BMC Plant Biology

RESEARCH

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Characterization of tae-miR156(s) and their response to abiotic stress in wheat (*Triticum aestivum* L.)

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Abstract

The microRNA156 (miR156) has been widely studied in plants, however, the characterization of the miR156 family of genes in wheat and their expression patterns under abiotic stress are not completely clear. In this study, a total of 20 miR156 family members, referred to as tae-miR156a to tae-miR156t, were identified in wheat with their loci mapped to various chromosomes. These members were divided into five subgroups: miR156a/b/c/d/e/f, miR156g/h/i, miR156j/k, miR156l/m/n/o/p/q, and miR156r/s/t. They were highly conserved during evolution. The prediction of *cis*-elements in the *tae-MIR156(s)* promoter region revealed that the *tae-MIR156(s)* had diverse *cis*-acting elements; of these, 15 *tae-MIR156(s) and* 6 *tae-MIR156(s)* were found to be drought-responsive elements and cold-responsive elements, respectively. And the prediction target genes of tae-miR156(s) are mainly *SPL* transcription factor genes. Expression analysis based on quantitative real-time polymerase chain reaction (qRT–PCR) showed that miR156(s) have different expression levels in the various wheat tissues, and the subgroups' response to abiotic stress varied. Among them, miR156g/h/i were strongly induced in the root of cold and heat stress, and miR156a/b/c/d/e/f were significantly increased in roots after drought stress, whereas miR156r/s/t were highly inhibited in leaves and roots after salt stress. These findings imply that tae-miR156(s) are involved in stress response in wheat, and they provide new fundamental knowledge for further analysis of the function of miR156 and its regulatory mechanism in response to abiotic stress.

Keywords MicroRNA, Wheat, MiR156, Expression, Abiotic stress

Background

MicroRNAs (miRNAs) are small non-coding RNAs of approximately 18–24 nucleotides in length that are now increasingly recognized as regulators of gene expression at the post-transcriptional level and have the ability to impact many biological processes [1]. MicroRNA156 (miR156) is widely found in monocots, dicots, and lower ferns and mosses [2–5]. A growing body of evidence shows that miR156 plays a significant role in regulating plant fitness, biomass, and yield [6]. Plant miRNAs from the same family share a high degree of sequence similarity, but they may have diverse roles in different plant species [7, 8]. For example, in rice (*Oryza sativa*), the deletion

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of miR156d/e/f/g/h/i suppressed ineffective tillering, increased plant height, grain length, and grain weight, improved stem resistance, and exhibited ideal plant morphology without affecting seed dormancy, whereas the miR156a/b/c/k/l mutant significantly improved seed dormancy and inhibited spike germination [9]. During symbiotic nodulation in legumes, miR156 family members were variably expressed, and miR156b inhibited nodulation by negatively regulating the expression of *GmSPL9d* (Soybean SQUAMOSA promoter-binding protein-like 9d) [10]. Overexpression of tae-miR156 resulted in increased tiller number and severe defects in spikelet generation for bread wheat (*Triticum aestivum* L.) [11]. MiR156 could impact fruit size and yield by regulating inflorescence architecture in tomatoes (*Solanum lycopersicum* L.) [12], as well as plant architecture and tuber development through starch accumulation in potatoes (*Solanum tuberosum* L.) [13], and somatic embryogenesis in citrus (*Citrus reticulata* Blanco) [14]. These findings indicated that miR156 plays a significant role in modifying plant growth and development.

Plants are constantly exposed to adverse conditions such as extreme temperatures, high salinity, and drought. These abiotic stresses are major factors limiting the geographical distribution of plants and their corresponding crop yields [15]. However, to reduce the adverse effects of these abiotic stresses, plants have plasticity in their defense mechanisms, enabling them to tolerate and survive stressful conditions [16]. For example, miR156 negatively impacted cold tolerance and positively regulated drought tolerance, while the overexpression of miR156a weakened salt resistance in apple (*Malus pumila* Mill.) [17–19]. MiR156 also exhibited a positive effect in *Nicotiana tabacum* subjected to cold stress [20]. Overexpression of miR156k resulted in lower tolerance to cold stress in rice [21]. Decreased expression of miR156 affected sugarcane (*Saccharum officinarum* L.) metabolism and growth after low-temperature treatment and enhanced cold tolerance by negatively regulating squamosa promoter binding protein-like (SPL) transcription factor [22]. Overexpression of miR156 could also improve drought and heat stress tolerance in alfalfa (*Medicago sativa*) [23, 24] while ahy-miR156 down-regulated target genes to enhance the level of drought resistance in groundnut legume (*Arachis hypogaea*) [25]. Newly published studies have also shown that in apple calli and *Arabidopsis*, *MdmiR156n* could regulate drought tolerance by inducing flavonoid accumulation and promoting reactive oxygen species (ROS) scavenging [26]. The *miR156/SPL9* pathway suppressed anthocyanin production and significantly improved *Arabidopsis* durability to salt and drought stress [27]. Heterogeneous expression of *Osa-MIR156bc* increased abiotic stress resistance and forage quality in alfalfa [28]. In brief, miR156 exhibits diverse actions in various species and is vital for responding to unfavorable stress [29, 30]. However, not much is known about how miR156 is regulated and the role it plays in wheat under abiotic stress.

Wheat is an important staple crop globally and provides approximately 20% of the global dietary energy [31]. Although wheat is grown in large areas, its total production remains the lowest amongst cereals and rice. This is mainly a consequence of abiotic stressors such as drought, salt, and high temperatures which are the main contributors to losses in wheat production [32]. Bread wheat is a hexaploid whose genome size is estimated to be about 17 Gb. This large and complex genome poses a great challenge for the mining and application of stress-resistance genes as well as the cultivation and improvement of resistant varieties of wheat. MicroRNAs are short non-coding RNAs that have generated much interest in biological research due to their role as major regulators of gene expression at the post-transcriptional level. One key miRNA identified in plants is miR156 with its functions and molecular mechanisms well described for rice, *Arabidopsis*, and other plants. Although the presence of miR156 in wheat has been reported, the specific regulatory functions and mechanisms that involve miR156 have not been fully resolved and verified due to the large and complex wheat genome. Nevertheless, current advances in genome sequencing and chromosomal fine mapping can facilitate investigations into and validation of the roles of miR156 in wheat (*Triticum aestivum* L.). To this end, we analyzed the chromosomal localization, evolution, sequence conservation, *cis*-acting regulatory elements, target genes, spatiotemporal expression patterns, and response to different abiotic stresses for miR156(s) in wheat. The results of this study provide comprehensive information to understand tae-miR156(s) and lay the foundation for functional research on abiotic stresses.

Methods

Identification and chromosomal localization of miR156(s) in wheat

The precursor sequences, mature sequences, and chromosomal location information of miR156(s) for wheat (*Triticum aestivum* L.) were downloaded from the PmiREN (Plant miRNA ENcyclopedia) database (https://www.pmiren.com/) [33].

Phylogenetic analysis of the *tae-MIR156(s)* in wheat

To construct the phylogenetic tree of plant miR156(s), we downloaded the precursor sequences of miR156(s) for wheat, rice, *Zea mays*, and *Arabidopsis* from the PmiREN database and performed multiple sequence alignments using the MEGA 11 software [34]. The neighbor-joining

method was used to construct the phylogenetic tree with the bootstrap value set to 1000 [35].

Prediction of *cis*-acting regulatory elements and target genes

The 2000 bp upstream sequences of *tae-MIR156(s)* and their *cis*-elements were predicted using the PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [36], and targets of tae-miR156(s) were predicted using the PsRNATarget (https://www.zhaolab.org/psRNATarget/) based on a *T. aestivum* cDNA library (EnsemblPlant, v.43) [37].

Plants and treatments

The hexaploid common wheat (*Triticum aestivum* L.) cultivar 'Fielder', an American, soft, white, pastry-type wheat, known for its amenability to *Agrobacterium tumefaciens*-mediated transformation and genome editing [38], was used for expression analysis and stress treatment. For spatial–temporal expression analysis, Fielder seeds (spring wheat) were directly sown in pots (30 cm height × 27 cm diameter) filled with a peat moss substrate (Pindstrup, Denmark) and mixed fertilizers (Osmocote Extract, Heerlen, the Netherlands) in a growth chamber set at 22 °C, a photoperiod regime of 16 h light/8 h darkness, and 45% humidity, different tissue samples were collected from Fielder plants at different developmental stages based on Zadoks' scale [39], there were three independent biological replicates for each sample.

For abiotic stress expression analysis, Fielder seeds of the same size with full grains were selected and surface-sterilized in 1% sodium hypochlorite for 15 min, rinsed in distilled water, and germinated at room temperature [40], after which the seeds were placed in black plant hydroponic box with 96 holes in hydroponics containing Hoagland's nutrient solution, placed in an artificial climate chamber (22 °C light for 16 h, 18 °C dark for 8 h) for cultivation, and the nutrient solution was changed every two days. After normal growth of 14 days, they were subjected to stress treatments (cold, heat, salt, drought) separately. For cold and heat treatments, the temperature of the incubator was set at 4℃ and 42℃ [41], respectively. Drought was established with PEG6000 (20%) and NaCl (200 mM) solution was used to mimic salt stress [42, 43]. The leaves and roots were collected as experimental materials after 0 h (means no treatment), 3 h, 6 h, 12 h, 24 h, and 48 h. The collected samples were immediately frozen in liquid nitrogen and stored at − 80 °C for subsequent analysis. The leaves and roots of 15 seedlings were mixed as one sample, respectively. There were three independent biological replicates for each sample.

RNA extraction and qRT-PCR

The miRcute Plant miRNA Isolation Kit (Tiangen, Beijing, China) was used to extract miRNA from Fielder seedlings, roots, stems, and other tissues according to the manufacturer's instructions. The miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) (Vazyme, Nanjing, China) was used for reverse transcription of the extracted RNA into cDNA. Quantification of miR156(s) expression by qRT-PCR was performed using a miRNA Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The wheat *U6* gene was used as the internal reference. The $2^{-\Delta\Delta CT}$ method was used to calculate expression levels. All assays were performed in three independent experiments. Primers used in this study are listed in Supporting Information Table S1.

Statistical analysis

Statistical analyses were performed using GraphPad Prism9 (https://www.graphpad.com/scientific-software/prism/) by one-way ANOVA. All data are presented as mean ± SD. Differences between the means were compared using the Tukey's test. Lowercase letters represent significant differences at $P < 0.05$.

Results

Chromosome mapping and conserved analysis of miR156 in wheat

Based on the PmiREN database, a total of 20 wheat *tae-MIR156* genes were identified. Among them, *tae-MIR156r/s/t* were localized on chromosomes 2A, 2B, and 2D, respectively. *Tae-MIR156a/l*, *tae-MIR156b/m*, and *tae-MIR156c/n* were localized on chromosomes 3A, 3B, and 3D, respectively. *Tae-MIR156j/o*, *tae-MIR156k/p*, and *tae-MIR156q* were localized on chromosomes 5A, 5B, and 5D, respectively. *Tae-MIR156d/g*, *tae-MIR156e/h,* and *tae-MIR156f/i* were localized on chromosomes 6A, 6B, and 6D, respectively (Table 1). Among these *tae-MIR156*, only *tae-MIR156j* and *tae-MIR156k* produced mature sequences of 20 nucleotides in length, while the rest produced mature sequences of 21 nucleotides. The mature miR156 members miR156a/b/c/d/e/f, miR156g/h/i, miR156j/k, miR156l/m/n/o/p/q, and miR156r/s/t have identical sequences (Fig. 1A). MiRNAs originate from primary precursor transcripts (pre-miRNAs) containing hairpin structures. However, we noted that the sequences and lengths of the pre-miRNA156(s) were highly varied, with the maximum sequence conservation noted for pre-miRNAs located in the stem section of the hairpin structures where mature miR156(s) are produced (Fig. S1). Phylogenetic analysis of miR156 family members in wheat, rice, maize, and *Arabidopsis* was performed. We noted that the different plant species have

Table 1 Information on the members of the miR156 family in wheat

miRNA	miRNA locus	Gene location	Mature sequence
tae-miR156a	*tae-MIR156a*	Chr3A: 76,564,147–76,564,167 (+)	UUGACAGAAGAGAGUGAGCAC
tae-miR156b	*tae-MIR156b*	Chr3B: 109,363,904–109363924 (-)	UUGACAGAAGAGAGUGAGCAC
tae-miR156c	*tae-MIR156c*	Chr3D: 65,610,352–65610372 (-)	UUGACAGAAGAGAGUGAGCAC
tae-miR156d	*tae-MIR156d*	Chr6A: 127,182,601–127182621 (+)	UUGACAGAAGAGAGUGAGCAC
tae-miR156e	*tae-MIR156e*	Chr6B: 191,531,743–191,531,763 (+)	UUGACAGAAGAGAGUGAGCAC
tae-miR156f	*tae-MIR156f*	Chr6D: 104,978,824–104978844 (+)	UUGACAGAAGAGAGUGAGCAC
tae-miR156g	*tae-MIR156g*	Chr6A: 437,637,057–437637077 (-)	UGACAGAAGAGAGUGAGCACA
tae-miR156h	*tae-MIR156h*	Chr6B: 448,349,738–448,349,758 (+)	UGACAGAAGAGAGUGAGCACA
tae-miR156i	*tae-MIR156i*	Chr6D: 287,888,860–287888880 (+)	UGACAGAAGAGAGUGAGCACA
tae-miR156j	*tae-MIR156j*	Chr5A: 75,281,037–75281056 (-)	UGACAGAAGAGAGCGAGCAC
tae-miR156k	*tae-MIR156k*	Chr5B: 51,122,100–51122119 (-)	UGACAGAAGAGAGCGAGCAC
tae-miR156l	*tae-MIR156l*	Chr3A: 76,564,340–76564360 (+)	CUGACAGAAGAGAGUGAGCAC
tae-miR156m	*tae-MIR156m*	Chr3B: 109,363,710–109363730 (-)	CUGACAGAAGAGAGUGAGCAC
tae-miR156n	*tae-MIR156n*	Chr3D: 65,610,164–65610184 (-)	CUGACAGAAGAGAGUGAGCAC
tae-miR156o	*tae-MIR156o*	Chr5A: 440,504,399–440504419 (-)	CUGACAGAAGAGAGUGAGCAC
tae-miR156p	*tae-MIR156p*	Chr5B: 398,367,514–398,367,534 (-)	CUGACAGAAGAGAGUGAGCAC
tae-miR156q	*tae-MIR156q*	Chr5D: 339,371,652–339,371,672 (-)	CUGACAGAAGAGAGUGAGCAC
tae-miR156r	*tae-MIR156r*	Chr2A: 599,784,458–599,784,478 (-)	GACAGAAGAGAGUGAGCACAC
tae-miR156s	*tae-MIR156s*	Chr2B: 537,835,523–537,835,543 (+)	GACAGAAGAGAGUGAGCACAC
tae-miR156t	*tae-MIR156t*	Chr2D: 456,504,616–456504636 (-)	GACAGAAGAGAGUGAGCACAC

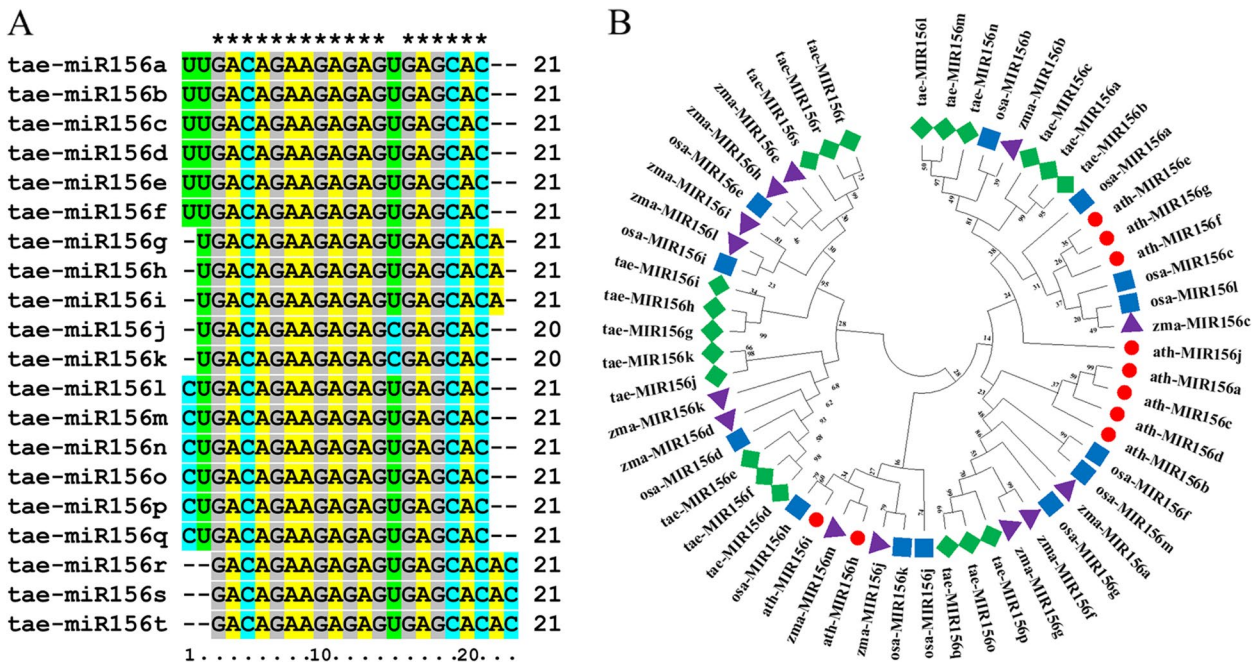

Fig. 1 Sequence alignment and phylogenetic relationship analysis. **A** All 20 sequences of the wheat miR156 family members were aligned using the MEGA 11 software. Asterisks represent conserved nucleotides in all mature miRNAs. **B** Phylogenetic analysis of 56 precursor sequences of miR156 family members from four plant species. The pre-miRNA sequences of ten miR156 family members in *Arabidopsis thaliana* (red circle), 13 in *Oryza sativa* (blue square), 13 in *Zea mays* (purple triangle), and 20 in wheat (green square) were used for the alignment, and the phylogenetic neighbor-joining tree was reconstructed using MEGA 11 phylogenetic analysis software. Bootstrap values (percentages of 1000 replicates) are indicated on the nodes

markedly different family numbers (Fig. 1B). Wheat has the largest number of miR156 genes (20 miR156s), while the other species have c. 10 miR156 family members. The phylogenetic analysis revealed that the miR156 family is conserved among *Oryza sativa*, *Zea mays*, and *Arabidopsis thaliana* (Fig. 1B).

Analysis of *cis*-acting elements in *tae-MIR156(s)* promoter regions in wheat

Plant gene promoter regions contain several critical *cis*-acting elements that are key to the regulation of gene expression [44]. The identification and analysis of the *cis*-acting elements of miRNA promoters will aid our understanding of the molecular mechanism by which miRNA regulates gene expression in plants. Therefore, to identify the function of *tae-MIR156(s)*, 2000 bp upstream regions of *tae-MIR156(s)* in wheat were used as putative promoter regions for the prediction of *cis*-acting elements. Of the 20 *tae-MIR156(s)* promoters, all contained the core promoter element TATA-box and the common CAAT-box elements (Table S2). In addition, 44 specific promoter *cis*-acting elements were identified and divided into the following four categories: hormone-responsive, stress-responsive, light-responsive, and biosynthesis and metabolism related *cis*-acting elements (Fig. 2). Among them, the light-responsive category was shared among the 20 *tae-MIR156(s)*, and five stress-response elements, namely, 'anaerobic induction element', 'low-temperature responsive element', 'defense and stress responsive element', 'drought inducibility element', and 'anoxic specific inducibility element', were identified. Among these stress response elements, a total of 15 *tae-MIR156(s)*(*tae-MIR156d/e/f/g/h/i/j/k/m/n/o/p/r/s/t*) were identified as possessing drought-responsive elements (MBS), and a total of 6 *tae-MIR156(s)*(*tae-MIR156e/f/g/h/k/s*) were identified as having low-temperature response elements (LTR).

Tissue specific expression analysis of miR156 in wheat

Constitutive expression of miR156 was previously reported to prolong the juvenile stage in *Arabidopsis thaliana,* rice, and tobacco, and influence resistance to abiotic stress [45–48]. Therefore, we analyzed spatial and temporal expression patterns of wheat tae-miR156(s) in different tissues using quantitative real-time PCR (qRT-PCR) and found that tae-miR156(s) were constitutively expressed in diverse wheat tissues at different developmental stages. Notably, tae-miR156g/h/i showed the highest expression levels in young roots and young leaves, while tae-miR156a/b/c/d/e/f exhibited the highest expression in stems at the heading stage (when the ear emerges from the flag leaf sheath). Interestingly, the expression of miR156j/k and miR156r/s/t in these tissues was limited (Fig. 3A-F). As the sequences of mature miR156(s) (miR156a/b/c/d/e/f, miR156g/h/i, miR156j/k, miR156l/m/n/o/p/q, and miR156r/s/t) were almost

Fig. 2 Promoter analysis of *tae-MIR156(s)* in wheat

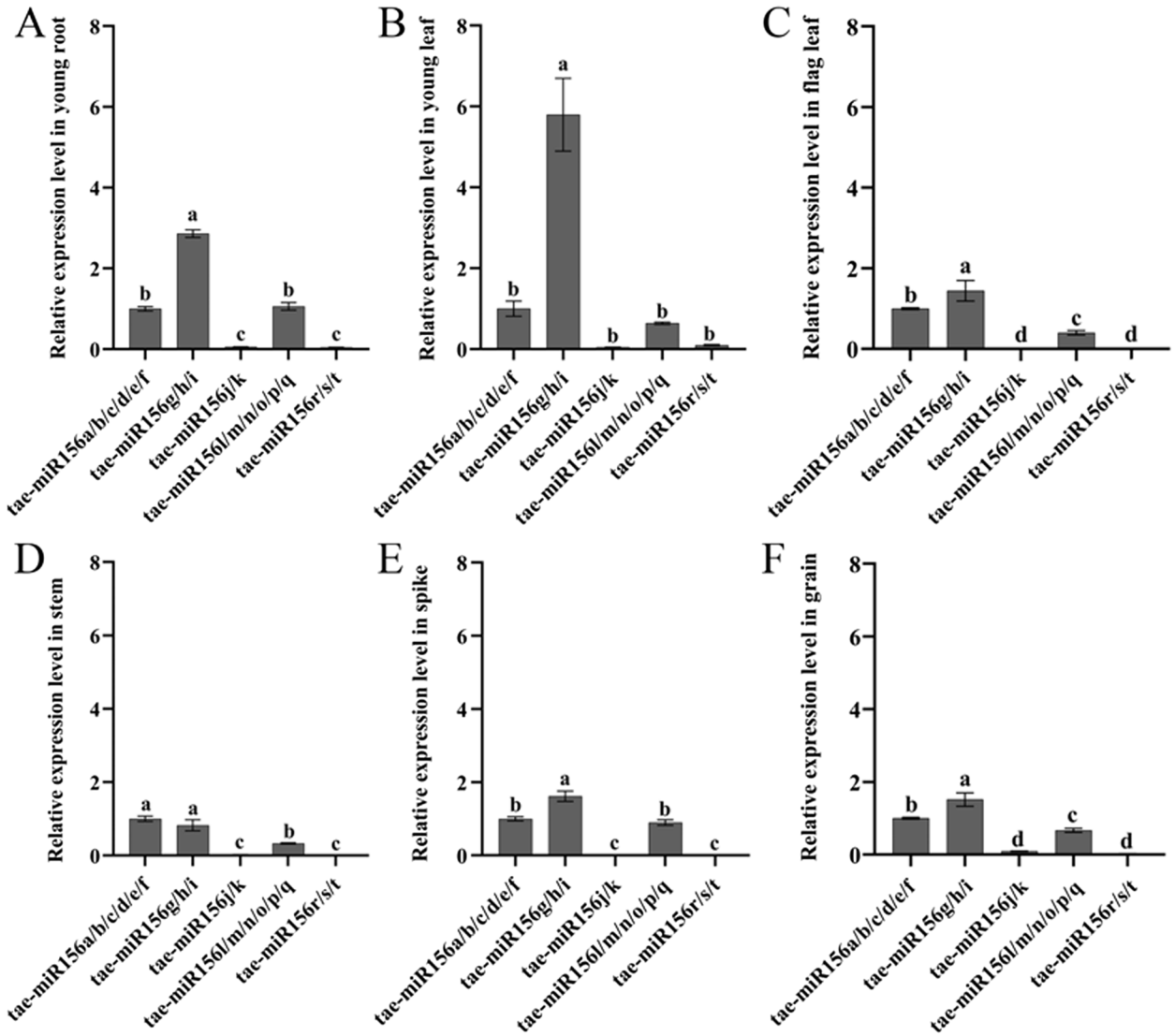

Fig. 3 Spatial and temporal expression patterns of tae-miR156(s) in wheat. Expression of tae-miR156(s) in (**A**) young root and leaf (**B**) at seeding stage (Zadok's growth stage, ZGS13); **C** flag leaf; **D** stem (ZGS37); **E** young spikes of 4 cm in length (ZGS37); (F) developing grains (ZGS77). Data are the mean of three replicates ± SD. Lowercase letters represent significant differences at $P < 0.05$

identical, the expression of these miR156(s) was the sum of the identical mature miR156(s). These results suggest that although miR156(s) from the same family are relatively conserved among themselves, they are expressed at different levels in various wheat tissues.

Expression pattern of tae-miR156(s) in response to abiotic Stress

Roots and leaves are the initial organs that sense the signals of environmental stresses. To further assess the functions of tae-miR156(s), we analyzed their expression levels in response to cold, heat, drought, and salt treatments. In leaves of wheat subjected to cold treatment (Fig. 4), it was observed that expression levels of miR156a/b/c/d/e/f and miR156l/m/n/o/p/q showed similar patterns following cold stress. The expression of both subgroups increased significantly at 6 h ($p < 0.05$) and then decreased significantly at 12 h ($p < 0.05$), followed by another increase in expression levels over the subsequent treatment times. The most significant increase in expression levels was detected for miR156l/m/n/o/p/q, at 1.76 times higher than that of the untreated control after 6 h of cold treatment ($p < 0.05$). The expression levels of miR156g/h/i and miR156r/s/t increased after cold treatment for 3 h and significantly decreased during the rest of the period, with the greatest decrease at 48 h. miR156r/s/t showed

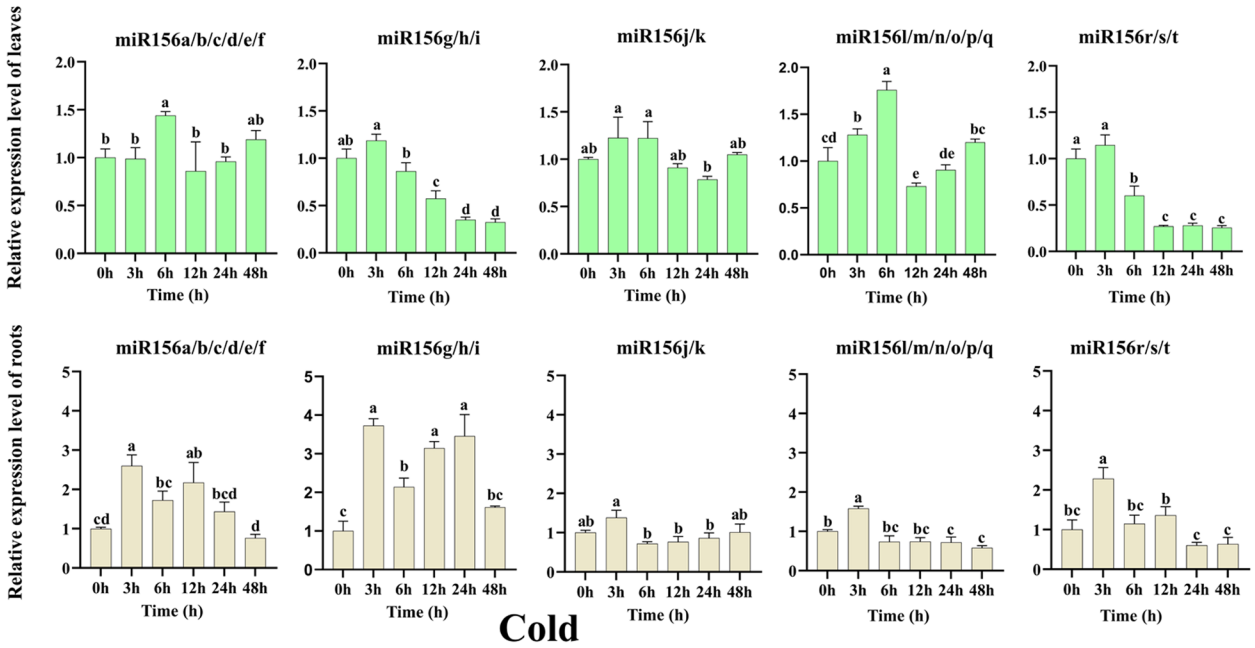

Fig. 4 The relative expression levels of tae-miR156(s) in leaves and roots after cold treatment as determined by qRT-PCR analysis. Data are the mean of three replicates ± SD. Lowercase letters represent significant differences at $P < 0.05$

the most significant decrease in expression level, which was 0.26 times that of the untreated control after 48 h of cold treatment. Compared to untreated leaves, the expression levels of miR156j/k increased after 3 h, 6 h, and 48 h of cold stress and decreased at 12 h and 24 h. In cold-treated root tissues, expression levels of all tae-miR156(s) were significantly increased after 3 h and then significantly decreased after 6 h. The upregulation of miR156g/h/i expression levels was the most significant, which was 3.72 times that of untreated, while miR156a/b/c/d/e/f, miR156j/k, miR156l/m/n/o/p/q, miR156r/s/t expression levels were 2.60, 1.38, 1.58, and 2.28 times higher than that of untreated control, respectively. The expression levels of miR156a/b/c/d/e/f were down-regulated at 48 h and increased at other time points, while that of miR156g/h/i were significantly up-regulated at all time points compared to the untreated control. However, the expression levels of miR156j/k, miR156l/m/n/o/p/q, and miR156r/s/t were significantly up-regulated ($p < 0.05$) after 3 h of cold treatment and there was no significant change in expression levels at the other time points (Fig. 4). These results indicated that cold stress affects the expression levels of tae-miIR156(s) in leaves and roots, but miR156g/h/i tends to be more sensitive in cold stressed root tissues.

Under heat treatment (Fig. 5), expression of all tae-miR156(s) was significantly down-regulated ($P < 0.05$) at 6 h in leaves, and the down-regulation was most pronounced at 12 h. Interestingly, except miR156a/b/c/d/e/f which demonstrated down-regulation in gene expression, the expression levels of other miR156(s) were higher after 3 h of heat treatment in leaves compared to the control. In roots subjected to heat treatment, the expression levels of miR156a/b/c/d/e/f, miR156g/h/i, and miR156j/k increased at 3 h, 6 h, 12 h, and 24 h but decreased significantly at 48 h compared to the untreated root samples. The most significant increase in expression was detected for miR156g/h/i, which was 1.70 times higher than that of the untreated control after 3 h of heat treatment. The most significant decrease in expression levels was observed for miR156a/b/c/d/e/f, which was 0.53 times that of the untreated control after 48 h of heat treatment. The expression of miR156l/m/n/o/p/q increased first and then decreased, but there was no significant difference between treatment and control. The expression of miR156r/s/t initially decreased, then increased to its highest level at 12 h after heat treatment but subsequently decreased at later time points. These results indicated that heat stress significantly affects the expression levels of tae-miR156(s).

Under drought treatment (Fig. 6), the expression levels of miR156a/b/c/d/e/f, miR156j/k, miR156l/m/n/o/p/q, and miR156r/s/t increased significantly after 3 h, most significantly in miR156j/k, which was 2.90 times higher than that of the no treatment control. On the other hand, expression levels of miR156g/h/i decreased over all time points after drought treatment and were most significant after 48 h of treatment. In root tissues subjected to

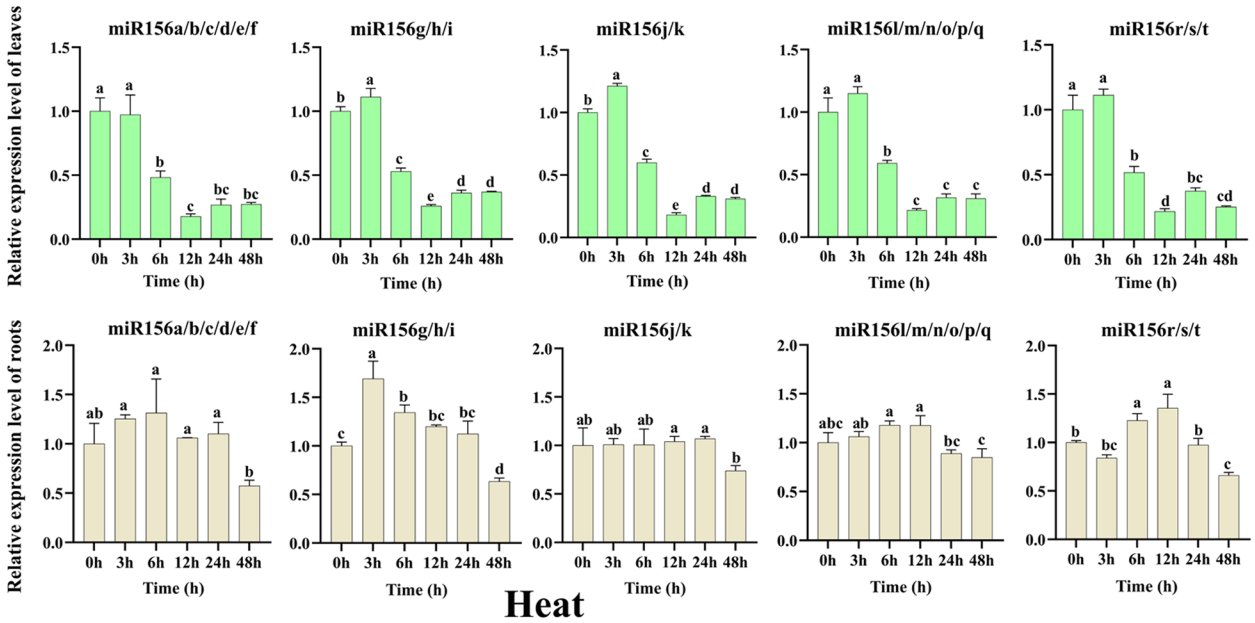

Fig. 5 The relative expression levels of tae-miR156(s) in leaves and roots after heat treatment as determined by qRT-PCR analysis. Data are the means of three replicates ± SD. Lowercase letters represent significant differences at $P < 0.05$

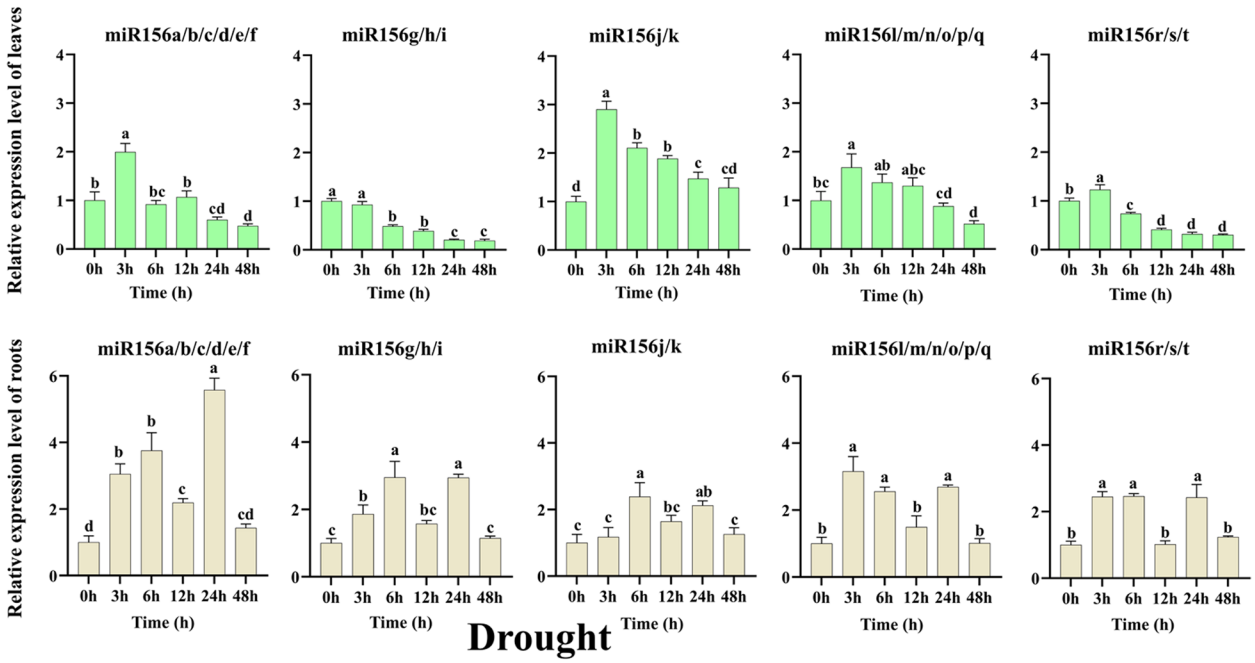

Fig. 6 The relative expression levels of tae-miR156(s) in leaves and roots after drought treatments by qRT-PCR analysis. Data are the mean of three replicates ± SD. Lowercase letters represent significant differences at $P < 0.05$

drought treatment, expression levels of all miR156(s) increased at different periods of treatment compared to untreated roots. The expression levels of miR156a/b/c/d/e/f were most significantly elevated after 24 h of treatment at 5.58 times higher than that of untreated. Expression levels of miR156g/h/i and miR156j/k were most significantly elevated after 6 h of treatment, at 2.95 and 2.40 times higher than that of untreated, respectively.

miR156l/m/n/o/p/q and miR156r/s/t expression levels were significantly elevated after 3 h of treatment, which were 3.16 and 2.45 times higher than untreated control before treatment, respectively. These results indicate that all members of the wheat miR156 family are involved in the drought stress response, but the root tissues were more affected compared with the leaves.

Under salt treatment (Fig. 7), all tae-miR156(s) showed a similar expression pattern with a significant increase after 3 h, followed by a decrease up to 48 h compared to untreated leaves. The most significant overexpression and suppression in expression levels were noted for miR156r/s/t, with expression levels 1.71-fold and 0.03-fold higher than untreated after 3 h and 48 h of treatment, respectively. Interestingly, in the root tissues under salt stress treatment, expression levels of miR156r/s/t decreased over the entire treatment period, but the most significant decrease was at 48 h, which was 0.19-fold of untreated. On the contrary, expression of miR156a/b/c/d/e/f increased over different periods compared to untreated, and the most significant increase was at 12 h, at 1.72 times higher than that of untreated. While the expression level of miR156g/h/i increased most significantly after 3 h of treatment (fold change of 1.58 times that of untreated), expression levels of miR156j/k and miR156l/m/n/o/p/q increased most significantly after 6 h of treatment at 1.37 and 1.48 times that of pre-treatment, respectively. These results indicated that the expression of miR156(s) was different in leaves and roots under the influence of salt stress.

Prediction of tae-miR156(s) target genes

MicroRNA156 plays a key role in plant growth, development, and response to stress through the precise regulation of its target genes [6]. To further explore the function of miR156 in wheat, the possible targets of tae-miR156(s) were predicted by PsRNATarget [37]. Of them, tae-miR156a/b/c/d/e/f had the highest number of presented target genes (56) (Table S3), followed by tae-miR156g/h/i (39) (Table S4), tae-miR156j/k (37) (Table S5), tae-miR156l/m/n/o/p/q (34) (Table S6), and miR156r/s/t (32) (Table S7). However, the majority of each tae-miR156's targets were from the *SPLs* family, with 27 genes (Table 2), revealing that *SPL* transcription factors are essential in wheat. In addition, the genes encoding Beta-galactosidase, Heparanase, Pectate lyase, Serine protease, SNF1 protein kinase, Xyloglucan endotransglucosylase hydrolase, Beta-carotene hydroxylase, Kinesin-related protein, Pentatricopeptide repeat-containing protein, Mitochondrial carrier-like protein, Chaperone protein DnaJ, Metal tolerance protein, Transcriptional corepressor SEUSS, Mitochondrial carrier-like protein, Auxilin-related protein 1, F-box protein family, Peptide chain release factor 1, Exocyst complex component, transcription factor *GTE10/8* and so on were also putative targets. The results indicated that tae-miR156(s) may

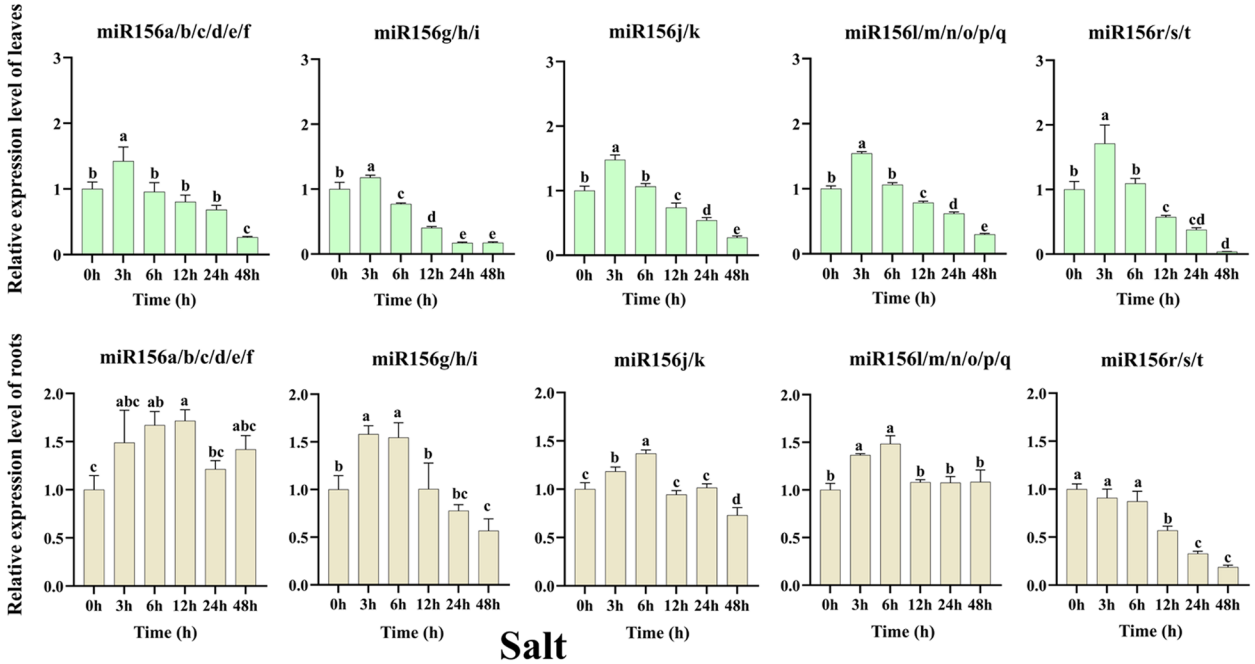

Fig. 7 The relative expression levels of tae-miR156(s) in leaves and roots after salt treatment as quantified by qRT-PCR analysis. Data are the mean of three replicates ± SD. Lowercase letters represent significant differences at $P < 0.05$

Table 2 Prediction of tae-miR156(s) target genes in wheat

Number	Gene name	Gene locus	Direction	Binding sequences	Function
I	miR156a/b/c/d/e/f		3′–5′	CACGAGUGAGAGAAGACAGUU	
II	miR156g/h/i		3′–5′	ACACGAGUGAGAGAAGACAGU	
III	miR156j/k		3′–5′	CACGAGUGAGAGAAGACAGU	
IV	miR156l/m/n/o/p/q		3′–5′	CACGAGUGAGAGAAGACAGUC	
V	miR156r/s/t		3′–5′	CACACGAGUGAGAGAAGACAG	
1	*TaSPL2-A*	*TraesCS3A02G432500*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage
2	*TaSPL2-B*	*TraesCS3B02G468400*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage
3	*TaSPL2-D*	*TraesCS3D02G425800*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage
4	*TaSPL3-A*	*TraesCS6A02G110100*	5′–3′	CAUGCUCUCUCUCUUCUGUCA	Cleavage
5	*TaSPL3-B*	*TraesCS6B02G138400*	5′–3′	CAUGCUCUCUCUCUUCUGUCA	Cleavage
6	*TaSPL3-D*	*TraesCS6D02G098500*	5′–3′	CAUGCUCUCUCUCUUCUGUCA	Cleavage
7	*TaSPL4-A*	*TraesCS6A02G155300*	5′–3′	CGUGCUCUCUCUCUUCUGUCA	Cleavage
8	*TaSPL4-B*	*TraesCS6B02G183400*	5′–3′	CGUGCUCUCUCUCUUCUGUCA	Cleavage
9	*TaSPL4-D*	*TraesCS6D02G145200*	5′–3′	CAUGCUCUCUCUCUUCUGUCA	Cleavage
10	*TaSPL7-A*	*TraesCS2A02G413900*	5′–3′	GGUGCUCUCUCUCUUCUGUCA	Cleavage
11	*TaSPL7-B*	*TraesCS2B02G432700*	5′–3′	GGUGCUCUCUCUCUUCUGUCA	Cleavage
12	*TaSPL7-D*	*TraesCS2D02G410700*	5′–3′	GGUGCUCUCUCUCUUCUGUCA	Cleavage
13	*TaSPL13-A*	*TraesCS2A02G232400*	5′–3′	CAUGCUCCCUCUCUUCUGUCA	Cleavage
14	*TaSPL13-B*	*TraesCS2B02G250900*	5′–3′	CAUGCUCCCUCUCUUCUGUCA	Cleavage
15	*TaSPL13-D*	*TraesCS2D02G232800*	5′–3′	CAUGCUCCCUCUCUUCUGUCA	Cleavage
16	*TaSPL14-A*	*TraesCS7A02G246500*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage
17	*TaSPL14-B*	*TraesCS7B02G144900*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage
18	*TaSPL14-D*	*TraesCS7D02G245200*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage
19	*TaSPL16-A*	*TraesCS7A02G260500*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage
20	*TaSPL16-B*	*TraesCS7B02G158500*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage
21	*TaSPL16-D*	*TraesCS7D02G261500*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage
22	*TaSPL17-A*	*TraesCS5A02G265900*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage
23	*TaSPL17-B*	*TraesCS5B02G265600*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage
24	*TaSPL17-D*	*TraesCS5D02G273900*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage
25	*TaSPL18-A*	*TraesCS5A02G286700*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage
26	*TaSPL18-B*	*TraesCS5B02G286000*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage
27	*TaSPL18-D*	*TraesCS5D02G294400*	5′–3′	UGUGCUCUCUCUCUUCUGUCA	Cleavage

interact with these possible target genes to regulate various biological processes in wheat.

Discussion

The miR156 gene family is widely distributed in plants, such as the rice miR156 family which has 12 members (miR156a to miR156l) [49], the *Arabidopsis thaliana* miR156 family with 10 members (miR156a *to* miR156j) [50], and the soybean miR156 family, comprising of 25 members (miR156a *to* miR156y) [51]. While many studies have reported on the function of miR156(s) in other plant species, the large and complex wheat genome has prevented similar studies in wheat. Nevertheless, current whole genome sequencing and assembly capabilities provide a platform to further explore the characteristics and functions of wheat *MIR156* gene family members. In this study, we identified 20 *MIR156* genes in wheat which are located on different chromosomes (Table 1), and have been largely conserved during the evolution (Fig. 1). However, they exhibited distinct spatiotemporal expression patterns at various stages of development (Fig. 3), demonstrating that they may have diverse regulatory functions. Whilst it has previously been demonstrated that *MIR156a/b/c* controlled the architecture of bread wheat [11], other miRNA members from the same family are proposed to have diverse functions [9, 52]. As the roles of tae-miR156(s) in other aspects of wheat have not been well described, the expression patterns of tae-miR156(s) described in our study provide a reference for future functional studies in wheat.

In many plants, miR156(s) respond to a wide range of abiotic stresses, showing complex and diverse functions and expression patterns, which are important to ensure plant adaptation to environmental stresses. And *cis*-acting elements play a crucial function in the regulation of gene expression. Previous studies have shown that promoters of *MIR156(s)* in apple and tea plants (*Camellia sinensis* (L.) O. Kuntze) harbor drought responsive elements known as MBS. Notably, during drought stress, the expression of csn-miR156f-2-5p was down-regulated in tea plants, but the expression of mdn-miR156ab was up-regulated in apples, indicating that various species had different expression patterns under drought stress [44, 53]. In our study, a total of 15 *tae-MIR156(s)* promoters possess drought-responsive elements (Fig. 2), implying that tae-miR156(s) may play a significant role in drought stress. In addition, *tae-MIR156e/f/g/h/k/s* contain low-temperature response elements (LTR), suggesting that they may be involved in cold stress (Fig. 2).

Abiotic stress affects miR156 expression in various plants. In *Arabidopsis* and sugarcane, low-temperature stress decreased miR156 expression and extended the nutritional growth phase, resulting in delayed growth metabolism [22, 45]. In young spikes of common wheat exposed to 48 h of cold treatment, miR156(s) were down-regulated [54] On the other hand, overexpression of *OsmiR156k* inhibited seedling growth under cold stress at the early development stage [21]. In our investigation, only expression of miR156g/h/i and miR156r/s/t were down-regulated in young leaves after 48 h of cold treatment when compared to untreated leaves, but in young root tissues, miR156g/h/i were up-regulated after 48 h of cold treatment. Almost all tae-miR156(s) were highly expressed following 3 h of cold stress treatment, with the most substantial up-regulation occurring in root tissues. These results proposed that tae-miR156(s) expression differed between leaves and roots during cold stress treatment (Fig. 4). Notably, the expression of tae-miR156g/h/i was up-regulated in root tissues at different time points compared to that of roots not exposed to the low temperature (Fig. 4), indicating that in wheat, tae-miR156g/h/i may be the most sensitive to cold stress of all miR156 members.

It has been demonstrated that without carbon dioxide fertilization, effective adaptation, and genetic improvement, global yields of wheat, rice, maize, and soybeans declined by an average of 6.0%, 3.2%, 7.0%, and 3.2%, respectively, for every 1 degree Celsius increase in global average temperature [55]. MiR156(s) were also involved in heat stress response in a variety of plants. In *Arabidopsis* seedlings, expression levels of miR156c, miR156d, and miR156h were up-regulated under heat stress proposing that overexpressing miR156 could enhance tolerance to heat stress [56]. Overexpression of miR156 also increased alfalfa's resistance to heat stress by boosting anthocyanin and chlorophyll accumulation [23]. As miR156 and its target genes are generally conserved in plants, it was proposed that the function of miR156 in heat stress memory may also be conserved in plants [57]. In our study, expression of miR156g/h/i, miR156j/k, miR156l/m/n/o/p/q, and miR156j/k were up-regulated in leaves after 3 h of heat treatment. Nonetheless, expression levels of all tae-miR156(s) in the leaves decreased sharply after 6 h of heat treatment and continued to decrease to a minimum level after 12 h (Fig. 5), suggesting that tae-miR156(s) have a similar response to heat stress. In contrast, in heat-treated roots, the expression of miR156g/h/i were most significantly up-regulated after 3 h of treatment compared with the other miR156s (Fig. 5). This suggested that among all the miR156 members in wheat, tae-miR156g/h/i were most likely implicated in resistance to heat stress.

The expression levels of miR156 may change in the presence of drought or high salt environments, allowing plants to adjust their growth strategies and improve drought and salt tolerance by regulating the expression of target genes. Under drought stress, the expression levels of miR156 were up-regulated in *Brachypodium distachyon* [58], maize (*Zea mays*) [59], tomato (*Solanum lycopersicum*) [60], and apple (*Malus domestica*) [26], but suppressed in rice [61], Chinese white poplar (*Populus tomentosa*) [62], and rape (*Brassica napus*) [63]. In addition, overexpression of miR156 enhanced tolerance to drought stress in alfalfa [64], tomato [60], and apple [26]. In this study, only miR156g/h/i expression in leaves was suppressed over all drought stress treatment time points while the expression levels of the other seventeen miR156(s) significantly increased after 3 h of drought treatment. And tae-miR156(s) were strongly induced by drought stress in root tissues compared to leaves (Fig. 6). These findings show that miR156 is differentially expressed in roots and leaves, implying that they serve separate functions during drought stress in wheat. Similar phenomena have been observed in plants such as cotton [65]. Of note, the expression levels of miR156a/b/c/d/e/f were significantly elevated after 24 h of treatment at 5.58 times higher than that of untreated (Fig. 6), showing that tae-miR156a/b/c/d/e/f may be the most susceptible to drought stress of all miR156 members in wheat.

For crops, salinity is one of the major abiotic stresses that often leads to reduced yields [66]. Under salt stress, the expression levels of miR156 in plants such as rice [67] and *Arabidopsis* [68] were up-regulated but the opposite was reported for expression in maize [69] and cotton [65]. Overexpression of miR156 reduced salt tolerance in apple seedlings [17]. MicroRNA156 positively regulates

the physiological responses of *Alfalfa* under salinity stress [70] and manipulating the expression of ZmmiR156 in tobacco could enhance the salt tolerance of transgenic plants without affecting plant structure [48]. These studies demonstrated that miR156 was also involved in the response to salt stress. Previous genomics-based analyses showed that miR156(s) expression levels were down-regulated in the roots of salt-tolerant wheat cultivars after 24 h of salt stress treatment [71]. Meanwhile, in our study, only the expression of miR156g/h/i and miR156r/s/t declined after 24 h of salt stress in the wheat plant roots, suggesting a differential response of diverse tae-miR156 to salt stress. In contrast, all tae-miR156(s) showed a similar expression profile in leaves under salt treatment, with a significant increase at 3 h, followed by a decrease, and a minimum at 48 h compared with the untreated at the corresponding time point, with the most significant fluctuation in expression observed for miR156r/s/t. Moreover, the expression level of tae-miR156r/s/t in root tissues was inhibited in salt-treated root tissues (Fig. 7). These results indicate that tae-miR156r/s/t may be more sensitive to salt stress.

MicroRNA156 has been reported to be a "superstar microRNA", involved in a variety of biological processes and stress responses in plants [72]. Under stress conditions, miR156 is induced to maintain the juvenile state for a longer period, while under favorable conditions, miR156 is inhibited to accelerate developmental transitions [73]. These studies suggest that the expression pattern of miR156 is critical to plant growth and development and response to stress. Hence, the expression of miR156 can be manipulated for genetic improvement of plant resistance to various abiotic stresses [48]. Previous transcriptome analysis revealed that wheat miR156 was implicated in stress response, but no specific members were identified [40, 71, 74]. Although miR156 is highly conserved throughout plants, its function varies among members of the same family, demonstrating functional differentiation within the family [8, 9]. Therefore, it is crucial to identify which miRNA family members are most vulnerable to unfavorable stress.

The *SPL* transcription factors are well known to play conserved roles in regulating diverse developmental processes and responses to biotic and abiotic stresses in various plant species [6, 75, 76]. And miR156 targets a large number of *SPL* genes across a wide range of plant species, indicating that the *miR156/SPLs* regulatory module has evolved to regulate diverse processes in the plant kingdom [6]. Among the 17 *Arabidopsis SPLs*, 11 are miR156 targets [77]. In rice, there are 19 *OsSPL* genes, of which 11 *SPL* genes are predicted targets of miR156 [49]. The wheat genome has 56 *TaSPL* genes [78, 79]. In this study, 27 *SPLs* target genes were predicted of miR156 (Table 2). Among these *TaSPLs* target genes, *TaSPL3*, *TaSPL13*, *TaSPL14*, and *TaSPL17* have been revealed to be targets of miR156, which may interact with miR156 to regulate plant architecture and improve agronomic traits in wheat [11, 79–82]. However, the interactions between wheat miR156 and other *SPL* target genes, as well as the molecular mechanisms of the *miR156/SPLs* pathway in regulating abiotic stresses still need to be further explored in wheat.

Conclusions

In this study, 20 miR156 family members (miR156a to miR156t) were identified in wheat. Although they are relatively conserved, the *cis*-elements of promoters and expression patterns were noted to be different. In addition, tae-miR156(s) were involved in abiotic stress response in wheat, and these miR156(s) showed different degrees of response in leaf and root tissues. Specifically, miR156g/h/i, miR156a/b/c/d/e/f, and miR156r/s/t may be the most effective molecular targets for inducing wheat stress resistance. Taken together, the miR156(s) despite originating from the same family, are involved in different responses to adverse conditions and may play a role in one of the most critical defense systems for wheat biotic stress tolerance. This lays the foundation for revealing the precise biological activities and molecular basis of adversity stress and will help to accelerate the breeding of stress-tolerant wheat varieties.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05899-4.

Supplementary Material 1.

Supplementary Material 2.

Acknowledgements

We thank the useful technical assistance provided by our colleagues in the laboratory. We also thank the rigorous evaluation of the manuscript made by the editors and reviewers.

Authors' contributions

The experimental design and execution of this study were led by SR and HS. SR and JL (Juan Lin) were primarily responsible for conducting the experiments and participated in data analysis. TL, YW, CX, LM, and WL also contributed to experimental operations and data analysis. Following the completion of the experiments, SR drafted the initial manuscript. CC, JL (Jie Lu), CM, and HS provided in-depth improvements to the manuscript. All authors have read and agreed to the final version of the manuscript.

Funding

The work was supported by a grant from The Agriculture Research System (CARS-03), Anhui Provincial and Ministerial Collaborative Innovation Center for Food Crops (APMCIC-FC), and Jiangsu Collaborative Innovation Center for Modern Crop Production (JCIC-MCP).

Data availability

All the supporting data are included within the article and its additional files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 19 June 2024 Accepted: 29 November 2024
Published online: 04 December 2024

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