miR2119, a Novel Transcriptional Regulator, Plays a Positive Role in Woody Plant Drought Tolerance by Mediating the Degradation of the CkBI-1 Gene Associated with Apoptosis

Furong Liu 1, Puzhi Zhang 1,*, Jiayang Li 2, Tianxin Zhang 1, Lifang Xie 1 and Chunmei Gong 2,*

1 College of Life Sciences, Northwest A&F University, Yangling, Xianyang 712100, China; 17196028015@163.com (F.L.); wellbeingzpz@163.com (P.Z.); tianxinzhang@nwafu.edu.cn (T.Z.); xlf8163@163.com (L.X.)
2 College of Horticulture, Northwest A&F University, Yangling, Xianyang 712100, China; jiayang@nwafu.edu.cn
* Correspondence: gcm228@nwafu.edu.cn

Abstract: Caragana korshinskii, an important vegetation restoration species with economic and ecological benefits in the arid region of northwest China, is characterized by significant drought tolerance. However, the underlying molecular mechanisms by which miRNAs confer this trait in C. korshinskii are unclear. Here, we investigate the effect of CkmiR2119 on drought tolerance and identified its target gene, CkBI-1. A negative correlation of CkmiR2119 and CkBI-1 in both stems and leaves in a drought gradient treatment followed by target gene validation suggest that CkmiR2119 might negatively regulate CkBI-1. Consistently, a decrease in the expression of the CkBI-1 gene was observed after both transient transformation and stable transformation of CkamiR2119 in tobacco (Nicotiana tabacum). Moreover, the physiological analysis of CkamiR2119 and CkBI-1 transgenic plants further indicate that CkmiR2119 can enhance the drought tolerance of C. korshinskii in two aspects: (i) downregulating CkBI-1 expression to accelerate vessel maturation in stems; (ii) contributing to a higher level of CkBI-1 in mesophyll cells to inhibit programmed cell death (PCD). This work reveals that CkmiR2119 can increase plants’ drought tolerance by downregulating the expression of CkBI-1, providing a theoretical basis to improve plants’ ability to withstand stress tolerance by manipulating miRNAs.

Keywords: Caragana korshinskii; drought; CkamiR2119; CkBI-1

1. Introduction

Drought is one of the major abiotic stresses affecting the growth and development of plants [1] through prolonged shortages in the soil water supply [2], causing massive damage to the ecological environment and water resources [3,4]. To adapt to this extreme stress, over time plants evolved many mechanisms at the molecular level, which may act as a useful strategy for plants’ normal growth and development in a drought environment. Recently, researchers unraveled some mechanisms of plant tolerance through a variety of approaches, including genetic engineering, marker-assisted selection [5], and microRNAs. MicroRNAs are a type of short single-stranded conserved noncoding RNAs (approximately 20–24 nucleotides in length). They derive from stem-loop regions of endogenous precursor transcripts [6,7] and participate in the modulation of many biological processes by regulating target mRNAs [8,9]. Many miRNAs play an important role in environmental stress tolerance [10,11]. Currently, some drought stress-regulated miRNAs are identified; for example, in Arabidopsis thaliana, overexpression of the miR398-resistant form of CSD2 suppresses the downregulation of CSD2 expression by miR398 and accumulates more mRNA in response to stress, thereby enhancing the scavenging effects of plant clearance ROS (reactive oxygen species) [12]. In addition, miR393 is positively regulated...
by dehydration, high salt, low temperature, and ABA induction, and can mediate auxin expression [13]. In rice, miR169g expression may be regulated directly by CBF/DREBs to respond to drought [14]. In Glycine max, it was recently shown that gma-miR398c can negatively regulate soybean drought tolerance, providing a new site to screen for a drought tolerance resource using CRISPR [15]. In Zanthoxylum bungeanum, some newly discovered miRNAs can act as important regulators in the antioxidant system by inhibiting relevant genes’ expression [16]. However, the underlying mechanisms of miRNAs that respond to drought stress remain largely uninvestigated. Our previous studies found that many miRNAs may respond to drought stress in C. korshinskii [17]. Among them, miR2119 is a novel miRNA reported only in leguminous plants [18]. A recent study shows that a reduction in miR2119 expression in drought stress can increase the accumulation of its target gene ADH1, thereby being more responsive to this stimuli [19]. In our previous study, we predicted that CkBI-1 may be a target gene in the drought stress regulation of CkmiR2119 [17].

Given their sessile lifestyles, plants must constantly accept environmental changes and rapidly adjust their metabolism and gene-expression profiles to adapt to inconstant conditions such as drought, extreme temperatures, salinity, nutrient imbalance, and toxic metals [20]. In past decades, various studies indicated that plants evolved sophisticated and effective systems for endomembrane trafficking in response to stress conditions, which is indispensable for the response and adaptation to environmental stresses [21]. Bax inhibitor-1 (BI-1) is an evolutionarily conserved endoplasmic reticulum localization protein crucial to the maintenance of endoplasmic reticulum homeostasis. It is widely recognized that BI-1 proteins mediate programmed cell death and resist ROS accumulation when facing biotic and abiotic stress [22]. Overexpression of the BI-1 gene can improve the ability of plants to resist drought, high temperature, and other adverse stresses [23].

C. korshinskii is a main feed crop in China, with small and pinnate leaflets; it is widely distributed in the arid and semiarid regions of northwest China [24,25]. C. korshinskii is a suitable shrub for plantation/afforestation to restore deserted and degenerated land, and was widely planted on the Loess Plateau as part of the project to restore farmland and preserve the ecological environment [26,27]. Some recent studies found that C. korshinskii has the typical morphological characteristics and physiological mechanisms of drought-tolerant plants [28,29], which provides a theoretical basis for soil and water conservation, vegetation restoration, and ecosystem reconstruction in arid and semiarid areas.

In this study, we found that CkmiR2119 can downregulate CkBI-1 in response to drought. To further identify the function of CkmiR2119, we cultivated transgenic tobacco that overexpressed CkmiR2119 and confirmed its drought tolerance using a leaf-disc tobacco experiment and other relevant plant physiological experiments. In summary, we report the differential expression of CkmiR2119 between the leaves and stems of plants in response to drought; this provides a new perspective for studies on plant drought tolerance.

2. Results

2.1. Protein Structure Prediction and Subcellular Localization of CkBI-1

To clarify the basic properties of CkBI-1, we first analyzed the three-dimensional structure of CkBI-1. The result of the structural prediction analysis indicated that the transmembrane region of CkBI-1 has a seven-fold transmembrane domain (Figure 1A,B). To investigate the distribution of CkBI-1 in leaves, the recombinant plasmid 35S: CkBI-1-GFP was constructed and transiently transformed into tobacco using Agrobacterium GV3101. The subcellular localization analysis suggested that CkBI-1 was mainly located in the endoplasmic reticulum membrane (Figure 1C–G). These results indicate that CkBI-1 has the same localization and function as other recognized BI-1 family members.
2.2. Different Expression Patterns of CkmiR2119 and CkBI-1 in Stems and Leaves of C. korshinskii

To verify the relationship between CkmiR2119 and CkBI-1, we first analyzed the expression patterns of CkmiR2119 and CkBI-1 in C. korshinskii under gradient drought treatment using qPCR. The expression tendency of CkmiR2119 and CkBI-1 is in the opposite direction in both stems and leaves. In stems, the relative expression of CkmiR2119 gradually increased by approximately 10 times with the increase in drought, whereas the expression of CkBI-1 gradually decreased by 85% under the same condition. However, the relative expression of CkmiR2119 and CkBI-1 in leaves showed a contrary tendency to that in stems (Figure 2). The results showed that CkmiR2119 was negatively correlated with CkBI-1 in C. korshinskii under drought stress. Therefore, CkmiR2119 may be involved in the negative regulation of CkBI-1.

Figure 1. Structural analysis and subcellular localization of CkBI-1 protein. (A) Three-dimensional structure of CkBI-1 protein. The two atom layers represent the upper and lower surfaces of the cell membrane. (B) Analysis of the transmembrane domain of CkBI-1 protein. (C) Prediction of subcellular localization of CkBI-1 protein. (D–G) Subcellular localization of CkBI-1 protein. (D) GFP excitation wavelength. (E) mCherry excitation wavelength. (F) Bright field. (G) Displayed as an overlay of three channels. Scale bars, 25 µm.
Figure 2. Expression trends of CkmiR2119 and CkBI-1 in the stems and leaves of C. korshinskii under drought gradients. Data are shown as the mean ± SD of three independent experiments. One-way ANOVA was performed for the statistical analysis, where different letters represent significant differences (Ducan, p < 0.05).

2.3. CkmiR2119 Can Cleave CkBI-1 and Reduce Its Expression Level

To study the function of CkmiR2119, 35S: CkBI-1-GUS and 35S: CkamiR2119 expression vectors were constructed. In the transient expression experiments, tobacco transformed with the empty vector 35S: GUS was used as a positive control (Figure 3A); 35S: CkBI-1-GUS and 35S: CkamiR2119-GUS were injected alone (Figure 3B,C) and co-injected in tobacco leaves (Figure 3D). As expected, 35S: GUS showed a blue signal in the whole tobacco leaf, and 35S: CkamiR2119-GUS showed no blue signal. CkamiR2119 undergoes several cleavages during processing and maturation, resulting in the cleavage of the following GUS gene failing to display a blue signal. At the same time, the GUS staining of tobacco leaves injected with 35S: CkBI-1-GUS alone was darker and distributed over entire leaves, while the staining degree of tobacco leaves injected with 35S: CkamiR2119 and 35S: CkBI-1 was relatively light, indicating that CkmiR2119 can downregulate the expression of CkBI-1.
To obtain the cleavage site between CkamiR2119 and CkBI-1, we obtained the RNA of C. korshinskii. The cleaved CkBI-1 was amplified using nested PCR by adding an adaptor to the 5′ end of the fragmented RNA and using random primers for reverse transcription. Its second-round nested PCR amplified band was approximately 300 bp (Figure 3E). After purification and recovery, it was linked to T vector for blue-white screening (Figure 3F,G). The cleaved CkBI-1-GUS cotransformation. (E) RLM-5′RACE nested PCR second-round amplification results. (F,G) Screening of positive clones after the amplified product is connected to T vector. (H) The cleavage site of CkmiR2119. Scale bars in (A–D), 10 mm.

Figure 3. Validation of the targeting relationship between CkamiR2119 and CkBI-1. (A–D) Verification of the targeting relationship between CkamiR2119 and CkBI-1 transient expression in tobacco leaves. (A) 35S: GUS. (B) 35S: CkBI-1-GUS. (C) 35S: CkamiR2119-GUS. (D) 35S: CkamiR2119 and 35S: CkBI-1-GUS cotransformation. (E) RLM-5′RACE nested PCR second-round amplification results. (F,G) Screening of positive clones after the amplified product is connected to T vector. (H) The cleavage site of CkmiR2119. Scale bars in (A–D), 10 mm.

To obtain the cleavage site between CkamiR2119 and CkBI-1, we obtained the RNA of C. korshinskii. The cleaved CkBI-1 was amplified using nested PCR by adding an adaptor to the 5′ end of the fragmented RNA and using random primers for reverse transcription. Its second-round nested PCR amplified band was approximately 300 bp (Figure 3E). After purification and recovery, it was linked to T vector for blue-white screening (Figure 3F,G). After the alignment of CkBI-1 sequencing with miR2119, the result showed that the cleavage site of CkmiR2119 and CkBI-1-mRNA was located at the 10–11th nucleotide at the 5′ end of the CkmiR2119 complementary sequence (Figure 3H). Phylogenetic tree analysis and multiple sequence alignment showed that the target sequence of CkmiR2119 was relatively conserved in many legumes (Figure S1). In conclusion, the above experiments show that CkmiR2119 can cleave the transcript of CkBI-1, thereby negatively affecting the expression of CkBI-1, and that this mechanism is relatively conserved in legumes.

2.4. CkBI-1-OE Line Has the Effect of Inhibiting Cell Death

To verify whether CkBI-1 can negatively regulate PCD, CkamiR2119, and CkBI-1, transgenic tobacco plants were obtained using A. tumefaciens GV3101-mediated callus culture (Figure S2). Three types of 25-day-old tobacco leaves were stained with trypan blue staining. The results showed that the staining of WT and transgenic empty vector tobacco were obviously darker than that of transgenic CkBI-1, which suggested that overexpression of CkBI-1 can significantly reduce PCD of mesophyll cells (Figure 4).
in the stems of WT and two transgenic tobacco were detected using qPCR. The result showed that the expression level of the tobacco wild-type and empty vector plants. Moreover, after drought treatment, the relative expression of the tobacco wild-type and empty vector plants. Additionally, we also calculated the ratios of the xylem staining area and the number of vessels in the stems of WT and two transgenic tobacco were detected using qPCR. The result was consistent with the previous section’s observation; the proportion of the xylem area and the number of vessels in the stems of the CkamiR2119-OE line were significantly higher than those of the wild-type and empty vector plants. Moreover, after drought treatment, the relative expression of the tobacco BI-1 gene of the CkamiR2119-OE lines was significantly lower than that of the other two types of plants (Figure 5F). Therefore, these results suggest that overexpression of CkamiR2119 significantly reduces the expression of the BI-1 gene and promotes xylem formation in stems.

Figure 4. Trypan blue staining of tobacco CkBI-1-OE line leaves. (A) CkBI-1-OE line. (B) WT. (C) Empty vector. (D) Analysis of the degree of trypan blue staining. Data are shown as the mean ± SD of three independent experiments. One-way ANOVA was performed for the statistical analysis, where different letters represent significant differences (p < 0.05). Scale bars, 100 μm.

2.5. CkamiR2119-OE Line Has More Developed Stem Xylems

Sections of CkamiR2119-OE line, wild-type, and transgenic empty vector tobacco were stained with safranin and observed under a microscope. The results showed that the CkamiR2119-OE line had significantly larger stem xylem areas and more stem vessels than the other two types of tobacco (Figure 5A–C). Additionally, we also calculated the ratios of the xylem staining area and the number of vessels of CkamiR2119-OE to WT tobacco in the above sections using ImageJ. The result was consistent with the previous section’s observation; the proportion of the xylem area and the number of vessels in the stems of the CkamiR2119-OE line were significantly higher than those of the wild-type and empty vector plants. Moreover, after drought treatment, the relative expression of the tobacco BI-1 gene of the CkamiR2119-OE lines was significantly lower than that of the other two types of plants (Figure 5F). Therefore, these results suggest that overexpression of CkamiR2119 significantly reduces the expression of the BI-1 gene and promotes xylem formation in stems.

Figure 5. Safranin staining of freehand sections of stems of CkamiR2119-OE line under drought stress. (A) WT. (B) Empty vector. (C) CkamiR2119-OE line. (D) The relative area of the xylem in stems. (E) The ratio of CkamiR2119-OE line and empty vector plants to the number of vessels in the wild-type. (F) The relative expression of tobacco BI-1 gene in tobacco plants after drought treatment. Data are shown as the mean ± SD of three independent experiments. One-way ANOVA was performed for the statistical analysis, where different letters represent significant differences (p < 0.05). Scale bars, 300 μm.
2.6. Drought Tolerance Analysis of CkmiR2119 Transgenic Plants under Drought Stress Treatment

Phenotypic analyses of transgenic and WT tobacco were performed under normal conditions and a follow-up drought treatment. In normal cultivation, CkamiR2119-OE lines had a slightly better growth state than that of the wild-type and the transgenic empty vector plants (Figure 6A). However, after drought treatment, the growth phenotype of CkamiR2119-OE lines, which had more new leaves and a lower withering rate, was obviously better than that of the other plants (Figure 6A).

![Drought Tolerance Analysis of CkmiR2119 Transgenic Plants under Drought Stress Treatment](image)

**Figure 6.** The phenotype of CkamiR2119 transgenic plants under drought treatment. (A) Phenotypic observations of drought treatment. (B) Relative water content of transgenic tobacco plants. (C) Relative conductivity of transgenic tobacco plants. Data are shown as the mean ± SD of three independent experiments. One-way ANOVA was performed for the statistical analysis, where different letters represent significant differences (p < 0.05).

Next, we collected the leaves of these three types of tobacco and analyzed their physiological parameters. The relative water content measurements revealed that there was no significant difference between the three types of tobacco when grown under normal conditions, whereas the relative water content of CkamiR2119-OE lines decreased much less than those of the other two types of tobacco after drought treatment (Figure 6B). In addition, the results of the relative electrical conductivity tests were similar to the detection of relative water content, which was a much lower increase in CkamiR2119-OE after drought treatment.
compared with the other two types of tobacco (Figure 6C). Therefore, these results suggest that overexpression of CkamiR2119 can improve the drought tolerance of plants.

3. Discussion

*C. korshinskii* has a strong drought tolerance and constitutes an important genetic resource. Plant miRNAs are critical post-transcriptional regulators, and can regulate the expression of their target genes in plants’ growth and development. At present, many miRNAs are proven to regulate plants’ life activities in extreme environments. [30,31]. In our previous study, high-throughput sequencing of miRNA and GO (Gene Ontology) enrichment analyses of *C. korshinskii* leaves under a natural precipitation gradient showed that the expression of CkmiR2119 decreased with an increase in drought gradient [17]. Surprisingly, in this study, we found that the expression tendency of CkmiR2119 in *C. korshinskii* stems was contrary to that in leaves after drought gradient treatment (Figure 2). Therefore, we first tried to identify the relationship between CkmiR2119 and CkmiR2119. Our results show that the expression of CkmiR2119 and CkBI-1 is negatively correlated in both leaves and stems in *C. korshinskii*. In addition, the tobacco transient transformation experiment and the verification of restriction sites suggest that CkmiR2119 might be a negative regulator of CkBI-1. These findings suggest that CkmiR2119 can potentially have value in responding to drought, which is crucial for plants’ survival in extreme climates.

In higher plants and animals, the BI-1 gene (*Bax inhibitor-1*) is an evolutionally conserved apoptotic inhibitor [32]. We found that CkBI-1 has seven transmembrane domains, and it is mainly located in endoplasmic reticulum (Figure 1C–G), which is consistent with the basic features of other members in the BI-1 family [33–35]. Our results show that CkBI-1 can be the target gene of CkamiR2119 (Figure 3), suggesting that BI-1 might play an important role in the drought tolerance of plants, which is consistent with other results [22,23,32]. The BI-1 protein is involved in endoplasmic reticulum stress [32] and maintains the homeostasis of the endoplasmic reticulum by binding to other functional proteins and inhibiting the cells’ PCD process. In our research, the results of trypan blue staining on leaves of CkBI-1-OE lines prove that CkBI-1 can inhibit cell death (Figure 4). In the relative expression experiment of CkamiR2119-OE lines, overexpression of CkamiR2119 can downregulate the expression of tobacco BI-1 in stems (Figure 5F). Furthermore, the observation of Safranin O sections suggests that CkamiR2119-OE lines tobacco have a better formation of vascular bundles than other types of plants (Figure 5A–C).

Water transport in plants is a unified dynamic continuous system of mutual feedback: a soil–plant–atmosphere continuum (SAPC). Plants can absorb water through their roots and transport water to leaves and other organs for growth and development through sophisticated xylem systems. Extra water drafts out from the stomata and participates in the turbulent exchange in the atmosphere. As the main water conduction tissue in plants, the water transport capacity of xylem is mainly determined by the number, diameter, and length of vessels. Normally, wide vessels have a more efficient ability to transport water [36]. The formation of vessel molecules is a typical PCD process in plant growth and development [37–39]. Therefore, we hypothesized that overexpression of CkmiR2119 can downregulate the expression of CkBI-1 in stems, so that it can induce more PCD and contribute to the formation of vascular bundles in plants [40], which will boost the water retention capacity, thus enhancing plants’ drought tolerance. As expected, the tobacco CkamiR2119-OE lines exhibited higher adaptation to drought stress, with significantly better detection of phenotypic and physiological indexes under drought stress. Transgenic plants had a higher relative water content in their leaves and lower conductivity than vector transgenic plants and WT plants (Figure 6).

In summary, this work reports the drought-resistant functions of CkmiR2119 in *C. korshinskii*. The results indicate that CkmiR2119 can repress the expression of CkBI-1, which may contribute to the drought tolerance of both stems and leaves. Based on our findings, we provide a hypothesis to explain the different expression patterns of CkmiR2119 in stems and leaves. CkmiR2119 can downregulate the expression level of CkBI-1 by target-
ing CkBI-1-mRNA and reducing the inhibitory effect of CkBI-1 on PCD, which promotes the formation of xylem vessel molecules and increases the efficiency of water transport in stems, thereby enhancing drought tolerance. In addition, in the leaves of C. korshinskii, the lower expression pattern of CkmiR2119 reduces the inhibition on the higher expression of CkBI-1, which can inhibit the occurrence of PCD in mesophyll cells under drought stress (Figure 7). This work elucidates an important function of plant miRNAs in drought stress, and provides more evidence regarding the theoretical basis for the disclosure of drought tolerance mechanisms for improved use in arid areas to reduce the phenomenon of land desertification.

Figure 7. Model of CkmiR2119 regulating CkBI-1 gene expression.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

Seeds of C. korshinskii were obtained from the Loess Plateau in Shaanxi Province and Inner Mongolia, Northwest China [41]. Sterilized seeds were germinated in the dark at 28 °C and were cultured in moist soil.

All the C. korshinskii were grown in a greenhouse with a cycle of 16 h light at 25 ± 2 °C and 8 h dark at 18 ± 2 °C. We chose 50-day-old seedlings with the same growth status and treated them with different extents of drought in different basins for 25 days; the basins were divided into three gradients, where soil water contents were maintained at 75% (suitable water availability), 55% (moderate drought), and 35% (severe drought), respectively; the soil water content (weight%) = (initial soil weight - dry soil weight) / dry soil weight × 100% = water weight / dry soil weight × 100%.

4.2. Protein Structure Prediction

The sequence of the CkBI-1 protein was obtained from the C. korshinskii transcriptome database of our laboratory. We predicted the subcellular localization of CkBI-1 protein using an online website (https://predictprotein.org/get_results?req_id=644255) (accessed on 19 August 2021). We predicted the transmembrane domain of CkBI-1 based on SOSUI (http://harrier.nagahama-i-bio.ac.jp/sosui/) (accessed on 25 March 2022). The 3D structure of CkBI-1 was predicted using SWISS-MODEL (https://swissmodel.expasy.org/interactive) (accessed on 26 March 2022).
4.3. Relevant Genes Cloning and Plasmids Construction and Subcellular Localization

The overexpression of CkmiR2119 was achieved by constructing artificial miRNA [42]. The miR319 precursor sequence of A. thaliana acted as the molecular skeleton of CkamiR2119. Three pairs of overlap PCR primers were designed using the online website WMD3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) (accessed on 25 May 2020) for sequence substitution. Total DNA was extracted from the A. thaliana seedlings using the CTAB method for cloning the AtmiR319 precursor sequence. The primers for cloning CkBI-1 were designed using Primer 5. Both CkmiR2119 and CkBI-1 genes were inserted into the vector pCAMBIA1304 between the Bgl II and NcoI I restriction sites. The relative primers are listed in Table S1.

The constructed pCambia1304-CkBI-1 vector was used for subcellular localization observation. At the same time, the pCambia1304 vector was used as a control. The freeze–thaw method was used to transform relevant plasmids into A. tumefaciens strain GV3101. Then, we injected the bacterial liquids using 1 mL disposable syringes from the back of leaves into 4-week-old tobacco. We cultured the transgenic plants at 25 °C in the dark for one day and then had a light culture in the growth chambers at 25 °C with a 16 h photoperiod for one day. The GFP signal was observed using a confocal laser scanning microscope (Leica TCS SP8, Wetzlar, German) three days after infiltration.

4.4. Verification of CkBI-1 Cleaves Sites

Plant growth and RNA extraction were performed as described previously [41]. The mRNA from C. korshinskii leaves was purified using the PloyATtract® mRNA Isolation system IV Kit (Promega, Madison, WI, USA). We designed RLM-5′ RACE reaction primers with reference to the FirstChoice™ RLM-RACE Kit (Invitrogen, Carlsbad, CA, USA); the 5′ junction sequence was synthesized by the Beijing Qingke Biotechnology Co., Ltd. (Beijing, China); the other primers were synthesized by the Beijing Oke Dingsheng Biotechnology Co., Ltd. (Beijing, China). The 5′ RACE connector was connected using T4 RNA ligase (Thermo Fisher Scientific, Waltham, MA, USA), and the target fragments obtained using nested PCR were connected to the pGEM-T vector (Promega, Madison, WI, USA) with the pGEM-T Easy carrier junction kit. The positive clones were screened using LB solid culture containing 40 mg/L X-Gal, 0.2 mM IPTG, and 100 mg/L Amp. We selected the positive bacteria to culture in the LB liquid medium overnight, followed by sequencing using Xi’an Optic Zesi Biotechnology Co., Ltd. (Xi’an, China).

4.5. Tobacco Transient Expression Experiment and Identification of Stable Expression Transgenic CkmiR2119 and CkBI-1 Tobacco Plants

All the plasmids were transformed into A. tumefaciens strain GV3101 using the freeze–thaw method. In the transient expression experiment, the A. tumefaciens solution was used to transform into 4-week-old Nicotiana benthamiana seedlings’ leaves with 1 mL disposable syringes. Three replicates were used for each treatment, including pCAMBIA1304-CkBI-1, pCAMBIA1304-CkamiR2119, pCAMBIA1304-CkamiR2119, pCAMBIA1304-CkBI-1 cotransformation, and an empty vector infection. Then, we cultured the injected tobacco in the dark for one day followed by light culture for approximately 60 h. We clipped down the transformed leaves and soaked them in GUS staining solution for 37 °C overnight; then, we rinsed them with ethanol in gradients of 55%, 75%, and 100%, respectively.

Stable genetic transformation of CkamiR2119 and CkBI-1 genes in Nicotiana benthamiana by A. tumefaciens mediated leaf disc transformation. The sterilized tobacco leaf discs (0.5 cm × 0.5 cm) were precultivated at 25 °C for 3 d on the MS medium containing 1 mg/L 6-BA and 0.1 mg/L NAA. The pretreated leaf discs were infected with A. tumefaciens by soaking in transformed bacterial solution for 10 min and then moved to an MS medium containing 1 mg/L 6-BA and 0.1 mg/L NAA at 22 °C for 2 d in the dark. Then, we transferred the discs to a shoot-inducing medium (MS medium with 1 mg/L 6-BA, 0.1 mg/L NAA, 250 mg/L Crab, and 25 mg/L Hyg) for shoot differentiation, and then transferred them to a root-inducing medium (1/2MS medium with 0.1 mg/L NAA, 250 mg/L Crab,
and 25 mg/L Hyg). The seedlings were planted in soil when they were 5–10 cm, and we identified the transgenic plants by genomic sequencing thereafter.

4.6. Quantitative Real-Time PCR (qRT-PCR) Assay

The samples of stems and leaves (approximately 0.1 g) were ground into a fine powder in liquid nitrogen and transferred to a precooled 1.5 mL EP tube. Total RNA was extracted from the seedlings using the TRIzol method [43]. The quantity and integrity of the total RNA were determined by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Shanghai, China) and agarose gel electrophoresis, respectively. The cDNA sequences were obtained by a stem-loop primer using a cDNA reverse transcription kit (Takara, Tokyo, Japan). Quantitative analysis on the expression of relevant genes was performed using the TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Tokyo, Japan) and the quantitative PCR amplifier CFX96 (Biorad, Hercules, CA, USA). The qRT-PCR reaction conditions were as follows: pre-denaturation at 95 °C for 1 min, denaturation at 95 °C for 5 s, annealing and extension at 60 °C for 30 s, and amplification for 40 cycles. In this experiment, u6 and CkUB were used to design primers as the internal reference genes of miR2119 and CkBI-1, respectively. The $2^{-\Delta\Delta CT}$ method and GraphPad Prism 5 software were used to calculate the relative expression of the target gene and mapping, respectively. Quantitative primers are listed in Table S2.

4.7. Physiological Analysis of the Transgenic and WT Plants under Drought

All transgenic and WT seeds were cultivated in chambers under the conditions described above. Drought treatment was applied after 30 days of cultivation. The relative water content (RWC) was calculated as $(M_1 - M_3)/(M_2 - M_3) \times 100\%$, where $M_1 =$ fresh weight of tobacco leaves, $M_2 =$ turgid weight (we soaked the blades in distilled water for 12 h, dried the surface of the blades with filter paper, and weighed them), and $M_3 =$ dry weight (we dried M2 in an 80 °C oven for 48 h to a constant weight). We calculated the relative water content of the leaves.

Washed fresh tobacco leaves were cut into 0.5 cm pieces and placed in a test tube with 6 mL of deionized water. All the samples were extracted with a vacuum pump and immersed. After 3 h, we measured the conductivity (E1) of the solution using a conductivity meter. After boiling the samples for 30 min, we measured the conductivity of the solution (E2) when it cooled to room temperature. We calculated the relative electrical conductivity (EC) of leaves (relative electrical conductivity = E1/E2 × 100%).

4.8. Safranin Staining

Transgenic tobacco stem samples were stained with Safranin O solution for 5 min after manual sectioning. After repeated decolorization with 95% ethanol, the result was observed under a light microscope.

4.9. Trypan Blue Staining

Leaf samples from 20-day-old tobacco seedlings of transgenic CkBI-1 gene, transgenic vector, and the WT group were immersed in trypan blue staining and boiled for 1 min. After 15 min of standing, we washed off the floating color with distilled water. The staining condition was observed under a microscope and analyzed using ImageJ.

4.10. Statistical Analysis

The data are presented as the mean values ± SDs (standard deviation). All the experimental data were analyzed with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). One-way ANOVA was used to compare the statistical difference in the mean among the plant lines under different treatments based on Duncan’s multiple range test (DMRT) at a significance level of $p < 0.05$. 
Supplementary Materials: Supplementary materials are available online at https://www.mdpi.com/article/10.3390/ijms31163606/s1.

Author Contributions: Conceptualization, C.G., T.Z. and F.L.; methodology, F.L. and L.X.; software, J.L.; validation, F.L.; formal analysis, F.L.; data curation, C.G.; writing—original draft preparation, P.Z.; writing—review and editing, J.L.; supervision, C.G.; project administration, C.G.; funding acquisition, C.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (No. 31770648 and 31070538), Key R&D Projects in Shaanxi Province (2020NY-190).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the article or Supplementary Materials.

Acknowledgments: We earnestly thank everyone in the Tea Plant Development and Stress Biology Laboratory of Northwest A&F University for their assistance. We are grateful to Beibei He, Minrong Luo and Fei Zhang (Horticulture Science Research Center, Northwest A&F University, Yangling, China) for their assistance with microscopy and other analyses.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Sternberg, T. Regional drought has a global impact. Nature 2011, 472, 169. [CrossRef]
2. Xu, E.; Fan, G.; Niu, S.; Zhao, Z.; Deng, M.; Dong, Y. Transcriptome-wide profiling and expression analysis of diploid and autotetraploid Paulownia tomentosa × Paulownia fortunei under drought stress. PLoS ONE 2014, 9, e113313. [CrossRef]
3. Daryanto, S.; Wang, L.; Jacinthe, P.A. Global synthesis of drought effects on cereal, legume, tuber and root crops production: A review. Agric. Water Manag. 2016, 179, 18-33. [CrossRef]
4. Brendan, C.; Brodribb, T.J.; Brodersen, C.R.; Duursma, R.A.; Rosana, L.; Medlyn, B.E. Triggers of tree mortality under drought. Nature 2018, 558, 531–539. [CrossRef]
5. Oladosu, Y.; Rafii, M.Y.; Samuel, C.; Fatai, A.; Magaji, U.; Kareem, I.; Kamarudin, Z.S.; Muhammad, I.; Kolapo, K. Drought resistance in rice from conventional to molecular breeding: A review. Int. J. Mol. Sci. 2019, 20, 3519. [CrossRef]
6. Bartel, D.P. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 2004, 116, 281–297. [CrossRef]
7. Ambros, V. The functions of animal microRNAs. Nature 2004, 431, 350–355. [CrossRef]
8. Zhang, B.; Pan, X.; Cobb, G.P.; Anderson, T.A. Plant microRNA: A small regulatory molecule with big impact. Dev. Biol. 2006, 289, 3–16. [CrossRef]
9. Shukla, L.I.; Chinnumasy, V.; Sunkar, R. The role of microRNAs and other endogenous small RNAs in plant stress responses. Biochim. Biophys. Acta 2008, 1779, 743–748. [CrossRef]
10. Sun, G. MicroRNAs and their diverse functions in plants. Plant Mol. Biol. 2012, 80, 17–36. [CrossRef]
11. Liu, Q.; Yan, S.; Yang, T.; Zhang, S.; Chen, Y.Q.; Liu, B. Small RNAs in regulating temperature stress response in plants. J. Integr. Plant Biol. 2017, 59, 774–791. [CrossRef] [PubMed]
12. Sunkar, R.; Kapoor, A.; Zhu, J.K. Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. Plant Cell 2006, 18, 2051–2065. [CrossRef] [PubMed]
13. Sunkar, R.; Zhu, J.K. Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. Plant Cell 2004, 16, 2001–2019. [CrossRef]
14. Zhao, B.; Liang, R.; Ge, L.; Li, W.; Xiao, H.; Lin, H.; Ruan, K.; Jin, Y. Identification of drought-induced microRNAs in rice. Biochem. Biophys. Res. Commun. 2007, 354, 585–590. [CrossRef]
15. Zhou, Y.; Liu, W.; Li, X.; Sun, D.; Xu, K.; Feng, C.; Kue Foka, I.C.; Kethehouli, T.; Gao, H.; Wang, N.; et al. Integration of sRNA, degradome, transcriptome analysis and functional investigation reveals gma-miR398c negatively regulates drought tolerance via GmCSDs and GmCCS in transgenic Arabidopsis and soybean. BMC Plant Biol. 2020, 20, 190. [CrossRef]
16. Fei, X.; Li, J.; Kong, L.; Hu, H.; Tian, J.; Liu, Y.; Wei, A. miRNAs and their target genes regulate the antioxidant system of Zanthoxylum bungeanum under drought stress. Plant Physiol. Biochem. 2020, 150, 196–203. [CrossRef]
17. Ning, P.; Zhou, Y.; Gao, L.; Sun, Z.; Zhou, W.; Liu, F.; Yao, Z.; Xie, L.; Wang, J.; Gong, C. Unraveling the microRNA of Caragana korshinskii along a precipitation gradient on the Loess Plateau, China, using high-throughput sequencing. PLoS ONE 2017, 12, e0172017. [CrossRef] [PubMed]
18. Arenas-Huertero, C.; Perez, B.; Rabanal, F.; Blanco-Melo, D.; De la Rosa, C.; Estrada-Navarrete, G.; Sanchez, F.; Covarrubias, A.A.; Reyes, J.L. Conserved and novel miRNAs in the legume Phaseolus vulgaris in response to stress. Plant Mol. Biol. 2009, 70, 385–401. [CrossRef]
19. De la Rosa, C.; Covarrubias, A.A.; Reyes, J.L. A dicistronic precursor encoding miR398 and the legume-specific miR2119 coregulates CSD1 and ADH1 mRNAs in response to water deficit. Plant Cell Environ. 2019, 42, 133–144. [CrossRef]

20. Yu, F.; Xie, Q. Non-26S Proteasome Endomembrane Trafficking Pathways in ABA Signaling. Trends Plant Sci. 2017, 22, 976–985. [CrossRef]

21. Rosquete, M.R.; Drakakaki, G. Plant TGN in the stress response: A compartmentalized overview. Curr. Opin. Plant Biol. 2018, 46, 122–129. [CrossRef]

22. Watanabe, N.; Lam, E. BAX inhibitor-1 modulates endoplasmic reticulum stress-mediated programmed cell death in Arabidopsis. J. Biol. Chem. 2008, 283, 3200–3210. [CrossRef]

23. Isbat, M.; Zeba, N.; Kim, S.R.; Hong, C.B. A BAX inhibitor-1 gene in Capsicum annum is induced under various abiotic stresses and endows multi-tolerance in transgenic tobacco. J. Plant Physiol. 2009, 166, 1685–1693. [CrossRef]

24. Jian, S.; Zhao, C.; Fang, S.; Yu, K. Soil water content and water balance simulation of Caragana korshinskii Kom. in the semiarid Chinese Loess Plateau. J. Hydrol. Hydromech. 2014, 62, 89–96. [CrossRef]

25. Yang, Q.; Yin, J.; Li, G.; Qi, L.; Yang, F.; Wang, R.; Li, G. Reference gene selection for qRT-PCR in Caragana korshinskii Kom. under different stress conditions. Mol. Biol. Rep. 2014, 41, 2325–2334. [CrossRef]

26. Tao, Q.; Yuanchun, Y.U.; Zhang, W.; Gao, H.; Chen, R.; Bai, L. Pelleting and germination of Caragana korshinskii and Caragana microphylla seeds. J. Anhui Agric. Univ. 2015, 42, 60–64. [CrossRef]

27. Fu, B.; Wang, S.; Liu, Y.; Liu, J.; Liang, W.; Miao, C. Hydrogeomorphologic ecosystem responses to natural and anthropogenic changes in the Loess Plateau of China. Annu. Rev. Earth Planet. Sci. 2016, 45, 223–243. [CrossRef]

28. Li, Y.J.; Zhao, Z.; Sun, D.X.; Han, G. Hydrological physiological characteristics of Caragana korshinskii under water stress. J. Northwest For. Univ. 2008, 3, 1–4.

29. Zhou, M. The Adaptation of Epidermal Micromorphology and Physiological Mechanism of Caragana Fabr. to Drought Stress; The College of Life Science of Lanzhou University: Lanzhou, China, 2016.

30. Chou, T.J.; Aung, K.; Lin, S.I.; Wu, C.C.; Chiang, S.F.; Su, C.L. Regulation of phosphate homeostasis by MicroRNA in Arabidopsis. Plant Cell 2006, 18, 412–421. [CrossRef]

31. Chen, L.; Luan, Y.; Zhai, J. Sp-miR396a-5p acts as a stress-responsive genes regulator by conferring tolerance to abiotic stresses and susceptibility to Phytophthora nicotianae infection in transgenic tobacco. Plant Cell Rep. 2015, 34, 2013–2025. [CrossRef]

32. Watanabe, N.; Lam, E. Bax Inhibitor-1, a conserved cell death suppressor, is a key molecular switch downstream from a variety of biotic and abiotic stress signals in plants. Int. J. Mol. Sci. 2009, 10, 3149–3167. [CrossRef] [PubMed]

33. Xu, Q.; Reed, J.C. Bax inhibitor-1, a mammalian apoptosis suppressor identified by functional screening in yeast. Mol. Cell 1998, 1, 337–346. [CrossRef]

34. Kawai-Yamada, M.; Jin, L.; Yoshinaga, K.; Hirata, A.; Uchimiya, H. Mammalian bax-induced plant cell death can be down-regulated by overexpression of Arabidopsis Bax Inhibitor-1 (AtBI-1). Proc. Natl. Acad. Sci. USA 2001, 98, 12295–12300. [CrossRef] [PubMed]

35. Bolduc, N.; Lamb, G.N.; Cessna, S.G.; Brisson, L.F. Modulation of Bax Inhibitor-1 and cytosolic Ca2+ by cytokinins in Nicotiana tabacum cells. Biochimie 2007, 89, 961–971. [CrossRef]

36. Tyree, M.T. The Cohesion-Tension theory of sap ascent: Current controversies. J. Exp. Bot. 2002, 48, 1753–1765. [CrossRef]

37. Greenberg, J.T. Programmed cell death: A way of life for plants. Proc. Natl. Acad. Sci. USA 1996, 93, 12094–12097. [CrossRef] [PubMed]

38. Fukuda, H.; Koizumi, K.; Motomatsu, K.; Motose, H.; Sugiyama, M. Molecular mechanisms of vascular pattern formation. Prog. Biotechnol. 2001, 18, 53–61.

39. Obara, K.; Kuriyama, H.; Fukuda, H. Direct evidence of active and rapid nuclear degradation triggered by vacuole rupture during programmed cell death in Zinnia. Plant Physiol. 2001, 125, 615–626. [CrossRef] [PubMed]

40. Sarkar, P.; Gladish, D.K. Hypoxic stress triggers a programmed cell death pathway to induce vascular cavity formation in Pisum sativum roots. Physiol. Plant. 2012, 146, 413–426. [CrossRef]

41. Liu, F.; Xie, L.; Yao, Z.; Zhou, Y.; Gong, C. Caragana korshinskii phenylalanine ammonialyase is up-regulated in the phenylpropanoid biosynthesis pathway in response to drought stress. Biotechnol. Biotechnol. Equip. 2019, 33, 842–854. [CrossRef]

42. Sablok, G.; Pérez-Quintero, A.L.; Hassan, M.; Tatarinova, T.V.; López, C. Artificial microRNAs (amiRNAs) engineering—On how microRNA-based silencing methods have affected current plant silencing research. Biochem. Biophys. Res. Commun. 2011, 406, 315–319. [CrossRef] [PubMed]

43. Chomczynski, P.; Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 1987, 162, 156–159. [CrossRef]