

1 **Temporal Small RNA Expression Profiling Under Drought Reveals a Potential**
2 **Regulatory Role of snoRNAs in Drought Responses of Maize**

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26 **Abstract**

27 Small RNAs (sRNAs) are short noncoding RNAs that play roles in many biological
28 processes, including drought responses in plants. However, how the expression of sRNAs
29 dynamically changes with the gradual imposition of drought stress in plants is largely
30 unknown. We generated time-series sRNA sequence data from maize seedlings under
31 drought stress and under well-watered conditions at the same time points. Analyses of
32 length, functional annotation, and abundance of 736,372 non-redundant sRNAs from
33 both drought and well-watered data, as well as genome copy number and chromatin
34 modifications at the corresponding genomic regions, revealed distinct patterns of
35 abundance, genome organization, and chromatin modifications for different sRNA
36 classes of sRNAs. The analysis identified 6,646 sRNAs whose regulation was altered in
37 response to drought stress. Among drought-responsive sRNAs, 1,325 showed transient
38 down-regulation by the seventh day, coinciding with visible symptoms of drought stress.
39 The profiles revealed drought-responsive microRNAs, as well as other sRNAs that
40 originated from ribosomal RNAs (rRNAs), splicing small nuclear RNAs, and small
41 nucleolar RNAs (snoRNA). Expression profiles of their sRNA deriviers indicated that
42 snoRNAs might play a regulatory role through regulating stability of rRNAs and splicing
43 small nuclear RNAs under drought condition.

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45

46

47 **Introduction**

48 Physiological responses to drought in plants are complex and regulated through an
49 interplay of a network of genetic components and chromatin structure. One component is
50 comprised of drought-responsive small RNAs (sRNAs) (KHRAIWESH *et al.* 2012). sRNAs
51 are short noncoding RNAs, predominately 20 to 24 nt in length, which function as
52 sequence-specific regulators in a wide variety of biological processes, including DNA
53 methylation, RNA degradation, translation regulation, and histone modification
54 (KHRAIWESH *et al.* 2012; AXTELL 2013a). Plant sRNAs are typically categorized into two
55 major groups, which are distinguished by the structure of the sRNA precursors. The first
56 group consists of microRNAs (miRNAs), which are predominately 21 nt in length and
57 processed from single-stranded precursor RNA, or pri-sRNA, are transcribed by RNA
58 polymerase II (PolII) and contain a hairpin structure. The second group is comprised of
59 small interfering RNAs (siRNAs) are derived from DICER/DICER-like processing of
60 double-stranded RNAs (dsRNAs).

61

62 MiRNAs function in drought stress responses (COVARRUBIAS AND REYES 2010; SHUAI *et*
63 *al.* 2013) and are, conceptually, categorized into three functional categories -
64 homeostasis, detoxification, and growth regulation (ZHU 2002) and function largely
65 through the destabilization of various transcription factors (RHOADES *et al.* 2002; DING *et*
66 *al.* 2013; FERDOUS *et al.* 2015; ZHANG 2015). The function of miRNAs in the regulation
67 of transcription factors places miRNAs at the hubs of gene regulatory networks for
68 drought responses. Whereas miRNAs primarily act in the posttranscriptional regulation
69 of gene expression, siRNAs regulate gene transcription through both guiding DNA

70 methylation by the pathway of RNA-directed DNA methylation (RdDM) and
71 posttranscriptional destabilization of transcripts in a sequence-specific manner (ONODERA
72 *et al.* 2005; WIERZBICKI *et al.* 2008). Small interfering RNAs can be further sub-grouped
73 into heterochromatic siRNAs, secondary siRNAs, and natural antisense transcripts
74 siRNAs (NAT-siRNA). Heterochromatic siRNAs, typically, are 23-24 nt in length and
75 require RNA-dependent RNA polymerase (RDR) and RNA polymerase IV (PolIV) for
76 biogenesis. Heterochromatic siRNAs were documented to be derived from
77 transposable/repetitive elements located at heterochromatic regions of nuclear DNA
78 (MEYERS *et al.* 2008; NOBUTA *et al.* 2008). Secondary siRNAs include trans-acting
79 siRNAs (ta-siRNA), which are formed through cleavage of capped and polyadenylated
80 siRNA transcripts by specific miRNAs, followed by conversion into dsRNAs by RDR
81 (VAZQUEZ *et al.* 2010). NAT-siRNAs are derived from dsRNAs formed by annealing of
82 natural sense and antisense transcripts from the same or separate nearly identical genomic
83 regions (VAZQUEZ *et al.* 2010).

84

85 Small RNAs can also originate from ribosomal RNAs (rRNAs), transfer RNAs (tRNAs),
86 small nucleolar RNAs (snoRNAs), and small nuclear RNAs associated with mRNA
87 splicing (splicing snRNAs) and are respectively referred to as rsRNAs, tsRNAs, sno-
88 sRNAs, and splicing sn-sRNAs hereafter (VAZQUEZ *et al.* 2010). The rsRNAs, tsRNAs,
89 sno-sRNAs, and splicing sn-sRNAs play regulatory roles in cellular processes (MORRIS
90 AND MATTICK 2014). In barley, tsRNAs and sno-sRNAs tended to be up-regulated and
91 down-regulated, respectively, under drought conditions (HACKENBERG *et al.* 2015). In
92 maize, miRNA biosynthesis and regulation under drought stress has been explored (LI *et*

93 *al.* 2013; LIU *et al.* 2014; WANG *et al.* 2014). However, the regulatory functions of
94 sRNAs other than miRNAs are largely unknown.

95

96 To understand sRNA function and regulation in the drought response of maize, we
97 sequenced sRNAs from maize seedlings over a period of 3 to 11 days after withholding
98 water along with sRNAs from well-watered plants or drought treated plants that
99 recovered after watering. The sRNAs were categorized with respect to length and
100 functional classification, and the genomic organization of sRNAs was analyzed. An
101 attempt was made to classify drought-responsive sRNAs using cluster and network
102 analyses on the time-series expression patterns, providing clues of destabilization of
103 ribosome RNA and splicing small nuclear RNAs under drought condition.

104

105 **Materials and Methods**

106 **Plant materials and drought treatments**

107 Seeds of the maize (*Zea mays*) inbred line B73 were surface-sterilized and germinated on
108 the wet rolled brown paper towel at 28°C for 48h, and eighteen germinated seeds were
109 selected and transplanted in a plastic pot (17×12×10cm) filled with nutrient soil (1:1 peat
110 moss and vermiculite). Three-day seedlings after germination were subjected to drought
111 stress up to 10 days by withholding water (10 DAW), and the control plants were well
112 watered. The plants were grown on the controlled conditions (27 °C day/23 °C night, 16 h
113 photoperiod, from 6 am to 10 pm, 300 μ mol m⁻² s⁻¹ photons, 30-50% relative humidity).
114 Treatment (drought stress) and the control pots were randomly laid in growth chamber.
115 Eighteen seedlings were planted in a pot. For every harvest and sample time, five pots

116 were used for a drought treatment and other 5 pots were used as a control. At 10 DAW,
117 drought treated seedling plants were divided into two groups: a group of seedlings kept
118 under drought stress without watering and the other group of seedlings that were re-
119 watered. In summary, 36 samples of soils and leaf tissues were collected: i) 32 samples
120 resulting from 2 treatments (drought stress (DS) and well watered (WW)) x 8 days (from
121 day 3 to day 10) x 2 biological replicates; ii) 4 samples resulting from 2 treatments (DS
122 and WW at day 11th of plants previously subjected to 10 days of DS) x 2 biological
123 replicates.

124

125 **Measurement of soil SWC, leaf RWC, and leaf REC**

126 Soil samples and leaf tissues for measuring SWC (soil water content), RWC (relative
127 water content), and REC (Relative electrical conductivity) were daily collected at around
128 9:30 am. Five independent replicates were performed for the SWC measurement, and five
129 biological replicates were performed for RWC and REC measurements. SWC, RWC, and
130 REC were carried out according to the previously described method (ZHENG *et al.* 2010).
131 Briefly, the soil SWC was the percentage of the weight loss of soils after drying. The
132 RWC of the fresh leaves was calculated using the formula of $(FW-DW)/(TW-DW)$
133 $\times 100\%$, where FW is the weight of fresh leaves, TW is the leaf weight after saturated in
134 water for 8 h, and DW is the leaf dry weight. REC was calculated using $Ec1/Ec2 \times 100\%$,
135 where Ec1 is electrical conductivity of fresh leaves after saturated in water for 3 h and
136 Ec2 is electrical conductivity of the same leaf samples after boiled in a water bath.

137

138 **sRNA sequencing experiment**

139 The above ground tissues of five seedlings of each treatment at each day were collected
140 at approximately 10 am each day and immediately frozen in liquid nitrogen. Total RNA
141 was isolated from harvested samples using TRIzol reagent (Invitrogen). A standard
142 Illumina small RNA library preparation kit was used to prepare small RNA sequencing
143 libraries from total RNAs. Briefly, a total of 2 µg sRNAs in the size range of 15 to 30
144 nucleotides were purified and ligated to 3' adaptor, and isolated by 15% denaturing
145 polyacrylamide gel electrophoresis gels to eliminate un-ligated 3' adaptors. The products
146 were ligated to 5' adaptor and then were used to conduct reverse transcription PCR. The
147 final PCR product was isolated by 3.5% agarose gel electrophoresis and served as a small
148 RNA library for the sequencing. The libraries were quantified and sequenced at
149 HiSeq2000 analyzer to produce single-end 50 bp reads. Two biological replicates were
150 employed in the sRNA sequencing experiment.

151

152 **sRNA data process**

153 Trimmomatic (version 0.32) was used to trim the adaptor sequence of sRNA reads. The
154 parameters used for the trimming is: “ILLUMINACLIP:adaptor_seq:2:30:7:
155 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:13 MINLEN:16”. The adaptor
156 sequence (adaptor_seq) includes a sequence of
157 “CTGTAGGCACCATCAATCAGATCGGAAGAGCACACGTCTGAACCTCCAGTCA
158 C”. These parameters were used to perform both adaptor and quality trimming. Although
159 quality trimming could shorten actual sRNAs, the percentage of reads subjected to
160 quality trimming is only ~0.3%. Therefore, quality trimming was applied to remove the

161 low quality of nucleotides at the marginal compromise of changing sRNA lengths. At
162 least 16 nt in size was required for clean reads.

163

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

171

172 **Functional annotation of sRNAs**

173 The small RNA annotation database was downloaded from Rfam 11.0 (BURGE *et al.*
174 2013). sRNAs generated from this experiment were aligned to Rfam 11.0 database using
175 Blastn (BLAST 2.2.29+) with the following parameters (-evalue 1e-1 –word_size 10 –
176 perc_identity 0.89 –strand plus –best_hit_overhang 0.2 –best_hit_score_edge 0.1 –outfmt
177 6 –max_target_seqs 10). The sRNAs was functionally annotated only if they were
178 unambiguously hit an Rfam family.

179

180 **Alignment to the reference genome to determine copy number of sRNA regions**

181 Each sRNA was aligned to the B73 reference genome (RefGen2 and 4) using bwa
182 (version 0.7.5a-r405) (LI AND DURBIN 2010). The command parameters were “bwa aln –l
183 18 –k 0 –t 48 –R 22500” followed by “bwa samse –n 22500”. The alignments were then

184 parsed with the stringent criteria: perfect match with at least 18 bp matching length.

185 These alignment and parsing criteria allow the maximal 22,500 perfect hits.

186

187 **K-mer analysis using sequencing data to determine copy number of sRNA genomic
188 regions**

189 B73 whole genome shotgun Illumina sequencing data were downloaded from Genbank

190 (SRR444422). Trimmomatic (version 0.32) was used for the adaptor and quality

191 trimming with the same parameters to those used in the sRNA data trimming. The

192 adaptor sequences used for the adaptor trimming are

193 (TACACTTTCCCTACACGACGCTCTCCGATCT and

194 GTGACTGGAGTTCAGACGTGTGCTCTCCGATCT). The clean data were

195 subjected to error correction using the error correction module (ErrorCorrectReads.pl) in

196 ALLPATHS-LG (BUTLER *et al.* 2008) with the parameters of “PHRED_ENCODING=33

197 PLOIDY=1”. We then used the corrected sequencing data to perform k-mer counting

198 using the count function in JELLYFISH (MARCAIS AND KINGSFORD 2011) with the

199 parameters of “-m k-mer -L 2 -s 100M -C”, where the k-mer was from 18 to 30 nt. Once

200 the read depth of each k-mer from 18 to 30 nt was counted, the read depth of a

201 corresponding sRNA can be determined. The highest density of k-mer counts was located

202 at 26.96 for a set of known single copy k-mers determined by reference genome

203 alignments, indicating approximately 26.96x sequencing depth was obtained. This

204 number was used as the base of read depths of a single copy to adjust counts of each k-

205 mer to roughly represent its genome copy number.

206

207 **Determination of mean levels of various histone modifications**

208 ChIP-Seq data of H3K27me3, H3K36me3, H3K4me3, and H3K9ac (WANG *et al.* 2009),
209 and H3K9me2 (WEST *et al.* 2014) of 14-day B73 seedlings were downloaded from
210 Genbank. To match sequencing data to sRNA sequences, ChIP-Seq data were subjected
211 to k-mer counts at different k-mer lengths from 18 to 26 nt using JELLYFISH (MARCAIS
212 AND KINGSFORD 2011). Through k-mer counts, read counts from ChIP-Seq data of each
213 sRNA sequence was determined. Using sequencing read counts of whole genome
214 sequencing (WGS) data of sRNA sequences as the control, the histone modification
215 signal of each sRNA, represented by ChIP read count divided by WGS read count of an
216 sRNA, was calculated. Due to the lack of biological replication and the limited
217 sequencing depth of ChIP-Seq data, we did not attempt to assess the histone modification
218 level of each sRNA. Instead, mean of histone modification levels of all sRNAs in a
219 certain functional group (e.g., miRNA, rsRNA) were determined and used as the
220 modification level of that sRNA group for the comparison between functional groups.
221 Comparisons were only performed within the same length of sRNAs.

222

223 To enable the comparison of histone modification levels among different lengths of
224 sRNAs, all lengths of sRNAs from 18 to 26 nt were converted to 18 nucleotide fragments
225 and the average signal of five epimarks were determined separately. As a control, 18 nt of
226 different genic regions, promoters, first exons, internal exons, introns, and last exons,
227 were sampled and the average histone modification levels of five epimarks of each genic
228 region were calculated.

229

230 **Identification of drought-responsive sRNAs**

231 A generalized linear model was fitted for each sRNA to identify drought responsive
232 sRNAs. The response variable in the model is the read count of an sRNA, which were
233 assumed to follow negative binomial distribution. The model contains two factors, DAW
234 (day) and treatment, and their interactions. The DAW has eight factor levels (from 3 to
235 10) and the treatment has two factor levels (DS and WW). A deviance test of no
236 interaction effect between DAW and treatment was conducted for each sRNA. The
237 generalized linear model fit and test, assuming a negative binomial distribution for read
238 counts, were implemented in DESeq2 (LOVE *et al.* 2014). sRNAs having at least five
239 reads on average per sample were used for the statistical test, resulting in a p-value from
240 each sRNA. A false discovery rate (FDR) approach was applied to account for multiple
241 comparisons (BENJAMINI AND HOCHBERG 1995). Significant sRNAs were declared using
242 the 5% FDR as the cutoff. The script was deposited at GitHub
243 (https://github.com/liu3zhenlab/sRNAs_drought).

244

245 **Clustering of drought-responsive sRNAs**

246 Drought responsive sRNAs were subjected to clustering analysis using mclust (FRALEY
247 AND RAFTERY 2007). For each drought-responsive sRNA, the Log2 of the ratio of the
248 mean of DS expression (the normalized value) to the mean of WW expression (the
249 normalized value) at a certain DAW (day) was determined, which represents the Log2 of
250 the fold change in expression between DS and WW. Log2 ratio values were then used for
251 the clustering analysis. The script was deposited at GitHub
252 (https://github.com/liu3zhenlab/sRNAs_drought).

253

254 **Identification of significantly differentially expressed sRNAs between DS and water
255 recovery**

256 To test the null hypothesis that no difference in sRNA expression between two groups at
257 11 DAW, DR and water recovery (DWR), generalized linear model for the read count of
258 each sRNA implemented in the DESeq2 package (version 1.4.5) was used (LOVE *et al.*
259 2014). A false discovery rate (FDR) approach was used to account for multiple tests
260 (BENJAMINI AND HOCHBERG 1995). The FDR 5% was used as the cutoff for declaration
261 of differential expression.

262

263 **Enrichment analysis**

264 The enrichment analyses were performed for determining if a certain type of category,
265 such as a member of sRNA functional families, is over-represented in a selected group of
266 sRNAs. To account for the biases read depth that influences the selection of members in a
267 certain group, the resampling method in the GOSeq enrichment test (YOUNG *et al.* 2010)
268 with the bias factor of read depth, total reads across all the samples of a certain sRNA,
269 was applied to enrichment analyses.

270

271 **Analysis of sRNA co-expression network**

272 Drought-responsive sRNAs (FDR < 1%) were used to build co-expression sRNA
273 network using Bioconductor package WGCNA (v1.51) (LANGFELDER AND HORVATH
274 2008). WW sRNA network was built using sRNA expression profiles in WW samples,

275 and DS sRNA network was constructed using sRNA expression profiles in DS samples.
276 The package WGCNA uses an appropriate soft-thresholding power to construct a
277 weighted gene network. Modules of highly correlated sRNAs were identified using
278 topological overlap measure (TOM) implemented in WGCNA. Module preservation
279 analysis was also performed using WGCNA, with DS network as a test and WW network
280 as a reference, and vice versa. An R script for network analysis has been deposited at
281 GitHub (https://github.com/liu3zhenlab/sRNAs_drought).

282

283 **Identification of miRNAs**

284 The database of mature miRNAs was downloaded from miRBase v22
285 (<ftp://mirbase.org/pub/mirbase>). In total, 325 mature B73 maize miRNAs from 174
286 miRNA genes were extracted. Any sRNAs discovered in this study identical to these
287 mature miRNAs were annotated as known miRNAs.

288

289 ShortStack (v3.8.5) was used to *de novo* identify a set of miRNAs with the parameters (–
290 dicermin 18 –dicermax 30 –mismatches 0 –mincov 0.5rpm), and using B73Ref4
291 (version 4) as the reference genome (AXTELL 2013b). ShortStack identified novel
292 miRNA loci that did not overlap with any known miRNA genes. Any mature miRNAs
293 from novel miRNA loci were referred to as novel miRNAs. Some mature miRNAs from
294 ShortStack are not known miRNAs but from known miRNA genes. Combining both
295 known mature miRNAs and all newly discovered mature miRNAs by ShortStack using
296 our massive sRNA datasets, we updated the miRNA set, referred to as
297 B73miRBase22plus.

298

299 **Identification of IsomiRs**

300 IsomiRs are variants of the reference mature miRNAs (MORIN *et al.* 2008). An isomiR in
301 this study is a small RNA perfectly matching a pri-miRNA but with a different sequence
302 from mature miRNAs in the B73miRBase22plus. Only 20-22 nt sRNAs identical to the
303 plus-stranded sequence of a region of pri-miRNAs were referred to as isomiRs.

304

305 **Identification of ta-siRNAs**

306 sRNAs matching ta-siRNA downloaded from tasiRNADB
307 (<http://bioinfo.jit.edu.cn/tasiRNADatabase/>) were defined as known tasiRNA. Also
308 sequences of maize trans-acting siRNA 3 (TAS3) were retrieved from Dotto *et al.*
309 (DOTTO *et al.* 2014).

310

311 **Degradome analysis of drought-responsive miRNAs**

312 Degradome raw reads were obtained from a previous maize miRNA study (LIU *et al.*
313 2014). After removing adaptor sequences and low-quality sequencing reads, clean reads
314 were used to identify cleavage sites based on B73 cDNA sequences (5b+). CleaveLand
315 4.0 was implemented for degradome analysis with the default parameters (ADDO-QUAYE
316 *et al.* 2009), which provides evidence for gene targeting by miRNAs or isomiRs.

317

318 **Prediction of miRNA targeted genes and GO enrichment analysis of targeted genes**

319 psRNATarget (<http://plantgrn.noble.org/psRNATarget/>) was used to predict miRNA
320 target genes (DAI AND ZHAO 2011) . Gene targets of miRNAs were predicted based on

321 B73 AGPv3.22 annotated transcript sequences with the expectation value no more than
322 1.5. Gene ontology (GO) enrichment of predicted miRNA-targeting genes was analyzed
323 with AgriGO (TIAN *et al.* 2017).

324

325 **Transposable element analysis of 24 nt genomic loci**

326 sRNA genomic clusters, from the ShortStack result, predominant by 24-nt sRNAs were
327 referred to as 24-nt genomic loci. RepeatMasker (open-4.0.5) was used to identify
328 sequences matching transposable elements with the maize transposon database. As a
329 control, the “shuffle” module in the bedtools was employed to randomly select intervals
330 simulating the number and sizes of genomic intervals of 24-nt loci.

331

332 **Data Availability**

333 The datasets supporting the conclusions of this article are included within the article and
334 its supplemental materials. Supplemental files available at FigShare. All sRNA
335 sequencing raw data were deposited at Sequence Read Archive (SRA) (accession
336 number: SRP081275).

337

338 **Results**

339 **Physiological changes of seedlings under drought conditions**

340 Maize seedlings were subjected to drought over a period of nine days (**Figure 1A**).
341 Three-day-old B73 seedlings after germination were subjected to two treatments, drought
342 stress (DS) and well-watered (WW). Above ground tissues (referred to here as leaves)
343 were collected at 3 to 10 Days After Withholding water (DAW) or with watering with two

344 biological replicates at each day. At 10 DAW, some seedlings from the DS treatment
345 group were subjected to two treatments: continuously withholding water (DS) and re-
346 watering, both of which were sampled at the 11th day. Two biological replicates were
347 collected, resulting in two additional DS samples on day 11 and two re-watering samples
348 at one day after addition of water at day 10. A total of 36 plant samples were processed.
349 Compared to WW seedlings, DS-treated seedlings showed severe stressed phenotype by
350 8 DAW. Soil water content (SWC) decreased in the DS treatment from ~60% to 20% in
351 the same period (**Figure 1B**). Leaf relative water content (RWC) of DS seedlings also
352 decreased upon the drought treatment, at a low declining rate from 3 to 7 DAW and a
353 high rate after 7 DAW (**Figure 1C**). Leaf relative electrical conductivity (REC), which is
354 a measure of cellular damage, exhibited strongest response to drought between 8 and 9
355 DAW (**Figure 1D**), indicating that leaf cells began to experience damage after 8 DAW
356 under drought conditions. The DS-treated seedlings showed visible stressed phenotypes
357 after 10 DAW. When re-watered at 10 DAW, the DS-treated plant seedlings were visibly
358 recovered at 11 DAW.

359

360 **Characterization of sRNAs**

361 The 36 RNA samples were extracted for sRNA sequencing, resulting in more than 886.6
362 millions of 50 bp single-end reads, from 20.5 to 34.2 millions reads per sample. On
363 average, 97.5% of reads were retained after adaptor and quality trimming of each sample
364 (**Table S1**). The majority of sRNAs were between 18 and 26 nucleotides (nt). The 24-nt
365 sRNA length class was the largest, followed by the 21 nt and 22 nt sRNA classes (**Figure**

366 **2A).** The same pattern of length distribution was observed across all the samples,
367 indicating that the drought treatment did not alter the global pattern of sRNA lengths.
368

369 All sRNA reads from the 36 samples were merged, and sRNAs with at least 72 reads
370 were retained. Removing redundant reads with the same sequence for each sRNA
371 resulted in a non-redundant sRNA (NR-sRNAs) set of unique 736,372 sRNAs (134,283
372 NR-sRNAs used in the later time-series statistical analysis were listed in **Table S2** and
373 **Table S3**. The NR-sRNAs set was annotated using the Rfam database (Rfam11.0). 12.4%
374 (91,473) of the NR-sRNAs could be unambiguously annotated with regard to function
375 (see Methods). Among the Rfam-annotated subset of sRNAs, rsRNAs, tsRNAs, and
376 miRNAs are the most abundant, comprising of 40%, 27%, and 7%, respectively (**Figure**
377 **2B**). The rsRNA and tsRNAs represented nearly 70% of all annotated NR-sRNAs, while
378 miRNAs distributed in a slightly narrower length range of 18 to 24 nt and a peak length
379 at 21 nt (**Figure 2C**). Of 21 nt NR-sRNAs, 22% are miRNAs from approximately 65% of
380 the total 21-nt sRNA reads (redundant sRNAs), indicating that some 21 nt miRNAs were
381 highly expressed (**Figure 2C, 2D**). Indeed, the single sRNA showing the highest
382 abundance is a miR159, with 14.8 million reads.

383

384 **Genome organization of NR-sRNAs in B73**

385 The copy number of individual NR-sRNAs in the B73 genome was estimated by both
386 mapping reads to the B73 reference genome (reference-based) and analyzing sequences
387 present in whole-genome-shotgun sequence reads (WGS-based) (see Methods). A small
388 number of NR-sRNAs, 20,452, were excluded based on alignment to either chloroplast or

389 mitochondria DNA. Among the remaining NR-sRNAs (N=705,920), perfect matches for
390 93.2% of the NR-sRNAs were identified in either the B73 reference genome or the B73
391 WGS data. The absence of perfect matches for 6.8% of the NR-sRNAs was attributed to
392 incomplete B73 genome assembly, contamination, sequencing errors, and/or RNA editing
393 (LIANG AND LANDWEBER 2007; SCHNABLE *et al.* 2009). The estimations of copy number
394 from the two approaches were largely consistent (**Figure S1**). Both estimations indicated
395 that most NR-sRNAs are from low-copy genomic loci (1-2 copies) except for NR-sRNAs
396 from rRNA and tRNA (**Figure S2**). NR-sRNAs of differing lengths exhibit varying
397 mixtures of low- and high-copy loci (**Figure S3**). The 24 nt sRNAs are mostly single
398 copy in the genome, while a high proportion of 21-23 nt sRNAs are derived from either
399 low-copy or very-high-copy genomic loci. Outside of the 21-24 sRNA range, NR-sRNAs
400 from highly repetitive genomic regions are dominant (**Figure S3**).

401
402 A linear association between expression level and genomic copy number of sRNAs was
403 not observed (**Figure S4**). Genomic single-copy NR-sRNAs can be highly expressed. For
404 example, the single copy miR168 locus was expressed at a high level (138,292 reads).
405 Conversely, the expression of most genomic high-copy NR-sRNAs was low. Some high-
406 copy NR-sRNAs were highly expressed, such as rsRNAs. Analysis of sRNA expression
407 profiles based on functional classes also showed that high proportions of splicing sn-
408 sRNAs and sno-sRNAs exhibit low expression, while many rsRNAs were expressed at a
409 high level (**Figure 2E**). The 23 and 24 nt sRNAs, regardless of functional classes, were
410 mostly expressed at a low level, while 20-22 nt sRNAs tended to be expressed at

411 relatively higher levels (**Figure 2F**). Compared to 21-24 nt sRNAs as a whole, a higher
412 proportion of 20 nt sRNAs were highly expressed (**Figure 2F**).

413

414 **Distinct histone modifications at genomic regions of different classes of sRNAs**

415 Histone modification status at genomic regions of sRNAs was collected from genome-
416 scale data repositories for B73 seedlings, including multiple histone modifications:
417 H3K27me3, H3K36me3, H3K4me3, H3K9ac, and H3K9me2. The lack of biological
418 replication and low depth of most chromatin modification data limited assessment of
419 histone modification levels for each sRNA locus. Therefore, the mean of histone
420 modification levels of genomic regions in each functional sRNA class was used to
421 represent the overall genomic modification level of each sRNA functional class. To avoid
422 systematic biases, we compared histone modifications among different functional classes
423 at the same sRNA length (**Figure 3, Figures S5-S8 and Table S4**). Average histone
424 modification levels on different functional classes showed that both miRNAs and sno-
425 sRNAs in size of 20, 21, and 23 nt were predominately found in open chromatin regions,
426 which were characterized by high modification levels of two hallmarks of open
427 chromatin regions, H3K4me3 and H3K9ac. The H3K4me3 signal at sno-sRNA genomic
428 regions was much higher than those of genomic regions of any other functional sRNA
429 classes across all lengths from 20 to 24 nt. Genomic regions of 21 nt miRNAs and sno-
430 sRNAs, overall, had moderate levels of a silent chromatin mark H3K9me2, and genomic
431 regions of 20 and 23 nt miRNAs and sno-sRNAs exhibited low levels of H3K9me2
432 signals. H3K9me2 is associated with CHG (where H is A, T, or C) cytosine methylation
433 (STROUD *et al.* 2013; WEST *et al.* 2014), indicating that genomic regions producing

434 miRNAs and sno-sRNAs, on average, exhibited low CHG cytosine methylation. At the
435 lengths of 20, 21, 23 nt, miRNA genomic regions had high levels of H3K27me3, a
436 repressive chromatin mark associated with gene silence (GAN *et al.* 2015), and relatively
437 low H3K36me3 levels that are generally positively associated with transcriptional
438 activity but also were found to be enriched at heterochromatin regions (CHANTALAT *et al.*
439 2011). The sno-sRNA genomic regions, relative to miRNA genomic regions, exhibited
440 the opposite modification pattern with low H3K27me3 levels and high H3K36me3 levels.
441 Genomic regions of rsRNAs, splicing sn-sRNAs, and tsRNAs had similar histone
442 modification patterns, namely, low levels of H3K27me3, H3K36me3, H3K4me3 and
443 H3K9ac and a high level of H3K9me2 across all lengths from 20 to 24 nt.

444
445 Through converting all lengths of sRNAs to the same 18 nt length, average signals of
446 histone modifications were compared among genomic regions producing different
447 lengths of sRNAs. As the control, 18 nt DNA fragments were also randomly sampled
448 from different genic regions, and their mean signals of histone modifications were
449 determined. The results showed that, except for H3K36me3, all epimarks shared a similar
450 trend that the modification signals were at a relatively high level for genomic regions of
451 small lengths of sRNAs and gradually decrease until at 24 or 22 nt, followed by elevated
452 modification signals (**Figure S9**). For open chromatin marks H3K4me3 and H3K9ac, on
453 average sRNAs exhibited lower levels relative to promoters and first exons but similar
454 levels to internal exons, introns and last exons. Our result showed that H3K9me2 was
455 generally at much higher levels on sRNA genomic regions relative to genic regions, of
456 which 24 nt sRNAs whose genomic regions had the closest H3K9me2 signal to genic

457 regions. That indicated that the CHG cytosine DNA methylation at genomic regions of 24
458 nt sRNAs is generally low.

459

460 **Identification of drought-responsive sRNAs**

461 A statistical test was performed to detect any interaction between drought stressed and
462 well-watered plants for each sRNA that had a minimum five sRNA reads per sample over
463 the 3 to 10 DAW period. The analysis revealed that 6,646 of the total 134,283 sRNAs
464 exhibited interactions between the DAW and the treatments at the 5% false discovery rate
465 (FDR) level (**Table S3**). Interacting sRNAs showing different responses under DS and
466 WW conditions at certain DAWs were scored as drought-responsive sRNAs. The
467 rsRNAs and 22 nt sRNAs are the two predominant groups in the drought-responsive
468 sRNA set (**Figure S10**). The DS-to-WW ratios of sRNA expression were further
469 subjected to cluster analysis using mclust (FRALEY AND RAFTERY 2007), resulting in 10
470 clusters. The sRNAs of clusters 3, 4, 5, 7, and 9 exhibited a pattern of up-regulation
471 under drought stress (**Figure 4A-F**), while sRNAs of clusters 1 and 8 showed a pattern
472 for down-regulation (**Figure 4G-I**). More than five times up-regulated sRNAs (N=4,373)
473 were detected than down-regulated sRNAs (N=816) under drought stress (**Figure 4**). The
474 enrichment analyses indicate that rsRNAs and splicing sn-sRNAs were over-represented
475 in up-regulated sRNAs, while miRNAs and sno-sRNAs were over-represented in down-
476 regulated sRNAs. Additionally, sRNAs of clusters 2 and 6 exhibited transiently down-
477 regulation on drought (transiently down-regulation group, N=1,325), which were down-
478 regulated at around 7 DAW when drought stress became intense, followed by a gradual

479 recovery of expression (**Figure 4J-L**). The enrichment analysis indicates that miRNAs
480 and sno-sRNAs are significantly over-represented in transiently down-regulated sRNAs.

481

482 A comparison of sRNA expression was performed between two additional seedling
483 groups at 11 DAW, DS and drought water recovery (DWR), which was re-watered on 10
484 DAW. Using the 5% FDR cutoff, 7,140 sRNAs were differentially expressed between
485 two groups, of which 2,264 and 4,876 sRNAs were up-regulated and down-regulated in
486 DWR relative to DS, respectively, and 486 were identified as drought-responsive sRNAs
487 in the time-series analysis (**Table S3, S5**). The 473 sRNAs (out of 486) were classified
488 into three groups in the time-series analysis: Down-regulated (N=43), up-regulated
489 (N=426), and transiently down-regulated (N=4). All 43 sRNAs from the down-regulated
490 group were up-regulated after DWR. Of 426 sRNAs in the up-regulated group, 76.3%
491 (325/426) sRNAs showed decreased expression in DWR, while 23.7% (101/426) were
492 continuously up-regulated even with water recovery. All four sRNAs in the transiently
493 down-regulated response group were up-regulated after re-watering. Overall, the
494 expression levels of most drought-responsive sRNAs were restored towards levels of
495 well-watered plants upon re-watering.

496

497 **Characteristics of co-expression networks of drought-responsive sRNAs**

498 DS and WW weighted co-expression networks were constructed using WGCNA
499 (LANGFELDER AND HORVATH 2008). Both networks consist of a subset of drought-
500 responsive sRNAs with the FDR cutoff of less than 1% from the drought response
501 statistical test. The DS and WW networks were built using normalized sRNA counts of

502 DS and WW samples, respectively (**Figure 5A, 5B, Table S3**). Network statistics
503 indicate intrinsic differences between the two networks (**Table S6**). Although the DS and
504 WW networks share similar network clustering coefficients, network centralizations, and
505 network densities, the DS network (**Figure 5B**) has the smaller network diameter and
506 lower heterogeneity, indicating expression of these drought-responsive sRNAs were more
507 correlated upon drought stress or tended to be co-expressed in response to drought stress.
508
509 Modularity analysis in the DS network and the WW network further revealed that the two
510 networks have different topology structures. Modularity analysis included two steps:
511 module identification and module preservation analysis. Modules are sub-networks,
512 consisting of co-expressed sRNAs. The sRNAs in the same module are similar in
513 expression to some degree, thereby are likely associated each other. Module preservation
514 analysis is used to determine if the topology of a network module identified in one
515 network changes in the other network. For example, a module is considered preserved in
516 the DS network, if its topology, based on preservation statistics, largely remains in the
517 WW network. The module preservation analysis identified a preserved module (blue
518 module) in the DS network compared to the WW networks (**Figure 5C**) and a preserved
519 module (blue module) in the WW network in comparison to the DS networks (**Figure**
520 **5D**). Most sRNAs (N=546) in two blue modules overlapped, of which more than 95% are
521 from the transiently down-regulated group (**Table S3**). The result indicated transiently
522 down-regulated sRNAs tended to be co-regulated in both drought and well-watered
523 conditions. On the other hand, these sRNAs exhibited a transient down-regulation to
524 drought, which might serve as the signal to induce downstream drought responses. Of

525 546 overlapping sRNAs, 343 and 178 are 22 nt and 24 nt sRNA, respectively, and a few
526 were functional annotated with the Rfam database (6 miRNAs and 9 sno-sRNAs). The
527 module preservation analysis also revealed differences between modules in the DS and
528 WW networks. The yellow module in the DS network is the least preserved module,
529 indicating sRNAs of the module were perturbed in response to drought stress (**Figure**
530 **5C**). Indeed, the yellow module consists of 38 sRNAs that were down-regulated upon
531 drought stress. In the WW network, the green module is the least preserved one, and most
532 sRNAs were up-regulated upon drought.

533

534 **Identification of drought-responsive miRNAs and the corresponding targeted genes**
535 sRNA homologous to Rfam miRNAs were referred to as miRNAs hereinbefore. We
536 refined the miRNA set based on the dedicated miRNA database, miRBase (KOZOMARA
537 AND GRIFFITHS-JONES 2014), and *de novo* discovery of miRNAs from our massive
538 datasets. We employed the ShortStack pipeline (AXTELL 2013b) and identified 53
539 miRNA loci of which 47 loci are known maize miRNA genes in miRBase (v22)
540 containing 174 miRNA genes. We found 59 new mature miRNAs from, including 47
541 mature miRNAs from known miRNA loci but with different sequences of mature
542 miRNAs, as well as 12 mature miRNAs from 6 novel miRNA loci. Requiring at least an
543 18 nt match with at least 90% identity, homologs of miRNAs from three novel miRNA
544 loci (Cluster_23765, Cluster_27697, and Cluster_45700) were identified in MIR1878,
545 MIR156c, and MIR166d, respectively. We combined both known and newly discovered
546 mature miRNAs to create a new miRNA set referred to as B73miRBase22plus (**Table**
547 **S7**) that contains 180 miRNA genes producing 392 mature miRNAs, of which 244 are

548 non-redundant miRNAs (**Table S8**). We also identified 608 isomiRs that are in length of
549 20-22 nt and identical to a region of a pri-miRNA sequence, but different from 392
550 mature miRNAs in sequence (**Table S9**).

551

552 Some miRNAs were highly expressed. The top eight most highly expressed miRNAs
553 belong to six families: miR159, miR168, miR396, miR156, miR169, and miR167 (**Table**
554 **S8**). Although highly expressed miRNAs, statistically, are most likely to be detected,
555 none of the top 25 miRNAs showed evidence of regulation under drought condition,
556 indicating that expression levels of most highly expressed miRNAs were kept at
557 relatively stable levels under drought stress. In total, 21/244 miRNAs and 18/608 isomiRs
558 showed significantly drought responses (**Table 1**). Most drought-responsive miRNAs
559 (N=13) were down-regulated by drought treatment, while four were up-regulated. The
560 remaining four were not categorized to any of the three major cluster groups. The 21
561 drought-responsive miRNAs belong to 13 families, including miR1432, miR156,
562 miR164, miR166, miR167, miR168, miR171, miR319, miR390, miR398, miR399,
563 miR408, and miR528 (**Table 1**). The miR390a-3p or miR390b-3p (miR390a/b-3p) of the
564 miR390 family was drought responsive. But no significant regulation on drought was
565 observed for miR390a/b-5p (AAGCUCAGGAGGGAUAGCGCC) that cleaves trans-
566 acting siRNA 3 (TAS3) loci to produce ta-siRNAs (ALLEN *et al.* 2005; WILLIAMS *et al.*
567 2005; DOTTO *et al.* 2014; XIA *et al.* 2017). Predicted TAS3 ta-siRNAs triggered by
568 miR390a/b-5p were either low expressed or with no significant regulation upon drought
569 stress (**Table S10**). For isomiRs, 7, 8, and 3 were in down-regulation, up-regulation, and
570 uncategorized groups, respectively, adding two additional miRNA families, miR396 and
571 miR444, showing drought responses. Notably, multiple isomiRs, and mirR156i-3p, from

572 the miR156 family were up-regulated on drought (**Table 1**). However, miR156j-3p was
573 down-regulated, implying that family members play divergent regulatory roles.

574

575 Targeted protein-coding genes of 21 miRNAs and 18 isomiRs responded to drought were
576 predicted with the psRNATarget tool (DAI AND ZHAO 2011). In total, 67 pairs of gene-
577 miRNA, including 43 non-redundant genes, were predicted to be targeted by 18 drought-
578 responsive miRNAs and isomiRs (**Table S11**). GO enrichment analysis showed that 43
579 miRNA-targeting genes are highly enriched in DNA binding function (GO:0003677, p-
580 value = 2.1E-16) and nucleus cell component (GO:0005634, p-value = 6.1E-16) (**Table**
581 **S12**), suggestive of considerable impacts of miRNAs on the genes regulating
582 transcription under drought stress. Nearly half of targets (18/43) are putative SPL
583 (Squamosa promoter binding protein-like) transcription factors, and 17/18 are targeted by
584 two isomiRs of the miR156 (GACAGAAGAGAGUGAGCACA and
585 UGACAGAAGAGAGUGAGCACA). SPL genes have been reported to be associated
586 with miR156 under drought condition in multiple plant species, such as rice (NIGAM *et al.*
587 2015) cotton (WANG *et al.* 2013), alfalfa (ARSHAD *et al.* 2017), and maize (MAO *et al.*
588 2016). In our result, both SPL-targeting miR156 were up-regulated upon drought (**Figure**
589 **6**), indicating the possible regulation in expression of SPL genes through miRNAs during
590 drought treatment. Another drought-responsive miRNA miR319a/b-3p
591 (UUGGACUGAAGGGUGCUCCC) was predicted to target one MYB and two TCP
592 transcription factors (GRMZM2G028054, GRMZM2G089361, GRMZM2G115516)
593 (ZHANG *et al.* 2009; LIU *et al.* 2014). This miR319a/b-3p remained at a low expression
594 level under high drought stress (**Figure S11**). Presumably, the expression of targeted

595 genes was under a low level of suppression imposed by miR319 under drought condition.
596 Indeed, one of three genes GRMZM2G115516 was up-regulated >4 times on drought
597 (**Table S11**) (LIU *et al.* 2015). The transcriptional regulation of genes targeted by
598 isomiRs of miR156 and miR319a/b-3p was well supported from degradome sequencing
599 data (**Table S11**), which were used to identify miRNA cleavage sites (SHEN *et al.* 2013;
600 ZHAI *et al.* 2013; LIU *et al.* 2014).

601

602 **Discussion**

603 In this study, sRNA sequencing was performed on samples of maize seedlings under
604 drought stress (DS) and well-watered (WW) conditions. The sRNAs were characterized
605 with respect to sRNA lengths, functional class, as well as copy number and epigenetic
606 modifications of sRNA genomic regions. Genomic copy number analysis indicates that
607 most 18-20 nt and 25-30 nt NR-sRNAs and approximately half of the 21-23 nt NR-
608 sRNAs are derived from high-copy genomic repeats. The 24 nt sRNAs were the
609 predominate species among single-copy sRNAs in this study, which is inconsistent with
610 the observations in most other plant species. In fact, 24 nt sRNAs are generally referred
611 to as heterochromatic siRNAs and are primarily derived from intergenic and/or repetitive
612 genomic regions (DUNOYER *et al.* 2007; KASSCHAU *et al.* 2007; AXTELL 2013a).
613 However, 24 nt sRNAs were also recently shown to be enriched in euchromatic regions
614 with low DNA cytosine methylation in an independent maize study (HE *et al.* 2013),
615 which is consistent with our observation. Based on ShortStack sRNA genomic mapping,
616 24-nt sRNA genomic loci were largely located at intergenic regions, but closer to protein-
617 coding genes compared to randomly shuffled simulated loci (**Figure S12**). The proximity

618 of 24-nt sRNA genomic loci to protein-coding genes, particularly highly expressed genes,
619 was previously observed (LUNARDON *et al.* 2016), and the 24-nt sRNA was proposed to
620 function to reinforce silencing of transposable elements close to active genes (LI *et al.*
621 2015a). Our transposon analysis found that 24-nt sRNA genomic loci were over-
622 represented at regions containing DNA transposon elements but under-represented at
623 regions containing LTR retrotransposon elements, *Copia* and *Gypsy* (**Table S13**),
624 suggesting the 24-nt sRNA might be more critical for silencing of DNA transposon
625 elements. Compared to other lengths of sRNAs, genomic regions generating 24 nt sRNAs
626 exhibited low histone modification levels for all histone epimarks examined. Given that
627 most 24 nt sRNAs are generated by PolIV, heavy nucleosome loading and/or strong
628 histone modifications of examined epimarks are likely not prerequisites for transcription
629 via PolIV (LI *et al.* 2015b; LUNARDON *et al.* 2016).

630

631 High proportions of sRNAs with two genomic copies were found in 21 and 22 nt sRNAs
632 but not in 23 or 24 nt sRNAs. Production of most 23 and 24 nt sRNAs requires RNA-
633 dependent RNA polymerase 2 (RDR2) to form dsRNAs and do not require multiple
634 genome copies for optimal function (NOBUTA *et al.* 2008). Two identical copies in the
635 genome could increase the chance for the expression of sense and antisense transcripts to
636 form NAT-siRNAs, which indicates that many 21 and 22 nt sRNAs might be NAT-
637 siRNAs. Genomic regions of 21 and 22 nt sRNAs have the highest modification levels of
638 H3K36me3 among all lengths of sRNAs, resembling H3K36me3 modification levels of
639 internal genic regions (internal exons and introns). The high H3K36me3 signals of 21 and
640 22 nt sRNAs genomic regions are likely contributed by sno-sRNA genomic regions,

641 which exhibited the highest H3K36me3 modification levels. Our results revealed the
642 complexity of histone modifications of plant sRNA genomic regions. However, the lack
643 of high depth of epimark data as well as the different experimental sources between
644 epimark information and sRNA expression data restrict the conclusion about their
645 correlation at a single locus level. Future stratification based on sRNA length, function,
646 as well as genomic and more informative epimark information of sRNA genomic regions
647 would be useful for understanding biogenesis and cellular function as well as further
648 classification of sRNAs.

649

650 Characterization of drought-responsive sRNAs indicates that sRNAs are differentially
651 expressed in response to drought stress. The miRNAs of maize were clustered into three
652 groups based on expression patterns, namely, up-regulated, down-regulated, and
653 transiently down-regulated upon drought stress and over-represented in the down-
654 regulated group, in which miRNAs were approximately 4.8x enriched. The miRNAs and
655 cognate gene targets are involved in drought stress responses in many plant species such
656 as *Arabidopsis* (BUTLER *et al.* 2008), rice (ZHOU *et al.* 2010; FANG *et al.* 2014), soybean
657 (AXTELL 2013b) and poplar (SHUAI *et al.* 2013). Drought-induced miRNAs suppress
658 their target mRNAs, while down-regulated miRNAs result in the de-repression of the
659 target mRNAs (FERDOUS *et al.* 2015). The miRNAs may exhibit distinct responses to
660 drought stress in different plant species (ZHAI *et al.* 2015). For example, miR168a/b
661 down-regulated on drought in rice (ZHOU *et al.* 2010), but was induced in response to
662 drought stress in maize. We have identified 39 drought-responsive miRNAs or isomiRs,
663 as well as their potential gene targets. Detailed studies on their regulatory networks and

664 their functional divergence among species or genotypes within a species would be
665 valuable to modulate miRNA-mediated pathways for improving drought tolerance of
666 plants.

667

668 In addition to miRNAs, sRNAs derived from rRNAs, tRNAs, snoRNAs, and splicing
669 snRNAs were also differentially regulated under drought condition. rRNAs are an
670 essential component of ribosomes and catalyzes protein assembly. rsRNAs (small RNAs
671 derived from rRNAs) were over-represented in the up-regulated sRNA group. rsRNAs
672 were significantly enriched in down-regulated sRNAs after addition of water at 10 DAW.
673 Thus, drought response involves an increase of rsRNAs, which is, in turn, suppressed
674 when water was supplied. Transfer RNAs (tRNAs) play an essential role in protein
675 synthesis. Although tsRNAs (small RNAs derived from tRNAs) are not enriched in either
676 up- or down-regulated sRNAs groups, up-regulated tsRNAs are almost seven times more
677 represented than down-regulated tsRNAs (148/22), which is higher than the ratio of all
678 up-regulated sRNAs to all down-regulated sRNAs (4,373/816). A barley sRNA study
679 also found that tsRNAs, overall, have a tendency to be up-regulated under drought
680 condition (HACKENBERG *et al.* 2015). Both rsRNA and tsRNA were abundant at all
681 lengths from 18 to 27 nt, implying that the cleavage activity of rRNA and tRNA is not
682 size-specific. Likely an unknown RNase III member is involved in rRNA or tRNA
683 cleavage, producing sRNAs with a broad range of lengths (WU *et al.* 2000). Splicing sn-
684 sRNAs, derived from splicing snRNAs that are involved in pre-mRNA splicing, were
685 over-represented in up-regulated sRNAs on drought. Alternative splicing of pre-mRNA
686 splicing under drought stress was observed in multiple tissues, particularly in the leaf and

687 ear (THATCHER *et al.* 2016), which might partially attributes to amount and stability of
688 various splicing sn-RNAs.

689

690 The snoRNAs primarily include two classes of sRNAs, box C/D and box H/ACA
691 snoRNAs, which guide methylation and pseudouridylation of other RNAs, respectively
692 (BACHELLERIE *et al.* 2002; KISS 2006). The snoRNA-mediated chemical modifications of
693 rRNAs and splicing snRNAs have been demonstrated to be essential for ribosomal
694 function as well as mRNA splicing and maturation (MORRIS AND MATTICK 2014;
695 DUPUIS-SANDOVAL *et al.* 2015). The sno-sRNA was over-represented in both down-
696 regulated and transiently down-regulated sRNA groups under drought stress. Down-
697 regulation of sno-sRNAs may be the result of the reduction of snoRNAs, which would
698 reduce the activity of methylation and pseudouridylation of rRNAs and splicing snRNAs.

699 Given the reduction of sno-sRNAs and the increase of rsRNA and splicing sn-sRNAs
700 upon drought stress, it is tempting to speculate that rRNAs and splicing snRNAs are
701 destabilized with decreased methylations or pseudouridylation as mediated by snoRNAs.

702 Both changes in chemical modification, presumably, and the quantity of rRNAs upon
703 drought stress could effectively alter the activity of the protein synthesis machinery. The
704 observation of sRNA changes related to rRNAs and splicing snRNAs indicates the post-
705 transcriptional regulation is an important mechanism for adaptive response to drought
706 stress. snoRNAs exhibiting responses to drought were also found in another plant species
707 (HACKENBERG *et al.* 2015). Recently, snoRNAs were also found to be involved in
708 metabolic stress responses, including oxidative stress in human cells (MICHEL *et al.* 2011;
709 CHU *et al.* 2012; YOUSSEF *et al.* 2015). Taken together, we propose that snoRNAs play a

710 role to regulate biological processes under drought stress through chemical modifications
711 of rRNAs and splicing snRNAs.

712

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721

722 **Authors' contributions**

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

727

728 **Competing interests**

729 The authors have declared that no conflict of interests exists.

730

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913

914

915 **Table 1.** The list of drought-responsive miRNAs

miRNA sequence	Len (nt)	Total reads ¹	Genomic copy ²	miRNA gene	miRNA type	Cluster group ³
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
ACGGCGCGACGAACGACAUAGC	22	1,417	1	MIR156d	isomiR	Others
GCUCACUGCUCUAUCUGUCAUC	22	14,829	1	MIR156i	3p	UP
GCUCACUGCUCUAUCUGUCAU	21	1,116	1	MIR156i	isomiR	UP
GCUCUCUGCUCUACUGUCAUC	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
GGAAUGUGUCUGGGCGAGA	21	416	1	MIR166i	5p	DOWN
GGUUUGUUUGUCUGGUUCAAGG	22	2,613	1	MIR166j	5p	DOWN
GGAAUGUUGGCUGGGCUCGAGG	21	2,563	2	MIR166m, Cluster_45700	5p	DOWN
GAUCAUGCUGUGGCAGCCUACU	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
CGCUUUGGUGCAGAACGGGAC	20	19,995	2	MIR168a,b	isomiR	UP
UCGUUUGGUGCAGAACGGGAC	20	294,017	2	MIR168a,b	isomiR	UP
UCGUUUGGUGCAGAACGGGAC	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	Others
CGCUAUCAUCCUGAGCUCCA	21	9,684	2	MIR390a,b	3p	DOWN
CAGCUUUCUUGAACUUCUUCU	21	823	2	MIR396e,f	isomiR	DOWN
GGGGCGAACUGAGAACACAUG	21	5,992	1	MIR398a	5p	DOWN
AUGUGUUCUCAGGUCGCCCCG	22	1,920	2	MIR398a,b	isomiR	Others
GGGGCGGACUGGGAACACAUG	21	53,148	1	MIR398b	5p	DOWN
GGCGGACUGGGAACACAUGG	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
CAGGGGAUGAGACAGAGCAUG	20	12,523	1	MIR408a	isomiR	DOWN
CAGGGGAUGAGACAGAGCAUGG	21	51,773	1	MIR408a	isomiR	DOWN
CAGGGACGAGGCAGAGCAUGG	21	6,822	1	MIR408b	5p	DOWN
CAGGGACGAGGCAGAGCAUG	20	10,218	1	MIR408b	isomiR	Others
UGCAAGUUGUGCAGUUGUUGU	21	2,125	3	MIR444a,b	isomiR	UP
CCUGUGGCCUGCCUCUCCAUAU	21	8,186	2	MIR528a,b	3p	DOWN
CUGUGGCCUGCCUCUCCAUAU	20	1,137	2	MIR528a,b	isomiR	DOWN

916 ¹total sRNA reads from all 36 samples

917 ²Genomic DNA copy number using the reference-based method

918 ³Clustering group from the mclust analysis. Down and Up represent down-regulated and up-
919 regulated groups respectively on drought stress. Others represent the group that does not belong
920 to down-regulated, up-regulated, or transiently down regulated groups.

921

922 **Figure legends**

923 **Figure 1.** Morphological and physiological changes of maize seedlings during drought
924 stress. (A) Three-day-old B73 seedlings were subjected to gradual drought stress or well-
925 watered conditions. The photos were taken at each day from 3 to 11 days. Bar=5cm. (B)
926 The changing curves of water content of soil (SWC) from five replicated pots of each
927 data point. (C) Leaf relative water content (RWC) of seedlings along days. (D) Leaf
928 relative electrical conductivity (REC) of seedlings along days. Red and green curves
929 represent plants under drought stress and well water, respectively. Five seedlings were
930 pooled as one replicate, and five independent biological replicates were conducted to
931 determine RWC and REC. Vertical lines represent standard errors.

932

933 **Figure 2.** Characterization of sRNAs. (A) Proportions of sRNAs of different lengths in
934 all samples. Each curve represents a sample. WW, DS, and DWR, represent well-watered,
935 drought stress, and drought water recovered plants, respectively. (B-D) Overview of
936 genomic copy number, lengths, functional categories, and expression of NR-sRNAs from
937 all the samples. (B) Pie chart of distribution of different classes of sRNAs. Others
938 represent sRNAs that were not unambiguously categorized. (C) Stacked barplot of
939 different functional classes of NR-sRNAs at varying sizes of sRNAs from 18 to 30 nt. (D)
940 Stacked barplot of different functional classes of sRNA reads, representing expression
941 levels, at varying lengths of sRNAs from 18 to 30 nt. (E) Density plots of expression
942 levels of different functional classes of sRNAs. Density on the y-axis represents the
943 probability of sRNA occurrences. (F) Density plots of expression levels of different
944 lengths of sRNAs. Density on the y-axis represents the probability of sRNA occurrences.

945

946 **Figure 3.** Modification levels of five epimarks on sRNA genomic regions. The average
947 ChIP-Seq signals, represented by read depths of ChIP-Seq, of five epimarks were
948 determined and normalized by sequencing library sizes separately. Heights of bars
949 represent relative histone modification levels. The general function of each epimark is
950 briefly described in the subtitle of each barplot.

951

952 **Figure 4.** Major clusters of drought-responsive sRNAs. Drought-responsive sRNAs were
953 subjected to clustering using the software mclust, which ended with 10 clusters. Nine
954 major clusters (A-E, G, H, J, K) were classified into three groups, up-regulated (light
955 blue), down-regulated (light orange), and transiently down-regulated (light purple). Each
956 curve represents an average sRNA expression ratio of drought stress to well-watered with
957 a Log2 transformation from two biological replicates along DAW. Three pie charts
958 designate proportions of different classes of sRNAs that were functionally annotated in
959 each of the three clustering groups: up-regulated (F); down-regulated (I); transiently
960 down-regulated (L).

961

962 **Figure 5.** sRNA co-expression networks

963 (A) Visualization of the DS network using Cytoscape, each node represents an sRNA and
964 each line is the edge connecting sRNA nodes. Five modules (sub-network) were
965 highlighted by different colors. (B) Visualization of the WW network. Six modules (sub-
966 networks) were highlighted by different colors. Note that assignment of colors in (A) and
967 (B) are sort of independent. The same color might not represent the same group of

968 sRNAs. (C) Result of the module preservation analysis performed to evaluate whether a
969 module identified in the DS network is preserved in the WW network. The color code
970 corresponds to that used in (A). (D) Result of the module preservation analysis of the
971 WW network in comparison with the WW network. The color code corresponds to that
972 used in (B).

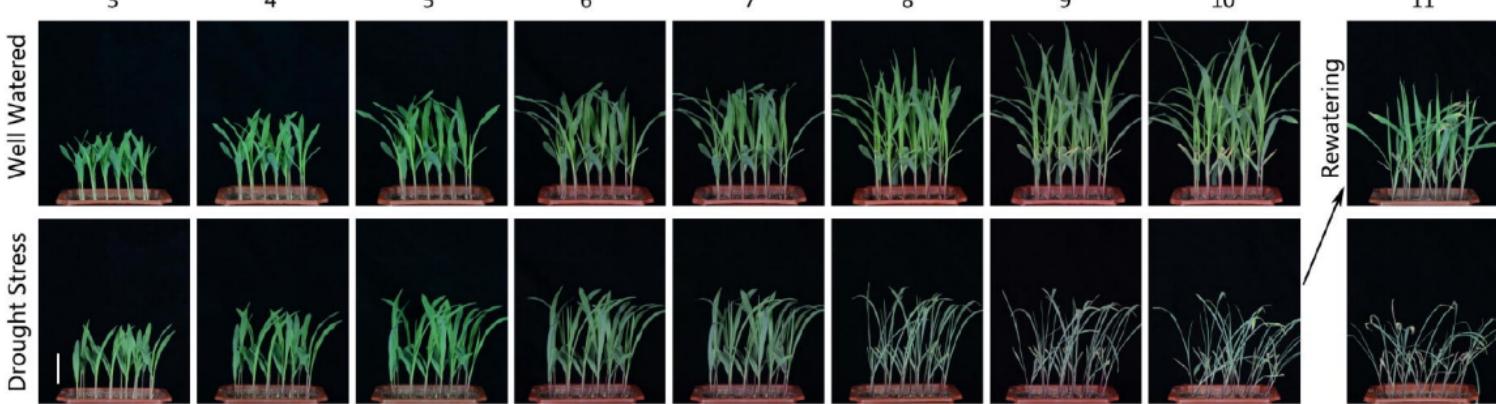
973

974 **Figure 6.** Time-series expression profiles of two miR156 targeting SPL genes
975 (A, B) Normalized counts of each miR156 (y-axis) were plotted along 3-11 DAW. WW,
976 DS, DWR represent well-watered, drought stress, drought water recovery, respectively. A
977 sequence on the top of each plot is the miR156 sequence. Each error bar represents the
978 range of a standard error above and below each mean value.

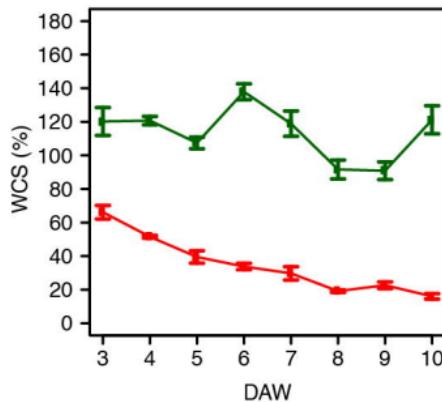
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A

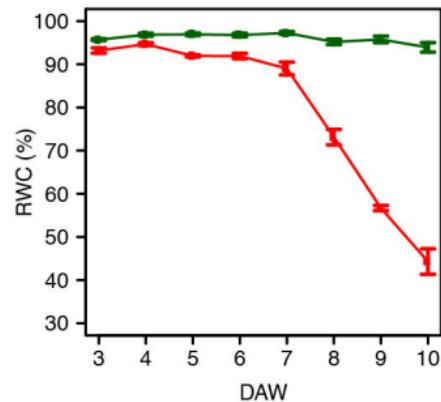
Days After Withholding water (DAW) for drought treatment

**B**

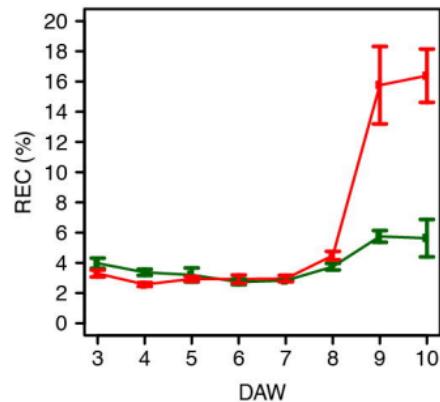
Soil water content (SWC)

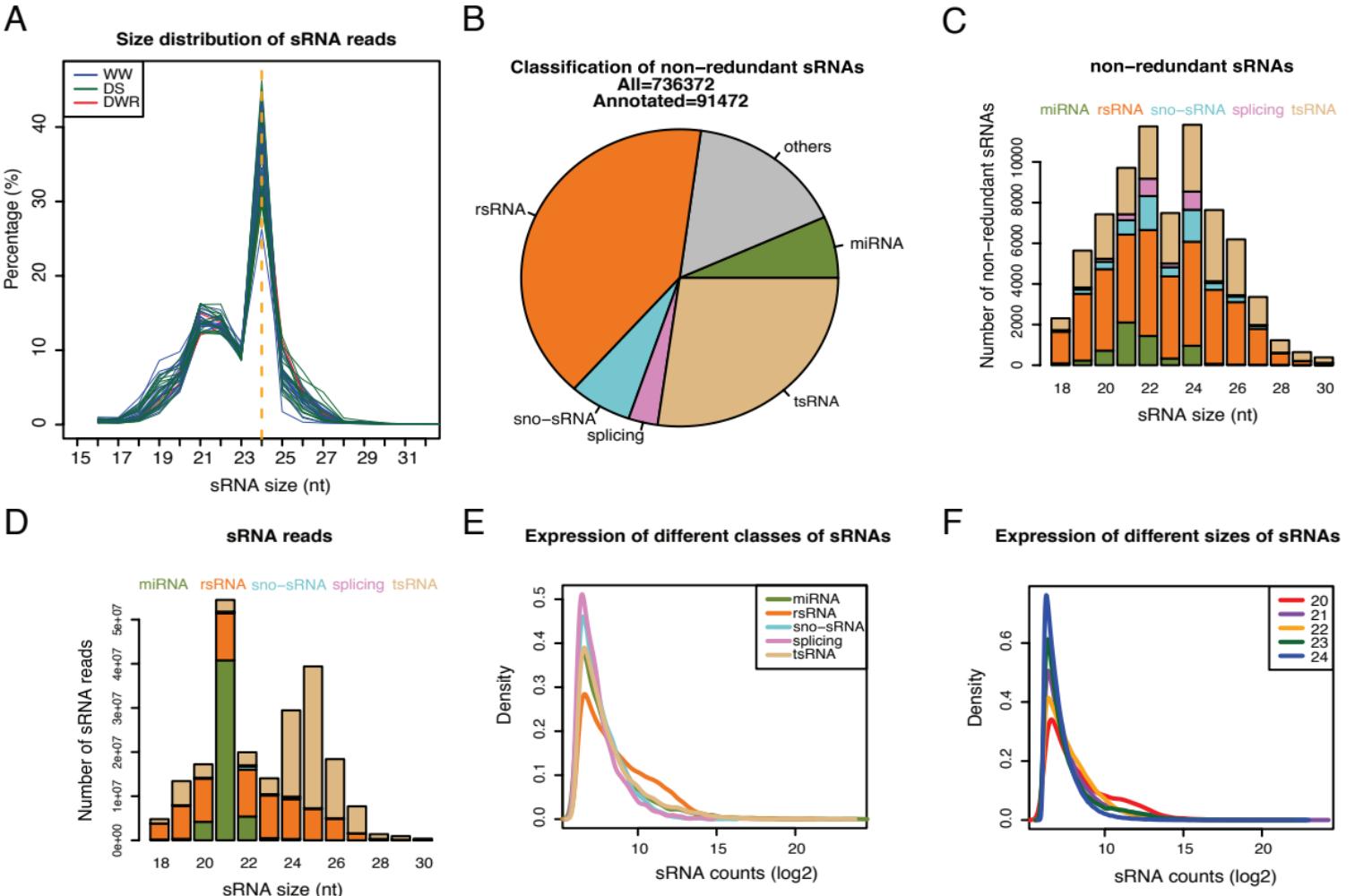
**C**

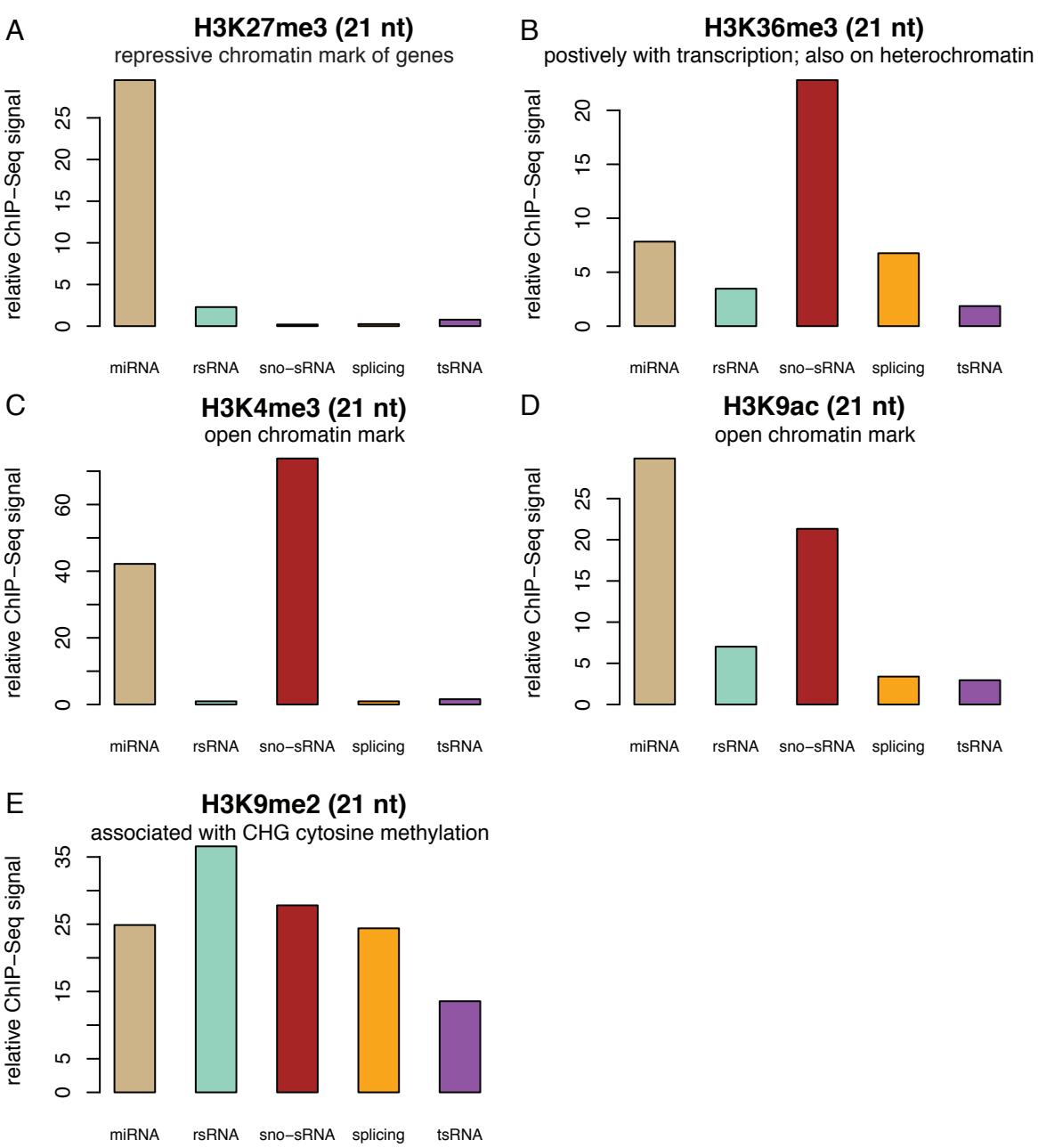
Leaf relative water content (RWC)

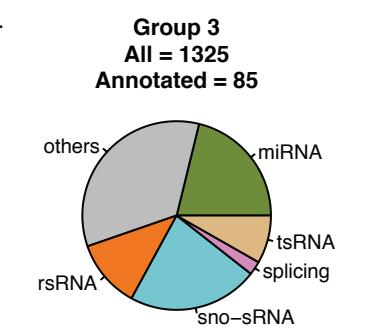
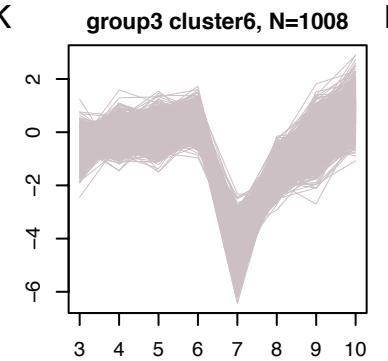
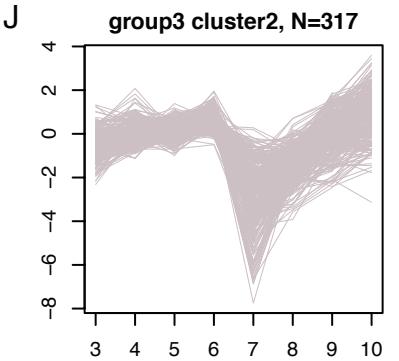
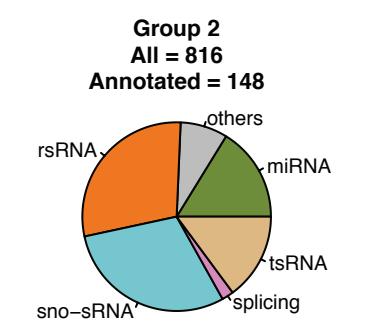
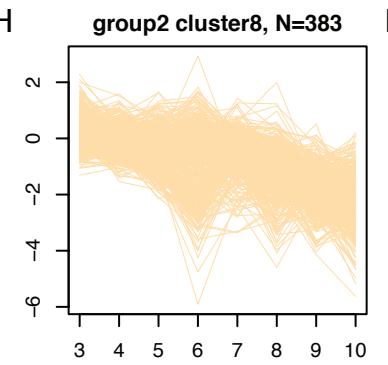
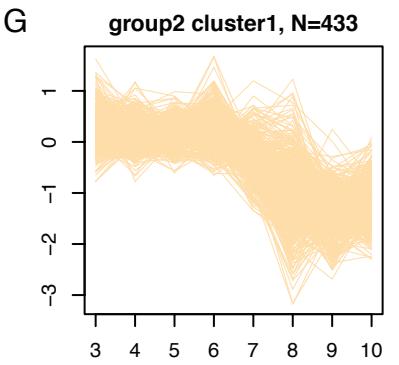
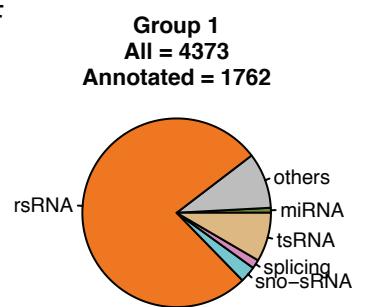
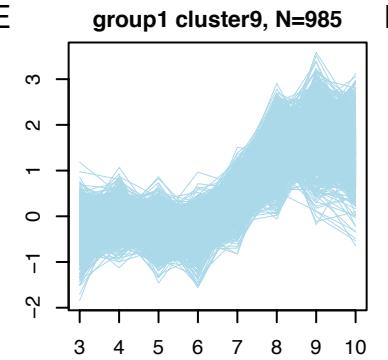
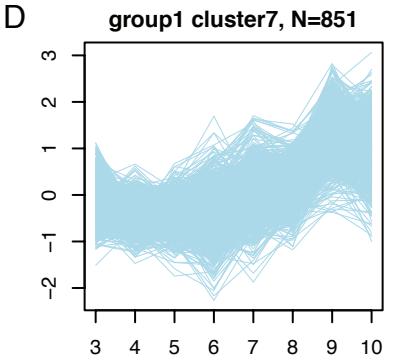
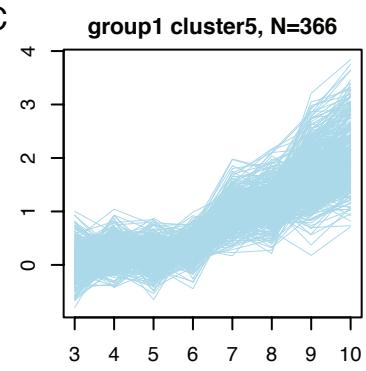
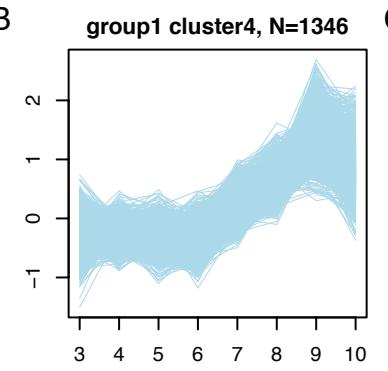
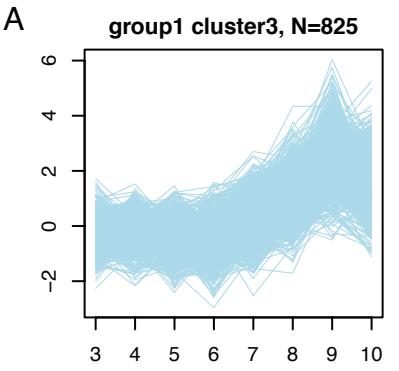
**D**

Relative electrical conductivity (REC)

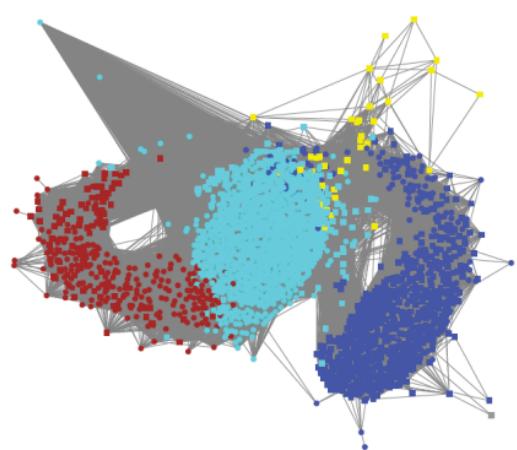




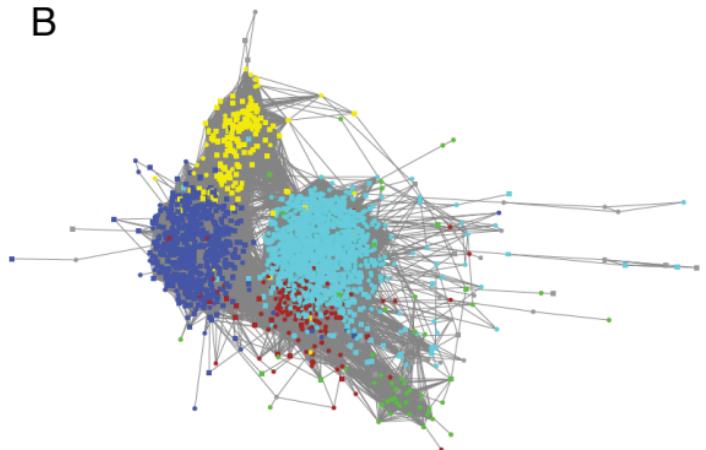




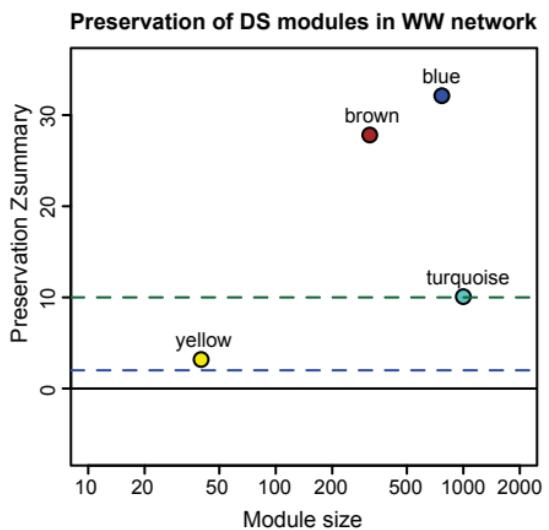
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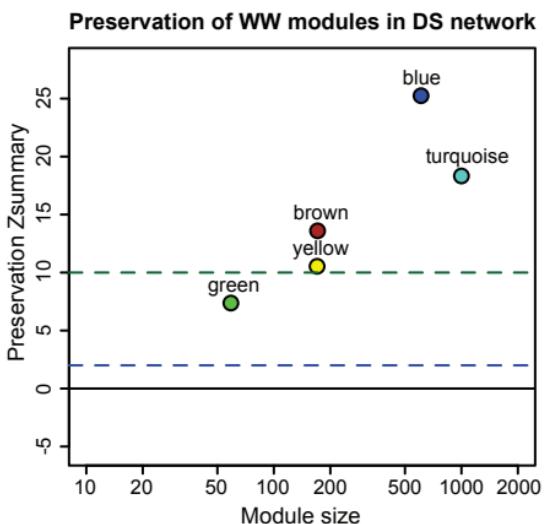
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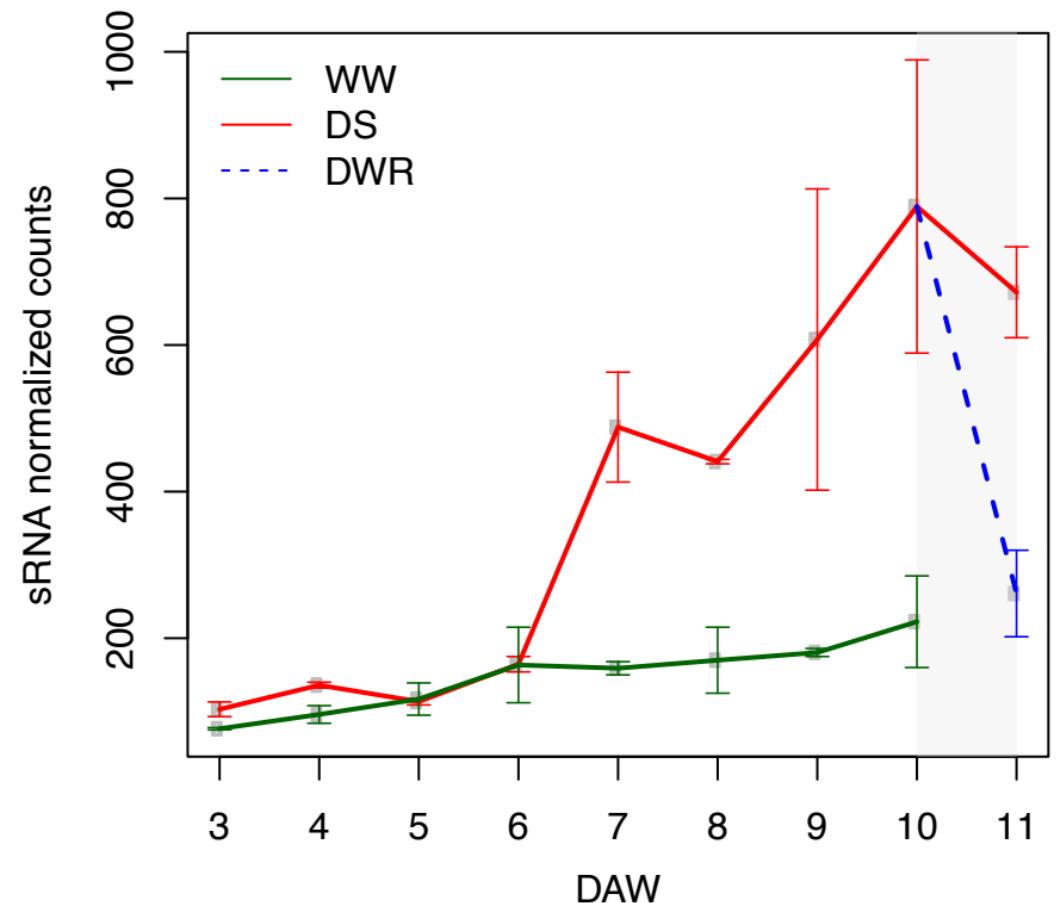
C



D



A GACAGAAGAGAGUGAGCACA



B UGACAGAAGAGAGUGAGCACA

