BMC Plant Biology

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BAP regulates lateral bud outgrowth to promote tillering in *Paphiopedilum callosum* (Orchidaceae)

Yuying Yin[1], Ronghui Zhong[2], Yefei Li[1], Beiyi Guo[1], Lin Li[1], Guohua Ma[1], Kunlin Wu[1], Lin Fang[1*] and Songjun Zeng[1*]

Abstract

Background *Paphiopedilum* orchids have a high ornamental value, and flower abundance is a key horticultural trait. Most *Paphiopedilum* plants exhibit weak tillering ability, with their tiller buds often entering a dormant state post-formation. Tiller production plays a crucial role in enhancing flower abundance and is potentially regulated by plant hormones. However, the effect of hormones on tillering in *Paphiopedilum* plants is still unclear.

Results In this study, we investigated the promotion of tillering in *P. callosum* through exogenous root irrigation of benzylaminopurine (BAP). We observed a dose-dependent promotion of tiller production by BAP, with the strongest effect observed at a concentration of 400 mg/L. By comparing the expression of key genes in *P.* 'SCBG Yingchun' (with strong tiller ability) and *P callosum* (with weak tiller ability), we found that BAP promotes tillering by interacting with abscisic acid (ABA). This interaction involves down-regulation of the ABA degradation gene *PcCYP707A*, leading to a reduction in ABA content, and the subsequent down-regulation of dormancy-associated genes (*PcDRMH1*, *PcSVP*) to release bud dormancy. Additionally, BAP promotes sustained outgrowth of tiller buds by increasing the level of indole-3-acetic acid (IAA) through up-regulation of the IAA synthesis gene *PcYUC2* and the transport gene *PcPINIC*.

Conclusions Our results indicated that the application of BAP promotes lateral bud outgrowth and increases tiller production in *P. callosum*. Through transcriptome analysis, we found that the BAP-promotion of tillering involves not only changes in endogenous IAA, ABA, and CTKs content but is also associated with the regulation of metabolism-related genes and dormancy-associated genes. This study presents the first comprehensive report of BAP-promoted tillering in *P. callosum*, providing a foundational basis for further mechanistic studies on tiller development in *Paphiopedilum* species and other non-model plants.

Keywords *Paphiopedilum*, Cytokinin, Tillering regulation, Transcriptome, Gene expression

*Correspondence:
Lin Fang
linfang@scib.ac.cn; linfang@scbg.ac.cn
Songjun Zeng
zengsongjun@scib.ac.cn
[1]Guangdong Provincial Key Laboratory of Applied Botany and Key Laboratory of South China Agricultural Plant Molecular Analysis and Gene Improvement, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China
[2]Guangdong Key Laboratory of Ornamental Plant Germplasm Innovation and Utilization, Environmental Horticulture Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China

Introduction

Paphiopedilum is one of the most favored groups of orchids in horticulture due to their rarity in the wild and unique pouch-shaped lip of the flower. This genus comprises 109 species, with 34 species recorded in China [1]. Unlike *Phalaenopsis*, *Paphiopedilum* exhibits a sympodial growth habit, characterized by limited growth potential. Normally, a tiller bud is formed at the base of each leaf on the mother stem. It is enclosed by the first leaf primordium and eventually develops into a mature bud with several young leaves. Different *Paphiopedilum* species vary in their tillering abilities. *P. purpuratum*, *P. wardii*, *P. callosum*, and *P. dianthum* have weaker tillering abilities, and their tillering buds enter a dormant state after formation. These buds can break dormancy and resume growth only after the apical meristem of the main stem undergoes the transition from vegetative to reproductive growth stage and bolting (Supplementary Data Figure S1 a–d). Consequently, these species exhibit a single-branch flowering phenotype. On the other hand, *P. spicerianum*, *P. insigne*, *P.* 'SCBG Yingchun', and *P.* 'Yuanchun' possess stronger tillering abilities, and the development of their tillering buds is not restricted by the main stem. Once mature, these buds can continue to grow alongside the main stem without entering a dormant phase (Supplementary Data Figure S1 e–h). As a result, these species eventually display a multi-branched flowering phenotype. The abundance of flowers is a key horticultural characteristic that significantly influences the ornamental and economic value of orchids. The final number of tillers, determined by the outgrowth of the lateral buds in *Paphiopedilum*, is one of the important factors influencing flower abundance.

Outgrowth of lateral buds consists of two successive processes, the activation of the lateral bud and sustained branch growth [2]. Cytokinins (CTKs) are well-known hormones that play a positive role in regulating lateral bud outgrowth. They are primarily synthesized in the root and can be translocated through xylem and phloem [3]. Various synthetic CTKs exist, among which Benzylaminopurine (BAP) is the first synthetic CTK and widely used in agriculture and horticulture [4, 5]. In perennial woody plants like apple and sweet cherry, the exogenous application of BAP effectively stimulate the activity of lateral buds and increased the total number of lateral branches per young tree [6, 7]. Additionally, CTKs treatment is commonly employed in annual or perennial grass species such as wheat, oats, barely and tall fescue to enhance tiller production [8–11]. Based on this, researchers have proposed a hypothesis that CTKs, acting as a second messenger, can directly enter the axillary bud to regulate bud activity. Once the axillary bud detects this signal, it breaks dormancy and triggers activation. The activated axillary bud then rapidly exports auxin to the main stem, establishing auxin canalization, and leading to sustained outgrowth as a new shoot [12, 13]. In the model plant *Arabidopsis* and pea, many studies have revealed the interactions between auxin and CTKs in the regulation of axillary bud outgrowth. On one hand, BAP treatment enhanced the accumulation of PIN3, PIN4, and PIN7 in the main stem, contributing to increased shoot branching [14]. On the other hand, auxin, through the classical AXR1-AFB (auxin resistance protein 1-auxin signalling f-box protein)-dependent auxin signalling pathway, degrades active CTKs by regulating the expression of genes such as *adenosine phosphate-isopentenyltransferase* (*IPT*) and *cytokinin oxidase* (*CKX*), thereby inhibiting the outgrowth of axillary buds [15, 16].

Orchids play a crucial role in the horticulture and florist industries, particularly as perennial grass species. While many studies have focused on the effects of plant hormones on in vitro shoot multiplication [17], the understanding of how plant hormones control tillering in seedlings remains limited. A recently study demonstrated that 100 mg/L BAP treatment induced the emergence of lateral shoots in *Phalaenopsis*, with 68.3% of plants producing one or two lateral shoots [18]. *Phalaenopsis* is a typical monopodial orchid, and the shoot apex has unlimited growth potential. Due to its single growing axis, it usually does not produce tillers at the base. Base on this, we predict that BAP treatment has similar effects on *Paphiopedilum*, breaking dormancy and reactivating lateral buds, ultimately increasing the number of tillers and the rate of tiller production. Understanding the mechanisms that control lateral bud outgrowth is crucial for improving flower abundance in plants. In this study, we explore the role of BAP in regulating tiller growth of *P. callosum*. Firstly, we conducted morphological observations of tiller growth in *P. callosum* under different BAP concentrations. Secondly, we analyzed the effects of BAP on endogenous hormone levels associated with tiller bud outgrowth. Thirdly, we performed transcriptome analysis to examine the expression profiles of genes involved in hormone metabolism during tiller development under BAP treatment. Finally, we conducted validation and comparative analysis of gene expression associated with dormancy and hormone metabolism in both *P. callosum* and *P.* 'SCBG Yingchun'.

Result

Formation and growth of tiller buds in *Paphiopedilum*

Mature *Paphiopedilum* plants typically have 4–5 distichous leaves that obscure the stem bases. Tiller buds could arise from the axial of each leaf on its abbreviated stem. Figure 1 shows the successive steps of tiller formation in two *Paphiopedilum* plants with different tillering abilities. Firstly, a tiller bud formed

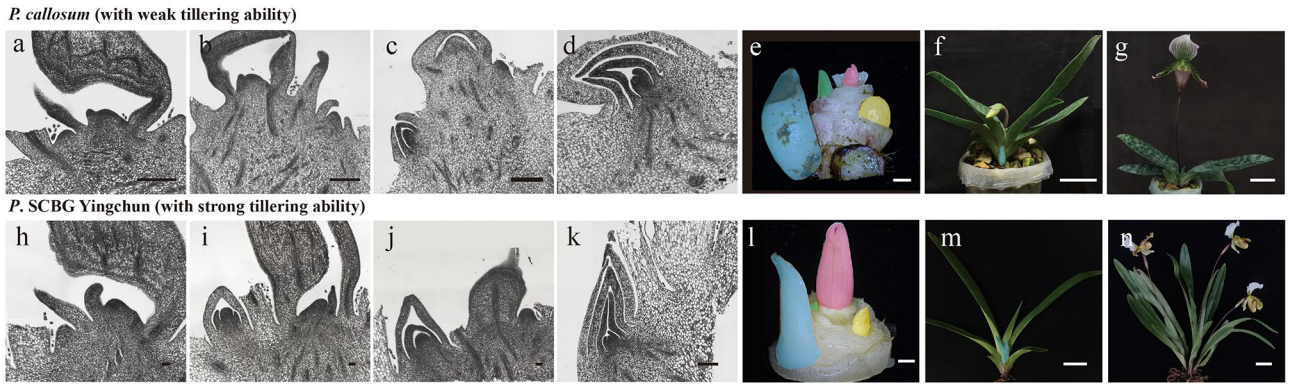

Fig. 1 Development of tiller buds at the main stem in *P. callosum* (with weak tillering ability)and *P.* 'SCBG Yingchun' (with strong tillering ability). **a–d**. The arrows indicate the progression of *P. callosum*'s tiller bud formation from leaf axils (**a**), through the stage with the first leaf primordium (**b**), two leaf primordia (**c**), to the stage with three leaf primordia (**d**); (**e**). Tiller buds at the internode of *P. callosum*; (**f**). The first tillering bud produced after bolting of *P. callosum*; (**g**). *P. callosum* with one-branch flowering; **h–k**. The arrows indicate the progression of tiller bud formation from leaf axils in *P.* 'SCBG Yingchun' (**h**), through the stage with the first leaf primordium (**i**), two leaf primordia (**j**), to the stage with three leaf primordia (**k**); (**l**). Tiller buds at internode of *P.* 'SCBG Yingchun'; (**m**). The first tillering bud of *P.* 'SCBG Yingchun' could grow without dormancy; (**n**). *P.* 'SCBG Yingchun' with three-branch flowering; The blue area indicate the primary tiller bud, the yellow area indicate the second tiller bud, the green area indicate the third tiller bud, the pink area indicate the shoot apex of the main stem; Scale a–d = 0.5 mm; e and **h**–**l** = 1 mm, **f**–**g** and **m**–**n** = 5 cm

from the axillary meristem (Fig. 1a, h). Then the tiller bud developed the first, second, and third leaf primordium step by step (Fig. 1b–d, i–k). When the tiller bud developed the fourth leaf primordium (indicated by blue markers in Fig. 1e, l), it has matured and acquired the ability to rapidly elongate, break through the mother plant, and become a normal tiller. Each tiller bud develops sequentially as the leaves grow, with the tillering bud at the lowest stem node (designated as tillering bud A, marked in blue in Fig. 1) forming and maturing first, followed by tiller bud B (marked in yellow) and tiller bud C (marked in green) further up the stem. The period from formation to maturation of a tiller bud typically takes 3–4 months, and the ability of the mature tiller bud to outgrow is crucial for determining the number of tillers. The outgrowth of a tiller bud in *Paphiopedilum* involves two consecutive processes: breaking dormancy and sustained outgrowth. For *P. callosum*, the tiller ability is weak, and tiller buds after maturation enter a dormant period of 5–7 months. After the apical meristem (Fig. 1marked in pink) changed from vegetative growth to reproductive growth and bolting, the mature tiller buds began to outgrowth, breaking through the mother plant (Fig. 1f). As a result, *P. callosum* usually exhibits a one-branch flowering phenotype. (Fig. 1g). In contrast, *P.* 'SCBG Yingchun', which has a stronger tillering ability, shows continuous elongation and growth of tiller buds after maturation, in sync with the growth of the main stem leaves, without dormancy (Fig. 1m). Before the formation of the flower organs, 2–3 tiller seedlings could be produced, ultimately resulting in a multi-branched flowering phenotype (Fig. 1n).

Exogenous BAP application could effectively promote both the tiller buds and roots development of *P. callosum*

Exogenous BAP treatment at concentrations of 100, 200, 400, and 1000 mg/L can promote the outgrowth of tiller buds in *P. callosum*. After 100 d of treatment, the formation of tiller buds can be observed in the mother plants (Fig. 2). Different concentrations of exogenous BAP have different promoting effects on the growth of tiller buds. At a BAP concentration of 400 mg/L, the number of tiller buds significantly increased to 3.41 after 100 d of treatment, with an average height of the first tiller bud at 4.32 cm. Increasing the BAP concentration to 1000 mg/L did not enhance the promotion effect on tiller buds compared to the 400 mg/L treatment (the number of tillering buds was 2.83, and the height of the first tiller bud was 3.30 cm at 100 days after treatment) (Table 1). Therefore, we selected the 400 mg/L BAP treatment as the optimal treatment for promoting tillering growth in *P. callosum*.

After 30 d of 400 mg/L BAP treatment, four protruding bud points can be seen on the basal stem of *P. callosum* after peeling off the outermost 1–2 leaves. This stage is referred to as the dormancy release stage (Fig. 2d-1), Subsequently, these tiller buds continue to expand and turn green, breaking through the mother stem at 60 days. This stage is denoted as the expansion stage (Fig.2d-2). Tiller buds continue their sustained outgrowth, following the development of new leaves, ultimately becoming new tiller shoots (Figs. 2d and 3), which is referred to as the sustained outgrowth stage. Low concentrations (100 and 200 mg/L) of BAP treatment can promote the production of new roots, although the promotion effects on tiller buds are not as significant as with the high concentrations. Plants subjected to 100 and 200 mg/L BAP

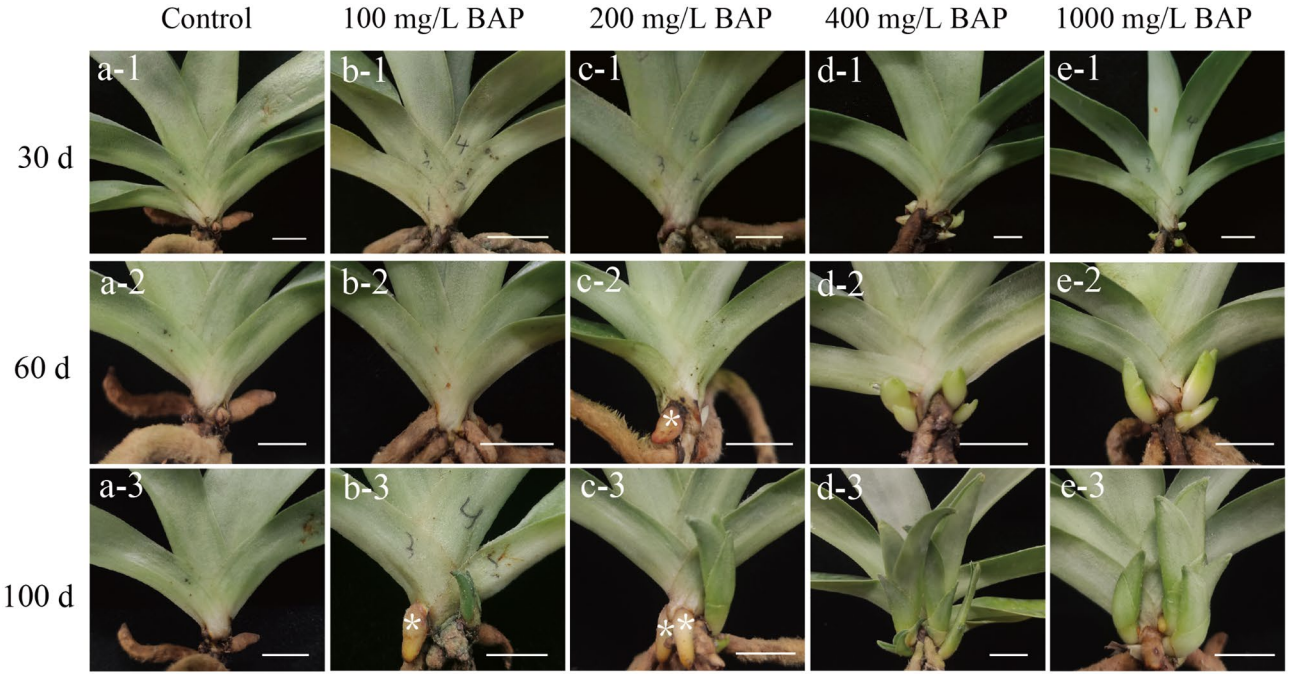

Fig. 2 Effects of different concentration of BAP on the growth of tiller bud and root in *P. callosum*. (**a**-1, 2, 3) Control plants at 30 d,50 d, and 100 d; (**b**-1, 2, 3) plants at 30 d,50 d, and 100 d of 100 mg/L BAP treatment; (**c**-1,2,3) plants at 30 d,50 d, and 100 d of 200 mg/L BAP treatment; (**d**-1,2,3) plants at 30 d,50 d, and 100 d of 400 mg/L BAP treatment; (**e**-1,2,3) plants at 30 d, 50 d, and 100 d of 1000 mg/L BAP treatment. * Indicated the initiated new root. Scale bars = 1 cm

Table 1 Effect of exogenous BAP pot irrigation on the number of tiller buds and the length of the first tiller bud

Days	Tiller bud number					1st Tiller bud length (cm)*				
	0 mg/L	100 mg/L	200 mg/L	400 mg/L	1000 mg/L	0 mg/L	100 mg/L	200 mg/L	400 mg/L	1000 mg/L
30 d	0.00±0.00 b	0.00±0.00 b	0.00±0.00 b	3.30±0.26 a	2.90±0.23 a	0.00±0.00 B	0.00±0.00 B	0.00±0.00 B	0.57±0.05 A	0.42±0.03 A
60 d	0.00±0.00 d	0.00±0.00 d	0.40±0.16 c	3.29±0.17 a	2.76±0.16 ab	0.00±0.00 C	0.00±0.00 C	0.37±0.08 B	0.97±0.14 A	1.18±0.19 A
100 d	0.00±0.00 d	0.48±0.10 cd	0.96±0.16 c	3.41±0.11 a	2.83±0.14 b	0.00±0.00 D	1.23±0.07 D	2.10±0.11 C	4.32±0.16 A	3.30±0.10 B

$N \geq 3$, values are mean ± standard error. Values followed by different lower-case letters within a column indicated significant difference of tiller bud number at $P < 0.05$ according to Duncan's multiple range test. Values followed by different upper-case letters within a column indicated significant difference of 1st tiller bud length at $P < 0.05$ according to Duncan's multiple range test. * Indicated the first tiller bud produced from the main stem

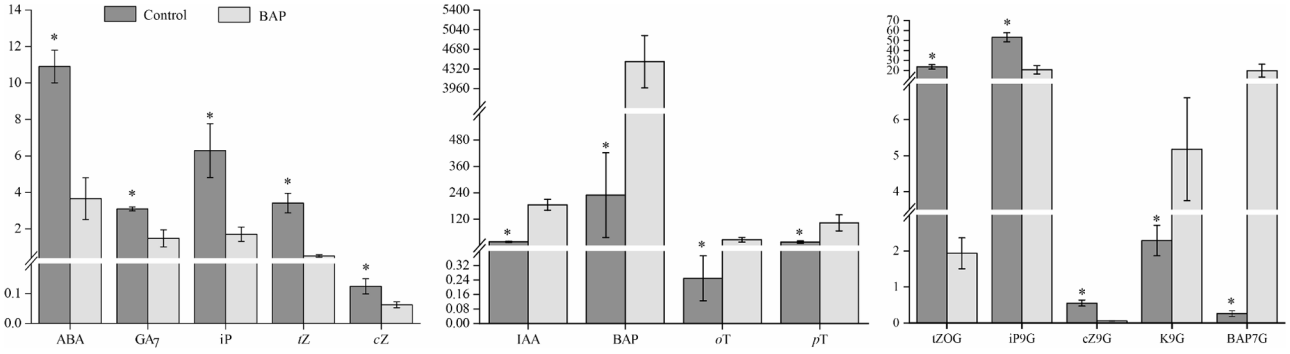

Fig. 3 Measurement of IAA, ABA, GA_7, CTKs content at 30 d in control and BAP-treated *P. callosum*. The results represent the means ± SE of three biological replicates, with 1.0 g fresh weight (FW) for each replicate. Hormone levels are indicated as ng/g fresh weight. * Indicates a significant difference from the control group (Student's t-test, $P < 0.05$)

treatment at 100 d can produce new roots, the number of the new root per plant being 1.25 ± 0.16 and 1.46 ± 0.14, respectively (Fig. 2b-3, c-3).

Effects of 400 mg/L BAP treatment on endogenous hormone content at dormancy release stage

Morphological results of exogenous BAP treatment showed that tiller buds could break dormancy and start to grow after 30 days of 400 mg/L BAP treatment. To determine whether the promotion of tiller growth by BAP is associated with changes in endogenous levels of key hormones, we quantified the content of various hormones at 30 days in both control and BAP-treated plants (Fig. 3, Supplementary Data Table S1).

The content of ABA, GA_7, and isoprenoid CTKs (iP, *t*Z, and *c*Z) was significantly reduced (by 66%, 52%, 73%, 85%, 49%) due to BAP application compared to the control plants. However, the content of IAA and aromatic CTKs (BAP, *o*T, and *p*T) in the BAP-treated stems was significantly higher compared to that of the control plants, which were 10.48, 19.40 and 107.53-fold of those in control plants, respectively. Additionally, the content of cytokinin glucosides (iP9G, cZ9G, K9G, and BAP7G) showed variable responses, with BAP treatment leading to significant increases in iP9G and K9G, while cZ9G and BAP7G contents were reduced compared to the control.

Transcriptomic analysis of tiller bud development in 400 mg/L BAP treatment

Based on the morphological characterization of tiller buds under 400 mg/L BAP treatment, we assigned the stages of tiller buds treated with BAP at 0d, 30d, 60d and 100d as stages 0, 1, 2, 3, respectively. To further investigate the transcriptomic regulation mechanisms of tiller growth by BAP treatment, RNA-seq analysis was performed at these four developmental stages. Clean data (78.94 Gb in total) were obtained from 12 libraries (1 treatment × 3 biological replicates × 4 developmental stages). Detailed statistics of the clean reads are listed in Supplementary Data Table S2. By de novo assembly of the total clean reads, we obtained 54,728 unigenes with an average length and N50 length of 1866.14 and 2692 bp, respectively (Supplementary Data Table S3). Among these unigenes, 36,408 (66.53%) were annotated with at least one putative function in publicly available databases (Supplementary Data Table S4). Samples were separately clustered based on the developmental stages in the principal component analysis (PCA) (Supplementary Data Figure S2a). Sequence comparisons showed a high similarity between *P. callosum* transcripts and those of *Dendrobium catenatum* (41.48%), *Phalaenopsis equestris* (11.18%), and *Apostasia shenzhenica* (9.66%) (Supplementary Data Figure. S2b).

Differentially expressed genes (DEGs) were detected using a screening criteria of False Discovery Rate (FDR) < 0.01 and log2 ratio ≥ 1.0 (Fold Change ≥ 2.0). Three comparison groups were designed between successive tiller growth stage. The S1 versus S2 group had the fewest DEGs, with 1227 DEGs, consisting of 809 up-regulated and 418 down-regulated genes. The S2 versus S3 group had the highest number of DEGs, with 2097 in total, including 1009 up-regulated and 1088 down-regulated genes (Fig. 4a). There were 80 overlapped DEGs shared by the three comparison groups, which could be play an essential role in tiller bud development (Fig. 4b).

KEGG enrichment analysis was conducted on the three comparison groups. In the S0 versus S1 group, the activation of pathways related to plant hormone signaling transduction, ribosome, and DNA replication was observed, suggesting an enhancement in the plant's activity, potentially promoting the activation of axillary buds (Fig. 4c). In the S1 versus S2 group, the significant enrichment of genes associated with starch and sucrose metabolism, nitrogen metabolism, and fatty acid degradation implies the involvement of sugars, nitrogen, and fatty acids in the process of tiller bud emergence from the mother stem and further elongation (Fig. 4d). In the S2 versus S3 group, pathways related to photosynthesis, photosynthesis-antenna proteins, and phenylpropanoid biosynthesis were highly enriched, indicating an increased energy requirement for the sustained growth of tiller buds (Fig. 4e).

Gene expression trend analysis was used to identify the common expression patterns of DEGs. In this study, we analyzed 3,797 genes across three comparison groups using the K-means algorithm, which led to the identification of 15 distinct clusters (Fig. 5a, Supplementary Data Table S5). Clusters K1 and K2 comprised 143 and 545 genes, respectively. These genes demonstrated a consistent upregulation trend during S1–S3 stage compared to the S0 stage. In contrast, the K3 gene cluster (encompassing 122 genes) and the K4 gene cluster (comprising 519 genes) exhibited a stable downregulation trend during the S1–S3 stage. Based on these observations, we hypothesize that the genes in these four clusters are likely involved in regulating the growth and development of tillering buds in response to exogenous BAP treatment. To gain further insight into the effect of DEGs on the regulation of tillering, we conducted a Gene Ontology (GO) enrichment analysis on a total of 1,329 genes from these four clusters (Fig. 5b). The significantly enriched GO terms included cytokinin metabolic process, cytokinin dehydrogenase activity, and abscisic acid binding. These findings suggest that CTKs are not only involved in the development of tillering buds in *P. callosum* but also indicate that ABA may play a crucial role.

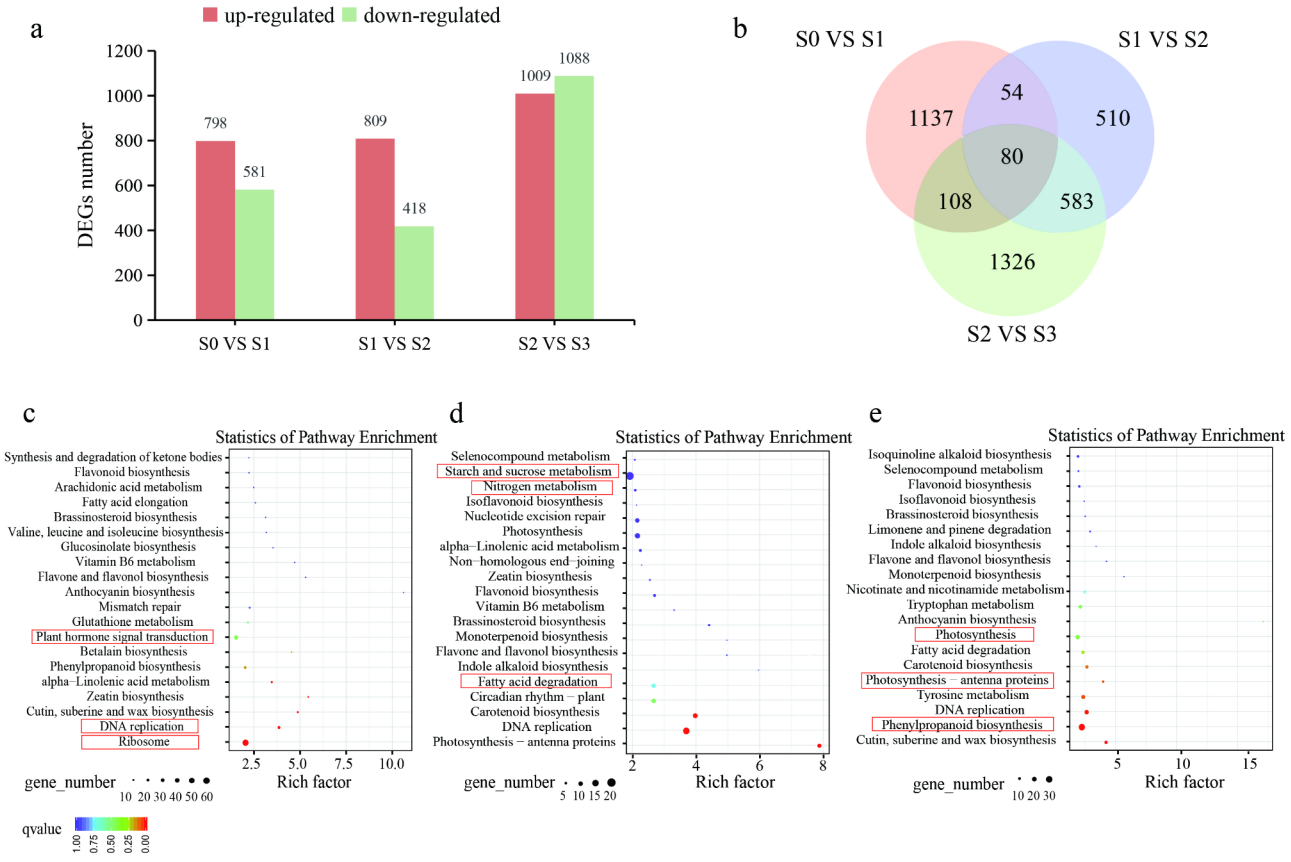

Fig. 4 Analysis of DEGs from samples at four developmental stages in *P. callosum* under 400 mg/L BAP treatment. (**a**) Histogram of the number of up-regulated and down-regulated DEGs in each comparison group; (**b**) Venn diagram of the number of DEGs in each comparison group; (**c**) KEGG enrichment analysis with the DEGs between S0 and S1; (**d**) KEGG enrichment analysis with the DEGs between S1 and S2; (**e**) KEGG enrichment analysis with the DEGs between S2 and S3

Expression profile of CTKs biosynthesis, degradation and signaling genes

CTKs are synthesized by Adenylate isopentenyltransferases (IPTs), cytochrome P450 monooxygenase (CYP735A) and riboside 5'-monophosphate phosphoribohydrolase (LONELY GUY, LOG) [19–21]. The deactivation of CTKs is accomplished by cytokinin oxidase/dehydrogenase (CKX) [22]. Cytokinin signaling is mediated by a two-component system. A- or B-type response regulators (A-type RRs or B-type RRs, respectively) negatively regulated CTKs signal transduction [23].

We found 4 homologs of IPT, 9 homologs of CKX, but only one homolog of CYP735A and LOG (Fig. 6). Three cytokinin-synthesis genes, *IPT1*, *CYP735A1*, and *LOG3* shows differentially expressed patterns. Expression level of *IPT1* was significantly down-regulated at S3, while *CYP735A1* exhibited the opposite trend of expression. *LOG3* was significantly up-regulated by BAP treatment at S1 and S3. Most members of *CKX* gene family exhibited up-regulating responses to BAP treatment.

P. callosum has nine paralogs of genes for *HK*, five for HP, seven for A-type RRs and nine for B-type RRs (Fig. 6, Supplementary Data Table S6). Our transcriptome profiling demonstrated that six genes coding for B-type RRs were significantly up-regulated by BAP treatment at S1, S2 and S3. However, expression level of two A-type RR genes significantly down-regulated at S1, S2 and S3. The decreased level of isoprenoid CTKs (Fig. 3) at S1, corresponded to the up-regulation of CTKs catabolism genes and CTKs signaling genes at S1.

Expression profile of DEGs related to biosynthesis, degradation and signaling of auxin and ABA

Besides CTKs, auxin and ABA also play pivotal roles in tiller development [24, 25]. A total of 28 DEGs involved in auxin biosynthesis, degradation, transport and signaling transduction were annotated (Fig. 7 and Supplementary Data Table S7). The IPA pathway is a mainly conserved pathway for most auxin synthesized in plants [26]. Flavin monooxygenase (YUCCA) and tryptophan amino acid transferase-1 (TAA1) are two key enzymes that regulated *via* the IPA pathway in a two-step process [27]. Dioxygenase of auxin oxidation (DAO) proteins is essential for keeping auxin homeostasis, which is responsible for

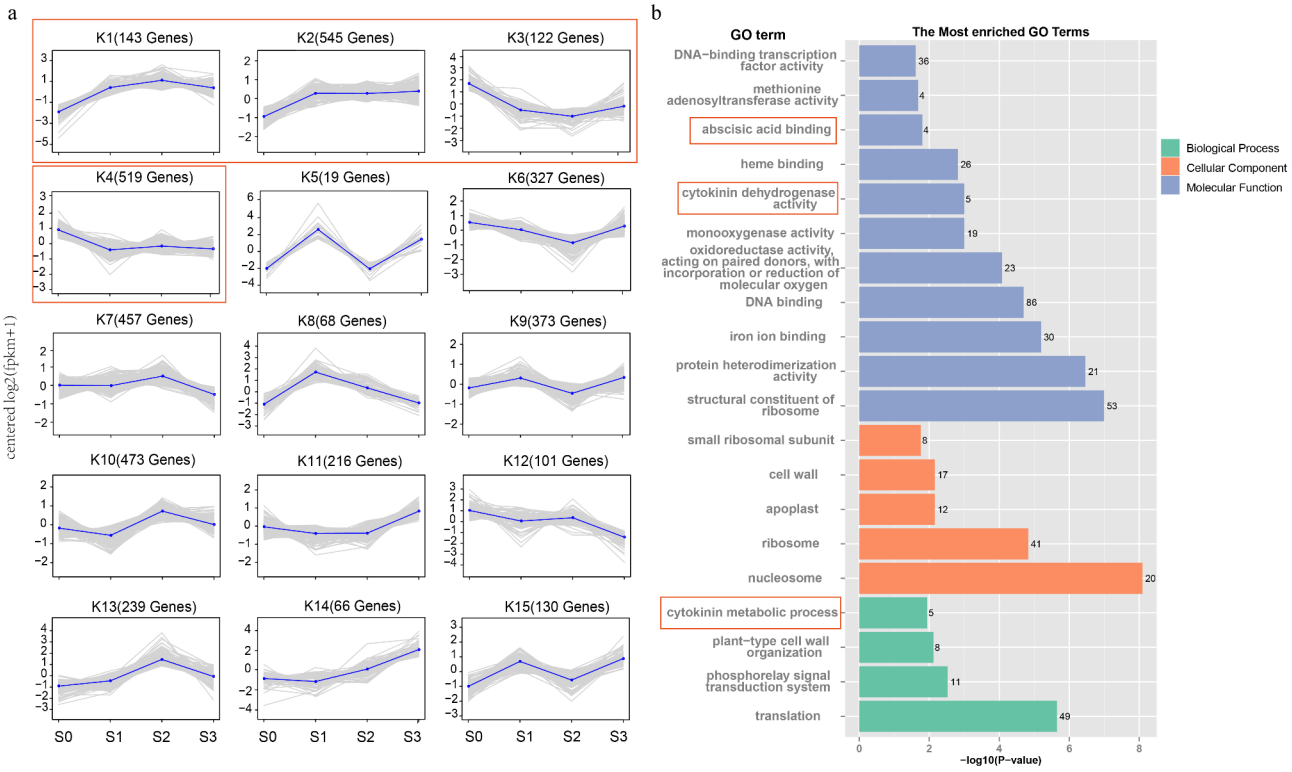

Fig. 5 Gene expression trend and GO enrichment analysis of DEGs. (**a**) k-Means cluster analysis on DEGs among three comparison groups. The x axis S0–S3 represents the status of tiller buds treated with BAP at 0 d, 30 d, 60 d and 100 d and the y axis depicts the centered $\log_2$ (FPKM + 1); (**b**) GO enrichment analysis of DEGs of K1–K4 cluster

catalyzing the conversion of active IAA into biologically inactive 2-oxoindole-3- acetic acid (OxIAA) [28]. PIN-formed (PIN) proteins, ATP-binding cassette subfamily B (ABCB) proteins, auxin resistant1/like auxin resistant 1 (AUX1/LAX)proteins and PIN-likes (PILS) proteins are key auxin transporters involved in diverse transporter systems that control short-range and long-range auxin transport [29, 30]. Our studies showed that a single gene for *YUCCA* were up-regulated during S1–S3, while a single gene for *DAO* exhibited the opposite trend of expression. Four homologs of auxin transporter genes (*PIN1C*, *ABCB19*, *PILS1*, *LAX3*) were all up-regulated in response to BAP during S1–S3.

Auxin-responsive gene expression relies on the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) pathway to initiate auxin signaling [31]. The downstream of IAA signaling pathway includes the *auxin response factor* (*ARF*), *Small Auxin Up RNA* (*SAUR*), *auxin/indole-3acetic acid* (*Aux/IAA*), *Gretchen Hagen3* (*GH3*) genes [32]. There are eight homologs for *IAA/AUX*, eight homologs for *ARF*, three homologs of *GH3* genes and three homologs of *SAUR* genes. The expression of these genes displayed a complex expression profile. One gene for *AFR* (*ARF19*) was significantly down-regulated during S1–S3, while other homologs of *AFR* genes did not show synchronized expression profile. High level expression of IAA synthesis and transport genes and low-level expression of degradation gene *DAO* during S1–S3 suggests that IAA functions actively in tiller outgrowth of *P. callosum*.

Zeaxanthin epoxidase (ZEP) and 9-cis-epoxycarotenoid dioxygenase (NCED) are two enzymes that regulate ABA biosynthesis. ABA levels in plants are not only regulated by synthesis but also by metabolism. CYP707A encoded ABA-8'-hydroxylase, which deactivated ABA [33]. PYR/PYL/RCARs, as ABA receptors, bind ABA in a complex with ABI1 or other phosphoprotein phosphatase 2Cs (PP2Cs). SNF1-related protein kinases (SnRKs) and ABRE-binding factors (ABFs) are core components that complete the downstream of ABA signaling [34]. In our analysis, there are three DEGs for ABA biosynthesis, two genes for *ZEP* up-regulated during S1–S3, however, one gene for *NCED* down-regulated during S1–S3. Four *CYP707A* family genes had different expression profile during S0–S3, in which *CYP707A7* was significantly up-regulated during S1–S3. Six DEGs in the ABA signaling pathway showed complex expression patterns. The expression level of gene coding for *PLY4* and *SAPK7* showed synchronized expression patterns, which were up-regulated during S1–S3, while one gene for *ABF2* exhibited the opposite trend of expression (Fig. 7 and Supplementary Data Table S7).

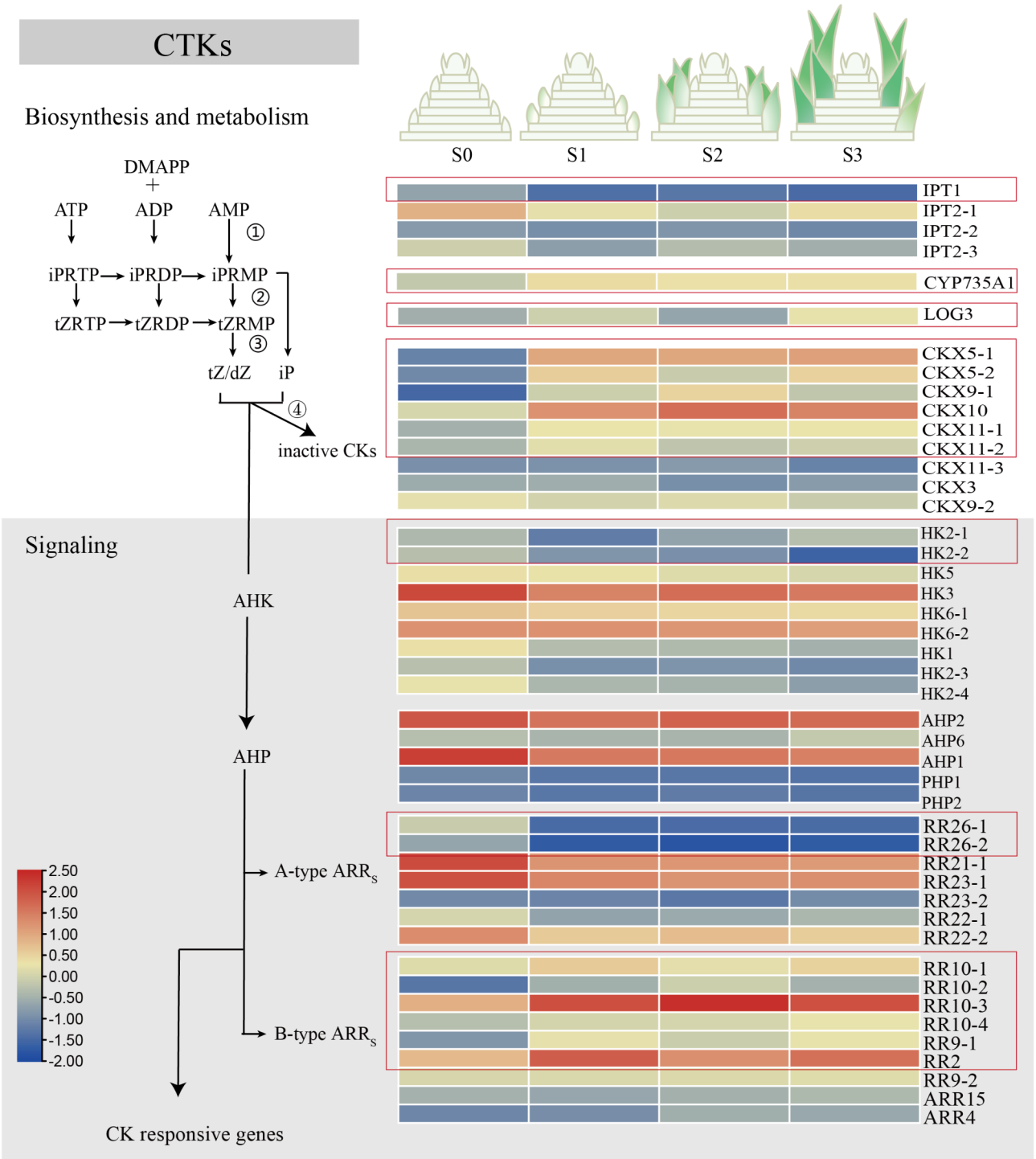

Fig. 6 Heatmap of genes related to CTKs biosynthesis, degradation and signaling. Heatmap of genes related to CTKs biosynthesis, deactivation, and signaling. Blue squares indicate down-regulation, whereas red squares indicate up-regulation. The color scale corresponds to the average log2 (FPKM + 1) and Z-score value (normalized by R software). S0–S3 represents the status of tiller buds treated with 400 mg/L BAP at 0d, 30d, 60d and 100d

Expression profile of DEGs involved in tiller bud outgrowth

In addition to hormone-related genes, genes associated with dormancy, sugar, and energy metabolism may also play a crucial role in the growth and development of tillers under BAP treatment [2, 35]. As depicted in Fig. 8 (Supplementary Data Table S8), *Dormancy-associated gene-1* (*DRM1*) and *dormancy-associated MADS-box/SHORT VEGETATIVE PHASE-like genes* (*DAM/SVP*), which are known as negative regulators of axillary bud outgrowth and branching

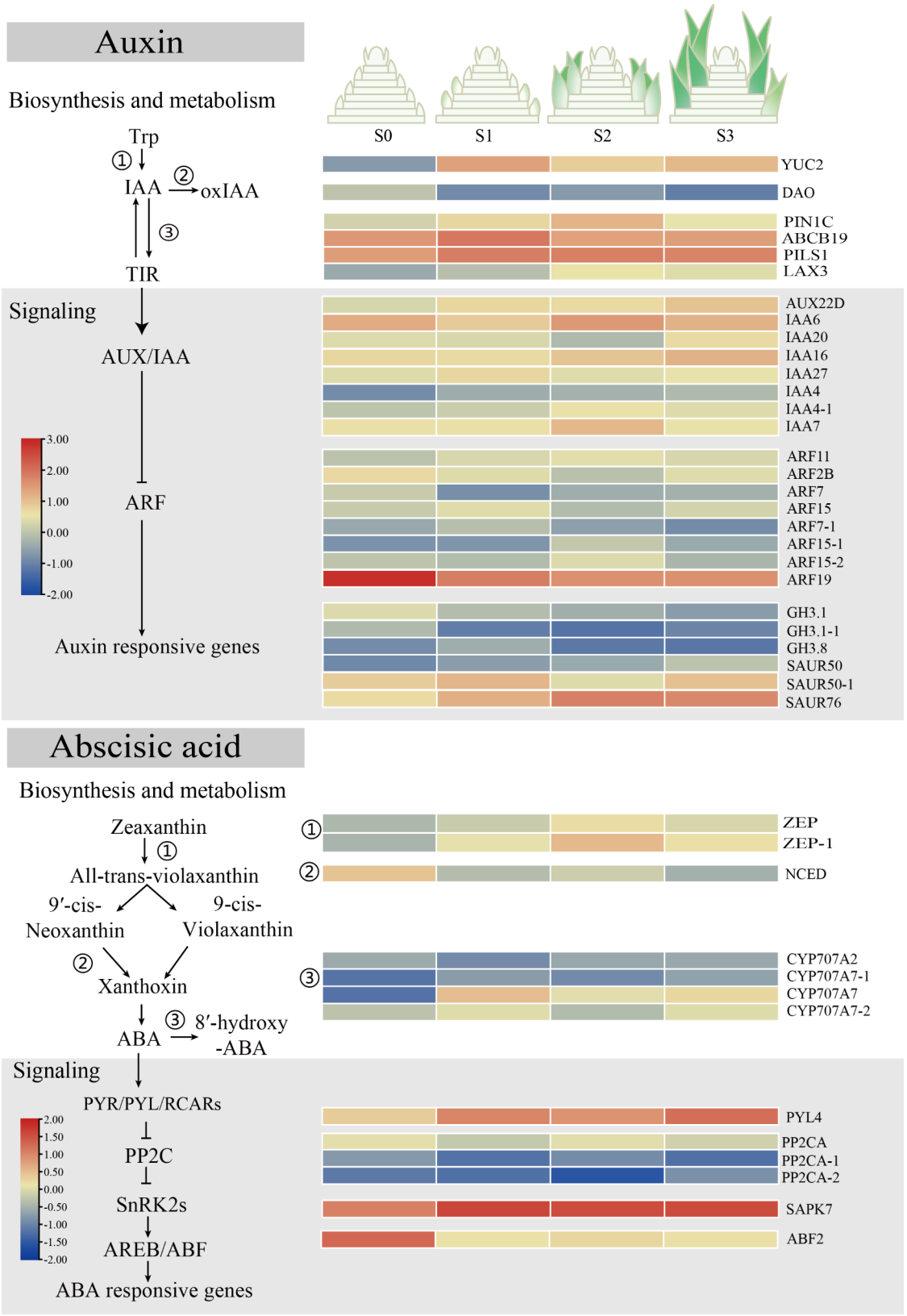

Fig. 7 Heatmap of DEGs related to auxin and ABA biosynthesis, degradation and signaling. Heatmap of DEGs related to auxin and ABA biosynthesis, degradation and signaling. Blue squares indicate down-regulation, whereas red squares indicate up-regulation. The color scale corresponds to the average log2 (FPKM + 1) and Z-score value (normalized by R software). S0–S3 represents the status of tiller buds treated with 400 mg/L BAP at 0d, 30d, 60d and 100d

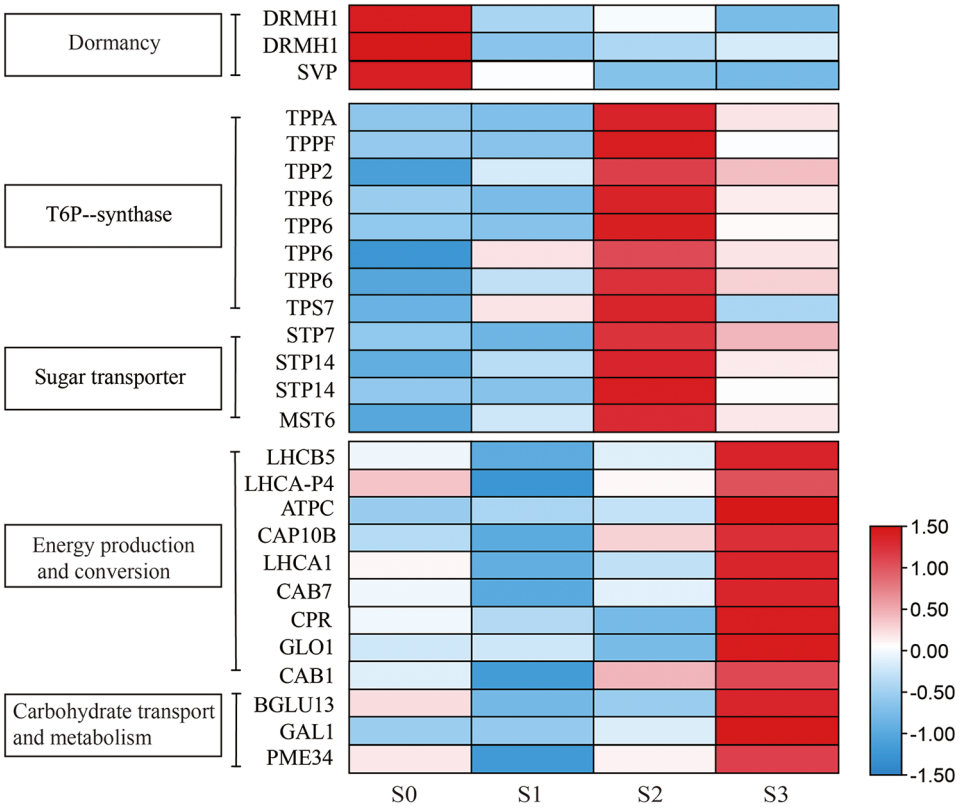

Fig. 8 Heatmap of DEGs related to tiller bud outgrowth subjected to 6-BA treatment in stem. Blue squares indicate downregulation, whereas red squares indicate upregulation. The color scale corresponds to the average log2 (FPKM + 1) and Z-score value (normalized by R software). S0–S3 represents the status of tiller buds treated with 400 mg/L BAP at 0d, 30d, 60d and 100d

[36, 37]. One homolog of the *SVP* gene and two homologs of *DRMH1* genes exhibited down-regulating responses to BAP treatment during S1–S3 stage. This suggests that BAP treatment may release tiller bud dormancy, thereby promoting their development. Furthermore, one homolog of *Trehalose-6-Phosphate Synthase 1* (*TPS1*) gene and seven homologs of *trehalose-6-phosphate phosphatase* (*TPP*) genes, which are involved in the formation of trehalose 6-phosphate (T6P) and regulate local and systemic sugar levels to drive bud outgrowth [38, 39], were significantly up-regulated at S2 among the four developmental stages under BAP treatment. Additionally, four DEGs encoding sugar transport protein were also up-regulated at S2 stage, indicating that CTKs may interact with sugars to regulate the transition of tiller buds from S1 to S2. Moreover, genes related to energy production and conversion, as well as carbohydrate transport and metabolism according to a database of orthologous groups of genes (eggNOG) class annotation, were significantly up-regulated at S3 stage. These results suggest that to promote the growth of the first and second true leaves and develop into a productive tiller, these buds enhance their competition for nutrients in the stem.

Comparison of expression patterns of tiller-related candidate genes in two *Paphiopedilum* species with different tillering abilities

Based on the results from the analysis of DEGs, we selected *P.* 'SCBG Yingchun', notable for its strong tillering ability, as the control. The continuous growth processes of the primary tiller bud in *P.* 'SCBG Yingchun' were found to be similar to those observed in *P. callosum* treated with 400 mg/L BAP, as depicted in Supplementary Data Figure S3. Quantitative Real-Time PCR (qRT-PCR) was subsequently employed to verify and compare the expression patterns of genes during the development of tillers in these two *Paphiopedilum* plants.

As shown in Fig. 9, compared to the non-BAP-treated plants of *P. callosum*, BAP-treated plants of *P. callosum* showed decreased expression of these two dormancy-associated genes during bud outgrowth process. In *P.* 'SCBG Yingchun', the relative mRNA level of *PcDRMH1* was 1/7 of that in the control plants of *P. callosum* during stage 0–3, and the expression level of *PcSVP* gene followed the same trend as that in BAP-treated plants of *P. callosum.* This indicated that BAP may induce the release of dormancy in tiller buds and promote tiller development. Furthermore, the expression of the gene involved in ABA catabolism (*PcCYP707A*) was initially below the

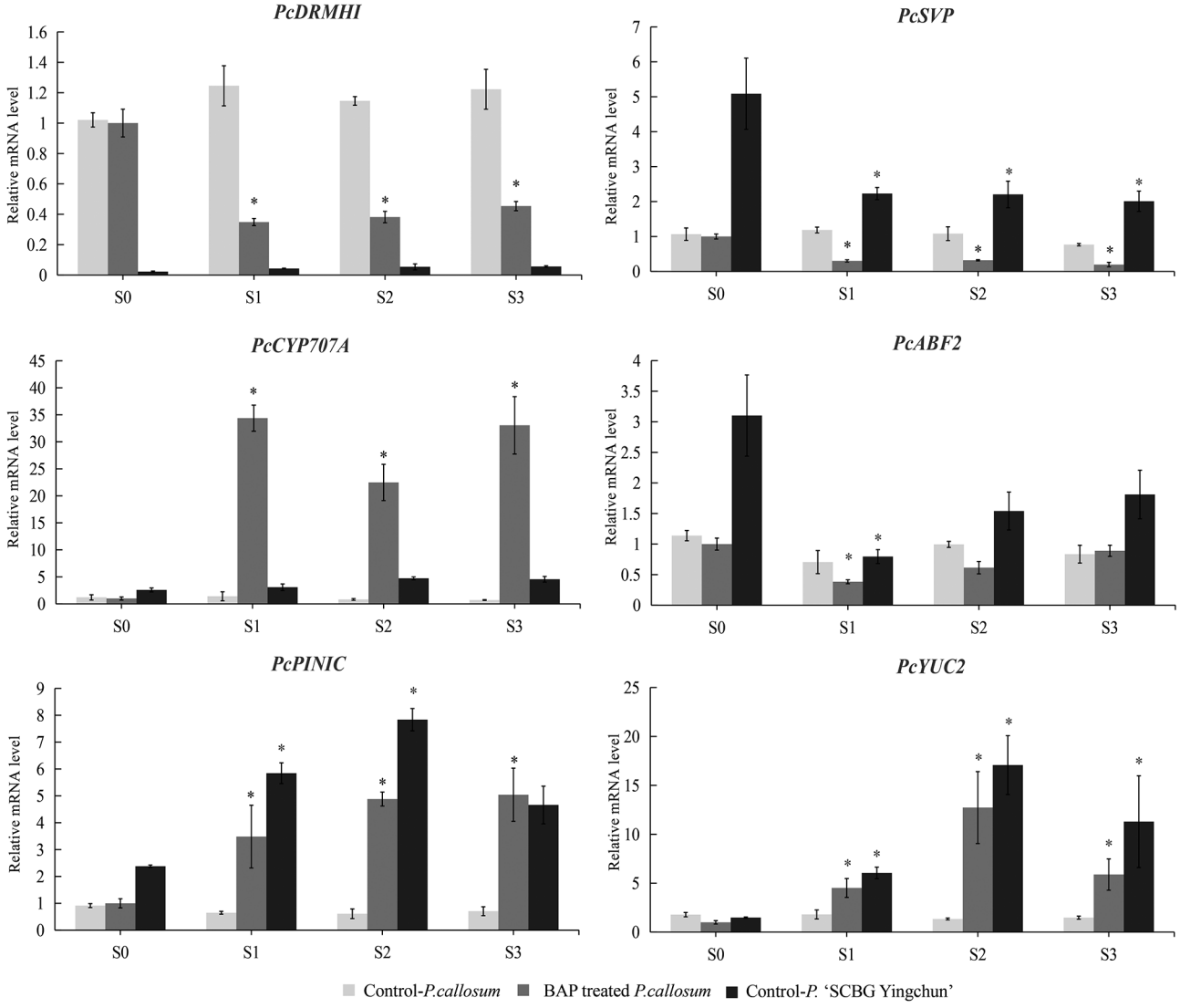

Fig. 9 Comparative study on the expression patterns of tillering-related candidate genes in two *Paphiopedilum* plants. RNA was extracted from control or 400 mg/L BAP treated plants of *P. callosum* and non-treated plants of *P.* 'SCBG Yingchun' during four development stages. *SPT6* (BMK_Unigene_064712) as the reference gene, and the relative expression levels were the means of three biological replicates (three plants for each replicate), with each replicate containing four technical replicates. Error bars represent the standard deviation of the mean ($n = 3$); An asterisk indicates a significant difference from the S0 stage in each treatment. (Student's t-test, $P < 0.05$)

baseline level at S0, but experienced a dramatic increase following BAP treatment. In contrast, the expression level of *PcCYP707A* in control plants of *P. callosum* and *P.* 'SCBG Yingchun' did not exhibit significant differences during stage 0–3. Conversely, the relative mRNA level of *PcABF2*, which codes for a key ABA response regulator, displayed an opposite expression pattern. It was down-regulated at the S1 stage in both in BAP-treated plants of *P. callosum* and control plants of *P.* 'SCBG Yingchun'. The auxin transporter gene *PcPIN1* and auxin biosynthesis gene *PcYUC2* exhibited a significantly up-regulated expression pattern during the tiller outgrowth process in BAP-treated *P. callosum* and control plants of *P.* 'SCBG Yingchun', compared with the control plants of *P. callosum*. This suggests that BAP affects the regulation of ABA catabolism and IAA transport during tiller bud outgrowth stage.

Validation of gene expression using qRT-PCR

We selected 12 DEGs associated with endogenous hormone and sugar synthesis, metabolism, transport, and axillary bud formation and maintenance. The accuracy of the transcriptome data was then verified through qRT-PCR, as shown in Fig. 10. Consistent with the transcriptome data, two CTKs catabolism genes (*CKX5* and *CKX10*), along with *RR10*, a negative regulator of the CTKs signaling pathway, exhibited significant upregulation following 400 mg/L BAP treatment. Similarly,

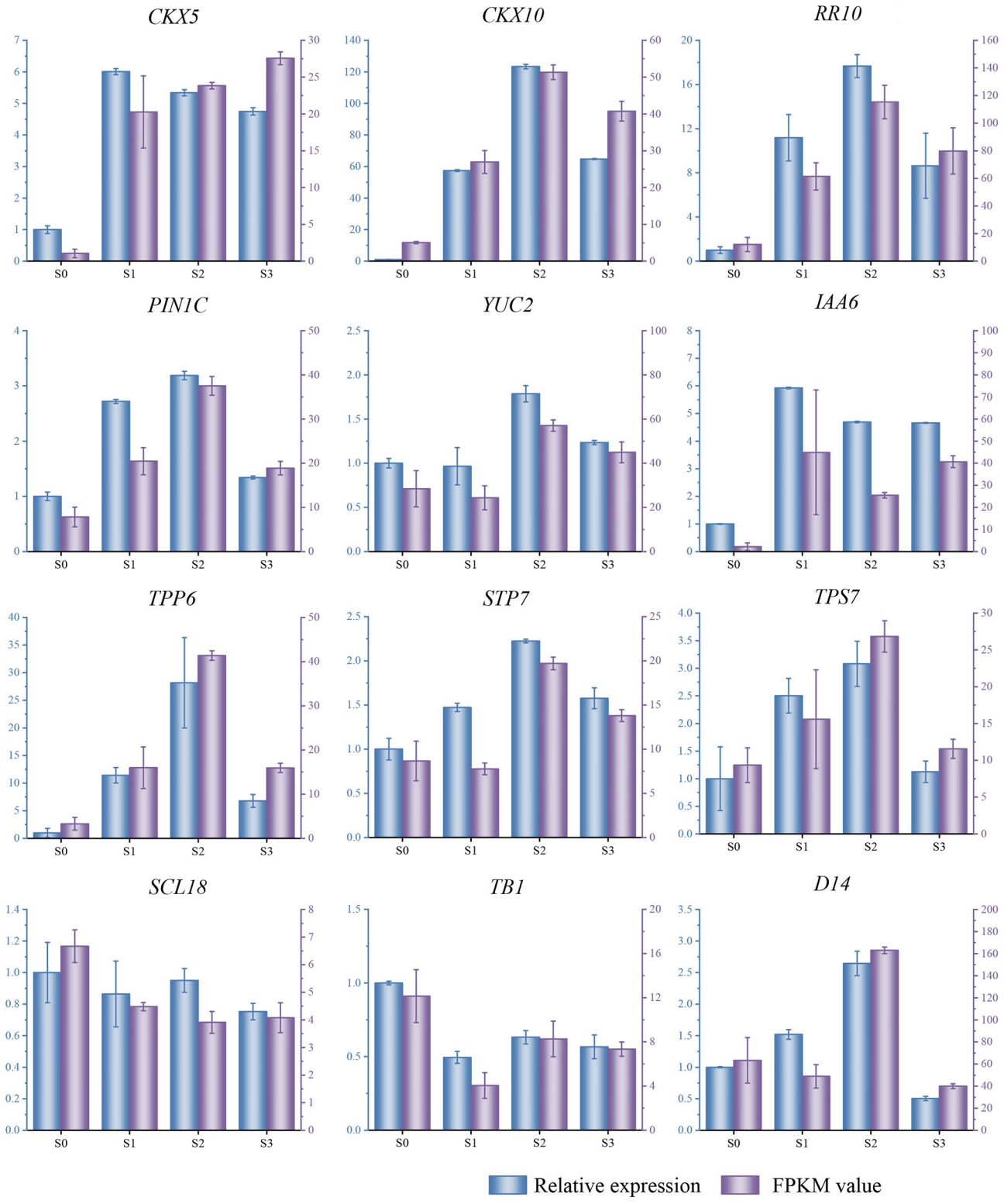

Fig. 10 qRT-PCR relative expression levels and FPKM values of tillering-related candidate genes. The reference gene was *SPT6* (BMK_Unigene_064712). The samples used for the RNA extraction came from three biological replicates. The x-axis represents the stages since the application of 400 mg/L BAP treatment when the stems were harvested. The left y-axis represents the FPKM values, and the right y-axis represents the relative expression levels. Error bars indicate the standard deviation of the mean ($n=3$)

three genes related to IAA synthesis (*YUC2*), transport (*PIN1C*) and singnal (*IAA6*) displayed the same trend. Furthermore, qRT-PCR analysis revealed that the expression levels of one gene involved in strigolactones (SLs) biosynthesis (*D14*), and three genes involved in sugar biosynthesis and transport (*TPP6*, *STP7*, *TPS7*), were the highest in S2 stage among the four tiller bud development stages. *SCL18*, a gene related to axillary bud formation, exhibited no significant differences in relative expression across the four stages of tiller bud development according to qRT-PCR results. The relative qRT-PCR expression of the gene *Teosinte Branched1 (TB1)*, which inhibits axillary bud elongation and growth, was significantly down-regulated at S1 stage. The relative expression levels of the target genes obtained by qRT-PCR were consistent with the FPKM (Per kilobase per million mapped reads) values of each gene.

Discussion

Exogenous BAP application promotes tiller bud outgrowth and increases the number of tillers

BAP is one of the plant growth regulators that has been widely used in agriculture and horticulture. Exogenous BAP can be applied through spraying, injection, or pot irrigation. Typically, 0.1 to 5 mg/L of BAP is sufficient to induce bud differentiation and proliferation. Exogenous foliar spraying of 10 to 100 mg/L BAP is primarily used to promote tillering, flowering, and fruit development in plants. However, higher concentrations can lead to toxicity and/or unwanted somaclonal variation, which poses significant challenges for subsequent commercial use [4, 5]. In our previous study, exogenous spraying and injection of BAP at different concentrations 20, 40, 80 mg/L did not have a significant effect on tiller bud growth in *P. callosum* (data unpublished). However, pot irrigation with 400 mg/L of BAP on *P. callosum* shoots every 2 weeks effectively promoted tiller bud outgrowth, leading to a significant increase in the number of tiller buds per plant compared to the control group. Similarly, in other Orchidaceae species such as *Dendrobium*, *Phalaenopsis*, and *Phaius*, BAP concentrations in the hundreds of mg/L have been shown to enhance flowering and tillering [18, 40–42]. In our study, Subsequent observations revealed that *P. callosum* seedlings treated with 400 mg/L BAP developed lateral buds that grew normally and flowered (Supplementary Data Figure S4). However, at 1000 mg/L BAP, lateral bud growth was slower, flowering was delayed, and flowering rates were reduced, suggesting that while higher concentrations may inhibit normal development, concentrations up to 400 mg/L are relatively safe for *P. callosum*.

Analysis of mutant phenotypes in *Arabidopsis* plants has demonstrated that CTKs can be synthesized in roots and act as long-distance signals transported to shoots through an efficient transport system [43]. After pot irrigation with exogenous BAP in *P. callosum*, the roots accumulated a substantial amount of CTKs, which were then transported to the shoot stem. This activation of tiller buds and promotion of tiller bud outgrowth ultimately resulted in the plants having more tillers. The promotion effects of BAP on tillering were more pronounced at a concentration of 400 mg/L BAP, supporting the second messenger model [44]. The total number of tillers produced by a plant is controlled by the number of tiller buds initiated from the stem and the rate of outgrowth of these buds. Promotion of either tiller bud initiation or outgrowth can lead to a higher tiller production [11]. In rice, *MONOCULM1(MOC1)* and *LAX PANICLE1 (LAX1)* are key genes involved in the formation and maintenance of lateral buds [45, 46]. Additionally, *TB1* in maize and its ortholog gene in *Arabidopsis BRANCHED 1* (*BRC1*), act as negative regulators, inhibiting bud outgrowth and modulating branching. Overexpression of *OsTB1* gene in rice resulted in a significant decrease in tiller number [47]. In *Arabidopsis*, exogenous CTKs treatment could down-regulate the *BRC1* gene at the transcriptional level to control bud outgrowth [48]. In our study, we also determined the expression levels of *PcSCL18* and *PcTB1*, which are orthologous genes of *LAX1* in rice and *BRC1* in *Arabidopsis*, using qPCR. Consistent with the RNA-Seq data, qPCR analysis showed that the expression level of *PcSCL18* was not significantly altered by exogenous BAP treatment. However, the expression level of *PcTB1* was significantly down-regulated at the S1 stage. This suggests that the promotion of tiller production by BAP is mainly due to its effect on promoting tiller bud outgrowth rather than bud initiation in *P. callosum*.

Effects of BAP on endogenous hormone content and the expression of genes related to hormone metabolism and transduction

The levels of free active compounds (iP, tZ, and cZ) and their glycosylated forms (tZOG, iP9G, and cZ9G) were found to decrease 30 d after exogenous BAP treatment, while the levels of BAP, oT, pT, and their glycosylated forms (K9G and BAP7G) increased. In Arabidopsis thaliana, exogenous BAP may inhibit the biosynthesis of endogenous cytokinins and actively promote their degradation through a feedback mechanism [49]. In our results, the expression level of IPT1 was significantly downregulated at the S3 stage, and most members of the CKX gene family exhibited up-regulation in response to BAP treatment. This may have led to the observed decrease in the levels of these free hormones and their glycosylated forms.

ABA is a key phytohormone that inhibits bud outgrowth by regulating bud dormancy [50]. Overexpression of the ABA degrading gene *CYP707A* has been

shown to reduce the content of endogenous ABA [62], breaking bud dormancy and promoting their growth and development in pear and grapevine [51, 52]. The endogenous content of ABA was significantly reduced in the stem of *P. callosum* with exogenous BAP application (Fig. 3). The reduction in ABA level corresponds to the up-regulation of the ABA catabolism gene Pc*CYP707A* at S1, supporting the role of ABA as a negative regulator of bud outgrowth in *P. callosum*. In rose, researchers have also observed an antagonistic interaction between CTKs and ABA in regulating tiller development. However, the underlying mechanism of how ABA and CTKs interact to control bud outgrowth is not well understood [53]. While, in *Arabidopsis*, it has been reported that the interaction between ABA and CTKs during seed germination is mediated by A-type transcriptional regulators, such as ARR4, ARR5 and ARR6, which could block the ABA-induced expression of ABI5 or other ABRE-binding factors (ABFs) [54]. In *P. callosum* subjected to BAP application at S1, 9 homologs of A-type *RRs* genes exhibited up-regulation, while one gene for *ABF2* showed down-regulation, suggesting a similar mechanism might exist.

Auxin is well known for inhibiting the outgrowth of axillary buds [55]. However, recently studies have shown that exporting auxin from dormant bud to the main stem by establishing a polar auxin transport (PAT) stream is crucial for sustained tiller bud outgrowth [44]. CTKs are considered the main regulators of this process as they can trigger auxin canalization and establish the PAT stream. In pea, direct application of BAP increased the expression of *PsAUX1* and *PsPIN1*, and induced polarization of the PsPIN1 protein, ultimately promoting bud outgrowth [56] In *Arabidopsis*, exogenous CTKs treatment was found to enhance the accumulation of auxin transport proteins PIN3, PIN4 and PIN7 in the main stem, leading to increased branching [14]. In *P. callosum*, we also found up-regulation of the IAA synthesis gene *YUC2* (BMK_Unigene_180410) and the transport gene *PINIC* (BMK_Unigene_391062) following BAP treatment, and the IAA level was significantly increased in the stem at S1. This finding is consistent with the importance of auxin-cytokinin crosstalk in the regulation of bud outgrowth.

Comparative study on gene expression of bud dormancy and sustained outgrowth between *P. callosum* and *P.* 'SCBG Yingchun'

The DRM1/ARP gene family is a unique and highly conserved protein found in higher plants, which plays an important role in maintaining bud dormancy and serves as a good dormancy marker [36]. Studies on wheat *tin* mutants have shown that increased expression of the *DRM1/ARP* gene inhibits elongation and outgrowth of tiller buds, resulting in reduced tillering [57]. The relative expression level of *DRMH1* gene in *P. callosum* in the untreated group was more than 7 times that of *P.* 'SCBG Yingchun', where the tiller buds did not require dormancy. Exogenous BAP treatment significantly down-regulated the *DRMH1* gene in *P. callosum*. These results indicated that the tiller buds of *P. callosum* did enter a dormant state when they matured, and that CTKs could break their dormancy. The DAM (DORMANCY-ASSOCIATED MADS-BOX) transcription factor belongs to the MIKC^{C}-type MADS-box family and is a key factor regulating the periodic dormancy of perennial plants [37, 58]. The *DAM* gene is highly homologous to the *SVP* gene and *AGL24* (*AGAMOUS-LIKE 24*) gene in *Arabidopsis.* In pear, it has been found that the expression of *DAM* genes is regulated by endogenous ABA. After long-term low temperature accumulation, the expression of ABA metabolism gene *PpyCYP707A3* was continuously up-regulated, leading to a decrease in ABA content. At this stage, the downstream response protein PpyABF3 of ABA is quickly degraded through 26 S proteasome and other pathways, thereby down-regulating the expression of *PpyDAM3* and releasing dormancy [51]. No homologous genes of *DAM* have been found in *P. callosum*, but one *SVP* (BMK_Unigene_347994) gene was significantly down-regulated after exogenous BAP treatment. The gene was also significantly down-regulated at the S1 stage of outgrowth of mature tiller buds of *P.* 'SCBG Yingchun', indicating that it may have a similar function as the DAM gene as a negative regulator of tiller bud outgrowth. In the genome analysis of *Cymbidium sinense*, it was found that it has not evolved the *DAM* gene, which regulates dormancy in perennial woody plants, and instead the *SVP* gene plays a major role [59].

Conclusions

The promotion of tillering by BAP in *P. callosum* may involve interactions with ABA and IAA, as evidenced by the decreased ABA content and up-regulation of the ABA degradation genes *PcCYP707A*, as well as the increased IAA content and up-regulation of the IAA biosynthesis gene *PcYUC2* in BAP-treated plants. Additionally, BAP may down-regulate the expression of dormancy genes *PcDRM1* and *PcSVP*, reactivating the tiller bud, and up-regulate the expression of the IAA transport gene *PcPIN1C* to support the sustained outgrowth of the tiller bud in *P. callosum*.

Materials and methods

Plant materials, growth conditions, and BAP treatment

The *P. callosum* and *P.* 'SCBG Yingchun' plants were maintained in a greenhouse at the South China Botanical Garden in Guangzhou, China. The seed parent and pollen parent of *P.* 'SCBG Yingchun' were *P. spicerianum* and *P. villosum var.annamense.* The *P. callosum* seedlings

were potted in a substrate of Zhijing stone suitable for orchids (Northridge Enterprise Co. Ltd, Taiwan, China), while the *P.* 'SCBG Yingchun' seedlings were potted in a mixed medium consisting of Zhijing stone for orchids and shattered fir bark in a ratio of 3:1 (v/v). The plants received natural light with an intensity of no more than 800 μmol m^{-2} s^{-1} (controlled by a sunshade net). The average temperature in the greenhouse ranged from 10 to 32 °C, and the relative humidity was maintained between 70% and 98%.

For this study, we employed a total of 225 *P. callosum* plants that were all uniformly grown and consisting of 5–8 leaves each. The plants were randomly divided into five groups, with 45 plants per group ($n = 45$). Each plant was placed in an individual pot, and sets of 15 pots were arranged on brackets. The BAP (Macklin, Shanghai, China) was dissolved in 1 M NaOH. To investigate the effects of different BAP concentrations on tiller development, the plants were root irrigated with 100, 200, 400, and 1000 mg/L BAP once every two weeks. The control plants were treated with distilled water. For each treatment, a 5 L BAP solution with the respective concentration was prepared in a 40 cm × 70 cm square plastic box. Each group of plants on the brackets was sequentially placed in the corresponding concentration of the solution, ensuring that the solution level reached just above the plastic pots, and each soaking lasted for 3 min.

Measurement of morphological characteristics and histological observations

Tiller bud number and the length of the first tiller bud were measured in both the BAP-treated and control plants. Each measurement was conducted in triplicate. To observe the development of primary tillers at the shoot apex of the main stem in *Paphiopedilum* plants, we collected the terminal buds of *P. callosum* and *P.* 'SCBG Yingchun' every month from May to December 2021. Paraffin sectioning was performed to prepare the samples for histological observations. The buds were immersed in formaldehyde-acetic acid-alcohol solution (FAA) [65]. A vacuum pump was employed to ensure that the materials were fully submerged in the liquid. After fixation for 24 h, the samples underwent dehydration through a graduated series of alcohol concentrations (70%, 83%, 95%, and twice in 100%; v/v), each for 30 min. Subsequently, the samples were immersed in a series of ethanol-xylene mixtures (2:1, 1:1, and 1:2; v/v) for 10 min, followed by immersion in xylene solution for 20 min. Paraffin was gradually added to the xylene solution and allowed to dissolve slowly at a constant temperature of 42 °C in a metal bath overnight. The temperature was then adjusted to 60 °C to ensure the complete dissolution of the paraffin. Following this, the xylene-paraffin mixture was replaced with melted paraffin three times, each for a duration of 3 h. The resulting wax blocks were sectioned into 8 μm slices using a microtome (RM2016, Leica). The sections were observed and photographed under biological microscope (Niikon, E200).

Measuring levels of endogenous plant hormones

For the extraction of IAA, ABA, GAs and CTKs, fresh main stem tissues were collected from plants treated with 400 mg/L BAP treatment for 30 d and from those in the control group. Each treatment consisted of three biological replicates, with each replicate weighing 0.6 g, composed of the main stems from 3 to 5 plants. Supplementary Data Figure S3 a–b showed the samples collected for hormone analysis. The samples were ground in liquid nitrogen and then extracted with 1 mL of a methanol/water/formic acid solution (15:4:1, V/V/V). After evaporation, reconstitution, and filtration, the sample extracts were analyzed using a UPLC-ESI-MS/MS system (UPLC, ExionLC™ AD; MS, Applied Biosystems 6500 Triple Quadrupole) by MetWare. The results were reported as the means of three replicates. Method validation included linearity, precision, accuracy, and reproducibility, with standard curves showing high correlation coefficients ($R^2 > 0.99$). Limits of detection (LOD) and quantification (LOQ) were determined for each hormone. Internal standards, sourced from Olchemim Ltd. (Czech Republic) and isoReag (Shanghai, China) with >98% purity, were used to ensure accuracy and precision in quantification (Supplementary Data Table S1).

RNA isolation and cDNA library construction

RNA was extracted from samples of *P. callosum* collected at 10 a.m. on days 30, 60, and 100 after 400 mg/L BAP treatment. The morphology of these collected main stems is detailed in Supplementary Data Figure S3 a–c. RNA was extracted from samples of *P.* 'SCBG Yingchun' at four corresponding developmental stages, with the morphology of these stems detailed in Supplementary Data Figure S3 e–h. The extraction was performed using RNAiso Plus (TaKaRa, Dalian, China), strictly adhering to the manufacturer's instructions. To prevent genomic DNA contamination, RNase-free DNaseI (TaKaRa, Dalian, China) was used. Three biological replicates were collected for each developmental stage in both species. The extracted RNA was assessed for quality using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA), while the quantity was determined using a NanoDrop ND2000 spectrophotometer (NanoDrop Thermo Scientific, Wilmington, DE, USA). Subsequently, we generated 12 normalized cDNA libraries of *P. callosum* using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations.

Transcriptome sequencing, unigene annotation and differentially expressed gene analysis

The cDNA libraries of *P. callosum* were sequenced by Illumina Solexa HiSeq 2000 sequencing system (Biomarker Technologies Co., China). Raw data is filtered using fastp software to remove reads with low-quality and contaminated adapters. The remaining high-quality data were used to construct unigenes on the Trinity software platform, using default parameter settings [60, 63]. For functional annotations and classification, the unigene sequences were subjected to BLASTX (http://blast.ncbi.nlmm.nih.gov/Blast.cgi) search in eight public databases: NR (National Center for Biotechnology Information (NCBI) non-redundant protein database), Swiss-Prot, COG (Clusters of Orthologous Groups), KOG (euKaryotic Orthologous Groups), eggNOG, Pfam (Protein family), GO (Gene Ontology), and KEGG (Kyoto Encyclopedia of Genes and Genomes).

The obtained reads from sequencing were aligned with the Unigene library using the Bowtie software (http://bowtie-bio.sourceforge.net/index.shtml). Based on the alignment information, the expression level was estimated using RSEM (http://deweylab.github.io/RSEM/). The reads per kilobase per million mapped reads (FPKM) method was employed to calculate unigene expression. To identify differentially expressed genes (DEGs) between libraries, we utilized the DESeq2 tool was utilized (http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html). DEGs were defined as unigenes with a False Discovery Rate (FDR) < 0.01 and a fold change ≥ 2. The resulting DEGs underwent GO enrichment and KEGG enrichment analyses to gain insights into their functional annotations and pathways.

Verification of gene expression using qRT-PCR

The 12 RNA-Seq samples of *P. callosum*, 12 RNA samples from four stages of untreated plants of *P. callosum*, and 12 RNA samples from four stages of *P.* 'SCBG Yingchun' were subjected to reverse transcription into cDNA using the One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen, Beijing, China) [63]. Five housekeeping genes (*ASHH2*, *UBC23*, *APRL3*, *ALDH7B4*, *SPT6*) were selected as appropriate reference genes in the four development stages and three treatment groups between two species (BAP-treated *P. callosum* × 3 biological replicates × 4 developmental stages, control *P. callosum* × 3 biological replicates × 4 developmental stages, control *P.* 'SCBG Yingchun' × 3 biological replicates × 4 developmental stages). The candidate genes showed a mean threshold (CT) ranging from 19.92 to 24.59 (Supplementary Data Table S9). Among the five candidate reference genes, SPT6 (BMK_Unigene_064712) exhibited the highest stability, and thus it was selected for the formal analysis in this study. Gene-specific primers for qRT-PCR were designed using Primer 5.0 and can be found in Supplementary Data Table S10. Relative expression levels were calculated using the $2^{\Delta\Delta CT}$ method [61, 64].

Abbreviations

BAP	Benzylaminopurine
IAA	Indole-3-acetic acid
ABA	Abscisic acid
CTKs	Cytokinins
GA	Gibberellin
cZ	Cis-Zeatin
iP	N6-isopentenyladenine
tZ	Trans-Zeatin
oT	Ortho-Topolin
HPLC	High performance liquid chromatography
IPT	Adenosine phosphate-isopentenyltransferase
CKX	Cytokinin oxidase
CYP735A	Cytochrome P450 monooxygenase
LOG	Riboside 5'-monophosphate phosphoribohydrolase
ZEP	Zeaxanthin epoxidase
NCED	9-cis-epoxycarotenoid dioxygenase
PP2C	Phosphoprotein phosphatase 2 C
ABFs	ABRE-binding factors
SnRKs	SNF1-related protein kinases
AXR1-AFB	Auxin resistance protein 1-auxin signalling f-box protein
DAO	Dioxygenase of auxin oxidation
ABCB	ATP-binding cassette subfamily B
AUX1/LAX	Auxin resistant1/like auxin resistant 1
PILS	Pin-likes
YUCCA	Flavin monooxygenase
PIN	Pin-formed
ARF	Auxin response factor
SAUR	Small Auxin Up RNA
Aux/IAA	Auxin/indole-3acetic acid
GH3	Gretchen Hagen3
DRMH	Dormancy-associated gene
SVP	Short vegetative phase gene
RNA-seq	RNA sequencing
PCA	Principal component analysis
DEGs	Differentially expressed genes
FPKM	Per kilobase per million mapped reads
FDR	False Discovery Rate
KEGG	Kyoto Encyclopedia of Genes and Genomes
eggNOG	Orthologous groups of genes
qRT-PCR	Quantitative Real-Time PCR
CT	Cycle threshold

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06256-9 .

Supplementary Material 1

Supplementary Material 2

Acknowledgements

The authors would like to thank Raozhen Jia for assistance with graphics production.

Author contributions

Y.Y.Y., L.F. and S.J.Z. conceived, designed, and assessed the experiments; R.H.Z., Y.F.L. and B.Y.G. performed the experiments; L.L., K.L.W. and G.H.M. analyzed the data; Y.Y.Y., L.F. and S.J.Z wrote the manuscript. All authors reviewed the manuscript, approved all edits in the final version and take public responsibility for the content.

Funding
This work was funded by Guangdong Basic and Applied Basic Research Foundation (2024A1515012952), Guangdong Science and Technology Plan Project(2023B1212060046), Science and Technology Projects in Guangzhou (E33309), Guangzhou Science and Technology Program (2023E04J0004, 2024B03J1129), Guangzhou Agricultural Research Program (23106468), Linzhi Science and Technology Program (2023-QYCX-02), and Forestry Grassland Ecological Protection and Restoration Program (E436070011).

Data availability
The raw data of the 12 samples used in the present study were submitted to the NCBI Short Read Archive (SRA) under the BioProject accession number PRJNA877420.

Declarations

Ethics approval and consent to participate
The collection of plant materials used in this study complied with institutional and national guidelines. The field studies were conducted in accordance with local legislation.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 9 November 2023 / Accepted: 13 February 2025
Published online: 24 February 2025

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