Genome-wide identification and expression profiling of DREB genes in Saccharum spontaneum

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Abstract

Background: The dehydration-responsive element-binding proteins (DREBs) are important transcription factors that interact with a DRE/CRT (C-repeat) sequence and involve in response to multiple abiotic stresses in plants. Modern sugarcane are hybrids from the cross between Saccharum spontaneum and Saccharum officinarum, and the high sugar content is considered to be the attribution of S. officinarum, while the stress tolerance is attributed to S. spontaneum. To understand the molecular and evolutionary characterization and gene functions of the DREBs in sugarcane, based on the recent availability of the whole genome information, the present study performed a genome-wide in silico analysis of DREB genes and transcriptome analysis in the polyploidy S. spontaneum.

Results: Twelve DREB1 genes and six DREB2 genes were identified in S. spontaneum genome and all proteins contained a conserved AP2/ERF domain. Eleven SsDREB1 allele genes were assumed to be originated from tandem duplications, and two of them may be derived after the split of S. spontaneum and the proximal diploid species sorghum, suggesting tandem duplication contributed to the expansion of DREB1-type genes in sugarcane. Phylogenetic analysis revealed that one DREB2 gene was lost during the evolution of sugarcane. Expression profiling showed different SsDREB genes with variable expression levels in the different tissues, indicating seven SsDREB genes were likely involved in the development and photosynthesis of S. spontaneum. Furthermore, SsDREB1F, SsDREB1L, SsDREB2D, and SsDREB2F were up-regulated under drought and cold condition, suggesting that these four genes may be involved in both dehydration and cold response in sugarcane.

Conclusions: These findings demonstrated the important role of DREBs not only in the stress response, but also in the development and photosynthesis of S. spontaneum.

Keywords: Saccharum spontaneum, DREB, Phylogenetic analysis, Gene expression, Dehydration response

Background

Plants are exposed to various abiotic stresses such as drought, salinity, and extreme temperature, which cause adverse effects on their growth and yield [1]. A number of genes are induced or repressed by these stresses to help plants to survive from these bad conditions, which can be divided into the genes coding stress tolerance proteins and the other coding regulatory proteins [2, 3]. Transcription factors (TFs) are necessary for regulating the expression of stress-responsive genes. Dehydration responsive element binding proteins (DREBs) are the important TFs that regulate stress-responsive genes expression in the abscisic acid (ABA)-independent pathway [4]. DREBs belong to a subfamily of the APETALA2/ethylene-responsive element-binding protein (AP2/
ERFBP) superfamily of TFs, and can bind a dehydration-responsive element (DRE) with the core motif A/GCCGAC that was found in the promoter of many dehydration- and cold stress-inducible genes [1, 5]. Each DREB protein contains a conserved AP2/ERF DNA-binding domain, which consist of ~60 amino acids [6, 7]. The three-dimensional structure of AP2/ERF domain revealed this domain comprises a three-strand antiparallel β-sheet and an α-helix packed similarly parallel to the β-sheet [8]. Two amino acids, the 14th valine (V14) and 19th glutamic acid (E19) in the AP2/ERF domain of DREB proteins are conserved and play a central role in determining the DNA-binding specificity of DREB proteins [1]. On the basis of the similarities in the AP2/ERF domain, DREB subfamily has been divided into 6 subgroup (A-1 to A-6), and the canonical DREB proteins belong to subgroups A-1 (DREB1) and A-2 (DREB2) [1, 9].

Though DREB genes are mainly involved in the process regulating the drought stress, other functions have been noted for some DREB genes. Previous studies have demonstrated that DREB genes can be induced by various abiotic stresses, including drought [10–13], low temperatures [14–17], heat stress [18–20] and high salt [21–23]. Overexpressing OsDREB2A in soybean enhanced salt tolerance by accumulating osmolytes and improving the expression levels of some stress-responsive genes and TFs [24]. In transgenic Salvia miltiorrhiza, AtDREB1A and AtDREB1B both play a positive role in plant drought stress tolerance [12, 25]. PvDREB1C gene is transcriptionally down-regulated in response to salt stress, whereas PvDREB1C overexpression improves plant salt tolerance in transgenic tobacco. On the other hand, ectopic overexpression of PvDREB1C has been characterized as a negative regulator of cold stress response [16]. StDREB2 has been reported to play an important role in the drought stress tolerance of cotton (Gossypium barbadense L.) [26].

Sugarcane (Saccharum spp.) is a major crop mostly grown in tropical and subtropical regions worldwide, and adversely affected by drought, salinity, low temperature, high temperature, etc. Modern sugarcane cultivars are complex autoploidy and aneuploidy of interspecific hybrids derived mainly from S. officinarum and S. spontaneum. For Saccharum hybrid, S. officinarum was assumed to contribute to genetic background of high sugar content, and S. spontaneum contributed to the stress tolerance and pest and disease resistance [27]. In China, over 70% of sugarcane were cultivated in the hilly area which contained a low level of soil water content during the drought season. Thus, enhancing drought tolerance has been an important target for improving the yield of sugarcane in field. According to previous researches, transgenic sugarcane transformed with AtDREB2A CA showed the enhanced drought tolerance without biomass penalty [28].

Overexpression of EaDREB2 (Erianthus arundinaceus DREB2) in sugarcane enhances the drought and salinity tolerance, what’s more, co-transformation of EaDREB2 and PDH4S (pea DNA helicase gene) shows lower drought tolerance but higher salinity tolerance than EaDREB2 alone [29]. Huang et al. recently had analyzed the DREB subfamily in S. spontaneum [30], here, we focused on the canonical DREB genes (DREB1s and DREB2s) and discriminated the genes and their alleles. We also explored the gene function based on large scales of expression profiles from RNA-seq data sets including leaf developmental gradient, diurnal cycle, development stage, drought stress and cold stress. Thus, this study may provide insights into the polyploid characterizes for the DREBs and function relative to photosynthesis and plant development beside the drought stress.

**Results**

**Identification of SsDREB genes in S. spontaneum genome**

A total of 277 proteins containing AP2/ERF domain(s) were originally obtained in the sugarcane S. spontaneum AP85–441 (1n = 4x = 32) genome. Based on the classification of the AP2/ERF superfamily in Arabidopsis [1], 54 of them, containing multiple AP2/ERF domains, were classified into the AP2 subfamily. Thirteen of these proteins, possessing both AP2/ERF and B3 domains, were belonging to the RAV subfamily. Thirty-one proteins lacked a conserved WLG motif. Of the remaining 179 proteins, containing only one AP2/ERF domain with a conserved WLG motif, 83 were classified into the DREB subfamily (Group A) and 96 were classified into the ERF subfamily (Group B). Two canonical subgroups of DREBs (DREB1 and DREB2) were 20 and 10 proteins in S. spontaneum, respectively. After re-annotating manually with the assistance of FGENESH (http://www.softberry.com/berry.phtml?topic=fgenes&group=programs&subgroup=fgind) [31], one protein in DREB1 subgroup was identified without AP2/ERF domain and deleted for further researching. Furthermore, these DREB genes have 1 to 4 alleles, including 1 gene with four alleles, 2 genes with three alleles, and 4 genes with two alleles (Additional File 1). Based on their chromosomal locations, we renamed these DREB1s and DREB2s as SsDREB1A to SsDREB1L, and SsDREB2A to SsDREB2F, respectively, and additional −1 to −4 were added to the gene name for their alleles (Additional File 1).

Gene characteristics, including the length of protein sequences (AA), the molecular weight (MW), the theoretical isoelectric point (pI), the aliphatic index (AI), the grand average of hydropathicity (GRAVY), and the instability index (II) were analyzed (Additional File 1). The
protein length were ranged from 186 to 390 aa, while the MW of the proteins from 20,362.44 Da to 41,745.7 Da, and the pI from 4.78 to 10.53 (Additional File 1).

Multiple sequence alignment and phylogenetic analysis of SsDREBs
All SsDREB protein sequences were found to have an AP2/ERF domain, with a highly conserved WLG motif (Additional File 2). Additionally, SsDREB2 proteins possessed a conserved 14th valine (V14) and a 19th glutamic acid (E19), whereas SsDREB1A to SsDREB11 did not have the glutamic acid in the E19 position (Additional File 2). In DREB1 subgroup, a nuclear localization signal (NLS) sequence ‘P/KKR/KP/RA/TGRT/ KKRETRETP’ and a DSAW motif nestled up to the AP2/ERF domain in the upstream and downstream, respectively. The LWSY motif was found at the end of the C-terminal region in most SsDREB proteins, except for SsDREB1A-1, SsDREB1A-3, SsDREB1F-2 and SsDREB1J (Additional File 2). In comparison with DREB1s, all DREB2 protein contained a CMIV-1 ([K/R]GKGGPxN) motif, and a PKK-like NLS sequence ‘RKxPAKKGSKKCMxGKGGPENxx’ was found at the upstream of AP2/ERF domain except SsDREB2E (Additional File 2).

In this study, we collected the DREB orthologous in Arabidopsis, rice, maize and sorghum (Table 1). It’s worth noting that there are two more DREB1 genes and one less DREB2 genes in S. spontaneum than that in the proximal species sorghum. A phylogenetic tree of the SsDREB proteins and their orthologous was constructed (Fig. 1). Interestingly, the AtDREB proteins were clustered separately from the proteins which were derived from monocots in the DREB1-type genes, while clustered together with other proteins in the DREB2-type genes. A DREB2-type gene ABI4 belongs to the A-3 subgroup, and those identified in Arabidopsis, rice, maize and sorghum were formed a clade, but not found in S. spontaneum (Fig. 1), indicating that ABI4 gene may be lost after the species divergence between S. spontaneum and sorghum.

| Species        | DREBS/CBFs  | Total |
|---------------|-------------|-------|
| Arabidopsis   | 6           | 15    |
| Z.mays        | 10          | 20    |
| O.sativa      | 10          | 16    |
| S.bicolor     | 10          | 17    |
| S.spartaneum  | 12(19)      | 6(10) | 18(29) |

The numbers in parenthesis detail the number of alleles of SsDREBs in S. spontaneum

Location and duplication events among SsDREB genes
The genome chromosome location information of SsDREBs showed that these 29 DREB alleles were unevenly distributed on the 14 chromosomes of S. spontaneum (Fig. 2a). Chromosome 2 (2A, 2B, 2C and 2D) contained the largest number of SsDREB genes, in addition to chromosome 7A with two SsDREB2 genes and other chromosomes only with one SsDREB2 gene (Fig. 2a).

Furthermore, according to the methods of Holub [32], a chromosomal region within 200 kb containing two or more genes is defined as a tandem duplication event. We identified 12 SsDREB1 allele genes (SsDREB1A-2/1C-1/1D, SsDREB1A-3/1E, SsDREB1B-3/1G/1F-2/1H/1A-4, and SsDREB1C-2/1H), which were clustered into four tandem duplication event regions by BLASTP and MCScanx software, these tandemly duplicated regions were distrusted on the chromosome 2B, 2C and 2D (Table 2). Chromosome 2D had two clusters, indicating a hot spot of DREB gene distribution. What’s more, 17 SsDREB allele genes were results of the segmental duplication or whole-genome duplication events, including all SsDREB2 genes (Additional File 3).

Among these tandemly duplicated gene pairs, SsDREB1C-2 and SsDREB1I, SsDREB1C-1 and SsDREB1D, possessed only one orthologous gene SbDREB1A, while the orthologous SbDREB genes of SsDREB1A-3/1E and SsDREB1B-3/1G/1F-2/1H/1A-4 were also identified as tandemly duplicated gene pairs (Fig. 2b), indicating that tandem duplication events of SsDREB1C-2 and SsDREB1I, SsDREB1C-1 and SsDREB1D may happened after the divergence between S. spontaneum and sorghum. We therefore estimated the divergence time between tandemly distributed SsDREB genes and their orthologous SbDREBs based on the pairwise Ks (Table 3). The divergence time between S. spontaneum and its closest related diploid species sorghum had been estimated by Zhang et al. [33], it is 7.779 million years ago (Mya). In the current study, the divergence time between tandem-duplicated SsDREBs was ranged from 6.487 Mya to 18.874 Mya. In addition, the divergence time of SsDREB1C-2 and SsDREB1D with their orthologous were 6.487 Mya and 6.496 Mya, respectively, which are shorter than that of S. spontaneum and sorghum (7.779 Mya).

Gene structure and motif composition analysis of SsDREBs
The exon-intron organizations and motifs of all SsDREBs genes were examined in S. spontaneum. As shown in Fig. 3, all SsDREB genes had no intron except SsDREB1L, SsDREB2F and SsDREB2B with only one intron. The number and size of exon/intron among SsDREB alleles were highly conserved, while those in...
SsDREB2F, SsDREB2F-2’s intron were larger than other alleles. In addition, ten conserved motif sequences were detected (Fig. 3). All SsDREB genes contained Motif 1 and 2, which were related with AP2/ERF domain structure. Motif 3, 4 and 6 were only found in DREB1 genes, whereas Motif 7 was unique to DREB2 genes.

To identify the evolutionary forces acting on the SsDREB genes with alleles, the ratio of the non-synonymous substitution rate to the synonymous substitution rate (Ka/Ks) was calculated. The Ka/Ks ratios between SsDREB1A-3 and SsDREB1A-4, SsDREB2F-2 and SsDREB2F-3 were 1.401 and 2.450, respectively (Fig. 4), indicating that positive selection may be the dominant force driving the evolution of these two SsDREB genes.

Expression analysis of SsDREB genes in S. spontaneum

The expression patterns of SsDREB genes in different tissues and developmental stages of S. spontaneum were investigated by using transcriptomic data. The RNA-seq results of SsDREB1E, SsDREB1F, SsDREB1H and SsDREB2F were corroborated by real time quantitative reverse transcription-PCR (qRT-PCR) in three tissues (the first, 6th and 15th segments of 11-day-old second leaves) of S. spontaneum (Additional File 4). There is a significant positive relationship ($R^2 = 0.7491$) between the relative expression level and the Fragments Per transcript Kilobase per Million fragments mapped (FPKM) value (Additional File 4), supporting the reliability of the gene expression based on RNA-seq.

Among the 18 SsDREB genes, 4 genes (SsDREB1I, SsDREB2A, SsDREB2B and SsDREB2C) were expressed at very low levels or undetectable in all examined tissues (Fig. 5). Transcripts of SsDREB2D was constitutively expressed in all these 12 tissues. The expression levels of SsDREB1E, SsDREB1F, SsDREB1H and SsDREB2F in leaves were higher than those in the stalks at different developmental stages. SsDREB1A exhibited much higher transcript levels in the leaves at maturing stage compared to other stages. The expression level of SsDREB1L increased with the maturity of the leaves, and gradually decreased from the top to bottom of the stem (Fig. 5). To further investigate the functions of DREB genes in the photosynthesis tissues of S. spontaneum. We exploited the continuously developmental gradient of
the leaf to analyze the transcriptome of \textit{SsDREBs}. Similarly to the maize [34], the leaf of \textit{S. spontaneum} can be divided into four zones, including a basal zone (base, 1 cm above the leaf two ligule, sink tissue), a transitional zone (5 cm, 1 cm below the leaf one ligule, undergoing the sink-source transition), a maturing zone (10 cm, 4 cm above the leaf one ligule) and a mature zone (tip, 1 cm below the leaf two tip, fully differentiated and active \textit{C4} photosynthetic zones). Five genes (\textit{SsDREB1C}, \textit{SsDREB1D}, \textit{SsDREB1I}, \textit{SsDREB2A} and \textit{SsDREB2B}) displayed undetectable or very low levels, suggesting that these genes play a very limited role in the developmental leaves in \textit{S. spontaneum}. \textit{SsDREB1A}, \textit{SsDREB1E}, \textit{SsDREB1F} and \textit{SsDREB1H} showed higher expression levels in mature zone than those in other zones of the leaf, whereas \textit{SsDREB1L} displayed higher expression levels in the transitional zone, \textit{SsDREB1J} and \textit{SsDREB1K} showed higher transcript levels in the basal zone (Fig. 6). For the \textit{SsDREB2}-type genes, \textit{SsDREB2F}'s transcript abundance gradually increased from the base to tip of the leaf, while the expression level of \textit{SsDREB2D} gradually decreased from the base to tip of the leaf in \textit{S. spontaneum} (Fig. 6). Additionally, we also collected samples for RNA-seq analysis at 2-h intervals over a 24-h period and 4-h intervals over an additional 24-h in \textit{S. spontaneum}. \textit{SsDREB2F} showed higher expression in the

\begin{figure}
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\includegraphics[width=\textwidth]{fig2.png}
\caption{Chromosome distribution of \textit{SsDREB} genes and gene model. \textbf{a} The chromosome distribution of \textit{SsDREB} genes. The chromosomal position of \textit{SsDREB} was mapped according to the \textit{S. spontaneum} genome. The chromosome numbers were shown at the top of each chromosome. The scale is in mage bases (Mb). The green lines indicate the tandem duplication regions. \textbf{b} Gene model of the tandemly duplicated regions. The colored boxes and lines indicate \textit{DREB} genes and chromosomes, respectively.}
\end{figure}
light period than that in the dark period over these two 24-h cycles, indicating this gene may play an important role in diurnal rhythms (Fig. 7).

Furthermore, the transcriptome data of all SsDREB genes were analyzed in the primary meristem of the heart leaf in three drought-stressed sugarcane varieties. As illustrated in Fig. 8a, two SsDREB1 genes and two SsDREB2 genes were observed in response to drought stress, while the expression levels of SsDREB1A were slightly up-regulated after re-watering in three sugarcane varieties. SsDREB1F displayed similar expression patterns in these three sugarcane varieties, and its expression was gradually decreased with the increases of drought stress (Fig. 8b). What’s more, the greatest drought-inducible gene was found in SsDREB1F under the mild drought stress. The expression of SsDREB1F was up-regulated by dehydration in the drought-tolerant F172, which was also induced by the mild drought stress in other two varieties. Interestingly, the transcript abundances of SsDREB1L was increased slightly after re-watering under the moderate and severe drought stress conditions in GT31. Two SsDREB2 genes, SsDREB2D and SsDREB2F, showed similar expression patterns with high expression levels. In contrast to the expressions under the normal growing conditions, the expressions of these two genes were up-regulated in response to dehydration, and then decreased after re-watering in all sugarcane varieties (Fig. 8b).

Finally, in order to investigate the response of SsDREB genes in cold stress, we analyzed the transcriptome expression profiles of all these genes in S. spontaneum under cold stress. Six SsDREBs were induced by cold stress in hyperploid clone 15–28 (2n = 92) of S. spontaneum, and eight SsDREB genes were up-regulated in hypoploid clone 12–23 (2n = 54) (Fig. 8a). The greatest cold-inducible response was observed in SsDREB1F, whose expression was up-regulated more than 200-fold both in clone 15–28 and clone 12–23, in compare with expression under normal growing conditions. The induction response of SsDREB1A, SsDREB1B, SsDREB1E and SsDREB1F in clone 15–28 were higher than that in clone 12–23, while the expression levels of SsDREB1L and SsDREB2F in clone 12–23 were higher than that in clone 15–28 under cold stress. The expression of SsDREB1H and SsDREB2D were only up-regulated in response to cold stress in clone 12–23.

For the genes in tandemly duplicated regions, SsDREB1F-2 and SsDREB1H showed higher expression levels in leaves than those in stalks at different developmental stages, moreover, the expression levels of SsDREB1F-2, SsDREB1H and SsDREB1A-4 gradually increased from the base to tip of the leaf, whereas SsDREB1B-3 and SsDREB1G displayed a lower levels in all tissues (Additional File 5). In addition, the expression of SsDREB1F-2 was significantly up-regulated in response to dehydration in three sugarcane varieties, while other SsDREB1 genes in tandemly duplicated clusters were expressed at very low levels or undetectable (Additional File 5).

### Discussion

The DREB-type transcription factors have been recently identified in many plants, for instance, Arabidopsis [1], Brassica rapa [36], rice [37, 38], barley [39], sorghum [40], and maize [9]. DREB genes also play a key role in plant response to multiple abiotic stresses [41]. Thus, it’s understandable that DREB genes may contribute to the enhanced stress tolerance and the improved production of sugarcane in field. However, the DREB genes have not been systematically studied in sugarcane because of its complex genetic background. In this study, 18 typical DREB genes in the S. spontaneum genome were identified and analyzed using a bioinformatics approach to

### Table 2 Tandem duplication events in the SsDREB genes

| Cluster number | Gene name     | Chromosome | Start site   | End site   |
|----------------|---------------|------------|--------------|------------|
| 1              | SsDREB1C-2    | Chr2D      | 22,208,944   | 22,209,639 |
| 2              | SsDREB1I      | Chr2D      | 22,226,936   | 22,227,607 |
| 3              | SsDREB1B-3    | Chr2D      | 22,092,888   | 22,093,103 |
| 4              | SsDREB1A      | Chr2D      | 22,117,638   | 22,118,345 |
| 5              | SsDREB1G      | Chr2D      | 22,107,211   | 22,108,036 |
| 6              | SsDREB1F-2    | Chr2D      | 22,113,187   | 22,113,903 |
| 7              | SsDREB1H      | Chr2D      | 22,117,638   | 22,118,345 |
| 8              | SsDREB1A-4    | Chr2D      | 22,126,308   | 22,127,015 |
| 9              | SsDREB1B-2    | Chr2B      | 26,564,597   | 26,565,409 |
| 10             | SsDREB1C-1    | Chr2B      | 26,597,272   | 26,598,093 |
| 11             | SsDREB1D      | Chr2B      | 26,612,975   | 26,613,670 |
| 12             | SsDREB1A-3    | Chr2C      | 30,543,015   | 30,543,701 |
| 13             | SsDREB1E      | Chr2C      | 30,554,216   | 30,554,923 |
| 14             | SsDREB1G      | Chr2D      | 22,107,211   | 22,108,036 |

### Table 3 The divergence time between tandem-duplicated SsDREB genes and their orthologous SbDREBs

| Gene pairs          | Ks    | Divergence time (Mya) |
|---------------------|-------|-----------------------|
| SbDREB1A-SsDREB1C-2 | 0.079 | 6.487                 |
| SbDREB1A-SsDREB1D   | 0.079 | 6.496                 |
| SbDREB1A-SsDREB1I   | 0.096 | 7.841                 |
| SbDREB1D-SsDREB1E   | 0.133 | 10.017                |
| SbDREB1D-SsDREB1H   | 0.140 | 11.496                |
| SbDREB1A-SsDREB1C-1 | 0.170 | 13.902                |
| SbDREB1B-SsDREB1B-2 | 0.180 | 14.747                |
| SbDREB1B-SsDREB1B-1 | 0.183 | 15.038                |
| SbDREB1B-SsDREB1G   | 0.191 | 15.868                |
| SbDREB1E-SsDREB1A-4 | 0.213 | 17.444                |
| SbDREB1E-SsDREB1A-2 | 0.230 | 18.874                |
Fig. 3 Phylogenetic relationships, gene structures and conserved protein motifs for the SsDREB genes. The phylogenetic tree was constructed based on the full-length protein sequences of 29 SsDREB alleles using MEGA 7.0. Exons and introns are represented by black boxes and lines, respectively. The AP2 domains are highlighted by red boxes. The numbers 1–10 of motifs are displayed in different color boxes.

Fig. 4 The Ka/Ks of SsDREB alleles and SsDREB-SbDREB. The blue boxes indicate the Ka/Ks of SsDREB allele genes, the red boxes indicate the Ka/Ks of orthologous between sorghum and S. spontaneum. The p-value < 0.05 is indicated by *. The p-value < 0.01 is indicated by **
provide the clues for further functional investigations of SsDREB genes.

In the present study, 12 SsDREB1 genes and six SsDREB2 genes were identified in the S. spontaneum genome, respectively. As a proximal species of sugarcane, there are ten DREB1-type genes and seven DREB2-type genes in the sorghum genome (Table 1). Phylogenetic analysis showed that a DREB2-type gene, ABI4, lost the orthologous gene in S. spontaneum, which explains the reason why S. spontaneum have one less DREB2 gene, relative to the number of DREB2 gene in sorghum. Previous research has reported that DREB1 family

![Fig. 5](image)

The expression pattern of SsDREBs based on FPKM in different tissues of different stages in S. spontaneum.

![Fig. 6](image)

The expression pattern of SsDREBs based on FPKM across leaf gradients in S. spontaneum.

| Seedling | Pre-mature | Mature |
|----------|------------|--------|
| Leaf     | Stem       | Leaf   | Stem   |
| SsDREB1A | 6.86       | 13.12  | 2.27   |
| SsDREB1B | 5.86       | 12.34  | 2.37   |
| SsDREB1C | 5.34       | 11.86  | 2.42   |
| SsDREB1D | 5.02       | 11.24  | 2.22   |
| SsDREB1E | 4.89       | 11.06  | 2.18   |
| SsDREB1F | 4.73       | 10.90  | 2.13   |
| SsDREB1G | 4.54       | 10.70  | 2.10   |
| SsDREB1H | 4.34       | 10.51  | 2.00   |
| SsDREB1I | 4.14       | 10.31  | 1.90   |
| SsDREB1J | 3.94       | 10.12  | 1.80   |
| SsDREB1K | 3.74       | 9.92   | 1.70   |
| SsDREB1L | 3.54       | 9.72   | 1.60   |
| SsDREB2A | 8.34       | 16.68  | 3.34   |
| SsDREB2B | 8.14       | 16.48  | 3.22   |
| SsDREB2C | 7.94       | 16.28  | 3.10   |

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were expanded by the process of gene duplications [38]. Eleven tandemly duplicated SsDREB1 allele genes were found in this paper, among them, the divergence times of SsDREB1C-2 and SsDREB1D with their orthologous SbDREB are shorter than that of S. spontaneum and sorghum, suggesting that these two genes originated from tandem duplication after species differentiation between S. spontaneum and sorghum. Although all SsDREB2 genes were derived from segmental duplication or whole-gnome duplication events, no gene expansion has occurred compared with sorghum. That is tandem duplication events, instead of segmental duplication or whole-gnome duplication events contributed to the expansion of DREB genes in S. spontaneum. Previous studies have been reported that duplications resulting in some gene families represented functional redundancy and/or divergence [42–44]. In this study, of the SsDREB1 genes in Cluster 2 tandemly duplicated gene pairs, SsDREB1F-2 and SsDREB1H have the same expression pattern at different developmental stages and leaf gradient segments (Additional File 5), whereas SsDREB1B-3 and SsDREB1G expressed at very low levels, suggesting these tandemly duplicated genes may exist functional redundancy.

The V14 and E19 in the AP2/ERF domain are highly conserved and play a major role in the recognition and binding specificity of DRE cis-acting element [1]. However, some studies have reported that E19 is not conserved in DREB1 proteins in rice, wheat, barley and rye [41, 45]. Moreover, Dubouzet et al. reported that OsDREB1A do not has the glutamic acid in the E19 position, but binds specifically to the DRE element in the promoter of rd29A genes [46]. These results indicate that V14 may be more important than E19 for the recognition of DNA-binding sequence in DREB1 proteins. That is, deletion of this glutamic acid in the E19 position of SsDREB1A-SsDREB1D genes (Additional File 2) has little effect on their functions. TFs only function in the nucleus, the regulation of their entry into nucleus is significant for their function. The NLS mediates the entry of TFs, and the TFs without NLS enters the nucleus by the interaction with the TFs with NLS [45]. The DREB1-type transcription factors are distinctly different from other subgroup DREBs, they have a NLS sequence ‘PKK/RPAGxKxKFxETRHP’ at the upstream of AP2/ERF domain, while DREB2 proteins possess a PKK-like NLS sequence ‘RKxPAKxGxSKxKxGxCMxGxKGGxPENx’ immediately upstream of the AP2/ERF domain [47, 48]. In the current study, all SsDREB1 proteins had a NLS sequence ‘PKxKR/KxP/RxA/TGRxKFxETRHP’, and most SsDREB2 proteins possessed a PKK-like NLS, except for SsDREB2E (Additional File 2), indicating that SsDREB2E might function differently from other SsDREB2 genes.

DREBs are considered as the master regulators of various abiotic stress responses, and also involved in the developmental processes of plants. For instance, OsDREB2A, OsDREB2B, and OsAIB4 were reported to involve in the embryo and endosperm development in rice [49]. In Arabidopsis, ABI4 was involved in the seed development and the lateral root formation [50, 51]. Given gene expression patterns are highly corrected with their functions in plants [52], SsDREB1E, SsDREB1F,
SsDREB1H, and SsDREB2F displayed higher expression levels in leaves than culms at different developmental stages of *S. spontaneum* in the present study (Fig. 5), suggesting these four genes might play a major role in the leaf development throughout the plant life cycle, while SsDREB1A showed related to the leaf development only at maturing stage. And the gradually increased expression pattern from the near-terrestrial end to the distal of steam at the stage of sugar accumulating suggested that SsDREB1L may play role relative to sugar accumulation.
metabolism. Constitutively expression of SsDREB2D in all tissues indicated that this gene may have a central role throughout S. spontaneum life cycle.

Li et al. reported that in the region of transition from the sink to source tissues of the leaf, the transcript abundance of genes associated with the photosynthetic machinery is increased [34], SsDREB1L exhibited higher expression levels in this transitional zone than other regions (Fig. 6), suggesting that this gene could be associated with the photosynthetic machinery of S. spontaneum. The peak expression levels of SsDREB1J, SsDREB1K and SsDREB2D in the undifferentiated basal region suggested that these three genes may regulate the basic cellular activities. The distal region of the leaf was fully differentiated with highest levels of photosynthesis, thus, the dominant expression levels of SsDREB1A, SsDREB1E, SsDREB1F, SsDREB1H and SsDREB2F in the distal region of the leaf indicated that these five genes may be highly correlated with the photosynthetic reactions in leaves. Previous researches have documented that the expression levels of some plant genes are effected by circadian rhythm, which gives plants the innate ability to measure time, and allows them to anticipate daily changes in the environment and to coordinate the developmental and metabolic processes induced by the environmental factors [53–58]. However, only SsDREB2F showed varied expression in the mature leaf during the diurnal cycle (Fig. 7), indicating that this gene might regulate the photosynthesis in mature sugarcane.

Previous efforts have been made to demonstrate that DREB1s or DREB2s involve the process regulating the stress in a number of plants, including maize, rice, cotton, and S. miliorrhiza [19, 25, 26, 46, 59]. In this work, expression profile analysis revealed that SsDREB1F, SsDREB1L, SsDREB2D, and SsDREB2F were induced by drought stress in three sugarcane varieties (Fig. 8), suggesting these four DREB genes might play a key role in response to dehydration in sugarcane. The expression level of SsDREB1L was increased after re-watering in GT31, a sugarcane variety which is not drought-tolerant with the good recovering ability. This result indicated that this gene might help plant recover from drought stress in sugarcane. In addition, the expression levels of SsDREB1A, SsDREB1B, SsDREB1E, SsDREB1F, SsDREB1H, SsDREB1L, SsDREB2D and SsDREB2F were up-regulated under cold stress in S. spontaneum, indicating that these eight genes may be involved in responding to cold stress in S. spontaneum.

Conclusions
In summary, the present study identified 12 DREB1 genes and 6 DREB2 genes from S. spontaneum, and renamed them as SsDREB1A-SsDREB1L, and SsDREB2A-SsDREB2F on the basis of their chromosomal locations. Phylogenetic analysis based on the orthologous from sorghum, rice, maize, and Arabidopsis revealed that a DREB2-type gene, ABI4, was lost during the evolution of S. spontaneum. Analysis of gene duplication showed that tandem duplication events contributed to the expansion of DREB1-type gene in S. spontaneum. In addition, these genes showed functional role in the sugarcane growth and development, photosynthesis, dehydration and cold stress response. However, how these DREB genes participate in the processes of development, and stress response remains to be further elucidated. Our present findings offer a useful information to understand the physiological functions of SsDREBs in sugarcane.

Materials and methods
Plant materials
The founding Saccharum species, S. spontaneum SES208 (originated in USA) was used in this paper [60]. The plant material was identified by Irvine JE [61], and the Saccharum species was planted in the campus of Fujian Agricultural and Forestry University (Fuzhou, China). The collection of S. spontaneum and the performance of experimental research on such plant were complied with the national guidelines of China.

The tissues of S. spontaneum at different developmental stages were obtained from leaf roll, leaf, top immature internode (Stem-3), premature internode (Stem-9) and mature internode (Stem-15) as previously described [62–64]. For leaf gradient experiment, S. spontaneum plants were grown under the following conditions: light intensity of 350 μmol/m²/s, 14:10 L/D, 30 °C L/22 °C D and 60% relative humidity. 11-day-old second leaves were collected after planting 3 h into the light period and were cut into 15 1-cm segments followed the approach described by Li et al. [34]. The mature leaves of S. spontaneum for the diurnal cycle experiment were collected at 12 time points (6 a.m., 8 a.m., 10 a.m., noon, 2 p.m., 4 p.m., 6 p.m., 8 p.m., 10 p.m., midnight, 2 a.m., 4 a.m.) and 7 time points (6 a.m., 10 a.m., 2 p.m., 6 p.m., 10 p.m., 2 a.m., 6 a.m.) from March 2 to 3, 2017 [64, 65].

The transcriptome data of sugarcane with drought treatment were obtained from Sugarcane Research Institute of Guangxi Academy of Agricultural Sciences (Nanning, China). We collected the first leaves of three 7-month-old sugarcane cultivars (F172, strong drought tolerance; GT31, middle drought tolerance; GZ18, drought sensitivity) under normal conditions, mild drought stress, moderate drought stress, severe drought stress and re-watering for RNA-seq library construction. The transcriptome data of S. spontaneum under cold stress were obtained from Yang et al [35].
**Identifications of SsDREB genes**

A Hidden Markov Model (HMM) profile of the AP2/ERF domain (PF00847) was obtained from the Pfam protein family database (http://pfam.xfam.org/) [66] and used to identify the proteins which contain AP2/ERF domain(s). Then we obtained DREB genes based on the similarities of the amino acid sequence and the number of AP2/ERF domains. Finally, the physical and chemical properties including AA, MW, theoretical pl, GRAVY, AI, and II of putative SsDREB proteins were calculated by the online ExPASy-ProtParam tool (http://web.expasy.org/protparam/). Manual annotation was performed for the genes that were incorrectly predicted. Additionally, DREB orthologous genes from sorghum, rice, maize, and Arabidopsis were collected [9, 37, 38, 40, 67].

**Sequence analysis**

The AP2/ERF domain sequences of identified 29 SsDREB proteins were included in multiple sequence alignments using DNAMAN with default parameters. The exon-intron organization of SsDREB genes was determined based on their coding sequence alignments and respective genomics sequences using the online program Gene Structure Display Server (GSDS: http://gsds.gao-lab.org/) [68]. Conserved motif in SsDREB proteins were predicted using TBtools software with number of motifs to find: 10 and minimum-maximum width to find: 6–50 [69]. The non-synonymous (Ka) and synonymous (Ks) substitution ratios were calculated by the easy_KaKs calculation program [70]. The divergence time (T) was calculated by T = Ks/ (2 × 6.1 × 10^-9) × 10^-6 Mya [71].

**Phylogenetic analysis**

The sequences of DREB proteins were aligned using MUSCLE in MEGA (version 7.0) with default parameters [72]. A phylogenetic tree based on the alignment was constructed using MEGA (version 7.0) with the neighbor-joining (NJ) method with the bootstrap test replicated 1000 times, the Poisson model, and Pairwise deletion [73, 74]. The result was imported into the Interactive Tree Of Life (iTOL) program to create the phylogenetic tree [75].

**Chromosomal distribution and gene duplication**

The physical location of SsDREBs on the chromosomes was obtained from the database of S. spontaneum genome. MapInspect software (http://www.softsea.com/download/MapInspect.html) was employed to visualize the chromosomal distribution of deduced SsDREB genes according to their initial position and length of chromosome. To analyze the duplication pattern for each SsDREB gene, the BLASTP program (E-value < 10^-5) and Multiple Collinearity Scan toolkit (MCScanX) were used [76].

**Expression profiling analysis of DREBs in S. spontaneum based on RNA-seq**

HiSeq™ 2500 platform (Illumina Inc., CA, USA) by the Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). The reads obtained from the sequencing instruments under drought and cold stress were filtered to remove adapters and low-quality reads by Trimomatic [77]. The reference genome of S. spontaneum AP85–441 (v20180123) is constructed to be indexed for further analysis by the align_and_estimate_abundance.pl of Trinity (version 2.8.5) [78]. Transcript expression levels of individual genes were quantified using FPKM values (fragments per kilobase of exon per million fragments mapped) by align_and_estimate_abundance.pl in Trinity [78] and value of the gene was calculated using the RNA-Seq by the Expectation-Maximization (RSEM) method.

**Experimental validation of DREB gene expression level by qRT-PCR**

RNA of each sample was in reverse transcription with the StarScript II First-strand cDNA Synthesis Mix with gDNA Remover (GenStar, A224–10) following the manufacturer’s instructions. The qRT-PCR amplification was carried out using 2 × RealStar Green Fast Mixture (GenStar, A301–10) on a Multicolor Real-Time PCR Detection System (Bio-Rad) and the reaction program was refer to the two steps method of the protocol from this kit: 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The expression of glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) and Eukaryotic elongation factor 1a (eEF-1a) were used as internal control [79] and the primers of DREB genes are listed in Additional File 6.

**Abbreviations**

TFs: Transcription factors; DREBs: Dehydration responsive element binding proteins; ABA: Abscisic acid; AP2/ERFBP: APETALA2/ethylene-responsive element-binding protein; AA: The length of protein sequences; MW: The molecular weight; pl: The theoretical isoelectric point; AI: The aliphatic index; GRAVY: The grand average of hydropathicity; II: The instability index; NLS: Nuclear localization signal; Ka: Non-synonymous; Ks: Synonymous; Mya: Million years ago; qRT-PCR: Real time quantitative reverse transcription-polymerase chain reaction; PKM: Fragments Per Transcript Kilobase per Million fragments mapped; RSEM: RNA Sequencing; HMM: Hidden Markov Model; NJ: Neighbor-joining; RSEM: RNA-seq by the Expectation-Maximization; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; eEF-1a: Eukaryotic elongation factor 1a

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12864-021-07799-5.

**Additional file 1** Sequence features of DREBs in S. spontaneum.
We generated in our own laboratory (accession numbers in Genbank: phylows/study/TB2:S28119). The genomic data of S. spontaneum deposited in the Treebase repository (http://purl.org/phylo/treebase).

Availability of data and materials
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Authors’ contributions
Z.L. and J.Z. conceived the study and designed the experiments. Z.L., G.W., X.L., Z.W., M.Z., and J.Z. carried out the experiments and analyzed the data. J.Z. and Z.L. wrote the manuscript. All authors read and approved the final paper.

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Additional file 2. Alignment of the deduced protein sequences of SsDREB1A, SsDREB1B and SsDREB1C with other species. The rectangle in black and red represent AP2/ERF domain and motifs (DASW, LWSY, CMV-I), respectively, the black line represent the NLS sequence.

Additional file 3. The segmental or whole-genome duplicated DREB genes in S. spontaneum.

Additional file 4. qRT-PCR verification of SsDREB2 genes in S. spontaneum. a Comparison of qRT-PCR and RNA-seq data of SsDREB2 genes. b Correlation coefficient between RNA-seq (X axis) and qRT-PCR (Y axis) of four SsDREB2 genes.

Additional file 5. Expression pattern of SsDREB2 genes in tandemly duplicated regions in S. spontaneum.

Additional file 6. Gene primers used for qRT-PCR analysis.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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