

Bioinformatics Analysis of Cellulose Synthase *CesA* Gene from *Miscanthus lutarioriparius*

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Abstract Based on 3 generations transcriptome sequencing technology-PacBio SMRT single-molecule real-time sequencing method, supplemented with 2 generation sequencing method, a complete and accurate unigene library was constructed. After comparing with closely related species, the cellulose synthase gene (*CesA*) 4, 7, 9 gene sequences of *Miscanthus lutarioriparius* were spliced and named as *MICesA4*, *MICesA7* and *MICesA9*. Then, Bioinformatics analysis software was used to construct phylogenetic trees of proteins, to predict and analyze the phosphorylation sites of post-translational modifications of amino acids, and to predict and analyze conserved domains of proteins. The analysis results showed that the sequence lengths of *MICesA4*, 7, 9 genes were 2980 bp, 3310 bp, and 3208 bp, respectively, which were closely related to *Sorghum bicolor* and *Zea mays*; cellulose synthase of *Miscanthus lutarioriparius* (*MICesA*) *MICesA4* and *MICesA9* have 43 phosphorylation sites, *MICesA7* has 48 phosphorylation sites; *MICesA4* is an unstable protein with 6 transmembrane domains, 4 domains are outside the membrane and 3 are inside the membrane.; *MICesA7* and *MICesA9* are stable proteins, both of which have 8 transmembrane domains, 5 domains are outside the membrane and 4 are inside the membrane. The three are all hydrophilic proteins, and the secondary structure is dominated by α -helix and random coils.

Keywords *Miscanthus lutarioriparius*; Cellulose synthase gene (*CesA*); Bioinformatics analysis

Cellulose is an important component of the cell wall of higher plants. It is usually in the form of tiny filaments. The basic unit of cellulose is pyranoid d-glucose, which is connected to the long chain structure by -1, 4 glycosidic bonds. Many genes are involved in the biosynthesis of plant cellulose, the most important of which is cellulose synthase gene Cellulose synthase (*CesA*) belongs to the glycosyltransferase-2 superfamily. Cellulose synthases, which are usually located on the cytoplasmic membrane, have eight transmembrane domains, including six at the carboxyl end and two at the amino end. The amino terminus of cellulose synthase contains four cysteine domains with CxxC motif, which is conducive to the formation of zinc finger structure (Taylor, 2008). In addition, there are two conserved substrate binding sites and catalytically active sites (McFarlane et al., 2014). These domains and active sites interact with other enzymes or proteins in the process of cellulose biosynthesis and participate in different synthesis processes. In 1996, Peal et al cloned the -1, 4-glucosidase transferase gene encoding CESA catalytic subunit from cotton for the first time by using cDNA library random sequencing and sequence analysis (Pear et al., 1996). Since then, cellulose synthase genes in other species have been reported successively, among which the model plant *Arabidopsis Thaliana* has the most mature research on cellulose synthase genes (Richmond, 2000). There are 10 known cellulose synthase genes in *Arabidopsis*, *AtCesA1*, *AtCesA3*, *AtCesA6* and *AtCesA10* are involved in the formation of primary cell walls. *AtCesA4*, *AtCesA7*, and *AtCesA8* are only expressed in the secondary cell wall and coordinate the formation of the secondary cell wall (Taylor et al., 2003); *AtCesA2*, *AtCesA5*, *AtCesA9* and *AtCesA6* are partially redundant in function (Desprez et al., 2007). Subsequently, *Oryza sativa* (Hazen et al., 2002), *Zea mays* (Appenzeller et al., 2004), *Hordeum vulgare* (Burton et al., 2004), *Populus trichocarpa* (Djerbi et al., 2005), *Boehmeria nivea* (Tian et al., 2008), *Phyllostachys edulis* (Zhang et al., 2010) and other *CesA* genes have also been cloned. *Miscanthus Lutarioriparius*, a perennial C4 plant belonging to *Miscanthus*, has the characteristics of fast growth, high yield, low cost, easy reproduction

and wide genetic range, which can preserve water and soil, prevent erosion and maintain the surrounding ecological environment. It is a kind of plant resources with both ecological and economic benefits (Yi, 2012). The fiber cells in the stems of *Miscanthus Lutarioriparius* stalks account for about half of the total number of cells, and the cellulose content is high, and they are often widely used as extremely high-quality papermaking materials (Liu et al., 2001). As a special biomass raw material crop in China, *Miscanthus Lutarioriparius* is attracting more and more researchers' attention.

1 Results and Analysis

1.1 Acquisition of *MICesA* gene fragment

Based on 3 generations transcriptome sequencing technology-PacBio SMRT single-molecule real-time sequencing method, supplemented with 2 generation sequencing method, a complete and accurate unigene library was constructed. After comparing with closely related species, the cellulose synthase gene (*CesA*) 4, 7, 9 gene sequences of *Miscanthus lutarioriparius* were spliced and named as *MICesA4*, *MICesA7* and *MICesA9*. According to the transcriptome information, the gene sequence of *CesA4*, 7 and 9 was extended by kmer, and the length of kmer was set as 57. The length of *MICesA4* gene was 2 980 bp, *MICesA7* gene was 3 310 bp, and *MICesA9* gene was 3 208 bp. The RNA in *M. Lutarioriparius* sample was extracted and measured by agarose gel electrophoresis and ultrahigh ultraviolet spectrophotometer. The 28s and 18s RNA bands were clear (Figure 1) and the value of OD_{260}/OD_{280} was 2.18, indicating that the RNA extraction effect was good and the integrity was high, and the subsequent library construction could be carried out. According to the effective concentration of the library and data output, the Pacbio Sequel platform was used for transcriptome sequencing.

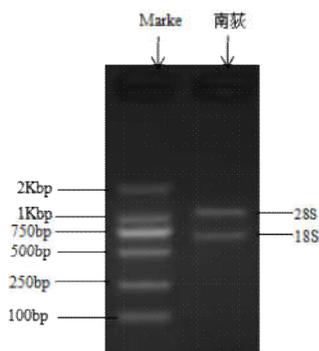


Figure 1 Electrophoresis map of total RNA of *Miscanthus lutarioriparius*

1.2 Analysis of the open reading frame of *MICesA* gene

The ORF Finder software in NCBI was used to analyze the open reading frame of *MICesA4,7,9* gene sequence. The longest open reading frame of *MICesA4* is Frame+3, and its coding region is between 12~2 960 bp, with a total of 2 949 bp and encodes 982 amino acid residues. The longest open reading frame of *MICesA7* is Frame+1, and its coding region is between 22~3 285 bp, with a total of 3 264 bp and encodes 1087 amino acid residues. The longest open reading frame of *MICesA9* is Frame+2, and its coding region is between 35~3 190 bp, with a total of 3 156 bp and encodes 1051 amino acid residues.

1.3 Construction of phylogenetic tree and motif analysis of *Miscanthus lutarioriparius* cellulose synthase (*MICesA*)

The three gene sequences obtained were translated into protein sequences. Through Blastp homologous sequence alignment, it was found that *Miscanthus lutarioriparius* *CesA4* has high similarity with *Sorghum bicolor* *CesA4* (XP_002456361.1), *Zea mays* *CesA11* (NP_001105236.2) and *Panicum miliaceum* *CesA4* (RLM93083.1). *Miscanthus lutarioriparius* *CesA7* has high similarity with *Miscanthus x giganteus* *CesA7* (AMQ81247.1), *Sorghum bicolor* *CesA3* (XP_021309425.1) and *Zea mays* *CesA7* (PWZ13382.1). *Miscanthus lutarioriparius* *CesA9* has high similarity with *Sorghum bicolor* *CesA9* (XP_002460229.1), and *Zea mays* *CesA12* (NP_001105532.1). The results of Blast amino acid homology sequence alignment were combined with MEGA software to construct phylogenetic tree (Figure 2). According to the phylogenetic tree, *Miscanthus lutarioriparius*

CesA is very similar to CesA of sorghum and corn. It is preliminary estimated that *Miscanthus lutarioriparius* is a relative species of sorghum and corn.

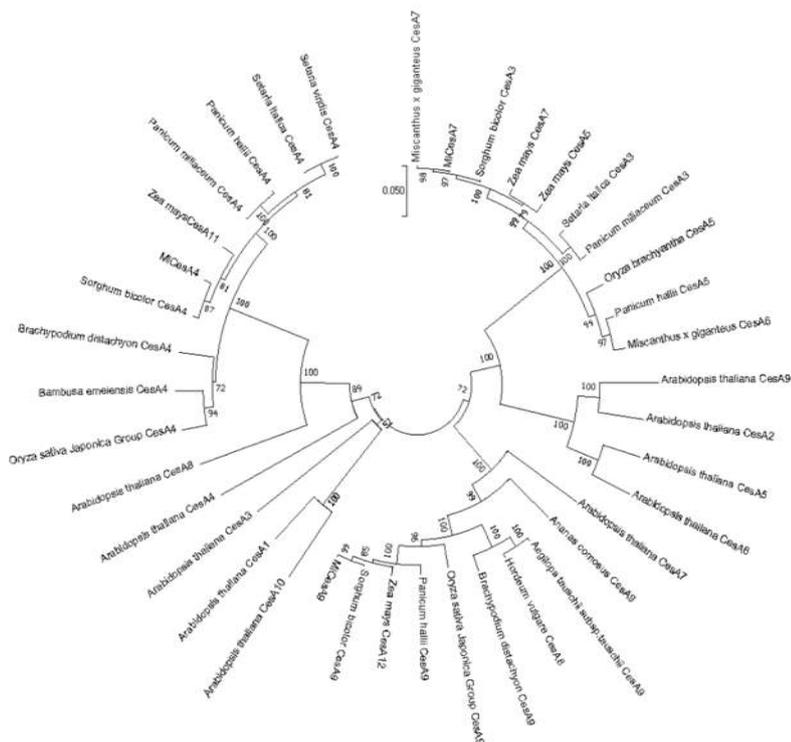


Figure 2 MICesA phylogeny tree

According to the results of the phylogenetic tree, the sequence similar to MICesA4, 7, 9 and the model plant *Arabidopsis* CesA were selected for motif analysis. According to the analysis results of MEME software, there are 15 Motif structures, and MICesA4, 7 and 9 all contain Motif1~Motif15 (Figure 3). Compared with the model plant *Arabidopsis*, MICesA4, 7 and 9 motifs showed little volatility, indicating that their structure was more conservative. MICesA4 repeated Motif3 structure, may be some overlap on the function, MICesA7 and MICesA9 Motif structure is very similar.

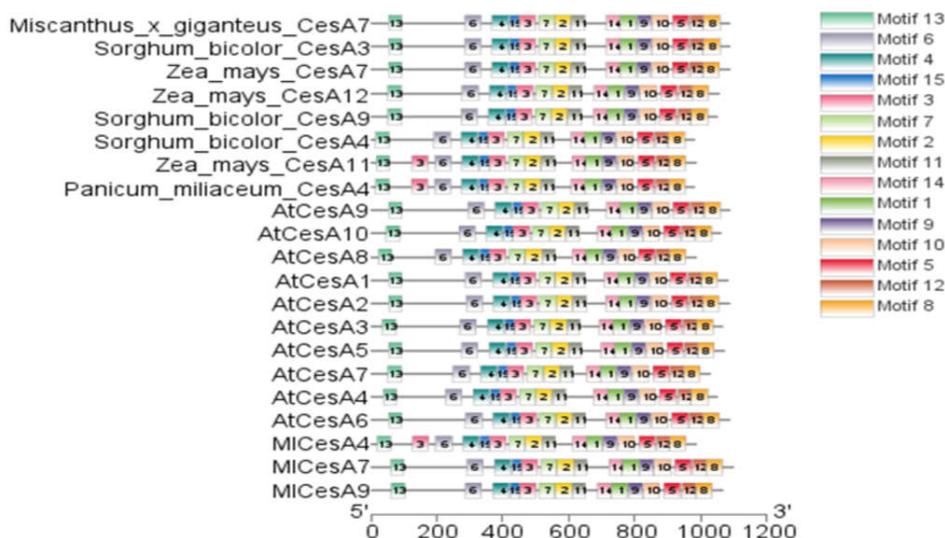


Figure 3 MICesA motif analysis

1.4 Prediction and analysis of basic physicochemical properties and post-translational modifications of amino acids of MICesA protein

After analysis by ProtParm tool software, the basic physicochemical properties of MICesA4, 7,9 proteins were obtained (Table 1).

Table 1 Analysis of the basic physical and chemical properties of MICesA protein

	Number of amino acids	Total number of positively charged amino acids	Total number of negatively charged amino acids	Formula	Isoelectric point	Instability index	Grand average of hydropathicity (GRAVY)
MICesA4	985	104	113	C ₄₉₇₅ H ₇₆₅₆ N ₁₃₁₈ O ₁₄₂₁ S ₅₈	6.11	40.94	-0.103
MICesA7	1094	120	127	C ₅₅₇₆ H ₈₅₃₀ N ₁₄₈₀ O ₁₅₇₆ S ₅₇	6.37	39.86	-0.248
MICesA9	1062	123	125	C ₅₃₈₆ H ₈₃₃₇ N ₁₄₄₅ O ₁₅₂₄ S ₅₆	6.93	36.76	-0.174

According to the data in the Table 1, MICesA4 is an unstable protein, MICesA7 and MICesA9 are stable proteins, and the average coefficient of their total hydrophilicity is negative, all of which are hydrophilic proteins.

Protein phosphorylation is one of the most common and important covalent modifications in organisms, which can regulate the activity and function of proteins. Protein phosphorylation can occur on a variety of amino acids, of which protein phosphorylation on the side chains of serine (Ser), threonine (Thr) and tyrosine (Tyrosine, Tyr) residues is the main one (Yuan et al., 2020). The prediction and analysis of amino acid post-translational modification were performed on the entire polypeptide chain of MICesA4, 7, and 9 through Net Phos 2.0 Server software (Table 2). The results showed that there were 43 amino acid sites with MICesA4 score above the threshold of 0.5, indicating that there were 43 phosphorylation sites of MICesA4. There were 48 amino acid sites with MsCesA7 score above the threshold of 0.5, and 48 phosphorylation sites. There were 43 amino acid sites with MsCesA9 score above the threshold of 0.5, and 43 phosphorylation sites.

Table 2 Prediction and analysis of post-translational modification of amino acids

	Serine	Threonine	Tyrosine	Total phosphorylation sites
MICesA4	21	10	12	43
MICesA7	18	14	16	48
MICesA8	22	10	11	43

1.5 Prediction and analysis of conservative domains

Using the Conserved Domain tool in NCBI to predict the conserved domains of the proteins encoded by the MICesA4, 7, and 9 genes, the results showed that the protein encoded by the MICesA4 gene has a superfamily conserved region PLN02195 super family (cl33434), and the protein encoded by the MICesA7 gene has a superfamily conserved region PLN02436 super family (cl33490), the protein encoded by the MICesA9 gene has superfamily conserved regions PLN02189 super family (cl33433) These conserved regions belong to the cellulose synthase family.

1.6 Prediction and analysis of MICesA protein transmembrane domain and signal peptide

The online software analysis of TMHMM Server v.2.0 transmembrane domains (Figure 4) shows that MICesA4 has 6 transmembrane domains, of which 4 are outside the membrane and 3 are inside the membrane; MICesA7 and MICesA9 have 8 transmembrane domains, of which 5 are outside the membrane and 4 are inside the membrane. According to Signal P-5.0, the potential signal peptide cleavage site in the amino acid sequence of MICesA4, 7, 9 protein was predicted, and the result showed that there was no signal peptide sequence.

1.7 Structural prediction of MICesA protein

The secondary structure of MICesA4, 7,9 protein was predicted by SOPMA software (Figure 5), the results showed that MICesA4 protein is composed of 39.80% α -helix, 4.67% β -turn, 13.60% extended chain, and 41.93% random coil structure. MICesA7 protein is composed of 33.69% α -helix, 5.30% β -turn, 16.00% extended chain, and 45.34% random coil structure; MICesA9 protein is composed of 33.99% α -helix, 4.80% β -turn, 15.25% of the

extended chain, 45.95% of random coil structure composition. The results show that α -helix and random coil structure are the main secondary structure elements in the protein structure of MICesA4,7,9.

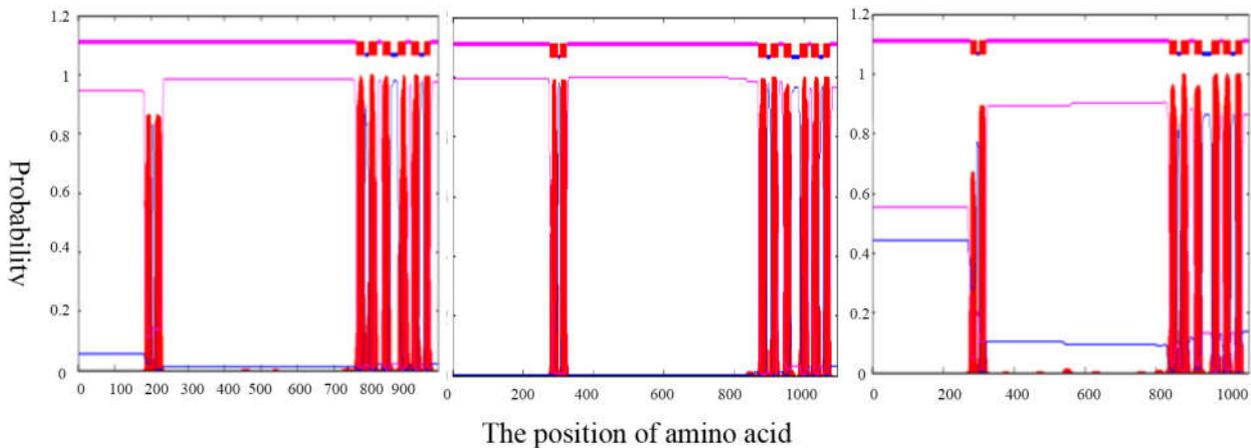


Figure 4 Predictive analysis of the transmembrane domain of MICesA protein

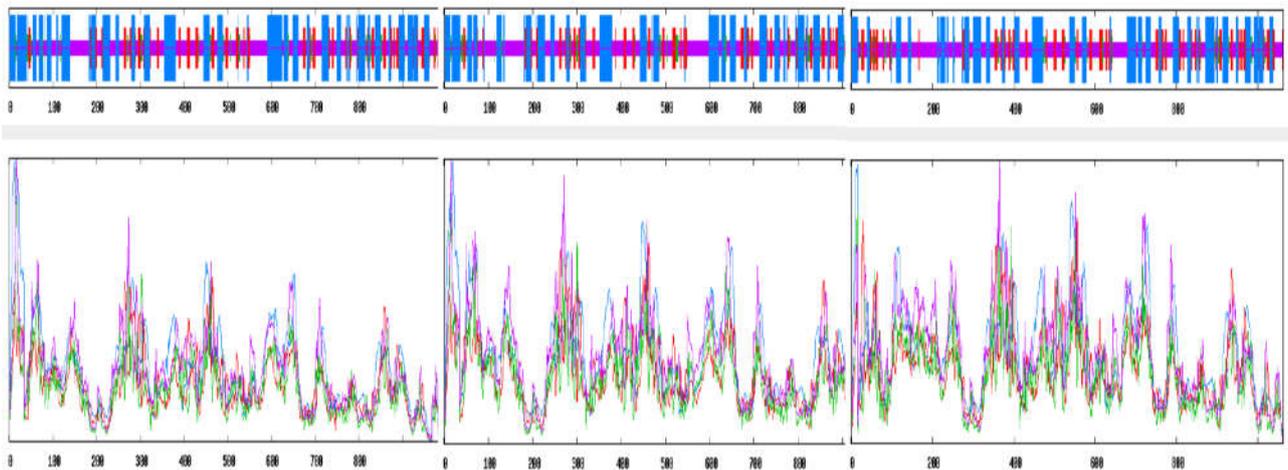


Figure 5 MICesA protein secondary structure prediction

Note: Blue represents: α -helix; Green represents: β -turn; Purple: Random curl; Red: Extended chain

The SWISS-MODE tool in ExPasy was used to predict the three-dimensional structure of MICesA4, 7, and 9 proteins online (Figure 6). Using poplar cellulose synthase (registration number 6wlb.1.A) as a homologous template, the protein sequences of MICesA4, 7, and 9 were compared, and their amino acid sequence similarities were 81.12%, 69.25% and 72.37 %, respectively.; Model Quality Estimation (GMQE) is 0.62, 0.53 and 0.53, respectively; Qualitative Model Energy Analysis (QMEAN) is -3.87, -3.49 and -3.36.

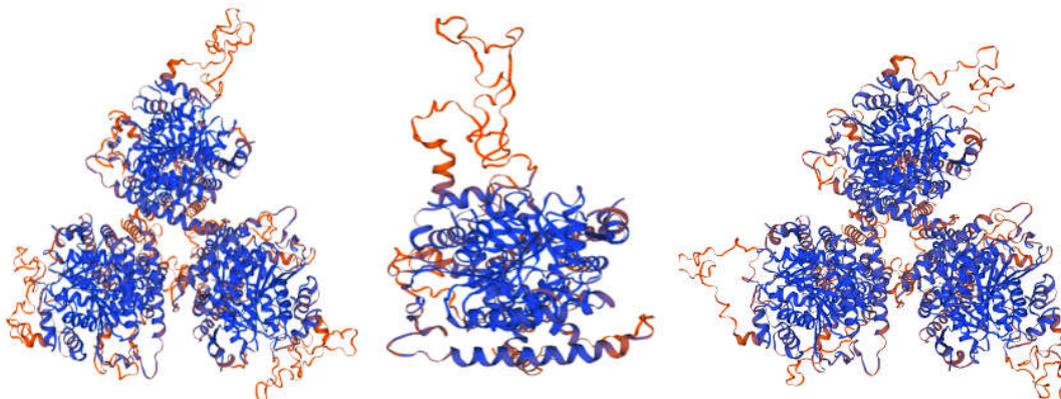


Figure 6 MICesA protein tertiary structure prediction

2 Discussion

2.1 The effect of *CesA* gene on plant growth and development

In recent years, with the rapid development of genetic engineering and bioinformatics, new *CesA* genes have been discovered in succession, and relevant studies on plant *CesA* genes have become more and more comprehensive. Studies have shown that the enzymes involved in cellulose synthesis are different in different tissues and different growth and development stages of plants. In gramineous rice, *OsCesA4*, *OsCesA7* and *OsCesA9* genes were found to be cooperatively expressed in seedlings, stems, immature panicles and roots, and jointly participate in the formation of secondary cell walls (Tanaka et al., 2003). In *Miscanthus × giganteus*, *MgCesA4* and *MgCesA7* are involved in the formation of primary cell walls, and *MgCesA10*, *11* and *12* are involved in the biosynthesis of secondary cell walls, and a cellulose synthase complex with a ratio of 1:1:1 is formed (Zeng et al., 2020). *CesA1*, *CesA3* and *CesA6* genes of sorghum are involved in the formation of primary cell walls and play the same role as those of Arabidopsis. *CesA4*, *CesA7* and *CesA9* genes are highly homologous to the formation of rice secondary cell walls and *CesA4*, *CesA7* and *CesA8* genes of Arabidopsis (Brian et al., 2016). According to the evolution and relationship between the species, *MICesA7* gene may be involved in the formation of primary cell wall, while *MICesA4* gene and *MICesA9* gene may be involved in the formation of secondary cell wall.

2.2 Expression regulation of *CesA* gene

Cellulose synthesis can be regulated at the transcriptional level through *CesA*, and the biggest difference between different *CesA* genes is the presence or absence of introns in the coding sequence and the position of introns in these sequences (Richmond, 2000). *Triticum aestivum CesA1*, *2* and *6* genes are involved in the formation of PCW with 13 introns, while *TaCesA4*, *7* and *8* are involved in the formation of SCW with 7, 12 and 9 introns respectively (Kaur et al., 2016). The number of introns of *CesA* gene in wheat PCW was higher than that of *CesA* gene in SCW. Previous studies have shown that introns in animal and plant genomes can enhance gene expression (Shaul, 2017), and genes containing introns have higher transcription levels (Tang and Gou, 2019). This indicates that in the expression of plant *CesA* genes, the transcription level of *CesA* genes involved in SCW formation is higher than that of *CesA* genes involved in PCW formation.

In addition to the regulation of *CesA* gene at the transcriptional level, the post-transcriptional level of *CesA* gene also affects the synthesis of cellulose. In *Hordeum vulgare*, the small RNA produced by *HvCesA6* can selectively attenuate the expression of *CesA* gene, and this expression may affect the expression of other cell wall biosynthesis genes, thereby greatly affecting the content of barley cellulose (Michael et al., 2008). In the plant cellulose synthesis pathway, in addition to *CesA* gene, the expression and mutation of *CSL* gene (Cao et al., 2019), *KORRIGAN* gene (Aggarwa et al., 2015), *COBRA* gene (Et al., 2020) and *SUSY* gene (Ahmed et al., 2019) in plants can also affect the synthesis of cellulose.

3 Materials and Methods

3.1 Experimental materials

The experimental material *Miscanthus lutarioriparius* (04081) was collected from Changsha, Hunan (113.07 E°, 28.18° N), and is now planted in the *Miscanthus* germplasm resource nursery of Hunan Agricultural University.

3.2 Extraction of total RNA from *Miscanthus lutarioriparius*

The young plants of *Miscanthus lutarioriparius* were selected, frozen in liquid nitrogen and ground, and the total RNA of *Miscanthus lutarioriparius* was extracted with an RNA extraction kit. Take 1 µL of the extracted total RNA and test it after electrophoresis on a 1.0% agarose gel under 180 v voltage for 16 min. After that, Nanodrop, Qubit 2.0, and Agilent 2100 were used to detect the purity, concentration, and integrity of RNA samples.

3.3 Acquisition of gene sequences

Based on 3 generations transcriptome sequencing technology-PacBio SMRT single-molecule real-time sequencing method, supplemented with 2 generation sequencing method, a complete and accurate *Miscanthus lutarioriparius* unigene library was constructed (Altschul et al., 1997). After comparing with closely related species, Use kmer to

extend the gene sequences of *Miscanthus lutarioriparius* *CesA4*, 7, 9 and name them *MICesA4*, *MICesA7*, *MICesA9*.

3.4 Bioinformatics analysis of *CesA* gene

Blast, MEME, ProtParm tool, ORF Finder and Net Phos 2.0 Server were used respectively to analyze and predict the sequence alignment, motif, basic physicochemical properties, open reading frame and amino acid post-translational modifications of the obtained MICesAs gene sequences.

The Conserved Domain, transmembrane Domain, and signal peptides of proteins were predicted and analyzed using the Software TMHMM Server V. 2.0, and SignalP-5.0 Server respectively.

SOPMA and Swiss-Mode analysis software were used to predict the secondary structure and tertiary mechanism of proteins.

Authors' contributions

Yao Li is the experimental designer and executor of this study, who has completed the data analysis and the writing of the first draft of the paper. Yancen He, Yuhuan Lin and Ting Wan participated in experimental design and analysis of experimental results. Zhiyong Chen and Meng Li are the thinkers and principals of the project, guiding experimental design, data analysis, paper writing and modification. All authors read and approved the final manuscript.

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