Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

ESTs identification and construction of normalized cDNA libraries of *Miscanthus lutarioriparius* across combinations of salt and drought stresses

Jianfeng Liao[1, 3, 4], Zili Yi[3, 4], Qian Sun[1,*], Yulai Han[2,*]

[1] *College of Health Science and Environmental Engineering, Shenzhen Technology University, 3002 Lantian Road, Pingshan District, Shenzhen, Guangdong 518118, P. R. China*
[2] *College of New Materials and New Energies, Shenzhen Technology University, 3002 Lantian Road, Pingshan District, Shenzhen, Guangdong 518118, P. R. China*
[3] *College of Bioscience & Biotechnology, Hunan Agricultural University, No.1 Nongda Road, Furong District, Changsha, Hunan 410128, P. R. China*
[4] *Hunan Engineering Laboratory of Miscanthus Ecological Applications, Hunan Agricultural University, No.1 Nongda Road, Furong District, Changsha, Hunan 410128, P. R. China*

Article Info

Article history:

Received: June 23, 2024
Accepted: April 17, 2025
Published: May 31, 2025

Use your device to scan and read the article online

Abstract

Miscanthus lutarioriparius is a perennial C_4 herb with high biomass production and is widely utilized as a non-food biobased material for bioproduction. This study successfully constructed two high-quality full-length normalized cDNA libraries from distinct salt-tolerant accessions of *M. lutarioriparius* under salt, drought, and combined salt-drought stress conditions. The study identified 420 high-quality Expressed Sequence Tags (ESTs) primarily associated with signal transduction mechanisms, post-translational modifications, energy production and transformation, as well as the synthesis, transport, and metabolism of amino acids, carbohydrates, and secondary metabolites. A total of 1370 Gene Ontology (GO) terms were obtained from two accessions, mainly related to cellular process, metabolic process, response to stimulus, biological regulation, biological regulation, cellular anatomical entity, binding, and catalytic activity. Five GO terms from the Biological Process Ontology consistently exhibited high *P*-values in both accessions, primarily associated with responses to exogenous substances and metabolic processes. The significant enrichment of genes associated with cellular components such as the chloroplast matrix, cytoplasm, and plastid matrix from the Cellular Component Ontology may explain the salt-drought tolerance mechanism of *Miscanthus*. This study is expected to deepen our understanding of the functional genes in *Miscanthus* plants and may provide a reference for screening salt and drought-resistance genes.

Keywords: Duplex-specific nuclease, Expressed Sequence Tags (ESTs), *Miscanthus spp.*, Normalized cDNA library, Salt-drought stress combinations.

1. Introduction

Salt and drought stress represent significant soil degradation issues and are considered major factors affecting agricultural production, food security, and sustainable development in arid and semi-arid regions globally [1-4]. According to the Food and Agriculture Organization of the United Nations (FAO), salinized soils covered 397 million hectares worldwide, primarily in low-lying basins and arid plains. Indeed, more than 75 countries were affected by salt stress, with a concentration in Africa, Asia, and the Americas. This stress impacted over 20% of the world's arable land and 33% of crop production in irrigated agricultural areas [5, 6]. Meanwhile, the global extent of saline land is increasing at a rate of 10% per year due to reduced precipitation, increased evaporation, and irrational irrigation resulting from global warming [7]. It is expected that 50% of global arable land will be affected by soil salinization by 2050 [8]. Cultivating crops in saline and arid areas entails significant costs, including soil remediation, irrigation infrastructure, and crop management. These expenses often exceed the potential profits, resulting in economic losses for farmers and agricultural enterprises in these regions. Nevertheless, these regions may be ideal for cultivating specialized bioenergy crops, as growing energy crops in these areas can reduce competition for arable land, decrease soil water evaporation, enhance soil properties and microclimate, and inhibit soil salt reversion [9-11].

Miscanthus lutarioriparius L. Liu ex Renvoize & S. L. Chen is a perennial C_4 herb belonging to the Gramineae family (Poaceae) and is a *Miscanthus* plant endemic to China. *M. lutarioriparius* is a valuable source of lignocellulosic bioethanol and biomass material, with versatile applications in various fields, including papermaking, biofuel production, and synthesis of high-polymer materials

* Corresponding author.
E-mail address: sunqian2@sztu.edu.cn (Q.Sun), hanyulai@sztu.edu.cn (Y. Han).
Doi: http://dx.doi.org/10.14715/cmb/2025.71.5.3

[12-14]. It possesses advantageous characteristics such as efficient photosynthesis, robust stress resistance, lower nutrient requirements, and long-term harvestability [15, 16]. Previous studies have indicated that *M. lutarioriparius* exhibited a higher average annual dry biomass yield compared to *M. sinensis*, *M. sacchariflorus*, and *M. floridulus* [17]. In recent years, the cultivation potential of *M. lutarioriparius* on underutilized or abandoned marginal lands has garnered significant attention as a national non-food biomass feedstock for energy production [18]. Physiological and biochemical responses of *Miscanthus* to individual abiotic stressors have been extensively investigated, including heavy metal stress (Cd, Hg, Pb, Zn, Cu), temperature stress, drought stress and salt stress [19-23]. However, literature addressing the physiological and biochemical responses of *Miscanthus* to combined multiple abiotic stresses is scarce. Only Stavridou *et al.* (2019) investigated the physiological responses of *Miscanthus* spp. (*M. sinensis*, *M. floridulus*, and *M. giganteus*) to both single and combined stresses induced by salt and drought [24]. Moreover, the molecular protective mechanisms of *M. lutarioriparius* in response to salt and drought abiotic stresses remain unclear. Hence, further research is warranted to comprehensively understand the effects of these stresses on *M. lutarioriparius*.

ESTs are complementary DNA (cDNA) sequences that are reverse transcribed from mRNA and represent the gene expression sequences of specific tissues of organisms at specific developmental stages or environmental conditions [25, 26]. With the development of gene sequencing technology and the decrease in sequencing costs, large-scale cDNA sequencing and EST analysis have become effective ways to rapidly and efficiently screen target genes and find unknown genes, which provided the basis for studying the genetic components and physiological functions of various genes [27, 28]. More than 21 million sequences of ESTs are currently present in all plant species [29]. The transcriptional abundance of mRNA in biological cells can be classified into three categories: high, medium, and low expression. Additionally, majority of genes fall into the medium or low expression categories [29]. For a single-tissue cDNA library, the presence of numerous highly abundant genes can lead to unnecessary waste during gene screening and identification, especially in the context of conducting large-scale EST sequencing. The normalized cDNA library is considered an effective strategy to overcome the barriers of functional gene screening and identification caused by differences in gene transcript levels. This process effectively reduced the abundance of duplicate cloned genes, thereby improving the efficiency of random sequencing to help discover new transcripts [30]. A cDNA library of *M. sinenesis* (Eulalia) was constructed by Seong *et al* (2015) after 48 h treatment under UV-B [31]. However, the construction of a normalized full-length cDNA library for *M. lutarioriparius* under conditions of salt, drought and salt-drought stress remains unexplored.

In this study, two accessions of *M. lutarioriparius* were selected as experimental materials to construct normalized full-length cDNA libraries for investigating the molecular mechanisms of stress responses, particularly those induced by salt, drought, and the combination of both stresses. Functional annotation and classification were conducted on ESTs obtained from the cDNA libraries. This study aims to establish a molecular foundation for isolating and screening *M. lutarioriparius* genes associated with salt-drought stress by employing EST sequencing and subsequent gene function analyses.

2. Materials and Methods

2.1. Seedling cultivation and stress treatment

Two accessions of *M. lutarioriparius* (salt-tolerant C0133 and salt-sensitive A0106) were obtained from the Nursery of *Miscanthus* Garden at Hunan Agricultural University. The stalk cuttings with buds were placed in a seedling substrate composed of a mixture of vermiculite and perlite (in a ratio of 4:1 by volume), which was sterilized with carbendazim. The seedling pots were placed in a climatic incubator for cultivation. The average day-night temperature was set to 25/18°C. The day-night photoperiod was set to 16/8 hours. The environment humidity was maintained at 70%, and intensity of photosynthetically active radiation (PAR) was set to 500 μmol/mˆ2/s. The plant seedlings were transplanted into soilless culture containers after reaching the stage of four fully expanded leaves. The roots of the seedlings were rinsed with sterile water to remove adhered soil and then disinfected with 5% NaClO for 5 seconds. Any remaining NaClO solution on the roots was washed away with sterile water. The soil-free culture medium consisted of a 1/2-strength Hoagland nutrient solution, which was refreshed weekly. Upon reaching the 8-leaf stage, *M. lutarioriparius* seedlings exhibiting consistent growth trends were selected for stress treatments, including control check (CK), 150 mM NaCl salt stress (SS), 15% PEG 6000 drought stress (DS), and 150 mM NaCl + 15% PEG 6000 salt-drought stress (SDS). Six seedlings were planted in each treatment group, and three biological replicates were established. After 8 days of treatment, seedlings were transferred to centrifuge tubes, and submerged in liquid nitrogen for storage.

2.2. Extraction and purification of total RNA

Total RNA of the *M. lutarioriparius* seedlings was extracted using the Trizol method (Trizol, Sangon Biotech Co., Ltd., Shanghai, China). The RNA concentration was determined using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., USA), and the integrity of the RNA was checked by agarose electrophoresis. mRNA was isolated using FastTrack® MAG mRNA isolation Kit (Thermo Fisher China Scientific (China) Co., Ltd., Shanghai, China) according to the kit manufacturer's requirements.

2.3. Construction and quality evaluation of normalized cDNA library

The first strand of cDNA was synthesized using the isolated and purified mRNA as the template. According to the kit (Superscript full-length library construction kit, Thermo Fisher China Scientific (China) Co., Ltd., Shanghai, China), first-strand cDNA was enriched using cap-antibody magnetic beads, which was then utilized as a template for the synthesis of the second strand cDNA. The normalized cDNA library was obtained using the Duplex-Specific Nuclease (DSN) method to degrade high-abundance DNA molecules of the cDNA library [32]. Different fragment size separation of the cDNA library was carried out in stages using cDNA Size Fractionation Column (Thermo Fisher China Scientific (China) Co., Ltd., Shanghai, China). The cDNA fragment above 500bp was inserted into the pDONR222 plasmid vector by BP recom-

bination. The initial normalized cDNA library was obtained by introducing the recombinant plasmid into competent *Escherichia coli* cells (DH10B) using Neon N×T Electroporation System (Thermo Fisher China Scientific (China) Co., Ltd., Shanghai, China). The 10 µl bacterial suspension from 2 ml of the original library was diluted 1000-fold, followed by culturing 50 µl of the diluted bacterial suspension using the LB spread plate method for overnight incubation at 37°C. The quality of the constructed cDNA library was assessed using the following calculation formulas: (1) cDNA library titer (CFU/mL) = Number of clones / 50 × 1000 × 1000 mL, and (2) cDNA library total clones (CFU) = cDNA library titer (CFU / mL) × total volume of library solution (mL). Twenty-four clones were randomly selected for PCR amplification to determine the size of the inserted fragment. PCR amplification was performed using pDONR222F/R primers (pDONR222 Primer F: 5′-TCCCAGTCACGACG TTGTAAAACGACGGCCAGTCTT-3′, pDONR222 Primer R: 5′-AGAGCTGCCAGG AAACAGCTATGACCATGTAATACGACTC-3′), and the PCR product fragment size was detected by 3% agarose gel electrophoresis.

2.4. EST Sequencing and data analysis

According to the instructions of commercial kits (Applied Biosystems Sanger Kits, Thermo Fisher China Scientific (China) Co., Ltd., Shanghai, China), 250 positive clones were randomly chosen from each library, resulting in a total of 500 positive clones selected for Sanger sequencing. The sequencing results were assembled and aligned using the *Seqman* software package of *DNAstar*. The vector sequence and the nucleic acid sequence shorter than 150 bp were removed by the *VecScreen* program from NCBI (https://www.ncbi.nlm.nih.gov/tools/vecscreen/). The refined high-quality EST sequences were stored in *Fasta* format for gene function analysis. Based on the *Nr* database, homology analysis and sequence alignment of EST sequences were performed using the *Blast X* software package on the NCBI website. Function annotation and classification of single genes were carried out using the *EggNOG* online function annotation database [33]. GO annotation and enrichment mapping of ESTs sequence were categorized and counted using *TBtool-II* software [34].

3. Results

3.1. RNA and cDNA quality identification

The results of agarose gel electrophoresis are displayed in Fig. 1. The electrophoresis bands for all samples were clear, and the brightness of the 28S electrophoretic band was about twice that of the 18S band. The total RNA concentrations were 1020.7 ng/µl (A0106) and 1021.0 ng/µl (C0133), and the ratios of A260/A280 were 2.14 and 2.15, respectively. These results indicated that the concentration and quality of the sample RNA were high and not contaminated by organic matter such as protein. Therefore, these RNA samples could serve as templates for constructing the library.

The abundance of double-stranded cDNA before and after normalization was detected by 3 % agarose gel electrophoresis (Fig. 2), it could be found that the length of full-length cDNA was mainly distributed between 500-2000 bp, which was consistent with the length range of mRNA. The electrophoretic bands in regions of high-abundance gene showed a uniform diffuse band after normalization. Comparatively, the size and range of the bands did not change significantly, which indicated that the normalized effect of cDNA library was as expected (Fig. 2).

3.2. Identification of normalized cDNA library quality

As shown in Fig. 3, the statistical results of culture plates showed that the monoclonal colony numbers of the cDNA library were 319 with A0106 and 371 with

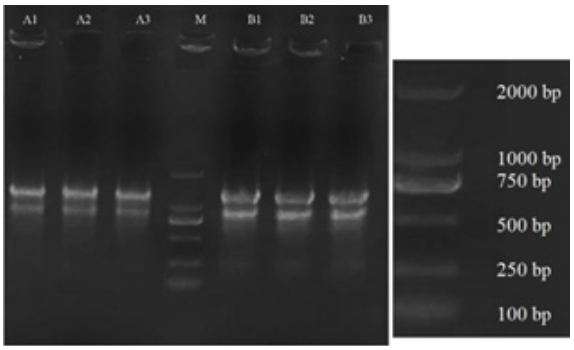

Fig. 1. Total RNA electrophoresis of the two plant accessions (A1 / A2 / A3 were three biological repeats of A0106, B1 / B2 / B3 were three biological repeats of C0133).

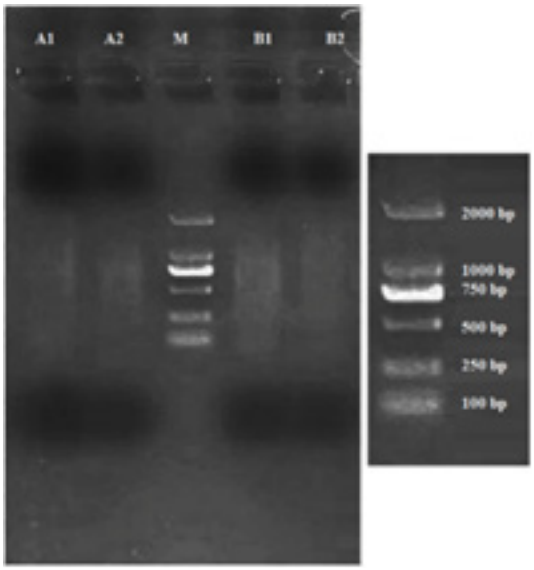

Fig. 2. Agarose gel electrophoresis of non-normalized and normalized amplified SMART-prepared cDNA (A1: cDNA of A0106 before normalization, A2: cDNA of A0106 after normalization, B1: cDNA of C0133 before normalization, B2: cDNA of C0133 after normalization).

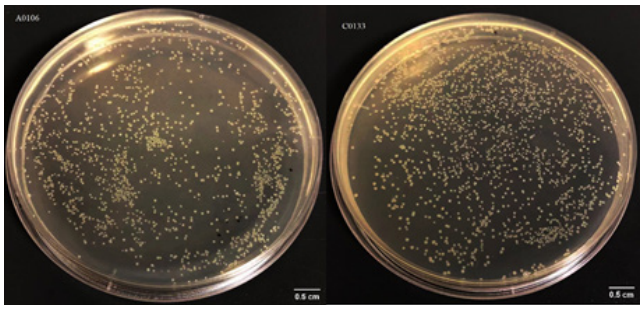

Fig. 3. Spread plate culture map of normalized cDNA library (The white dots represent monoclonal colonies, with A0106 on the left and C0133 on the right).

C0133, respectively. The cDNA library titers for A0106 and C0133 were 6.3×10^6 CFU/mL and 7.4×10^6 CFU/mL, respectively. The cDNA library storage capacity was 1.2×10^7 CFU and 1.4×10^7 CFU, respectively.

As shown in Fig. 4, all lanes of the electropherogram showed clear bands. This means that during the recombination with cDNA library construction, the recombination rate of cDNA library was 100 % (24 / 24). It could be found also that the size of the insertion fragments was concentrated between 500-2000 bp, and the average length was about 1500 bp.

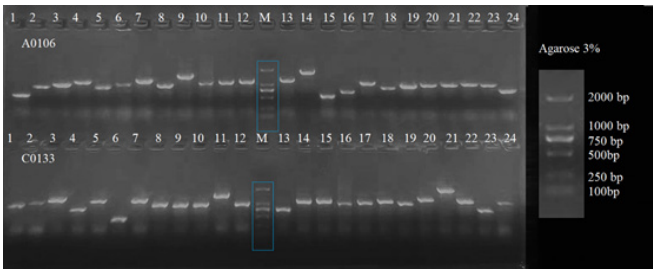

Fig. 4. Agarose gel electrophoresis results of the insert fragment size of two normalized cDNA libraries (M represents DL2000 DNA Marker, and numbers 1 to 24 represent different random clones).

3.3. EST Sequencing and function annotation

A total of 420 effective ESTs were obtained (A0106: 207, C0133: 213) after removing the vector and low-quality sequences (<150bp). This list of GenBank accession numbers for both A0106 and C0133 were PP145384-145590 and PP145591-145803, respectively. The GC content was 54.12 % (A0106) and 54.24 % (C0133), and the average length was 964 bp (A0106) and 878 bp (C0133) (Table 1), respectively. The redundancy of the library was 17.20 % (A0106) and 14.80 % (C0133), respectively.

Table 1. Number of cDNAs sequenced and contig assembly results.

	A0106	C0133
Number of EST sequencing	250	250
Number of unigene	207	213
Number of contigs	15	14
Number of singletons	192	199
Redundancy of cDNA library	17.20%	14.80%
GC%	54.12%	54.24%
Average of length/bp	964	878

Among the 420 sequences of two accessions, 350 sequences had homology, of which 294 sequences had known protein sequences, and 56 sequences had unknown protein sequences (Table 2). According to the known functional protein matching results in the NOG database, which can be classified into three categories (Table 2): information storage and processing (18.71 % in A0106 and 19.55 % in C0133), cellular processes and signaling (32.16 % in A0106 and 32.96 % in C0133), and metabo-

Table 2. Functional category of ESTs matched to known genes.

Classified information	A0106		C0133	
	Number of EST	Percentage of EST	Number of EST	Percentage of EST
Information storage and processing				
Translation, ribosomal structure, and biogenesis	9	5.26%	9	5.03%
RNA processing and modification	5	2.92%	6	3.35%
Transcription	12	7.02%	12	6.70%
Replication, recombination, and repair	5	2.92%	5	2.79%
Chromatin structure and dynamics	1	0.58%	3	1.68%
Cellular processes and signaling				
Cell cycle control, cell division, chromosome partitioning	\	\	3	1.68%
Defense mechanisms	4	2.34%	4	2.23%
Signal transduction mechanisms	19	11.11%	19	10.61%
Cell wall/membrane/envelope biogenesis	5	2.92%	5	2.79%
Cytoskeleton	5	2.92%	6	3.35%
Intracellular trafficking, secretion, and vesicular transport	4	2.34%	4	2.23%
Posttranslational modification, protein turnover, chaperones	18	10.53%	18	10.06%
Metabolism				
Energy production and conversion	11	6.43%	9	5.03%
Carbohydrate transport and metabolism	8	4.68%	9	5.03%
Amino acid transport and metabolism	12	7.02%	13	7.26%
Nucleotide transport and metabolism	1	0.58%	2	1.12%
Coenzyme transport and metabolism	2	1.17%	2	1.12%
Lipid transport and metabolism	6	3.51%	6	3.35%
Inorganic ion transport and metabolism	6	3.51%	6	3.35%
Secondary metabolites biosynthesis, transport, and catabolism	10	5.85%	10	5.59%
Poorly characterized				
Function unknown	28	16.37%	28	15.64%
Total	171	100%	179	100%

lism (32.75 % in A0106 and 31.84 % in C0133). Among them, genes related to *signal transduction mechanisms (STM), posttranslational modification, protein turnover, chaperones (PM/PT/C), amino acid transport and metabolism (AAT/M), transcription, energy production, and conversion (T/EP/C), translation, ribosomal structure, and biogenesis (T/RS/B), carbohydrate transport and metabolism (CT/M), and secondary metabolites biosynthesis, transport, and catabolism (SMB/TC/)* had the highest percentage, with a proportion of 57.89 % (A0106) and 55.31 % (C0133) (Table 2).

3.4. Gene ontology analysis

Through annotating the EST sequences, a total of 1370 GO terms were obtained, with 766 attributed to A0106 and 604 to C0133 (Fig. 5). The proportion of Biological Processes Ontology terms was significantly higher than those of Celluar Component and Molecular Function (Fig. 5). The statistical analysis of GO level 2 terms revealed predominant categories across Biological Process, Cellular Component, and Molecular Function. Specifically, cellular process (GO: 0009987), metabolic process (GO: 0008152), response to stimulus (GO: 0050896), and biological regulation (GO: 0065007) were notable in the Biological Process. In the Cellular Component, cellular anatomical entity (GO: 0110165) stood out, while binding (GO: 0005488) and catalytic activity (GO: 0003824) were prominent in Molecular Function. These categories collectively represented the largest proportion of all GO terms (Fig. 5).

The GO enrichment analysis was conducted using *TBtool-II* software, and the 30 GO terms with the highest *P*-values were selected for display (Fig. 6). According to the GO enrichment results, Biological Process was significantly enriched in the A0106 accession, while both Biological Process and Cellular Component were enriched in C0133 (Figure 6). In the Biological Process Ontology, two accessions consistently showed high *P*-values for five GO terms: response to xenobiotic stimulus (GO:0009410), cellular response to xenobiotic stimulus (GO:0071466), xenobiotic metabolic process (GO:0006805), response to antibiotic (GO:0046677), and regulation of cellular macromolecule biosynthetic process (GO:2000112). Additionally, the Cellular Component Ontology of C0133 showed significant enrichment for three GO terms: chloroplast stroma (GO:0009570), cytoplasm (GO:0005737), and plastid stroma (GO:0009532) (Fig. 6).

4. Discussion

4.1 Construction of cDNA library

The cDNA library contains all the mRNA information of a cell and is an essential tool for studying spatial and temporal-specific expression, gene regulation, gene function, *etc* [35]. During large-scale EST sequencing, highly expressed abundant genes may generate a significant number of redundant sequences, potentially leading to resource wastage in gene screening and identification [36]. By reducing the copy number of genes with high to medium abundance, the frequency of high, medium, and low abundance genes in the library can be made relatively consistent, resulting in what is also referred to as a normalized cDNA library [37]. Currently, the primary strategy for constructing a normalized cDNA library is based on DSN, which can selectively degrade DNA in double-stranded DNA and DNA-RNA hybrid complexes [30]. Utilizing DSN to normalize cDNA offers the advantages of high efficiency and simplicity, ensuring that the length of individual cDNA sequences remains constant. Zhulidov *et al.* (2004) first constructed a normalized full-length cDNA library of *Aplysia californica* (a popular model organism in neuroscience) using the DSN principle [30]. Therefore, this study employed the DSN method to generate normalized cDNA libraries for two accessions of *M. lutarioriparius*.

In the process of constructing a cDNA library, the presence of high-quality mRNA is essential to produce a qualified full-length cDNA library. In this study, the extracted mRNA electrophoretic bands were clear without protein contamination, which provided a solid foundation for the subsequent construction of a high-quality full-length cDNA library. Generally, a high-quality library should have a titer greater than 1×10^6 CFU/mL, a total capacity exceeding 5 million CFU, a positive cloning rate of over 95 %, and an average insertion fragment size greater than or equal to 1.5 kb [38]. In this study, the titer of the two cDNA libraries was more than 6.3×10^6 CFU/ mL, the total library capacity was more than 1.2×10^7 CFU, the average length of the inserted fragments was about 1.5 Kbp, and

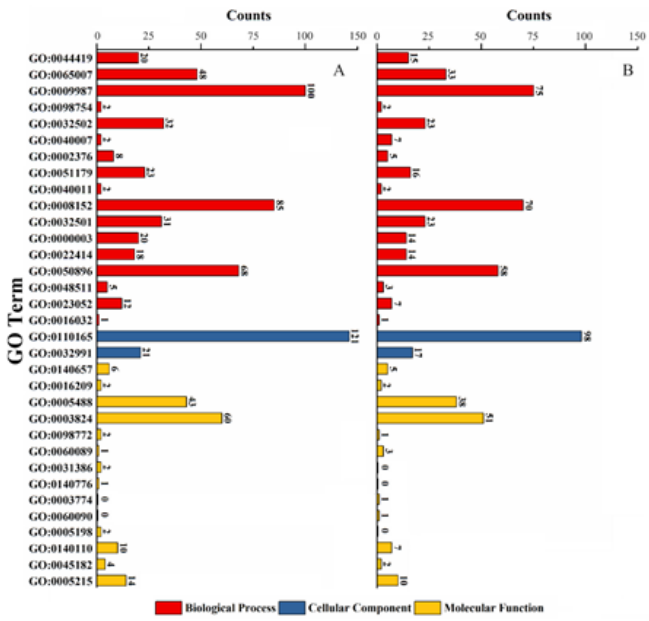

Fig. 5. Distribution of level 2 Gene Ontology (GO) categories by biological process, cellular component, and molecular function (A represents A0106 accession, B represents C0133 accession).

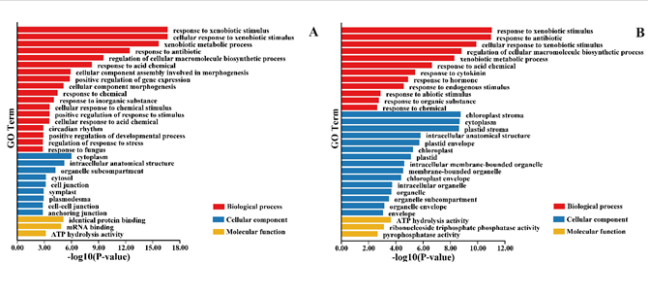

Fig. 6. Gene Ontology (GO) functional enrichment analysis of different salt-tolerant accessions of *M. lutarioriparius* across combinations of salt and drought stresses (the top 30 enriched GO terms were ranked according to their enrichment score, A represents A0106 accession, B represents C0133 accession).

the recombination rate of the library was 100 %. Therefore, the two cDNA libraries constructed in the study were high-quality libraries.

4.2. EST sequence analysis

In recent years, there has been rapid progress in genomics research, leading to a deeper understanding of the spatial and temporal patterns of gene expression [39, 40]. In genomic research, EST sequencing is one of the most cost-effective and efficient methods for obtaining genetic information [41, 42]. By analyzing the ESTs of the full-length cDNA library, the gene expression patterns of specific tissues or developmental stages could be detected, which could reflect the differential expression of genes caused by the environment [43]. Therefore, accurate analysis of EST sequences was essential to ensure the reliability of the research results. In this study, a total of 500 EST sequences were obtained from the two cDNA libraries, and *VecScreen* program was employed to remove vector sequences from the ESTs, thus preventing vector sequence contamination. The EST sequences were then assembled into contiguous sequences using the *Seqman* program. The putative encoded proteins of the ESTs were aligned using the *BlastX* program to identify the highest homology. Further functional annotation of these protein sequences was conducted using the *EggNOG* online tool and *TBtool-II* software. These methods serve to minimize data errors and ensure the reliability of the results.

Research has shown that when subjected to abiotic stresses such as salt and drought, *M. sinensis* relies on osmoregulatory substances (e.g., ion concentration, sucrose content, total free amino acid content, soluble protein content), as well as antioxidant systems, and ion channel activity in its leaves to protect itself from these stresses [20]. Płażek *et al.* (2014) demonstrated that *M. giganteus* accumulated significant amounts of potassium ions and proline in its leaves when subjected to 50 mM NaCl treatment, suggesting that the accumulation of these substances contributed to the tolerance of *M. giganteus* to salt stress [44]. Our study reveals that the proportion and quantity of gene expression in cellular processes, signaling, and metabolism accounted for over 60% of the total cDNA library EST sequences in the two libraries (Table 2, Fig. 5). Among these, genes related to protein turnover, chaperones, signal transduction mechanisms, and post-translational modifications account for the largest proportion, which is also essential for maintaining the normal life cycle of plants. Genes related to salt and drought tolerance, including amino acid transport and metabolism, energy production and conversion, carbohydrate transport and metabolism, secondary metabolite biosynthesis, transport and catabolism, lipid transport and metabolism, and inorganic ion transport and metabolism, were also highly enriched in two *M. lutarioriparius*.

Additionally, in Biological Processes Ontology, the top 5 significantly enriched GO terms of the two *M. lutarioriparius* accessions were consistent, primarily involving responses and metabolic processes triggered by xenobiotic substances. This implies that *M. lutarioriparius* might activate a variety of biosynthetic processes to cope with salt and drought stress. These activities primarily involve the activation of relevant genes and production of osmotic regulators. In Cellular Component Ontology, the chloroplast stroma (GO:0009570), cytoplasm (GO:0005737), and plastid stroma (GO:0009532) of C0133 accessions exhibited significant enrichment. While the cytoplasm (GO:0005737) was enriched to a certain extent in A0106, the degree of enrichment was significantly lower than that observed in C0133. This observation might explain the greater salt stress resistance of C0133 compared to A0106. This could be attributed to the higher concentration of osmotic regulators, such as soluble proteins and sugars, within the cytoplasm and chloroplast stroma of C0133. This could effectively enhance the osmoregulatory stress resistance of *M. lutarioriparius*. This study analyzed the gene expression patterns of *M. lutarioriparius* under salt-drought stress conditions. This aspect provided a deeper understanding of the molecular mechanisms underlying salt and drought tolerance in *Miscanthus spp*.

5. Conclusion

M. lutarioriparius, a C_4 herb belonging to the Poaceae family within the genus *Miscanthus*. It is considered one of the most promising energy crops due to its robust photosynthetic rate and high biomass yield. This study constructed two high-quality cDNA libraries for *M. lutarioriparius*, with cDNA library titers of 6.3×10^6 CFU/mL for A0106 and 7.4×10^6 CFU/mL for C0133, respectively. The average length of the inserted fragments was approximately 1.5 Kbp, with a recombination rate of 100% for the libraries. The ESTs gene functions corresponding to the two cDNA libraries are primarily associated with signal transduction mechanism, post-translational modification, energy production and transformation, and synthesis, transport and metabolism of amino acids, carbohydrates, and secondary metabolites. Biological Process ontology terms associated with stimulation by exogenous substances were significantly enriched in both accessions. Additionally, Cellular Component terms related to the cytoplasm and chloroplast were significantly enriched in the salt-tolerant accession C0133, potentially explaining its higher degree of salt tolerance compared to A0106. This study provides a molecular theoretical basis for the molecular breeding of *M. lutarioriparius* to enhance salt and drought tolerance.

Conflict of interest

All authors declare that No conflict of interest exists.

Availability of data and material

All data was embedded in the manuscript.

Author's contributions

Conceptualization, Q.S. and Y.H.; investigation, J.L., Z.Y. and Q.S.; resources, Z.Y. and Y.H.; writing—original draft preparation, J.L.; writing—review and editing, J.L., Q.S. and Y.H.; visualization, J.L.; supervision, Q.S. and Y.H.; project administration, Q.S.; funding acquisition, Q.S. All authors have read and agreed to the published version of the manuscript.

Funding

This work was supported by the Natural Science Foundation of Top Talent of SZTU (Grant no. 2019010801010).

References

1. Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant

to cell. Annals of Botany 103 (4): 551-560. doi: 10.1093/aob/mcn125
2. Ma Y, Dias MC, Freitas H (2020) Drought and Salinity Stress Responses and Microbe-Induced Tolerance in Plants. Frontiers in Plant Science 11: 591911. doi: 10.3389/fpls.2020.591911
3. Machado R, Serralheiro R (2017) Soil Salinity: Effect on Vegetable Crop Growth. Management Practices to Prevent and Mitigate Soil Salinization. Horticulturae 3 (2): 13. doi: 10.3390/horticulturae3020030
4. Madhulika, Singh, Jitendra, Kumar, Samiksha, Vijay, Pratap, Sheo (2015) Roles of osmoprotectants in improving salinity and drought tolerance in plants: a review. Reviews in Environmental Science & Biotechnology 14 (3): 407 - 426. doi: 10.1007/s11157-015-9372-8
5. Li JG, Pu LJ, Han MF, Zhu M, Zhang RS, Xiang YZ (2014) Soil salinization research in China: Advances and prospects. Journal of Geographical Sciences 24 (5): 943-960. doi: 10.1007/s11442-014-1130-2
6. Osman KT (2018) Saline and Sodic Soils. In: Osman KT (ed) Management of Soil Problems. Springer International Publishing, Cham, pp 255-298. doi: 10.1007/978-3-319-75527-4_10
7. Shrivastava P, Kumar R (2015) Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. Saudi Journal of Biological Sciences 22 (2): 123-131. doi: 10.1016/j.sjbs.2014.12.001
8. Jamil A, Riaz S, Ashraf M, Foolad MR (2011) Gene Expression Profiling of Plants under Salt Stress. Critical Reviews in Plant Sciences 30 (5): 435-458. doi: 10.1080/07352689.2011.605739
9. Tiedge K, Muchlinski A, Zerbe P (2020) Genomics-enabled analysis of specialized metabolism in bioenergy crops: current progress and challenges. Synthetic Biology 5 (1): ysaa005. doi: 10.1093/synbio/ysaa005
10. Oliver RJ, Finch JW, Taylor G (2009) Second generation bioenergy crops and climate change: a review of the effects of elevated atmospheric CO and drought on water use and the implications for yield. Global Change Biology Bioenergy 1 (2): 97-114. doi: 10.1111/j.1757-1707.2009.01011.x
11. Somerville C, Youngs H, Taylor C, Davis SC, Long SP (2010) Feedstocks for Lignocellulosic Biofuels. Science 329 (5993): 790-792. doi: 10.1126/science.1189268
12. Chung J-H, Kim D-S (2012) *Miscanthus* as a potential bioenergy crop in East Asia. Journal of Crop Science and Biotechnology 15 (2): 65-77. doi: 10.1007/s12892-012-0023-0
13. Wang S, Yi Z, Iqbal Y, Chen Z, Xue S, Fu T, Li M (2022) Polyploid *Miscanthus Lutarioriparius*: A Sustainable and Scalable Biomass Feedstock for Cellulose Nanocrystal Preparation in Biorefinery. Agronomy 12 (5): 1057. doi: 10.3390/agronomy12051057
14. Edmonds P, Franz KJ, Heaton EA, Kaleita AL, Soupir ML, VanLoocke A (2021) Planting *miscanthus* instead of row crops may increase the productivity and economic performance of farmed potholes. GCB Bioenergy 13 (9): 1481-1497. doi: 10.1111/gcbb.12870
15. Zheng C, Xiao L, Iqbal Y, Sun G, Feng H, Liu F, Duan M, Yi Z (2022) *Miscanthus* interspecific hybrids exceed the biomass yield and quality of their parents in the saline–alkaline Yellow River delta. Food and energy security 11 (1): 347. doi: 10.1002/fes3.347
16. Wang C, Kong Y, Hu R, Zhou G (2021) *Miscanthus*: A fast-growing crop for environmental remediation and biofuel production. GCB Bioenergy 13 (1): 58-69. doi: 10.1111/gcbb.12761
17. Xue S, Guo MQ, Iqbal Y, Liao JF, Yang S, Xiao L, Yi ZL (2020) Mapping current distribution and genetic diversity of the native *Miscanthus lutarioriparius* across China. Renewable & Sustainable Energy Reviews 134: 110386. doi: 10.1016/j.rser.2020.110386
18. Xue S, Lewandowski I, Wang XY, Yi ZL (2016) Assessment of the production potentials of *Miscanthus* on marginal land in China. Renewable & Sustainable Energy Reviews 54: 932-943. doi: 10.1016/j.rser.2015.10.040
19. Stavridou E, Hastings A, Webster RJ, Robson PRH (2017) The impact of soil salinity on the yield, composition and physiology of the bioenergy grass *Miscanthus* × *giganteus*. Global Change Biology Bioenergy 9 (1): 92-104. doi: 10.1111/gcbb.12351
20. Sun Q, Yamada T, Takano T (2014) Salinity Effects on Germination, Growth, Photosynthesis, and Ion Accumulation in Wild *Miscanthus sinensis* Anderss. Populations. Crop Science 54 (6): 2760-2771. doi: 10.2135/cropsci2013.09.0636
21. Wang J, Duan XC, Wang YZ, Sheng JJ (2022) Transcriptomic and physiological analyses of *Miscanthus lutarioriparius* in response to plumbum stress. Industrial Crops and Products 176: 114305. doi: 10.1016/j.indcrop.2021.114305
22. Wilkins C (1997) The uptake of copper, arsenic and zinc by *Miscanthus*-environmental implications for use as an energy crop. Aspects of Applied Biology: 335-340.
23. Zhang J, Yang S, Huang Y, Zhou S (2015) The Tolerance and Accumulation of *Miscanthus Sacchariflorus* (maxim.) Benth., an Energy Plant Species, to Cadmium. International Journal of Phytoremediation 17 (6): 538-545. doi: 10.1080/15226514.2014.922925
24. Stavridou E, Webster RJ, Robson PRH (2019) Novel *Miscanthus* genotypes selected for different drought tolerance phenotypes show enhanced tolerance across combinations of salinity and drought treatments. Annals of Botany 124 (4): 653-674. doi: 10.1093/aob/mcz009
25. Bhavsar M, Mangukia N, Kumar SP, Patel S, Rawal R, Mankad A (2023) Identification and characterization of known and new miRNAs from *Nicotiana tabacum* and nta-miR156's predictive role in Wnt Signalling Pathway. Advances in cancer biology - Metastasis 8: 100105. doi: 10.1016/j.adcanc.2023.100105
26. Mahalle RM, Bosamia TC, Chakravarty S, Srivastava K, Meena RS, Kadam US, Srivastava CP (2023) De Novo Mining and Validating Novel Microsatellite Markers to Assess Genetic Diversity in (F.), a Legume Pod Borer. Genes 14 (7): 1433. doi: 10.3390/genes14071433
27. Zhang P, Kratz AS, Salama M, Elabd S, Heinrich T, Wittbrodt J, Blattner C, Davidson G (2015) Expression screening using a Medaka cDNA library identifies evolutionarily conserved regulators of the p53/Mdm2 pathway. BMC Biotechnology 15: 92. doi: 10.1186/s12896-015-0208-y
28. Ochoa-Hernández ME, Reynoso-Varela A, Martínez-Córdova LR, Rodelas B, Durán U, Alcántara-Hernández RJ, Serrano-Palacios D, Calderón K (2023) Linking the shifts in the metabolically active microbiota in a UASB and hybrid anaerobic-aerobic bioreactor for swine wastewater treatment. Journal of Environmental Management 344: 118435. doi: 10.1016/j.jenvman.2023.118435
29. Blair MW, Fernandez AC, Ishitani M, Moreta D, Seki M, Ayling S, Shinozaki K (2011) Construction and EST sequencing of full-length, drought stress cDNA libraries for common beans (*Phaseolus vulgaris* L.) BMC Plant Biology 11 (1): 171 -171. doi: 10.1186/1471-2229-11-171
30. Zhulidov PA, Bogdanova EA, Shcheglov AS, Vagner LL, Khaspekov GL, Kozhemyako VB, Matz MV, Meleshkevitch E, Moroz LL, Lukyanov SA, Shagin DA (2004) Simple cDNA normalization using kamchatka crab duplex-specific nuclease. Nucleic Acids Research 32 (3): e37. doi: 10.1093/nar/gnh031
31. Seong, Yoo JH, Choi JH, Kim NJ, Yu CY (2015) Construction and classification of a cDNA Library from *Miscanthus sinensis* (Eulalia) treated with UV-B. Plant Omics 8 (3): 264-269.
32. Zhan XY, Yang S, Zhang Y, Yang X, Fu WL (2021) Construction of microRNA-21 terahertz metamaterial sensing method based

on duplex-specific nuclease triggered rolling circle amplification. Chinese journal of preventive medicine 55 (2): 212 -218. doi: 10.3760/cma.j.cn112150-20201111-01359

33. Huerta-Cepas J, Szklarczyk D, Heller D, Hernández-Plaza A, Forslund SK, Cook H, Mende DR, Letunic I, Rattei T, Jensen LJ, von Mering C, Bork P (2019) eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. Nucleic Acids Research 47 (D1): D309-D314. doi: 10.1093/nar/gky1085
34. Chen CJ, Wu Y, Li JW, Wang X, Zeng ZH, Xu J, Liu YL, Feng JT, Chen H, He YH, Xia R (2023) TBtools-II: A "one for all, all for one"bioinformatics platform for biological big-data mining. Molecular Plant 16 (11): 1733-1742. doi: 10.1016/j.molp.2023.09.010
35. Islam MS, Mohamed G, Polash SA, Hasan MA, Sultana R, Saiara N, Dong WB (2021) Antimicrobial Peptides from Plants: A cDNA-Library Based Isolation, Purification, Characterization Approach and Elucidating Their Modes of Action. International Journal of Molecular Sciences 22 (16): 8712. doi: 10.3390/ijms22168712
36. Liu YS, Beyer A, Aebersold R (2016) On the Dependency of Cellular Protein Levels on mRNA Abundance. Cell 165 (3): 535-550. doi: 10.1016/j.cell.2016.03.014
37. Chen YG (2003) Construction of a normalized cDNA library by mRNA-cDNA hybridization and subtraction. Methods Mol Biol 221: 33-40. doi: 10.1385/1-59259-359-3:33
38. Fülle H-J (2003) Quality Assessment of cDNA Libraries. In: Ying S-Y (ed) Generation of cDNA Libraries: Methods and Protocols. Humana Press, Totowa, NJ, pp 145-153. doi: 10.1385/1-59259-359-3:145
39. Cui T, Bai Q, Yu WQ, Guo DY, Ban YW, Chen K, Raza A, Zhou GH, Wu QF (2023) Chromosome-level genome assembly and population genomic analysis provide novel insights into the immunity and evolution of *Sogatella furcifera*. Genomics 115 (6): 110729. doi: 10.1016/j.ygeno.2023.110729
40. Gutierrez AP, Selly SLC, Pountney SM, Taggart JB, Kokkinias P, Cavrois-Rogacki T, Fernandez EJ, Migaud H, Lein I, Davie A, Bekaert M (2023) Development of genomic markers associated to growth-related traits and sex determination in lumpfish (*Cyclopterus lumpus*). Genomics 115 (6): 110721. doi: 10.1016/j.ygeno.2023.110721
41. Liao QY, Kong XJ, Luo GQ, Wu XY, Li YP, Liu QC, Tang C, Liu ZH (2022) Molecular Diversity of Peptide Toxins in the Venom of Spider *Heteropoda pingtungensis* as Revealed by cDNA Library and Transcriptome Sequencing Analysis. Toxins 14 (2): 40. doi: 10.3390/toxins14020140
42. Wang BX, Zhang MM, Zhang J, Huang LP, Chen X, Jiang MY, Tan MP (2020) Profiling of rice Cd-tolerant genes through yeast-based cDNA library survival screening. Plant Physiology and Biochemistry 155: 429-436. doi: 10.1016/j.plaphy.2020.07.046
43. Liu C, Wang X, Tian JB, Ma PY, Meng FX, Zhang Q, Yu BF, Guo R, Liu ZZ, Wang HL, Xie J, Cheng NL, Wang JH, Niu B, Sun GQ (2019) Construction of a cDNA library and preliminary analysis of the expressed sequence tags of the earthworm *Eisenia fetida* (Savigny, 1826). Molecular Medicine Reports 19 (4): 2537-2550. doi: 10.3892/mmr.2019.9938
44. Płażek A, Dubert F, Kościelniak J, Tatrzańska M, Maciejewski M, Gondek K, Żurek G (2014) Tolerance of *Miscanthus×giganteus* to salinity depends on initial weight of rhizomes as well as high accumulation of potassium and proline in leaves. Industrial Crops and Products 52: 278-285. doi: 10.1016/j.indcrop.2013.10.041