Analysis of controlling genes for tiller growth of *Psathyrostachys juncea* based on transcriptome sequencing technology

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Abstract

Tillering is an important trait of bunch grass that affects biomass and seed yield. *Psathyrostachys juncea* is a typical perennial bunch grass, and unraveling the regulatory mechanisms of tillering in *P. juncea* could be helpful to improve the yield of perennial gramineous forages. Hence, we selected the tiller node of *P. juncea* for transcriptome sequencing to determine the differentially expressed genes (DEG) between high and low tillering materials. The metabolic pathway was studied, candidate genes were screened, and reference genes stability were evaluated. The results showed that approximately 5466 DEGs were identified between two *P. juncea* genotypes that significantly differed in tiller number. Pathway enrichment analysis indicated that DEGs related to the biosynthesis of three classes of phytohormones, i.e., strigolactones (SLs), auxin (IAA), and cytokinin (CTK), as well as "nitrogen metabolism" and "biosynthesis of lignin" dominated the differences between the dense and sparse tillering genotypes. Meanwhile, the reference gene *Actin1*, having with the best stability, was screened from twelve highest expression level genes and was used in verification of ten tillering candidate genes. The candidate genes revealed in our research are involved in the regulation of tillering in perennial grasses and are available for new breeding resources establishment for high-yield perennial grasses.

Introduction

Tillering is a very complicated process controlled by a variety of internal factors and external environmental conditions, including natural conditions, endogenous hormones, genetic features and other factors. It has been reported that the growth of tiller buds is closely related to nutrition. When the plant nutrition is sufficient, it can promote the growth of tiller buds, in which nitrogen can regulate the number of tillers (Bauer 2020). Transcriptome analysis can provide basic information for the genetic characteristics of species without detailed genomes sequence information, particularly in the research of tillering-related genes (Rosli 2013; Pombo 2017). Therefore, next-generation sequencing technology was used in this research to study the differentially expressed genes (DEG) related to tillering of *P. juncea* to identify the candidate genes that may regulate tillering in perennial grasses, and to study the metabolic pathways corresponding to the regulation of tillering related genes. Furthermore, based on the RNA-seq datasets screened candidate genes of *P. juncea*, the reliability of the transcriptome data was verified by qRT-PCR. The stability of the reference gene for expression verification was comprehensively evaluated to establish appropriate reference genes for *P. juncea* genome. This study provides basic data for genotype selection, molecular marker-assisted breeding, and breeding efficiency acceleration of *P. juncea* and other perennial grass on biomass yield traits.

Methods

P. juncea plant materials used in this study were mainly came from two national plant germplasm organizations. In total, 17 accessions were used based on field tillering phenotypic evaluation.

Tillering traits were measured on the materials of 60 individual plants in two samples at two locations for three consecutive years. The ploidy of *P. juncea* was identified by Beckman Coulter (Beckman Kurt Co., Ltd, USA) flow cytometry. A group of 30 plants with dense tillers and another group of 30 plants with sparse tillers were selected from the same 17 accessions (named DT and ST, respectively). Total RNA was extracted from tissue using the rapid plant RNA extraction kit. Equal amounts of RNA of the 30 plants of each group were pooled to establish DT and ST samples with three repetitions per sample. The cDNA library was constructed and sequenced by the Biomarker Biotechnology Corporation (Beijing, China). All unigene sequences were compared with NR, Swiss-Prot, GO, KOG/COG, Pfam, KEGG databases using BLAST. Finally, annotation information of unigene was obtained. To identify DEGs between dense and sparse tiller samples, the FPKM method was used to analyze gene expression levels in transcriptome sequencing data. Using RNA-seq data from *P. juncea* with different tiller numbers, we chose those with high expression level genes (mostly, FPKM >10) (Shen 2020). Finally, 12 genes were selected as candidate reference genes. To validate the RNA-Seq expression data, 10 selected DEGs involved in tillering traits were determined by one-step qRT-PCR.

Results and Discussion

Phenotypic analysis of the dense and sparse tillering trait of P. juncea

Dense and sparse tillering materials formed an average of 51 and 25 new tillers in 2019, respectively. Therefore, there was a significant difference in the number of tillers between DT and ST ($P < 0.05$) (Fig. 1). The flow cytometry analysis of the two samples showed that both DT and ST were diploid genotypes. The ploidy identification results show that the difference between dense tillering and sparse tillering was not caused by ploidy.

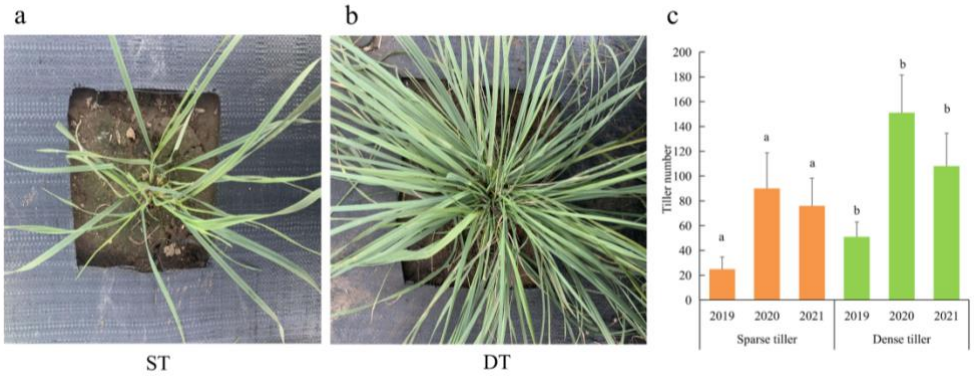

Fig. 1 The phenotype photographs of ST and DT. (a), sparse tillering genotype in the experimental field. (b), dense tillering genotype in the experimental field. (c), the tiller number of ST and DT in three years ($P<0.05$).

Gene annotation of the P. juncea transcriptome

A total of 100,560 unigenes were identified, of which 49,363 unigenes were annotated in at least one database using BLASTx (E-value < 1×10^{-5}) and HMMER (E-value < 1×10^{-10}). In addition, 145, 588, 1815, 319, 330 and 4833 unigenes were annotated to the COG, SwissProt, Pfam, KOG, KEGG and Go databases. About half (48,512/100,560) of the unigenes could be annotated by BLASTx (E-value <1E-5) using the NCBI Nr database. Based on the E-value distribution, approximately 30% of unigenes showed homology (1E-30 < E-value ≤ 1E-5), 30% of unigenes showed strong homology (1E-100 <E-value ≤ 1E-30), and 40% of the unigenes showed extremely strong homology (E-value <1E-100) to available plant genome sequences.

Functional characterization of DEGs among the DT and ST genotypes

The comprehensive analysis of the KEGG pathway and GO functional enrichment showed that there were DEGs related to carbon metabolism, photosynthesis and DNA replication in eukaryotes between DT and ST. In DT vs ST upregulation comparison, metabolic processes of "biosynthesis of amino acids" and "plant hormone signal transduction" had relatively high q value, and this suggested that these processes were involved in tillering regulation of *P. juncea* (Fig. 2b).

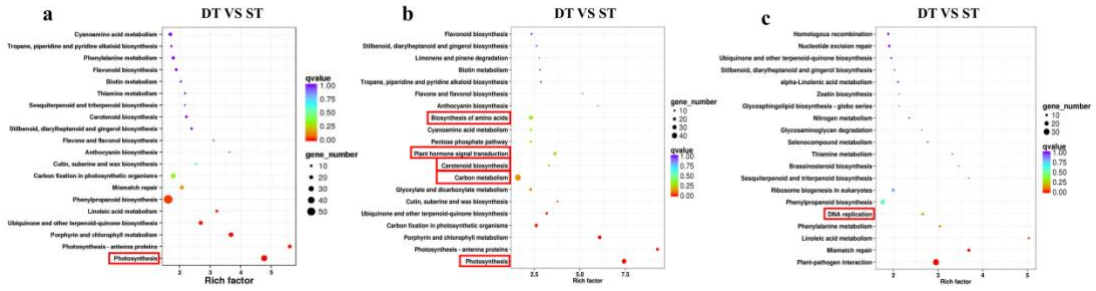

Fig. 2 Scatter plot of KEGG functional classification of DEGs. Including (a), all annotation. (b), up annotation (c), down annotation. The larger the rich factor, the more significant the enrichment level of DEGs in the pathway; The color of the dot represents the q value, and the smaller the q value, the more reliable the enrichment significance of DEGs in the pathway; larger dots represent more DEGs.

KEGG pathway enrichment analysis of DEGs with tillering

To provide further insight into the effect of DEGs on tillering regulation, we performed an enrichment analysis of DEGs based on the KEGG database. SLs and auxin have been reported to inhibit tiller shoot formation, while cytokinin can promote tiller formation. Thus, we first compared SL biosynthesis pathway. Overall, the DEGs encoding five enzymes were up-regulated in DT. In the ABA biosynthesis pathway, ABAH expression was down-regulated in DT. Second, we compared 3-Methyldioxyindole biosynthesis in the tryptophan metabolism pathway. Overall, 1 DEG encoding enzymes *YUCCA* was downregulated, and 1 DEG encoding enzyme ALDH was upregulated; The third pathway we compared was cytokinin biosynthesis in the zeatin biosynthesis pathway, one DEG encoding enzymes *CKX* was downregulated, and one DEG encoding enzyme *IPT* was upregulated. The fourth compared nitrogen metabolism pathway, Nrt, Nr, GLU, and GLUD were upregulated in tiller buds of DT relative to ST, indicating that DT could have a higher nitrogen utilization rate. The fifth pathway we compared was lignin biosynthesis in the phenylpropanoid biosynthesis pathway, wherein 4 DEGs encoding enzymes *PAL*, C4H, *4CL* and *CCR* were downregulated in DT and COMT was upregulated in DT.

Stability analysis of different reference genes

We selected 12 protein-coding genes in *P. juncea* with the highest expression levels as candidate reference genes. RefFinder recommended the gene expressional stability rankings in the order *Actin1 > GAPDH > Actin97 > EF-1α > αTUB > βTUB >18S rRNA > UBC17 > CYP >UBC28 > UBI >UBC2.* Irrespective of program used, the least stable genes were *UBI* and *UBC2.*

RNA-seq expression validation of DEGs by quantitative real-time PCR analysis

We selected 10 genes (*IPT, CKX4, CKX5, D27,CCD7, CCD8, YUCCA, CCR, 4CL* and *PAL*) involved in the four pathways discussed in the previous section for further verification. Only *IPT* had higher expression levels in DT than ST among the candidate DEGs, while the other nine genes all had lower

expression levels in DT (Fig. 3). The expression levels of all genes detected by qRT-PCR were highly consistent with transcriptome data, which proved the accuracy and effectivity of the transcriptome data.

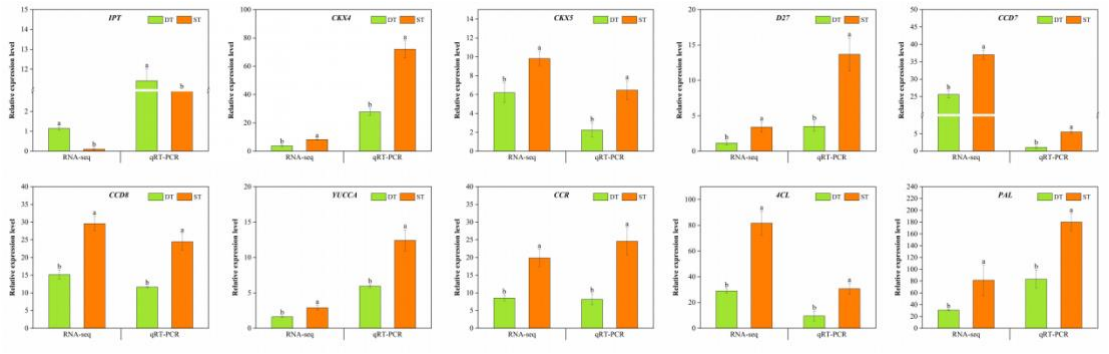

Fig. 3 Expression of tillering-related unigenes of *P. juncea* quantified by RNA-Seq and qRT-PCR analysis.

Conclusions

In this research, the tillering mechanism of perennial grass *P. juncea* was expounded by transcriptome analysis. We demonstrated that dense-tillering genotypes may be distinguished by their low expression patterns of genes involved in SL, IAA, and high expression patterns of genes involved in CTK biosynthesis at the tillering stage. Furthermore, we found that nitrogen metabolism and lignin biosynthesis can also affect the number of tillers. In the tissue of tiller nodes of *P. juncea* with different tiller numbers, the screened candidate reference gene *Actin1* had the best stability. The candidate genes revealed in our study are involved in the regulation of tillering of perennial gramineous forages. These genes provide breeding resources for the establishment of high-yield and high-quality forage grasses, and will also provide a theoretical basis for the molecular breeding of *P. juncea* in the future.

Acknowledgements

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