Published February 14, 2019

Temporal Small RNA Expression Profiling under Drought Reveals a Potential Regulatory Role of Small Nucleolar RNAs in the Drought Responses of Maize

Jun Zheng, Erliang Zeng, Yicong Du, Cheng He, Ying Hu, Zhenzhen Jiao, Kai Wang, Wenxue Li, Maria Ludens, Junjie Fu, Haiyan Wang, Frank F. White, Guoying Wang,* and Sanzhen Liu*

J. Zheng, Y. Du, Z. Jiao, K. Wang, W. Li, J. Fu, G. Wang, Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing 100081, P.R. China; E. Zeng, M. Ludens, Dep. of Biology, Univ. of South Dakota, Vermillion, SD, 57069; E. Zeng, Dep. of Computer Science, Univ. of South Dakota, Vermillion, SD, 57069; E. Zeng (current address), Division of Biostatistics and Computational Biology, College of Dentistry, Univ. of Iowa, Iowa City, IA 52242; C. He, S. Liu, Y. Hu, Dep. of Plant Pathology, Kansas State Univ., Manhattan, KS, 66506; Y. Hu (current address), Horticultural Sciences Dep., Univ. of Florida, Gainesville, FL 32611; H. Wang, Dep. of Statistics, Kansas State Univ., Manhattan, KS, 66506; F.F. White, Dep. of Plant Pathology, Univ. of Florida, Gainesville, FL, 32611.

ABSTRACT Small RNAs (sRNAs) are short noncoding RNAs that play roles in many biological processes, including drought responses in plants. However, how the expression of sRNAs dynamically changes with the gradual imposition of drought stress in plants is largely unknown. We generated time-series sRNA sequence data from maize (*Zea mays* L.) seedlings under drought stress (DS) and under well-watered (WW) conditions at the same time points. Analyses of length, functional annotation, and abundance of 736,372 nonredundant sRNAs from both DS and WW data, as well as genome copy numbers at the corresponding genomic regions, revealed distinct patterns of abundance and genome organization for different sRNA classes. The analysis identified 6646 sRNAs whose regulation was altered in response to drought stress. Among drought-responsive sRNAs, 1325 showed transient downregulation by the seventh day, coinciding with visible symptoms of drought stress. The profiles revealed drought-responsive microRNAs, as well as other sRNAs that originated from ribosomal RNAs (rRNAs), splicing small nuclear RNAs, and small nucleolar RNAs (snoRNA). Expression profiles of their sRNA derivers indicated that snoRNAs might play a regulatory role through regulating the stability of rRNAs and splicing small nuclear RNAs under drought condition.

Abbreviations: DAW, days after withholding water; DS, drought stress treatment; DWR, drought–water recovery; FDR, false discovery rate; GO, gene ontology; miRNA, microRNA; NR-sRNA, nonredundant sRNA; nt, nucleotides; RDR, RNA-dependent RNA polymerase; REC, leaf relative electrical conductivity; rRNA, ribosome RNA; rsRNA, small RNA derived from rRNA; RWC, leaf relative water content; siRNA, small interfering RNA; snoRNA, small nucleolar RNA; sno-sRNA, small RNA derived from snoRNA; SPL, Squamosa promoter binding protein-like; sRNA, small RNA; SWC, soil water content; ta-siRNA, *trans*-acting siRNA; tRNA, transfer RNA; tsRNA, small RNA derived from tRNA; WGCNA, Weighted Gene Correlation Network Analysis; WW, well-watered.

CORE IDEAS

- Thousands of drought-responsive small RNAs (sRNAs) were identified.
- More microRNAs and sRNAs from small nucleolar RNAs (snoRNAs) are downregulated under drought.
- Most 24-nt sRNAs are from low-repetitive intergenic regions closer to genes.
- The abundance of sRNAs from ribosome RNA increases under drought.
- Ribosome RNA might be destabilized by drought through reduced snoRNA activity.

Physiological responses to drought in plants are complex and regulated through the interplay of a network of genetic components. One component comprised drought-responsive sRNAs (Khraiwesh et al., 2012). Small RNAs are short noncoding RNAs, predominately 20 to 24 nt in length, which function as sequence-specific regulators in a wide variety of biological processes, including DNA methylation, RNA degradation, translation regulation, and histone modification (Khraiwesh et al., 2012; Axtell, 2013a). Plant sRNAs are

Citation: Zheng, J., E. Zeng, Y. Du, C. He, Y. Hu, Z. Jiao, K. Wang, W. Li, M. Ludens, J. Fu, H. Wang, F.F. White, G. Wang, and S. Liu. 2019. Temporal small RNA expression profiling under drought reveals a potential regulatory role of small nucleolar RNAs in the drought responses of maize. Plant Genome 12:180058. doi: 10.3835/plantgenome2018.08.0058

Received 24 Aug. 2018. Accepted 31 Oct. 2018.
*Corresponding authors (wangguoying@caas.cn, liu3zhen@ksu.edu).

typically categorized into two major groups, which are distinguished by the structure of the sRNA precursors. The first group consists of microRNAs (miRNAs), which are predominately 21 nt in length and processed from single-stranded precursor RNA that are transcribed by RNA polymerase II and contain a hairpin structure. The second group comprised small interfering RNAs (siRNAs) that are derived from DICER/DICER-like processing of double-stranded RNAs.

Micro-RNAs function in drought stress responses (Covarrubias and Reyes, 2010; Shuai et al., 2013) and are conceptually categorized into three functional categories: homeostasis, detoxification, and growth regulation (Zhu, 2002) and function largely through the destabilization of various transcription factors (Rhoades et al., 2002; Ding et al., 2013; Ferdous et al., 2015; Zhang, 2015). The function of miRNAs in the regulation of transcription factors places miRNAs at the hubs of the gene regulatory networks for drought responses. Whereas miRNAs primarily act in the post-transcriptional regulation of gene expression, siRNAs regulate gene transcription through both guiding DNA methylation by the pathway of RNA-directed DNA methylation and post-transcriptional destabilization of transcripts in a sequence-specific manner (Onodera et al., 2005; Wierzbicki et al., 2008). Small interfering RNAs can be further subgrouped into heterochromatic siRNAs, secondary siRNAs, and natural antisense transcript siRNAs. Heterochromatic siRNAs are typically 23 to 24 nt in length and require RNA-dependent RNA polymerase (RDR) and RNA polymerase IV for biogenesis. Heterochromatic siRNAs have been documented to be derived from transposable or repetitive elements located at heterochromatic regions of nuclear DNA (Meyers et al., 2008; Nobuta et al., 2008). Secondary siRNAs include *trans*-acting siRNAs (ta-siRNAs), which are formed through cleavage of capped and polyadenylated siRNA transcripts by specific miRNAs, followed by conversion into double-stranded RNAs by RDR (Vazquez et al., 2010). Natural antisense transcript siRNAs are derived from double-stranded RNAs formed by annealing of natural sense and antisense transcripts from the same or separate nearly identical genomic regions (Vazquez et al., 2010).

Small RNAs can also originate from ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nucleolar RNAs (snoRNAs), and small nuclear RNAs associated with mRNA splicing (splicing snRNAs), which are referred to as rsRNAs, tsRNAs, sno-sRNAs, and splicing sn-sRNAs respectively hereafter (Vazquez et al., 2010). The rsRNAs, tsRNAs, sno-sRNAs, and splicing sn-sRNAs play regulatory roles in cellular processes (Morris and Mattick, 2014). In barley (*Hordeum vulgare* L.), tsRNAs and sno-sRNAs tended to be upregulated and downregulated, respectively, under drought conditions (Hackenberg et al., 2015). In maize, miRNA biosynthesis and regulation under drought stress has been explored (Li et al., 2013; Liu et al., 2014; Wang et al., 2014); however, the regulatory functions of sRNAs other than miRNAs are largely unknown.

To understand the function and regulation of sRNA in the drought response of maize, we sequenced sRNAs from maize seedlings over a period of 3 to 11 d after withholding water, along with sRNAs from well-watered plants or drought-treated plants that recovered after watering. The sRNAs were categorized with respect to length and functional classification and the genomic organization of sRNAs analyzed. An attempt was made to classify drought-responsive sRNAs through the use of cluster and network analyses of the time-series expression patterns. Examining sRNAs other than miRNAs identified increased degradation of ribosome RNA and splicing small nuclear RNAs under drought condition as well as a possible mechanism for their destabilization.

MATERIALS AND METHODS

Plant Materials and Drought Treatments

Seeds of the maize inbred line 'B73' were surface-sterilized and germinated on a wet rolled brown paper towel at 28°C for 48 h. Eighteen germinated seeds were selected and transplanted into a plastic pot (17 by 12 by 10cm) filled with nutrient soil (1:1 peat moss and vermiculite). After germination, 3-d-old seedlings were subjected to drought stress up to 10 d by withholding water (10 d after withholding water [DAW]); the control plants were well-watered. The plants were grown under controlled conditions (27:23°C day/night, a 16-h photoperiod from 6:00 AM to 10:00 PM, 300μmol m^{-2} s^{-1} photons, 30–50% relative humidity). The treatment (DS) and the control pots were randomly set out in the growth chamber. Eighteen seedlings were planted per pot. For every harvest and sample time, five pots were used for a drought treatment and another five pots were used as a control. At 10 DAW, drought-treated seedling plants were divided into two groups: a group of seedlings kept under DS without watering and the other group of seedlings that were rewatered. In summary, 36 samples of soils and leaf tissues were collected: (i) 32 samples resulting from two treatments (DS and WW) × 8 d (from Day 3 to Day 10) × 2 biological replicates; (ii) four samples resulting from two treatments (DS and WW at Day 11 for plants previously subjected to 10 d of DS) × 2 biological replicates.

Measurement of Soil Water Content, Leaf Relative Water Content, and Leaf Relative Electrical Conductivity

Soil samples and leaf tissues for measuring SWC (soil water content), RWC (relative water content), and REC (relative electrical conductivity) were collected daily at around 9:30 AM. Five independent replicates were performed for the SWC measurement, and five biological replicates were performed for the RWC and REC measurements. Soil water content, RWC, and REC were performed according to the method described previously (Zheng et al., 2010). Briefly, the soil SWC was the percentage of the weight loss of soils after drying. The RWC of the fresh leaves was calculated using the formula (FW – DW)/(TW – DW) × 100%, where FW is

the weight of fresh leaves, TW is the leaf weight after saturated in water for 8 h, and DW is the leaf dry weight. Relative electrical conductivity was calculated as Ec1/Ec2 × 100%, where Ec1 is the electrical conductivity of fresh leaves after saturation in water for 3 h and Ec2 is the electrical conductivity of the same leaf samples after they had been boiled in a water bath.

The sRNA Sequencing Experiment

The aboveground tissues of five seedlings of each treatment at each day were collected at approximately 10:00 AM each day and immediately frozen in liquid N. Total RNA was isolated from harvested samples with the TRIzol reagent (Invitrogen, Waltham, MA). A standard Illumina sRNA library preparation kit was used to prepare sRNA sequencing libraries from the total RNA. Briefly, 2 μg of sRNAs in the size range of 15 to 30 nucleotides (nt) were purified and ligated to the 3′ adaptor and isolated by 15% denaturing polyacrylamide gel electrophoresis gels to eliminate unligated 3′ adaptors. The products were ligated to 5′ adaptor and were then used to conduct reverse transcription polymerase chain reaction. The final polymerase chain reaction product was isolated by 3.5% agarose gel electrophoresis and served as a sRNA library for the sequencing. The libraries were quantified and sequenced on the HiSeq2000 analyzer (Illumina, San Diego, CA) to produce single-end 50-bp reads. Two biological replicates were used in the sRNA sequencing experiment.

The sRNA Data Process

Trimmomatic version 0.32 (Bolger et al., 2014) was used to trim the adaptor sequence of the sRNA reads. The parameters used for the trimming were: "ILLUMINACLIP:adaptor_seq:2:30:7: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:13 MINLEN:16". The adaptor sequence (adaptor_seq) includes a sequence of "CTGTAGGCACCATCAATCAGATCGGAAGAGCACACGTCTGAACTCCAGTCAC". These parameters were used to perform both adaptor and quality trimming. Although quality trimming could shorten the actual sRNAs, the percentage of reads subjected to quality trimming was only ∼0.3%. Therefore, quality trimming was applied to remove the low-quality nucleotides at the marginal compromise of changing sRNA lengths. At least 16 nt in size was required for clean reads.

A nonredundant sRNA (NR-sRNA) set was obtained by pooling sRNAs from all the samples and removing the redundancy. To remove most of the sRNA sequences that carried sequence errors, only sRNAs that were shown in at least two different samples and at least twice in each sample were included in the unique sRNA set. After determining the read counts of each sRNA from all 36 samples, a further reduction was performed to keep only sRNAs with at least 72 reads summed from all the samples, equivalent to 0.08 reads per million of total reads, resulting in a NR-sRNA set.

Functional Annotation of sRNAs

The sRNA annotation database was downloaded from Rfam version 11.0 (Burge et al., 2013). The sRNAs generated from this experiment were aligned to the Rfam version 11.0 database with Blastn (BLAST version 2.2.29+) with the following parameters (-evalue 1e-1 -word_size 10 -perc_identity 0.89 -strand plus -best_hit_overhang 0.2 -best_hit_score_edge 0.1 -outfmt 6 -max_target_seqs 10). The sRNAs were functionally annotated only if they were unambiguously aligned to sequences from an Rfam family.

Alignment to the Reference Genome to Determine the Copy Number of sRNA Regions

Each sRNA was aligned to the B73 reference genome (RefGen versions 2 and 4; Schnable et al., 2009) via Burrows–Wheeler alignment (version 0.7.5a-r405) (Li and Durbin, 2010). The command parameters were "bwa aln -l 18 -k 0 -t 48 -R 22500" followed by "bwa samse -n 22500". The alignments were then parsed with the stringent criterion of a perfect match with at least 18 bp matching length. These alignment and parsing criteria allowed the maximum of 22,500 perfect hits.

K-mer Analysis with Sequencing Data to Determine the Copy Number of sRNA Genomic Regions

B73 whole-genome shotgun Illumina sequencing data were downloaded from Genbank (SRR444422) (www.ncbi.nlm.nih.gov/sra, accessed 21 Jan. 2019). Trimmomatic version 0.32 (Bolger et al., 2014) was used for the adaptor and quality trimming with the same parameters as those used for sRNA data trimming. The adaptor sequences used for the adaptor trimming were TACACTCTTTCCCTACACGACGCTCTTCCGATCT and GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT. The clean data were subjected to error correction via the error correction module (ErrorCorrectReads.pl) in ALLPATHS-LG (Butler et al., 2008) with the parameters of "PHRED_ENCODING=33 PLOIDY=1". We then used the corrected sequencing data to perform k-mer counting by using the count function in JELLYFISH (Marçais and Kingsford, 2011) with the parameters of "-m k-mer -L 2 -s 100M -C", where the k-mer was from 18 to 30 nt. Once the read depth of each k-mer from 18 to 30 nt was counted, the read depth of a corresponding sRNA could be determined. The highest density of k-mer counts was located at 26.96 for a set of known single copy k-mers determined by reference genome alignments, indicating that approximately 26.96× sequencing depth was obtained. This number was used as the base of read depths of a single copy to adjust the counts of each k-mer to roughly represent its genome copy number.

Identification of Drought-Responsive sRNAs

A generalized linear model was fitted for each sRNA to identify drought-responsive sRNAs. The response variable in the model was the read count of an sRNA, which was assumed to follow negative binomial distribution.

The model contained two factors, DAW and treatment, and their interactions. The DAW factor had eight factor levels (from 3 to 10) and the treatment had two factor levels (DS and WW). A deviance test of no interaction effect between DAW and treatment was conducted for each sRNA. The generalized linear model fit and test, assuming a negative binomial distribution for the read counts, were implemented in DESeq2 (Love et al., 2014). Small sRNAs with at least five reads on average per sample were used for the statistical test, resulting in a *p*-value from each sRNA. A false discovery rate (FDR) approach was applied to account for multiple comparisons (Benjamini and Hochberg, 1995). Significant sRNAs were determined with 5% FDR as the cutoff. The script was deposited at GitHub (https://github.com/liu3zhenlab/manuscripts, accessed 21 Jan. 2019).

Clustering of Drought-Responsive sRNAs

Drought-responsive sRNAs were subjected to clustering analysis via mclust (Fraley and Raftery, 2007). For each drought-responsive sRNA, the $\log_2$ of the ratio of the mean of DS expression (the normalized value) to the mean of WW expression (the normalized value) at a certain DAW was determined, which represents the $\log_2$ of the fold change in expression between DS and WW. Log_2 ratio values were then used for the clustering analysis. The script was deposited at GitHub (https://github.com/liu3zhenlab, accessed 11 Jan. 2019).

Identification of Significantly Differentially Expressed sRNAs between DS and Water Recovery

To test the null hypothesis that no difference in sRNA expression between two groups, DS and drought–water recovery (DWR), at 11 DAW, a generalized linear model for the read count of each sRNA implemented in the DESeq2 package (version 1.4.5) was used (Love et al., 2014). A FDR approach was used to account for multiple tests (Benjamini and Hochberg, 1995). A FDR of 5% was used as the cutoff for determining of differential expression.

Enrichment Analysis

The enrichment analyses were performed for determining if a certain type of category, such as a member of a sRNA functional family, was over-represented in a selected group of sRNAs. To account for the biases from read depths that may have influenced the selection of members in a certain group, the resampling method in the GOSeq enrichment test (Young et al., 2010) with the bias factor of read depth, namely total reads across all the samples of a certain sRNA, was applied to the enrichment analyses.

Analysis of sRNA Coexpression Networks

Drought-responsive sRNAs (FDR < 1%) were used to build coexpression sRNA networks in the Bioconductor package Weighted Gene Correlation Network Analysis version 1.51 (WGCNA) (Langfelder and Horvath, 2008). The WW sRNA network was built from the sRNA expression profiles in WW samples; the DS sRNA network was constructed from the sRNA expression profiles in DS samples. The package WGCNA used an appropriate soft-thresholding power to construct a weighted gene network. Modules of highly correlated sRNAs were identified via the topological overlap measure implemented in WGCNA. Module preservation analysis was also performed with WGCNA, with the DS network as a test and the WW network as a reference, and vice versa. An R script for the network analysis has been deposited at GitHub (https://github.com/liu3zhenlab, accessed 11 Jan. 2019).

Identification of miRNAs

The database of reference mature miRNAs was downloaded from miRBase version 22 (ftp://mirbase.org/pub/mirbase, accessed 11 Jan. 2019). The database is referred to as B73miRBase22. In total, 325 reference mature B73 maize miRNAs from 174 miRNA genes are in the B73miRBase22 database. Any sRNAs discovered in this study identical to these reference mature miRNAs were annotated as known mature miRNAs.

ShortStack (version 3.8.5) was used to identify a set of miRNAs de novo with the parameters (-dicermin 18 -dicermax 30 -mismatches 0 -mincov 0.5rpm), with B73Ref4 (version 4) as the reference genome (Axtell, 2013b). ShortStack identified novel miRNA loci that did not overlap with any known miRNA genes in the B73miRBase22 database. Any miRNAs from novel miRNA loci were referred to as novel miRNAs. Some miRNAs annotated from ShortStack were from known miRNA genes but with difference sequences from known mature miRNAs in the B73miRBase22 database. Combining both known mature miRNAs and all miRNAs newly discovered by ShortStack in our massive sRNA datasets, we updated the miRNA set, referred to as B73miRBase22plus.

Identification of IsomiRs

IsomiRs are variants of the reference miRNAs (Neilsen et al., 2012). An isomiR in this study is a 20-22-nt RNA identical to the plus-stranded sequence of a region of primary miRNAs and at least 12 nt overlapping with a miRNA in the B73miRBase22plus database.

Identification of ta-siRNAs

Small RNAs matching ta-siRNA downloaded from tasiRNAdb (http://bioinfo.jit.edu.cn/tasiRNADatabase/, accessed 21 Jan. 2019) were defined as known tasiRNAs. Furthermore, sequences of maize *trans*-acting siRNA 3 (*TAS3*) were retrieved from Dotto et al. (2014).

Degradome Analysis of Drought-Responsive miRNAs

Raw degradome reads were obtained from a previous maize miRNA study (Liu et al., 2014). After adaptor sequences and low-quality sequencing reads had been removed, clean reads were used to identify cleavage sites on the basis of the B73 cDNA sequences (5b+).

CleaveLand version 4.0 was implemented for degradome analysis with the default parameters (Addo-Quaye et al., 2009), which provides evidence for gene targeting by miRNAs or isomiRs.

Prediction of miRNA Targeted Genes and Gene Ontology Enrichment Analysis of Targeted Genes

psRNATarget (http://plantgrn.noble.org/psRNATarget/, accessed 11 Jan. 2019) was used to predict miRNA target genes (Dai and Zhao, 2011). Gene targets of miRNAs were predicted based on B73 AGPv3.22 annotated transcript sequences with an expectation value less than 1.5. Gene ontology (GO) enrichment of the predicted miRNA-targeting genes was analyzed with AgriGO (Tian et al., 2017).

Transposable Element Analysis of 24-nt Genomic Loci

Small RNA genomic clusters from the ShortStack result, which were predominantly 24-nt sRNAs, are referred to as 24-nt genomic loci. RepearMasker (version open-4.0.5) was used to identify sequences matching transposable elements in the maize transposon database. As a control, the "shuffle" module in the bedtools package was used to randomly select intervals simulating the number and sizes of genomic intervals of 24-nt loci.

Data Availability

The datasets supporting the conclusions of this article are included within the article and its supplemental materials. All sRNA sequencing raw data were deposited the Sequence Read Archive (accession number: SRP081275).

RESULTS

Physiological Changes of Seedlings under Drought Conditions

Maize seedlings were subjected to drought over a period of 9 d (Fig. 1A). Three-day-old B73 seedlings after germination were subjected to two treatments: DS and WW. Aboveground tissues (referred to here as leaves) were collected at 3 to10 DAW or with watering, with two biological replicates taken each day. At 10 DAW, some seedlings from the DS treatment group were subjected to two treatments: continuously withholding water (DS) and rewatering, both of which were sampled on Day 11. Two biological replicates were collected, resulting in two additional DS samples on Day 11 and two rewatering samples at 1 d after addition of water at Day 10. Thirty-six plant samples were processed. Compared with WW seedlings, DS-treated seedlings showed a severe stressed phenotype by 8 DAW. Soil water content decreased in the DS treatment from ~60 to 20% in the same period (Fig. 1B). The leaf RWC of DS seedlings also decreased under the drought treatment at a low declining rate from 3 to 7 DAW and a high rate after 7 DAW (Fig. 1C). Leaf REC, which is a measure of cellular damage, exhibited the strongest response to drought between 8 and 9 DAW (Fig. 1D), indicating that leaf cells began to experience damage after 8 DAW under drought conditions. The DS-treated seedlings showed visibly stressed phenotypes after 10 DAW. When rewatered at 10 DAW, the DS-treated plant seedlings had visibly recovered at 11 DAW.

Characterization of sRNAs

The 36 RNA samples were extracted for sRNA sequencing, resulting in more than 886.6 million 50-bp single-end reads, from 20.5 to 34.2 million reads per sample. On average, 97.5% of the reads were retained after adaptor and quality trimming of each sample (Supplemental Table S1). Most sRNAs were between 18 and 26 nt. The 24-nt sRNA length class was the largest, followed by the 21- and 22-nt sRNA classes (Fig. 2A). The same pattern of length distribution was observed across all the samples, indicating that the drought treatment did not alter the global pattern of sRNA lengths.

All sRNA reads from the 36 samples were merged and sRNAs with at least 72 reads were retained. Removing redundant reads with the same sequence for each sRNA resulted in a set of NR-sRNAs (N = 736,372). The NR-sRNAs set was annotated with the Rfam database (Rfam version 11.0) (Burge et al., 2013). Here, 12.4% (91,473) of the NR-sRNAs could be unambiguously annotated with regard to their function (see Methods). Among the Rfam-annotated NR-sRNAs, the rsRNAs, tsRNAs, and miRNAs were the most abundant, comprising 40, 27, and 7%, respectively (Fig. 2B). Compared with rsRNAs and tsRNAs, the miRNAs were distributed across a slightly narrower length range of 18 to 24 nt and had a peak length at 21 nt (Fig. 2C). In terms of the read abundance of each annotated group, rsRNAs had a more homogenous size distribution between 18 and 26 nt, whereas tsRNAs were mostly 24 to 26 nt in length (Fig. 2D). Of the 21-nt NR-sRNAs, 22% were miRNAs from approximately 65% of the total 21-nt sRNA reads (redundant sRNAs), indicating that some 21-nt miRNAs were highly expressed (Fig. 2C, D). Indeed, the single sRNA showing the highest abundance was a miR159, with 14.8 million reads.

Genome Organization of NR-sRNAs in B73

The copy number of individual NR-sRNAs in the B73 genome was estimated by both mapping reads to the B73 reference genome (reference-based) and by analyzing the sequences present in whole-genome shotgun sequence reads (see Methods). Among the NR-sRNAs (N = 705,920) that were not mapped to either chloroplast or mitochondrial DNA, 93.2% were perfectly mapped to either the B73 reference genome or the B73 whole-genome shotgun data. The absence of perfect matches for 6.8% of the NR-sRNAs was attributed to incomplete B73 genome assembly, contamination, sequencing errors, and/or RNA editing (Liang and Landweber, 2007; Schnable et al., 2009). Both estimations indicated that most NR-sRNAs were from low-copy genomic loci (one or two copies) except for NR-sRNAs from rRNAs and tRNAs (Supplemental Fig. S1). The

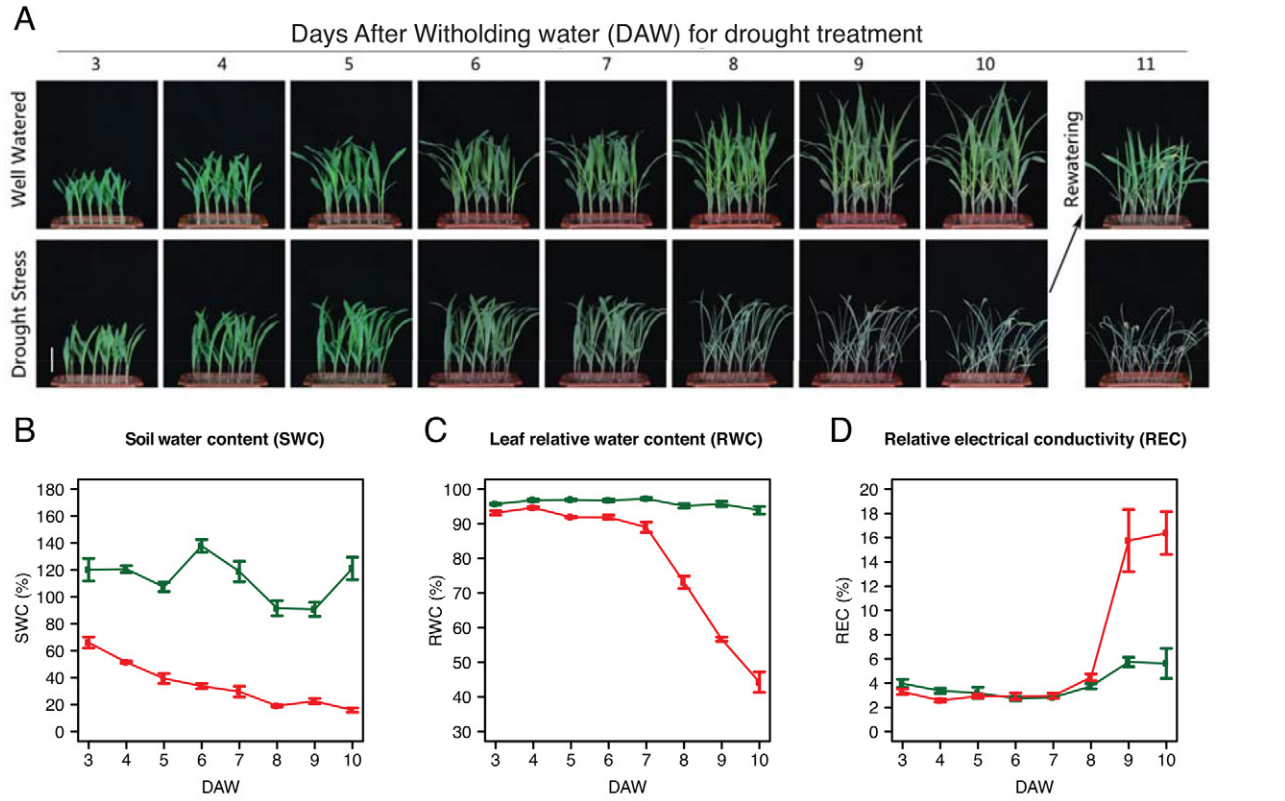

Fig. 1. Morphological and physiological changes of maize seedlings during drought stress. (A) Three-day-old B73 seedlings were subjected to gradual drought stress or well-watered conditions. The photos were taken each day from 3 to 11 d. Bar = 5 cm. (B) The changing curves of soil water content (SWC) from five replicated pots of each data point. (C) Leaf relative water content (RWC) of seedlings during the experiment. (D) Leaf relative electrical conductivity (REC) of seedlings during the experiment. Red and green curves represent plants under drought stress and well-watered plants , respectively. Five seedlings were pooled as one replicate; five independent biological replicates were conducted to determine RWC and REC. Vertical lines represent SEs.

NR-sRNAs of differing lengths exhibited varying mixtures of low- and high-copy loci (Supplemental Fig. S1). The 24-nt sRNAs were mostly single copies in the genome, but a high proportion of 21- to 23-nt sRNAs were derived from either low-copy or very-high-copy genomic loci. Outside the 21- to 24-nt sRNA range, NR-RNAs from highly repetitive genomic regions were dominant (Supplemental Fig. S2).

A linear association between the expression level and the genomic copy number of sRNAs was not observed (Supplemental Fig. S3). Genomic single-copy NR-sRNAs can be highly expressed. For example, the single-copy miR168 locus was expressed at a high level (138,292 reads). Conversely, the expression of most genomic high-copy NR-sRNAs was low. Some high-copy NR-sRNAs were highly expressed, such as rsRNAs. Analysis of sRNA expression profiles based on functional classes also showed that high proportions of splicing sn-sRNAs and sno-sRNAs exhibited low expression, whereas many rsRNAs were expressed at a high level (Fig. 2E). The 23- and 24-nt sRNAs, regardless of their functional classes, were mostly expressed at a low level, whereas 20- 22-nt sRNAs tended to be expressed at relatively higher levels (Fig. 2F). Compared to 21- to 24-nt sRNAs as a whole, a higher proportion of 20-nt sRNAs were highly expressed (Fig. 2F).

Identification of Drought-Responsive sRNAs

A statistical test was performed to detect any interaction between DS and WW plants for each sRNA that, on average, had a minimum five sRNA reads per sample over the 3- to 10-DAW period. The analysis revealed that 6646 of the 134,283 sRNAs exhibited interactions between the DAW and the treatments at the 5% FDR level (Supplemental Table S2, Supplemental Table S3). Interacting sRNAs showing different responses under DS and WW conditions at certain DAWs were scored as drought-responsive sRNAs. The rsRNAs and 22-nt sRNAs were the two predominant groups in the drought-responsive sRNA set (Supplemental Fig. S4). The DS-to-WW ratios of sRNA expression were further subjected to cluster analysis in mclust (Fraley and Raftery, 2007), resulting in 10 clusters. The sRNAs of Clusters 3, 4, 5, 7, and 9 exhibited a pattern of upregulation under drought stress (Fig. 3A–F), whereas sRNAs of lusters 1 and 8 showed a pattern for downregulation (Fig. 3G–I). Upregulated sRNAs (N = 4373) were detected five times more frequently than downregulated sRNAs (N = 816) under drought stress (Fig. 3). The enrichment analyses indicate that rsRNAs and splicing sn-sRNAs were over-represented in upregulated sRNAs, whereas miRNAs and sno-sRNAs were over-represented in downregulated sRNAs.

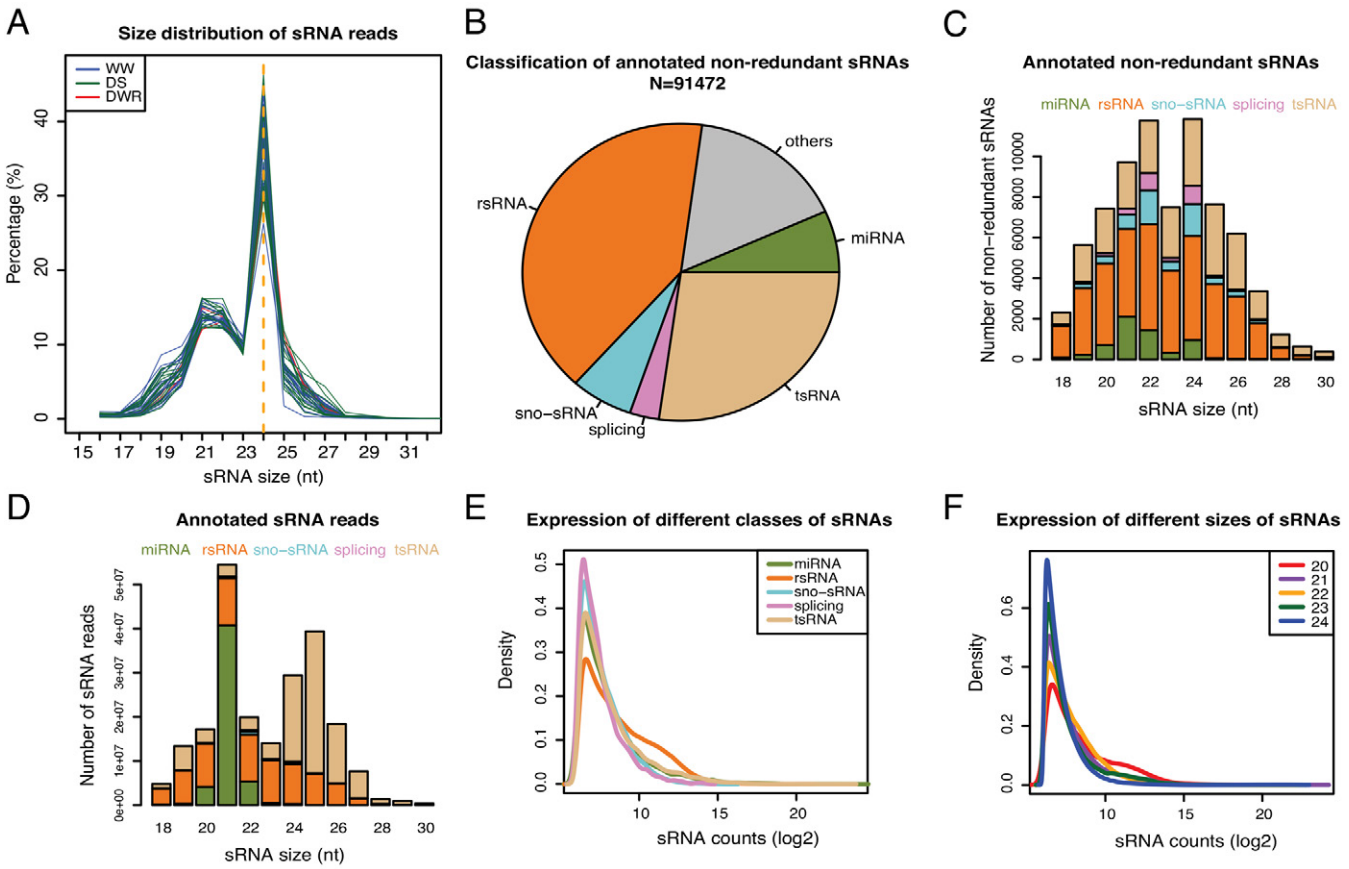

Fig. 2. Characterization of maize short RNAs (sRNAs). (A) Proportions of sRNAs of different lengths in all samples. Each curve represents a sample. WW, well-watered; DS, drought stress; DWR, drought–water-recovered plants. (B–D) Overview of genomic copy number, lengths, functional categories, and expression of nonredundant sRNAs (NR-sRNAs) from all the samples. (B) Pie chart of distribution of different classes of sRNAs. 'Others' represents sRNAs that were not unambiguously categorized. (C) Stacked barplot of different functional classes of NR-sRNAs at varying sizes of sRNAs from 18 to 30 nt. (D) Stacked barplot of different functional classes of sRNA reads, representing expression levels, at varying lengths of sRNAs from 18 to 30 nt. (E) Density plots of the expression levels of different functional classes of sRNAs. Density on the *y* axis represents the probability of sRNA occurrences. (F) Density plots of expression levels of different sRNA lengths. Density on the *y*-axis represents the probability of sRNA occurrences.

Additionally, sRNAs of Clusters 2 and 6 exhibited transient downregulation under drought (the transiently downregulated group, $N = 1325$), which were downregulated at around 7 DAW when drought stress became intense, followed by a gradual recovery of expression (Fig. 3J–L). The enrichment analysis indicated that miRNAs and sno-sRNAs were significantly over-represented in transiently downregulated sRNAs.

A comparison of sRNA expression was performed between two additional seedling groups at 11 DAW: DS and DWR, which was rewatered on 10 DAW. With the 5% FDR cutoff, 7140 sRNAs were differentially expressed between the two groups, 2264 and 4876 sRNAs of which were upregulated and downregulated in DWR relative to DS, respectively; 486 were identified as drought-responsive sRNAs in the time-series analysis (Supplemental Table S3, Supplemental Table S4). The 473 sRNAs (out of 486) were classified into three groups in the time-series analysis: Downregulated ($N = 43$), upregulated ($N = 426$), and transiently downregulated ($N = 4$). All 43 sRNAs from the downregulated group were upregulated after DWR. Of 426 sRNAs in the up-regulated group, 76.3% (325/426) sRNAs showed decreased expression in DWR, while 23.7% (101 out of 426) were continuously upregulated even with water recovery. All four sRNAs in the transiently downregulated response group were upregulated after rewatering. Overall, the expression levels of most drought-responsive sRNAs were restored toward the levels of WW plants on rewatering.

Characteristics of the Coexpression Networks of Drought-Responsive sRNAs

Drought-stressed and well-watered weighted coexpression networks were constructed with WGCNA (Langfelder and Horvath, 2008). Both networks consisted of a subset of drought-responsive sRNAs with a FDR cutoff of less than 1% in the drought response statistical test. The DS and WW networks were built from normalized sRNA counts of DS and WW samples, respectively (Fig. 4A,B, Supplemental Table S2). The network statistics indicated intrinsic differences between the two networks (Supplemental Table S5). Although the DS and WW networks

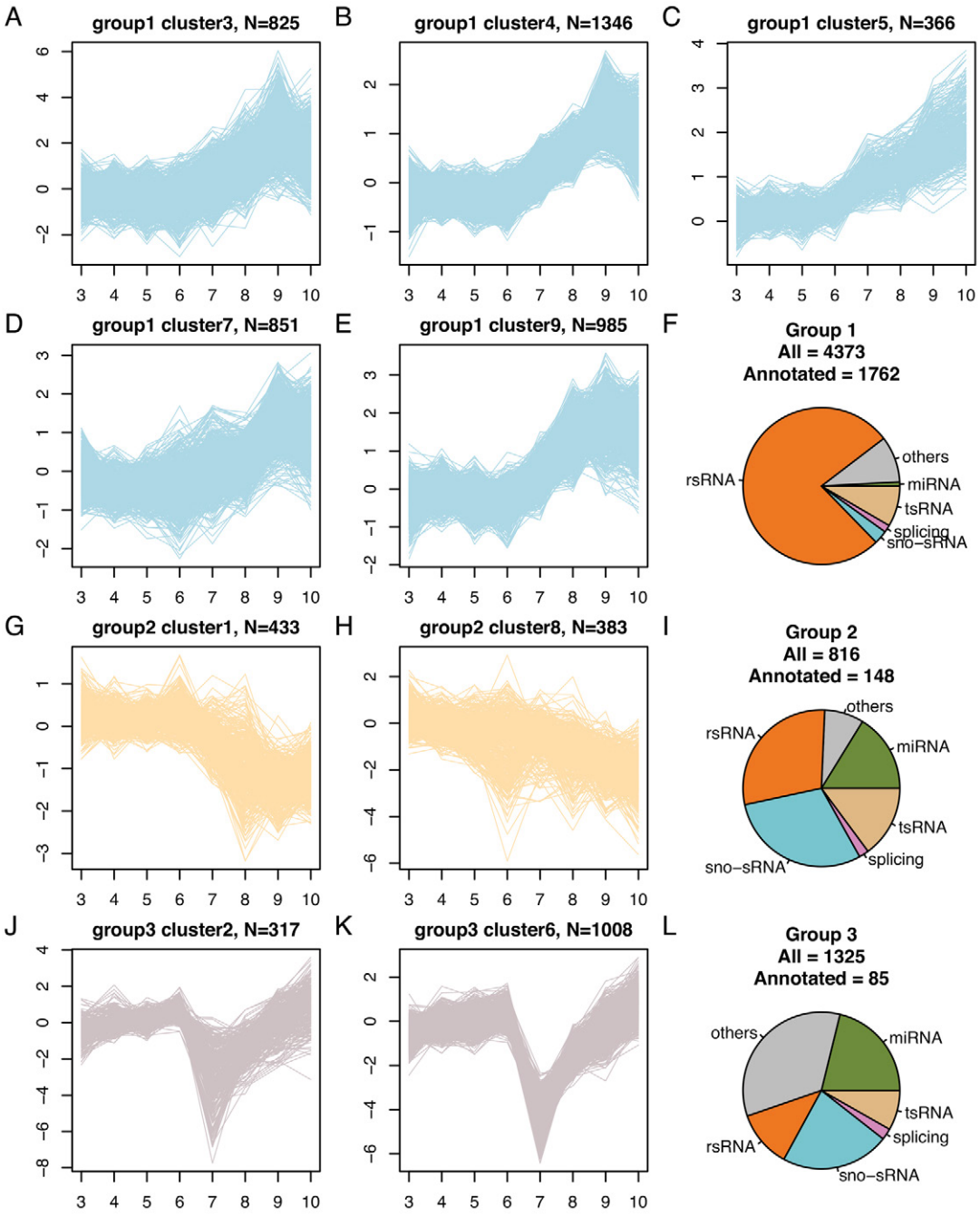

Fig. 3. Major clusters of drought-responsive maize short RNAs (sRNAs). Drought-responsive sRNAs were subjected to clustering with the software mclust package, which produced 10 clusters. Nine major clusters (A–E, G, H, J, K) were classified into three groups: upregulated (light blue), downregulated (light orange), and transiently downregulated (light purple). Each curve represents the average sRNA expression ratio of drought stress to well-watered with a $\log_2$ transformation from two biological replicates versus days after withholding water (DAW). The three pie charts show the proportions of different classes of sRNAs that were functionally annotated in each of the three clustering groups: upregulated (F), downregulated (I), and transiently downregulated (L).

shared similar network clustering coefficients, network centralizations, and network densities, the DS network (Fig. 4B) had the smaller network diameter and lower heterogeneity, indicating that the expression of these drought-responsive sRNAs was more correlated with drought stress or tended to be coexpressed in response to drought stress.

Modularity analysis in the DS network and the WW network further revealed that the two networks had different topology structures. Modularity analysis included two steps: module identification and module preservation analysis. Modules are subnetworks consisting of coexpressed sRNAs. The sRNAs in the same module were similar in expression to some degree and therefore were likely to be associated each other. Module preservation analysis was used to determine if the topology of a network module identified in one network changed in the other network. For example, a module

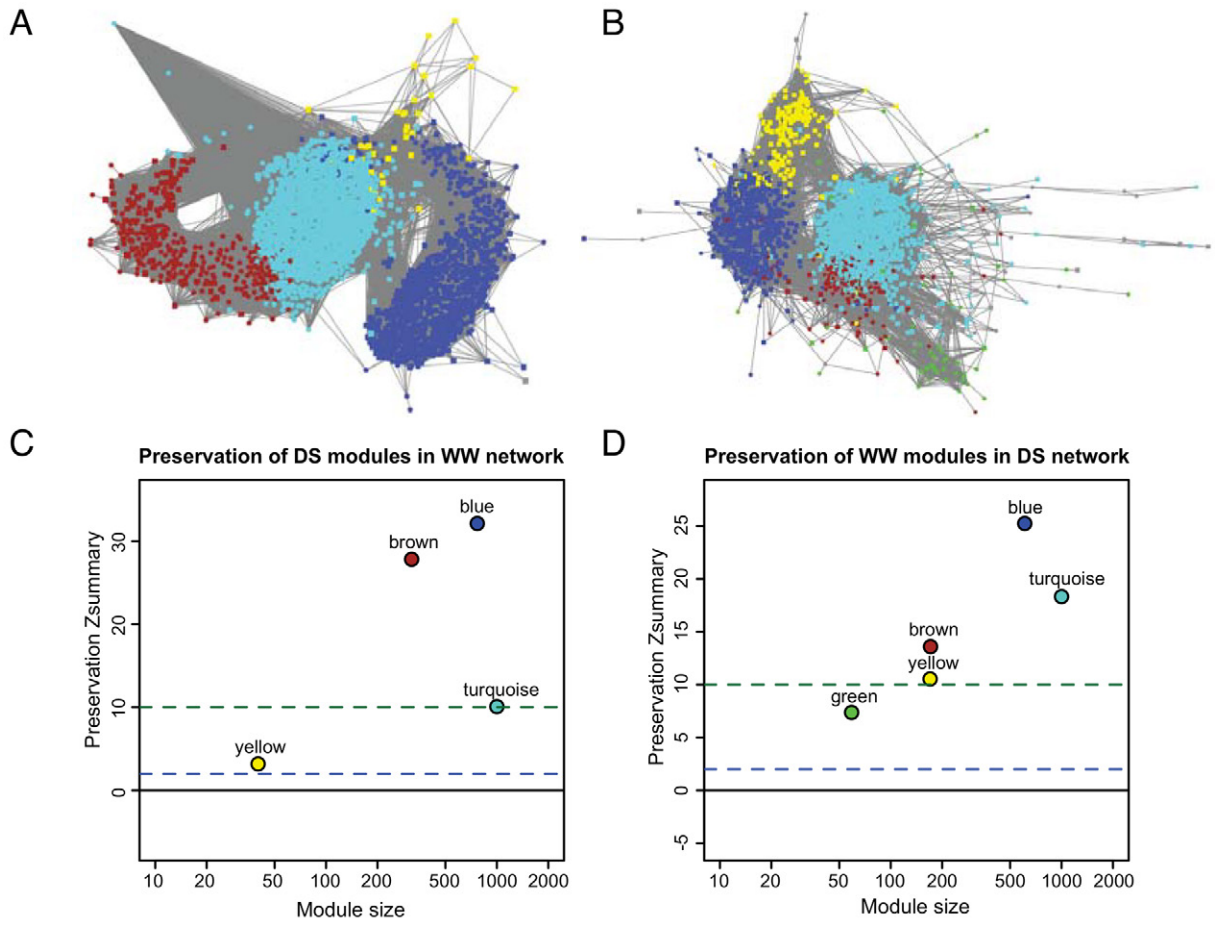

Fig. 4. Short RNA (sRNA) coexpression networks in maize. (A) Visualization of the drought stressed (DS) network from Cytoscape, where each node represents an sRNA and each line is the edge connecting the sRNA nodes. Five modules (subnetworks) are highlighted by different colors. (B) Visualization of the well-watered (WW) network. Six modules (subnetworks) are highlighted by different colors. Note that assignment of colors in (A) and (B) are mostly independent. The same color might not represent the same group of sRNAs. (C) Results of the module preservation analysis performed to evaluate whether a module identified in the DS network was preserved in the WW network. The color code is the same as that used in (A). (D) Results of the module preservation analysis of the WW network in comparison with the WW network. The color code corresponds to that used in (B).

was considered to be preserved in the DS network if its topology, based on the preservation statistics, largely remained in the WW network. The module preservation analysis identified a preserved module (blue module) in the DS network compared with the WW networks (Fig. 4C) and a preserved module (blue module) in the WW network in comparison with the DS networks (Fig. 4D). Most sRNAs ($N = 546$) in the two blue modules overlapped, more than 95% of which were from the transiently downregulated group (Supplemental Table S3). The result indicated that the transiently downregulated sRNAs tended to be coregulated in both the DS and WW conditions. On the other hand, these sRNAs exhibited a transient downregulation in response to drought, which might serve as the signal to induce downstream drought responses. Of 546 overlapping sRNAs, 343 and 178 are 22- and 24-nt sRNA, respectively, and a few were functional annotated with the Rfam database (six miRNAs and nine sno-sRNAs) (Burge et al., 2013). The module preservation analysis also revealed differences between modules in the DS and WW networks. The yellow module in the DS network was the least preserved module, indicating that sRNAs of the module were perturbed in response to drought stress (Fig. 4C). Indeed, the yellow module consisted of 38 sRNAs that were downregulated under drought stress. In the WW network, the green module was the least preserved one, and most sRNAs were upregulated in response to drought.

Identification of Drought-Responsive miRNAs and the Corresponding Targeted Genes

The sRNAs homologous to Rfam miRNAs were referred to as miRNAs. We refined the miRNA set on the basis of the dedicated miRNA database, miRBase (version 22) (Kozomara and Griffiths-Jones, 2014) and de novo discovery of miRNAs from our massive datasets. We used the ShortStack pipeline (Axtell, 2013b) and identified 53 miRNA loci, 47 of which loci are known maize miRNA genes in miRBase containing 174 miRNA genes. We found 59 new miRNAs, including 47 miRNAs from known miRNA loci but with different sequences of mature miRNAs, as well as 12 mature miRNAs from

six novel miRNA loci. When we required at least an 18-nt match with at least 90% identity, homologs of miRNAs from three novel miRNA loci (Cluster_23765, Cluster_27697, and Cluster_45700) were identified in MIR1878, MIR156c, and MIR166d, respectively. We combined both known and newly discovered mature miRNAs to create a new miRNA set, referred to as B73miRBase22plus (Supplemental Table S6), which contained 180 miRNA genes producing 392 mature miRNAs, 244 of which were nonredundant miRNAs (Supplemental Table S7). We also identified 510 isomiRs that were identical to a region of a primary miRNA sequence and overlapped with mature miRNAs of the B73miRBase22plus database (Supplemental Table S8).

Some miRNAs were highly expressed. The top eight most highly expressed miRNAs belong to six families: miR159, miR168, miR396, miR156, miR169, and miR167 (Supplemental Table S7). Although highly expressed miRNAs are statistically the most likely to be detected, none of the top 25 miRNAs showed evidence of regulation under drought conditions, indicating that the expression levels of the most highly expressed miRNAs were kept at relatively stable levels under drought stress. In total, 21 out of 244 miRNAs and 15 out of 510 isomiRs showed significantly drought responses (Table 1). Most drought-responsive miRNAs ($N = 13$) were downregulated by drought treatment and four were upregulated. The remaining four were not categorized to any of the three major cluster groups. The 21 drought-responsive miRNAs belonged to 13 families, including *miR1432*, *miR156*, *miR164*, *miR166*, *miR167*, *miR168*, *miR171*, *miR319*, *miR390*, *miR398*, *miR399*, *miR408*, and *miR528* (Table 1). The *miR390a-3p* or *miR390b-3p* (*miR390a/b-3p*) of the *miR390* family was drought-responsive, but no significant regulation under drought was observed for *miR390a/b-5p* (AAGCUCAGGAGGGAUAGCGCC), which cleaves *TAS3* loci to produce ta-siRNAs (Allen et al., 2005; Williams et al., 2005; Dotto et al., 2014; Xia et al., 2017). Predicted *TAS3* ta-siRNAs triggered by miR390a/b-5p were either seldom expressed or with no significant regulation under drought stress (Supplemental Table S9). For isomiRs, five , eight, and two were in the downregulation, upregulation, and uncategorized groups, respectively, adding two additional miRNA families, *miR396* and *miR444*, showed drought responses. Notably, multiple isomiRs and *mirR156i-3p* from the *miR156* family were upregulated under drought (Table 1). However, *miR156j-3p* was downregulated, implying that family members play different regulatory roles.

Targeted protein-coding genes of 21 miRNAs and 15 isomiRs that responded to drought were predicted with the psRNATarget tool (Dai and Zhao, 2011). In total, 66 gene–miRNA pairs were predicted, including 43 nonredundant genes targeted by 17 drought-responsive miRNAs or isomiRs (Supplemental Table S10). The GO enrichment analysis showed that 43 miRNA-targeting genes were highly enriched for the DNA binding function (GO:0003677, p-value = 2.1×10^{-16}) and nucleus cell component (GO:0005634, p-value = 6.1×10^{-16}) (Supplemental Table S11), suggestive of the considerable impact of miRNAs on the genes regulating transcription under drought stress. Nearly half of the targets (18 out of 43) are putative Squamosa promoter binding protein-like (SPL) transcription factors and 17 out of 18 are targeted by two isomiRs of the miR156 (GACAGAAGAGAGUGAGCACA and UGACAGAAGAGAGUGAGCACA). Squamosa promoter binding protein-like genes have been reported to be associated with miR156 under drought conditions in multiple plant species, such as rice (*Oryza sativa* L.) (Nigam et al., 2015), cotton (*Gossypium hirsutum* L.) (Wang et al., 2013), alfalfa (*Medicago sativa* L.) (Arshad et al., 2017), and maize (Mao et al., 2016). In our result, both SPL-targeting miR156 isomiRs were upregulated under drought (Fig. 5), indicating the possible regulation in expression of SPL genes through miRNAs during drought treatment. Another drought-responsive miRNA miR319a/b-3p (UUGGACUGAAGGGUGCUCCC) was predicted to target one MYB and two TCP transcription factors (*GRMZM2G028054*, *GRMZM2G089361*, and *GRMZM2G115516*) (Zhang et al., 2009; Liu et al., 2014). This miR319a/b-3p remained at a low expression level under high drought stress (Supplemental Fig. S5). Presumably, the expression of targeted genes was under a low level of suppression imposed by miR319 under drought conditions. Indeed, one of the three genes, *GRMZM2G115516*, was upregulated by over fourfold under drought (Supplemental Table S10) (Liu et al., 2015). The transcriptional regulation of genes targeted by isomiRs of *miR156* and *miR319a/b-3p* was well supported by degradome sequencing data (Supplemental Table S10), which were used to identify miRNA cleavage sites (Shen et al., 2013; Zhai et al., 2013; Liu et al., 2014).

DISCUSSION

In this study, sRNA sequencing was performed on samples of maize seedlings under DS and WW conditions. The sRNAs were characterized with respect to sRNA lengths, functional class, and copy number of sRNA genomic regions. Genomic copy number analysis indicates that most 18- to 20-nt and 25- to 30-nt NR-sRNAs and approximately half of the 21- to 23-nt NR-sRNAs are derived from high-copy genomic repeats. The 24-nt sRNAs were the predominant species among single-copy sRNAs in this study, which is inconsistent with the observations in many other plant species. In fact, 24-nt sRNAs are generally referred to as heterochromatic siRNAs and are primarily derived from intergenic and/or repetitive genomic regions (Dunoyer et al., 2007; Kasschau et al., 2007; Axtell, 2013a). However, 24-nt sRNAs were also recently shown to be enriched in euchromatic regions with low DNA cytosine methylation in an independent maize study (He et al., 2013). In maize *mop1–1* (RNA-dependent RNA polymerase *rdr2* homolog) mutants, 23- or 24-nt sRNAs were dramatically decreased relative to wild-types, and many more reduced 23- or 24-nt

sRNAs were from low-copy genomic regions, as opposed to high-copy regions, which is different from the observation in the comparison between *Arabidopsis thaliana* (L.) Heynh. *rdr2* mutants and wild-types (Nobuta et al., 2008). According to the ShortStack sRNA genomic mapping, 24-nt sRNA genomic loci were largely located at intergenic regions but closer to protein-coding genes than randomly shuffled simulated loci (Supplemental Fig. S6). The proximity of 24-nt sRNA genomic loci to protein-coding genes, particularly highly expressed genes, has previously been observed (Lunardon et al., 2016) and 24-nt sRNA was proposed to function in reinforcing the silencing of transposable elements close to active genes (Li et al., 2015a). Our transposon analysis found that 24-nt sRNA genomic loci were over-represented at regions containing DNA transposon elements but under-represented at regions containing long terminal repeats retrotransposon elements, *Copia* and *Gypsy* (Supplemental Table S12), suggesting that the 24-nt sRNAs might be more critical for silencing DNA transposon elements. Compared with other lengths of sRNAs, genomic regions generating 24-nt sRNAs exhibited low histone modification levels for many histone epimarks (data not shown). Given that most 24-nt sRNAs are generated by Polymerase IV, the heavy nucleosome loading and/or strong histone modifications of examined epimarks are not likely to be prerequisites for transcription via Polymerase IV (Li et al., 2015b; Lunardon et al., 2016).

Table 1. The list of drought-responsive maize microRNAs (miRNAs)

miRNA sequence	Length	Total reads†	Genomic copy‡	miRNA gene	miRNA type	Cluster group§
	nt					
UGGGUGUCAUCUCGCCUGAAGC	22	531	1	*MIR1432*	3p	Others
UCAGGAGAGAUGACACCGACG	21	9,059	1	*MIR1432*	5p	Up
GACAGAAGAGAGUGAGCACA	20	9,921	8	*MIR156a,b,c,d,e,h,i,l, Cluster_27697*	isomiR	Up
UGACAGAAGAGAGUGAGCACA	21	23,675	8	*MIR156a,b,c,d,e,h,i,l, Cluster_27697*	isomiR	Up
GCUCACUGCUCUAUCUGUCAUC	22	14,829	1	*MIR156i*	3p	Up
GCUCACUGCUCUAUCUGUCAU	21	1,116	1	*MIR156i*	isomiR	Up
GCUCUCUGCUCUCACUGUCAUC	22	607	1	*MIR156j*	3p	Down
CACGUGCUCCCCUUCUCCACC	21	499	1	*MIR164g*	3p	Down
GGAAUGUUGUCUGGUUCAAGG	21	40,096	2	*MIR166b,d*	5p	Down
GGAAUGUUGUCUGGUUCAAGGU	22	839	4	*MIR166b,d*	isomiR	Down
GGAAUGUCGUCUGGCGCGAGA	21	416	1	*MIR166i*	5p	Down
GGUUUGUUUGUCUGGUUCAAGG	22	2,613	1	*MIR166j*	5p	Down
GGAAUGUUGGCUGGCUCGAGG	21	2,563	2	*MIR166m, Cluster_45700*	5p	Down
GAUCAUGCUGUGGCAGCCUCACU	23	3,287	1	*MIR167c*	3p	Down
AGGUCAUGCUGUAGUUUCAUC	21	3,986	1	*MIR167g*	isomiR	Down
AGAUCAUGUGGCAGUUUCAUU	21	2,807	1	*MIR167j*	isomiR	Up
CCCGCCUUGCACCAAGUGAA	20	25,019	1	*MIR168a*	3p	Up
CGCUUGGUGCAGAUCGGGAC	20	19,995	2	*MIR168a,b*	isomiR	Up
UCGCUUGGUGCAGAUCGGGA	20	294,017	2	*MIR168a,b*	isomiR	Up
UCGCUUGGUGCAGAUCGGGACC	22	59,451	2	*MIR168a,b*	isomiR	Up
UGUUGGCUCGGCUCACUCAGA	21	21,299	2	*MIR171d,e*	5p	Down
UUGGACUGAAGGGUGCUCCC	20	62,868	4	*MIR319a,b,c,d*	3p	Other
CGCUAUCUAUCCUGAGCUCCA	21	9,684	2	*MIR390a,b*	3p	Down
CAGCUUUCUUGAACUUCUUCU	21	823	2	*MIR396e,f*	isomiR	Down
GGGGCGAACUGAGAACACAUG	21	5,992	1	*MIR398a*	5p	Down
AUGUGUUCUCAGGUCGCCCCCG	22	1,920	2	*MIR398a,b*	isomiR	Other
GGGGCGGACUGGGAACACAUG	21	53,148	1	*MIR398b*	5p	Down
GGGCGGACUGGGAACACAUGG	21	10,086	1	*MIR398b*	isomiR	Down
GGGUACGUCUCCUUUGGCACA	21	390	1	*MIR399c*	5p	Others
GGGCUUCUCUUUCUUGGCAGG	21	2,098	1	*MIR399e*	5p	Others
GGGCAACUUCUCCUUUGGCAGA	22	2,743	1	*MIR399f*	5p	Up
CAGGGACGAGGCAGAGCAUGG	21	6,822	1	*MIR408b*	5p	Down
CAGGGACGAGGCAGAGCAUG	20	10,218	1	*MIR408b*	isomiR	Other
UGCAAGUUGUGCAGUUGUUGU	21	2,125	3	*MIR444a,b*	isomiR	Up
CCUGUGCCUGCCUCUUCCAUU	21	8,186	2	*MIR528a,b*	3p	Down
CUGUGCCUGCCUCUUCCAUU	20	1,137	2	*MIR528a,b*	isomiR	Down

† Total short RNA reads from all 36 samples.

‡ Genomic DNA copy number using the reference-based method

§ Clustering group from the mclust analysis. Down and Up represent downregulated and upregulated groups respectively in response drought stress. "Other" represents the group that does not belong to the downregulated, upregulated, or transiently downregulated groups.

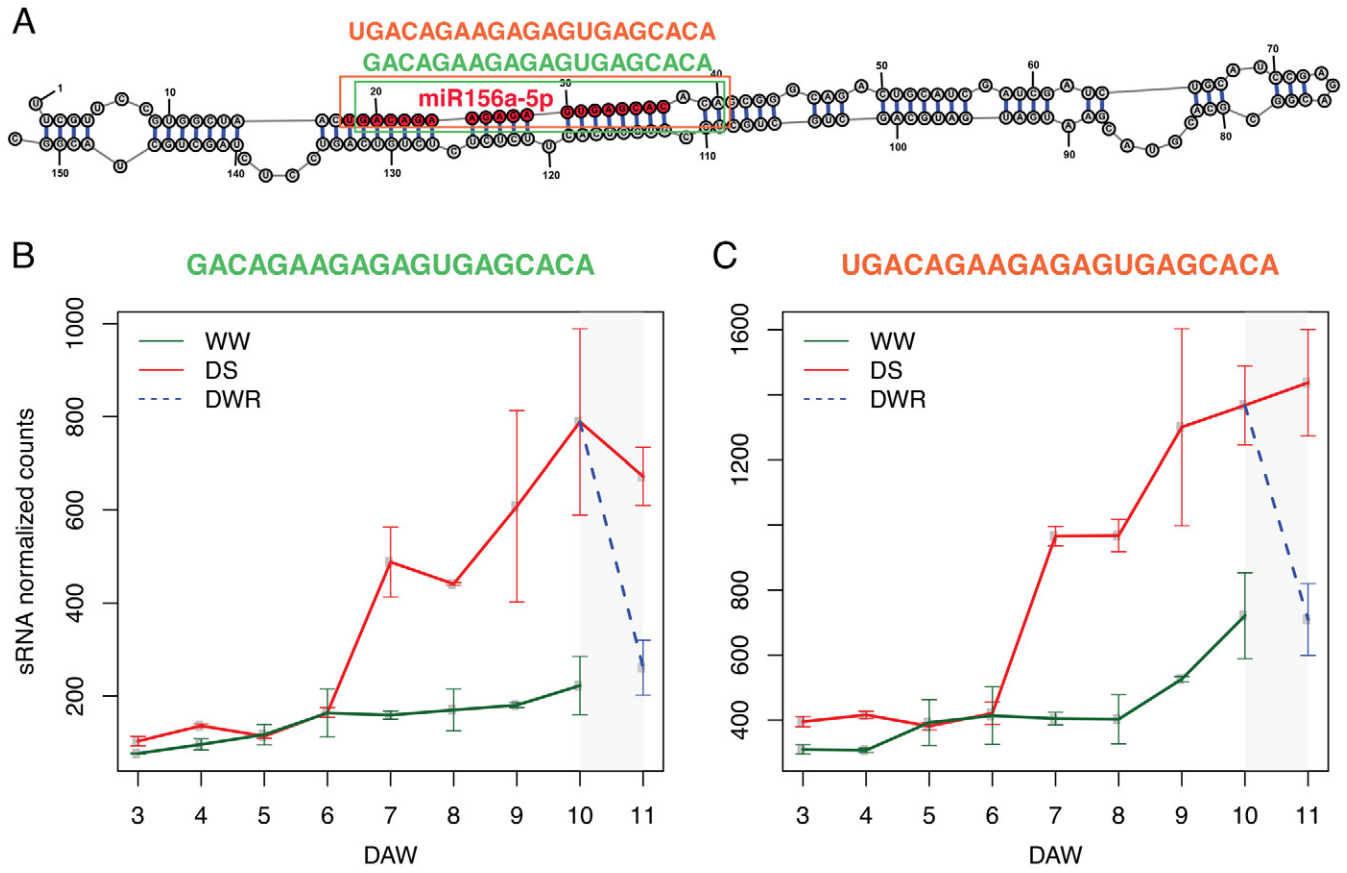

Fig. 5. Time-series expression profiles of two miR156 isomiRs targeting Squamosa promoter binding protein-like genes in maize. (A) Structure of primary miR156a. The reference miR156 reference mature sequence is highlighted in red. Two boxes indicate the regions for two miR156 isomiRs. (B, C) Normalized counts of each miR156 isomiR (*y*-axis) were plotted along 3–11 d after withholding water (DAW). WW, well-watered; DS, drought stress; DWR, drought–water recovery. The sequence on the top of each plot is the miR156 isomiR sequence. Each error bar represents the range of SE above and below the mean value.

Characterization of drought-responsive sRNAs indicates that sRNAs are differentially expressed in response to drought stress. The miRNAs of maize were clustered into three groups on the basis of their expression patterns: namely, upregulated, downregulated, and transiently downregulated on drought stress and over-represented in the downregulated group, in which miRNAs were enriched by approximately 4.8×. The miRNAs and cognate gene targets are involved in drought stress responses in many plant species such as *A. thaliana* (Butler et al., 2008), rice (Zhou et al., 2010; Fang et al., 2014), soybean [*Glycine max* (L.) Merr.] (Axtell, 2013b), and poplar (*Populus trichocarpa* Torr. & A.Gray ex Hook.) (Shuai et al., 2013). Drought-induced miRNAs presumably suppress their target mRNAs, whereas downregulated miRNAs result in the de-repression of their target mRNAs (Ferdous et al., 2015). The miRNAs may exhibit distinct responses to drought stress in different plant species (Zhai et al., 2015). For example, miR168a/b was downregulated under drought in rice (Zhou et al., 2010) but induced in response to drought stress in maize. We have identified 36 drought-responsive miRNAs or isomiRs, as well as their potential gene targets. Detailed studies of their regulatory networks and their functional divergence among species or genotypes within a species would be valuable to modulate miRNA-mediated pathways for improving the drought tolerance of plants.

In addition to miRNAs, sRNAs derived from rRNAs, tRNAs, snoRNAs, and splicing snRNAs were also differentially regulated under drought condition. rRNAs are an essential component of ribosomes and catalyze protein assembly. The rsRNAs (sRNAs derived from rRNAs) were over-represented in the upregulated sRNA group. rsRNAs were significantly enriched in downregulated sRNAs after addition of water at 10 DAW. Thus drought response involved an increase in rsRNAs, which is, in turn, suppressed when water was supplied. The upregulation of rsRNAs implied that the rRNA decay rate increases on drought. In bacteria, the degradation of rRNA increases under the conditions restricting cell growth (e.g., starvation) because of the need to reduce functional ribosomes (Maruyama and Mizuno, 1970; Basturea et al., 2011). A similar process could be involved with plants under abiotic stresses. rsRNAs from the rRNA were abundant and homogeneous at all lengths from 18 to 26 nt, implying that the cleavage activity of rRNA, such as RNase T2 from *A. thaliana* (Hillwig et al., 2011), is not size-specific. Transfer RNAs play an

essential role in protein synthesis. Although tsRNAs (sRNAs derived from tRNAs) were not enriched in either up- or downregulated sRNAs groups, upregulated tsRNAs were almost seven times more represented than downregulated tsRNAs (148:22), which was higher than the ratio of all upregulated sRNAs to all downregulated sRNAs (4373:816). A barley sRNA study also found that tsRNAs, overall, have a tendency to be upregulated under drought conditions (Hackenberg et al., 2015). Splicing sn-sRNAs, derived from splicing snRNAs that are involved in pre-mRNA splicing, were over-represented in upregulated sRNAs under drought. Alternative splicing of pre-mRNA splicing under drought stress was observed in multiple tissues, particularly in the leaf and ear (Thatcher et al., 2016), which might partially be attributed to the amount and stability of various splicing sn-RNAs.

The snoRNAs primarily include two classes of sRNAs, box C/D and box H/ACA snoRNAs, which guide the methylation and pseudouridylation of other RNAs, respectively (Bachellerie et al., 2002; Kiss, 2006). The snoRNA-mediated chemical modifications of rRNAs and splicing snRNAs have been demonstrated to be essential for ribosomal function as well as mRNA splicing and maturation (Morris and Mattick, 2014; Dupuis-Sandoval et al., 2015). The sno-sRNAs were over-represented in both the downregulated and transiently downregulated sRNA groups under drought stress. Downregulation of sno-sRNAs may be the result of a reduction of snoRNAs, which would reduce the activity of methylation and pseudouridylation of rRNAs and splicing snRNAs. Given the reduction in sno-sRNAs and the increase in rsRNA and splicing sn-sRNAs under drought stress, it is tempting to speculate that rRNAs and splicing snRNAs are destabilized with decreased methylations or pseudouridylations, which are mediated by snoRNAs. Both changes in chemical modification, presumably, and the quantity of rRNAs under drought stress could alter the activity of the protein synthesis machinery. The observation of sRNA changes related to rRNAs and splicing snRNAs indicates that the post-transcriptional regulation is an important mechanism for adaptive responses to drought stress. snoRNAs exhibiting responses to drought were found in another plant species (Hackenberg et al., 2015). Recently, snoRNAs were also found to be involved in metabolic stress responses, including oxidative stress in human cells (Michel et al., 2011; Chu et al., 2012; Youssef et al., 2015). Taking all these findings together, we propose that the snoRNA plays a role in regulating biological processes under drought stress through altering levels of chemical modifications of rRNAs and splicing snRNAs.

Authors' Contributions

JZ and GW designed the study. JZ, YD, ZJ, and KW performed experiments and generated the sequence data. SL, EZ, CH, JF, YH, ML, WL, and HW analyzed the data. SL, FW, JZ, and EZ wrote the manuscript. FW, HW, EZ, WL, and GW revised the manuscript. All authors reviewed and approved the final manuscript.

Supplemental Information

Figure S1. Density plots of genomic copy number of various sRNAs.

Figure S2. Density plots of sRNA distributions with different genomic copies.

Figure S3. The relation between sRNA expression levels and genome copies.

Figure S4. Pie charts of functional classes and lengths of drought-response sRNAs.

Figure S5. Time-series expression profile of a miR319.

Figure S6. Distances of 24 nt sRNA loci to the closest genes.

Table S1. Number of total reads and trimming summary of 36 samples.

Table S2. Normalized counts of 134,283 NR-sRNAs.

Table S3. Detailed information of 134,283 NR-sRNAs.

Table S4. Result of differential expression analysis between DS and DWR at 11 DAW.

Table S5. Network statistics.

Table S6. The list of miRNAs in MiRBase22plus.

Table S7. List of nonredundant miRNAs detected.

Table S8. List of isomiRs.

Table S9. Information on ta-siRNAs.

Table S10. Genes targeted by drought-responsive miRNAs.

Table S11. Gene ontology enrichment analysis of targeted genes by drought-responsive miRNAs.

Table S12. Transposon summary of 24-nt sRNA genomic loci

Conflict of Interest Disclosure

The authors declare that there is no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program of China (Grant No. 2016YFD0101002), the National Natural Science Foundation of China (Grant No. 31330056), the Agricultural Science and Technology Innovation Program of the Chinese Academy of Agricultural Sciences, and the US National Science Foundation (Awards No. 1238189 and 1741090). This work of HW was partly supported by a grant from the Simon Foundation (Award No. 246077). We are grateful for the support from the Kansas Agricultural Experiment Station (contribution No. 17-132-J).

REFERENCES

Addo-Quaye, C., W. Miller, and M.J. Axtell. 2009. CleaveLand: A pipeline for using degradome data to find cleaved small RNA targets. Bioinformatics 25:130–131. doi:10.1093/bioinformatics/btn604

Allen, E., Z. Xie, A.M. Gustafson, and J.C. Carrington. 2005. microRNA-directed phasing during *trans*-acting siRNA biogenesis in plants. Cell 121:207–221. doi:10.1016/j.cell.2005.04.004

Arshad, M., B.A. Feyissa, L. Amyot, B. Aung, and A. Hannoufa. 2017. MicroRNA156 improves drought stress tolerance in alfalfa (*Medicago sativa*) by silencing *SPL13*. Plant Sci. 258:122–136. doi:10.1016/j.plantsci.2017.01.018

Axtell, M.J. 2013a. Classification and comparison of small RNAs from plants. Annu. Rev. Plant Biol. 64:137–159. doi:10.1146/annurev-arplant-050312-120043

Axtell, M.J. 2013b. ShortStack: Comprehensive annotation and quantification of small RNA genes. RNA 19:740–751. doi:10.1261/rna.035279.112

Bachellerie, J.P., J. Cavaille, and A. Huttenhofer. 2002. The expanding snoRNA world. Biochimie 84:775–790. doi:10.1016/S0300-9084(02)01402-5

Basturea, G.N., M.A. Zundel, and M.P. Deutscher. 2011. Degradation of ribosomal RNA during starvation: Comparison to quality control during steady-state growth and a role for RNase PH. RNA 17:338–345. doi:10.1261/rna.2448911

Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate- a practical and powerful approach to multiple testing. J. R. Stat. Soc. B 57:289–300.

Bolger, A.M., M. Lohse, and B. Usadel. 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30(15):2114–2120. doi:10.1093/bioinformatics/btu170

Burge, S.W., J. Daub, R. Eberhardt, J. Tate, L. Barquist, E.P. Nawrocki, et al. 2013. Rfam 11.0: 10 years of RNA families. Nucleic Acids Res. 41:D226–D232. doi:10.1093/nar/gks1005

Butler, J., I. MacCallum, M. Kleber, I.A. Shlyakhter, M.K. Belmonte, E.S. Lander, et al. 2008. ALLPATHS: De novo assembly of whole-genome shotgun microreads. Genome Res. 18:810–820. doi:10.1101/gr.7337908

Chu, L., M.Y. Su, L.B. Maggi, Jr., L. Lu, C. Mullins, S. Crosby, et al. 2012. Multiple myeloma-associated chromosomal translocation activates orphan snoRNA ACA11 to suppress oxidative stress. J. Clin. Invest. 122:2793–2806. doi:10.1172/JCI63051

Covarrubias, A.A., and J.L. Reyes. 2010. Post-transcriptional gene regulation of salinity and drought responses by plant microRNAs. Plant Cell Environ. 33:481–489. doi:10.1111/j.1365-3040.2009.02048.x

Dai, X., and P.X. Zhao. 2011. psRNATarget: A plant small RNA target analysis server. Nucleic Acids Res. 39:W155–W159. doi:10.1093/nar/gkr319

Ding, Y., Y. Tao, and C. Zhu. 2013. Emerging roles of microRNAs in the mediation of drought stress response in plants. J. Exp. Bot. 64:3077–3086. doi:10.1093/jxb/ert164

Dotto, M.C., K.A. Petsch, M.J. Aukerman, M. Beatty, M. Hammell, and M.C.P. Timmermans. 2014. Genome-wide analysis of *leafbladeless1*-regulated and phased small RNAs underscores the importance of the *TAS3* ta-siRNA pathway to maize development. PLoS Genet. 10(12):e1004826. doi:10.1371/journal.pgen.1004826

Dunoyer, P., C. Himber, V. Ruiz-Ferrer, A. Alioua, and O. Voinnet. 2007. Intra- and intercellular RNA interference in *Arabidopsis thaliana* requires components of the microRNA and heterochromatic silencing pathways. Nat. Genet. 39:848–856. doi:10.1038/ng2081

Dupuis-Sandoval, F., M. Poirier, and M.S. Scott. 2015. The emerging landscape of small nucleolar RNAs in cell biology. Wiley Interdiscip. Rev. RNA 6:381–397. doi:10.1002/wrna.1284

Fang, Y., K. Xie, and L. Xiong. 2014. Conserved miR164-targeted NAC genes negatively regulate drought resistance in rice. J. Exp. Bot. 65:2119–2135. doi:10.1093/jxb/eru072

Ferdous, J., S.S. Hussain, and B.J. Shi. 2015. Role of microRNAs in plant drought tolerance. Plant Biotechnol. J. 13:293–305.

Fraley, C., and A.E. Raftery. 2007. Model-based methods of classification: Using the mclust software in chemometrics. J. Stat. Softw. 18:1–13. doi:10.18637/jss.v018.i06

Hackenberg, M., P. Gustafson, P. Langridge, and B.J. Shi. 2015. Differential expression of microRNAs and other small RNAs in barley between water and drought conditions. Plant Biotechnol. J. 13:2–13. doi:10.1111/pbi.12220

He, G., B. Chen, X. Wang, X. Li, J. Li, H. He, et al. 2013. Conservation and divergence of transcriptomic and epigenomic variation in maize hybrids. Genome Biol. 14:R57. doi:10.1186/gb-2013-14-6-r57

Hillwig, M.S., A.L. Contento, A. Meyer, D. Ebany, D.C. Bassham, and G.C. MacIntosh. 2011. RNS2, a conserved member of the RNase T2 family, is necessary for ribosomal RNA decay in plants. Proc. Natl. Acad. Sci. USA 108:1093–1098. doi:10.1073/pnas.1009809108

Kasschau, K.D., N. Fahlgren, E.J. Chapman, C.M. Sullivan, J.S. Cumbie, S.A. Givan, et al. 2007. Genome-wide profiling and analysis of *Arabidopsis* siRNAs. PLoS Biol. 5:e57. doi:10.1371/journal.pbio.0050057

Khraiwesh, B., J.-K. Zhu, and J. Zhu. 2012. Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. Biochim. Biophys. Acta Gene Regul. Mech. 1819:137–148.

Kiss, T. 2006. SnoRNP biogenesis meets pre-mRNA splicing. Mol. Cell 23:775–776. doi:10.1016/j.molcel.2006.08.023

Kozomara, A., and S. Griffiths-Jones. 2014. miRBase: Annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. 42:D68–D73. doi:10.1093/nar/gkt1181

Langfelder, P., and S. Horvath. 2008. WGCNA: An R package for weighted correlation network analysis. BMC Bioinformatics 9:559. doi:10.1186/1471-2105-9-559

Li, H., and R. Durbin. 2010. Fast and accurate long-read alignment with Burrows–Wheeler transform. Bioinformatics 26:589–595. doi:10.1093/bioinformatics/btp698

Li, J.S., F.L. Fu, M. An, S.F. Zhou, Y.H. She, and W. Li. 2013. Differential expression of microRNAs in response to drought stress in maize. J. Integr. Agric. 12:1414–1422. doi:10.1016/S2095-3119(13)60311-1

Li, Q., J.I. Gent, G. Zynda, J.W. Song, I. Makarevitch, C.D. Hirsch, et al. 2015a. RNA-directed DNA methylation enforces boundaries between heterochromatin and euchromatin in the maize genome. Proc. Natl. Acad. Sci. USA 112:14728–14733. doi:10.1073/pnas.1514680112

Li, S.F., L.E. Vandivier, B. Tu, L. Gao, S.Y. Won, S. Li, et al. 2015b. Detection of Pol IV/RDR2-dependent transcripts at the genomic scale in *Arabidopsis* reveals features and regulation of siRNA biogenesis. Genome Res. 25:235–245. doi:10.1101/gr.182238.114

Liang, H., and L.F. Landweber. 2007. Hypothesis: RNA editing of microRNA target sites in humans? RNA 13:463–467. doi:10.1261/rna.296407

Liu, H., C. Qin, Z. Chen, T. Zuo, X. Yang, H. Zhou, et al. 2014. Identification of miRNAs and their target genes in developing maize ears by combined small RNA and degradome sequencing. BMC Genomics 15:25. doi:10.1186/1471-2164-15-25

Liu, Y., M. Zhou, Z. Gao, W. Ren, F. Yang, H. He, et al. 2015. RNA-seq analysis reveals MAPKKK family members related to drought tolerance in maize. PLoS One 10:e0143128. doi:10.1371/journal.pone.0143128

Love, M.I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15:550. doi:10.1186/s13059-014-0550-8

Lunardon, A., C. Forestan, S. Farinati, M.J. Axtell, and S. Varotto. 2016. Genome-wide characterization of maize small RNA loci and their regulation in the *required to maintain repression6-1* (*rmr6-1*) mutant and long-term abiotic stresses. Plant Physiol. 170:1535–1548.

Mao, H., L. Yu, Z. Li, Y. Yan, R. Han, H. Liu, et al. 2016. Genome-wide analysis of the SPL family transcription factors and their responses to abiotic stresses in maize. Plant Genome 6:1–12. doi:10.1016/j.plgene.2016.03.003

Marçais, G., and C. Kingsford. 2011. A fast, lock-free approach for efficient parallel counting of occurrences of k-mers. Bioinformatics 27:764–770. doi:10.1093/bioinformatics/btr011

Maruyama, H., and D. Mizuno. 1970. Ribosome degradation and the degradation products in starved *Escherichia coli*. II. Changes in base sequence of ribosomal RNA during degradation induced by phosphate and magnesium starvation. Biochim. Biophys. Acta 199:166–175. doi:10.1016/0005-2787(70)90705-7

Meyers, B.C., M. Matzke, and V. Sundaresan. 2008. The RNA world is alive and well. Trends Plant Sci. 13:311–313. doi:10.1016/j.tplants.2008.05.003

Michel, C.I., C.L. Holley, B.S. Scruggs, R. Sidhu, R.T. Brookheart, L.L. Listenberger, et al. 2011. Small nucleolar RNAs U32a, U33, and U35a are critical mediators of metabolic stress. Cell Metab. 14:33–44. doi:10.1016/j.cmet.2011.04.009

Morris, K.V., and J.S. Mattick. 2014. The rise of regulatory RNA. Nat. Rev. Genet. 15:423–437. doi:10.1038/nrg3722

Neilsen, C.T., G.J. Goodall, and C.P. Bracken. 2012. IsomiRs—The overlooked repertoire in the dynamic microRNAome. Trends Genet. 28:544–549. doi:10.1016/j.tig.2012.07.005

Nigam, D., S. Kumar, D.C. Mishra, A. Rai, S. Smita, and A. Saha. 2015. Synergistic regulatory networks mediated by microRNAs and transcription factors under drought, heat and salt stresses in *Oryza sativa* spp. Gene 555:127–139. doi:10.1016/j.gene.2014.10.054

Nobuta, K., C. Lu, R. Shrivastava, M. Pillay, E. De Paoli, M. Accerbi, et al. 2008. Distinct size distribution of endogeneous siRNAs in maize: Evidence from deep sequencing in the *mop1-1* mutant. Proc. Natl. Acad. Sci. USA 105:14958–14963. doi:10.1073/pnas.0808066105

Onodera, Y., J.R. Haag, T. Ream, P. Costa Nunes, O. Pontes, and C.S. Pikaard. 2005. Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. Cell 120:613–622. doi:10.1016/j.cell.2005.02.007

Rhoades, M.W., B.J. Reinhart, L.P. Lim, C.B. Burge, B. Bartel, and D.P. Bartel. 2002. Prediction of plant microRNA targets. Cell 110:513–520. doi:10.1016/S0092-8674(02)00863-2

Schnable, P.S., D. Ware, R.S. Fulton, J.C. Stein, F. Wei, S. Pasternak, et al. 2009. The B73 maize genome: Complexity, diversity, and dynamics. Science 326:1112–1115. doi:10.1126/science.1178534

Shen, Y., Z. Jiang, S. Lu, H. Lin, S. Gao, H. Peng, et al. 2013. Combined small RNA and degradome sequencing reveals microRNA regulation during immature maize embryo dedifferentiation. Biochem. Biophys. Res. Commun. 441:425–430. doi:10.1016/j.bbrc.2013.10.113

Shuai, P., D. Liang, Z. Zhang, W. Yin, and X. Xia. 2013. Identification of drought-responsive and novel *Populus trichocarp*a microRNAs by high-throughput sequencing and their targets using degradome analysis. BMC Genomics 14:233. doi:10.1186/1471-2164-14-233

Thatcher, S.R., O.N. Danilevskaya, X. Meng, M. Beatty, G. Zastrow-Hayes, C. Harris, et al. 2016. Genome-wide analysis of alternative splicing during development and drought stress in maize. Plant Physiol. 170:586–599. doi:10.1104/pp.15.01267

Tian, T., Y. Liu, H. Yan, Q. You, X. Yi, Z. Du, et al. 2017. agriGO v2.0: A GO analysis toolkit for the agricultural community, 2017 update. Nucleic Acids Res. 45:W122–W129. doi:10.1093/nar/gkx382

Vazquez, F., S. Legrand, and D. Windels. 2010. The biosynthetic pathways and biological scopes of plant small RNAs. Trends Plant Sci. 15:337–345. doi:10.1016/j.tplants.2010.04.001

Wang, M., Q.L. Wang, and B.H. Zhang. 2013. Response of miRNAs and their targets to salt and drought stresses in cotton (*Gossypium hirsutum* L.). Gene 530:26–32. doi:10.1016/j.gene.2013.08.009

Wang, Y.G., M. An, S.F. Zhou, Y.H. She, W.C. Li, F.L. Fu. 2014. Expression profile of maize microRNAs corresponding to their target genes under drought stress. Biochem. Genet. 52:474–493. doi:10.1007/s10528-014-9661-x

Wierzbicki, A.T., J.R. Haag, and C.S. Pikaard. 2008. Noncoding transcription by RNA polymerase Pol IVb/Pol V mediates transcriptional silencing of overlapping and adjacent genes. Cell 135:635–648. doi:10.1016/j.cell.2008.09.035

Williams, L., C.C. Carles, K.S. Osmont, and J.C. Fletcher. 2005. A database analysis method identifies an endogenous *trans*-acting short-interfering RNA that targets the *Arabidopsis ARF2*, *ARF3*, and *ARF4* genes. Proc. Natl. Acad. Sci. USA 102:9703–9708. doi:10.1073/pnas.0504029102

Xia, R., J. Xu, and B.C. Meyers. 2017. The emergence, evolution, and diversification of the miR390-*TAS3*-*ARF* pathway in land plants. Plant Cell 29:1232–1247.

Young, M.D., M.J. Wakefield, G.K. Smyth, and A. Oshlack. 2010. Gene ontology analysis for RNA-seq: Accounting for selection bias. Genome Biol. 11:R14. doi:10.1186/gb-2010-11-2-r14

Youssef, O.A., S.A. Safran, T. Nakamura, D.A. Nix, G.S. Hotamisligil, and B.L. Bass. 2015. Potential role for snoRNAs in PKR activation during metabolic stress. Proc. Natl. Acad. Sci. USA 112:5023–5028. doi:10.1073/pnas.1424044112

Zhai, J., H. Zhang, S. Arikit, K. Huang, G.-L. Nan, V. Walbot, et al. 2015. Spatiotemporally dynamic, cell-type–dependent premeiotic and meiotic phasiRNAs in maize anthers. Proc. Natl. Acad. Sci. USA 112:3146–3151. doi:10.1073/pnas.1418918112

Zhai, L., Z. Liu, X. Zou, Y. Jiang, F. Qiu, Y. Zheng, et al. 2013. Genome-wide identification and analysis of microRNA responding to long-term waterlogging in crown roots of maize seedlings. Physiol. Plant. 147:181–193.

Zhang, B. 2015. MicroRNA: A new target for improving plant tolerance to abiotic stress. J. Exp. Bot. 66:1749–1761.

Zhang, L., J.M. Chia, S. Kumari, J.C. Stein, Z. Liu, A. Narechania, et al. 2009. A genome-wide characterization of microRNA genes in maize. PLoS Genet. 5:e1000716. doi:10.1371/journal.pgen.1000716

Zheng, J., J.J. Fu, M.Y. Gou, J.L. Huai, Y.J. Liu, M. Jian, et al. 2010. Genome-wide transcriptome analysis of two maize inbred lines under drought stress. Plant Mol. Biol. 72:407–421. doi:10.1007/s11103-009-9579-6

Zhou, L., Y. Liu, Z. Liu, D. Kong, M. Duan, and L. Luo. 2010. Genome-wide identification and analysis of drought-responsive microRNAs in *Oryza sativa*. J. Exp. Bot. 61:4157–4168. doi:10.1093/jxb/erq237

Zhu, J.K. 2002. Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. 53:247–273. doi:10.1146/annurev.arplant.53.091401.143329