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Expression of miR9863a in responding to drought stress in some wheat and *Aegilops* species

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Original paper

Abstract: MicroRNAs are small RNAs known for their essential roles in regulating both biotic and abiotic stress responses. Drought stress poses a significant challenge to wheat productivity in Iran. The present study evaluated the expression of miR9863a and its target genes in wheat, as well as three *Aegilops* species under drought stress. The results revealed differential expression of miR9863a in the shoots of the studied plants under drought stress conditions. Specifically its expression was increased in *Ae. tauschii* and *Ae. crassa*, while decreasing in *Ae. cylindrica*. The observed differential expression could be explained by the inherent nature of miRNA as a mediator molecule in various biological processes. Analyzing the expression pattern of miR9863a and its target genes in *Ae. tauschii* suggests that the effect of miR9863a in response to drought stress may be attributed to *PLGG1*, impacting glycerate/glycolate transfers and *SAR1A*, influencing trafficking of transcription factors from the endoplasmic reticulum to the nucleus. In addition to complementing previous studies on the role of miR9863 in countering plant diseases, the results presented here illustrate how this miRNA assists the abiotic stress-response mechanism in plants, particularly in the context of drought stress.

Keywords: crassa, cylindrica, *PLGG1*, qRT-PCR, *SAR1A*, tauschii.

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Introduction

Drought stands as the most limiting factor for crop productivity worldwide. Approximately 88% of the total arable area of Iran is located in arid and semi-arid regions (Vaghefi et al., 2019). Therefore, conducting research on the mechanisms of drought-stress tolerance is crucial. Plants employ various mechanisms to tolerate drought, often utilizing osmotic adjustment, desiccation tolerance, and antioxidant capacity (Zhang, 2007). Numerous genes contribute to drought tolerance, initially involving protein kinase-encoding genes and transcription factors (TFs) (Hadiarto and Tran, 2011). Beyond their defense function, drought-related genes can be categorized into three groups; transcriptional regulation, post-transcriptional RNA and osmoprotectant metabolism or molecular chaperons (Yang et al., 2010).

The plastidial glycolate/glycerate translocator 1 (*PLGG1*) gene is responsible for encoding a chloroplast protein that facilitates transports glycolate and glycerate- two of compounds that are required for the photorespiratory cycle to function appropriately (Pick et al., 2013). The *PLGG1* is involved in other major ABA responses, including ABA-inhibited seed germination, ABA-mediated stomatal movement, and drought tolerance (Dong et al., 2018). Secretion Associated Ras Related GTPase 1A (*SAR1A*) is a member of a large family of small GTP-binding proteins. These proteins exist in many organisms and regulate various processes such as signal transduction, cell duplication, cytoskeletal formation, and intracellular membrane exchange. Also, they are involved in the hydrolysis of GTP and transfers from the endoplasmic reticulum to the Golgi apparatus (Vernoud et al., 2003). Endomembrane trafficking is a fundamental cellular process in all eukaryotic cells, the regulatory mechanisms of which have been studied extensively (Wang et al., 2020). A higher amount of GTP-binding proteins can mediate plasma membrane trafficking, affect the plasma membrane proteome, and increase drought tolerance (Ambastha et al., 2021).

miRNAs are a class of short and non-coding RNAs. They are about 20 to 25 nucleotides in length and regulate gene expression in plants and animals. Generally, miRNAs are transcribed from DNA (pri-miRNAs) and processed into stem-loop regions (pr-miRNAs) and mature miRNAs (Ha and Kim, 2014). In plants, miRNAs are extremely complementary to target mRNAs and usually direct cleavage by silencing specific complexes (Bartel, 2004). miRNAs have important roles in gene regulation at different points of the plant life cycle and are involved in several biological processes such as plant development (Curaba et al., 2012), stress response (Budak et al., 2015) and pathogen resistance. The miR9863 family has been identified in *Aegilops tauschii* (Jia et al., 2013). Recently, miR9863a-3p (MI0031654) was reported in *Triticum aestivum* (Naghavi and Fard, 2021). The function of miR9863a-3p is not well-known but is reportedly associated with disease resistance (Liu et al., 2014; Tang and Chu, 2017) and abiotic stress tolerance (Ferdous et al., 2017).

Bread wheat (*T. aestivum*) is one of the most widely grown crops (Mochida and Shinozaki, 2013) and an important food source in the world (Kurtoglu et al., 2014). *T. aestivum* has a complex genome of 6x ploidy (AABBDD) (Marcussen et al., 2014). *Ae. tauschii* is the diploid (2n = DD) progenitor of the D genome of hexaploid wheat and is an important genetic resource in the wheat family (Luo et al., 2017).

Ae. cylindrica is a tetraploid species (CCDD) that shows meiotic pairing with the D genome chromosomes of hexaploid wheat (Dubcovsky and Dvorak, 1994). *Ae. crassa* is tetra- (DDMM) and hexaploid (DDDDMM) and is mainly distributed in Afghanistan, Iraq, Iran, Palestine, Syria, and Turkestan (Eig, 1929). Previous studies have demonstrated the most crucial pathways associated with drought-stress response in *Aegilops* species (Noori et al., 2015; Falaknaz et al., 2019; Zhao et al., 2020). As a wild relative of wheat, *Aegilops* species are regarded as an important source of drought-tolerance genes that can be transferred to wheat cultivars.

To date, only a limited number of studies have investigated the association between miR9863a and abiotic stress response, particularly in the context of drought. The present study aimed to identify and compare the expression of miR9863a-3p and its target genes (*PLGG1* and *SAR1A*) in response to drought stress across wheat and three species of *Aegilops*. The results may help illustrate the roles of miR9863a-3p in

drought-stress response in the *Triticeae* tribe.

Materials and Methods

Plant samples and drought stress treatments

An Iranian bread wheat cultivar (Qods) and three species of *Aegilops*, i.e. *Ae. tauschii* (Acc. No.: TN012189), *Ae. cylindrica* (Acc. No.: KC50180) and *Ae. crassa* (Acc. No.: TN01300), were obtained from the Seed and Plant Improvement Institute of Iran and the National Plant Gene Bank of Iran, respectively. The seed surface was sterilized by rinsing under tap water and then soaking them in 3% NaOCl for 10 minutes. After washing with distilled water, the seeds were transferred to petri dishes and incubated at 4°C for 72 h. Germinated seeds were planted in pots (12 cm in diameter) containing a mixture of loess and sand (5:1 v:v) for 1 month. After two weeks of normal irrigation, drought stress was applied by limiting the irrigation, maintaining 20% of field capacity for 14 days, followed by 3 days without water supply, (Supplementary Figure 1) (Zivcak et al., 2013). The plants were visually assessed for the effects of drought. After 30 days, shoot samples of drought-stressed and control plants were immediately frozen in liquid N_2 and stored at -80ºC.

RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from the shoots of plants at the 4-leaf stage by the CTAB method, with three repetitions (Barman et al., 2017). RNA quantity and quality were measured by NanoDrop 2000 (Thermo Co.). After the treatment of total extracted RNA with *DNase* I, the cDNA was synthesized using the YT4500 kit (YektaTajhizAzma Co.). Oligo dT primers were used for the first strand cDNA synthesis according to the manufacturer's instructions. According to the confidence sequence of *miR9863a* (Acc. No. MIMAT0037105) (tgagaaggtagatcataatagc), stem-loop RT, forward and universal primers were designed according to previously published protocols (Chen et al., 2005; Varkonyi-Gasic et al., 2007). 18s rRNA (Acc. No. AY059462.1) was used as an internal wheat control gene in each reaction (Paolacci et al., 2009; Safarzadeh et al., 2014). qRT-PCR was performed on cDNA samples of wheat and the three species of *Aegilops*, with three biological and two technical repeats using QIAGEN's Rotor-Gene Q and Ampliqon's RealQ Plus 2X Master Mix Green (Table 1).

Prediction of miRNA drought-related target genes

The psRNATarget software was used for predicting the target genes. The miR9863 sequence was used as a query against the *T. aestivum* cDNA library (Ensemble Plants, release 43) with default parameters, including a maximum expectation value of 3, a target accessibility of 25, a mismatch for translational inhibition between 9 and 11 nucleotides, and a maximum mismatch at the complementary site which was equal to or less than 4 without any gaps (Dai and Zhao, 2011). Relevant target genes for drought stress were selected from the results. Primers for qRT-PCR reactions were designed from the deduced sequence, corresponding to the two drought-related target genes (*PLGG1*and *SAR1A*) using the OLIGO 7 primer analysis software (Table 2).

Statistical analysis

Shoot samples from the control and drought-stress-treated plants were collected randomly. The relative level of gene expression for each gene of interest was calculated using the $2^{-\Delta\Delta}$ method described by the Relative Expression Software Tool (REST) (Pfaffl et al., 2002).

Table 1. List of the primers used in cDNA synthesis and qRT-PCR.

Primers	Sequences (5′–> 3′)
miR-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCTATT
miR-F	ATGCCGTGAGAAGGTAGATCA
Universal	GTGCAGGGTCCGAGGT
18s rRNA F	GATGAGCCAAGTGCATATCTCG
18s rRNA R	CTTGTCCGCTAAGTAGGTTGC

RT: stem-loop Reverse Transcription primer, F: Forward primer, R: reverse primer

Table 2. List of primer pairs used in the qRT-PCR target genes.

Gene Accession number	Gene name	Primers	Sequences (5′–> 3′)	Product length
XM_020343245.3	*PLGG1*	Forward Reverse	TCTCCGTGCTCCTCGTC AACCAGCCACCGAATGTG	202
XM_020327846.3	*SAR1A*	Forward Reverse	CTCCTCATCCTTGATGCTCTG TCACCTTACACACGAACACTC	138

Table 3. List of identified function for miR9863 reported in *Triticeae* and monocots.

Plant	Function	Reference
Aegilops	relative to MPV (mid-parent value)	Li et al. (2014)
wheat	predicted to target R gene analogs	Wei et al. (2009)
barley	confirmed to regulate distinct Mla alleles	Liu et al. (2014)
monocots	regulated the NB-LRR genes with CC domains (Disease resistance-associated)	Zhang et al. (2019)
barley	associated with fungal pathogen	Tang and Chu (2017)
barley	regulation under drought stress and their role in mediating expression of target genes for abiotic stress response	Ferdous et al. (2017)

Results and Discussion

Identification, confirmation and expression analysis of miR9863a

According to previous indications, the miR9863 family is mainly expressed in wheat and barley and might be *Triticeae*-specific (Table 3).

The miR9863 family was previously known in *Ae. tauschii*. The family has two members, ata-MIR9863a (Acc. No.: MI0031654) and ata-MIR9863b (Acc. No.: MI0031721). They are located on chromosome 1. (Jia et al., 2013). In particular, *miR9863a* was recently identified and confirmed to exist in *T. aestivum* (Naghavi and Fard, 2021). Despite the difference in the precursor sequence, the sequence of mature *miR9863a* was identical in both plants (Figure 1).

Identification of miR9863a target genes

The target genes were identified for miR9863a using the psRNATarget. Among the 208 genes, *PLGG1* and *SAR1A* were selected and regarded as effective in inducing the drought-stress response based on Gene Ontology (GO) analysis via the UniProt database. The alignment of target gene sequences with miR9863a is shown in Figure 2.

The expectation score to search targets having a poor complementary matching with miRNA or long gaps in alignment were 3 and 4 in the case of *PLGG1* and *SAR1A*, respectively. Unpaired energy (UPE) is defined as the energy required to open secondary structures around the target site on mRNA. The UPE was -1 in both target genes. A lower amount of energy represented a higher likelihood of being an effective target site because the secondary structures may prevent small RNAs and target sites from coming into contact with each other. From a total of 22 co-aligned sequences between *PLGG1* and miR9863a, there were 4 mismatches and two wobble base pairs (G–U). Also, there were 2 mismatches and 3 wobble base pairs between the *SAR1A* and miR9863a alignment. The protein coding region of *PLGG1* gene is located between 117 to 1712 nucleotides, therefore the target site in *PLGG1* gene is located in the coding region, while in *SAR1A* gene, the protein coding region is located from 209 to 790 nucleotide, and as a result, the target site is located in the 3'UTR region.

Quantitative analysis of miR9863a expression

The expression patterns of the *miR9863a* in the drought-stress treatment demonstrated an up-

regulation in *Ae. tauschii* and *Ae. Crassa*, whereas a down-regulation was observed in T. aestivum and *Ae. cylindrica* (Figure 3).

Different expressions of miR9863a were observed in plants under the drought stress treatment. For example, miR9863a was 6-fold up-regulated in Ae. tauschii during drought stress, whereas the miRNA in wheat was 2-fold down-regulated. There are many indications in the available literature that miRNAs have different expression patterns in response to abiotic stresses such as drought (Mehri et al., 1970; Anderberg and Walker-Simmons, 1992; Bakhshi et al., 2013; Hua et al., 2019). In response to drought stress, plants activate their drought-response mechanisms, involving morphological and structural changes, expressions of drought-resistant genes, synthesis of hormones, and osmotic regulatory substances that assist in alleviating drought stress (Yang et al., 2021).

It seems that miR9863a is an intermediate molecule which modulates other protein-coding genes and takes different functions among plants when drought stress is applied. Also, differential expression may due to the fact that overexpression in one tissue may be masked by down regulation in another one (Mone et al., 2018).

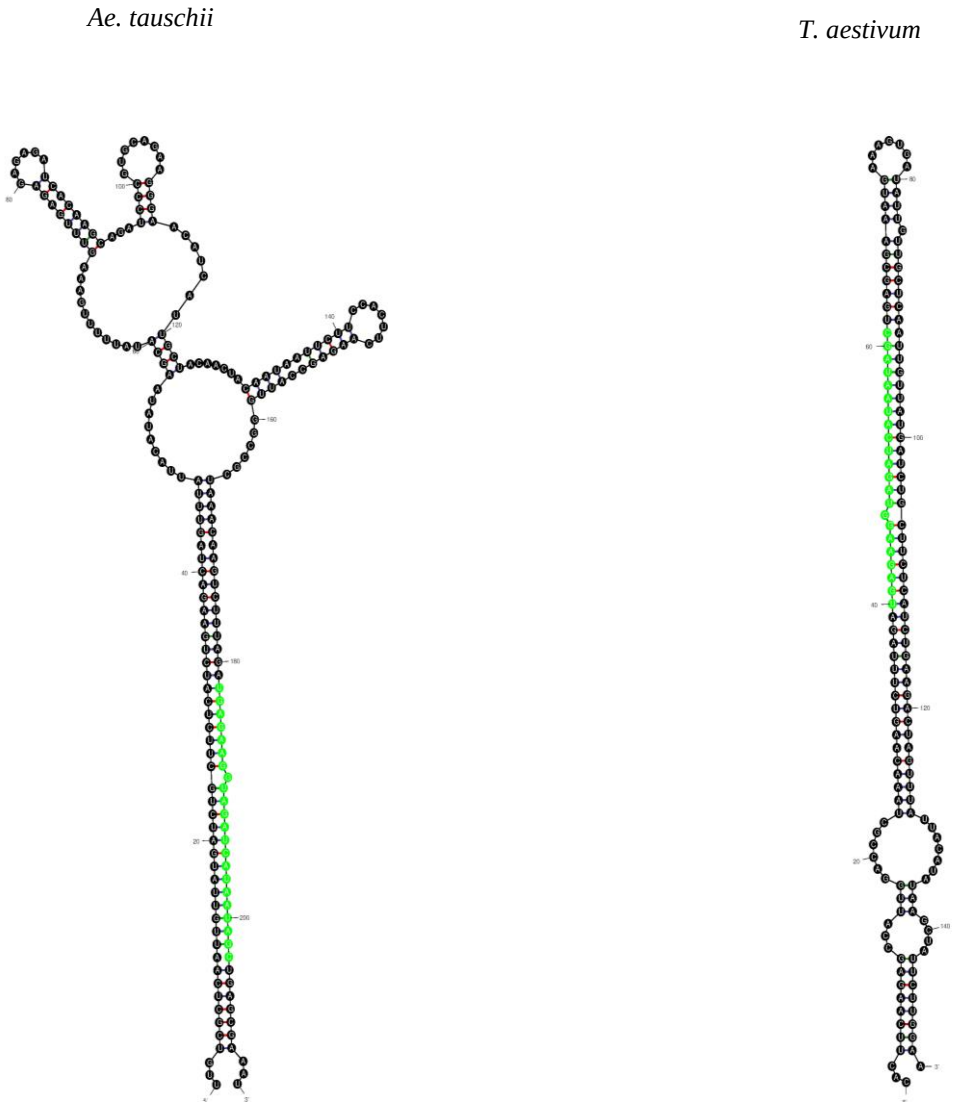

Figure 1. miR9863a structure in *Ae. tauschii* and *T. aestivum.* Identical mature sequences with a length of 22 nucleotides are marked with green spots.

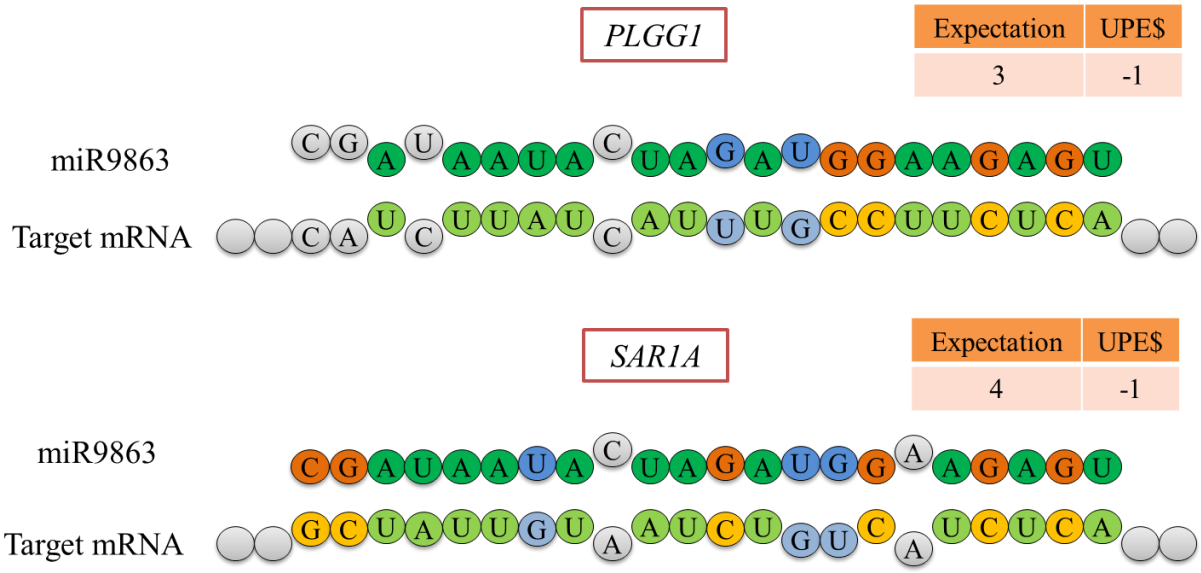

Figure 2. Alignment of *PLGG1* nd *SAR1A* with miR9863a sequences by psRNATarget software.

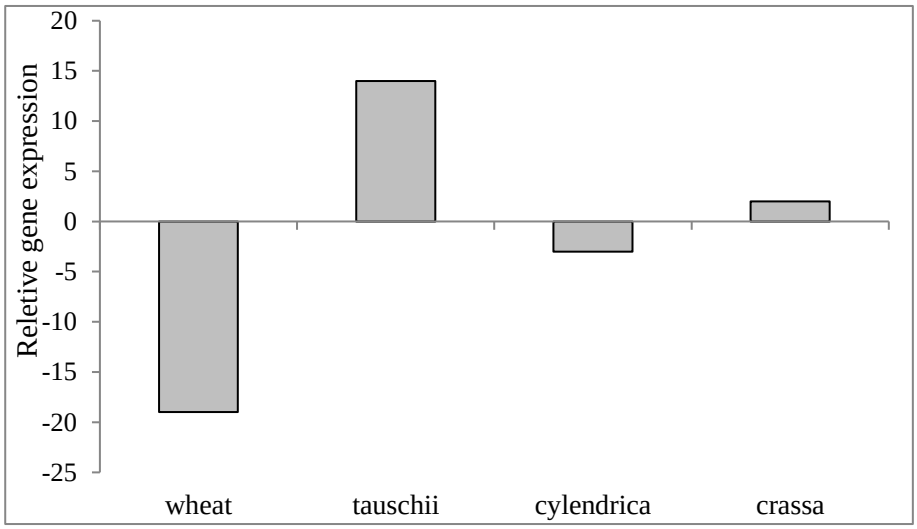

Figure 3. The relative expression rate of miR9863a in drought-stress treatments on *T. aestivum*, *Ae. tauschii*, *Ae. cylendrica* and *Ae. crassa*.

miR9863a expression and regulation of the glycolate/glycerate translocator in photorespiration

To evaluate miR9863 target genes, the relative expression of drought-related genes in wheat and the three *Aegilops* species was evaluated using qRT-PCR. The *PLGG1* was not amplified or had a small level of expression in wheat, *Ae. cylindrica*, and *Ae. crassa* under normal and drought stress conditions. In the *Ae. tauschii*, despite a high level of *PLGG1* expression in the normal condition, its expression was significantly suppressed under drought stress. Referring to the increase in miR9863a expression and the decrease in *PLGG1* expression in *Ae. tauschii* during drought stress, it seems that drought stress may cause an increase in miR9863a expression which suppressed its target gene.

The *PLGG1* gene encodes a chloroplastic protein named plastidial glycolate/glycerate translocator 1 which is required for photorespiration. During plant growth and development, drought stress is one of the most severe environmental restrictions that lead to the accumulation of ABA. Under drought stress, ABA regulates the transpiration

rate via guard cells by triggering stomatal closure and inhibiting stomatal opening. When the plant closes its stomata, carbon dioxide cannot enter the plant. As a result of the stomatal closure, while oxygen is produced, it is entrapped inside the plant. Thus, the amount of oxygen increases and photorespiration takes place. Photorespiration is a metabolic repair pathway that is activated in oxygenic photosynthetic organisms to degrade a toxic product of oxygen fixation. Within the metabolic pathway, energy is consumed and carbon dioxide is released (Dong et al., 2018). PR is frequently regarded as a wasteful process because the loss of CO2 and the occurrence of energy consumption can lower the photosynthetic efficiency and the plant yield. To mitigate the yield penalty, scientists have suggested different strategies, one of which is the synthetic bypass of PR to circumvent mitochondrial glycine decarboxylation, thereby avoiding the release of CO_2 (Figure 4). In this way, a large portion of the carbon in glycolate is released as CO_2 in close proximity to Rubisco within the chloroplast, thereby enhancing CO_2 fixation (Kuhnert et al., 2021).

In the present study, considering the miR9863a and *PLGG1* expression pattern in *Ae. tauschii*, it seemed that the drought stress induced the expression of miR9863a which then down-regulated the *PLGG1* expression. A decrease in *PLGG1* expression causes synthetic bypass. Also, *PLGG1* is involved in the drought-response via ABA (Pfaffl et al., 2002). Possibly, miR9863a is indirectly implicated in the drought-response by inhibiting the *PLGG1* gene.

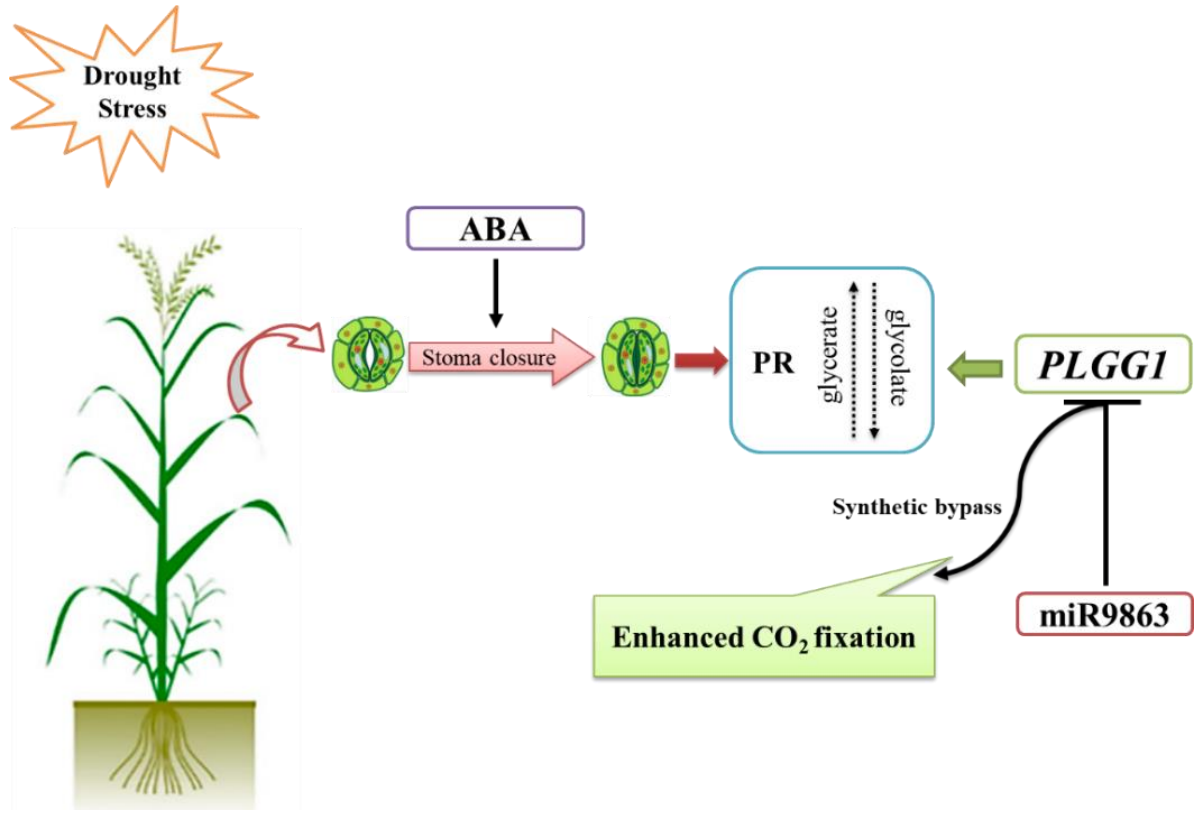

Figure 4. Significance of the *PLGG1* in anticipated flux distribution between native and synthetic route in PR metabolism. Silencing the plastidial glycolate/glycerate transporter allows synthetic bypass. Image adapted from (Nawkar et al., 2018; Wang et al., 2020).

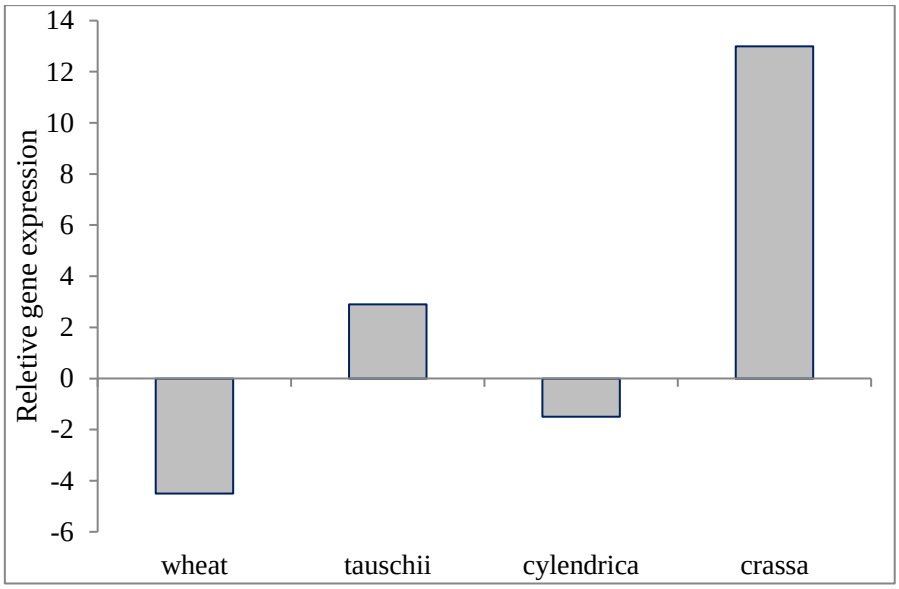

Figure 5. The relative expression rate of *SAR1A* target gene in response to the drought-stress treatment on *T. aestivum*, *Ae. tauschii*, *Ae. cylendrica* and *Ae. crassa*.

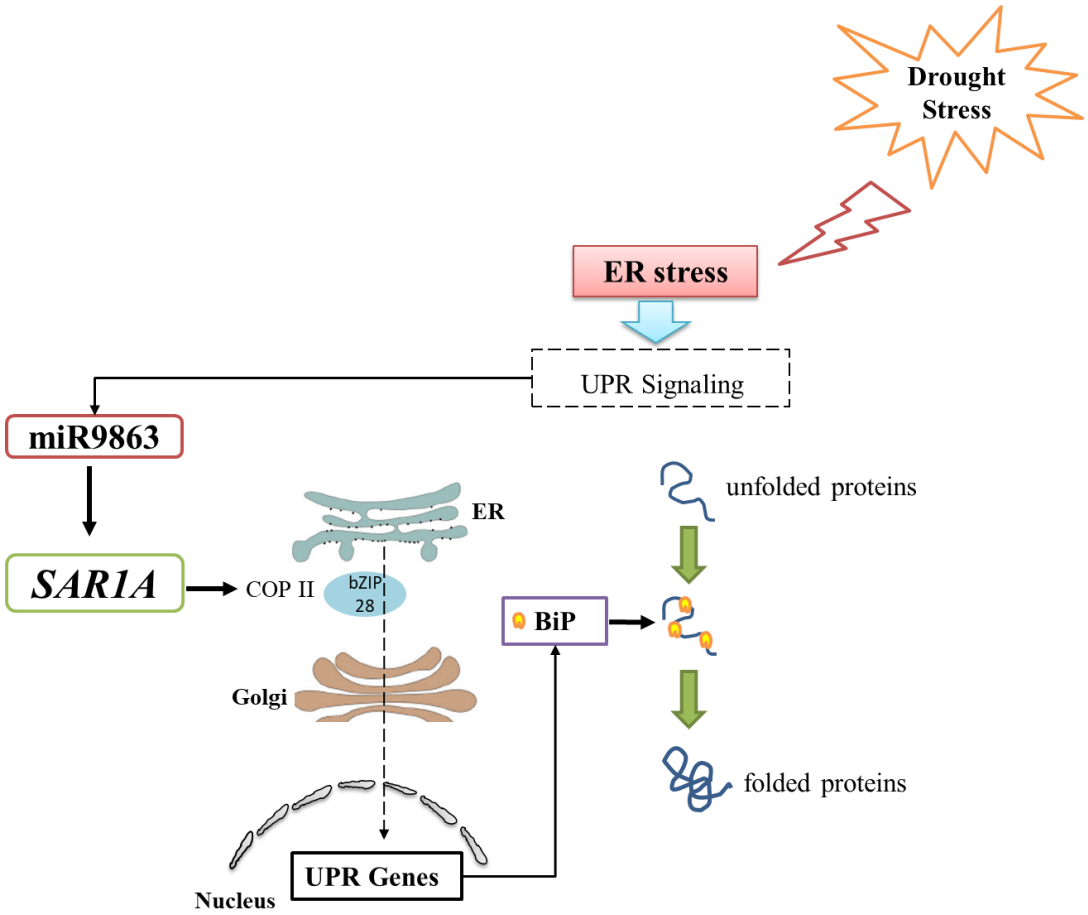

Figure 6. The role of miR9863a and its target gene in response to drought stress. Endoplasmic reticulum stress due to drought stress activates the Unfolded Protein Response (UPR) signaling and the *SAR1A* gene. The translocation of bZIP28 by COP II vesicles from ER to the nucleus induced responsive genes and the encoded BiP protein assisted in folding the proteins despite drought stress.Image adapted from (Nawkar et al., 2018; Wang et al., 2020).

miR9863a and regulation of intracellular protein trafficking

The results of qRT-PCR analysis for the relative expression of the *SAR1A* gene in the plants are shown in Figure 5. As it's depicted in Figure 5, *SAR1A* was up-regulated in *Ae. tauschii* and *Ae. crassa*, but was down-regulated in *T. aestivum* and *Ae. cylindrica* during drought stress. The expression pattern of *SAR1A* was similar to the expression pattern of miR9863a in all of the plants under drought stress. In other words, there was a direct relationship between miR9863a and the *SAR1A* target gene expression. The *SAR1A* gene encodes GTP-binding proteins that are involved in transport from the endoplasmic reticulum (ER) to the Golgi apparatus. Many small GTPases are involved in the regulation of intracellular protein trafficking (Wang et al., 2020). The SAR family of GTPases are essential for the formation of transport vesicles while having functions in the endoplasmic reticulum and assist in the ER-to-Golgi transport in plant cells. Endomembrane trafficking is a fundamental cellular process in all eukaryotic cells and signaling pathways. It mainly transducts signals during stress (Nawkar et al., 2018). *SAR1A* defines a specific colony of COPII vesicles which moderate the export of ER proteins to the Golgi apparatus. Drought stress leads to ER stress which disturbs protein folding and causes a set of signaling pathways, collectively called the Unfolded Protein Response (UPR). In the UPR mechanism, COPII vesicles by SAR1Aassist the export of bZIP28 as a transcription factor to the Golgi apparatus. The cytosolic part of bZIP28 can be translocated into the nucleus to activate the expression of ER stress-responsive genes (Wang et al., 2020). Binding proteins (BiP) encoded relevant responsive genes that assisted in the proper folding of the unfolded proteins (Nawkar et al., 2018) (Figure 6).

Therefore, considering the miR9863a and *SAR1A* expression pattern in *Ae. tauschii*, it seems that an increase in miRNA expression under drought stress signaled the increase in *SAR1A* expression and consequently produced small proteins that became bound to GTP, thereby becoming involved in endomembrane trafficking and the drought-stress response pathway.

Conclusion

While previous publications primarily focused on describing the involvement of miR9863 in biotic-stress response (Wei et al., 2009; Li et al., 2014; Liu et al., 2014; Zhang et al., 2019), the current research offers an analysis of miR9863a under drought stress conditions. In this research, we report that miR9863a exhibits distinct expression patterns in response to drought stress. Differential expression may result from the fact that overexpression in one tissue may be counteracted by down regulation in another (Mone et al., 2018). It will be the focus of future attempt to investigate expression variations tissue by tissue. In addition, when considering miR9863a and its target genes in *Ae. tauschii*, it is likely that miR9863a probably exerts its effects through a relationship between this miRNA and its target genes, regulating photorespiration and endomembrane trafficking in the drought-stress response pathway. As miR9863a is considered a newly explored miRNA, these results can illuminate the regulatory role of miR9863a in abiotic-stress response, particularly in the context of drought stress.

Supplementary Materials

The Supplementary Material for this article can be found online at: https://www.jpmb-gabit.ir/article_704821.html.

Supplementary Figure 1. Stages of cultivation and the application of drought stress on the plants.

Author Contributions

Investigation and writing, M.B.; supervision, M.R.A and M.R.N; project administration, E.M.F. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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بیان miR9863a در پاسخ به تنش خشکی در برخی از گونه‌های گندم و *Aegilops*

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چکیده: MicroRNAها، RNAهای کوچک حدود ۲۲ نوکلئوتیدی هستند که نقش مهمی در اثرات تنظیمی پاسخ به تنش‌های زیستی و غیرزیستی دارند. تنش خشکی یکی از مهمترین چالش‌های تولید گندم در ایران است. در مطالعه حاضر، بیان *miR9863a* و ژن‌های هدف در گندم و سه گونه اجیلوپس تحت تنش خشکی مورد بررسی قرار گرفت. نتایج نشان داد که *بیان miR9863a* در اندام هوایی گیاهان مورد مطالعه در شرایط تنش خشکی متفاوت بود به طوری که بیان آن در اجیلوپس تائوشی و کراسا افزایشی و در اجیلوپس سیلندریکا کاهشی بود. این اختلاف بیان در گیاهان تحت تنش می‌تواند به دلیل ماهیت miRNA به عنوان مولکولی واسطه‌ای در فرآیندهای مختلف زیستی باشد. با در نظر گرفتن الگوی بیان *miR9863a* و ژن‌های هدف در اجیلوپس تائوشی، می‌توان تا حدودی به نقش *miR9863a* در پاسخ به تنش خشکی از طریق ژن هدف *PLGG1* به واسطه نقش در نقل و انتقالات گلیسرات/گلیکولات و ژن هدف *SAR1A* به دلیل نقش داشتن در نقل و انتقالات عناصر تنظیمی از شبکه اندوپلاسمی به هسته متصور شد. همچنین با توجه به اینکه مطالعات گذشته بر ارتباط *miR9863a* با بیماری‌های گیاهی متمرکز بوده، نتایج مطالعه حاضر می‌تواند بر نقش این miRNA در مکانیسم‌های پاسخ به تنش‌های غیر زیستی با تأکید بر تنش خشکی کمک نماید.

کلمات کلیدی: تائوشی، کراسا، سیلندریکا، *PLGG1*، qRT-PCR.

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