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Full Length Article

Identification of Drought-Responsive CircRNAs in Leaves of *Brachypodium distachyon*

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Abstract

Brachypodium distachyon is currently recognized as a model organism in Gramineae, especially for temperate grains. Research on its dehydration response mechanism is beneficial for elucidating the complex drought regulation network in temperate grains. The circRNA is an important type of non-coding RNA in organisms, which could involve in various biological processes. By sequencing the transcriptome of the leaves of *B. distachyon* under desiccation treatment and normal condition, a total of 737 circRNAs were obtained in this study, of which 332 and 265 were up-regulated and 265 down-regulated, respectively, with 140 insignificant differences. There were 229 and 303 circRNAs specifically expressed under CK and drought, respectively and 204 were co-expressed. Bioinformatics methods were used to identify potential miRNA targets of differentially expressed circRNAs. Among them, 150 differentially expressed circRNAs had putative miRNA binding sites. According to the predicted miRNA with binding site, the target genes downstream of the miRNA were screened. In terms of the functional annotation of target genes, they are divided into seven categories: hormone-related, photosystem, stress response, regulation factor, ion transport, TF and ribosomal protein. These circRNAs involved in water deficit response may play a significant role in the drought response and defense of *B. distachyon*. This also provides new insights for the improvement of the dehydration regulation mechanism of *B. distachyon* and even temperate grains. © 2021 Friends Science Publishers

Keywords: *Brachypodium distachyon*; circRNAs; Drought; Illumina sequencing; Functional category

Introduction

Compared with the well-known model plants *Arabidopsis thaliana* and rice, *Brachypodium distachyon* (*B. distachyon*) is more closely related to many important cereal crops such as wheat, barley, oats, corn, and so on (Brkljacic *et al.* 2011; Catalan *et al.* 2015). Therefore *B. distachyon* is a currently recognized model organism for Gramineae, and its biological characteristics, such as easy cultivation, smaller habit, and short growth cycle (Scholthof *et al.* 2018), are conducive to in-depth research on the development, evolutionary biology and abiotic stress response of temperate grain crops.

Unlike linear RNA, circular RNA (circRNA) is a special member of the non-coding RNA family. Since circRNA forms a covalent closed loop structure and does not have a polyadenylic acid tail, it is resistant to RNase, which can effectively degrade linear RNA (Zhao *et al.* 2019). The discovery of circRNA has been for decades, and it was once considered a by-product of splicing errors (Sanger *et al.* 1976; Kos *et al.* 1986). However, with the continuous development of high-throughput deep sequencing technology and bioinformatics tools, a variety of circRNAs have been discovered and identified in many organisms, such as humans (Jeck *et al.* 2013), fruit flies (Westholm *et al.* 2014), mice (Fan *et al.* 2015), zebrafish (Shen *et al.* 2017), *Arabidopsis* (Ye *et al.* 2015), rice (Lu *et al.* 2015) and soybeans (Wang *et al.* 2020). These researches confirmed that circRNA is ubiquitous in eukaryotes. The recent research on circRNA is mostly concentrated in mammals (Memczak *et al.* 2013), whereas research in plants is still in its infancy. The whole genome identification of circRNA in plants was first carried out in *Arabidopsis thaliana* and *Oryza sativa*. Ye *et al.* (2015, 2016) identified 6,012 and 2,806 circRNAs from Arabidopsis leaves and rice seedling root tissues, respectively. In addition to these two well-known model plants, circRNA has also been identified in other monocot and dicot species, such as soybean (Wang *et al.* 2020), barley (Behrooz *et al.* 2016), and wheat (Xu *et al.* 2019). Similar to the expression pattern of circRNA in animals (Westholm *et al.* 2014; Fan *et al.* 2015), circRNA in plants also exhibits tissue specificity and responds to environmental stress (Lu *et al.* 2015; Behrooz *et al.* 2016).

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For example, rice has 27 different expressions of circRNA under conditions of sufficient phosphate and starvation (Ye *et al.* 2015). There are 163 circRNAs in tomato showing a cold-responsive expression pattern (Zuo *et al.* 2016). Increasing evidences indicate that circRNAs may play significant roles in a variety of biological processes, such as miRNA binding, protein binding and transcriptional regulation (Hansen *et al.* 2013; Memczak *et al.* 2013; Chen *et al.* 2016).

Drought is one of the most serious adversity stresses affecting plant growth. With the global warming, the shortage of water resources has become more and more serious, which has directly led to the expansion of arid areas and the increase of aridity. In the long-term evolutionary process, plants have formed their own defense response mechanisms to deal with various stresses including water privation, such as the accumulation of osmotic adjustment compounds (Sattar *et al.* 2020b), stoma regulation systems (Sattar *et al.* 2020a), and signal transduction pathways (Iqbal *et al.* 2017). However, the response of plants to drought stress is the result of multi-angle and multi-level interaction, including not only the transcription level and translation level, but also the regulation of the post-transcriptional level (Chen *et al.* 2017b). As a result, in addition to mRNA, there are also a large number of non-coding RNAs involved, such as miRNA, lncRNA and circRNA. The researches about the former two in drought stress have been widely reported (Ma *et al.* 2019; Nadarajah and Kumar 2019; Feng *et al.* 2020), whereas research on how circRNA functions in drought stress response and regulation is rare. Therefore, this study adopted high-throughput technology to sequence the transcriptome of the *B. distachyon* leaves under water shortage stress, and used bioinformatics tools to analyze and identify the sequencing results, intending to provide theoretical basis for further understanding and improvement of drought response in Gramineous crops.

Materials and Methods

Plant material, growing environment and drought treatment

B. distachyon Bd-21 was sprouted in a clean petri dish for 1 day and then transferred to a 4°C incubator for 8 days for vernalization. After vernalization, these seedlings were planted in a pot, placed in a light incubator at 25°C with photoperiod 16 h light/ 8 h dark. When the seedlings grew to the 7-leaf stage, they were subjected to drought treatment. The control samples were watered once every other day, and the experimental group was treated with water restriction in a drought treatment for 7 days. After the treatment, the leaves of the seedlings were quickly frozen in liquid nitrogen and stored in an ultra-low temperature refrigerator at -80°C for subsequent experiment.

Library construction, sequencing and circRNA identification

RNA sequencing is a transcriptomics research method based on next-generation sequencing technology. Each step of sample detection, library construction, and sequencing is strictly controlled to ensure the output of high-quality data. First, extract the total RNA of the biological sample, according to the Feng *et al.* (2020) description. Then remove the rRNA and linear RNA. The RNA obtained is theoretically only circular RNA, and then through the steps of reverse transcription and PCR, and finally the enriched cDNA is subjected to high-throughput sequencing. After base calling analysis, the original image data files obtained by Illumina HiSeqTM2500 sequencing platforms are converted into sequenced reads, namely raw reads. CircRNA from the sequencing results could be identified through find circ (Memczak *et al.* 2013), and the candidate circRNAs whose read count greater than or equal to 2 were taken as the identified circRNA.

Expression analysis of circRNAs

The expression level of known and new circRNA in each sample was counted, which was normalized by transcript per million tags (TPM) (Zhou *et al.* 2010). Differentially expressed genes sequencing (DEGseq) (Wang *et al.* 2010) was employed to analyze the differences in circRNA expression between different samples. The differential circRNAs were screened from two aspects of fold change and corrected significance level (q value). The default differential circRNA screening condition is: q value < 0.01 and $|\log2 \text{(fold change)}| > 1$.

Verification of circRNA

Since the splice junction mapping on the genome after reverse splice of circRNA is reversed, the specific primers for detecting circRNA are generally designed as divergent primers, which are correspond to the convergent primers used to detect linear genes. Divergent primers include two types. Type I is generally designed back-to-back so that the splice junction is included in the middle of the product (Fig. 1A). Type II is the junction overlapping divergent primers (JOD primers), which span the splice junction, that is, the splice junction needs to be placed at the 3'end of a primer so that the splice junction is included on the primer rather than included in the PCR product (Fig. 1B). During PCR, JOD primers can only be amplified normally when they match the target circRNA, and it cannot be amplified when there is a mismatch, which greatly improves the specificity. Convergent primers were also designed as positive controls for linear transcripts (Fig. 1C). The designed primers were amplified and verified using cDNA and gDNA as templates. In order to confirm the reliability of the transcriptome sequencing results, 8 circRNAs were randomly selected for

qRT-PCR to verify their expression patterns. The highly specific JOD primers were used as primers, and the fluorescence quantification system and procedures were described by Feng *et al.* (2019) and Ma *et al.* (2019).

Bioinformatics analysis of parent genes of circRNAs under drought treatment

The input data of circRNA differential expression was the read count obtained in the analysis of circRNA expression level. The software psRobot and psRNATarget were adopted to predict the miRNA binding site of circRNA (Hansen *et al.* 2013) and the target gene of miRNA (Dai and Zhao 2011), respectively. After the differentially expressed circRNAs between each group were acquired, according to the corresponding relationship between circRNA and predicted miRNAs with circRNA binding sites, as well as target genes downstream of miRNAs, the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were carried out (Zhang *et al.* 2017). GO (http://www.geneontology.org/) is an international standard classification system for gene function. KEGG is the major public database about pathway (Kanehisa *et al.* 2008). Pathway significant enrichment analysis takes KEGG pathway as the unit and applies hypergeometric test to find the pathway that is significantly enriched in the target gene compared with the entire genome background.

Results

Sequencing results statistics and circRNA identification

To identify the circRNAs in *B. distachyon* responsing to drought stress, we constructed the RNA-Seq library from drought-treated *B. distachyon* leaves and performed Illumina HiSeq high-throughput sequencing. The above library construction, sequencing and bioinformatics analysis were carried out by Beijing Novogene Bioinformatics Co., Ltd. Each step of sample detection, library construction, and sequencing is strictly controlled to ensure the output of high-quality data. The quality assessment of the original data of the sample sequencing output is shown in Table 1. The CK and clean reads under water loss are 122,429,492 and 111,457,314, respectively. Clean bases reached 18.36G and 16.72G, respectively. The length distribution of circRNA of all samples is shown in Fig. 2. It can be seen from the Fig. 2 that the length of circRNA obtained by sequencing is mainly concentrated below 1000nt, and the number gradually decreases as the length increases. According to the source of circRNA in the genome and its constituent sequence, it can be divided into exonic circRNAs, intronic circRNAs and intergenic circRNAs. Fig. 3 counts the sources of circRNA in CK and water scarcity. Among them, There were 870 and 1,111 exonic circRNAs in CK and drought, respectively, which are dominated (53.28 and 52.85%), and followed by intronic circRNAs with 710 and 920, and intergenic circRNAs are the least with 53 and 71, respectively.

Analysis of differentially expressed circRNAs under drought

The expression level of known and new circRNA in each sample was calculated, and the expression level was normalized by TPM (Zhou *et al.* 2010). The differential circRNA was screened through the fold change and the corrected significance level (padj/qvalue). Fig. 4 (volcano map) can infer the overall distribution of different circRNAs. Among them, 332 and 265 were up-regulated and down-regulated separately, and 140 were not dramatically different. The Venn diagram of differential expressed circRNAs under CK and drought treatment is shown in Fig. 5. There are 229 and 303 circRNAs specifically expressed under CK and drought, respectively, and 204 are co-expressed.

Validation of circRNA in *B. distachyon*

Fluorescence quantitative polymerase chain reaction (qRT-PCR) was performed to validate the 8 circRNAs randomly selected from the circRNA-Seq analysis. Using highly specific JOD primers to amplify circRNA, cDNA and gDNA were used as amplification templates, and gDNA was used as negative control (Fig. 6A). Since the JOD primer spans the circularization site, the corresponding fragment cannot be amplified by using gDNA as a template. β-actin was used as an internal reference gene to normalize the expression of these 8 circRNAs. The qRT-PCR results in Fig. 6B showed that the expression levels of these 8 circRNAs were consistent with the RNA-seq results, indicating the reliability of the RNA-seq results. All the primers were showed in the Supplementary Table 2.

miRNA binding site analysis

The circRNA can inhibit the function of miRNA by binding to miRNA (Hansen *et al.* 2013), that is, it can regulate gene expression by acting as a miRNA sponge. Therefore, the miRNA binding site analysis of the identified circRNA will help to further study the function of circRNA. To investigate whether the circRNA in *B. distachyon* can affect the post-transcriptional level of target genes by binding to miRNAs under dehydration treatment, a bioinformatics method was employed to identify potential miRNAs target position of circRNA that are differentially expressed in *B. distachyon*.

In this study, 150 differentially expressed circRNAs have putative miRNA binding sites, and each circRNA has at least one predicted miRNA binding site, and a single miRNA can be targeted by multiple circRNAs, and a single circRNA can also target different miRNAs (Supplementary Table 1). For example, the predicted bdi-miR5169a is targeted by 9 circRNAs, while bdi_circ_0000061 can target 3 miRNAs in *B. distachyon*. In addition, based on predicted

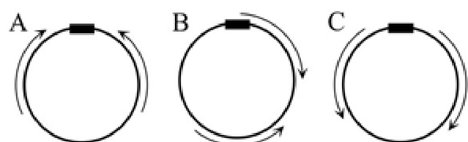

Fig. 1: Schematic diagram of divergent and convergent primers. **A**, type I divergent primer, the junction site is in the middle of the PCR product; **B**, type Ⅱ divergent primer, overlapping with the junction site; **C**, convergent primer, used to verify linear transcripts. The black rectangle represents the junction site

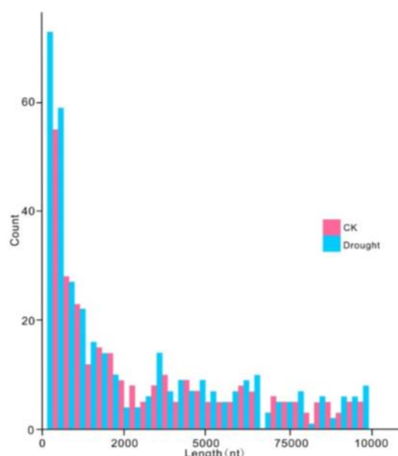

Fig. 2: The length distribution of circRNAs. circRNAs below 1000 nt are 132 and 159 in CK and drought samples, accounting for 40% and 43%, respectively

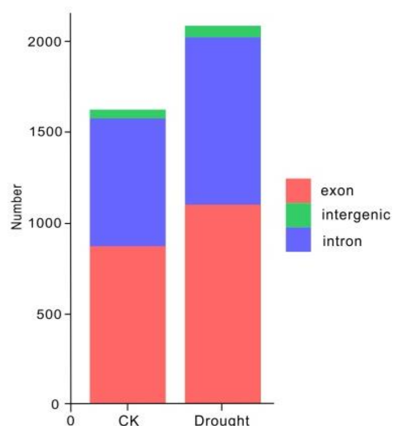

Fig. 3: Statistical analysis of exonic circRNAs, intergenic circRNAs, and intronic circRNAs in each sequenced sample. The above three circRNAs are 870, 53, 710 in CK, and 1111, 71, 920 in drought sample, respectively

miRNAs with binding sites, target genes downstream of miRNAs were screened. In line with function annotations, they are divided into seven categories: hormone-related, photosystem, stress response, regulation factor, ion transport, transcription factor (TF), and ribosomal protein (Table 2, Supplementary Table 2). Among the circRNAs corresponding to these seven types of target genes, the number of up-regulated expression was remarkably more than that of down-regulated expression (Table 3, Supplementary Table 2).

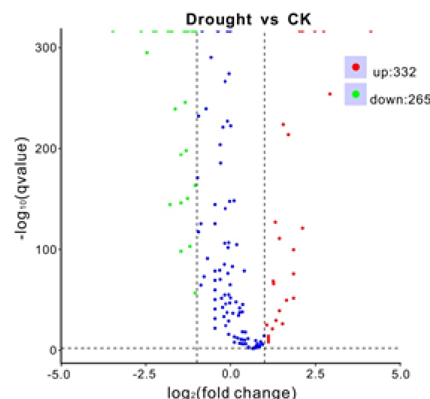

Fig. 4: Volcano plot of differentially expressed circRNA. The abscissa represents the fold change of circRNA expression in different samples, the ordinate represents the statistically significant degree of circRNA expression change, the scattered dots in the figure represent each circRNA, and the blue dots represent circRNAs with no significant differences, the red dots indicate significantly up-regulated circRNAs, and green dots indicate significantly down-regulated circRNAs

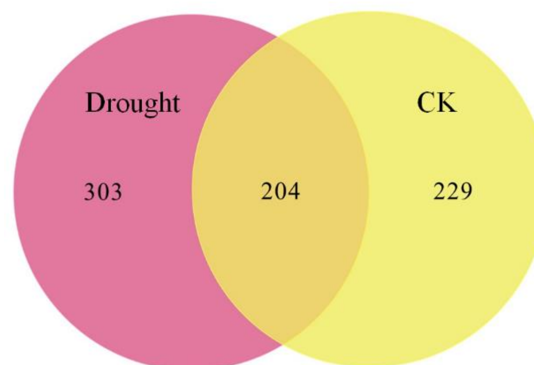

Fig. 5: Venn diagram of differential expressed circRNA. The number in each large circle represents the circRNA expressed in this sample, and the overlapping part of the circle represents the circRNA co-expressed between samples

Bioinformatics analysis of target genes predicted by circRNA under drought

According to the predicted miRNAs with circRNA binding sites, target genes downstream of miRNAs were screened, and Gene Ontology and KEGG enrichment analysis were performed on these target genes. The number of genes in each GO term that were notably enriched is shown in a histogram (Fig. 7). For biological processes, the category of metabolic process (GO: 0008152) is the richest in GO terms. For cell components, the target genes of circRNA are mainly involved in cell (GO: 0005623) and cell part (GO: 0044464). For molecular functions, the two most abundant categories are binding (GO: 0005488) and catalytic activity (GO: 0003824). The scatter plot of target gene KEGG

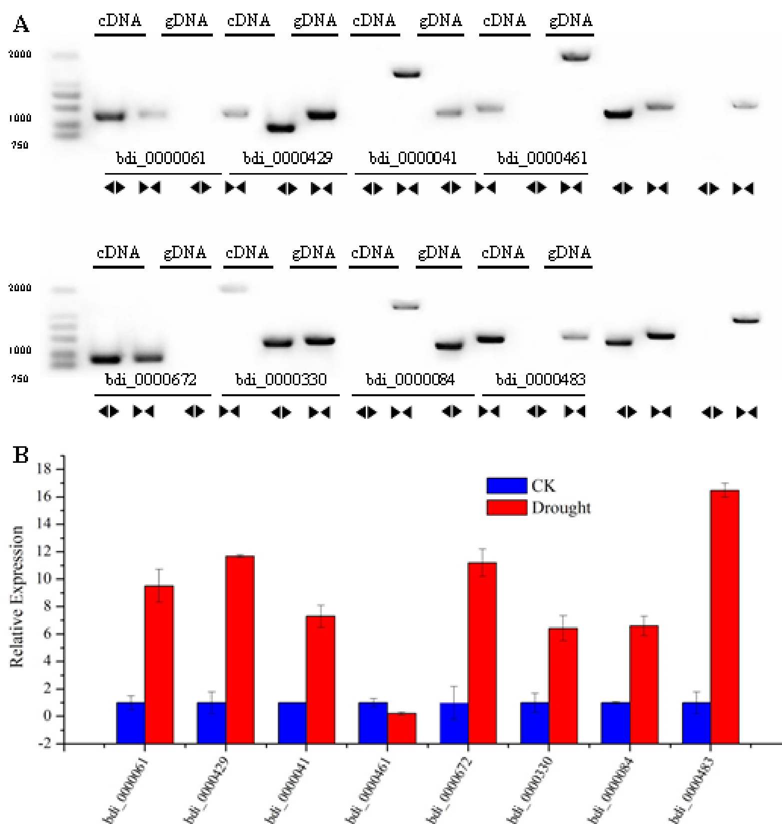

Fig. 6: Various experimental strategies validated the eight circRNAs from transcriptome sequencing. **A**, JOD primers (black back-to-back triangle pairs) and convergent primers (black opposing triangle pairs) were adopted to amplify eight circRNAs (bdi_0000061, bdi_0000429, bdi_0000041, bdi_0000461, bdi_0000672, bdi_0000330, bdi_0000084, bdi_0000483) in cDNA and gDNA. The former successfully amplified in cDNA but failed in gDNA, and the latter worked on both cDNA and gDNA; **B**, qRT-PCR validated the expression of the eight circRNAs

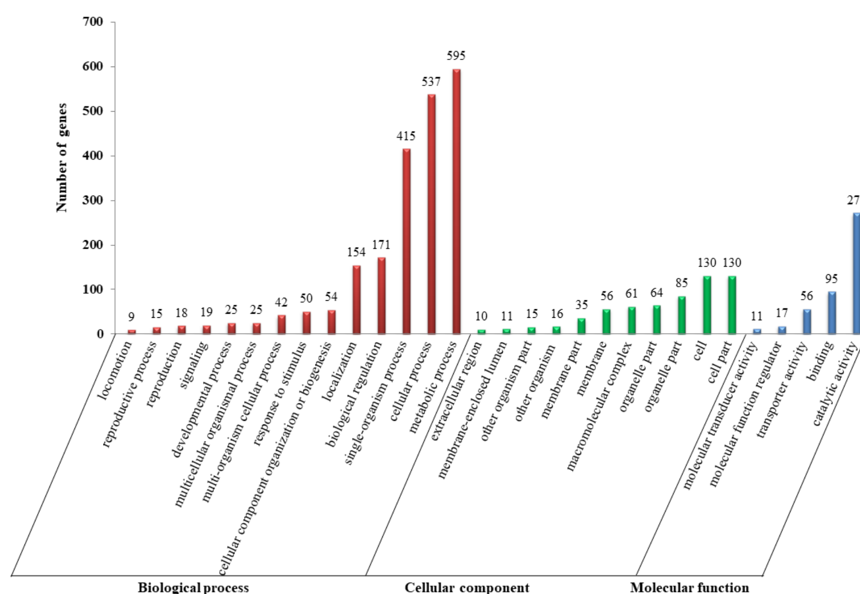

Fig. 7: GO enrichment histogram of target gene predicted by circRNA. The abscissa is the GO term of the three major categories of GO, and the ordinate is the number of target genes annotated to the term (including the sub-terms of the term)

Table 1: Statistics of sequencing data quality

Sample	Raw reads	Clean reads	Clean bases	Error rate (%)	Q20	Q30	GC content (%)
CK	128,474,682	122,429,492	18.36G	0.01	97.12	92.87	43.97
Drought	116,766,272	111,457,314	16.72G	0.01	97.21	92.92	44.70

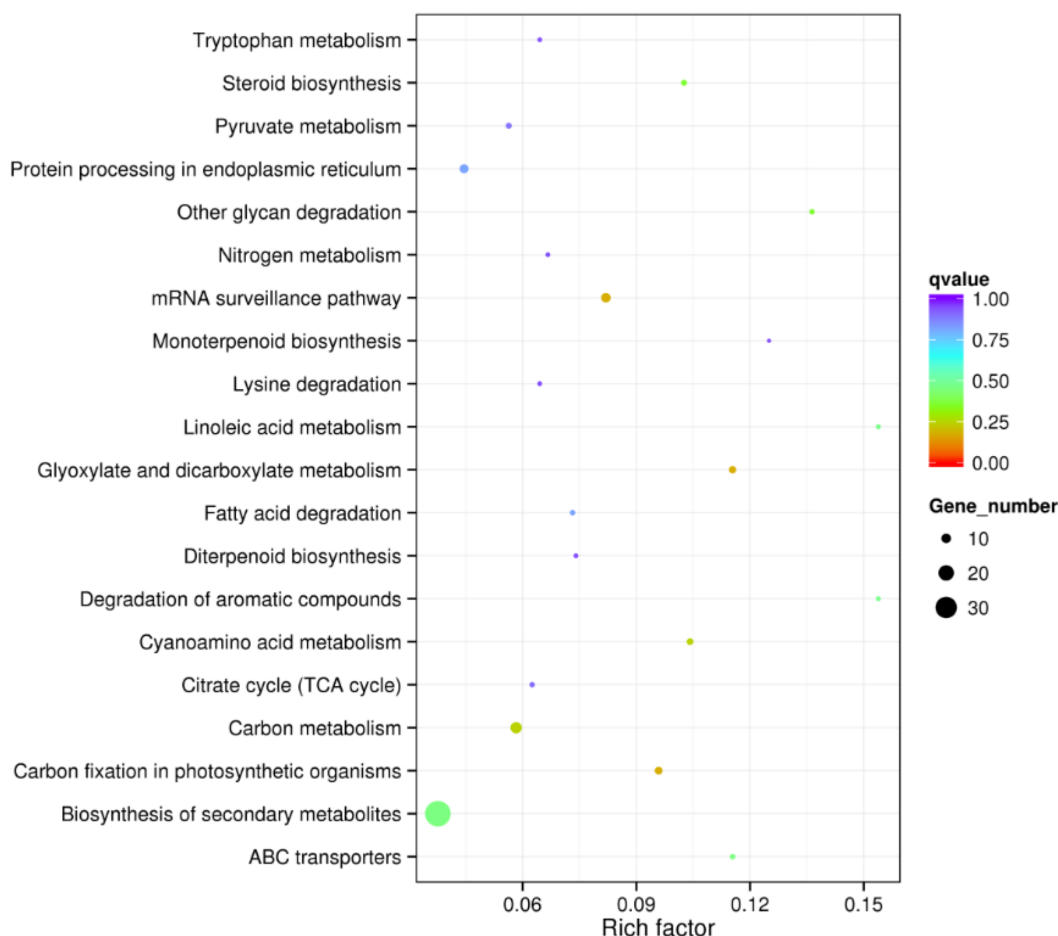

Fig. 8: KEGG enrichment scatter plot of target gene predicted by circRNA. The vertical axis represents the pathway, and the horizontal axis represents the rich factor. The size of the dot indicates the number of target genes in this pathway, and the color of the dot corresponds to different Q-value ranges

enrichment is a graphical display of KEGG enrichment analysis results (Fig. 8). The degree of KEGG enrichment is measured by rich factor, Q-value and the number of genes enriched in this pathway. Among them, rich factor refers to the ratio of the number of genes located in this pathway among differentially expressed genes to the total number of genes located in this pathway among all annotated genes. The greater the rich factor, the greater the degree of enrichment. Q-value is the p-value after multiple hypothesis testing and correction. The range of Q-value is [0, 1], which is closer to 0, the more significant. We selected the most enriched 20 pathways for display in Fig. 8, among which biosynthesis of secondary metabolites, carbon metabolism and mRNA surveillance pathway are the three pathways with the largest number of enriched genes.

Discussion

Previous studies believed that circRNA was a byproduct of the transcription process (Cocquerelle *et al.* 1993; Memczak *et al.* 2013), but more and more studies in the past decade have confirmed that circRNA is a very important type of non-coding RNA, which plays a crucial role in a variety of biological processes (Chen *et al.* 2017a). Most of the researches on the function of circRNA have focused on mammals (Salzman *et al.* 2012), however, circRNA has also been identified in some plants, including Arabidopsis (Chen *et al.* 2017a), rice (Lu *et al.* 2015), wheat (Wang *et al.* 2017), and soybean (Wang *et al.* 2020). In present study, transcriptome sequencing and identification of the circRNA in the leaves of *B. distachyon* under dehydrated stress were performed. A total of 737 circRNAs were obtained, of which

Table 2: Function classification of the predicted mRNA*

	circRNA ID	Binding site of	target gene ID	Function annotation
Hormone related	bdi_circ_0000045; bdi_circ_0000206; bdi_circ_0000214; bdi_circ_0000257; bdi_circ_0000471; bdi_circ_0000472; bdi_circ_0000483; bdi_circ_0000484; bdi_circ_0000485; bdi_circ_0000489; bdi_circ_0000559; bdi_circ_0000576; bdi_circ_0000581; bdi_circ_0000582; bdi_circ_0000629; bdi_circ_0000708	bdi-miR5174d-5p	Bradi3g40830.1	auxin-responsive family protein
	bdi_circ_0000045; bdi_circ_0000047; bdi_circ_0000206; bdi_circ_0000214; bdi_circ_0000257; bdi_circ_0000366; bdi_circ_0000471; bdi_circ_0000472; bdi_circ_0000483; bdi_circ_0000484; bdi_circ_0000485; bdi_circ_0000517; bdi_circ_0000559; bdi_circ_0000576; bdi_circ_0000581; bdi_circ_0000582; bdi_circ_0000629; bdi_circ_0000708	bdi-miR5174f		
	bdi_circ_0000130; bdi_circ_0000131; bdi_circ_0000214; bdi_circ_0000366; bdi_circ_0000471; bdi_circ_0000472; bdi_circ_0000576; bdi_circ_0000581; bdi_circ_0000582	bdi-miR5174a bdi-miR5174b-5p bdi-miR5174e-		
	bdi_circ_0000214; bdi_circ_0000471; bdi_circ_0000472; bdi_circ_0000576; bdi_circ_0000581; bdi_circ_0000582	bdi-miR5174c-5p		
	bdi_circ_0000045	bdi-miR7757-	Bradi1g46060.1	abscisic acid responsive elements-binding factor 2
	bdi_circ_0000144	bdi-miR7708a-3p	Bradi1g46060.2	
	bdi_circ_0000061	bdi-miR171c-5p	Bradi3g45880.9; Bradi3g45880.5;	auxin response factor
	bdi_circ_0000491; bdi_circ_0000492	bdi-miR5166	Bradi3g04920.20	
	bdi_circ_0000324; bdi_circ_0000325	bdi-miR9486b	Bradi2g44490.2; Bradi2g44490.1	auxin-like 1 protein
	bdi_circ_0000015	bdi-miR9491		
	bdi_circ_0000082	bdi-miR164f	Bradi1g22830.1	Auxin-responsive GH3 family
	bdi_circ_0000330; bdi_circ_0000331	bdi-miR528-3p	Bradi2g46190.3	Transcriptional factor B3 family protein / auxin-responsive factor AUX/IAA-related
Photosystem	bdi_circ_0000491; bdi_circ_0000492	bdi-miR5166	Bradi1g24870.6; Bradi1g24870.2; Bradi1g24870.5; Bradi1g24870.4; Bradi1g24870.3; Bradi1g24870.1	light harvesting complex photosystem II
	bdi_circ_0000366	bdi-miR9495	Bradi1g77340.1; Bradi1g77340.3	Mog1/PsbP/DUF1795-like photosystem II reaction center PsbP family protein
	bdi_circ_0000041; bdi_circ_0000072; bdi_circ_0000395; bdi_circ_0000429; bdi_circ_0000521; bdi_circ_0000531; bdi_circ_0000659; bdi_circ_0000660; bdi_circ_0000708	bdi-miR5169a bdi-miR5169b	Bradi4g19720.1 Bradi4g19720.1	photosystem II reaction center protein A
	bdi_circ_0000483; bdi_circ_0000484; bdi_circ_0000485; bdi_circ_0000632; bdi_circ_0000708	bdi-miR159a-5p	Bradi1g25430.4	
Stress response	bdi_circ_0000294	bdi-miR5064	Bradi1g28090.2	chloroplastic drought-induced stress protein of 32 kD
	CircRNA ID	Binding site of mRNA	Target gene ID	Function annotation
	bdi_circ_0000009	bdi-miR5281a bdi-miR5281b	Bradi2g28040.2	Drought-responsive family protein
	bdi_circ_0000041; bdi_circ_0000092	bdi-miR7726a-5p	Bradi3g34540.1; Bradi3g34540.5	early-responsive to dehydration stress protein (ERD4)
	bdi_circ_0000092		Bradi3g34540.4; Bradi3g34540.3	
	bdi_circ_0000308	bdi-miR827-5p	Bradi3g34540.6; Bradi3g34540.3; Bradi3g34540.4; Bradi3g34540.5; Bradi3g34540.1	
Ion transport	bdi_circ_0000617	bdi-miR9496	Bradi1g45940.7; Bradi1g45940.8; Bradi1g45940.2	high-affinity K^+ transporter 1
	bdi_circ_0000072	bdi-miR437	Bradi5g26820.1; Bradi4g01430.1	K^+ efflux antiporter 1
	bdi_circ_0000009	bdi-miR5281a bdi-miR5281b	Bradi4g01430.1	K^+ efflux antiporter 3
	bdi_circ_0000617	bdi-miR9496		
	bdi_circ_0000491; bdi_circ_0000492	bdi-miR5166	Bradi1g37860.9; Bradi1g37860.5; Bradi1g37860.6; Bradi1g37860.7; Bradi1g37860.8; Bradi1g37860.11; Bradi1g37860.10; Bradi1g37860.1; Bradi1g37860.12	K^+ efflux antiporter 4
	bdi_circ_0000708	bdi-miR169d	Bradi1g76640.5	K^+ efflux antiporter 5
Regulation factor	bdi_circ_0000461	bdi-miR396e-5p	Bradi1g09900.2; Bradi3g52547.1; Bradi5g20607.1; Bradi1g50597.1; Bradi3g57267.1; Bradi3g39620.3; Bradi3g39630.1; Bradi3g39590.1	growth-regulating factor
	bdi_circ_0000061	bdi-miR171c-5p	Bradi2g23250.1	heat shock protein 70
	bdi_circ_0000254	bdi-miR5184	Bradi5g24410.6; Bradi5g24410.3; Bradi5g24410.5; Bradi5g24410.1; Bradi5g24410.2	jasmonate-zim-domain protein 12
	bdi_circ_0000444	bdi-miR5178-3p	Bradi5g07080.3; Bradi5g07080.1; Bradi5g07080.4; Bradi5g07080.2	Oxidoreductase, zinc-binding dehydrogenase family protein

Table 2: Continued

Table 2: Continued

	CircRNA ID	Binding site of mRNA	target gene ID	Function annotation
	bdi_circ_0000444	bdi-miR5178-3p	Bradi5g07080.3; Bradi5g07080.1; Bradi5g07080.4; Bradi5g07080.2	Oxidoreductase, zinc-binding dehydrogenase family protein
	bdi_circ_0000461	bdi-miR7738-5p	Bradi2g58130.5; Bradi2g58130.4; Bradi2g58130.6; Bradi2g58130.3; Bradi2g58130.7	relative of early flowering 6
	bdi_circ_0000228	bdi-miR7774-5p	Bradi1g59560.4	splicing factor, putative
	bdi_circ_0000276	bdi-miR2118b	Bradi5g22310.7; Bradi5g22310.10;	starch synthase 3
	bdi_circ_0000367; bdi_circ_0000369; bdi_circ_0000370	bdi-miR7777-3p.1	Bradi5g22310.7; Bradi5g22310.10; Bradi5g22310.5; Bradi5g22310.8; Bradi5g22310.4; Bradi5g22310.6; Bradi5g22310.3; Bradi5g22310.9; Bradi5g22310.1; Bradi5g22310.2	
TF	bdi_circ_0000629	bdi-miR7782-3p	Bradi3g08280.2; Bradi3g08280.1; Bradi3g08280.5; Bradi3g08280.3; Bradi3g08280.4	basic helix-loop-helix (bHLH) DNA-binding family protein
	bdi_circ_0000214; bdi_circ_0000471; bdi_circ_0000472; bdi_circ_0000576; bdi_circ_0000581; bdi_circ_0000582	bdi-miR5174d-3p	Bradi3g39927.1	
	bdi_circ_0000617	bdi-miR9496		
	bdi_circ_0000366; bdi_circ_0000456; bdi_circ_0000489; bdi_circ_0000490; bdi_circ_0000491; bdi_circ_0000511; bdi_circ_0000531; bdi_circ_0000540	bdi-miR9493	Bradi1g54111.5	
	bdi_circ_0000072; bdi_circ_0000130; bdi_circ_0000131; bdi_circ_0000242; bdi_circ_0000439; bdi_circ_0000511; bdi_circ_0000520; bdi_circ_0000521; bdi_circ_0000607; bdi_circ_0000622; bdi_circ_0000623; bdi_circ_0000624; bdi_circ_0000659; bdi_circ_0000660; bdi_circ_0000664; bdi_circ_0000665; bdi_circ_0000682; bdi_circ_0000683; bdi_circ_0000733; bdi_circ_0000734; bdi_circ_0000735	bdi-miR5171a; bdi-miR5171b	Bradi1g05480.1	bZIP transcription factor family protein
	bdi_circ_0000045	bdi-miR7757-3p.1	Bradi5g23340.3	
	bdi_circ_0000092	bdi-miR7743-3p	Bradi1g46230.11; Bradi1g46230.8; Bradi1g46230.13; Bradi1g46230.14; Bradi1g46230.12; Bradi1g46230.9	global transcription factor group A2
	bdi_circ_0000041; bdi_circ_0000092	bdi-miR7726a-5p	Bradi3g05260.1	K-box region and MADS-box
	bdi_circ_0000708	bdi-miR169d	Bradi3g54720.4	Plant-specific GATA-type zinc finger transcription factor family protein
	bdi_circ_0000330; bdi_circ_0000331; bdi_circ_0000332; bdi_circ_0000333; bdi_circ_0000334; bdi_circ_0000335	bdi-miR5198		
	bdi_circ_0000396	bdi-miR5163a-3p	Bradi1g08106.2	WRKY DNA-binding protein
	CircRNA ID	Binding site of mRNA	Target gene ID	Function annotation
	bdi_circ_0000013; bdi_circ_0000041; bdi_circ_0000088; bdi_circ_0000144; bdi_circ_0000162; bdi_circ_0000164; bdi_circ_0000165; bdi_circ_0000166; bdi_circ_0000167; bdi_circ_0000211; bdi_circ_0000228; bdi_circ_0000334; bdi_circ_0000335; bdi_circ_0000344; bdi_circ_0000366; bdi_circ_0000458; bdi_circ_0000581; bdi_circ_0000582; bdi_circ_0000687; bdi_circ_0000721; bdi_circ_0000736	bdi-miR5175b	Bradi1g08106.4	
	bdi_circ_0000045	bdi-miR9494	Bradi2g19070.1	
	bdi_circ_0000061	bdi-miR171c-5p	Bradi4g33370.2; Bradi4g33370.9	
	bdi_circ_0000330; bdi_circ_0000331; bdi_circ_0000672	bdi-miR528-5p	Bradi4g28280.1	
	bdi_circ_0000045	bdi-miR9494	Bradi2g42023.1	
	bdi_circ_0000288; bdi_circ_0000366	bdi-miR164c-3p	Bradi2g30695.1	
	bdi_circ_0000172	bdi-miR7771-3p	Bradi2g30800.2	
Ribosomal protein	bdi_circ_0000172	bdi-miR7771-3p	Bradi3g09780.1	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
	bdi_circ_0000172	bdi-miR7771-3p	Bradi4g34750.1	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein
	bdi_circ_0000045	bdi-miR7757-	Bradi1g71200.1	Ribosomal protein S3Ae

*Part of the classification results is displayed. See Supplementary Table 2 for the complete results

exon circRNA exceeded 50% (Fig. 3). The high ratio of exon circRNA is similar with the Arabidopsis exon circRNA under high temperature (Pan *et al.* 2018). It is speculated that the increase in the number of exon circRNA may be due to adversity stress. The increase in exon circRNA not only responds to plant stress, but may also enhance stress resistance. It has been confirmed that circRNA in plants can respond to a variety of stresses. Zuo *et al.* (2016) verified that 163 circRNAs in tomato respond to cold stress, while 62 circRNAs in wheat are differentially expressed under drought stress compared to the control (Wang *et al.* 2017). There are 597 circRNAs with significant differential

Table 3: Statistics of circRNA expression in seven functional categories

Function classification	circRNA	Up-regulated circRNA	Down-regulated circRNA
Hormone related	124	78	46
Photosystem	47	27	20
Stress response	30	20	10
Ion transport	34	21	13
Regulation factor	94	56	38
TF	141	88	53
Ribosomal protein	130	79	51

expression in the leaves of *B. distachyon* under dehydration stress, which is consistent with previous research results, that is, circRNA can respond to multiple stresses and may further participate in the regulation of stress resistance.

The predicted target genes of circRNAs were analyzed by bioinformatics. The GO results showed that they were related to multiple functions, including different biological processes, cellular components, and molecular functions. EGG analysis enriched 20 pathways, most of which were involved in drought response. For example, the pathway with the largest number of enriched genes is biosynthesis of secondary metabolites, and there are as many as 89 hormone-related genes in the functional classification of target genes, including auxin-responsive family protein, auxin-responsive factor, and abscisic acid responsive elements-binding factor. Phytohormones are active substances induced by plant cells receiving specific environmental signals. They not only regulate plant growth and development, but also participate in a variety of stress responses, including low temperature (Xin *et al.* 2019), water deficit (Hu *et al.* 2018), salt (Yu *et al.* 2020) and so on (Wang *et al.* 2018). The exogenous application of ABA can increase the cold tolerance of rice plants during the flowering period and thus increase the seed setting rate (Xiang *et al.* 2019). Besides, exogenous application of gibberellin could relieve the adverse effects of salinity, drought, and heat stresses on the growth of *Capsicum annuum* L., including increase biomass and chlorophyll content, as well as improve photosynthetic efficiency (Khan *et al.* 2015). Since circRNA can act as a molecular sponge of miRNA and prevent it from regulating target mRNA and controlling gene expression (Memczak *et al.* 2013), the expression trend of most circRNAs is positively correlated with their corresponding target genes. Under drought treatment, there were more markedly up-regulated circRNA in the leaves of *B. distachyon* than down-regulated expression. For example, the circRNAs predicted to be hormone-related genes, 78 were up-regulated and 46 were down-regulated. Therefore, it is speculated that the circRNA corresponding to the hormone-related gene is involved in dehydrated response of *B. distachyon* in a positive or negative manner, and contributes to the regulation of its stress response.

In addition to hormone-related genes, the circRNA corresponding to target genes whose function is annotated as stress response, regulation factor, photosystem and ion transport are also up-regulated more than down-regulated. Drought is one of the common environmental stresses. Genes that respond to drought stress have always been research hotspots. With the continuous development of high-throughput sequencing technology, the function of non-coding RNA is gradually recognized by people. Apart from miRNA and lncRNA, circRNA in plants also play a significant role in stress resistance. The circRNA and its target genes obtained in this analysis can be divided into seven categories: hormone-related, photosystem, stress response, regulation factor, ion transport, TF and ribosomal protein according to their functional classification. Among them, photosystem- and hormone-pathway, as known regulation networks, participate in plant drought response (Chen *et al.* 2016; Rao and Chaitanya 2016), including a series of responses, like light harvesting complex photosystem II, photosystem II reaction center protein A, auxin response factor and auxin transport protein genes, are involved in dehydration response, which is consistent with previous studies (Chen *et al.* 2016; Rao and Chaitanya 2016). Furthermore, Rai *et al.* (2012) confirmed that early-responsive to dehydration stress protein (ERD4) is highly expressed in plant drought response and could effectively improve the desiccation adaptability of plants. ERD4 in the stress response classification was up-regulated under drought treatment, which was in line with the results of Rai et al. Under adversity stress, a large number of genes are up-regulated and expressed to positively regulate the stress resistance of plants, whereas these emergency response genes are inhibited by related mechanisms in normal growth conditions. During the evolution process, plants have established related mechanisms to inhibit the expression of stress-related genes under normal conditions. The main role of growth-regulating factors (GRFs) is to inhibit the stress response under non-stress conditions. Compared with wild-type Arabidopsis and *AtGRF7* overexpression lines, the Arabidopsis *atgrf7* mutant is more tolerant to salt and dehydrated stress (Kim *et al.* 2012), therefore, GRF is down-regulated under stress conditions to activate related stress genes to improve plant resistance. The circRNAs, whose target genes belong to the growth-regulating factor classification, were distinctly down-regulated under drought treatment, which is consistent with the negative regulation of plant stress resistance by GRF, which may be a crucial regulatory network of stress response in the plant.

The K^+ efflux antiporter (KEA), for K^+, is functioning

in maintaining pH, the active accumulation and balance of K^+ and ion homeostasis (Zhu *et al.* 2018). Additionally, soybean *GmKEAs* gene family up-regulated in the beginning and down-regulated later under excessive potassium stress (Tao *et al.* 2015). The response of *GmKEAs* gene to abiotic stress indicates that it is involved in soybean resistance. The target genes, whose function annotated as KEA1, KEA3, KEA4 and KEA5 in *B. distachyon*, their corresponding circRNAs were also up-regulated after water shortage, which may also be involved in the response to osmotic stress. WRKY TF exerts a critical role in a variety of biological processes, including plant growth, development and adversity stress. Gao *et al.* (2018) overexpressed *TaWRKY2* in wheat to enhance the dehydrated resistance of transgenic plants. Shirazi *et al.* (2019) cloned the stress response *BnWRKY57* in *Brassica napus* and analyzed its expression. Those results showed that *BnWRKY57* not only responded to drought and salt stress, but also enhanced the resistance of *Brassica napus* to the two stresses. The circRNA whose target gene function was annotated as WRKY DNA-binding protein was obviously up-regulated after drought treatment, and it was speculated that it not only participated in the drought response of *B. distachyon*, but also exerted an influence in stress defense.

Moreover, some of the other predicted target genes in the functional classification may also be involved in the drought response of *B. distachyon*. However, more evidence is needed to further prove its specific function, and the mechanism of action may depend on circRNA regulation.

Conclusion

Through transcriptome sequencing of *B. distachyon* leaves under drought treatment, a total of 737 circRNAs were obtained in this study, of which exon circRNA exceeded 50%. Differential expression analysis showed that there were 332 and 265 up-regulated and down-regulated expressions, respectively and 140 had no significant difference. There were 229 and 303 circRNAs specifically expressed under CK and drought, respectively, and 204 were co-expressed. Bioinformatics methods were used to identify potential miRNA targets of differentially expressed circRNAs. Among them, 150 differentially expressed circRNAs had putative miRNA binding sites. Based on the predicted miRNA with binding site, the target gene downstream of the miRNA was screened. Analysis of GO and KEGG shows that the categories of metabolic process (GO: 0008152), cell (GO: 0005623) and cell part (GO: 0044464), binding (GO: 0005488) and catalytic activity (GO: 0003824) are the most abundant entry in GO. Among the 20 most significant pathway entries, biosynthesis of secondary metabolites, carbon metabolism and mRNA surveillance pathway are the three pathways with the largest number of enriched genes. According to the functional annotation of target genes, they are divided into seven categories: hormone-related, photosystem, stress response, regulation factor, ion transport, TF and ribosomal protein. CircRNA is an important type of non-coding RNA in organisms, and these circRNAs involved in desiccation response may exert a crucial role in drought response and defense of *B. distachyon*. The valuable information also provides a theoretical basis for further identification of candidate genes participated in drought regulation.

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Author Contributions

Yalan Feng performed the experiments and wrote the manuscript; Shuang Zhou and Jun Zhang analyzed the experiment data and wrote the manuscript; Ke Xv, Yating Li and Mengzhen Zhang sampled material and analyzed the experiment data; Chao Ma and Youjun Li designed the research and revised the manuscript.

Conflict of Interest

Authors declare no conflict of interest.

Data Availability

The data and supplementary material are all available online.

Ethics Approval

This research does not involve the ethical approval.

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