Cloning and Functional Analysis of Tae-miR167 for Drought Resistance in Wheat Postprint

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Abstract

The miR167 family is a conserved miRNA family in the plant kingdom that plays important regulatory roles in plant growth, development, and stress response. To clarify the function of wheat Tae-miR167 under stress, this study identified and analyzed the sequences of the wheat Tae-miR167 family, and used qRT-PCR to analyze the differential expression of Tae-miR167 mature forms in different wheat organs and under stress conditions. The Tae-miR167c precursor sequence was cloned, an overexpression vector was constructed and transformed into Arabidopsis, and the function of Tae-miR167c-overexpressing lines in response to drought stress was studied. The results showed that: (1) The Tae-miR167 family contained 18 sequences with 3 types of mature sequences, all having typical stem-loop secondary structures. (2) The three Tae-miR167 mature forms were expressed in most wheat organs, with relatively high expression levels in roots, leaves, and seeds; the mature forms Tae-miR167b and Tae-miR167c could respond to low temperature and PEG stress treatments, respectively, with upregulated expression. (3) Arabidopsis lines overexpressing the Tae-miR167c precursor showed significantly improved germination rate and root length under drought stress; transgenic seedlings exhibited enhanced drought tolerance, with significantly increased water content, soluble sugar content, and chlorophyll content. (4) Target gene prediction revealed that Tae-miR167c could bind to F-box proteins and participate in the regulation of stress response. Collectively, these results indicate that wheat Tae-miR167c was significantly upregulated under drought stress, and transgenic lines overexpressing Tae-miR167c exhibited enhanced drought stress tolerance. These findings enrich the functional studies of Tae-miR167 and may provide new gene resources for wheat germplasm innovation.

Full Text

Preamble

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Title: Clone and Drought Function Analysis of Wheat Tae-miR167

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Abstract: The miR167 family is a conserved microRNA (miRNA) family in plants that plays a vital role in regulating plant growth, development, and stress responses. To clarify the function of wheat Tae-miR167 under abiotic stress, we identified and analyzed Tae-miR167 family sequences in wheat and examined the differential expression profiles of mature Tae-miR167 in various wheat organs and under abiotic stress conditions using quantitative reverse transcription polymerase chain reaction (qRT-PCR). The precursor sequence of Tae-miR167c was cloned, and its role in drought response was investigated through overexpression in *Arabidopsis thaliana*. The results were as follows: (1) The Tae-miR167 family comprised 18 members that produce three distinct mature miRNA sequences, all exhibiting characteristic hairpin secondary structures. (2) The three mature Tae-miR167 variants were expressed in most wheat organs, with relatively higher expression levels observed in roots, leaves, and seeds. Mature Tae-miR167b and Tae-miR167c were upregulated in response to low-temperature and PEG-induced drought stress, respectively. (3) *Arabidopsis* lines overexpressing Tae-miR167c showed significantly improved germination rates and root lengths under drought stress. The transgenic seedlings also exhibited enhanced drought tolerance, with significant increases in water content, soluble sugar content, and chlorophyll content. (4) Target gene prediction revealed that Tae-miR167c could bind to F-box proteins, suggesting its involvement in regulating stress responses. In conclusion, wheat Tae-miR167c was significantly upregulated under drought stress, and transgenic lines overexpressing Tae-miR167c showed enhanced drought tolerance. These findings enrich our understanding of Tae-miR167 function and provide novel genetic resources for wheat germplasm innovation.

Keywords: wheat, miRNA, drought, overexpression, function

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Introduction

Abiotic stress negatively impacts plant growth and development. To cope with adverse environments, plants have evolved a complex regulatory network in which microRNAs (miRNAs) play a crucial role. miRNAs are endogenous, non-coding, single-stranded small molecules with precursor sequences that form typical stem-loop structures. Mature miRNA sequences are approximately 22 nucleotides in length and are widely present in animals and plants, where they participate in gene regulation (Huang et al., 2019). Plant miRNAs primarily regulate target genes (including transcription factors and signaling proteins) at the post-transcriptional level by cleaving mRNA or blocking protein translation, thereby influencing various physiological processes (Wang et al., 2022). Recent studies have shown that miRNAs are ubiquitous in plants and play important regulatory roles in growth and development, stress responses, organogenesis, signal transduction, and apoptosis (Li et al., 2020).

Many conserved miRNA families in plants are involved in stress responses. For example, the miR398 family targets copper/zinc superoxide dismutase and regulates reactive oxygen species (ROS) to enable plants to respond to multiple stresses, while the miR164 family regulates plant resistance to pathogens by targeting NAC transcription factors (Šečić et al., 2021). The miR167 family is one of the most highly conserved miRNA families in plants, participating in the regulation of plant growth, development, and stress responses (Liu et al., 2021). Studies have shown that miR167 regulates plant vegetative and reproductive organ development, flowering time, and stress responses by targeting ARF6, ARF8, and IAR3 (Liu et al., 2021). Abiotic stresses such as drought and high salinity, as well as fungal or viral infections, can affect miR167 expression in wheat, rice, tomato, apple, and other plants, enabling them to respond to adverse conditions by regulating stomatal opening, inducing downstream defense gene expression, and modulating auxin homeostasis (Jodder et al., 2018; Qu et al., 2021). Wheat miR167 responds to water stress by regulating gene expression to induce stomatal closure and increase leaf water content (Fileccia et al., 2019). Overexpression of grape vvi-miR167 in *Arabidopsis* significantly improved the heat tolerance of transgenic lines, indicating that miR167 plays a positive regulatory role in grape thermostability (Zhang et al., 2023). miR167 expression varies across different species and under different stress conditions. Plants can either decrease miR167 expression to increase energy reserves for stress resistance or increase miR167 expression to reduce growth and enhance stress tolerance (Li et al., 2019).

Wheat is an important food crop, and extreme temperatures, high salinity, and drought can severely reduce its yield and quality. Studying the functions of

stress-related genes can facilitate the breeding of resistant wheat varieties (Hu et al., 2013). While miR167 has been extensively studied in model plants such as *Arabidopsis*, research in wheat remains limited, focusing primarily on gene identification and expression analysis. For example, novel miR167 sequences have been identified from wheat hybrid necrosis sequencing data (Zhou et al., 2013), and wheat miR167 has been identified and characterized in response to root rot and leaf spot diseases (Sharma et al., 2021; Samavatian et al., 2023). Additionally, wheat miR167d and miR167e have been studied for their expression patterns in wheat organs and under cold and osmotic stress conditions (Song et al., 2021; Chang et al., 2019). These studies demonstrate that wheat miR167 can respond to multiple stresses, and further functional characterization of this family may identify potential target genes for germplasm innovation.

In this study, we used bioinformatics tools to identify and analyze members of the wheat Tae-miR167 family, examining their sequence characteristics and secondary structures. We analyzed the expression patterns of Tae-miR167 in different wheat organs and under stress conditions using qRT-PCR and investigated its function in drought stress response by overexpressing Tae-miR167c in *Arabidopsis*. Specifically, we addressed: (1) sequence and structural differences among Tae-miR167 family members; (2) the response of mature Tae-miR167 to abiotic stress in wheat; and (3) the drought resistance function of Tae-miR167c-overexpressing lines. Our results provide a foundation for understanding Tae-miR167 function and offer genetic resources for developing stress-resistant wheat varieties.

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1 Materials and Methods

1.1 Plant Materials

Wheat (*Triticum aestivum*, Chinese Spring variety) and wild-type *Arabidopsis thaliana* (Columbia Col-0 ecotype) were used as experimental materials.

1.2 Identification and Bioinformatics Analysis of Wheat Tae-miR167

Tae-miR167 sequences were retrieved from the sRNAanno (http://plantsrnas.org/) and PmiREN databases and merged after redundancy removal. Mature Tae-miR167 sequences were aligned using ClustalW software, and precursor secondary structures were analyzed using the online tool RNAfold. Target genes were predicted using the online software psRobot (http://omicslab.genetics.ac.cn/psRobot/).

1.3 Drought Treatment and Expression Analysis

Wheat seeds were germinated and grown at 25 °C under a 16 h/8 h light/dark photoperiod. Ten-day-old seedlings were subjected to simulated osmotic

stress (20% PEG6000), salt stress (200 mmol · L^{-1} NaCl solution), and low-temperature stress (4 °C) to evaluate their responses to various abiotic stresses, with untreated materials serving as controls (Hu et al., 2013). Leaf samples were collected 6 hours after treatment for gene expression analysis under stress conditions. For organ-specific expression analysis, RNA was extracted from different wheat organs including roots, stems, leaves, spikes, and seeds [Tiangen Biotech (Beijing) Co., Ltd.].

cDNA was synthesized using a miRNA first-strand cDNA synthesis kit (Vazyme) and used as a template for qRT-PCR analysis. Specific primers were designed for the three mature Tae-miR167 sequences (Table 1), with wheat *Actin* as the internal reference. Relative gene expression levels were calculated using the 2^(-ΔΔCt) method. All samples were analyzed with three biological replicates.

1.4 Gene Cloning, Vector Construction, and Genetic Transformation

Wheat genomic DNA was extracted as a template (Tiangen), and specific primers (F: 5 -GGCAGTGTCACGAGGTGTGAG-3 ; R: 5 -CTGGGAGATTTTGTATGGAG-3) were used for PCR amplification. The PCR program consisted of: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s; and a final extension at 72 °C for 10 min. After sequencing verification, the plant expression vector pCAMBIA1304-miR167c was constructed. The recombinant vector was transformed into *Agrobacterium tumefaciens* strain GV3101 and used for *Arabidopsis* transformation via the floral dip method. Transgenic seeds were selected on 1/2 MS medium containing hygromycin, and rooted seedlings were transferred to soil. Leaf DNA was extracted from each line for PCR verification, and successfully identified lines were propagated for seed collection (Jia et al., 2020).

1.5 Germination Rate and Root Length Analysis

Normal 1/2 MS medium was used as a control, while 1/2 MS medium supplemented with 150 or 300 mmol · L^{-1} mannitol was used to simulate drought stress (Hu et al., 2013). Seeds from different transgenic lines were cultured on plates, stratified at 4 °C for 48 hours, and then germinated in a greenhouse at 22 °C. Germination was monitored every 1–3 days to calculate germination rates (Xu et al., 2020). After germination, seeds were transferred to plates with corresponding mannitol concentrations and grown vertically at 22 °C for approximately 10 days before root length measurement.

1.6 Stress Treatment and Physiological Index Determination

Arabidopsis seedlings were grown at 22 °C under a 16 h/8 h light/dark cycle. For drought stress treatment, water was withheld for 3 weeks before phenotypic observation and physiological index measurement. Water content (Ma et al., 2016) and chlorophyll a and b contents (Mulati et al., 2021) were determined

using previously described methods. Soluble sugar content was measured using the anthrone colorimetric method. Each group had three replicates, and statistical analysis was performed using SPSS software.

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2 Results

2.1 Sequence Identification of the Tae-miR167 Family

After redundancy removal, 18 members of the Tae-miR167 family were identified, distributed across chromosomes 5A, 5B, and 5D (4 members each), chromosomes 4A, 4D, 6A, and 6D (1 member each), and chromosome 6B (2 members) (Table 2). The family produced three mature sequences: Tae-miR167a (UGAAGCUGCCAGCAUGAUCUA), Tae-miR167b (UGAAGCUGCCAGCAUGAUCUGA), and Tae-miR167c (UGAAGCUGCCAGCAUGAUCUGC), with 9, 5, and 4 members, respectively. The sequences were 21, 22, and 22 nt in length, differing only at the 21st and 22nd nucleotide positions (Figure 1 [Figure 1: see original paper]A). Secondary structure analysis of randomly selected precursor sequences corresponding to the three mature variants revealed typical stem-loop hairpin structures with stable free energy. The mature miR167 sequences were all located on the 5 arm of the hairpin structure and showed strong conservation, while the loop regions exhibited more obvious differences due to sequence variation (Figure 1B).

2.2 Expression Analysis of Mature Tae-miR167

Organ-specific expression analysis revealed that all three mature Tae-miR167 variants were expressed in the examined wheat organs, with relatively higher expression levels in roots, leaves, and seeds, followed by spikes and stems (Figure 2 [Figure 2: see original paper]A–C). qRT-PCR analysis under different abiotic stress treatments showed that Tae-miR167 mature variants responded to PEG and low-temperature treatments (Figure 2D–F). Specifically, compared with untreated controls, Tae-miR167b expression was upregulated more than 2.5-fold under low-temperature stress, while Tae-miR167c expression was upregulated approximately 3.5-fold under PEG-simulated drought stress. These results suggest that Tae-miR167 may play a potential role in wheat stress responses.

- indicates significant correlation at the 0.05 level; ** indicates significant correlation at the 0.01 level. The same below.

Figure 2 [Figure 2: see original paper]: Relative expression of Tae-miR167 in different wheat organs (A–C) and under different stress treatments (D–F).

2.3 Cloning of Wheat Tae-miR167c Precursor and Construction of Overexpression Lines

To further investigate the function of Tae-miR167c in drought stress response, we designed primers to amplify a 400-bp sequence upstream and downstream of Tae-miR167c (located at wheat 6A: 149923007-149923139). The PCR product was cloned into the plant expression vector pCAMBIA1304-miR167c and transformed into *Arabidopsis thaliana*. Transgenic plants were selected on antibiotic-containing medium and grown under the same conditions as wild-type plants. After two generations of single-plant seed collection and transgenic positive plant screening, Tae-miR167c-overexpressing *Arabidopsis* lines were obtained. Compared with wild-type *Arabidopsis*, the transgenic plants showed no obvious changes in growth characteristics or morphological features.

2.4 Analysis of Germination Rate Under Osmotic Stress

To investigate the drought tolerance function of Tae-miR167c, we used mannitol to simulate osmotic stress and examined the germination rates of transgenic *Arabidopsis* lines. The results showed no significant differences in germination rates among different lines on normal medium. However, under 300 mmol $\cdot$ L^{-1} mannitol treatment, the germination rates of Tae-miR167c-overexpressing lines (OE2, OE9) were significantly higher than those of wild-type (WT) and empty vector control (VC) plants (Figure 3 [Figure 3: see original paper]).

2.5 Analysis of Root Length Under Osmotic Stress

After vertically growing seedlings of different *Arabidopsis* lines on plates with various mannitol concentrations for several days, no significant differences in root length were observed among lines on normal medium. Under 150 mmol $\cdot$ L^{-1} and 300 mmol $\cdot$ L^{-1} mannitol stress, root lengths of wild-type and empty vector controls showed no significant differences, while Tae-miR167c-overexpressing lines exhibited significantly longer roots than WT and VC controls (Figure 4 [Figure 4: see original paper]A and B).

2.6 Phenotypic Analysis of *Arabidopsis* Under Drought Stress

Before drought stress treatment, no obvious differences were observed among the different lines. After drought stress treatment, both wild-type and empty vector control plants showed withered states, while transgenic plants exhibited better drought resistance, maintaining normal growth and development despite showing some water-deficiency symptoms such as yellowing, curling, and wrinkling of leaves (Figure 5 [Figure 5: see original paper]A and B).

2.7 Physiological Index Analysis of *Arabidopsis* After Drought Stress Treatment

To analyze the function of Tae-miR167c in drought stress response, we measured various physiological indices after drought treatment (Figure 6 [Figure 6: see original paper]). The results showed no significant differences in physiological indices between WT and VC groups after drought treatment. However, overexpressing lines showed significantly increased water content, soluble sugar content, and chlorophyll content compared with WT and VC groups, indicating that Tae-miR167c overexpression in *Arabidopsis* enhances plant tolerance to drought stress.

2.8 Target Gene Prediction of miR167

Using Tae-miRNA target gene prediction software, we identified potential target genes of Tae-miR167c in wheat and screened them based on miRNA function. The results showed three target genes of Tae-miR167c: TraesCSU02G039500.1, TraesCS3B02G004400.1, and TraesCS3D02G275100.1, all encoding F-box proteins (Figure 7 [Figure 7: see original paper]).

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3 Discussion

3.1 Sequence Characteristics of the Tae-miR167 Family

The miR167 family is a highly conserved miRNA family in plants that functions by regulating auxin response factors (ARFs) and IAA-Ala Resistant 3 (IAR3) genes, thereby participating in plant development and stress responses (Liu et al., 2021). miR167 exhibits species specificity, with reports of 4 members in *Arabidopsis*, 10 in rice, and 11 in soybean (Liu et al., 2021). The 10 members of rice miR167 produce two mature forms that differ by only one nucleotide at the 3 end (Liu et al., 2012). The soybean miR167 family contains 11 members divided into two subgroups (miR167a/b/d/e/f/g/h/i/j/k and miR167c), with mature sequences differing by one or two nucleotides at the 3 end (Wang et al., 2015). In this study, we identified 18 members of the wheat Tae-miR167 family producing three mature sequences that differ by 1–2 nucleotides at the 3 end. These findings are consistent with previous reports in other species, indicating that while the miR167 family is conserved in plants, wheat miR167 also exhibits species-specific characteristics.

3.2 Tae-miR167 Response to Abiotic Stress

The miR167 family responds to various abiotic stresses. For example, poplar miR167 is upregulated under UV-B radiation (Jia et al., 2009), and salt-tolerant cotton varieties show upregulated miR167 expression under salt stress (Li et al., 2009). Studies on drought stress responses have reported upregulated miR167 expression in *Arabidopsis*, wheat, and maize (Pandey et al., 2013; Liu et al.,

2016). In this study, wheat Tae-miR167c was upregulated under PEG-simulated drought stress, consistent with previous reports. Regarding low-temperature stress responses, eggplant miR167c-3p was downregulated under cold treatment (Yang et al., 2017a), whereas miRNA sequencing analysis of cold-resistant and cold-sensitive sugarcane varieties showed upregulated miR167 expression in both cultivars under low temperature (Yang et al., 2017b). In our study, Tae-miR167b was upregulated under low-temperature stress, similar to reports in other species, indicating that miR167 can respond to cold stress, though expression patterns vary across species.

3.3 Drought Resistance Function of miR167c-Overexpressing Lines

The miR167 family is widely involved in plant responses to water stress, heat stress, and drought. Wheat miR167 responds to water stress by regulating gene expression to induce stomatal closure and increase leaf water content (Fileccia et al., 2019). Overexpression of grape vvi-miR167 in *Arabidopsis* enhanced the heat tolerance of transgenic lines, indicating that miR167 plays a positive regulatory role in grape thermostability (Zhang et al., 2023). Overexpression of poplar miR167a promoted lateral root development by regulating ARF8 expression, thereby affecting plant tolerance to environmental stresses (Cai et al., 2019). In this study, Tae-miR167c-overexpressing *Arabidopsis* showed significantly increased seed germination rates and root lengths under drought stress, with enhanced drought tolerance and significantly elevated water content, soluble sugar content, and chlorophyll content. These results are consistent with previous reports that miR167 responds to drought stress and improves plant heat and drought tolerance.

miRNAs exert their biological functions primarily by regulating target gene expression. Studies have shown that *Arabidopsis* miR167 mainly targets ARF6, ARF8, and IAR3, affecting root, stem, flower, and ovule development and participating in stress responses by activating auxin activity (Liu et al., 2021). Additionally, miR167 may target membrane transporter NRAMP (Meng et al., 2017), phospholipase PLD (Wei et al., 2009), ubiquitin ligases, and cell cycle proteins (Wang et al., 2021), thereby influencing plant responses to drought, high salinity, and heavy metal stress. In this study, target gene prediction analysis revealed that the three target genes of Tae-miR167c encode F-box proteins, which differs from previously reported target genes. F-box proteins, first discovered in cell cycle protein Cyclin F, contain a conserved F-box domain and primarily function by forming SCF complexes involved in ubiquitin-mediated protein degradation pathways, participating in plant growth and development, signal transduction, and responses to biotic and abiotic stresses (Xu et al., 2021). F-box proteins can directly or indirectly affect plant hormone signaling pathways, such as ABA and ET signaling, influencing plant drought and salt-alkali resistance. This regulation can be either positive or negative. For instance, overexpression of wheat F-box protein TaFBA1 improved plant drought tolerance but negatively regulated stomatal closure (An et al., 2019). The F-

box protein RIFP1 negatively regulated ABA signaling by degrading the ABA receptor RCAR3, and *rifp1* knockout mutants showed enhanced drought resistance (Li et al., 2016). Overexpression of *Arabidopsis* F-box protein AtPP2-B11 increased sensitivity to drought and salt stress by mediating the degradation of stress-resistant protein AtLEA14, playing a negative regulatory role in drought resistance (Li et al., 2014). Our study suggests that Tae-miR167c may enhance drought resistance in transgenic lines by targeting and regulating downstream negatively regulated F-box proteins, though further experimental validation of these target genes and detailed mechanistic studies are needed.

In conclusion, this study systematically identified the wheat Tae-miR167 family and demonstrated that mature Tae-miR167c is significantly upregulated under drought stress. *Arabidopsis* overexpressing Tae-miR167c showed significantly enhanced germination rates and root lengths under drought stress, with improved drought tolerance. These findings enrich our understanding of wheat Tae-miR167 function and provide new genetic resources for wheat germplasm innovation.

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Note: Figure translations are in progress. See original paper for figures.