Industrial Crops & Products 167 (2021) 113511

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Gibberellin A3 induces polyaerial shoot formation and increases the propagation rate in *Paris polyphylla* rhizomes

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ARTICLE INFO

Keywords:
Paris polyphylla var. *yunnanensis*
Rhizome development
Rhizome cutting
Rhizome dormancy
Polyaerial shoot
Gibberellin

ABSTRACT

Paris polyphylla var. *yunnanensis* is a rhizomatous geophyte in high demand for industrial and medicinal purposes. It grows very slowly, and thus, rhizome cutting is a promising propagation technique. However, whether and how polyapical shoots can be artificially produced with rhizomes is unclear. This study reported that a *P. polyphylla* rhizome mainly sprouts one shoot but could occasionally grow polyapical shoots. Application of phytohormones, including auxins, cytokinins, abscisic acids and gibberellins on *P. polyphylla* rhizomes showed that GA_3 could break dormancy in two weeks and induced polyapical shoots at a rate of 100 %. The apical shoot number per GA_3-treated rhizome reached 7.2. The GA_3-induced occurrence of polyapical shoots derived from the repetitive *de novo* formation of apical buds. GA_3 application changed endogenous auxin, cytokinin or ABA concentrations slightly or not at all but dramatically increased endogenous GA_3 and GA_1 levels in rhizomes. Transcriptional profiling showed there were few shared differentially expressed genes and significant differences in the expression patterns of hormone-related genes between rhizomes with mono- and polyapical shoots. These results suggest that *P. polyphylla* has the genetic potential to produce polyapical shoots. GA_3 treatment can quickly break dormancy and strongly induce polyapical shoots in this species, providing an efficient rhizome cutting technique for *P. polyphylla* propagation.

1. Introduction

Regardless of in vivo or vitro conditions, the cutting of underground storage organs is a commonly used propagation technique to produce a large number of propagules in geophytes with industrial applications (Kamenetsky and Okubo, 2015). Cuttings with axillary meristems have a greater tendency to produce roots and stem shoots and eventually develop into intact plants. Thus, this method is highly efficient for propagating geophytes, particularly those that are slow to produce offsets (Kamenetsky and Okubo, 2015). However, the multiplication rate is mainly restricted by the size of the axillary meristems in the underground storage organs. For the propagation of rhizomatous species, the multiplication rate depends on the number of rhizome cuttings and thus depends on the number of aerial shoots in one rhizome. The regulation of aerial shoot numbers in geophytes is of great commercial interest.

Paris polyphylla var. *yunnanensis* is a rhizomatous geophyte and belongs to the family Melanthiaceae. Its rhizome is a famous traditional medicine resource in China (Nguyen et al., 2009; Qin et al., 2016) and is in increasing demands for industrial production of medicinal, healthcare and daily household-use products (Cunningham et al., 2018; Heng et al., 2015). The growth of its rhizome is extremely slow, and an average of 7–8 years is required before harvest. Propagation of this species from seeds is also time-consuming because the seeds have to experience two winters to break their "double dormancy" (Heng, 1998). The total time from seed germination to rhizome harvest takes as long as 10 years. Rhizome cutting has become an important way to propagate *P. polyphylla* in recent years because it not only saves time but also produces strong seedlings. However, how the occurrence of aerial shoots is limited and whether the aerial shoot number can be controlled in *P. polyphylla* is unknown.

P. polyphylla has a special annual developmental cycle to adapt to the long dry season from September to June that is unique to Southwest China (Yang et al., 2016). Its cycle includes three stages (Fig. 1). The first stage is "sprouting" in June when rain starts. The scale leaves of

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https://doi.org/10.1016/j.indcrop.2021.113511
Received 24 November 2020; Received in revised form 3 April 2021; Accepted 5 April 2021
Available online 15 April 2021

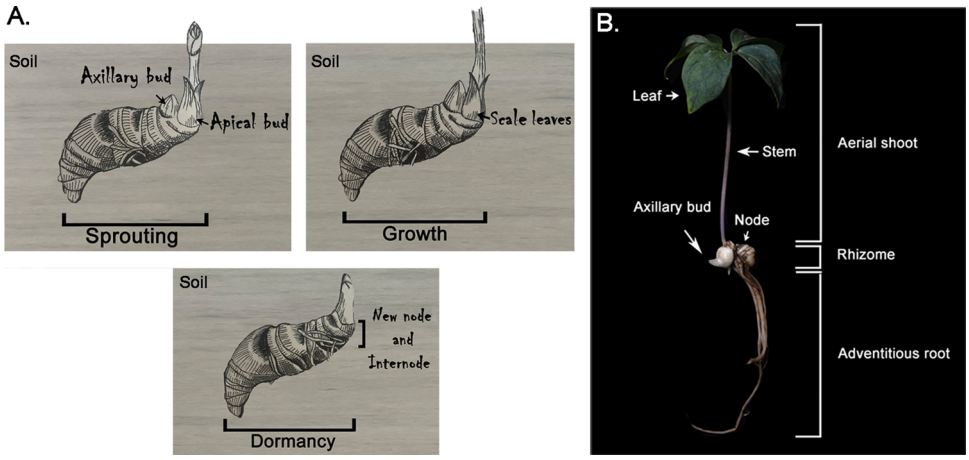

Fig. 1. Diagram of *P. polyphylla*. (A) Developmental cycle of a rhizome. (B) Structure of a whole plant.

P. polyphylla turn green, apical shoots start to elongate, and leaves differentiate and turn green. The resources for development were stored in the last cycle in the mother rhizome. The second stage is "growth" from June to September. Under appropriate hydrothermal conditions, apical leaves perform photosynthesis and make resources for rhizome storage, and consequently, the rhizome enlarges. The third stage is "dormancy" in September, when the environmental conditions become cold and dry. *P. polyphylla* aerial shoots die, and the rhizome gradually becomes dormant. The leaf primordium differentiates into apical bud and axillary bud. The mother rhizome forms new internode and node. The new internode becomes the daughter rhizome as a storage organ for the next cycle. Morphological observations of the developmental process suggest that apical buds form during rhizome dormancy. However, how their formation occurs and whether it can be regulated to produce multiple aerial shoots are still unclear.

The developmental cycle of geophytes is regulated by environmental signals and phytohormones (Kamenetsky and Okubo, 2015). The roles of phytohormones in dormancy regulation are well documented. Gibberellins and cytokinins generally function in breaking dormancy (Aksenova et al., 2013). Exogenous application of GA_3 broke corm dormancy and promoted shoot growth in gladiolus (Khan et al., 2013). After soaking in solutions containing 6-benzylaminopurine, rhizome dormancy was broken in *Zingiber zerumbet* (Goh et al., 2018). The combination of GA_3 with CK is necessary to break dormancy in potato (Hartmann et al., 2011). Abscisic acid inhibits dormancy breaks and antagonizes the effects of GA and CK. Hormones also regulate rhizome development. GA_3 is used to induce tillering in welsh onions (Yamazaki et al., 2015) and bud formation and an increase in clove number in garlic (Liu et al., 2019). GA and brassinolide positively regulate plant height (Tong et al., 2014), and their mixture promoted the growth of tulip shoots significantly more than GA alone (Wegrzynowicz-Lesiak, 2010). However, the effects of hormones on *P. polyphylla* rhizome development remain unknown.

The present study employed uniform *P. polyphylla* rhizomes grown with *in vitro* propagation to investigate the occurrence of aerial shoots, rhizome development, the effects of hormones on apical bud formation, and explored the transcriptional regulation of polyapical shoot occurrence. It revealed the developmental model of the *P. polyphylla* rhizome and found that GA_3 broke dormancy and induced polyshoots in a manner different from monoshoot induction.

2. Materials and methods

2.1. Plant materials and aerial shoot number statistics

The Kunming Institute of Botany, Chinese Academy of Sciences supplied *P. polyphylla* cultivars. "SL", "LC", and "MD" were from Shilin County (east to Yunnan Province), Lancang County (south Yunnan Province) and Mouding County (central Yunnan Province), respectively. The seedlings were all cultivated at 23 °C, 60 % relative humidity, and a light intensity of 300 μmol m^{-2} s^{-1}. The number of aerial shoots was counted in 50 randomly selected biennial plants from the SL, MD and LC cultivars, and this was repeated three times.

2.2. Phytohormone application

Sterilized SL biennial cultivars were used in this study and inoculated in 1/2 Murashige and Skoog (MS) medium containing 30 g/l sucrose, 3 g/l phytagel, and l g/L polyvinyl pyrrolidone in 480 mL plastic culture bottles supplemented with 1 mg/L indole-3-butyric acid (IBA), 0.2 mg/L kinetin (KT), 5 mg/L ABA (abscisic acid) or gibberellin A3 (GA_3) at different concentrations (5, 10, 20 mg/L). The hormone concentrations for dormancy break and polyaerial shoot induction were screened from pre-experiments after referring to previous reports (Hartmann et al., 2011; Suttle, 2004; Braide and Hamadina, 2018; Masuda et al., 2012; Uranbey et al., 2010). Half-strength MS medium was supplemented with IBA and KT before autoclaving. ABA and GA_3 were sterilized by sterile syringe filter (0.2 μm) under room temperature. The pH of the media was adjusted to 5.7–5.8 (using 1 M HCl or 1 M KOH) prior to autoclaving at 121 °C for 15 min. The cultures were maintained under 12/12 h day^{-1} (day/night) with 60 % relative humidity and a light intensity of 300 μmol $m^{-2}s^{-1}$. The temperature was 23 ± 2 °C. After 6 months of cultivation, the rate of sprouting, mean number of aerial shoots (ASs), fresh weight of rhizomes, number of adventitious roots (ARs) and fresh weight of ARs were measured. For the GA_3 treatment, the average number of AS events, maximum number of AS events, average AS length and maximum AS length were recorded 6 months after harvest.

Field tests were performed by transplanting rhizomes into breeding bags (18 cm height and 12 cm diameter) with soil (prepared with 25 % humus, 25 % peat and 50 % red soil). Plants were irrigated with 50 mL of water with or without GA_3 at the indicated concentration once a week

for 6 months. Twenty seedlings of each treatment were planted side-by-side in a greenhouse.

2.3. Paraffin sectioning

Dormant, mono- and polyaerial shoot plants were selected randomly, and the shoots were cut off from each rhizome for observation of the tissue structure. The shoots were fixed with FAA, dehydrated, waxed and embedded. The block was embedded with a Leica paraffin embedding machine (Leica, HistoCore Arcadia H). A paraffin slicer (Leica, RM2235) was used for cross-sectioning the swollen parts. The section thickness was approximately 5−10 μm. These sections were stained with safranine-fast green and sealed with neutral gum. Observations and imaging were performed with a microscope (Leica, DM5500B).

2.4. Hormone concentration analysis in P. polyphylla rhizomes by UHPLC-MS/MS

For analysis of hormone levels, the rhizomes of *P. polyphylla* in dormant and mono- and polyaerial shoot stages after 6 months of cultivation were collected and immediately ground into powder in liquid nitrogen and kept at −80 °C. Each sample was divided into 3 parts, one for hormone concentration analysis, one for RNA-Seq, and one for qRT-PCR. Then, a 25 mg aliquot of each individual sample was precisely weighed and transferred to an Eppendorf tube. After the addition of 1000 μL of extract solution (50 % acetonitrile in water, precooled at −40 °C, containing an isotopically labeled internal standard mixture), the samples were vortexed for 30 s, sonicated for 5 min in an ice-water bath, homogenized at 40 Hz for 4 min and sonicated for 5 min. The homogenization and sonication cycle were repeated twice. After centrifugation (10 min, 12,000 rpm, and 4 °C), an 800 μL aliquot of the supernatant was further purified with SPE. The SPE cartridges were washed with 1 mL of methanol and then equilibrated with 1 mL of 50 % ACN/H_2O (v/v). After loading the samples (supernatant obtained following the procedure described above), the flow-through fraction was discarded. The cartridge was then rinsed with 1 mL of 60 % ACN/H_2O (v/v). After this single-step SPE, the samples were evaporated to dryness under a gentle stream of nitrogen and reconstituted in 100 μL of 10 % ACN/H_2O (v/v). All the samples were vortexed for 30 s and sonicated for 5 min. After centrifugation (15 min, 12,000 rpm, and 4 °C), the clear supernatant was subjected to UHPLC-MS/MS analysis. Each treatment was replicated three times.

The auxins examined were methyl 3-indolylacetate, indole-3-acetic acid, indole-3-carboxaldehyde, and 3-indolebutyric acid (Fig. 5). The CTKs examined were N6-isopentenyladenosine, kinetin, trans-zeatin-riboside, N6-(delta 2-isopentenyl)-adenine, trans-zeatin, cis-zeatin, and DL-dihydrozeatin (Fig. 5). The GAs examined were gibberellin A1, gibberellin A3, gibberellin A4 and gibberellin A7 (Fig. 5). The contents of various hormones are shown in Table S1.

2.5. RNA isolation and RNA-Seq

Total RNA was extracted from rhizomes (three biological replicates) using TRIzol reagent (Tiangen, Beijing). The concentration of RNA was determined using a NanoDrop 2000 spectrophotometer, and its integrity was assessed on an Agilent 2100 Bioanalyzer. RNA degradation was monitored on agarose gels. Nine samples were all deemed high quality and used to construct transcriptome libraries.

Total mRNA was enriched using oligo (dT) magnetic beads. Taking these cleaved mRNA fragments as templates, first-strand cDNA was synthesized using random hexamers. Second-strand cDNA was then synthesized using DNA polymerase I, dNTPs, and RNaseH. These cDNA fragments were then subjected to end repair, poly(A) tailing was performed, and a ligation sequencing adapter was used. The required fragments were purified using agarose gel electrophoresis and purified via reagent. The recovered products were dissolved in EB solution. The Agilent 2100 Bioanalyzer and ABI Step One Plus Real-Time PCR System were used to evaluate the library quality. Nine libraries were sequenced using an Illumina NovaSeq 6000 by Wuhan Benagen Tech Solutions Company Limited.

2.6. RNA-Seq data analysis, de novo transcriptome assembly and annotation

Prior to the assembly process used to obtain the sequences of transcripts, sequence filtering was conducted. Raw reads were cleaned by removing sequencing adapters and trailing low-quality bases. Since there was no reference genome for this species, the trinity pipeline was deployed to perform *de novo* transcriptome assembly. To identify the putative functions of assembled transcripts of *P. polyphylla*, the unigene sequences were searched against the following public databases: NCBI nonredundant protein sequences (NR), UniProt Knowledgebase (UniProt), Gene Ontology (GO), eggNOG, Protein family (Pfam), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Table S5). The threshold values for NR and UniProt were cutoff e-values of $1^{e-5,}$ and for Pfam, it was a cutoff e-value of 0.01. WEGO software was then used to perform GO functional classification and enrichment analysis of all the unigenes to view the distribution of gene functions (Fig. S4). *P. polyphylla* transcripts were classified into three major gene function categories viz. cellular component (CC), molecular function (MF) and biological process (BP).

2.7. Differential gene expression analysis

Significant DEGs were screened using read count data of the gene expression in each sample obtained by expression quantification with DESeq2 software. Q value < 0.05 and $|\log2 \text{ (fold change)}| > 1$ were set as the thresholds between any two treatments (dormant vs mono-, dormant vs poly- and poly- vs monoaerial shoot rhizomes). Fold changes in the expression levels between samples were used as the criteria in the screening process. After merging the DEGs from dormant vs mono-, dormant vs poly- and poly- vs monoaerial shoot rhizomes, clustering analyses were performed using Cluster 3.0 and Java TreeView.

Fig. 2. (A) Images of three *P. polyphylla* cultivars LC, SL, and MD. (B) Polyaerial shoot rate of LC, SL, and MD. LC, cultivar from Lancang County; SL, cultivar from Shilin County; MD, cultivar from Mouding County.

Table 1
The effects of hormones on *P. polyphylla* rhizomes. Values are means ± SE (n = 5).

Treatment (mg/L)	Sprouting rate (%)	AS number per rhizome	Fresh weight of rhizomes (g)	AR number per rhizome	Fresh weight of ARs (g)
Control	20	0.20 ± 0.45^{a}	0.73 ± 0.24^{ab}	16.80 ± 4.49^{b}	0.81 ± 0.38^{a}
KT (0.2)	40	0.40 ± 0.55^{a}	1.14 ± 0.28^{b}	14.20 ± 2.59^{ab}	0.80 ± 0.31^{a}
IBA (1.0)	0	0^{a}	1.14 ± 0.55^{b}	16.80 ± 5.67^{b}	0.90 ± 0.40^{a}
GA_3 (10)	100	4.60 ± 1.14^{b}	1.05 ± 0.35^{b}	17.80 ± 1.92^{b}	0.83 ± 0.47^{a}
ABA (5)	0	0^{a}	0.53 ± 0.21^{a}	9.20 ± 3.56^{a}	0.51 ± 0.35^{a}

Different letters above the values indicate significant differences ($P \leq 0.05$) between different treatments. AS, aerial shoot; AR, adventitious root; KT, kinetin; IBA, 3-indolebutyric acid; GA_3, gibberellin A3; ABA, abscisic acid.

2.8. Quantitative real-time PCR (RT-qPCR) analysis of diff ;erentially expressed genes

To validate the expression profiling by RNA-Seq, 15 DEGs were selected for quantitative real-time PCR (qRT-PCR) analysis. Actin-3 was used as an internal reference gene. Primers for candidate genes were designed using Primer Premier 6 software. Genes and primer sequences can be found in Table S5. cDNA synthesis was performed with PrimeScript II 1 st Strand cDNA Synthesis SuperMix (Yesen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using abm® EvaGreen qPCR MasterMix-ROX on a QuantStudio 7 Flex System. The 20 μL reaction mixture contained 10 μL of MasterMix ROX, 2 μL of template DNA, 0.6 μL of each primer, and 6.8 μL of RNase-free double-distilled H_2O. All of the reactions were performed in triplicate three times. The relative expression levels for each gene were calculated using the comparative Ct method with normalization to the internal control gene.

2.9. Statistical analysis

Analysis of variance (ANOVA) was performed using IBM SPSS Statistics 19.0 software. The figures were generated using OriginPro 8.

3. Results

3.1. The occurrence of polyshoots in different cultivars of P. polyphylla

In nature, *P. polyphylla* rhizomes break dormancy and sprout, with each forming an apical shoot, in June (Fig. 1). However, two, three, or even more aerial shoots from a rhizome can be occasionally observed (Heng, 1998). To investigate the phenomenon of polyaerial shoots, uniform rhizomes were grown by *in vitro* propagation of three *P. polyphylla* cultivars, LC, SL, and MD, from three geographic locations with different environmental conditions and were cultured at room temperature for 6 months. Polyaerial shoots occurred in all cultivars but with different rates at 10, 20, and 50 % (Fig. 2). In particular, three and four aerial shoots were observed in MD. The order of the rates observed in LC, SL, and MD was consistent with observations in the field. The results suggest that *P. polyphylla* has genetic potential to form polyaerial shoots and that the number of aerial shoots could be affected by the physiological status of the rhizome.

Table 2
The effects of GA_3 dosage on *P. polyphylla* rhizomes. Values are means ± SE (n = 5).

GA_3 (mg/L)	AS number per rhizome	Max AS number	Fresh weight of ASs (g)	Average AS length (cm)	Max AS length (cm)
5	3.80 ± 0.45^{a}	4	0.38 ± 0.07^{a}	1.59 ± 0.77^{a}	2.7
10	5.00 ± 0.71^{b}	6	0.62 ± 0.08^{b}	2.75 ± 1.42^{b}	6.2
20	7.20 ± 0.84^{c}	8	0.43 ± 0.15^{a}	2.81 ± 1.09^{b}	5.5

Different letters above the values indicate significant differences ($P \leq 0.05$) between different treatments. AS, aerial shoot; GA_3, gibberellin A3.

Table 3
The effects of GA_3 watering on *P. polyphylla* rhizomes in pot culture under greenhouse conditions. Values are means ± SE (n = 20).

GA_3 (mg/L)	Sprouting rate (%)	AS number per rhizome	Max AS number
0	45	0.85 ± 0.88^{a}	3
100	75	2.40 ± 2.26^{ab}	8
200	85	4.05 ± 5.10^{b}	22
500	65	3.20 ± 3.72^{b}	15

Different letters above the values indicate significant differences ($P \leq 0.05$) between different treatments. AS, aerial shoot; GA_3, gibberellin A3.

3.2. Gibberellin breaks dormancy and induces polyaerial shoots in the P. polyphylla rhizome

To test whether the occurrence of polyapical shoots can be induced, the effects of hormones on the development of the *P. polyphylla* rhizome were examined. Exogenous auxin (indole-3-butyric acid, IBA), cytokinin (kinetin, KT), gibberellin (gibberellin A_3, GA_3), and abscisic acid (ABA) were respectively applied to the dormant rhizomes of *P. polyphylla* (SL cultivar). After incubated for 6 months, the rhizomes sprouted at the rates of 20 % in the control, 40 % under KT, 0% under IBA, 100 % under GA_3, and 0% under ABA (Table 1 and Fig. S1A). The results suggested that IBA and ABA promote dormancy, while KT and GA_3 break dormancy in *P. polyphylla* rhizomes. The effects of ABA, KT and GA_3 on

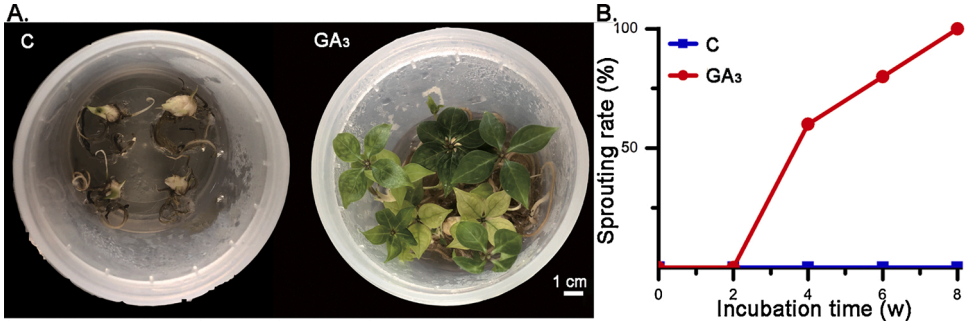

Fig. 3. (A) *P. polyphylla* rhizomes were cultured under control conditions (C) or 10 mg/L gibberellin A3 (GA_3) treatment for 8 weeks of culture. (B) Sprouting rate of rhizomes under the control (C) or 10 mg/L GA_3 treatment for the indicated times.

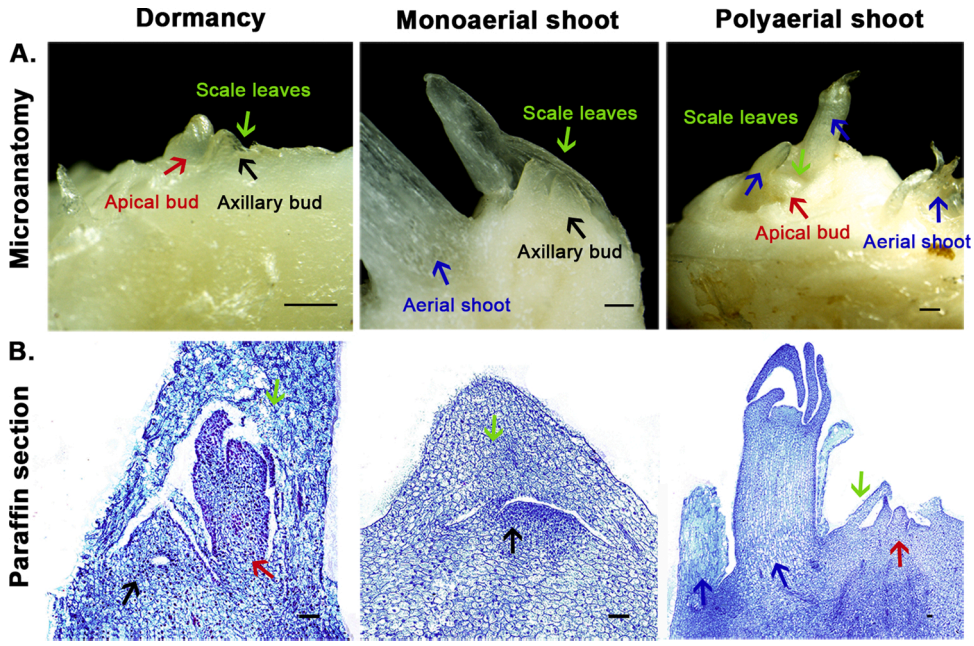

Fig. 4. Comparison of the histological structures between dormant, mono- and polyaerial shoot rhizomes. (A) Microscopy images. (B) Paraffin sections. Red arrows indicate apical buds. Black arrows indicate axillary buds. Green arrows indicate scale leaves. Blue arrows indicate aerial shoots. The scale bars in A and B are 500 μm and 80 μm in length, respectively.

dormancy were consistent with those in other species (Braide and Hamadina, 2018; Yamazaki et al., 1999; Goh et al., 2018; Kantar et al., 2012; Khan et al., 2013; Rentzsch et al., 2012; Rahman et al., 2006; Geng et al., 2005a). Interestingly, whereas the fresh weight and adventitious root number and weight were the same among the treatments, the average number of aerial shoots per rhizome under GA_3 was 4.6, which was dramatically greater than that of the control (0.2) and under KT (0.4) (Table 1). This phenomenon suggests that GA_3 plays two roles in the development of *P. polyphylla* rhizomes: breaking dormancy and inducing polyapical shoots.

To confirm the role of GA_3 in dormancy, the rhizome sprouting was investigated over a time course under GA_3 treatment (Fig. 3). Rhizomes started to sprout after 2–3 weeks and exhibited a 100 % sprout rate after 8 weeks under the GA_3 treatments. In contrast, rhizomes in the control did not sprout at all. To confirm the effects of GA_3 on the number of aerial shoots, the rhizome sprouting was investigated under a dose-dependent treatment and showed that both the average and maximum numbers of aerial shoots per rhizome significantly increased as the GA_3 concentration increased (Table 2 and Fig. S1B). The induction of poly-apical shoots by GA_3 was also proven by watering rhizomes with GA_3 in pot culture under greenhouse conditions (Table 3 and Fig. S2). These results demonstrate that gibberellin can break dormancy and induce polyaerial shoots in the *P. polyphylla* rhizome. The 100 % occurrence rate of polyapical shoots and the 3.8–7.2 multiplication rate under GA_3 treatment have great potential in industrial planting of this species.

3.3. The occurrence of polyaerial shoots results from repetitive de novo formation of apical buds

The occurrence of polyaerial shoots was explored at the histological level. The morphological structure of the *P. polyphylla* rhizome at the "sprouting" and "growth" stages constituted three organs representing three generations (Fig. 1A): a mother rhizome (previous one), apical bud (current one) and axillary bud (next one). In contrast, the rhizome at the dormancy stage constituted only two organs, the mother rhizome and a bud (Fig. 1A). This bud developed into two types of buds, another apical bud and an axillary bud, during sprouting (Fig. S3). This observation suggests that polyaerial buds must occur to produce polyaerial shoots in this process. Thus, the dormant, monobud and polybud rhizomes were dissected and compared by using paraffin sectioning and microanatomy to observe the histological occurrence of polybuds (Fig. 4).

The bud of the dormant rhizome was coated with scale leaves (Fig. 1). After the scales were removed, they showed an initiated leaf primordium of the apical bud and a dome-shaped axillary bud (Fig. 4). The axillary bud was composed of undifferentiated outer scales and meristem. Considering that apical and axillary buds are the organs of the current and next generations, respectively, there were actually two generations inside the bud of the dormant rhizome (Fig. S3). In other words, a dormant rhizome of *P. polyphylla* harbored three simultaneous generations, the mother rhizome, apical bud and axillary bud, similar to the sprouting and growing rhizomes (Fig. S3).

For the rhizome in the sprouting stage (Fig. 4), its axillary bud meristem was activated and further differentiated into finger-like and dome-like shapes, which developed into apical bud and lateral bud, respectively, in the next cycle. The inner epidermis of meristem cells differentiated into multiple scale leaves to protect the buds only once formation of the apical bud was observed.

For the rhizomes treated with GA_3 (Fig. 4), in addition to several distinguishable leaf primordia that differentiated into leaves or were still at the finger-like stage, the most obvious histological feature was that two apical buds had grown into apical shoots and three apical buds were forming. This means that repetitive *de novo* formation of apical buds occurred. This observation showed that the GA-induced occurrence of polyaerial shoots results from repetitive *de novo* formation of apical buds.

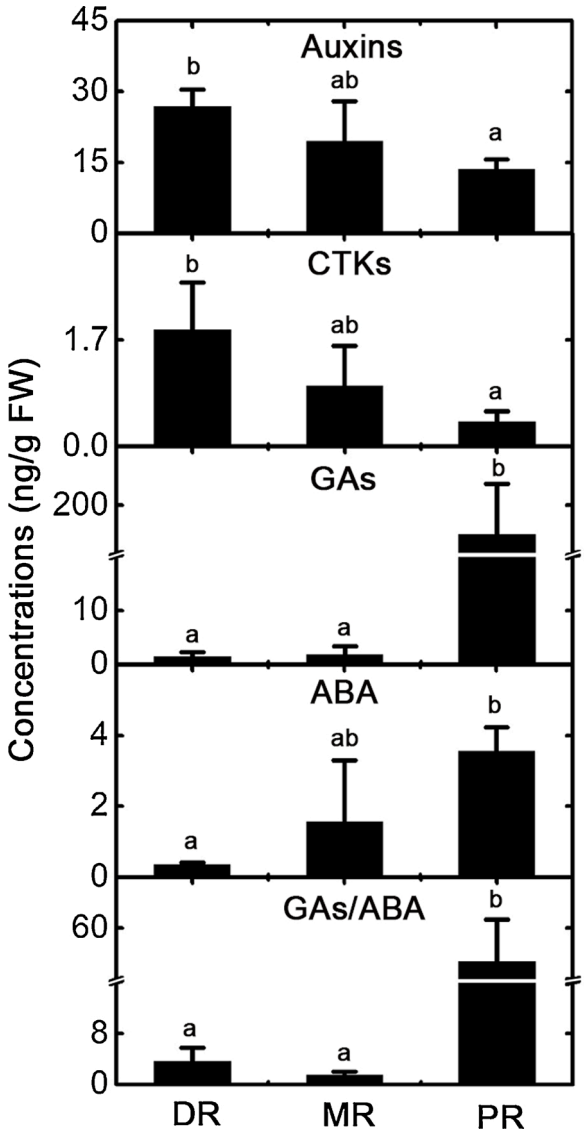

Fig. 5. Hormone concentrations measured in the dormant rhizome (DR), monoaerial shoot rhizome (MR) and polyaerial shoot rhizome (PR) of *P. polyphylla*. Auxins, cytokinins (CTKs), gibberellin acids (GAs), and abscisic acid (ABA). Data are means ± SE ($n = 3$). Different letters above the values indicate significant differences ($P \leq 0.05$).

3.4. Marked increase of gibberellins in the polyaerial shoot rhizome

To verify the effects of auxins, CKs, GAs and ABA on the occurrence of polyaerial shoots, their endogenous concentrations in the dormant rhizome (DR), monoaerial shoot rhizome (MR) and polyaerial shoot rhizome (PR) were examined (Fig. 5 and Table S1). The levels of auxins, CTKs and ABA in the PR were the same as or slightly lower than those in the DR or MR, except for GAs. The levels of GAs in the PR were dramatically higher than those in the DR or MR (Fig. 5). Interestingly, GA_3 was detected in the PR at 27.36 ng/g and was 20.27- and 15.37-fold higher levels than those in the DR and MR, respectively (Table S1). GA_1, another form of gibberellin, was detected at levels as high as 148.58 ng/g in the PR but not in the DR and MR. The results showed that the occurrence of polyaerial shoots was mainly associated with high GA levels and suggested that complicated responses of gibberellin metabolism took place in the polyapical shoot rhizome. This phenomenon also supported the previously mentioned results wherein GA_3 induced the occurrence of polyaerial shoots in *P. polyphylla*.

3.5. Transcriptome comparison between monoapical and polyapical shoot occurrences

To explore the potential mechanism underlying the occurrence of polyapical shoots, the transcripts of DRs, MRs and PRs were profiled and compared between the occurrences of MRs and PRs. The profiling provided 206,098 unigenes (Table S3) and showed 3860 differentially expressed genes (DEGs) (Fig. 6). The change from the DR to MR involved 2039 (53 %) DEGs. Among them, 1822 (47 %) genes were upregulated, but only 217 (6%) genes were downregulated. The number of upregulated genes was 8.4-fold that of the downregulated genes; in contrast, the change from DR to PR involved 1345 (35 %) genes, and the number of upregulated genes (894) was 2.0-fold that of downregulated genes (451) (Fig. 6A). The results suggest that the transcriptional activity for monoapical and polyapical shoot occurrences are markedly different and that the former is much more active than the latter. On the other hand, the changes from the DR to MR and from the DR to PR shared 248 genes, only 6% of the total DEGs, which was much less than that shared between DR/MR and MR/PR (1344, 35 %) and between DR/PR and MR/PR (469, 12 %) (Fig. 6B). This means that the occurrences of mono- and polyapical shoots might employ almost completely different mechanisms. Taken together, transcriptional regulation of monoapical shoot and polyapical shoot occurrences is different not only in activity but also in patterns.

3.6. Expression analysis of hormone-related genes during monoapical shoot and polyapical shoot occurrences

To examine the effects of hormones on the occurrence of polyaerial shoots at the transcriptional level, the expression of auxins, CTKs, GAs and ABA-related genes were compared among DRs, MRs and PRs. Thirty-three auxin-, nine cytokinin-, fourteen gibberellin-, and twenty-one ABA-related genes were identified and their relative expression were analyzed by hierarchal clustering (Fig. 7). The overall appreciation showed that the gene expression patterns in DRs, MRs and PRs were obviously different from each other. For the auxin-related genes, their expression patterns in MRs and PRs were clustered into the same group (Fig. 7); however, for the CKs, GAs and ABA-related genes, their expression patterns in MRs and PRs were clustered into different groups (Fig. 7). In other words, the difference in expression patterns between MRs and PRs was greater than that between DRs and MRs and that between DRs and PRs in most cases. The results suggest that monoapical shoots and polyapical shoots might occur through different hormonal regulation pathways.

4. Discussion

Rhizome cutting has recently been used for *P. polyphylla* propagation. The commercial success of this method will depend on whether polyapical shoots can be reliably produced in rhizomes. This study showed that *P. polyphylla* cultivars from different locations were able to produce both mono- and polyapical shoots, and the occurrence rate of polyapical shoots ranged from 10 to 50 %. *P. polyphylla* rhizomes always harbor three generations of organs: the mother rhizome of the previous generation, the apical bud of the current generation and the axillary bud of the next generation (Fig. 1). The application of the hormone GA_3

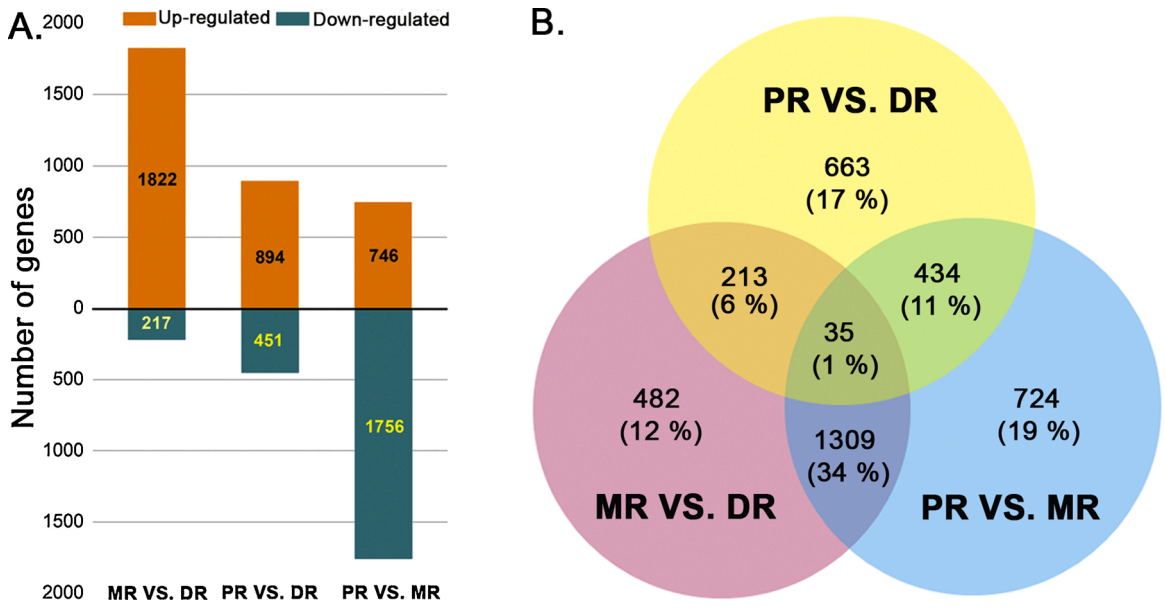

Fig. 6. mRNA expression in the *P. polyphylla* dormant rhizome (DR), monoaerial shoot rhizome (MR) and polyaerial shoot rhizome (PR). (A) Number of up- and downregulated genes in three pairwise comparisons. (B) Venn diagram. Numbers represent common and specific differentially expressed genes (DEGs) in each comparison between treatments.

could quickly break dormancy and induce 100 % occurrence of polyapical shoots in rhizomes. The average number of apical shoots in GA_3-treated rhizomes reached 7.2. The GA_3-induced occurrence of polyapical shoots derived from the repetitive *de novo* formation of apical buds. The application of GA_3 did not change or slightly changed auxin, CK and ABA concentrations but markedly increased GA_3 and GA_1 levels in rhizomes. The occurrences of mono- and polyapical shoots shared very few DEGs and exhibited different expression patterns of hormone-related genes. The effects of gibberellin on *P. polyphylla* rhizomes actually include two events. The first is the breaking of dormancy (Fig. 3), which is indicated by the sprouting of apical bud developed in the last cycle and occurs at the very early stage of sprouting. The second is the induction of repetitive *de novo* formation of buds, which are derived from the reactive meristem in the current cycle and occur at later stages of sprouting. The two events have a certain chronological order and can be separated at the histological level (Fig. 4). It is well known that developmental inhibition in dormant meristems results from the arrest of bud meristem cells in the G-1 phase of the cell cycle (Campbell et al., 1996). Cytokinins, such as those occurring in the geophytes *Zingiber*, tuberosum, *Helianthus*, and *Gladiolus*, can terminate the arrest and thus break rhizome dormancy. In our case of *P. polyphylla* rhizomes, cytokinin had a certain effect on breaking dormancy but not on the induction of polyapical shoots (Table 1). Gibberellin clearly functions in breaking seed dormancy, but its role in rhizome dormancy is controversial. Gibberellin is speculated to break rhizome dormancy in potato (Rentzsch et al., 2012), *Helianthus* (Kantar et al., 2012), *Gladiolus* (Khan et al., 2013), garlic (Rahman et al., 2006), and tulip (Geng et al., 2005b). However, in other studies (Hartmann et al., 2011; Suttle, 2004; Carrera et al., 2000), its role is to promote shoot elongation after dormancy is broken by cytokinin. Our results revealed, for the first time in *P. polyphylla*, that gibberellin not only breaks rhizome dormancy but also reactivates the meristem to form multiple apical buds.

P. polyphylla rhizomes were very sensitive to GA_3. They exhibited quick and dramatic changes at morphological and physiological levels in response to exogenous GA_3. Untreated rhizomes of *P. polyphylla* developed very slowly. At six months of culture, only 20 % of plants broke rhizome dormancy, and none produced polyapical shoots (Table 1 and Fig. S1). In contrast, GA_3-treated rhizomes developed extremely fast, showing dormancy break in two weeks and all presenting polyapical shoots (Table 1 and Fig. S1). The sensitivity of these rhizomes to GA_3 was also reflected at the metabolic and transcriptional levels. Polyapical shoot rhizomes had overwhelming endogenous GA_3 and GA_1 levels. Endogenous GA_3 could be directly taken up from exogenous GA_3, whereas GA_1 must be derived from GA_3 through the gibberellin metabolic pathway because no GA_1 was detected in dormant and monoapical shoot rhizomes (Table S1), and there might be a metabolic pathway among gibberellins (Gao et al., 2017). Considering the dramatic difference in the transcriptome, particularly the transcriptome related to hormonal metabolism, between mono- and polyapical shoot rhizomes (Fig. 7), hormonal metabolism in polyapical shoot rhizomes could be severely interrupted.

5. Conclusion

P. polyphylla has the genetic potential to produce polyapical shoots and that the occurrence of polyapical shoots in the *P. polyphylla* rhizome could be induced by GA_3. Through transcriptional regulation differing from that in normal sprouts, GA_3 treatment could break dormancy in a very short time and provide a high multiplication rate for *P. polyphylla* propagation with rhizome cutting.

CRediT authorship contribution statement

Mulan Wang: Investigation, Visualization, Writing. **Jiaqi Chen**: Investigation. **Xudong Zhang**: Methodology Development. **Shenghong Li**: Resources, Funding acquisition. **Tie Zhang**: Resources. **Weiqi Li**: Conceptualization ideas, Writing, Supervision. **Liang Lin**: Methodology Development, Resource.

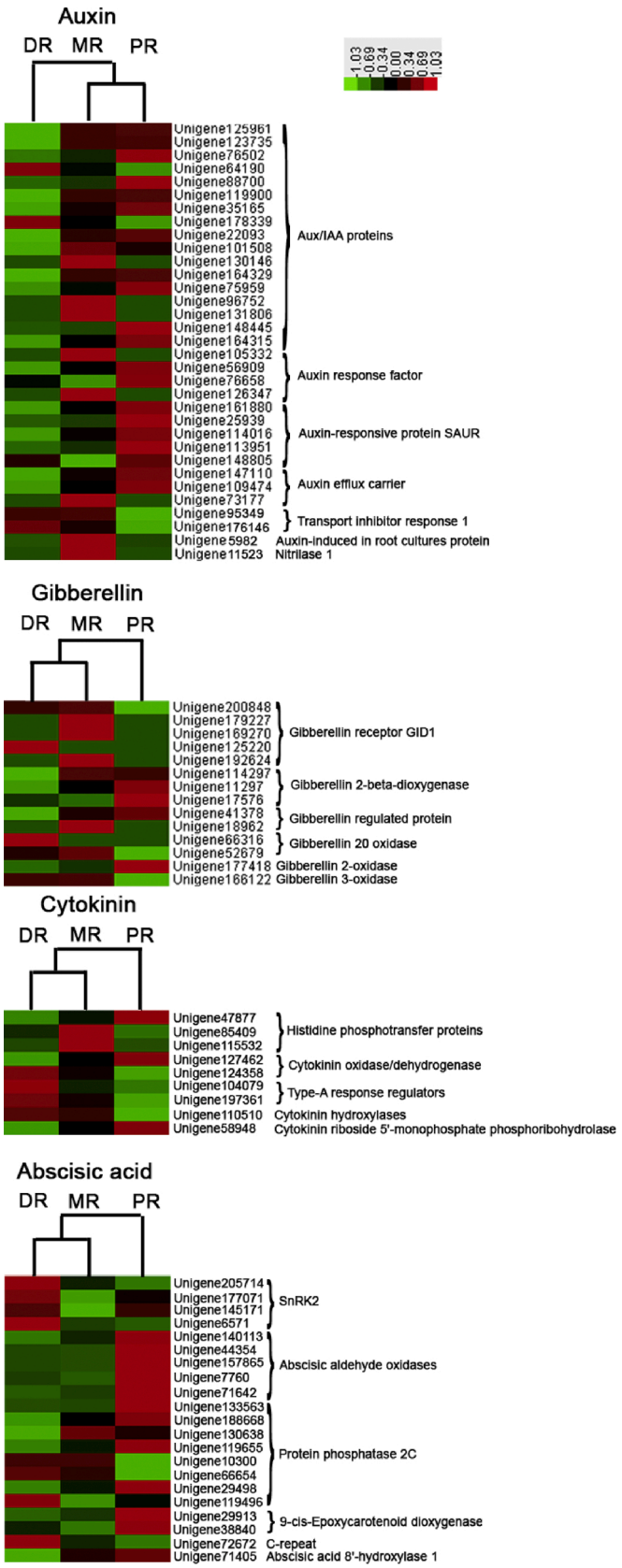

Fig. 7. Heat map of auxin-, gibberellin-, cytokinin- and abscisic acid-related gene expression in dormant rhizomes (DRs), monoaerial shoot rhizomes (MRs) and polyaerial shoot rhizomes (PRs). Red blocks indicate high expression, and green blocks indicate low expression.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

This research was funded by grants from the Yunnan Applied Basic Research Project (2017AB001 and 2017FB057), Yunnan Key Research and Development Program (2019ZF011-2), Join Fund of Yunnan Local College (2018FH001-029), National Natural Science Foundation (31770375), and "Cross-Cooperative Team" of the Germplasm Bank of Wild Species, Kunming Institute of Botany, CAS. The authors thank Biotree Biotech Co., Ltd. (Shanghai, China) for technical assistance in phytohormone analyses.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2021.113511.

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