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Role of cin-miR2118 in drought stress responses in *Caragana intermedia* and *Tobacco*

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A B S T R A C T

The miR2118 is highly conserved in leguminous plants. Its function is to regulate the expression of genes encoding the TIR-NBS-LRR resistance protein. In this study, cin-miR2118 from *Caragana intermedia* was functionally characterized, especially with regard to its role in drought stress resistance. Two target genes of cin-miR2118 were predicted and cloned, the occurrence of miR2118 target sequence in both genes indicated that they might be targets of cin-miR2118. We investigated the expression patterns of cin-miR2118 and its target genes in *C. intermedia* stems and found diverse changes in expression in response to drought stress. *CiDR1* was negatively correlated with corresponding miR2118 expression while *CiDR2* was positively correlated with cin-miR2118. For further study, induced tolerance was observed in the transgenic *Tobacco* with overexpression cin-miR2118 upon 140-min water deficiency. And the expression level of cin-miR2118 was dramatically increased under drought stress. These results reveal that cin-miR2118 exert positive effects on drought stress tolerance. In addition, our study unexpectedly found that overexpression of cin-miR2118 in *Tobacco* can cause phenotype changes, which suggested that cin-miR2118 may have a novel function as a growth regulator in *Tobacco*.

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1. Introduction

MicroRNAs (miRNAs) are ~21 nt endogenous RNAs that are highly conserved among higher plants (Chen and Rajewsky, 2007; Jones-Rhoades et al., 2006). They use base-pairing interactions to regulate mRNA expression (Bushati and Cohen, 2007) by cleaving the targeted transcript, or translational inhibition following imperfect pairing. Although both mechanisms have been observed in plants, cleavage is the more common (Arenas-Huertero et al., 2009; Brodersen et al., 2008). Numerous recent studies have shown that miRNAs and their targets play important roles in multiple biological processes, such as plant growth (Guo, 2005), organ development (Emery et al., 1768–1774; Kim et al., 2005), signal transduction (Wang et al., 2004), pathogen infection (Prokhnevsky and Chapman, 2004), and stress responses (Sunkar, 2001–2019). With the rapid development of molecular biology techniques, many novel miRNAs have been identified, although little is known about their functions or target genes (Sunkar, 2001–2019; He and Hannon, 2004).

Some miRNAs have been confirmed experimentally to be involved in a variety of abiotic stress responses, encompassing drought, salinity, extreme temperatures, nutrition stress, ultraviolet (UV)-B radiation, and oxidative stress. miR398 was the first miRNA reported to respond to abiotic stress. miR398-directed mRNA cleavage of target genes *CSD1* and *CSD2* fine-tunes their expression under oxidative stresses (Sunkar, 2006), while over-expressing osa-miR396c in rice and *Arabidopsis* reduced salt and alkali stress tolerance (Gao et al., 2010). Other studies found that miR399 is strongly induced by low phosphate stress, and that it controls phosphate homeostasis by regulating the expression of a putative ubiquitin-conjugating E2 enzyme, UBC24, in *Arabidopsis* (Fujii et al., 2005). miR169g and miR169n, which both target *NF-YA*, exhibited overlapping and distinct responses to drought and salt stresses (Zhao et al., 2009). Additionally, Zhou identified that 21 miRNA genes in 11 miRNA families are up-regulated under UV-B stress conditions in *Arabidopsis* (Zhou et al., 2007). Taken together, these data suggest that abiotic stresses have an important influence on miRNA expression.

Drought is one of the most common environmental stresses that threaten agroforestry and result in deterioration of the environment. Given the fact that drought and salinity are becoming particularly widespread in many regions, it has been predicted that more than 50% of all arable land will be affected by serious salinization by the year 2050 (Wang et al., 2003; Boyer, 1982). Therefore, more effective xerophytic breeding strategies are urgently required to improve plant survival under extremely harsh environments. Novel drought-related miRNAs have been identified using novel methodologies such as high-throughput sequencing and oligonucleotide microarray in *Arabidopsis* and other model plants such as *Oryza sativa*, *Populus euphratica*, and

Abbreviations: C. intermedia, *Caragana intermedia*; miRNA, microRNA; NCBI, National Center for Biotechnology Information; Pre, precursor; qRT-PCR, quantitative reverse transcription PCR; RLM-5′RACE, RNA ligase-mediated amplification of cDNA ends.

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Medicago truncatula. Thus, distinct responses to drought stresses were reported for miR393, miR397b, miR402, miR169a, and miR169c in *Arabidopsis* (Sunkar, 2001–2019; Li et al., 2008); miR170, miR172, miR397, miR408, miR529, and miR395 in *O. sativa* (Zhou et al., 2010); miR156a, miR162a, miR319a, miR396a, and miR166n in *Populus* (Li et al., 2011); and miR2118, miR399, miR164, miR169, miR171, miR396 in *M. truncatula* (Wang et al., 2011a).

Previous studies have demonstrated that miR2118, which guides the mRNA cleavage of Toll interleukin-1 receptor–nucleotide-binding site–leucine-rich repeat (TIR–NBS–LRR) resistance genes, is closely related to drought response in legumes (Arenas-Huertero et al., 2009; Wang et al., 2011a; Kulcheski et al., 2011). For example, Arenas-Huertero et al. reported that pvu-miR2118 was responsive to drought and salinity in the common bean (Arenas-Huertero et al., 2009); Wang et al. found that miR2118 was up-regulated by drought stress in *M. truncatula* (Wang et al., 2011a); and Luo et al. identified that miR2118, which targets four *TG01* genes, was strongly induced by drought stress (Luo, na). *Caragana intermedia*, of the Leguminosae family, is a deciduous, perennial shrub that is widespread in the sandy grassland and desert regions of northwest China and Mongolia (Wang et al., 2011b; Xu et al., 2007). It has important ecological and economic value in this area, including drought resistance, sand-fixation, water and soil conservation, as well as a nutritious pasture (Li et al., 2014; Xu et al., 2012). Because of its strong adaptability and stress tolerance, *C. intermedia* is an ideal candidate plant for studying the mechanisms of drought tolerance and creating drought-resistant forests in China. To date, however, only a few miRNAs have been identified in *C. intermedia*. 142 miRNAs were identified and 38 miRNA targets were predicted, 4 of which were validated in *C. intermedia*. Furthermore, the expression of 12 miRNAs was detected by qRT-PCR under salt stress, seven of which were upregulated, while one miRNA was downregulated (Zhu et al., 2013a).

In this study, we predicted two potential target genes of miR2118 from the *C. intermedia* transcriptome and cloned them. We also characterized the expression patterns of miR2118 and its target genes under drought stress in various tissues of *C. intermedia*. To further this analysis, we generated transgenic *Tobacco* plants constitutively overexpressing cin-miR2118, and found that they showed enhanced drought stress tolerance. The results of this study contribute to a better understanding of the role of miR2118 and its target genes in drought stress responses.

2. Materials and methods

2.1. Plant materials and drought stress treatment

C. intermedia seeds were washed three times in distilled water, soaked in distilled water for 6 h, then germinated in pots filled with sterilized soil in a growth chamber at 23 °C with a 16 h light/8 h dark photoperiod and relative humidity of 80%. Three-week-old seedlings were subjected to drought treatment by adding 15% polyethylene glycol (PEG) 6000 to 1/2 Hoagland's solution (Table S1) to mimic drought stress, then were sampled at 0, 2, 4, 8, 12, 24 and 48 h after treatment. The roots, stems, and leaves from 3-week-old seedlings and drought-stressed plants were harvested separately and stored at −80 °C for RNA isolation. Samples for tissue-specific expression analysis were collected from three different seedlings as three biological replicates.

Transgenic *Tobacco* drought tolerance assays were performed on the leaves of wild-type and transgenic seedlings of *Nicotiana benthamiana*. Seedlings were germinated on MS medium (Table S1) for 2 months, then the leaves were detached, placed at room temperature, and weighed on an electronic balance (Sartorius, Gottingen, Germany) every 20 min. Drought tolerance experiments were conducted in triplicate. The numerical data were subjected to statistical analysis using Excel 2010.

2.2. Full-length cDNA and sequence analysis

We obtained sequence data for mature miR2118 and pre-miR2118 from the small RNA library of *C. intermedia* (unpublished results). Mature miR2118 target sequence prediction was performed with the online psRNATarget server using relatively strict rules (http://plantgrn.noble.org/psRNATarget/) including a maximum expectation value of 3. Total RNA was prepared from *C. intermedia* using the RNAprep Pure Plant Kit (Tiangen Biotech, Beijing, China). First-strand cDNA was synthesized from total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA). Rapid amplification of cDNA ends (RACE) was carried out to obtain 3′ and 5′ end cDNA sequences using the SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA). RACE primers are described in Table S2. The open reading frame (ORF) was implemented using the NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Domain searching was performed with the conserved domain database program (NCBI), and multiple alignments were obtained using Clustal X. Other bioinformatics analyses of this study, including the theoretical isoelectric point (pI), were predicted by the ExPASY bioinformatics resource portal (http://web.expasy.org).

2.3. Quantification of gene expression (quantitative real-time PCR, qRT-PCR)

RNA and total miRNA were extracted from the treated or untreated leaves, stems, and roots of 3-week-old plants. Total RNA isolation and first-strand cDNA synthesis were performed as described above. Total miRNA was extracted using an miRcute miRNA Isolation Kit (Tiangen Biotech), and first-strand cDNA synthesis of miRNA was conducted using an miRcute miRNA first-strand cDNA synthesis Kit (Tiangen Biotech).

Transcript levels of the samples were detected by the CFX96™ real-time polymerase chain reaction (PCR) system (BioRad, Hercules, CA). The miRcute miRNA qPCR Detection Kit (SYBR Green) (Tiangen Biotech) was used for miRNA qRT-PCR with a forward primer designed according to the mature miR2118 sequence, and the reverse primer provided by the kit. *C. intermedia* 5.8S ribosomal RNA was selected as a reference gene (Table S2).

Table S2 shows the *CiDR1* and *CiDR2* qRT-PCR primers for mRNA analysis. Expression levels of all target genes were normalized to that of *UNK2* (Zhu et al., 2013b). SYBR Premix Ex Taq™ (Takara, Dalian, China) was used in the real-time PCR of target genes, and the set-up was according to the manufacturer's protocol. All reactions were repeated three times for each sample. Statistical analysis and sample comparisons were conducted using the $2^{-\triangle\triangle}$Ct method.

2.4. Plasmid constructs and the generation of transgenic Tobacco plants

The overexpression construct was made by inserting a 256 bp pre-miR2118 fragment into the plant vector pSuper1300+ (Huayueyang, Beijing, China). The pre-miR2118 sequence including the fold-back structure was amplified from *C. intermedia* genomic DNA using the gene-specific primers cin-QTF and cin-QTR (Table S2). The plasmid containing the cDNA was double digested with *Xba* I and *Kpn* I, then the excised fragment was transfered into Trans-T1 competence-cell. The resultant reconstructed plasmid was introduced into the *Agrobacterium* strain GV3101 carrying the 35S promoter, then named 35S::pre-MIR2118. Transformation was achieved using an *Agrobacterium*-mediated method as previously described (Dayan et al., 2010).

2.5. Anatomical observation

For histological analysis, fresh stems from the same phase of 2-month-old wild-type and transgenic tobacco plants were embedded in 3% agar. Approximately 20-μm-thick free-hand sections were cut

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