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Plant hormones determine the differentiation of callus tissue during tissue culture of mature and immature *Brachypodium distachyon* embryos

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Abstract

Brachypodium distachyon (*B. distachyon*) has emerged as a new model for the structural and functional genomics of temperate grasses. Somatic embryo culture serves as an effective method for the genetic transformation of *B. distachyon*. However, the role of regulatory genes in inducing somatic embryogenesis through endogenous hormone modulation in *B. distachyon* remains unexplored. This study investigated the endogenous hormone levels and transcriptome data of embryogenic calli (EC) derived from both mature and immature *B. distachyon* embryos. Our findings indicate that higher levels of IAA and GAs promote the formation of mature embryo-produced compact embryogenic calli (MEC), whereas higher tZR and lower ABA levels promote the development of immature embryo-produced compact embryogenic calli (IEC). The differential expression of genes involved in the biosynthesis and signal transduction of auxin (*YUCCA*, *ALDH*, *AUX/IAA*), trans-zeatin riboside (*CKX*), gibberellin (*GAO*, *GA3ox*), and abscisic acid (*AAO3*, *SnRK2*) influences the endogenous hormone concentrations in MEC and IEC of *B. distachyon*. Specifically, increased expression of *AUX/IAA* is linked to MEC formation. Thus, the differential expression of genes associated with the AUX, CK, GA, and ABA biosynthesis and signal transduction pathways may modulate endogenous hormone levels, steering the differentiation of MEC and IEC in *B. distachyon*.

Keywords *Brachypodium distachyon* · embryogenic calli · differentially expressed genes · endogenous hormone

Introduction

Brachypodium distachyon (*B. distachyon*) is a newly identified model system for studying grass biology. This species is closely related to major cereals such as wheat and barley, as well as forage grasses (Draper et al., 2001). The use of model organisms is invaluable for analyzing complex biological processes and can significantly accelerate scientific investigations.

In the past three decades, *Arabidopsis thaliana* (*Arabidopsis*) has served as a model system for understanding plant development, ecology, evolution, physiology, cell biology, and genetics (Koornneef et al., 2010). Although *Arabidopsis* exhibits excellent features and shares similarities with many crops, it has limitations in regard to studying monocot-specific processes. Grasslands, specifically monocot plants, are crucial in agriculture and provide a significant portion of global food calories. Maize, barley, and wheat have been proposed as model systems for grass biology, but they present challenges due to

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their large size, large genome size, long generation time, and limited access to the germplasm (Brkljacic et al., 2011).

In contrast, *B. distachyon* offers several advantages as an experimental model for investigating biological questions in grasses (Garvin et al. 2008). It is a small, fast-growing, and easily cultivable species that exhibits natural variation in important traits such as flowering time, vernalization responses, polyploidy, and seed size (Opanowicz et al. 2008). Its genome is one of the smallest among grasses (B275 Mb, harboring 26,000 genes excluding isoforms). Furthermore, *B. distachyon* shares a high degree of gene content similarity with wheat and maintains good syntenic associations (Griffiths et al. 2006).

One essential aspect of studying *B. distachyon* is the development of specific mutant resources and the validation of gene function, which require reliable genetic transformation technologies. The first step for successful plant transformation is the ability to regenerate plants in tissue culture. Transformation protocols rely on somatic embryogenesis (SE).

SE is a valuable morphogenetic pathway with significant biotechnological applications. It has wide-ranging uses in large-scale plant production, reproduction of transgenic plants, studies on cell differentiation, gene expression, and molecular genetics in plant development (Dela-Peña et al. 2015; Zimmerman 1993). The source of material used to produce embryogenic calli (EC) varies among species. The production of compact embryogenic calli (CEC) is particularly desirable for genetic transformation. In *Brachypodium*, immature embryos have been identified as the most suitable explants for callus induction (Christiansen et al. 2005; Griffiths et al. 2006; Vogel et al. 2008). However, good efficiencies in producing compact embryogenic calli from immature embryos (IEC) have also been achieved using whole seeds (Bablak et al. 1995; Jeon et al. 2010). Immature embryos are commonly selected as explants due to their high regeneration capacity, but they require a constant supply of healthy plants and access to controlled greenhouse conditions. Mature embryos, on the other hand, can be stored as seeds and are available throughout the year, making the production of compact embryogenic calli from mature embryos (MEC) easier. Therefore, a robust SE technique using mature embryos as the starting material is highly desirable.

Changes in plant age result from the differential regulation of developmental processes, including responses to environmental changes, as well as morphological, physiological, and metabolic processes (C. C. Chen et al. 2012; Jaikumar et al. 2016; Kus et al. 2002). An illustrative example is the effect of explant age on callus formation in *Arabidopsis*, where hypocotyl maturation increases callus formation (C. C. Chen et al. 2012). However, the effects of embryo maturity in *Brachypodium distachyon* on the efficiency of embryogenic callus formation and changes in endogenous hormone contents are still unknown.

Previous studies have demonstrated the significant role of plant endogenous hormones in the development and morphogenesis of explants. The levels of auxin and cytokinin have an impact on the dedifferentiation and redifferentiation of plant cells, dictating tissue regeneration and cell fate (Ikeuchi et al. 2019). For instance, *YUCCA*, is an important gene that regulates key enzymes in auxin biosynthesis, and it is essential for embryogenic calli formation in *Gossypium hirsutum* (Zheng et al. 2014), and somatic embryo development and maturation in *Musa spp* (Enriquez-Valencia et al. 2019). Additionally, the *AUX/IAA* gene, which is responsible for auxin signal transduction, exerts a significant regulatory effect on SE development; however, its expression patterns differ across different plants during the SE process (Y. Chen et al. 2020; Liu et al. 2019; Quintana-Escobar et al. 2019). In addition, Li et al. (Li et al. 2019) shows that gibberellin and abscisic acid content are very important for the maturation and germination of somatic embryos. The differential expression of *GA3ox* and *AAO3* related to GA and ABA

is highly important for the development of somatic embryos in *G. hirsutum* and *Medicago truncatula* (Rose 2019). All these genes mentioned above can regulate and influence the biosynthesis and signal transduction of endogenous hormones in plants. Therefore, studying the changes in endogenous hormones and analyzing transcriptome data are necessary to elucidate the mechanism by which endogenous hormones regulate SE in *Brachypodium distachyon*.

In this study, EC from *Brachypodium distachyon* subjected to SE was used as a test material to determine endogenous hormone levels via LC–MS/MS and to analyze RNA-Seq data. The aim of this study was to reveal the changes in endogenous hormone levels in MEC and IEC of *Brachypodium distachyon*, explore the molecular mechanisms underlying the formation of MEC and IEC, and provide a theoretical foundation for optimizing the SE system of mature and immature *Brachypodium distachyon* embryos.

Materials and methods

Plant material and production of mature and immature embryos from line Bd21

Distachyon (L.) P. Beauv. Seeds of the Bd-21 strain ($2n = 10$) were sown in 625-mL plastic pots containing a mixture of perlite, vermicompost, and peat soil. The seedlings were then placed in growth chambers, and the pots were maintained at 22°C with an irradiance of 150 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ under a 20:4 h (light: dark) photoperiod. The plants were grown for 7–9 weeks under a 20-h photoperiod to obtain immature embryos at the desired stage. Fully matured embryos could be obtained when the plant reached full maturity.

Production of embryogenic calli (EC) and histomorphological characterization

Isolated immature seeds collected two weeks after tasseling were placed in Petri dishes and surface-sterilized in a laminar flow cabinet. This was achieved by dipping the seeds in a 1.3% active chlorine solution for 3 minutes, followed by three rinses with sterilized distilled water. In the laminar flow cabinet, the immature embryos were carefully removed from the seeds using fine tweezers and a scalpel. The immature embryos were then cultured in MSB media consisting of MS salts, 30 g/L sucrose, 2.5 mg/L 2,4-D, 0.5 mg/L casein, 0.6 mg/L CuSO_4 , and 4 g/L Phytigel. The pH of the medium was adjusted to 5.8. The cultures were maintained at a temperature of 25°C in darkness. After 2 weeks, the IEC were fragmented and allowed to grow further for another 2 weeks in freshly prepared MSB media.

Similarly, the collected mature seeds were placed in Petri dishes and subjected to surface sterilization in a laminar flow cabinet. The seeds were dipped in a 1.3% active chlorine solution for 15 minutes, followed by three rinses with sterilized distilled water. The mature embryos were then removed from the seeds with the aid of fine tweezers and a scalpel. These embryos were cultured in MSB media and maintained at 25°C in the dark. After 2 weeks, the EC were allowed to grow further for another 2 weeks in freshly prepared MSB media, and the MEC were fragmented.

The IEC and MEC were photographed under a camera for observation.

Determination of endogenous hormone levels

High-performance liquid chromatography (HPLC)-mass spectrometry (MS)/MS analysis was conducted to determine the contents of four endogenous hormones (IAA, ABA, GA3, and tZR) (Pan et al. 2010; Wenhong et al. 2015). The endogenous hormones were extracted using an isopropanol/water/hydrochloric acid extraction method. In summary, approximately 1.0 g of fresh plant sample was ground in liquid nitrogen until a powder was obtained. Subsequently, the powder was mixed with 10 mL of isopropanol/hydrochloric acid buffer and incubated at 4°C for 30 minutes with oscillation. After adding 20 mL of dichloromethane, the sample was further incubated at 4°C for 30 minutes with

oscillation. The mixture was then centrifuged at 13,000 r/min at 4°C for 5 minutes, after which the organic phase in the lower layer was collected. The organic phase was air-dried in the dark using nitrogen. Next, the sediment was suspended in 400 µL of methanol (0.1% formic acid). The hormones in the solution were measured via HPLC–MS/MS after filtration through a 0.22 µm filter membrane. The endogenous hormone contents were determined using an Agilent 1290 HPLC system coupled with a reversed-phase column (Poroshell 120 SB-C18, 2.1 × 150, 2.7 µm) maintained at 30°C. Mobile phase A consisted of distilled water with 0.1% formic acid, while mobile phase B consisted of methanol with 0.1% formic acid. After separating the sample material using HPLC, an AB Qtrap 6500 mass spectrometer with electrospray ionization (ESI) as the ion source and multichannel detection (MRM) mode was used. The parent ion was specifically selected by the mass analyzer and then dissociated by collision induction in the collision reaction chamber to generate daughter ions. For most hormones, two or three daughter ions were detected, and the one with the highest response value was selected as the quantitative ion.

RNA extraction, library construction, and Illumina sequencing

Total RNA was extracted from the tissues using Trizol. The quality of the RNA was assessed using a 5300 Bioanalyzer (Agilent) and quantified using an ND-2000 (NanoDrop Technologies). mRNA was isolated using the polyA selection method with oligo(dT) beads and then fragmented using fragmentation buffer. Subsequently, double-stranded cDNA was synthesized using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, CA) with random hexamer primers (Illumina). The synthesized cDNA underwent end repair, phosphorylation, and 'A' base addition according to Illumina's library construction protocol. The libraries were size-selected for cDNA target fragments of 300 bp on 2% low-range Ultra agarose, followed by 15 cycles of PCR amplification using Phusion DNA polymerase (NEB). The libraries were quantified using a Qubit 4.0, and paired-end RNA-seq libraries were sequenced using a NovaSeq 6000 sequencer.

Assembly and gene function annotation

The raw paired-end reads were trimmed and subjected to quality control using fastp (S. Chen et al. 2018) with default parameters. The resulting clean reads were aligned to a reference genome using HISAT2 (Kim et al. 2015) software in orientation mode. StringTie (Pertea et al. 2015) was then used to assemble the mapped reads for each sample via a reference-based approach. The unigenes were aligned with the Swiss-Prot, COG/KOG, GO, KEGG, NR, and Trembl databases ($E \leq 1e-5$) using BLAST software. The amino acid sequences of the unigenes were predicted, and HMMER software was used to compare them with the Pfam database to obtain annotation information.

Differential gene analysis and functional enrichment

Fragments per kilobase of transcript per million mapped reads (FPKM) values were used to measure transcript or gene expression levels. The threshold for identifying DEGs was set at $|\log_2 \text{Fold Change}| \geq 1$ and $\text{FDR} < 0.05$. Functional enrichment analysis, including GO and KEGG enrichment analyses, was performed to determine which DEGs were significantly enriched in GO terms and metabolic pathways compared with the whole-transcriptome background using a Bonferroni-corrected P value ≤ 0.05 . GO functional enrichment analysis was conducted using Goatools, while KOBAS (Xie et al. 2011) was used for KEGG pathway analysis.

Quantitative real-time PCR

Twelve DEGs selected from the transcriptome sequencing data were subjected to relative expression level verification using quantitative real-time polymerase chain reaction (qRT–PCR). The sequences of

primers used for qRT–PCR are provided in Table 1. BdUBC18 served as the internal reference gene. qRT–PCR was carried out on a fluorescence quantitative PCR system (Analytikjena qTOWER3, Jena, Germany) with the following conditions: 95°C for 30 s; 40 cycles of 95°C for 10 s and 54°C for 30 s; and 72°C for 15 s. The relative gene expression levels were calculated using the DDCT method (Pfaffl 2001), and each expression level represented the mean of three biological replicates.

Statistical analysis

SPSS (SPSS Inc., Chicago, IL, USA) was used to identify differences between the MEC and IEC groups by Fisher's protected least significant difference test at $P < 0.05$. All the data are presented as the mean $\pm$ standard deviation (SD) of three replicates. The graphs were created with Origin 9.0 and Photoshop 2019 software.

Table 1 Primers for qRT–PCR.

Gene Name	Primer	Forward/Reverse primer
LOC100833271	F	GGGGAGAGGAAGCTAATGCC
	R	GGAAGTACAGACCCTTGCCC
LOC100839190	F	AGCTCAAGTCTCACCTGCTG
	R	CGCTGCCTTTCTCTTCTTGC
LOC100831978	F	GACGAAGACCAGGACACCAC
	R	CCCCGTCCATGCTAACCTTC
LOC104583275	F	AGCAACCCTGGAATGGTCAG
	R	GCTCATTTAGCAGGTTGGCG
LOC100823225	F	ACATCGAGCTACCTGCATCG
	R	AGCCGTCATTGAGCACAGAA
LOC100829850	F	CGCATCTTCCACCTCCTTGA
	R	CGATCTCCCGCCTGTACTTG
LOC100825535	F	CTCACGTTGGGGTCCATCTC
	R	TTGCCAGAAGCTTGGTCCTC
LOC100831525	F	GGAAGCAGGATCTCCACCAG
	R	GAGGATTTGAGCCGGAGGTC
LOC100843625	F	CCGTTCAAGGAGGTTGTCC
	R	CTGCTGGAAGAAGTAGCGGG
LOC100835718	F	TCTCCATCCTCACAAACGGC
	R	GAGGAAGTACCCGAGGGACA
LOC100831517	F	TTGGCTCGAGAACCATGGAG
	R	AGGCACCTTTGTCAGAACCA
LOC100838916	F	CGAGCGTTGCTGTTCAAGAC
	R	AAAAGCGCACCAAGCAAGAG
BdUBC18	F	GTCACCCGCAATGACTGTAAGTTC
	R	TTGTCTTGCGGACGTTGCTTTG

Results

Morphological analysis

Mature embryos were cultured for 2 weeks, resulting in the formation of embryogenic calli, which were

subsequently transferred to fresh medium and cultured for an additional 2 weeks, leading to the production of MEC. Immature embryos, on the other hand, began to form compact embryogenic calli after 2 weeks of culture, indicating a faster and greater production rate than that of mature embryos. By the time MEC was formed, the IEC had already started fragmenting and proliferating in large quantities (Fig. 1).

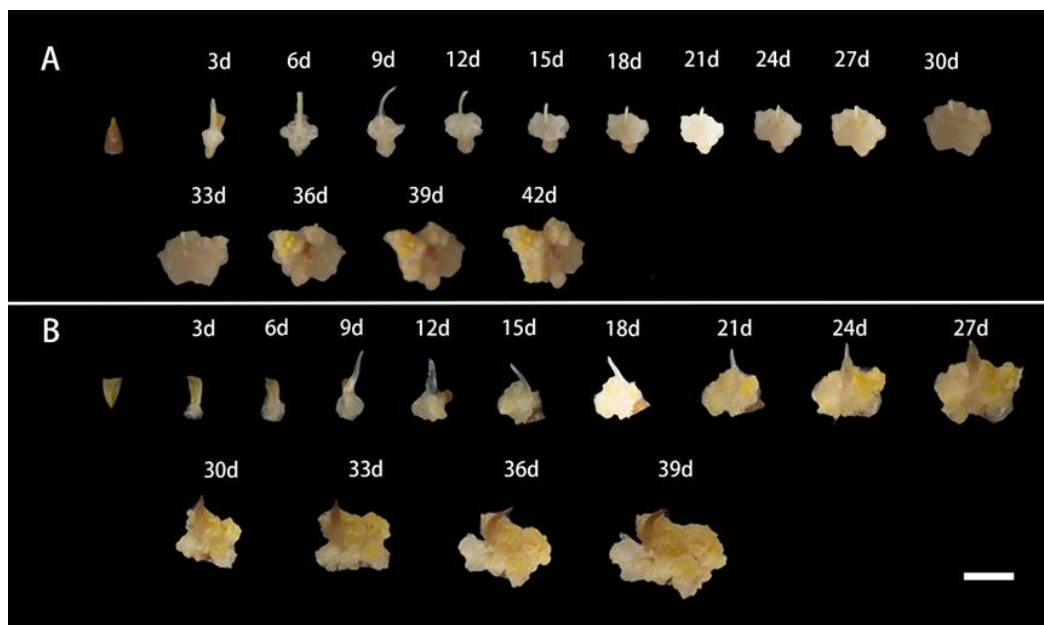


Fig. 1 Somatic embryogenesis in *B. distachyonis*. Photographic records every three days. (A) Mature embryo produced compact embryogenic calli (MEC); (B) Immature embryo produced compact embryogenic calli (IEC). Bar=0.5 cm.

Endogenous hormone contents

The presence of four common phytohormones, IAA, GA3, tZR, and ABA, was determined. The endogenous hormone levels in *B. distachyon* were significantly different between MEC and IEC, as depicted in Fig. 2. Notably, the tZR content was the lowest in MEC, whereas the ABA content was the highest. Additionally, the IAA and GA3 contents were greater in MEC than in IEC. Furthermore, the ABA content in MEC was significantly greater than that in IEC. These findings suggested that MEC formation was influenced by relatively low tZR levels and high IAA, GA3, and ABA contents. In contrast, IEC production was associated with the highest tZR content, which was low in MEC. Furthermore, the IAA, ABA, and GA3 contents in IEC were lower than those in MEC. These results clearly indicated significant differences in the endogenous hormone levels of the four aforementioned hormones between MEC and IEC, with each tissue possessing its own specific hormone content.

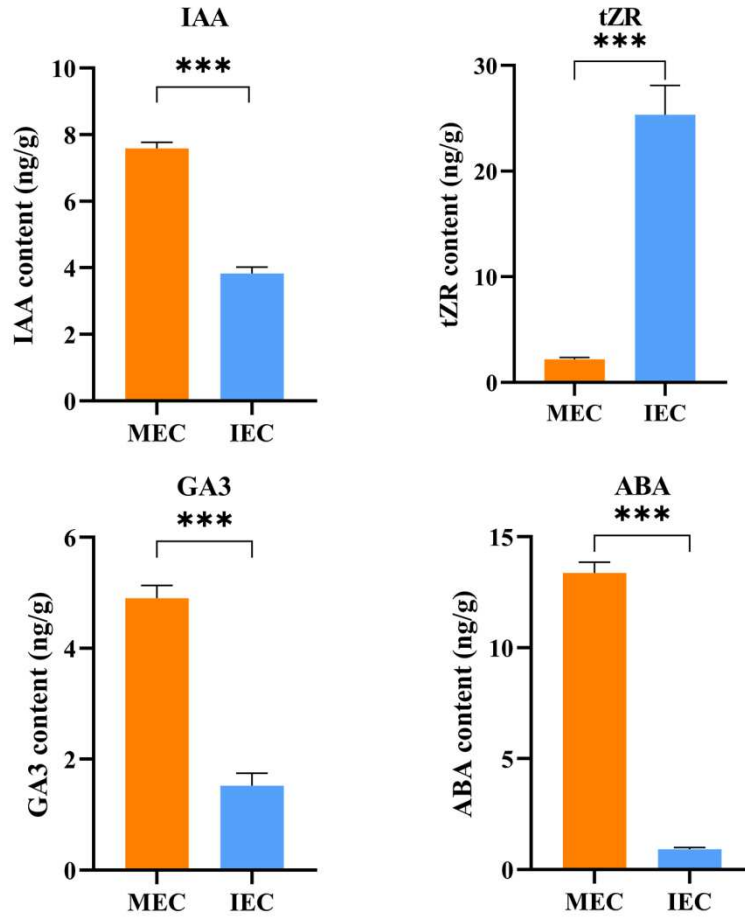


Fig. 2 Contents of phytohormones at 4 weeks age of MEC and IEC of *B. distachyonis*. IAA, auxin, tZR, trans-zeatin riboside, GA₃, gibberellin 3, ABA, abscisic acid. Bars, mean±SD; n=3; ***p≤0.01; **p≤0.01; *p≤0.05 (one-way ANOVA plus Dunnet's multiple comparisons test).

Gene ontology analysis of DEGs and KEGG metabolic pathways

There were 980 DEGs between MEC and IEC. Of these DEGs, 857 were downregulated and 123 were upregulated, with a significantly greater number of downregulated DEGs than upregulated DEGs (Fig. 3A, B). Fig. 3C shows that most of the GO terms were similar in the three sets. Based on the GO annotations, the unigenes were classified into different functional categories. The "molecular function" category contained the most DEGs in the terms "catalytic activity" and "binding". In the "cellular component" category, most DEGs were associated with "membrane part" and "cell part". The "biological process" category had the most DEGs associated with "metabolic process" and "cellular process" (Fig. 3C). The DEGs in MEC versus those in IEC were annotated in the KEGG database, resulting in 88 metabolic pathways. The significantly enriched metabolic pathways included "phenylpropanoid biosynthesis", "glutathione metabolism", "ascorbate and aldarate metabolism", "isoquinoline alkaloid biosynthesis", "plant hormone signal transduction", "cysteine and methionine metabolism", and "photosynthesis-antenna proteins" (Fig. 4A). Among these pathways, "phenylpropanoid biosynthesis" and "plant hormone signal transduction" were the most represented. The analysis revealed significant differences in the endogenous hormone contents between MEC and IEC. To understand how these changes in hormone contents are regulated by gene expression in plant hormone biosynthesis and signal transduction pathways, we focused on the biosynthesis and signal transduction of auxin, trans-zeatin riboside, gibberellin, and abscisic acid. The results showed that (1) tryptophan metabolism was annotated

to 2 DEGs, (2) zeatin biosynthesis was annotated to 3 DEGs, (3) diterpenoid biosynthesis was annotated to 2 DEGs, and (4) carotenoid biosynthesis was annotated to 2 DEGs. In terms of plant hormone signal transduction, 18 DEGs were expressed and annotated. These results indicate that the differences in endogenous hormone contents between MEC and IEC are regulated by genes encoding plant hormones.

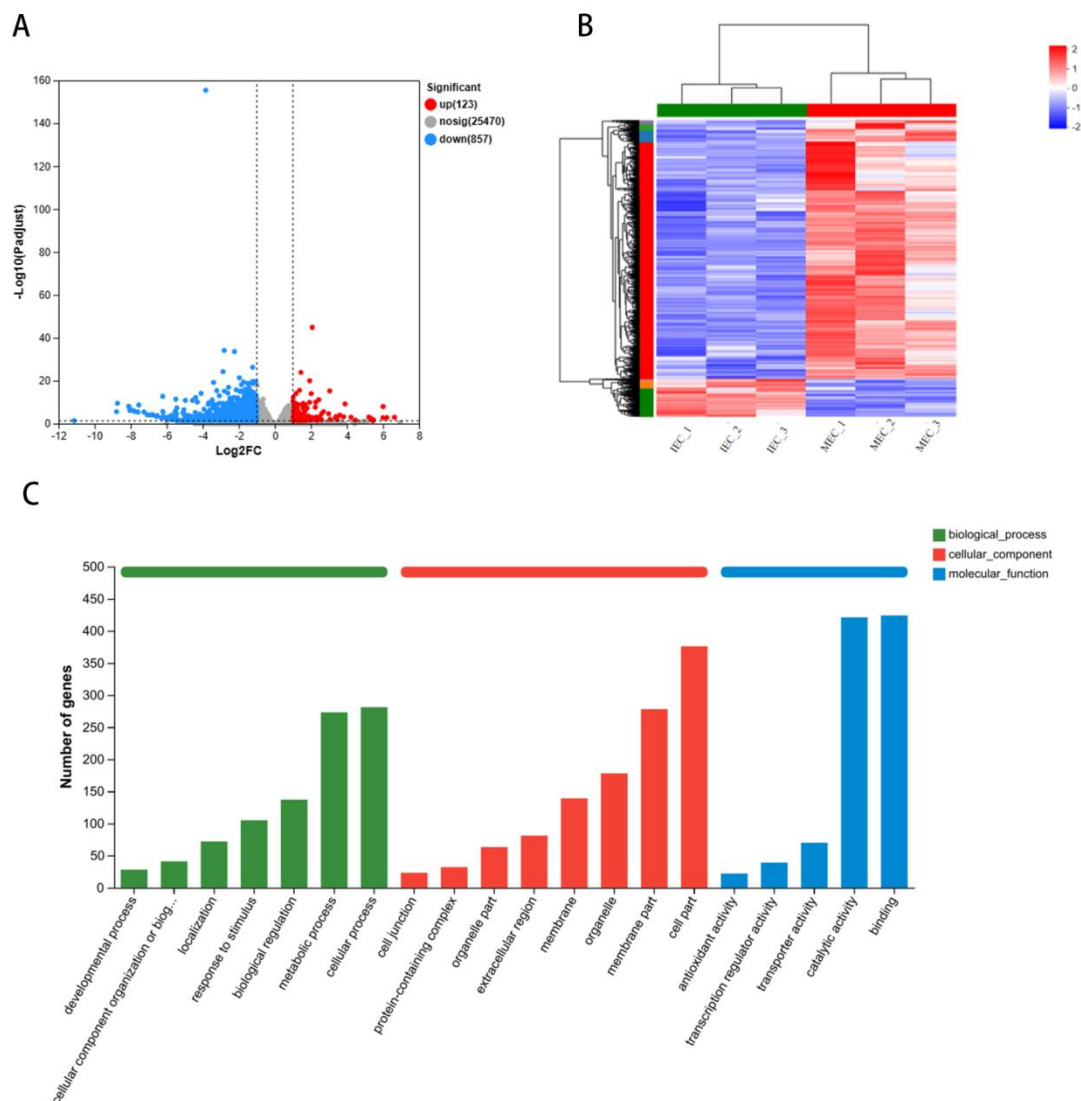


Fig. 3 The number of differentially expressed genes. (A) Statistic of up or down-regulated genes; (B) Cluster analysis of DEGs; (C) GO annotation of DEGs.

GO functional enrichment of DEGs

GO functional enrichment analysis of the DEGs revealed a total of 326 GO terms. Among these genes, several were associated with phytohormones, including brassinosteroid biosynthetic process (GO:0016132), steroid hormone biosynthetic process (GO:0120178), response to auxin (GO:0009733), regulation of growth (GO:0040008), hormone biosynthetic process (GO:0042446), cytokinin-activated signaling pathway (GO:0009736), response to hormone (GO:0009725), and response to cytokinin (GO:0009735). Cluster analysis of the DEGs revealed that most of the genes were upregulated in MEC (Fig. 4B). Among these DEGs, nine GO terms related to endogenous hormone biosynthesis and signal transduction were selected (Fig. 4C), and 20 DEGs were enriched. These genes are involved in biological processes such as hormone-mediated signaling pathways (GO:0009755) and hormone responses

(GO:0009725), which play a role in MEC and IEC development during somatic embryogenesis in both mature and immature *B. distachyon* embryos.

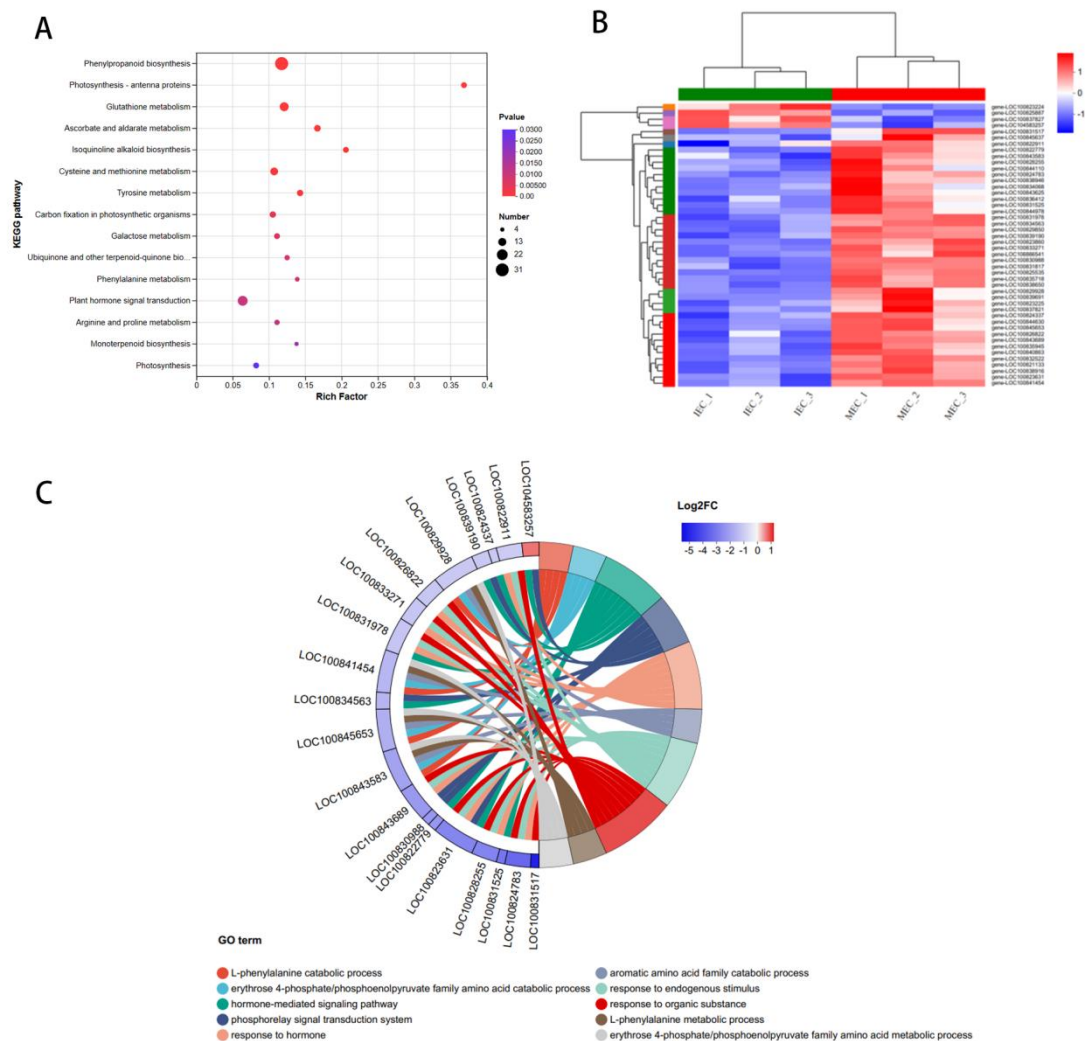


Fig. 4 KEGG and GO functional enrichment of DEGs. (A) KEGG enrichment analysis of the differential genes at MEC vs IEC. (B) Cluster analysis of genes enriched in phytohormones-associated KEGG. (C) Chordal diagram of GO terms from DEGs in Fig. 4B.

DEGs related to phytohormones in MEC and IEC of *Brachypodium distachyon*

To further investigate the genes associated with changes in endogenous hormones during somatic embryogenesis in mature and immature *B. distachyon* embryos, DEGs involved in plant hormone biosynthesis and signal transduction were analyzed using transcriptome data (Fig. 5). The expression patterns of the 12 selected genes, as analyzed by qRT-PCR and RNA-Seq, were consistent (Fig. 6), indicating the reliability of the transcriptome data.

In tryptophan metabolism, the auxin-related DEGs included *YUCCA* (indole-3-pyruvate monooxygenase) and *ALDH* (aldehyde dehydrogenase [NAD⁺]). One *ALDH* gene showed low expression in IEC and high expression in MEC, while one *YUCCA* gene was upregulated in IEC (Fig. 7A). In zeatin biosynthesis, one *CYP735A* gene and one *UGT73C* gene exhibited high expression in MEC and low expression in IEC, whereas one *CKX* gene showed high expression in IEC and low expression in MEC (Fig. 7B). In diterpene biosynthesis, one *GA3ox* gene and one *KAO* gene were highly

expressed in MEC and weakly expressed in IEC (Fig. 7C). The expression pattern of *AAO3* in carotenoid biosynthesis was similar to that of the *GA3ox* and *KAO* genes in the MEC and IEC of *B. distachyon* (Fig. 7D). With respect to plant hormone signal transduction, three small auxin-up RNAs (SAURs), three auxin-responsive proteins IAA (AUX-IAA), two indole-3-acetic acid-amido synthetases (GH3), five type-A Arabidopsis response regulators (A-ARRs), one histidine-containing phosphotransfer protein (AHP), and one serine/threonine-protein kinase 2 (SNRK2) were screened (Fig. 5B, Fig. 7E). These genes are believed to be involved in the biosynthesis of corresponding endogenous hormones and may impact the formation of CEC from mature and immature *B. distachyon* embryos.

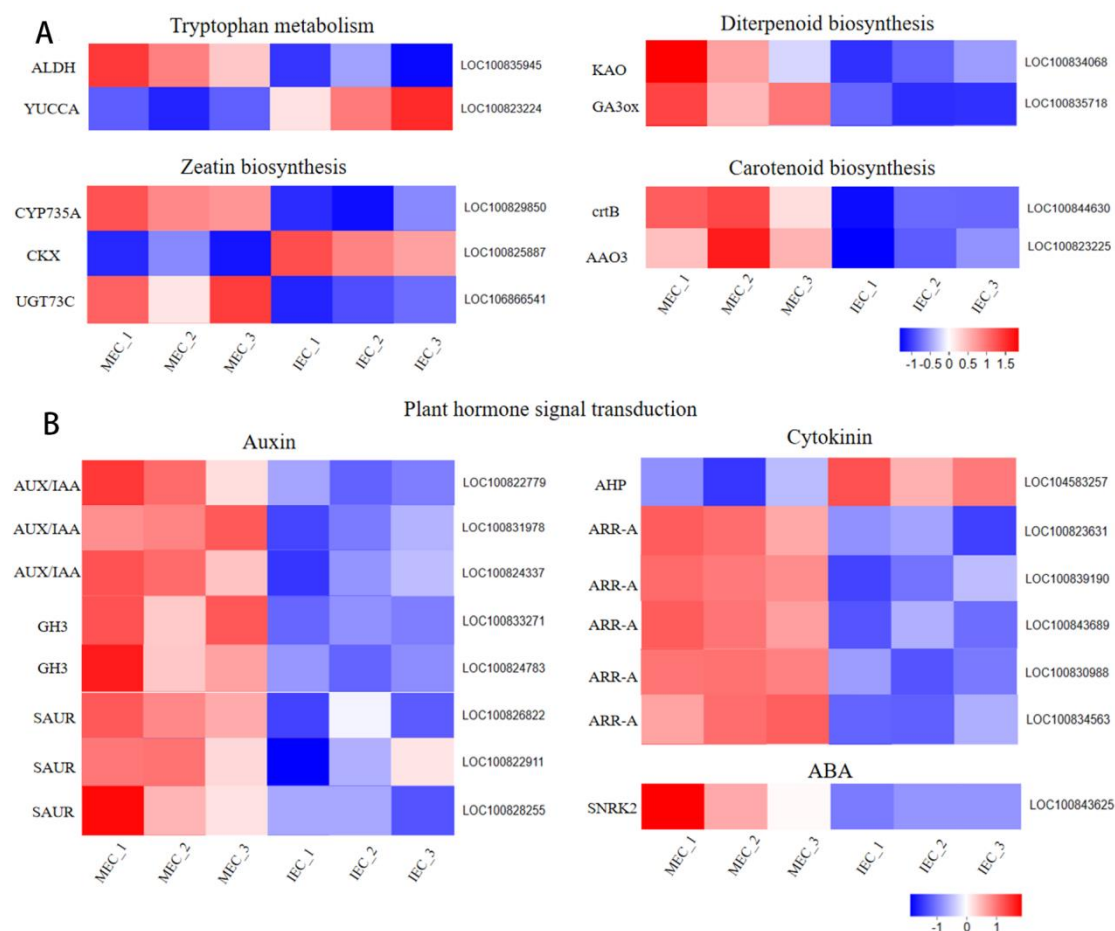


Fig. 5 Differentially expressed genes related to endogenous hormone biosynthesis and signal transduction in *B. distachyonis*.

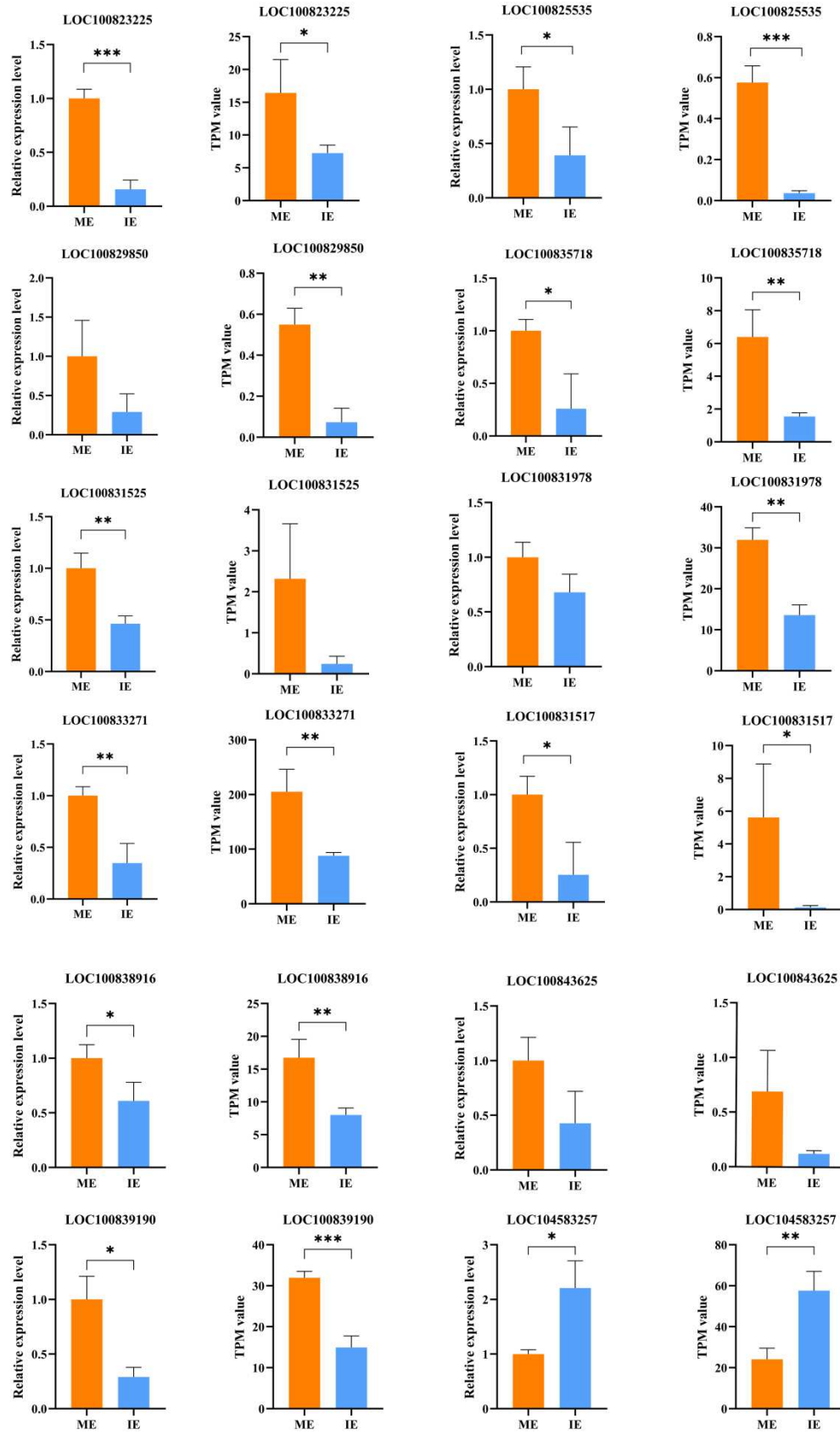


Fig. 6 Verification of relative expression level of 12 DEGs. Relative gene expression level was validated from qRT-PCR and transcripts per million (TPM) value was obtained from transcriptome sequencing.

embryo callus (IEC). The formation of IEC occurred earlier than that of MEC, and with the development of IEC, the IAA content decreased. Similar findings were reported for *Medicago truncatula* (Kępczyńska et al. 2021) and *Cyathea delgadii* (Grzyb et al. 2017). These results suggest that the increase in auxin concentration is associated with the establishment of embryo polarity, the initiation of stem cells, and the maintenance of the shoot apical meristem (Y. Chen et al. 2020; Vondrakova et al. 2018). Furthermore, *YUCCA* genes involved in auxin synthesis were found to be upregulated in early somatic embryos. In this study, both the *YUCCA* and *ALDH* genes involved in auxin biosynthesis exhibited differential expression, with *YUCCA* being upregulated in IEC and *ALDH* being upregulated in MEC. The content of IAA in IEC was lower than that in MEC. These findings suggest that the differential expression of the *YUCCA* and *ALDH* genes affects the level of IAA, thus playing a key role in the production of MEC and IEC in *B. distachyon*.

As a transcription factor, *AUX/IAA* plays a crucial role in plant growth and development. In this study, *AUX/IAA* genes involved in the SE process of *B. distachyon* were highly expressed in MEC and downregulated in IEC. The expression of *AUX/IAA* also decreased gradually during the SE process in coffee (Quintana-Escobar et al. 2019). It is speculated that the high expression of *AUX/IAA* promotes MEC formation in *B. distachyon*. Overall, exogenous auxin is considered the key hormone for inducing embryogenic callus formation, and the expression of the *AUX/IAA* gene plays a crucial role in the auxin signaling pathway.

Cytokinin plays a crucial role in promoting the cytoplasmic division, differentiation, and growth of multiple tissues, and it exhibits a cooperative effect with IAA. This phytohormone is involved in regulating the growth and development of plant cells, as supported by studies conducted by Hwang et al. (2012), Jameson et al. (2016), and Wybouw et al. (2019). Specifically, the active form of cytokinin, tZR, was found to have a lower content in the MEC of *B. distachyon* than in the IEC. Fraga et al. (2016) reported that a decrease in cytokinin levels was associated with reduced embryogenic potential. Cytokinin oxidase/dehydrogenase (CKX) is an enzyme responsible for irreversibly cleaving free cytokinins and cytokinin nucleosides, resulting in the reduction of biologically active CKs (L. Chen et al. 2020; Sakakibara 2006; Tuan et al. 2019). *CKX* overexpression decreased the levels of endogenous cytokinin, including tZR and iP, which are cleaved by CKX (Zalabák et al. 2014). In this study, the upregulation of *CKX* gene expression in MEC, along with the lower content of tZR compared to that in IEC, suggested that the differential expression of *CKX* affected tZR levels during MEC formation in *B. distachyon*. This, in turn, regulated MEC induction, differentiation, and cotyledon embryo germination. Gibberellins contribute to the regulation of plant growth and development through complex biosynthetic and signaling pathways. Additionally, GAs and ABA play important roles in adapting to stress responses and improving crop yield. According to Nolan et al., a high ABA:GA ratio in *Arabidopsis* synergistically stimulates somatic embryogenesis (SE). However, in *M. truncatula*, a low ABA:GA ratio was found to be effective in inducing and improving SE for regeneration in different plants. Interestingly, the contents of GA3 and ABA in the IEC were significantly lower than those in the MEC of *B. distachyon*. GA3 is a biologically active GA in plants, and the high ABA:GA3 ratio observed in *B. distachyon* contributes to the production of MEC. The *KAO* (*ent*-kaurenoic acid oxidase) gene is crucial for the presynthesis of gibberellins, and it was found to be upregulated in MEC but downregulated in IEC. *GA3ox* genes encode GA3-oxidase, which is involved in a series of reactions leading to the formation of GA3 (Appleford et al. 2006; Li Qiang 2014; Yamaguchi 2008). The upregulation of the *GA3ox* gene in MEC, as reported by Mitsuhashi et al. (2003), corresponded to a greater content of GA3 in MEC than in IEC. The expression patterns of the *KAO* and *GA3ox* genes were consistent with the GA levels, indicating their involvement

in GA3 biosynthesis in both MEC and IEC (Mitsuhashi et al. 2003). The differential expression of the *KAO* and *GA3ox* genes affected GA3 biosynthesis, ultimately leading to a high content of GA3 and playing a key role in the formation of MEC and IEC in *B. distachyon*.

Absciscic acid (ABA) is a pivotal phytohormone involved in seed development and germination regulation. Its activity level and signal transduction play crucial roles in seed development, including embryogenesis and maturation processes (Ali et al. 2022; Kozaki et al. 2022). ABA and gibberellins are known as major hormones that antagonize embryogenesis regulation (Sano et al. 2021; Shu et al. 2016). *GAST1*, the first identified family member in *Solanum lycopersicum*, was found to be induced by gibberellins but inhibited by ABA (Zhang et al. 2017). A higher concentration of ABA in MEC implied its inhibitory effect on MEC induction, as observed in *M. truncatula* (Kępczyńska et al. 2021). Absciscic aldehyde oxidase 3 (*AAO3*), identified in *Arabidopsis*, plays a role in the final two steps of ABA biosynthesis (Seo et al. 2004). The *AAO3* gene exhibited low expression in IEC and high expression in MEC of *B. distachyon*. This expression pattern was consistent with the levels of ABA, indicating a correlation between *AAO3* expression and ABA levels. *SnRK2*, a positive regulator of the ABA signaling network, is involved in various embryogenesis processes in *Arabidopsis* (Finkelstein et al. 2008). In this study, the *SnRK2* gene was significantly upregulated in MEC of *B. distachyon*. This finding aligns with the expression patterns of the *AAO3* gene, suggesting that the differential expression of *AAO3* and *SnRK2* collectively influenced ABA biosynthesis. These results establish a robust molecular foundation for explaining why high ABA concentrations can inhibit MEC induction in *B. distachyon*.

Conclusion

Changes in endogenous hormone levels serve as the fundamental basis for SE growth and morphogenesis in *B. distachyon*. The expression profiles of genes involved in plant hormone biosynthesis and signal transduction impact endogenous hormone levels and regulate MEC and IEC formation. Relatively high levels of IAA and gibberellins promote MEC formation, while high CKs and low ABA levels favor IEC formation. The differential expression of genes related to auxin biosynthesis and signal transduction (*YUCCA*, *ALDH*, *AUX/IAA*), trans-zeatin riboside (*CKX*), gibberellin (*KAO*, *GA3ox*), and absciscic acid (*AAO3*, *SnRK2*) influences endogenous hormone levels during MEC and IEC formation in *B. distachyon*. Upregulated expression of *AUX/IAA* stimulates MEC formation. Overall, these findings provide novel insights into the molecular mechanism underlying MEC and IEC formation in *B. distachyon*, serving as a technical and theoretical foundation for further research on the underlying mechanism and expansion of *B. distachyon* resources and propagation practices.

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Declarations

Conflict of interest The authors declare no competing financial interests.

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