRF1* and *RPL5A* genes increased by about 101- and 33-fold, respectively, after 72 hrs. The YAO gene's transcription level rose by about 42-fold after 24 hrs.

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Introduction

Global climate change has jeopardized crop plant productivity. Under abiotic stresses such as drought stress, metabolic reprogramming is key, and it regulates the cytosolic osmotic balance. It threatens sustainable crop production (Shaffique et al. 2024). Barley (*Hordeum vulgare*) is considered the fifth most widely cultivated crop plant. It is a multi-purpose cereal, valued in the food industry for malt and beverage production, as well as in both animal and human nutrition (Arendt and Zannini 2013, Akbari et al. 2023). Due to the multi-genic nature of drought tolerance and the limited information about the candidate genes efficiently involved, molecular breeding for drought tolerance/resistance is highly challenging. Evolutionarily, plants have adapted various defensive strategies to sense, respond, and cope with diverse abiotic stresses (He et al. 2018).

Drought tolerance in plants comes from multiple mechanisms, including osmotic adjustment, antioxidative defense systems, DNA methylation, and stress-related genes. These are physiological, biochemical, genetic, and epigenetic processes/mechanisms. Post-transcriptional gene expression is one key to the regulatory processes. It helps maintain cellular and molecular balance under environmental stress conditions (Hu et al. 2018). According to Kaur et al. (2023), microRNAs (miRNAs) have an important role in the regulation of involved genes in response to drought stress, suggesting that miRNAs play a modulator role by regulating the expression of multiple genes (Kaur et al. 2023). The barley (*Hordeum vulgare* L.) genotype "Nimrooz" represents a modern cultivated variety that has been evaluated for its adaptability under arid and semi-arid conditions. Breeding programs have highlighted Nimrooz as a promising genotype due to its relatively high yield stability and tolerance to environmental stresses such as drought and salinity, which are critical factors in the context of climate change (Rahimi et al. 2019). In contrast, the wild progenitor *Hordeum vulgare* ssp. Spontaneum represents the primary gene pool from which domesticated barley originated. This ecotype, distributed across the Fertile Crescent and parts of Central Asia, harbors extensive allelic diversity related to abiotic stress tolerance, flowering time, and disease resistance (Nevo and Chen 2010). Unlike modern genotypes such as Nimrooz, *H. spontaneum* populations demonstrate remarkable ecotypic differentiation, enabling survival under extreme environments, including deserts and saline soils (Nevo et al. 2012). The genetic richness of spontaneum remains essential for broadening the genetic base of cultivated barley and for introducing stress-resilient traits into breeding programs aimed at enhancing food security.

Plant miRNAs are 20-24 nucleotide-long post-transcriptional regulatory molecules (Kouhi et al. 2020). Conserved miRNAs are key in many systems of mature plants. They affect plant growth, development, and stress tolerance. Since miRNAs induce a range of diverse target gene expressions, the mechanism of miRNAs' interaction with their target gene is of particular significance (Ruan et al. 2024). A rising number of studies support the important role of miRNAs in coordinating important agronomic traits in crops.

Conserved miRNAs are key in many systems of mature plants. They affect plant growth, development, and stress tolerance. Several drought-related miRNAs have been identified in Arabidopsis, cowpea, rice, tobacco, and soybean, using global expression profiling (Basso et al. 2019, Chakraborty et al. 2020, Guleria et al. 2021). For example, miR156 expression can promote flowering under drought stress, which mitigates the damaging effects of drought stress (Bai et al. 2020). Similarly, the expression level of miR160 in transgenic tobacco is downregulated under drought stress, thereby enhancing its drought tolerance (Singh et al. 2023). Moreover, the expression levels of some conserved miRNAs, such as miR159, miR167, miR169, and miR397, are up- or down-regulated under drought or high salt stress conditions (Bakhshi and Fard 2023, Srivastava et al. 2023). In barely, we previously reported that miR414 and miR2102 regulate target genes associated with drought tolerance (Zare et al. 2019).

Interestingly, studies have found that miR414 primarily targets transcriptional regulators from the MYB, B3, AP2/ERF, and bZIP transcription factor families. These transcription factors play crucial roles in plant growth and development, physiological and morphological changes, secondary metabolism, and responses to diverse environmental stresses (Zinati et al. 2016, Zhang et al. 2017).

Previously, Zare et al. (2019) reported that the expression of N-butyl-N-methylpiperidinium (Pip1; 4) and a non-specific lipid transport protein (nsLTP) increased by 95.98-fold and 54.53-fold, respectively, after 72 hrs of drought stress. Additionally, a significant difference ($P < 0.05$) was observed between the two genotypes regarding the expression levels of these candidate genes (Zare et al. 2019). To elucidate the gene network associated with miR414 and miR2102, the gene network mapping of the target genes was deciphered.

Materials and Methods

The psRNATarget server was used to mine candidate miRNAs in the PMTED database. According to the software developer, the psRNATarget software performs two major tasks: (1) applying a proven scoring pattern to identify inverted associations between any miRNA and its target gene and (2) assessing target-region accessibility by calculating the unpaired energy (UPE) required to "open" the secondary structure around the miRNA target site on the mRNA (Dai et al. 2011). psRNATarget predicts target gene sequences based on two main principles: (1) calculating the degree of pairing between the microRNA sequence and the target sequence, and (2) analyzing the secondary structures of these sequences to determine if they can successfully bind to each other. The psRNA target algorithm predicts the target binding sites of plant miRNAs based on the complementary scoring schema and uncovers the inhibition pattern of cleavage. The software specifications for psRNATarget server were set as follows: the number of top targets was 20, expectation was set to 5, penalty for G : U pairs were set to 0.5, penalty for other mismatches was 1, extra weight in the seed region was 1.5, seed region spans 2-13

nt, a maximum of 2 mismatches were allowed in the seed region, maximum UPE is 25, penalty for opening a gap was set to 2, HSP (high-scoring pair) size was 19 and penalty for extending a gap was set to 0.5.

The protein-protein interaction (PPI) network was constructed using the STRING database (https://string-db.org/). The gene identifiers of *Arabidopsis thaliana* were used as input. A minimum required interaction score of 0.7 (high confidence) was applied to ensure that only reliable interactions were included. All other parameters were kept at the default settings of STRING. The resulting network was analyzed topologically, and the degree (number of direct connections of each node) was calculated for all genes.

A 2 × 2 factorial experiment arranged in a completely randomized design (CRD) with three replications was conducted to evaluate the expression profile of drought-responsive candidate genes by using real-time RT-PCR analysis. Nimruz cultivar and a wild-type diploid genotype from Spontaneum were treated in two different irrigation regimes according to Zare et al. (2019). Sampling was done at three levels of 0, 24, and 72 hrs time interval after drought stress. Briefly, seeds were planted in soil, and pots were irrigated based on the calculated FC until the 5-leaf stage, after which water treatment was applied. Leaf samples were collected, frozen in liquid nitrogen, and stored in a -70°C freezer.

Total RNA was extracted from leaf and root-frozen samples by using a Trizol reagent (Life Technology, Invitrogen, USA). The quantity and quality of the isolated total RNA were measured using a Nanodrop ND-1,000 (Nanodrop Technologies, Wilmington, DE, USA). RNA samples were treated with RNase-free DNase (Promega, USA) to remove possible genomic DNA contamination. cDNAs were synthesized using the iScriptcDNA Synthesis Kit (Bio-Rad, USA). Gene-specific Real-time RT-PCR primers were designed by Oligo v. 5.1 software and synthesized (MWG Co., Germany (Guo et al. 2009). Real-time RT-PCR was conducted in triplicate, using three biological cDNA replicates (iQSYBR Green Supermix kit, BIO-RAD, USA) on an iCycler iQ thermocycler Real-Time PCR Detection system (BIO-RAD, USA) according to the manufacturer's instructions (Internal control). Initial annealing was performed for 4 min at 95°C. Initial amplification was

Table 1. PCR primers for barley selected genes.

Gene	Primer (5'→3')	Annealing temp used (°C)	Product size (bp)
NUC-L1	F: ATGCTGGTGCTGATGTTGGA	57°C	220 bp
	R: CAGCAGGACAAAGCCTTCTT		
GRF1	F: GCTGAGGATGAGGAGGATGA	59°C	210 bp
	R: TCCAGGAGCAGCATCAGTTC		
YAO	F: ATGGGCTTCTTGGAGGAGAA	57°C	360 bp
	R: GCTTCTCCACCTTCTTGCAT		
RPL5A	F: ATGGAGAAGGCTGGTGGTAA	57°C	180 bp
	R: TTGATGCGGTTGGTAGCATT		

Analyses were performed using SAS version 9.1 software and mean comparisons were conducted using Duncan's multiple range test at the 5% significance level.

performed in 40 cycles, with the samples being subjected to 30 sec at the annealing temperature (95°C), 30 sec at the primer annealing temperature, and 30 sec at the strand elongation stage at 72°C, and the final strand elongation was performed for 5 min at 72°C. Finally, to plot the melting curve, the temperature was increased from 50°C to 95°C in 90 cycles of 10 sec, increasing by 0.5°C in each cycle, and the amount of light emitted from the samples was recorded at the end of 10 sec (Table 1). The $2^{(-\Delta\Delta Ct)}$ method was used to calculate gene expression levels of the genotypes under drought-stressed conditions, which were normalized to the control Ct value of spontaneum in the control condition.

Results and Discussion

Fig. 1 shows the gene network of *miR414* and *miR2102* target genes in the barley genome. The *NUC-L1*, *At5g14050 (GRF1)*, *YAO,* and *RPL5A* genes contrast most with other network genes. The constructed PPI network revealed several hub genes with central regulatory roles. Among them, four genes (NUC-L1, At5g14050/GRF1, YAO, and RPL5A) were selected for further expression analysis because of their high connectivity in the network and their potential involvement in drought stress response. NUC-L1, which showed the highest degree value (8), is a nucleolin-like protein known to be involved in ribosome biogenesis and nucleolar functions. Previous studies have reported its role in stress adaptation through the regulation of chromatin organization and transcriptional activity. Its central position in the network suggests that it may mediate broad transcriptional responses under drought conditions. At5g14050 (GRF1) encodes a growth-regulating factor that functions as a transcriptional regulator. As revealed in the PPI network, GRF1 interacts with multiple partners, placing it among the hub-like regulators. Its importance lies in coordinating growth and stress signaling, which is critical during drought when plants must balance growth reduction with stress tolerance. YAO, identified as a hub gene in the cell cycle/embryogenesis-related cluster, is involved in nucleolar function and developmental regulation. Its connectivity in the network and association with other nucleolar proteins highlight its possible role in stress-induced modulation of cell cycle and reproductive development under drought stress. RPL5A, a component of the ribosomal large subunit, clustered with other ribosomal proteins in the network. Ribosomal proteins are increasingly recognized not only as structural components of the translational machinery but also as stress-responsive regulators. The presence of RPL5A in the network cluster associated with translation emphasizes its potential role in maintaining protein synthesis capacity under drought stress.

The *NUCL1* gene, also known as NUCLEOSOME ASSEMBLY PROTEIN1 (NAP1), plays a crucial role in plant DNA repair and transcription regulation. Expression patterns of *NUCL1* and *GRF1* genes were investigated in two barley genotypes under normal and drought stress conditions. Relative expression showed that each genotype had a different expression pattern for genes. Nimruz cultivar had a higher expression as a tolerant cultivar. The transcription level of *NUCL1* increased by about 56-fold under drought

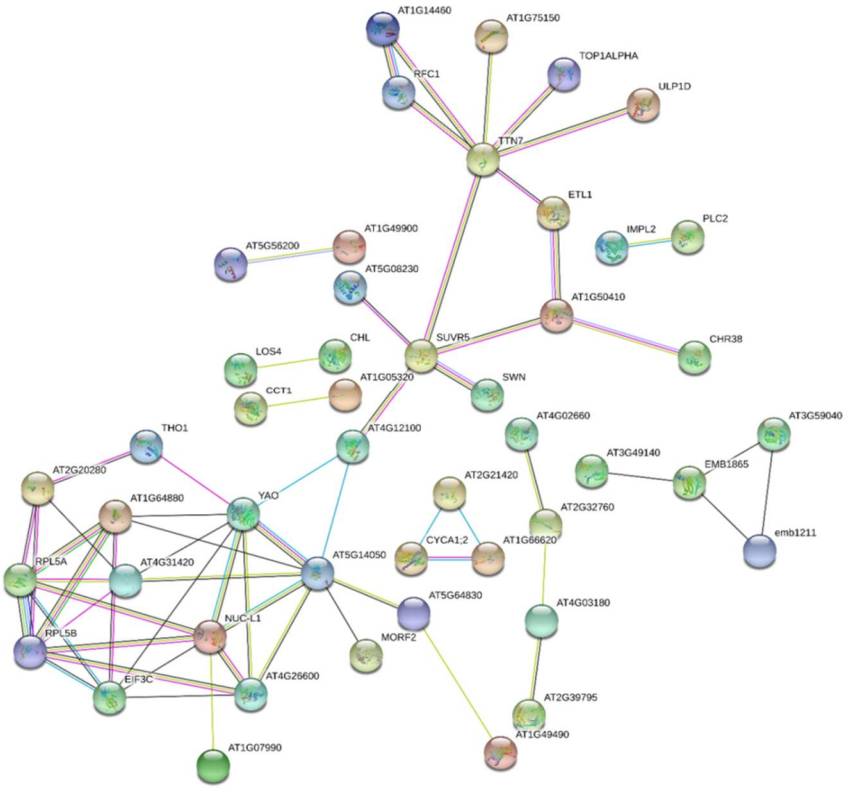

Fig. 1. The gene network between target genes.

stress in Nimruz after 72 hrs, while only slight or no significant elevations were observed in Spontaneum (Fig. 2). Based on the results, the expression of the *GRF1* gene was influenced by genotype and drought stress ($P < 0.05$), Generally, the Nimruz cultivar showed a higher expression than the genotype of spontaneum, while both genotypes showed increased expression under stress conditions. The transcription level of *GRF1* increased by about 101-fold under drought stress in Nimruz after 72 hrs (Fig. 2).

Expression patterns of the *YAO* and *RPL5A* genes were investigated in two barley genotypes under normal and drought stress conditions. Results showed that each genotype had a different expression pattern for the *YAO* and *RPL5A* genes under drought stress. Nimruz cultivar had a higher expression as a tolerant cultivar. In Nimruz cultivar, the transcription level of *YAO* significantly ($P < 0.05$) increased by about 42-fold after 24 hrs drought stress, while the transcription level of *RPL5A* increased by about 33-fold after 72 hrs drought stress (Fig. 3).

Literature indicates that NUCL1 is involved in the nucleotide excision repair (NER) pathway, which is essential for correcting DNA damage caused by environmental stressors (Liu et al. 2009). NUCL1 proteins are implicated in the assembly and disassembly of nucleosomes, facilitating the repair of damaged DNA (Dong et al. 2003).

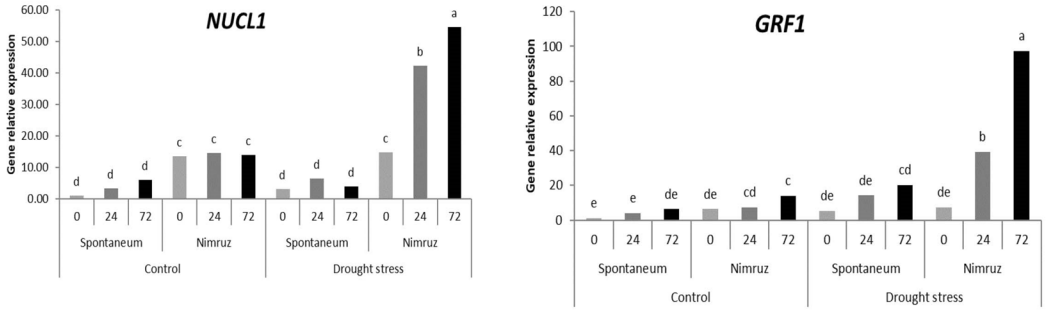

Fig. 2. Comparison of the gene relative expression; *NUCL1* and GRF1 in response to drought stress treatment after 0, 24, and 72 hrs (Different letters in each graph (a-e) indicate significant differences (P <0.05, ANOVA and Duncan's Multiple Range test).

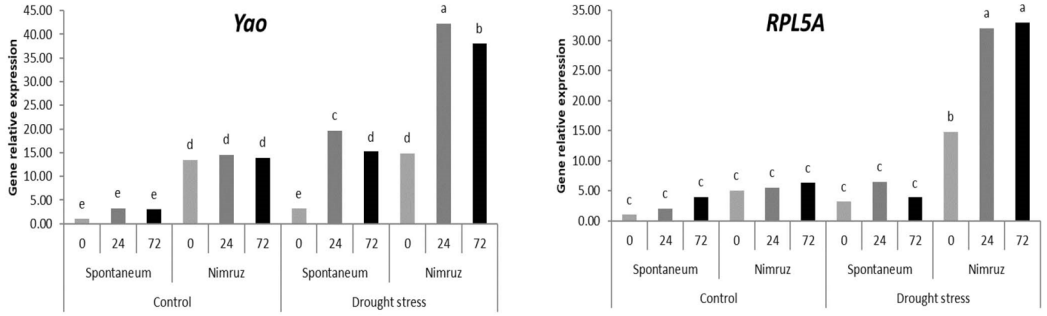

Fig. 3. Comparison of the gene relative expression; *RPL5A* and *YAO* in response to drought stress treatment after 0, 24 and 72 hrs (Different letters in each graph (a-e) indicate significant differences (P <0.05, ANOVA and Duncan's Multiple Range test).

Disruption of NUCL1 genes leads to hypersensitivity to DNA damage, highlighting their protective function against genotoxic stress (Jambunathan et al. 2010). NUCL1 also influences gene expression, as its absence results in altered transcriptional profiles, particularly for genes associated with the NER pathway (Liu et al. 2009). The protein's ability to bind chromatin suggests a direct role in regulating the accessibility of DNA for transcription and repair processes (Liu et al. 2009). The *GRF1* gene in Arabidopsis thaliana encodes a protein that plays a significant role in plant development and stress responses. Specifically, it is associated with the regulation of auxin homeostasis, which is crucial for vascular development. The *At5g14050* gene is part of the *GRF* family, which encodes inositol polyphosphate 5-phosphatases. These enzymes are vital for phosphatidylinositol metabolism, influencing various plant growth processes (Lin et al. 2005). Mutants lacking *GRF1* exhibit defects in cotyledon vein development, indicating its essential role in vascular patterning through auxin regulation (Lin et al. 2005). The gene's involvement in auxin homeostasis suggests it may also play a role in

plant responses to environmental stresses, such as drought, by modulating growth and development under adverse conditions (Kesari et al. 2012). In contrast, while *GRF1* is crucial for auxin regulation, other genes like *AtC3H14* also influence plant growth through different mechanisms, highlighting the complexity of gene interactions in developmental processes (Kim et al. 2014).

Results showed that each genotype had a different expression pattern for the *YAO* and *RPL5A* genes. Nimruz cultivar had a higher expression as a tolerant cultivar. The YAO gene, identified in Arabidopsis, encodes a nucleolar WD40-repeat protein essential for embryogenesis and gametogenesis. It plays a critical role in the correct positioning of the first zygotic division and is involved in the development of male and female gametophytes. Mutations in YAO lead to zygote arrest and misplacement of the cell plate, resulting in early embryo lethality and impaired gametophyte development (Li et al. 2010). YAO's expression is notably high in tissues undergoing active cell division, such as embryo sacs and pollen. Furthermore, YAO is hypothesized to be involved in rRNA processing, similar to its role in yeast and humans, which are components of the U3 snoRNP complex (Li et al. 2010). This suggests a broader functional role in cellular processes beyond gametogenesis. Conversely, while YAO is crucial for plant development, its role in other organisms remains less understood, indicating potential differences in function across species and highlighting the need for further research into its evolutionary significance.

The *RPL5A* gene plays a significant role in drought stress tolerance, primarily through its involvement in the regulation of ribosomal protein synthesis and nitric oxide (NO) accumulation. Research indicates that ribosomal proteins, including *RPL5A*, are up regulated in response to drought conditions, enhancing water-use efficiency (WUE) and stress tolerance in plants (Fig. 3). Specifically, RPL23A, a member of the ribosomal protein family has been shown to improve growth and yield under drought stress by promoting the expression of other ribosomal proteins and enhancing physiological traits such as chlorophyll content and root length (Moin et al. 2017). Additionally, the WD40-REPEAT 5a (WDR5a) gene, a homolog of RPL5A, regulates NO accumulation, which is crucial for stomatal closure during drought, further linking ribosomal proteins to drought response mechanisms (Liu et al. 2017). Overall, RPL5A and related genes contribute to a plant's adaptive responses to water scarcity through multiple pathways, including ribosomal activity and NO signaling.

In conclusion, drought stress is among the main agronomic challenges worldwide and has a profound effect on yield losses in crop plants such as barley. A single miRNA can regulate multiple target genes, and similarly, multiple miRNAs can target a single gene. This regulatory complexity allows miRNAs to participate in various biological processes, such as resistance to drought stress. This study concluded that *NUC-L1*, *GRF1*, *YAO,* and *RPL5A* genes have the highest relative expression contrast with other network genes as target genes of *miR414* and *miR2102* under drought stress in Nimruz barley cultivar.

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