

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

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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 400mg/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*. These findings provide insights into the regulatory mechanisms of BAP in tiller bud outgrowth in *P. callosum*.

Conclusions:

Our results indicated that the application of BAP promotes lateral bud outgrowth and increases tiller production in *P. callosum*. HPLC results showed exogenous BAP affects the levels of endogenous hormones, including IAA, CTKs, and ABA. Transcriptomic data combined with qPCR analyses suggest that the genes *PcCYP707*, *PcYUC2*, *PcPINIC*, *PcDRMH1*, *PcSVP* may play key roles in regulating tiller bud outgrowth. This study presents the first comprehensive report of BAP-promoted tillering in *P. callosum*, which should provide a foundational basis for further mechanistic studies on tiller development in *Paphiopedilum* species and other non-model plants.

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. dianthus* 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 Fig. 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 Fig. 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 axil 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 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. 1 marked in pink) changed from vegetative growth to reproductive growth and bolting, the mature tiller buds began to outgrow, 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. <link rid="fig2">2</link>d-2). Tiller buds continue their sustained outgrowth, following the development of new leaves, ultimately becoming new tiller shoots (Fig. 2d-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 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-<link rid="fig3">3</link>, c-<link rid="fig3">3</link>).

Effects of 400mg/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 400mg/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).

The content of ABA, GA₇, 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.

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

Based on the morphological characterization of tiller buds under 400mg/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 S1. 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 S2). Among these unigenes, 36,408 (66.53%) were annotated with at least one putative function in publicly available databases (Supplementary Data Table S3). Samples were separately clustered based on the developmental stages in the principal component analysis (PCA) (Supplementary Data Fig. 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 Fig. S2b).

Differentially expressed genes (DEGs) were detected using a screening criteria of False Discovery Rate (FDR) < 0.01 and log2 ratio $\geq$ 1.0 (Fold Change $\geq$ 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 (Fig. 4b), and 3797 DEGs were expressed in at least one comparison group. The expression patterns of these 3797 DEGs were distinct among the four stages (Fig. 4c). K-means clustering analysis revealed 15 distinct clusters, named K1-K15 (Fig. 4d, Supplementary Data Table S4), which provided insights into the potential roles of the DEGs. The highly expressed genes in S0 (represented by K1-K4), S1 (represented by K5-K8), S2 (represented by K9-K12), and S3 (represented by K13-K15) were primarily involved in DNA binding, ribosomes, starch and sucrose metabolism, and photosynthesis, respectively.

KEGG enrichment analysis of differentially expressed genes

KEGG enrichment analysis was conducted on the three comparison groups. In the S0 versus S1 group, the top-enriched KEGG pathways included ribosome, DNA replication, cutin, suberine and wax biosynthesis, and zeatin biosynthesis (Fig. 5a). Highly enriched pathways in the S1 versus S2 group were photosynthesis-antenna proteins, DNA replication, carotenoid biosynthesis, and circadian rhythm - plant (Fig. 5b). Highly enriched pathways in the S2 versus S3 group were cutin, suberine and wax biosynthesis, phenylpropanoid biosynthesis, DNA replication, and tyrosine metabolism (Fig. 5c).

These results indicate that after BAP treatment, the plant hormone signaling transduction, ribosome, and DNA replication pathways are activated in the early stage (S1), enhancing the plant's activity, which may promote the activation of axillary buds. In the mid-stage (S2), genes related to starch and sucrose metabolism, Nitrogen metabolism, and Fatty acid degradation were significantly enriched, suggesting that sugars, nitrogen, and fatty acid may be involved in the process of tiller bud breakthrough from the mother stem and further elongation in *P. callosum*. As the tiller buds enter the S3 stage, they require more energy for sustained growth, leading to the significant enrichment of pathways related to photosynthesis, photosynthesis-antenna proteins, and phenylpropanoid biosynthesis.

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 S5). 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. 7a and Supplementary Data Table S6). 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 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 RESISTANT1* (*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. 7b and Supplementary Data Table S6).

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

Unlike *P. callosum*, *P. 'SCBG Yingchun'* exhibits stronger tillering ability, where the outgrowth of tiller buds is not inhibited by the main shoot. When the main shoot grows to 5 leaves, the tiller buds can protrude from the main shoot and develop into normal tillers without the need for the main shoot to bolt or exogenous BAP treatment (Fig. 1). Figure 8 shows the continuous processes of the growth of the primary tiller bud in *P. 'SCBG Yingchun'*. Similar to *P. callosum* treated with 400 mg/L BAP, the growth of the tiller buds in *P. 'SCBG Yingchun'* can also be divided into four corresponding stages.

To investigate the expression of key genes during this process, we selected several DEGs that may be related to tiller bud outgrowth through in-depth analysis of transcriptome data. qRT-PCR was conducted in this study. *Dormancy-associated gene-1 (DRM1)* and *dormancy-associated MADS-box/SHORT VEGETATIVE PHASE-like genes (DAM/SVP)*, known as negative regulators for axillary bud outgrowth and branching [35, 36]. As shown in Fig. 8, 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* showed more than 7-fold lower than that in 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 below the initial level at S0 but rose dramatically after BAP treatment, while the expression level of *PcCYP707A* in control plants of *P. callosum* and *P. 'SCBG Yingchun'* did not show significant differences during stage 0–3. The auxin transporter gene *PcPIN1* exhibited a significantly up-regulated expression pattern during the tiller

outgrowth process in BAP-treated *P. callosum* and *P.* 'SCBG Yingchun', compared with the control plants of. 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 related to endogenous hormone and sugar synthesis, metabolism and transport, as well as axillary bud formation and maintenance, and verified the accuracy of transcriptome data by qRT-PCR (Fig. 9). Consistent with the transcriptome data, qPCR showed that the expression levels of two genes involved in SLs biosynthesis and signaling (*CCD8B*, *D14*), and 3 genes involved in sugar biosynthesis and transporter (*TPS7*, *TPP6*, *STP7*) were the highest in S2 stage among the four tiller bud development stages. We also used qPCR to evaluate the expression profiles of 4 genes involved in CTKs biosynthesis and catabolism (*LOG3*, *CKX10*, *CKX5*, *RR2*) and 3 genes involved in auxin biosynthesis and transport (*YUC2*, *PIN1C*, *ABCB19*). Two CTKs catabolism genes (*CKX5* and *CKX10*) and one gene *RR2*, which is a negative regulator of CTKs signaling pathway, showed significant up-regulation after 400mg/L BAP treatment. one IAA synthesis gene and two transport genes showed the same trend. The expression of *LOG3* increased first, then decreased and then increased in the four stages of tiller growth under 400mg/L exogenous BAP treatment. *SCL18* (BMK_Unigene_356084) is a gene related to axillary bud formation, and the relative expression of qRT-PCR showed no significant difference in the four stages of tiller bud. The relative qRT-PCR expression of the gene *TB1* (BMK_Unigene_308669), 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 FKPM 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 [5]. In our previous study, exogenous spraying and injection of BAP at different concentrations (100, 200, 400 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. 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 [37]. 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 [38]. 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 [39, 40]. Additionally, *Teosinte Branched1* (*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 [41]. In *Arabidopsis*, exogenous CTKs treatment could down-regulate the *BRC1* gene at the transcriptional level to control bud outgrowth [42]. In our study, we also determined the expression levels of *PcSCL18* (BMK_Unigene_356084) and *PcTB1* (BMK_Unigene_308669), 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

ABA is a key phytohormone that inhibits bud outgrowth by regulating bud dormancy [43]. Overexpression of the ABA degrading gene *CYP707A* has been shown to reduce the content of endogenous ABA, breaking bud dormancy and promoting their growth and development in pear and grapevine [44, 45]. 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 *PcCYP707A* (BMK_Unigene_370776) 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 [46]. 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) [47]. 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 [48]. 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 [38]. 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 [49]. 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 [50]. In *P. callosum*, we also found up-regulation of the IAA synthesis

gene *YUC2* (BMK_Unigene_180410) and the transport gene *PIN1C* (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 [35]. 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 [51]. 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 MIKCC-type MADS-box family and is a key factor regulating the periodic dormancy of perennial plants [36, 52]. 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 26S proteasome and other pathways, thereby down-regulating the expression of *PpyDAM3* and releasing dormancy [53]. 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 [54].

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 $800 \mu\text{mol m}^{-2} \text{s}^{-1}$ (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 2 weeks. The control plants were treated with distilled water. For each treatment, a 5L 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 minutes.

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, following the procedures described in [55].

Measuring levels of endogenous plant hormones

For the extraction of IAA, ABA, GA, and CTKs, 0.6 g of fresh main stem tissue was collected from the plants treated with 400 mg/L BAP for 30 d, as well as the control group. 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.

RNA isolation and cDNA library construction

RNA was extracted from samples of *P. callosum* collected at 0 d, 30 d, 60 d, and 100 d of treatment under 400 mg/L BAP, as well as the samples of four corresponding development stages of *P.* 'SCBG Yingchun' using RNAiso Plus (TaKaRa, Dalian, China) following 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). The raw data underwent filtering to remove reads with low quality and contaminated adaptors. The remaining high-quality data were used to construct unigenes on the Trinity software platform, using default parameter settings [56]. For functional annotations and classification, the unigene sequences were subjected to BLASTX (<http://blast.ncbi.nlm.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 (RPKM) 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). 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* $\times$ 3 biological replicates $\times$ 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 S7). 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 S8. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [57].

Abbreviations

BAP: Benzylaminopurine; IAA: Indole-3-acetic acid; ABA: Absciscic acid; CTKs: Cytokinins; GA: Gibberellin; HPLC: High performance liquid chromatography; RNA-seq: RNA sequencing; YUCCA: Flavin monooxygenase; PIN: PIN-FORMED; CYP707A: Cytochrome P450 monooxygenase; DRMH: Dormancy-associated gene; SVP: Short vegetative phase gene; IPT: adenosine phosphate-isopentenyltransferase; CKX: cytokinin oxidase; Cz: cis-Zeatin; IP: N6-isopentenyladenine; tZ: trans-Zeatin; oT: ortho-Topolin; 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; CT: Cycle threshold.

Declarations

Acknowledgements

Not applicable.

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., 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.

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Availability of data and materials

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.

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 conflicts of interest.

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Tables

Table 1 is not available with this version.

Figures

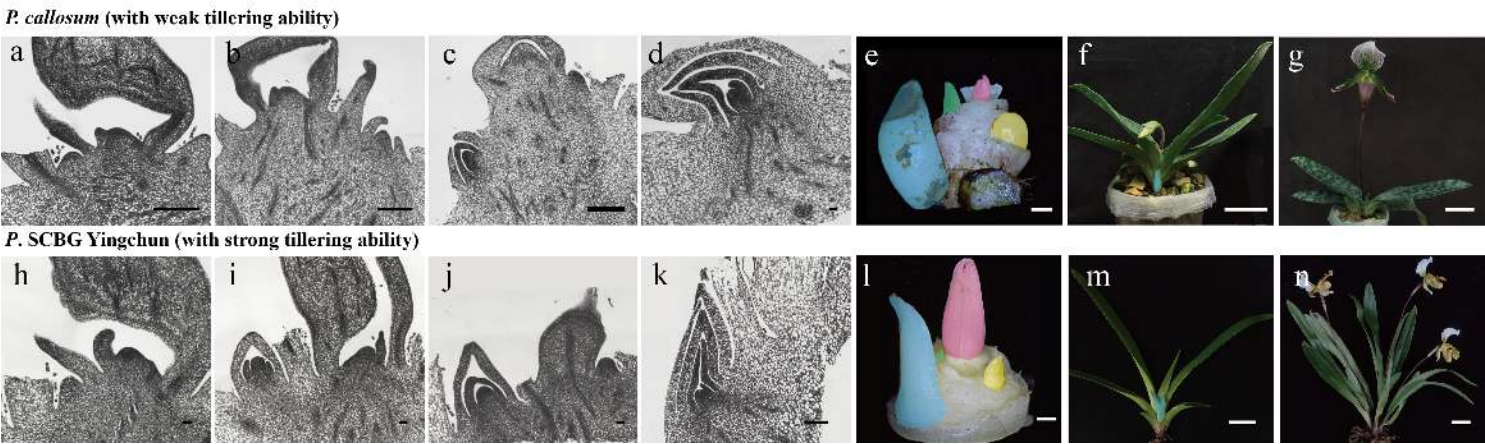


Figure 1

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 = 0.5mm; e and h-l=1 mm, f-g and m-n =5 cm.

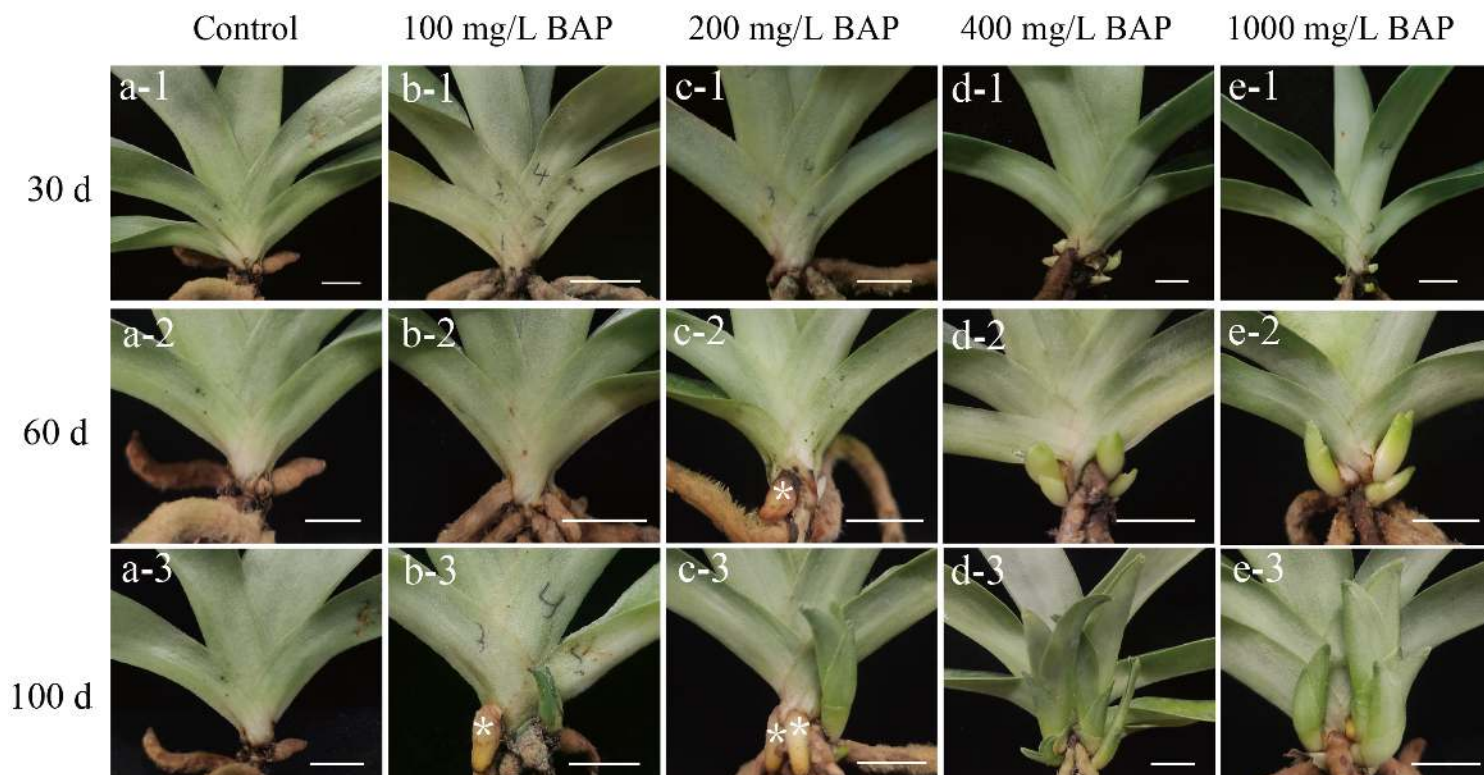


Figure 2

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=1cm.

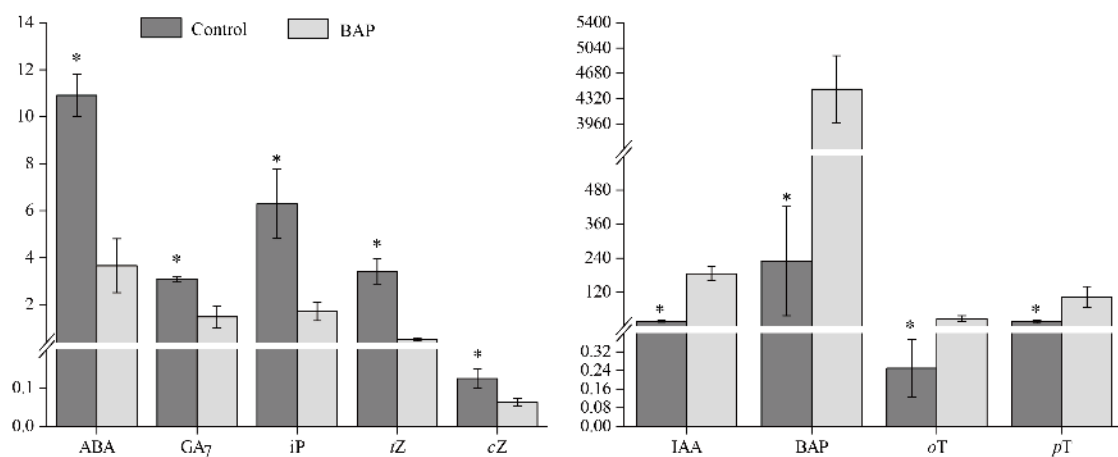


Figure 3

Measurement of IAA, ABA, GA₇, CTKs content at 30 d in control and BAP-treated *P. callosum*

The results represent the means $\pm$ 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, $P < 0.05$).

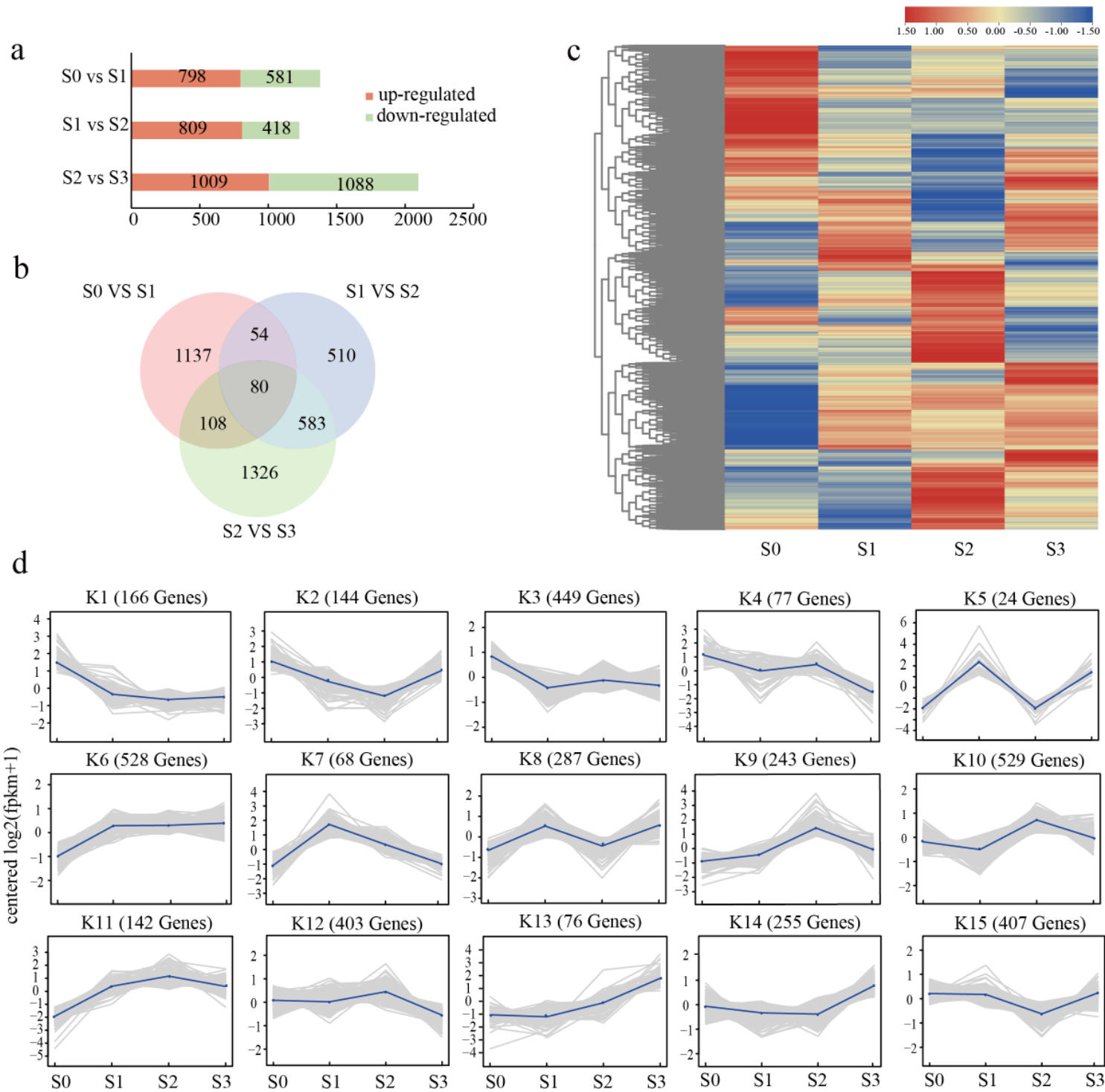


Figure 4

Global expression profile of 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) Heat map of 3797 DEGs from four developmental stages; (d) k-Means cluster analysis on DEGs. 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 log2 (FPKM+1; FPKM, fragments per kilobase of transcript per million mapped reads).

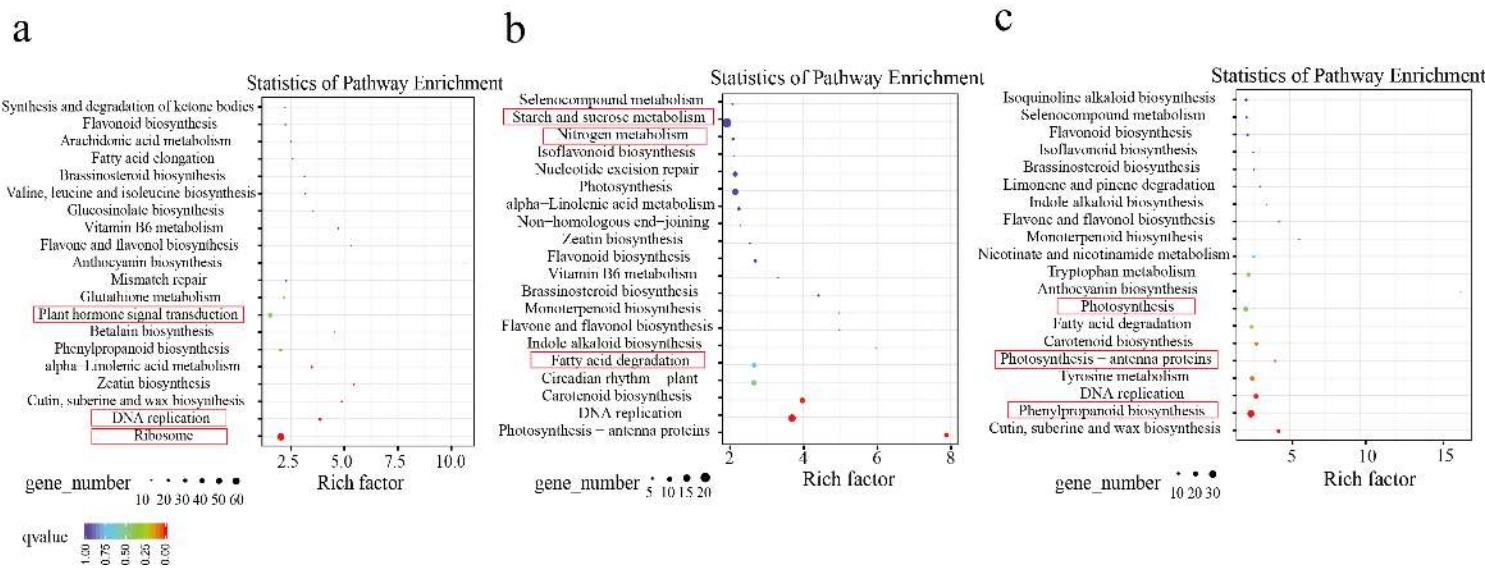


Figure 5

KEGG enrichment analysis of DEGs in three comparison groups

(a) KEGG enrichment analysis with the DEGs between S0 and S1; (b) KEGG enrichment analysis with the DEGs between S1 and S2; (c) KEGG enrichment analysis with the DEGs between S2 and S3

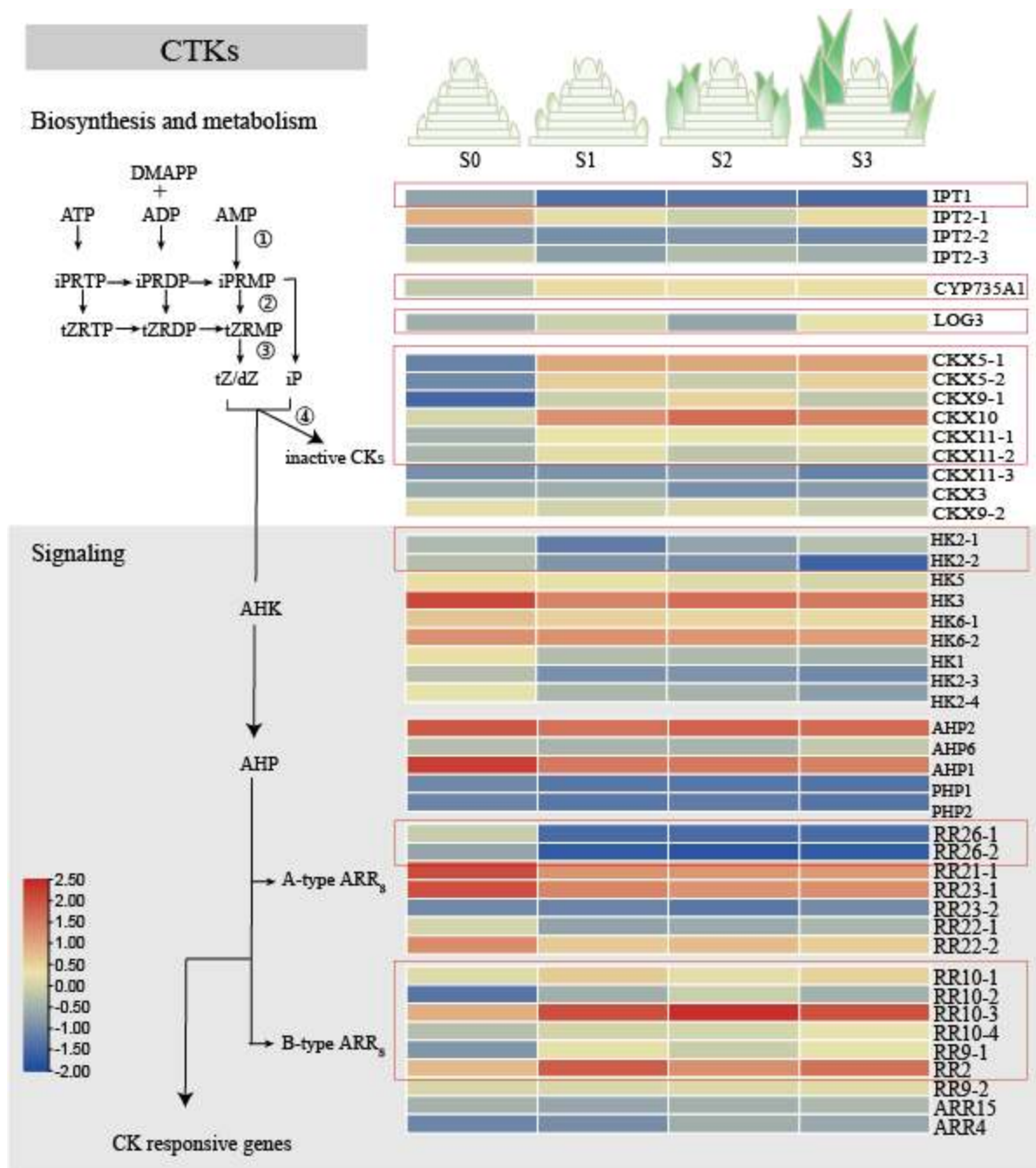


Figure 6

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 400mg/L BAP at 0d, 30d, 60d and 100d.

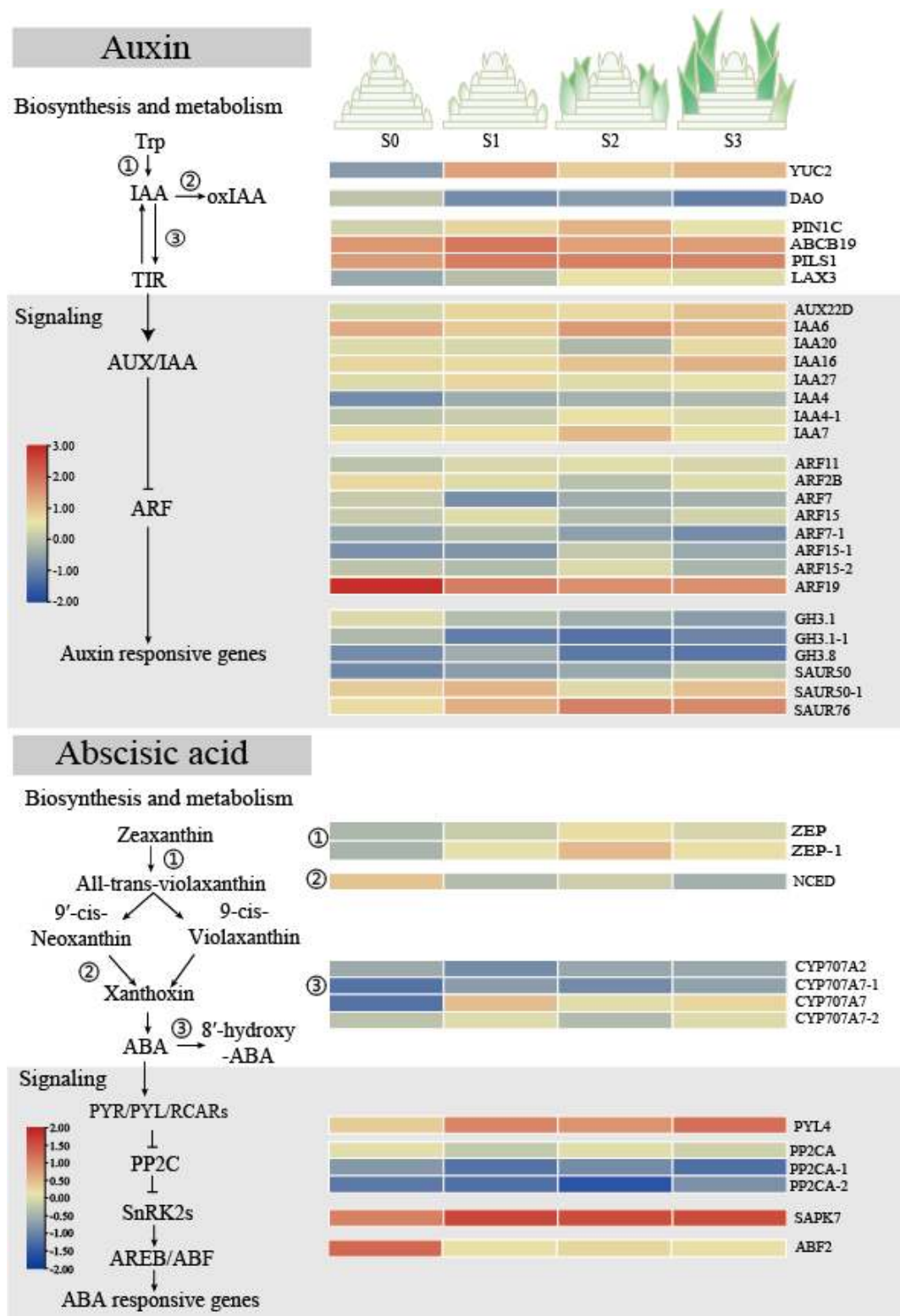


Figure 7

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 400mg/L BAP at 0d, 30d, 60d and 100d.

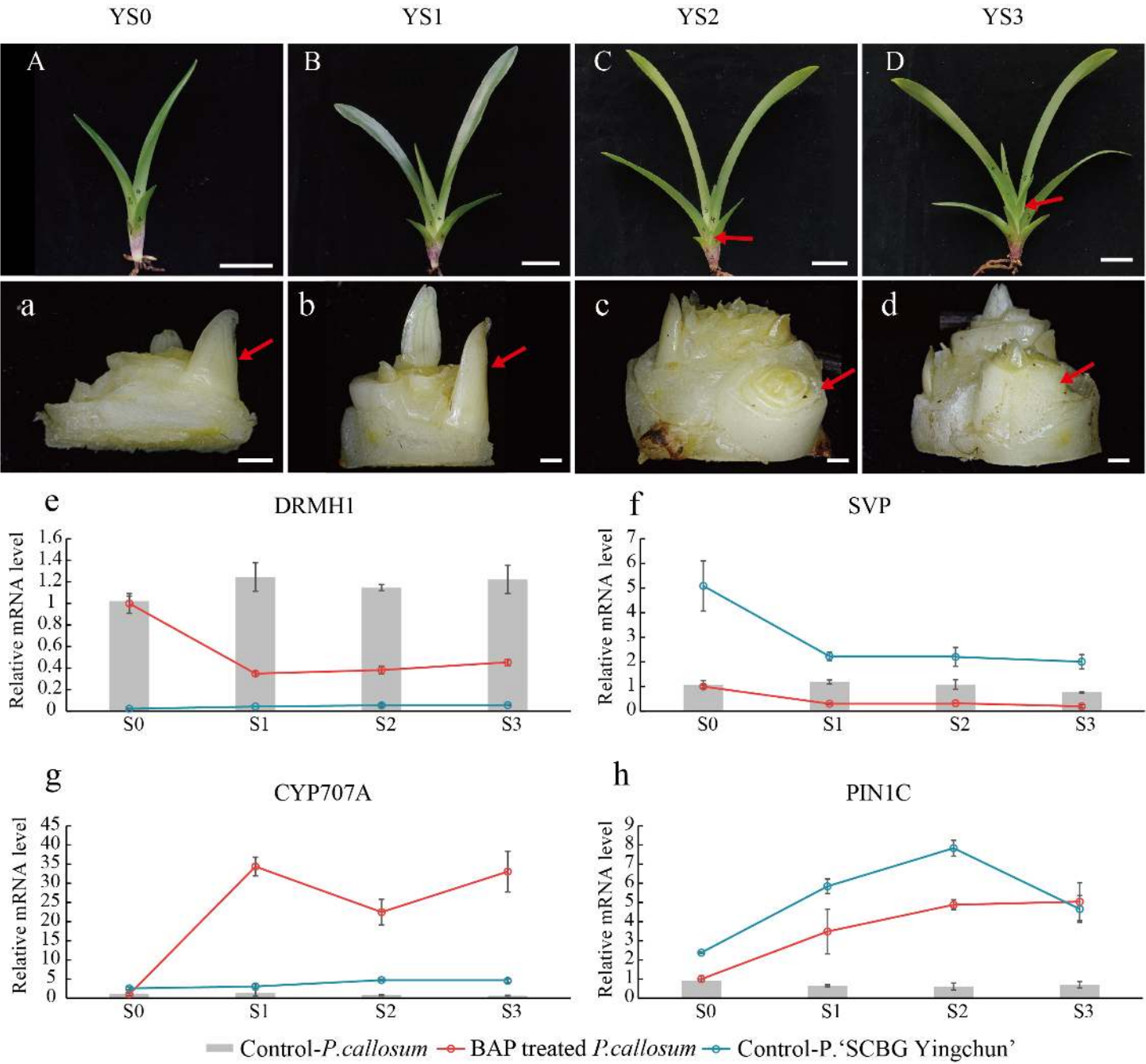


Figure 8

Comparative study on the expression patterns of tillering-related candidate genes in two *Paphiopedilum* plants

A-D: Four developing stages of the primary tiller bud on the main shoot in *P. 'SCBG Yingchun'*; Scale bars=5 cm. a-d: Observation of the main stem after peeling off all the leaves under a microscope; Scale bars = 0.1 cm. Stage 0 (YS0): the main shoot with four leaves (A), the primary tiller bud matured, forming an equilateral triangle (a); Stage 1 (YS1): the main shoot with five leaves (B), the primary tiller bud began

to outgrowth, forming an isosceles triangle (b); Stage 2 (YS2): the main shoot with five full mature leaves and the primary tiller bud protruding from the main shoot (C), the primary tiller bud with four leavesembraced (c); Stage 3 (YS0): the main shoot with six leaves and the primary tiller bud with several extended leaves (D), the growing primary tiller has formed its first tiller in leaf axil (d). e-h: 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 (3 plants for each replicate), with each replicate containing 4 technical replicates. Error bars represent the standard deviation of the mean; The arrow indicates the primary tiller bud of *P. 'SCBG Yingchun'*.

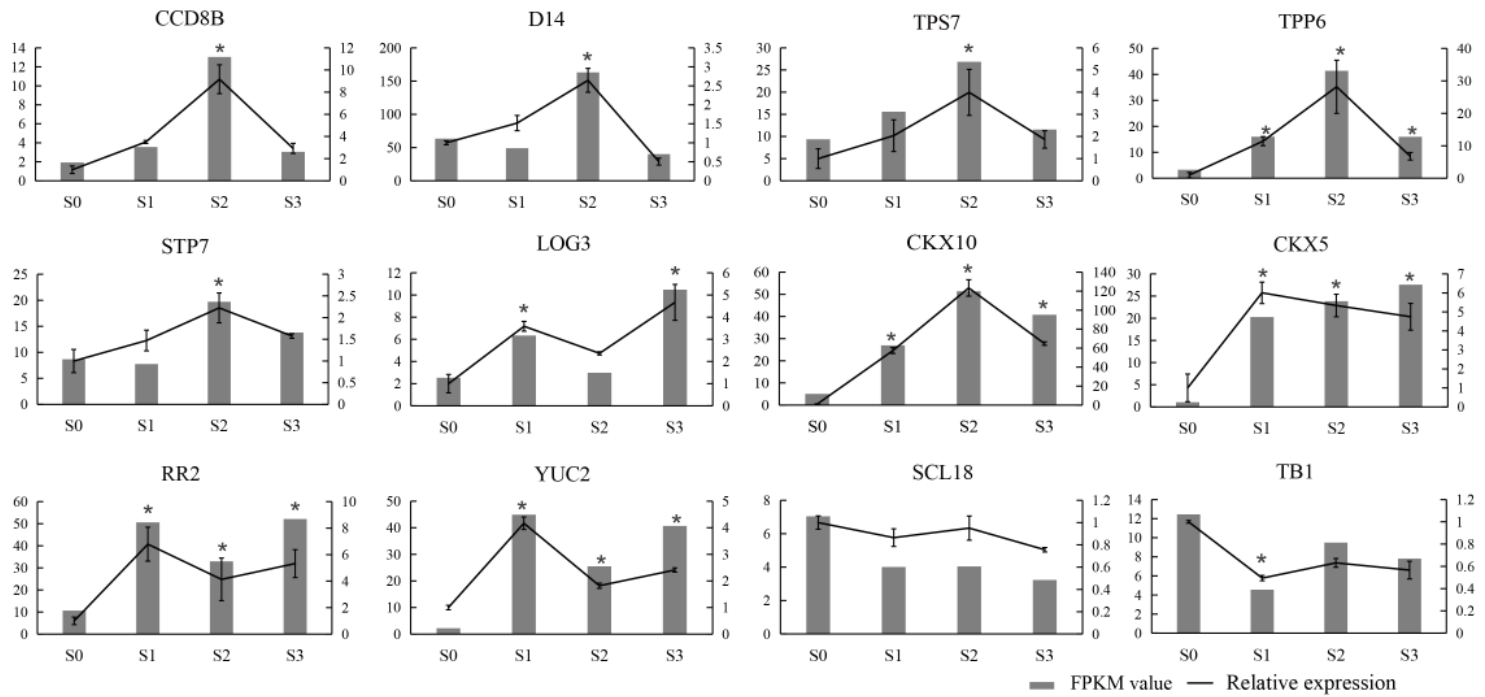


Figure 9

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 RPKM values, and the right y-axis represents the relative expression levels. Error bars indicate the standard deviation of the mean (n = 3). An asterisk indicates a significant difference from the S0 stage (Student's t-test, P < 0.05).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.xlsx](#)
- [SupplementaryinformationFigures.docx](#)

- [SupplementalTables.xlsx](#)