

## Article

# Genome-Wide Investigation of the MiR166 Family Provides New Insights into Its Involvement in the Drought Stress Responses of Tea Plants (*Camellia sinensis* (L.) O. Kuntze)

Caiyun Tian <sup>1,2</sup>, Chengzhe Zhou <sup>1,2,3</sup>, Chen Zhu <sup>1,2,3</sup>, Lan Chen <sup>1,4</sup>, Biying Shi <sup>1,2</sup>, Yuling Lin <sup>1,3</sup>, Zhongxiong Lai <sup>1,3</sup>  and Yuqiong Guo <sup>1,2,\*</sup>

<sup>1</sup> College of Horticulture, Fujian Agriculture and Forestry University, Fuzhou 350002, China; cytian1997@foxmail.com (C.T.); chengzhezhou@foxmail.com (C.Z.); zhuchen19921118@foxmail.com (C.Z.); clan1995@foxmail.com (L.C.); biyingshi1997@foxmail.com (B.S.); buliang84@163.com (Y.L.); laizx01@163.com (Z.L.)

<sup>2</sup> Tea Industry Research Institute, Fujian Agriculture and Forestry University, Fuzhou 350002, China

<sup>3</sup> Institute of Horticultural Biotechnology, Fujian Agriculture and Forestry University, Fuzhou 350002, China

<sup>4</sup> College of Horticulture, Hunan Agriculture and Forestry University, Changsha 410125, China

\* Correspondence: guoyq828@163.com

**Abstract:** MicroRNA166 (miR166) is a highly conserved plant miRNA that plays a crucial role in plant growth and the resistance to various abiotic stresses. However, the miR166s in tea (*Camellia sinensis* (L.) O. Kuntze) have not been comprehensively identified and analyzed. This study identified 30 mature miR166s and twelve pre-miR166s in tea plants. An evolutionary analysis revealed that *csn*-miR166s originating from the 3' arm of their precursors were more conserved than the *csn*-miR166s derived from the 5' arm of their precursors. The twelve pre-miR166s in tea were divided into two groups, with *csn*-MIR166 Scaffold364-2 separated from the other precursors. The Mfold-based predictions indicated that the twelve *csn*-MIR166s formed typical and stable structures comprising a stem-loop hairpin, with minimum free energy ranging from  $-110.90$  to  $-71.80$  kcal/mol. An analysis of the *CsMIR166* promoters detected diverse *cis*-acting elements, including those related to light responses, biosynthesis and metabolism, abiotic stress defenses, and hormone responses. There was no one-to-one relationship between the *csn*-miR166s and their targets, but most *csn*-miR166s targeted *HD-Zip III* genes. Physiological characterization of tea plants under drought stress showed that leaf water content proportionally decreased with the aggravation of drought stress. In contrast, tea leaves' malondialdehyde (MDA) content proportionally increased. Moreover, the cleavage site of the *ATHB-15-like* transcript was identified according to a modified 5' RNA ligase-mediated rapid amplification of cDNA ends. The RT-qPCR data indicated that the transcription of nine *csn*-miR166s was negatively correlated with their target gene.

**Keywords:** tea; miR166s; *HD-ZIP III* family; evolutionary characteristics; drought stress



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## 1. Introduction

Tea (*Camellia sinensis* (L.) O. Kuntze) is an important economic crop that originated in southwestern China. The environmental stresses associated with the frequent occurrence of extreme weather events may adversely affect tea plant growth and development. Drought stress, which is one of the major challenges faced by growers in the major tea-producing regions, seriously affects tea plant growth and development and tea yield and quality [1,2]. To cope with the drought stress, tea plants would use a series of mechanisms, including morphological, physiological (e.g., cellular membrane damage, photosynthesis changes, osmotic regulation, etc.) and molecular levels to adapt to changes in environmental [3–5]. Therefore, the mechanism mediating tea plant drought resistance should be thoroughly characterized.

MicroRNAs (miRNAs) are small endogenous single-stranded non-coding RNAs composed of 20–24 nucleotides (nt) that specifically regulate target messenger RNAs (mRNAs) at the post-transcriptional or translational level through complementary base pairing [6]. Previous research revealed that miRNAs mediate cellular and metabolic processes in almost all plants, including those that regulate growth and responses to diverse abiotic stresses [7–9]. Unlike animals, plants are sessile organisms unable to move away from exposures to various stresses. Instead, they evolved sophisticated systems that provide protection from the adverse effects of environmental stresses (e.g., drought). Specifically, miRNAs are important modulators of drought stress responses. Several studies have verified that many miRNAs, such as miR156 [10], miR166 [11], miR393 [12], and miR399 [13], are important for drought resistance because they regulate the expression of stress resistance-related target genes. Of these miRNAs, miR166 is a highly conserved drought-responsive miRNA [14–18] with important functions in diverse species. For example, miR166 expression is inhibited in *Sorghum bicolor* (L.) Moench under drought conditions [19], whereas it increases in wheat roots and leaves [16]. Additionally, miR166 mainly targets and controls the expression of *homeodomain-leucine zipper III* (*HD-ZIP III*) genes, which encode important regulators of crucial plant developmental processes [20–22]. Recent studies confirmed that members of this gene family are widely involved in the resistance to various stresses, including drought [11,23]. The overexpression of miR166 confers drought resistance to rice (*Oryza sativa* L.) by modulating the expression of the *HD-ZIP III* gene *OsHB4* to induce leaf rolling and altered stem xylem development [11]. In mulberry (*Morus alba* L.), the overexpression of miR166f can negatively regulate the expression of its target genes, thereby enhancing plant responses to drought stress [24]. We previously determined that two miR166s (miR166a and miR166g-3p) are critical for *C. sinensis* drought stress responses because of their negative feedback-based regulation of the expression of their targets *ATHB-14-like* and *ATHB-15-like*, which is the *HD-ZIP III* subfamily transcription factors and plays an important role in plant resistance to abiotic stress [2]. However, miR166 usually belongs to a multi-member family in the plant kingdom that includes multiple miR166s [25,26]. The underlying regulatory mechanism of all miR166 members and their targets in tea plants under drought conditions remains largely undetermined.

Because the tea genome has been completely sequenced [27–34], the genes targeted by csn-miR166s may be identified by analyzing the available tea reference genome. In this study, we provide a detailed overview of the evolution of miR166s and pre-miR166s in tea, also predicting and analyzing the csn-miR166 targets as well as the *cis*-acting regulatory elements in *CsMIR166* promoters. Additionally, malondialdehyde (MDA) is perceived as the product of lipid peroxidation. Its content can be used as an indicator to reflect the degree of oxidative damage in the plant cell membrane under stress and evaluate the drought resistance of the plant [35,36]. Thus, to investigate the physiological changes of tea plants under drought stress, a physiological characterization was carried out through measures of relative water content (soil water content and leaf water content) and malondialdehyde (MDA) content. The expression patterns of csn-miR166s and their targets (*HD-ZIP III* genes) in response to drought stress were characterized. Our study provided the foundation for investing in the role of csn-miR166 and their targets in drought resistance in tea plants.

## 2. Materials and Methods

### 2.1. Plant Materials and Treatments

Eight-year-old tea plants (*C. sinensis* cv. ‘Tieguanyin’) were cultivated in the conservatory of Fujian Agriculture and Forestry University, Fuzhou, China (E 119°14′, N 26°05′). Drought treatment was performed following the Guo et al. method [2]. Briefly, the tea plants were transplanted and grown in a greenhouse at 25 °C with a 12/12 h (day/night) photoperiod and 40–65% relative humidity in September 2021. After 15 days, the compensatory watering method was adopted for drought treatment. Tea plants were divided into four groups (normal water supply, CK; mild drought stress, T1; moderate drought stress, T2; severe drought stress, T3), in which the soil water content was maintained at 19.06%,

14.37%, 9.29%, and 5.34% under CK, T1, T2, and T3, respectively, and treatment lasted for ten days. The soil water holding capacity was 23.45%. Each treatment was analyzed by collecting three biological replicates, and each replicates in at least ten randomly selected leaves. Tender leaves randomly distributed in the canopies were collected and immediately frozen in liquid nitrogen and kept at  $-80\text{ }^{\circ}\text{C}$  until analyzed.

## 2.2. Identification of Csn-MiR166s in Tea Plant's Genome and Acquisition of Plant MiR166 Sequences

The csn-miR166 and pre-miR166 sequences were obtained based on small RNA sequencing results and previous reports [37–39]. The obtained sequences were named by combining the abbreviation for *Camellia sinensis* (L.) O. Kuntze (csn) and the common name for their highly homologous sequences and the pre-miR166 positions on scaffolds (e.g., csn-miR166a, b, and c and csn-MIR166 Scaffold1344, csn-MIR166 Scaffold1761, and csn-MIR166 Scaffold2148) (Additional file 1. Table S1 and Additional file 2. Table S2). As one of the highly conserved miRNAs, miR166s have been identified in 45 plant species (including monocotyledonous species and dicotyledonous species). To comprehensively understand the sequence and evolution characteristics of miR166 and pre-miR166, the miR166 and pre-miR166 sequences from *Arabidopsis thaliana* (L.) Heynh, *O. sativa*, *Zea mays* L., *Vitis vinifera* L., and *Malus domestica* B. were downloaded from the miRBase database (release 22.1) (<http://www.mirbase.org/>; accessed on 13 November 2020).

## 2.3. Bioinformatic Analyses of Csn-MiR166

The csn-miR166 and pre-miR166 sequences were aligned using DNAMAN (version 6.0, San Ramon, CA, USA). A phylogenetic tree was subsequently constructed according to the neighbor-joining algorithm (1000 bootstrap replicates) using the MEGA (version 7.0, Auckland, New Zealand). The default parameters of the Mfold 3.5 tool were used to assess the secondary structure.

The transcription start site (TSS) sequences of the *CsMIR166* genes were predicted using the TSSP tool of Softberry (<http://linux1.softberry.com/berry.phtml?topic=tssp&group=programs&subgroup=promoter>; accessed on 29 October 2020). To predict the *cis*-acting elements, we extracted the 1500-bp sequence upstream of the TSS of 12 *CsMIR166* genes as the hypothetical promoter region for an analysis using the PlantCARE server (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>; accessed on 29 October 2020).

Based on the genome sequences of 'Shuchazao' [29] and 'Yunkang 10' cultivars [27], the csn-miR166 targets were predicted using the psRNATarget (<http://plantgrn.noble.org/psRNATarget/?function=3>; accessed on 26 April 2020) online tool. The default parameters were applied, with the exception of the expected value, which was set to 3.0 [25]. Additionally, the Cytoscape (version 3.9.1, Boston, MA, USA) analyzed the potential regulatory relationship of csn-miR166s and their targets in tea plants.

## 2.4. Physiological Characterization of the Tea Plant under Different Drought Degrees

The soil water content was determined according to the method for determining soil water content (NY/T 52-1987). In brief, 20 g of soil samples were taken from the potting and oven-dried at  $105 \pm 2\text{ }^{\circ}\text{C}$  for 12 h. Once the soil samples have cooled to room temperature, it is weighed. Soil water contents were determined as the difference between the fresh and dry soil mass, expressed as a percentage. The leaf water content was determined according to the method for tea-determination of moisture content (GB/T 8304-2002). The Malondialdehyde (MDA) content of tea leaves was measured using the MDA Assay Kit (Solabiol Technology Co., Ltd., Beijing, China).

## 2.5. Total RNA Isolation and cDNA Synthesis

Total RNA was extracted from the samples mentioned above using Transzol UP (TransGen Biotech, Beijing, China). The quality and concentration of the extracted RNA were respectively determined with a 1% agarose gel electrophoresis and the NanoDrop

2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The ratios of  $OD_{260}/OD_{280}$  between 1.90 and 2.10 indicated the samples were of sufficient quality for reverse transcribed to cDNA for target genes of miR166s and first-strand cDNA of miRNA using the TransScript<sup>®</sup> Uni One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen) and the TransScript<sup>®</sup> miRNA First-Strand cDNA Synthesis SuperMix (TransGen) kits, respectively.

#### 2.6. Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) Analyses

The Primer3 (<https://bioinfo.ut.ee/primer3/>; accessed on 9 January 2021) online tool was used to design RT-qPCR primers specific for the csn-miR166s, potential targets, and reference genes (Additional file 3. Table S3). The RT-qPCR analysis of the csn-miR166s and their targets were conducted using the TransStart<sup>®</sup> Tip Green qPCR SuperMix (TransGen) and the LightCycler 480 system (Roche Applied Sciences, Basel, Switzerland). Relative expression levels were calculated according to the  $2^{-\Delta\Delta C_t}$  method [40]. Each reaction was performed independently in triplicate. Additionally, *U6 small nuclear RNA (U6 snRNA)* and *SAND* were selected as the reference genes for the csn-miR166s and their targets, respectively.

#### 2.7. Cleavage Site Identification on the Basis of a Modified 5' RNA Ligase-Mediated Rapid Amplification of cDNA Ends (RLM-RACE)

Equal amounts of the total RNA extracted from the samples mentioned above were mixed and then reverse transcribed for a modified RLM-RACE using the FirstChoice<sup>™</sup> RLM-RACE Kit (Invitrogen, CA, USA) [41]. The target gene cleavage primers were designed using the Primer3 (<https://bioinfo.ut.ee/primer3/>; accessed on 13 November 2021) online tool (Additional file 3. Table S3). Two rounds of nested PCR amplification were performed using the gene-specific and universal primers. The amplified products were analyzed by 1.5% agarose gel electrophoresis. The target fragments were purified from the gel using the EasyPure<sup>®</sup> Quick Gel Extraction Kit (TransGen) and cloned into the T1 vector (TransGen). Single colonies of putative transformants were selected for sequencing (BioSune, Fuzhou, China).

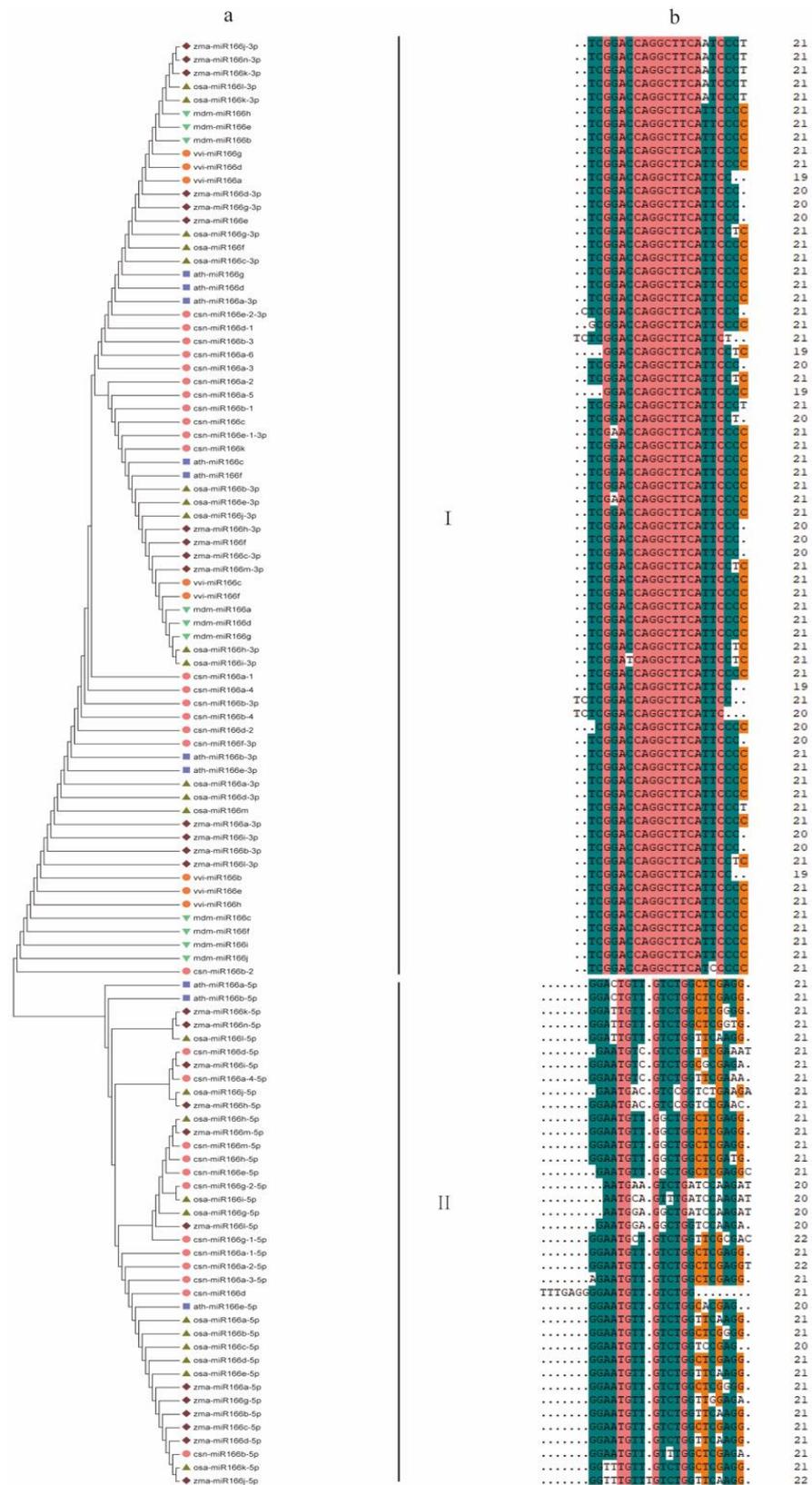
#### 2.8. Statistical Analysis

All dates were provided herein as the mean  $\pm$  standard deviation. The SPSS (version 25.0, Armonk, NY, USA) software was used for a one-way analysis of variance followed by Tukey's post-hoc test. Differences were considered significant at  $p < 0.05$ .

### 3. Results

#### 3.1. Analysis of MiR166 Sequences in Plants

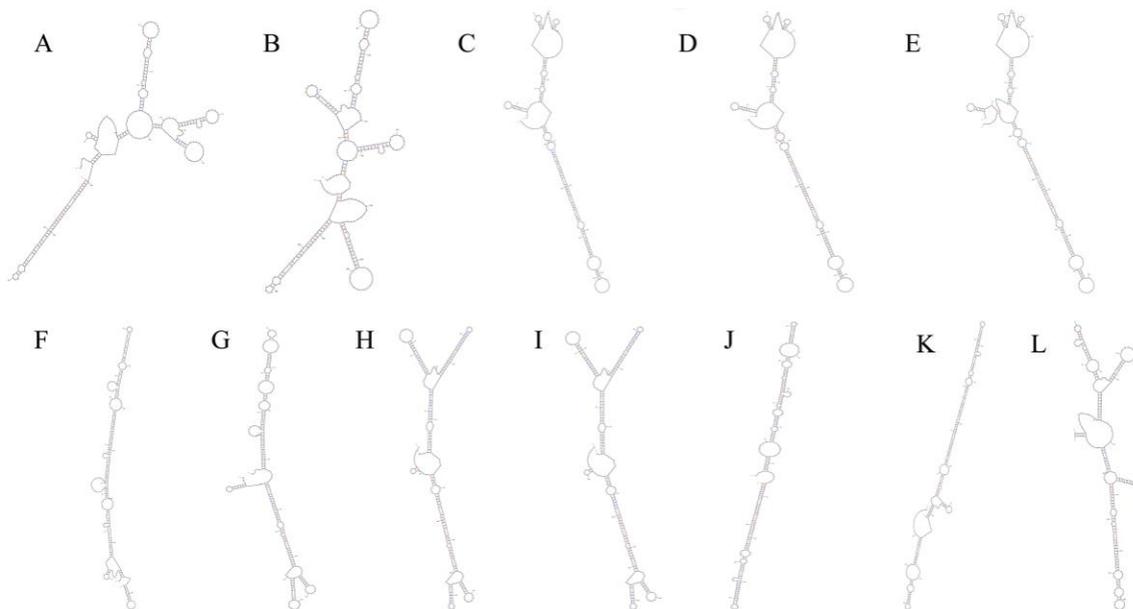
To investigate the evolutionary relationships among miR166s, we used the miR166s from *C. sinensis*, *A. thaliana*, *O. sativa*, *Z. mays*, *M. domestica*, and *V. vinifera* to construct a phylogenetic tree (Figure 1a). All miR166s were classified into two groups according to whether they derived from the 5' arm or 3' arm of their precursors. Mutations, deletions, and insertions were detected in the miR166 sequences at nucleotide positions 1, 2, 4, 6, 17, 19, 20, and 21; the remaining nucleotides were highly conserved (Figure 1b). Further analyses revealed fewer mutations, deletions, and insertions at the 3' end than at the 5' end of the csn-miR166 precursors.



**Figure 1.** Analysis of mature miR166 sequences in selected plants. **(a)** Phylogenetic relationships analysis of csn-miR166s. **(b)** Sequence alignment analysis of csn-miR166s. vvi. *Vitis vinifera* L.; mdm. *Malus domestica* B.; osa. *Oryza sativa* L.; csn. *Camellia sinensis* (L.) O. Kuntze; ath. *Arabidopsis thaliana* (L.) Heynh; zma. *Zea may* L. Different highlight colors represent different homology levels of sequence. Pink.  $\geq 100\%$ ; Green.  $\geq 75\%$ ; Orange.  $\geq 50\%$ .

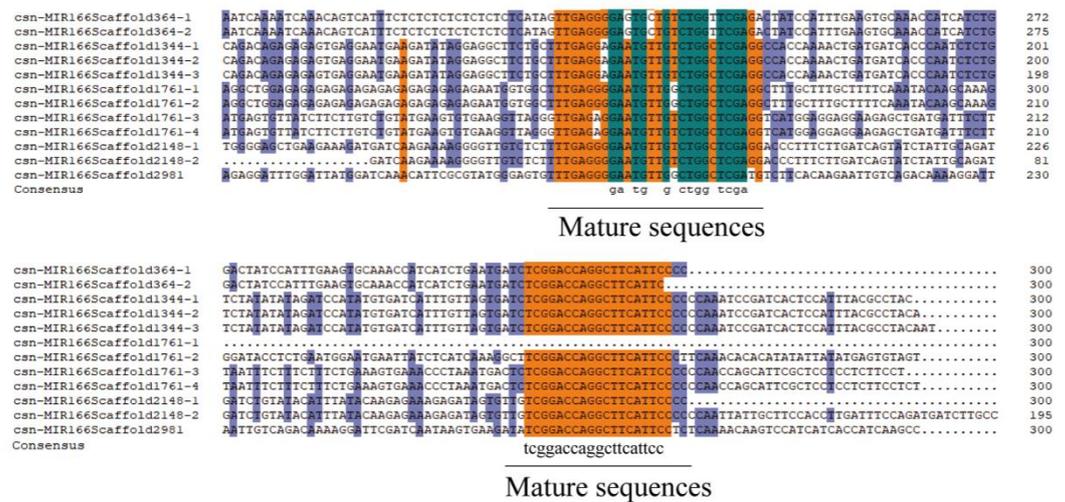
### 3.2. Sequence Analysis and Predicted Secondary Structure Analysis of Pre-MiR166s in Tea Genome

Twelve precursors were named according to the pre-miR166 scaffold positions (Additional file 2. Table S2 and Additional file 4. Table S4). The identified pre-miR166s comprised 300 nt, within the normal range for plant miRNA precursor lengths (55–930 nt) [42]. The Mfold 3.5 predictions (Figure 2) indicated that all pre-miR166s formed typical and stable structures with a stem-loop hairpin. The minimum folding free energy of the pre-miR166s in tea varied considerably, ranging from  $-110.90$  (csn-MIR166 Scaffold1761-2) to  $-71.80$  (csn-MIR166 Scaffold364-2) kcal/mol.



**Figure 2.** Stem-loop structures of twelve pre-miR166s in *C. sinensis*. (A–L) correspond to csn-MIR166 Scaffold364-1, 364-2,1344-1, 1344-2, 1344-3, 1761-1, 1761-2, 1761-3, 1761-4, 2148-1, 2148-2, and 2918.

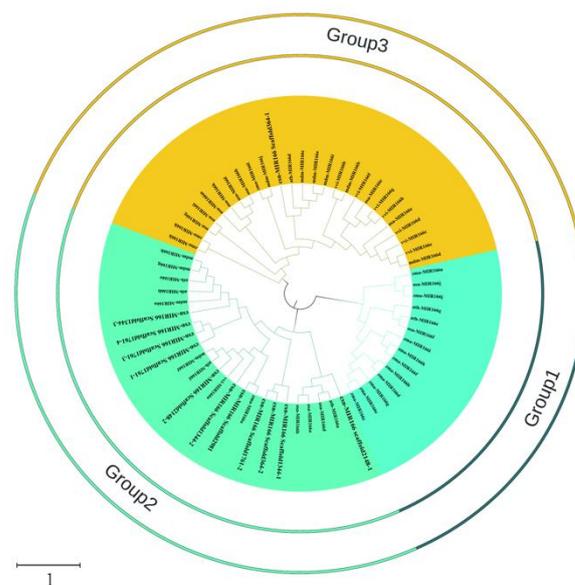
To further clarify the origins of csn-miR166s, a multi-sequence alignment analysis was performed to compare the mature miR166s and pre-miR166s in tea (Figure 3), which revealed overlapping sites between the precursor and mature sequences. Additionally, the nucleotides were more conserved in the 3' arm than in the 5' arm, which is consistent with the observation that the csn-miR166s derived from the 3' arm of their precursors were more conserved than the csn-miR166s derived from the 5' arm of their precursors. Further analyses of the origins of csn-miR166s indicated that most of the mature csn-miR166s (e.g., miR166a-1, a-2, a-3, a-4, a-5, b-3p, b-4, d-2, and e-5p, etc.) were associated with twelve pre-miR166s (Additional file 4. Table S4). However, some csn-miR166s (e.g., miR166b-2, b-3, d-1, e-1-3p, a-4-5p, b-5p, d-5p, g-1-5p, g-2-5p, and h-5p) did not exactly match precursor sequences, possibly reflecting the complexity of csn-miR166 evolution.



**Figure 3.** Sequences alignment among *C. sinensis* pre-miR166. Different highlight colors represent different homology levels of sequence. Green.  $\geq 100\%$ ; Orange.  $\geq 75\%$ ; Blue.  $\geq 50\%$ .

### 3.3. Evolutionary Relationships among Pre-MiR166s in Plants

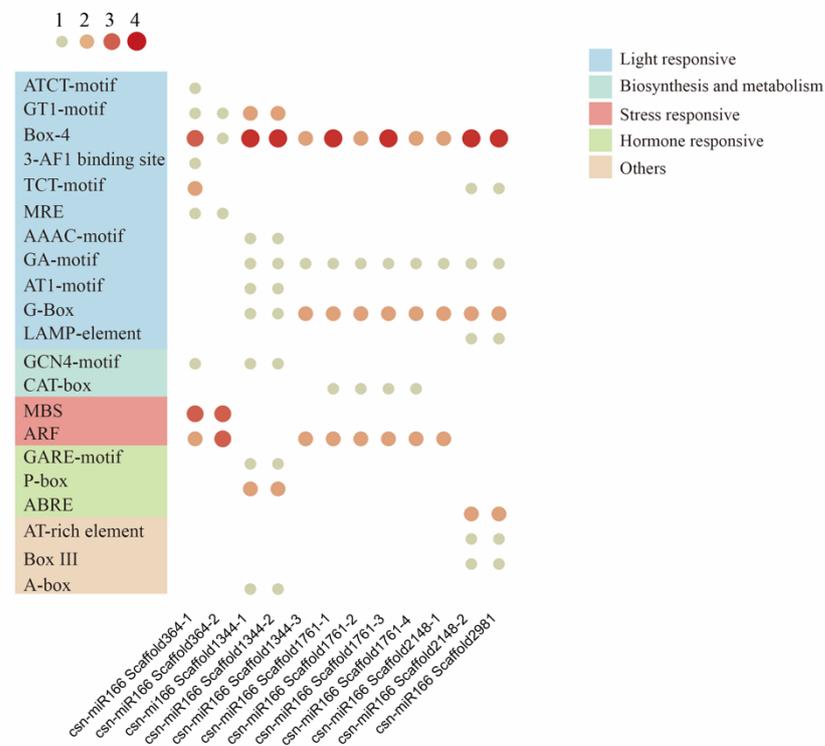
The evolutionary relationships among the pre-miR166s in different plant species were investigated by examining a phylogenetic tree constructed using the pre-miR166 sequences from *C. sinensis*, *A. thaliana*, *O. sativa*, *Z. mays*, *M. domestica*, and *V. vinifera* (Figure 4). The pre-miR166s from the analyzed plant species were classified into three groups. Specifically, group 1 was composed of ten zma-MIR166s, two osa-MIR166s, and two ath-MIR166s. 24 pre-miR166s were clustered in group 2 comprising eleven csn-MIR166s (csn-MIR166 Scaffold364-2, 1344-1, 1344-2, 1344-3, 1761-1, 1761-2, 1761-3, 1761-4, 2148-1, 2148-2, and 2981), four ath-MIR166s (ath-MIR166a, f, b, and e), three osa-MIR166s (osa-MIR166d, a, and b), four mdm-MIR166s (mdm-MIR166f, a, g, and h), one vvi-MIR166 (vvi-MIR166a), and one zma-MIR166 (zma-MIR166a). The other pre-miR166s from the six plant species were clustered in group 3. Furthermore, csn-MIR166 Scaffold364-2 belonged to a separate branch that did not include any other tea csn-pre-miR166s.



**Figure 4.** Phylogenetic relationships of pre-miR166 sequences in plants. vvi. *Vitis vinifera* L.; mdm. *Malus domestica* B.; osa. *Oryza sativa* L.; csn. *Camellia sinensis* (L.) O. Kuntze; ath. *Arabidopsis thaliana* (L.) Heynh; zma. *Zea may* L.

### 3.4. Analysis of Cis-Acting Elements in CsMIR166 Promoter Regions

To identify the *cis*-acting elements in *CsMIR166* promoter sequences, the TSSP tool of Softberry was used to predict the TSS of *CsMIR166s* and the 1500-bp region upstream of the TSS of *CsMIR166* genes (i.e., hypothetical promoter region) was analyzed using the PlantCARE server. The core promoter element TATA-box and the common CAAT-box element were detected in the promoter and enhancer regions of all *CsMIR166* genes. The other identified elements in *CsMIR166* promoters were divided into the following five categories: light response-, biosynthesis and metabolism-, abiotic defense-, and hormone response-related *cis*-acting elements (Figure 5). The *cis*-acting elements were unequally distributed among the *CsMIR166* genes. The light-responsive elements were the most abundant (all promoters contained photoresponsive elements), followed by the anaerobic responsive elements (66.7%). In contrast, some *cis*-acting elements, such as meristematic tissue expression elements (CAT-box, 33.3%), endosperm expression elements (GCN4, 25.0%), drought response elements (MBS, 16.7%), gibberellin response elements (GARE and P-box, 16.7%), and abscisic acid response elements (ABRE, 16.7%), were identified in no more than three *CsMIR166* genes.

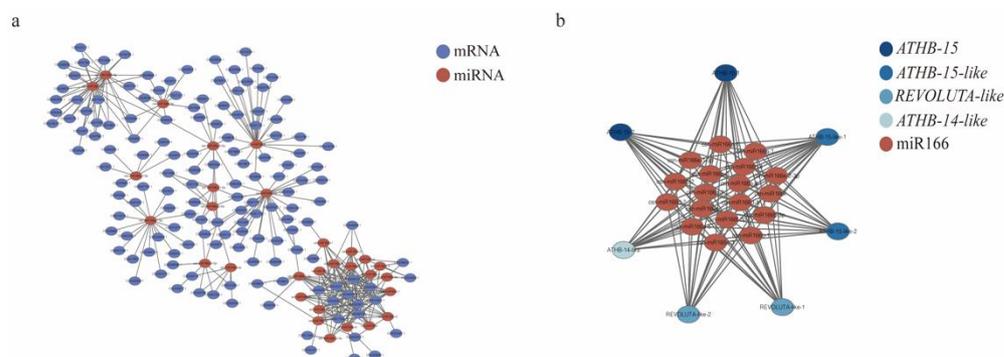


**Figure 5.** Promoter analysis of *MIR166* genes in *Camellia sinensis* (L.) O. Kuntze.

### 3.5. Prediction of *csn*-miR166 targets

To explore the biological functions of *csn*-miR166s, based on the tea reference genome, potential target genes of *csn*-miR166s were predicted using the psRNATarget tool, which resulted in a total of 151 potential target genes were predicted (Figure 6a and Additional file 5. Table S5). The regulatory relationships between the *csn*-miR166s and their characteristic target genes revealed a lack of a one-to-one relationship between the regulation of *csn*-miR166s and their respective targets. The *HD-Zip III* family, the primary gene family controlled by miR166s, was simultaneously targeted by fifteen *csn*-miR166s. The targets included one *ATHB-14-like* (*CSS049178.1*), two *REVOLUTA-like* (*CSS017546.1* and *CSS042067.1*), two *ATHB-15* (*CSS002611.1* and *CSS043540.1*), and two *ATHB-15-like* (*CSS012922.1* and *CSS044030.1*) genes (Figure 6b and Additional file 6. Table S6). Further analyses indicated that *csn*-miR166s from the 3' arm of their precursors could target *HD-Zip III* genes, whereas

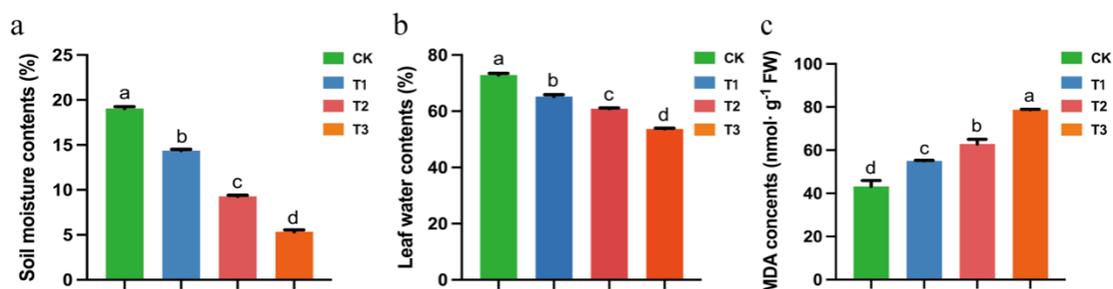
csn-miR166s from the 5' arm of their precursors could target genes that did not belong to the *HD-Zip III* family.



**Figure 6.** Regulatory network of the csn-miR66s and its targets in the tea plant. (a) Regulatory network of the csn-miR66s and its targets. (b) miRNA-mRNA modules related to the *HD-ZIP III* family.

### 3.6. Analysis of Tea Plants Relative Water Content and Malondialdehyde Content under Drought Stress

To explore the physiological changes in tea plants under the different drought degrees, the relative water content (soil water content and leaf water content) and malondialdehyde content were determined (Figure 7). With the aggravation of drought stress, the soil water content and leaf water content proportionally decreased in T1, T2, and T3 with respect to the normal water supply (CK), whereas the malondialdehyde content proportionally increased.

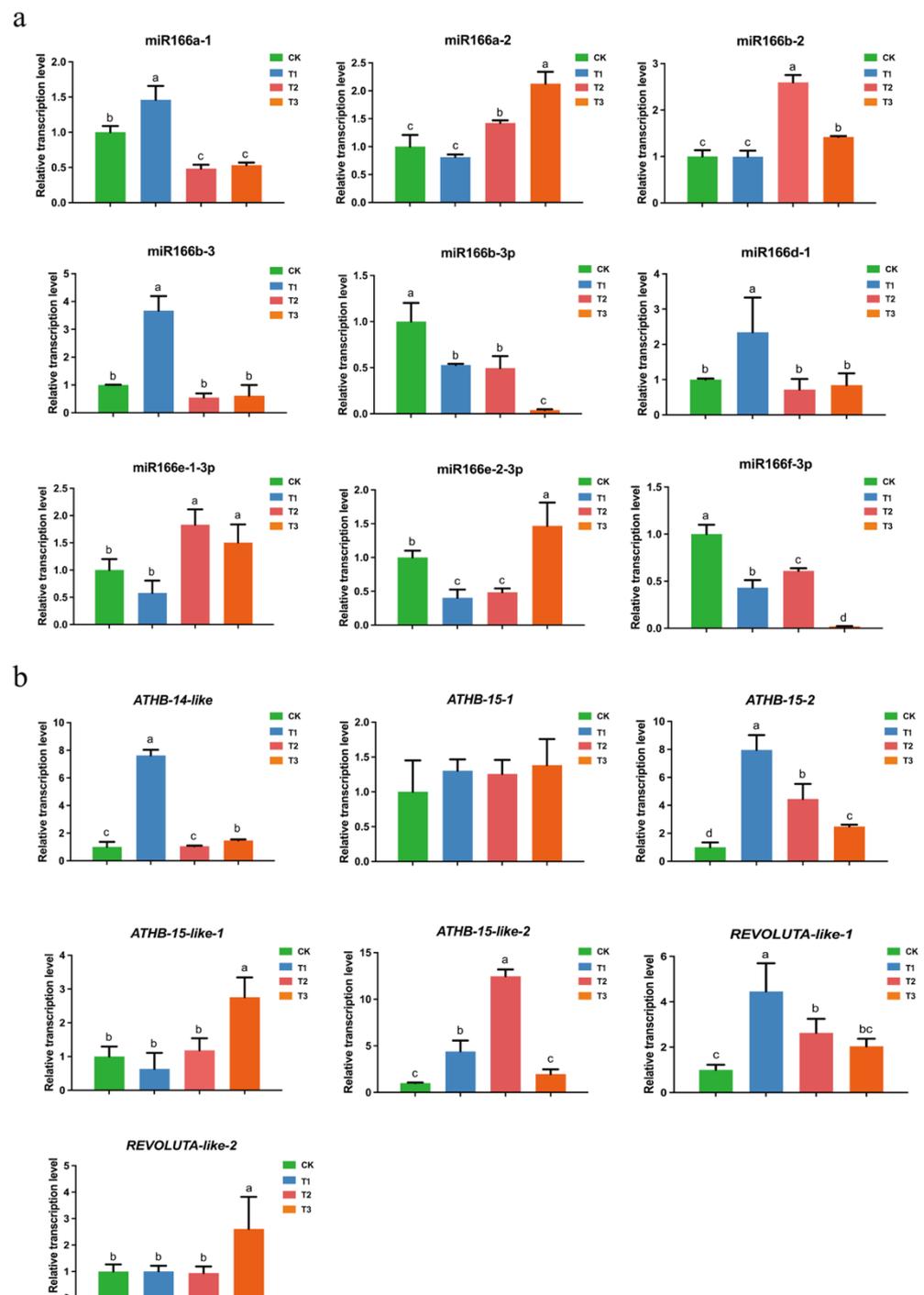


**Figure 7.** (a) Soil water content in different drought degrees. (b) Leaf water content of tea plant in different drought degrees. (c) MDA content of tea plant in different drought degrees. Data were the mean of three independent replicates  $\pm$  standard deviation (SD). Different lowercase letters indicate significant differences at different drought degrees ( $p < 0.05$ ).

### 3.7. Transcription Profiling and Cleavage Site Identification of Csn-MiR166s and Their Targets under Different Drought Degrees

To determine the regulatory effects of csn-miR166s on *HD-Zip III* genes under different drought degrees, the transcription profiles of nine csn-miR166s (miR166a-1, a-2, b-3p, b-2, b-3, d-1, e-1-3p, e-2-3p, and f-3p) were examined (Figure 8a). The csn-miR166 family transcription patterns displayed a different change in response to different drought stress degrees. More specifically, the transcription levels of three miR166s (miR166a-1, b-3, and d-1) showed a similar tendency. Their transcription levels were up-regulated under T1 and down-regulated under T2 and T3. In contrast, the transcription levels of miR166a-2, b-2, and e-1-3p were down-regulated under T1 and up-regulated under T2 and T3, as compared with those in CK. Additionally, the transcription levels of miR166b-3p and miR166f-3p were down-regulated under T1, T2, and T3, whereas miR166e-2-3p's transcription level was similar to miR166b-3p and miR166f-3p in T1 and T2 but down-regulated under T3. To functionally characterize the *HD-Zip III* genes regulated by csn-miR166s, the transcription patterns of seven *HD-Zip III* family members following different drought degrees were

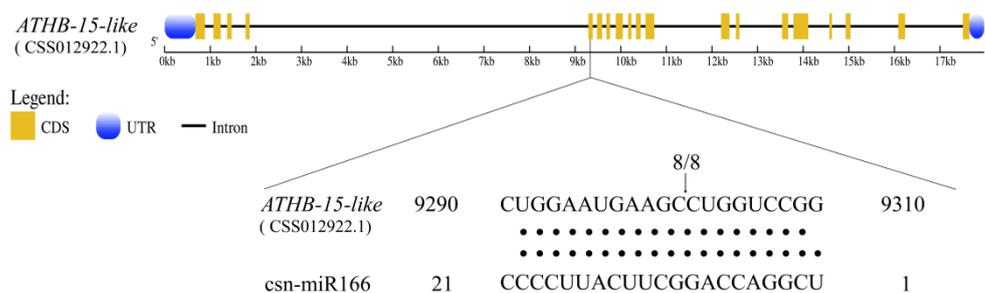
explored (Figure 8b). Compared with CK, the transcription levels of *ATHB-14-like*, *ATHB-15-1*, *ATHB-15-like-2*, *REVOLUTA-like-1*, *ATHB-15-like-2*, and *ATHB-15-2* were up-regulated under T1, T2, and T3, whereas the transcriptions of *ATHB-15-like-1* and *REVOLUTA-like-2* were down-regulated in T1 and T2, respectively.



**Figure 8.** (a) Transcription patterns of the miR166s under different drought degrees. (b) Transcription patterns of the targets under different drought degrees. Data were the mean of three independent replicates  $\pm$  standard deviation (SD). Different lowercase letters indicate significant differences at different drought degrees ( $p < 0.05$ ).

The miR166–*HD-Zip III* gene pairs in tea showed that the transcription of *csn-miR166s* was negatively correlated with the transcription of the corresponding targets under the drought stress and displayed the diversity in regulating corresponding targets in responding to the different drought degrees (under T1, T2, and T3) (Figure 8 and Additional file 7. Table S7). The miR166a-1, b-3, and d-1 would negatively regulate the transcriptions of *ATHB-15-like-1* and *ATHB-14-like* under drought stress. The miR166b-3p and miR166f-3p transcription levels were inversely correlated with the transcriptions of *ATHB-15-1*, *REVOLUTA-like-1*, *ATHB-15-like-2*, and *ATHB-15-2* under drought stress. Additionally, *REVOLUTA-like-1* would be negatively regulated by miR166a-2, b-2, and e-1-3p under T1 and T2 and negatively regulated by miR166a-1, b-3, b-3p, d-1, and f-3p under the T3. The miR166e-2-3p negatively regulated the transcriptions of *ATHB-14-like*, *ATHB-15-1*, *REVOLUTA-like-1*, *ATHB-15-like-2*, and *ATHB-15-2* under T1 and T2.

Subsequently, the cleavage site of the *ATHB-15-like* transcript was identified based on a modified 5' RLM-RACE analysis (Figure 9). The transcription of *ATHB-15-like* was regulated via the cleavage of the binding region between nucleotides 12 and 13 from the 5' end of the *csn-miR166s*. Hence, miR166s can regulate the *HD-Zip III* family by modulating the transcription of related target genes following exposure to drought stress.



**Figure 9.** *ATHB-15-like* cleavage sites of *csn-miR166s* identified by 5' RLM-RACE.

## 4. Discussion

### 4.1. Evolutionary Characteristics of *Csn-MiR166s*

The miR166s are highly conserved miRNAs that are widely distributed in 45 plant species, including angiosperms, gymnosperms, ferns, and mosses [25,26,43]. The information available in the miRBase database indicates that miR166s are abundant in many plant species, including *Glycine max* (L.) Merr (26), *Z. mays* (26), *O. sativa* (24), *Brachypodium distachyon* (L.) Beauv (18), and *Populus trichocarpa* (Torr & Gray) (17). Based on the miRBase database, the *G. max* and *Z. mays* had the highest number of miR166 members among plants, each containing 26 miR166 members. In the present study, 30 *csn-miR166s* were identified, more than the number of miR166 in *G. max* and *Z. mays* to our knowledge. Increases in the number of *csn-miR166* genes may have been facilitated by whole-genome duplication events [30] in the large tea genome (approximately 3 Gb) [27–29,33]. Additionally, conserved miRNAs reportedly underwent both conserved and diverse evolutionary processes [44]. We identified 30 *csn-miR166s* and divided them into two categories (Figure 1). The *csn-miR166s* derived from the precursor 3' arm were more conserved than the *csn-miR166s* originated from the precursor 5' arm (Figure 1b). The more conserved *csn-miR166s* had the same putative target genes in the *HD-ZIP III* family (Figure 6b). In contrast, the *csn-miR166s* derived from the precursor 5' arm targeted many other genes encoding functionally diverse proteins, including ABC transporter and proteins responsive to stress and auxin (Additional file 5. Table S5). A previous investigation proved that miR166s are highly complex miRNAs regarding their evolution. There is no one-to-one relationship between the number of miR166s in a species and their corresponding loci. Different mature sequences may have originated from the same or multiple loci [26]. For example, the miR166s of *G. max* (26), *Z. mays* (26), *O. sativa* (24), *B. distachyon* (18), and *P. trichocarpa* (17) are respectively associated with 21, 14, 13, 10, and 13 genomic loci. In tea, 30 *csn-miR166s*

were revealed to be associated with 12 loci. Interestingly, of the 30 *csn-miR166s*, some *csn-miR166s* (e.g., *miR166b-2*, *b-3*, *d-1*, *e-1-3p*, *a-4-5p*, *b-5p*, *d-5p*, *g-1-5p*, *g-2-5p*, and *h-5p*) did not precisely match the precursor sequences, suggesting that the *csn-miR166* family has been affected by mutations during its formation and may have more undiscovered or unconfirmed loci. This hypothesis needs to be confirmed by further experiments. Additionally, *csn-MIR166 scaffold364-2* was separated far from eleven pre-*miR166s* (Figure 4), suggesting that tea pre-*miR166s* may have complex and distinct evolutionary rates and diverse modes of evolution. Based on that evidence, we speculated that *csn-miR166s* are both conservation and diversification in evolution and function.

#### 4.2. Cleavage of *HD-ZIP III* Transcripts by *Csn-MiR166s* Is a Crucial Mechanism Underlying Tea Plant Drought Tolerance

Examining the putative promoter regions is likely to provide clues regarding the *csn-miR166* regulatory mechanism. *Cis*-regulatory elements are crucial for the transcriptional regulation of *MIR* genes [6]. We observed that putative *CsMIR166* promoter regions include many *cis*-regulatory elements, including MBS and ABRE elements (Figure 5). The *miRNA* genes (e.g., *MIR167*, *MIR168*, and *MIR396*) with promoter regions that contain ABRE elements are important for drought stress responses [45,46]. Similarly, *miR166* genes with MBS and ABRE elements in their promoter regions might also play crucial roles during plant responses to drought stress. As expected, the transcription patterns of *csn-miR166s* are affected by drought stress (Figure 8a). Previous studies reported that different *miR166* members possibly present a special manner or regulatory role in withstanding the abiotic stresses [2,25]. In this study, the transcription patterns of *csn-miR166* members differed, and the regulation of *csn-miR166s* on their targets displayed diversity under the different drought degrees (Figure 8 and Additional file 7. Table S7). It is indicated that the regulation of *csn-miR166* family members on their targets under the different drought degrees may be complex, with the division of roles.

MicroRNAs can regulate the expression of their target genes by cleaving the corresponding mRNA or inhibiting translation according to the perfect or near-perfect complementarity principle [25]. Cleavage is one of the main *miRNA* modes of action for silencing their target genes [6]. The *HD-ZIP III* genes, which are critical for plant responses to various abiotic stresses, are the main targets of *miR166s* [2,47,48]. In this study, the prediction of *csn-miR166* targets showed its role in inhibiting the transcription of their putative targets (*HD-ZIP III* family members) primarily via transcript cleavage (Additional file 6. Table S6). This was indeed the case since the *csn-miR166s* can cleave the *ATHB-15-like* transcript by applying a modified 5' RLM-RACE strategy (Figure 9). This is consistent with the previous report that *miR166* could respond to drought stress by cleaving *HD-ZIP III* family members [11,25]. Thus, we speculated that *csn-miR166*-mediated *HD-ZIP III* cleavage could be an important mechanism for mediating tea plant drought resistance. The *HD-ZIP III* genes encode transcription factors that regulate the expression of many downstream stress-associated genes [23]. In future studies, a systematic exploration of the specific mechanism by which *csn-miR166s* control the transcription of downstream genes through their regulatory effects on *HD-ZIP III* genes to protect tea plants from drought stress will help to improve our understanding of tea plant resistance to drought stress.

## 5. Conclusions

In this study, *csn-miR166s* and *csn-pre-miR166s* were analyzed to elucidate the characteristics and effects of *csn-miR166s* on tea plant drought resistance. We revealed the conserved and diverse evolution and functions of *csn-miR166s*. Moreover, the RT-qPCR analysis indicated that *csn-miR166* family members in responding to drought stress might have the division of labor and complementary function. The *csn-miR166*–target gene pairs confirmed that *csn-miR166s* regulate the transcription of *HD-Zip III* family members. More specifically, they control target gene expression levels to mediate drought stress resistance.

Our study provides crucial insights into the role of the csn-miR166-mediated regulatory mechanism contributing to tea plant resistance to drought stress.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/f13040628/s1>, Table S1. The sequences of csn-miR166, pre-miR166, and the target genes of csn-miR166s, Table S2. Naming information of csn-miR166s and pre-miR166s, Table S3. Primers for RT-qPCR, Table S4. The location information of the pre-miR166s scaffold, Table S5. Prediction of the csn-miR166 target gene, Table S6. Naming information of csn-miR166 target gene, Table S7. The specific functions of different csn-miR166 members under the different drought degrees.

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