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Original article

Gibberellin disturbs the balance of endogenesis hormones and inhibits adventitious root development of *Pseudostellaria heterophylla* through regulating gene expression related to hormone synthesis

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ABSTRACT

The adventitious roots of some plants will develop into tuberous roots which are widely used in many traditional Chinese medicines, including *Pseudostellaria heterophylla*. If adventitious root development is inhibited, the yield of Chinese medicinal materials will be reduced. Gibberellic acid is an important phytohormone that promotes plant growth and increases the resistance to drought, flood or disease. However, the effects of gibberellic acid on adventitious roots of *Pseudostellaria heterophylla* are not clear. Here, we reports GA3 suppressed adventitious root development of *Pseudostellaria heterophylla* by disturbing the balance of endogenesis hormones. By detecting the contents of various endogenous hormones, we found that the development of adventitious roots negatively correlated with the content of CA3 in tuberous roots. Exogenous GA3 treatment decreased the diameter of adventitious roots, but increased the length of adventitious roots of *Pseudostellaria heterophylla*. In contrast, blocking the biosynthesis of GA3 suppressed stem growth and promoted the xylem of tuberous roots development. Moreover, exogenous GA3 treatment resulted in imbalance of endogenesis hormones by regulating their synthesis-related genes expression in xylem of tuberous roots. These results suggest GA3 broke the established distribution of hormones by regulating synthesis, transport and biological activation of hormones to activate the apical meristem and suppress lateral meristem. Regulating GA3 signaling during adventitious roots development would be one of the possible ways to increase the yield of *P. heterophylla*.

1. Introduction

Pseudostellaria heterophylla (*P. heterophylla*) has strengthening spleen-stomach, anti-fatigue, anti-hypoxia, anti-inflammation, anti-aging, enhancing immunity, anti-cough and anti-virus effects (Pang et al., 2011; Wang et al., 2013). The original roots of *P. heterophylla* wilted and germinated adventitious roots from its underground stem at 2 months after flowering. These adventitious roots evolve into spindle-shaped tuberous roots (Han et al., 2008). Development of tuberous roots involves with the formation of secondary xylem and secondary phloem from vascular cambium. However, the molecular mechanism for regulating the development of tuberous roots of *P. heterophylla* is not clear. Studying the mechanism which tuberous roots development will help to increase the output of *P. heterophylla*.

The demand of plants for endogenous hormones is diverse in different growth parts and periods (Murcia et al., 2016). For example, indoleacetic acid (IAA) is produced at the tip of plant meristem and bud, which mainly promotes the elongation and growth of plants (Utami et al., 2018). Cytokinin (CTK) is produced in the root tips of plants and mainly promotes cell division and expansion (Fukudome and Koiwa, 2018). Gibberellic acid (GA3) is produced in part of the stem region, which mainly promotes cell elongation and thus leads to increased stem height. Abscisic acid (ABA) is often used as an inhibitor of plant growth, which can significantly promote root growth by inhibiting ethylene synthesis in the absence of water (Liu and Hou, 2018). Root tuber formation is

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Table 1
Primer sequence for normal PCR.

Gene name	Forward primer sequence (5′ → 3′)	Reverse primer sequence (5′ → 3′)
TMC	GCTTGGTTTTTATTCGGCAACATTG	TTTTTGAGGTTGGATTAGAAGGTGG
GH3.1	AATCATAATGGTTGGGGTTTCAGTG	ATTTCGTTTCTGGTTAGTAGCAAGG
GH3.2	ATGGCAGTGTACGACGATGTGAAAG	GGTGGCTTAGTTGAAATCACGAGTG
GH3.3	ATGACGGTCCACGATGATAATAACG	CAAGTTTCAACACCAAATCACACAC
GA20ox1	TAGTGCTGATGATACACCCTTAAAC	CCATAAAGGTATCACCAATGTTCAC
GA20ox2	CTTACCATGAATCCAAAAACACACC	TTAAATATTAAGTCCATGCATGGC
GID1a	TTTACTATGGCTGGAAATAATGAAG	GTACTACCGGTCACACTTATAAAAC
GID1b	TGTCTCCTTTTCTAACCACATCACC	CCAAGGGCTATTCACAGTCAGGATT
A-IPT1	GAACTCCCCTCTTAATACTACAATC	GTCTCCAAGGTCATCATTTCTAAAC
A-IPT2	AAACGAGTACCTAAACAAACGAGTC	GAGATCGAAGAAGAGCTGTAAAAAG
CYP735A	AATGAGTCCTATTTTTCAATTTGTG	TGATAACATTTCAATTTCTCATAGT
tRNA-DMATase	AGACCGAAATAGGAAAGATGAGTGG	TCTATAATGTACTGCTGCAGGGTTG
CKX1	AGAAAGAAATAACGAGGTACGATCC	CATGTCCCTAGATTATATGGAGCTT
CKX2	CTCCTATTTAAACCCCCCACTCAAC	ACTAATTTGACGAGCCCTAGAGAAG
ACO1	AACTATTACTTATACAAAGGGCCAG	CACGTATGCATGTGTCTAATATTTG
ACO2	AATGACTAACACAGACATGATCCCA	CCATATTACAAACGGAAGTAGCTCG
ACO3	ACTATGAGAGTGAGAGTTTGTGGTC	CAAATCTTACATCTGTTCGTCCTTC
ACS1	ATCCACACACTTTCCTAAGCTCCCT	AGTAGTTCCCGTGCACGATATCTTC
ACS2	AGAAATGCCGTGGCAAGATTTATGG	CTACGTTCGAGCTCGAACGAGAGC
SAMS	CTCGCCTTACCAGTCTAAACAAACG	GCTGTAGTCTGTCGGTTGTGATTGC
ZEP1	AGGCTAAGGTTACATTGTGGGGGAG	CTGCCTACGTACACTGTGACTGATG
ZEP2	GCTGAAAATGGAAGTAAATGTGGAT	TAGCTTCACCGATCAAGAACGAGTT
AAO	TCTGAAGCCGAGATGTCTATAGCCG	GTATGTTTCAACAATATCGAACCCAC
CYP707A1	AAAGAAAAGGAAGAAAGTGGGGAAG	TGTGACATCCACTATTGACTTTTGC
CYP707A2	GCCACGCTTAACGAATACACAAATC	GATAAAGGAACACAAGCATCATGGC

Table 2
Primer sequence for quantitative RT-PCR.

Gene name	Forward primer sequence (5′ → 3′)	Reverse primer sequence (5′ → 3′)
TMC	TTACGTCGAATGGTGTCGAG	GTGATGCTGCCTTTGGAAAT
GH3.1	GGAAATGTCGTGGCAAAGTT	CCAACGTCACGATGTCACTC
GH3.2	AAACCCAAATCACATTCCGA	GGAGACGGTTGTGACAGGTT
GH3.3	GATGAGGATCACACATCGGA	ACGGTCGTGTCAGGTTCAAT
GA20ox1	GCCTCATTATTGAATCATTTCCTT	GAATTCGGGAGGGTGTATGA
GA20ox2	ATTTCGCCAAATTTCAATGC	ACGGCTCTATGCAAGCAACT
GID1a	TTTACTATGGCTGGAAATAATGAAG	GTACTACCGGTCACACTTATAAAAC
GID1b	TAATGAAATCAACACAAATGAATGC	CGATCTAAATATTCATTCAGTTCCC
A-IPT1	TCCCTTATTTGCATCCAGGT	TAACCTCTTCGACCAAGCCT
A-IPT2	GGAGGGTGGGAGCTACATAA	CCTCCAAGAACGTCTTCACA
CYP735A	GAAGATGGGATGGGTATTCAA	GTCGATCCATACGTTTGTGC
tRNA-DMATase	GAAGTGTATGCGGAGCTTGA	ATTTAGCCCTTGGGTCTCCT
CKX1	TAAAGGACACAAGCAATGGG	CACCGCAGATGTAAGGAATG
CKX2	AAATTATTCGGCATTGCACA	TCTGGACCATTGATTTCCAA
ACO1	TCATTGGGTCGATGTTCCTT	TGCTGCCATTGAAACTCTTG
ACO2	AGAACGGAGGTGATGGAATG	GTCAAGTACGATTCCGCGTT
ACO3	ACCCAACCCGAATAACAGTG	CACCATGGTTCACCACTTGA
ACS1	CGCAAACTCAACACATGCTT	TTAGGCATCGGATTCTGACC
ACS2	CGCGTGATCATCAATGAAGT	CGTGTCATCGCTCATATTGG
SAMS	TCGTCCAGGTCTCCTATGCT	TGGCCTGAAGTCGAAGTTCT
ZEP1	GGACAGCACAAATGGGACTT	TCGAAATACTGCCAGATTGGT
ZEP2	TAGGCCTCCATGGCAAATAC	CCATCCTCAAGAGCACAACA
AAO	CATGCTTCTCAGTGCGGCTT	CCGGCTATAGACATCTCGGCTT
CYP707A1	ATGGTGATCATATTTGTAGCTTTG	ATCGAACCTGGATGGATTGA
CYP707A2	TGGTTTCCAAGGGTTTCACT	GATAAAGGAACACAAGCATCATGGC
PYR1	CGCTTGTTTGCGGATACGGT	GCACTCCCACTACCCACTCC

the result of multiple hormone interactions (Li et al., 2017). ABA signaling reduces plant response to IAA and promotes root branching (Dinis et al., 2018). It also can significantly increase root growth by suppressing ethylene synthesis in the absence of water (Wang et al., 2015). Under normal physiological conditions, stable growth of plants requires a dynamic balance of hormones. Under stress or disease, hormones homeostasis is broken, and the various parts of plant are out of sync and so are not developing in a co-ordinated manner (Shao et al., 2016). When the ZR/IAA ratio is high, it mainly promotes the differentiation and growth of buds. On the contrary, it promotes the growth and differentiation of roots.

GA3, acts as a natural plant growth regulator, play an important role in plant growth and development (Gupta and Chakrabarty, 2013). Researches showed that GA3 have diverse effects on hairy root of many plant species. Therefore, the effect of GA3 on the adventitious roots development in *P. heterophylla* is unclear. Here we showed that GA3 activated the apical meristem but suppressed the lateral meristem in *P. heterophylla* by targeting the gene expression which related plant hormones regulation. This reveals a biological mechanism, by which GA3 Gibberellin disturbs the balance of endogenesis hormones and stunts tuberous roots expanding in *P. heterophylla*.

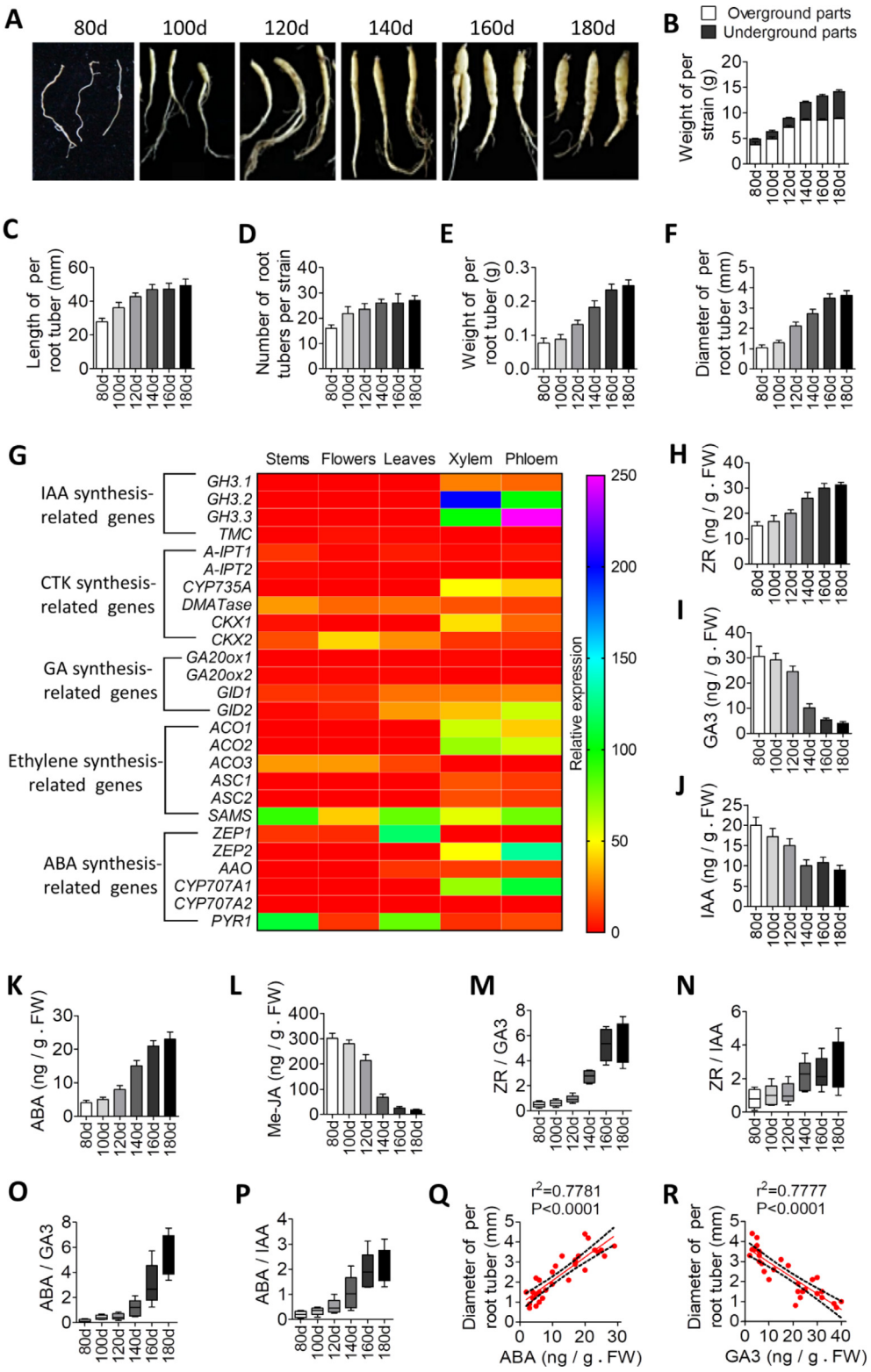


Fig. 1. The tuberous roots expanding was negatively correlated with the content of GA3 in *P. heterophylla*. (A) Photos represented the changes of tuberous roots of *P. heterophylla* at different time points after seedling emergence. Scale bar, 5 mm. (B) The changes of the overground parts weight and underground parts weight of *P. heterophylla* at different time points after seedling emergence. Data represents mean ± sem. n = 9–12 strain/group. (C -F) The changes of the length of per root tuber, number of root tuber per strain, weight of per root tuber and the diameter of per root tuber at different time points after seedling emergence. All tuberous roots of per strain were measured, and their means were used for statistical chart. Data represents mean ± sem. n = 9–12 strain/group. (G) Hot-map revealed the plant hormones-related genes expression in leaves, stems, flowers, xylems and phloem of root tuber of *P. heterophylla*. (H -L) The changes of the content of zeatin-riboside (ZR), gibberellic acid (GA3), indoleacetic acid (IAA), abscisic acid (ABA) and methyl ester-jasmonic acid (Me-JA) in root tuber of *P. heterophylla* at different time points after seedling emergence. Data represents mean ± sem. n = 9–12 strain/group. (M -P) The changes of the ratio of ZR/GA3, ZR/IAA, ABA/GA3 or ABA/IAA in root tuber of *P. heterophylla* at different time points. Data represents mean ± sem. n = 9–12 strain/group. (Q) Correlation analysis between diameter per root tuber and the content of ABA in root tuber of *P. heterophylla*. Six independent samples were collected at each time point for correlation analysis, totaling 36 samples. $r^2 = 0.7781$, $P < 0.0001$. (R) Correlation analysis between diameter per root tuber and the content of GA3 in root tuber of *P. heterophylla*. Six independent samples were collected at each time point for correlation analysis, totaling 36 samples. $r^2 = 0.7777$, $P < 0.0001$.

2. Materials and method

2.1. Plant materials

The seeds of *P. heterophylla* were collected from Shibing county, Guizhou province of China (108°7′12″N, 27°1′48″E). These seeds are dried and sealed in an airtight pouch for storage. After sand laminated processing for 70d at 0–4 °C, these seeds were used for sowing.

2.2. Growth conditions

Seeds were sowed in greenhouse under at 18/25 °C. The relative humidity was 60–88%, and the average illumination was 17.06–31.42 KLX. All experiments were carried out in the growth conditions.

2.3. Pharmacological treatments

60-day-old seedlings were transplanted individually to flowerpot (diameter: 20 cm; high: 30 cm). Allow these plants to adapt to the new environment for 40 days. GA3 was purchased from TAKARA (Japan), and PBZ was purchased from Beijing Solarbio Science & Technology Co., Ltd (China). GA3 were applied in DMSO, dilute with water to 20 mg/L, 75 mg/L and 150 mg/L respectively. PBZ were applied in DMSO, dilute with water to 20 mg/L. Each plant is watered with 200 mL GA3 or PBZ solution once a week. The control plants were watered with 200 mL vehicle which keeps up with treatment group.

2.4. Characters analysis

The stem length, number of cleistocarp, number of leaves, the weight of overground part, the diameter of per root tuber, weight of the underground part, weight of tuber, the length of per root tuber were measured after treatment for 20d, 40d or 60d. All tuberous roots and stems of per strain were measured, and their means were used for statistical chart. The length measurement is accurate to 0.01 mm and the weight accurate to 0.01 g.

2.5. Paraffin section

Eight tuberous roots were selected from each strain for observation of tissue structure. The roots were fixed by FAA, and these roots were dehydrated, waxed and embedded. The paraffin slicer (Leica, RM2245) was used for cross-cutting the swollen parts. The section thickness is 8–10 μm. These sections were stained with safranine- fast green, and sealing with neutral gum. Observing and imaging with a microscope (Olympus, BX41). Count and measure the number of cell layer and thickness of secondary phloem, cambium and secondary xylem under the microscope.

2.6. Quantification of endogenous hormones

Lyophilized aerial tissue (100 mg dry weight) was used for quantification of ABA, ZR, GA3, IAA and Me-JA. The extracted sample was transferred into the round bottom flask, vacuum concentration and drying. The sample was diluted to 2 mL. The Enzyme Linked Immunosorbent Assay (ELISA) kits for ABA, ZR, GA3, IAA and Me-JA were provided by Crop Chemical Control Laboratory, Agricultural University of China. The detection limit was 1 ng/mL.

2.7. RNA extraction, PCR, and quantitative RT-PCR.

Total RNA, PCR, and Quantitative RT-PCR of *P. heterophylla* was followed the previous descripted (Wang and Ng, 2001). All genes were normalized control PhACT2. The primer sequences are listed in Tables 1 and 2.

2.8. Statistical analyses

All of the results are expressed as the Means ± SEM. Graph drawing and statistical analyses were performed using GraphPad Prism (version 7.0). Potential differences between the mean values were evaluated using Independent-Sample Test and one/two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for post hoc comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

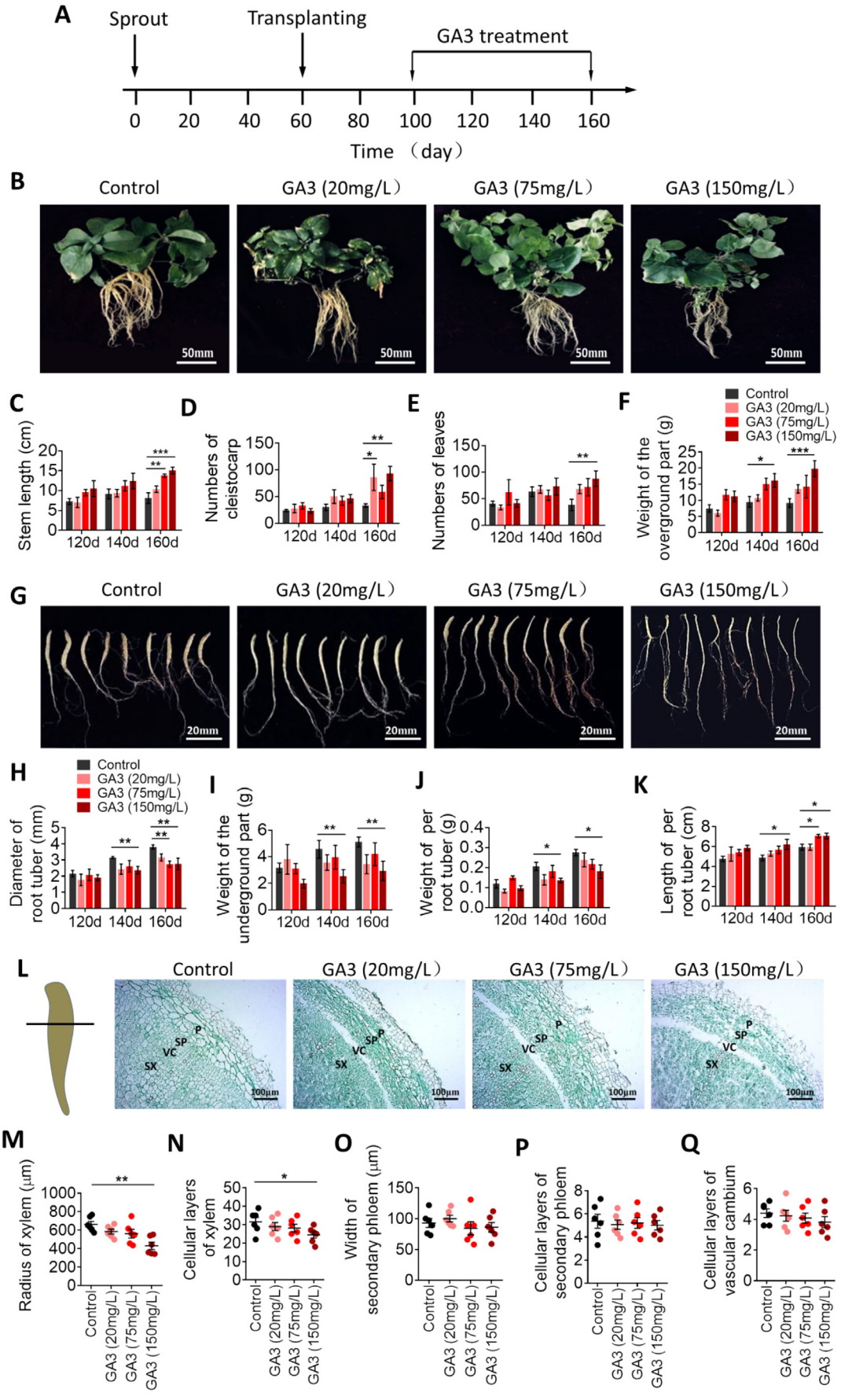
3.1. The tuberous roots expanding was negatively correlated with the content of GA3 in P. heterophylla

To confirm the time-axis which tuberous roots formation of *P. heterophylla*, we collected tuberous roots at different time points after seedling emergence and compared the changes of tuber roots of *P. heterophylla* (Fig. 1A). We found that the weight of overground parts of *P. heterophylla* did not increase any more, but the weight of underground parts of *P. heterophylla* progressively increased after 140 day of seedling emergence (Fig. 1B). Further analysis revealed that the length of per root tuber and the number of tuberous roots per strain were did not increase significantly after 140 days of seedling emergence (Fig. 1C and D). However, the weight of each root tuber of *P. heterophylla* keeps increasing during this period of time, as well as the diameter of per root tuber (Fig. 1E and F).

The plant hormones-related genes were investigated in leaves, stems, flowers, xylems and phloem of root tuber of *P. heterophylla*

Table 3
Key enzyme gene screening for ABA, GA, CTK, IAA and ethylene from the transcriptome database of *Pseudostellaria heterophylla*.

Plant hormones	Key enzyme gene name	Mnigene number	Length/bp
IAA	*TMC*	c49937_g1	974
	GH3.1	c37777_g1	1103
	GH3.2	c43412_g1	2364
	PhGH3.3	c43146_g1	2301
GA	*GA20ox1*	c63354_g1	486
	GA20ox2	c51030_g1	1249
	GID1	c49885	486
	GID2	c46739	692
CTK	*A-IPT1*	c43666_g1	2043
	A-IPT2	c33221_g1	1127
	CYP735A	c52234_g1	1805
	tRNA-DMATase	c45128_g1	2854
	CKX1	c34678_g1	2472
	CKX2	c47706_g1	2045
Ethylene	*ACO1*	c47464_g1	1413
	ACO2	c41278_g1	2357
	ACO3	c50466_g1	2252
	ACS1	c55186_g1	1999
	ACS2	c60708_g1	1621
	SAMS	c57892_g1	1661
ABA	*ZEP1*	c88335_g1	540
	ZEP2	c65454_g1	1263
	AAO	c59545_g1	3564
	CYP707A1	52289_g1	500
	CYP707A2	c65149_g5	509
	PYR1	C55366_g1	777


A
Sprout
Transplanting
GA3 treatment
0 20 40 60 80 100 120 140 160
Time （day）
B
Control
GA3 (20mg/L）
GA3 (75mg/L）
GA3 (150mg/L）
50mm
C
Stem length (cm)
D
Numbers of cleistocarp
E
Numbers of leaves
F
Weight of the overground part (g)
Control
GA3 (20mg/L)
GA3 (75mg/L)
GA3 (150mg/L)
120d 140d 160d
G
20mm
H
Diameter of root tuber (mm)
I
Weight of the underground part (g)
J
Weight of per root tuber (g)
K
Length of per root tuber (cm)
L
P
SP
VC
SX
100μm
M
Radius of xylem (μm)
N
Cellular layers of xylem
O
Width of secondary phloem (μm)
P
Cellular layers of secondary phloem
Q
Cellular layers of vascular cambium

based on previous transcriptome databases (Li et al., 2016). Search results showed in Table 3. We found that most IAA, CTKs, GAs, ethylene and ABA synthesis-related genes are active in xylems and phloem of root tuber of *P. heterophylla* at 140 days after seedling emergence (Fig. 1G). These genes were augmented by PCR, and determined the molecular weight by gel electrophoresis (Figure S1). We further examined the content of several major plant hormones in root tuber of *P. heterophylla* by ELASA. We found that the content of Zeatin-riboside (ZR) and ABA increased gradually following root tuber expanding of *P. heterophylla.* In contrast, the content of GA3, IAA and methyl ester-jasmonic acid (Me-JA) were decreased gradually following root tuber expanding of *P. heterophylla* (Fig. 1H–L). The ratio of ZR/GA3, ZR/IAA, ABA/GA3 and ABA/IAA were decreased gradually in root tuber of P. heterophylla in a time-dependent manner (Fig. 1M–P). The results from correlation analysis showed that the diameter of per root tuber was positively associated with the content of ABA but negatively associated with the GA3 in root tuber of P. heterophylla (Fig. 1Q and R).

3.2. GA3 treatment restrains the root tuber expanding

To determine if exogenous gibberellin treatment inhibits root tuber expanding, 100 days-old *P. heterophylla* are watered with different concentrations of GA3 (Fig. 2A). The results showed that 150 mg/L GA3 treatment for 60d significantly increased stem length, the number of cleistocarp, leaf numbers and the weight of the overground part of *P. heterophylla* (Fig. 2B–F). However, 75 mg/L GA3 treatment for 40d or 60d significantly decreased the diameter of root tuber, the weight of the underground part and the weight of per root tuber of *P. heterophylla* (Fig. 2G–J). And interestingly enough, 75 mg/L GA3 treatment for 40d or 60d significantly increased length of root tuber of *P. heterophylla* (Fig. 2K).

Next, we examined the changes of tissue structure in the expanded portion of root tuber of GA3-treated *P. heterophylla* (Fig. 2L). The results showed that 75 mg/L GA3 treatment markedly increased the radius of xylem and the cellular layers of xylem (Fig. 2M and N). But the GA3 treatment had no significant effect on the width of secondaryphloem, cellular layers of secondaryphloem and vescular cambium (Fig. 2O–Q).

3.3. Blocking the biosynthesis pathway of GA3 accelerated root tuber expanding

To detect the effect of endogenous GA3 on root tuber expanding, paclobutrazol (PBZ) was used to block the biosynthesis of gibberellin in *P. heterophylla* (Fig. 3A). The results showed that PBZ treatment for 60d significantly decreased stem length, the number of cleistocarp, leaf numbers and the weight of the overground part of *P. heterophylla* (Fig. 3B–F). However, PBZ treatment for 40d or 60d significantly decreased the diameter of root tuber, the weight of the underground part and the weight of per root tuber of *P. heterophylla* (Fig. 2G–J). But PBZ treatment did not effect on the length of root tuber of *P. heterophylla* (Fig. 2K).

The results from tissue structure research showed that PBZ treatment increased the radius of xylem and the cellular layers of xylem (Fig. 2M and N), but had no effect on the secondaryphloem and vescular cambium (Fig. 2O–Q).

3.4. Blocking the synthesis of gibberellin after exogenous gibberellin treatment could get tuberous roots expanding back on track again

We further determined that whether blocking the synthesis of gibberellin after exogenous gibberellin treatment could eliminate the inhibition of root tuber expanding in *P. heterophylla*. The 100d old *P. heterophylla* was watered with 75 mg/L GA3 for 30d, and then, watered the PBZ for another 30 days (Fig. 4A). The GA3-treated *P. heterophylla* showed decrease in stem length, the number of cleistocarp, leaf numbers and the weight of the overground part when compared with control group (Fig. 4B–F). The GA3 + PBZ-treated *P. heterophylla* showed no statistical significance in stem length, the number of cleistocarp, leaf numbers and the weight of the overground part when compared with GA3-treated group (Fig. 4B-F). However, PBZ treatment reversed the GA3-induced decrease in diameter of root tuber, the weight of the underground part and the weight of per root tuber in *P. heterophylla* (Fig. 4G–J). The results from tissue structure research showed that PBZ treatment reversed the GA3-induced decrease in the radius of xylem and the cellular layers of xylem in *P. heterophylla* (Fig. 4K and L), but had no effect on the secondary phloem and vescular cambium of root tuber (Fig. 4N–O).

3.5. GA3 disturbs the balance of endogenesis hormones in xylem of tuberous roots

Plant endogenous hormones in the xylem of roots tuber of *P. heterophylla* from different independent experiments were tested by ELISA. The results showed that exogenous GA3 treatment upregulated ZR, GA3 and IAA, but decreased ABA and Me-JA in xylem of root tuber of *P. heterophylla* in a concentration dependent manner (Fig. 5A–E). In contrast, inhibiting gibberellin synthesis could increase the content of ZR and ABA, and suppress the GA3 and IAA synthesis in xylem of root tuber of *P. heterophylla* in a time-dependent manner (Fig. 5F–J). PBZ treatment reversed the GA3-induced increase in the content of ZR, IAA and GA3 in xylem of root tuber of *P. heterophylla*. On the other hand, PBZ treatment reversed the GA3-induced decrease in content of ABA in xylem of root tuber of *P. heterophylla* (Fig. 5K–O). In addition, GA3 treatment decreased the ratio of ZR/IAA, ZR/GA3, ABA/IAA and ABA/GA3 in xylem of root tuber of *P. heterophylla*. Nevertheless, PBZ treatment reversed the GA3-induced decrease in content of ABA in xylem of root tuber of *P. heterophylla* (Fig. 5P–S).

Fig. 2. GA3 treatment restrains the root tuber expanding. (A) Time-line for GA3 treating to *P. heterophylla*. (B) Photos represented the changes of appearance of *P. heterophylla* after treating 20 mg/L, 75 mg/L or 150 mg/L GA3 for 60d. Scale bar, 50 mm. (C -F) The changes of stem length, number of cleistocarp, number of leaves and the weight of overground part after treating 20 mg/L, 75 mg/L or 150 mg/L GA3 for 20d, 40d or 60d. Data represents mean ± sem. n=11 strain/group. Statistical analysis was performed using one way ANOVA followed by Tukey's multiple comparison test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. (G) Photos represented the changes of root tuber of *P. heterophylla* after treating 20 mg/L, 75 mg/L or 150 mg/L GA3 for 60d. Scale bar, 20 mm. (H -K) The changes of the diameter of per root tuber, weight of the underground part, weight of per root tuber, the length of per root tuber after treating 20 mg/L, 75 mg/L or 150 mg/L GA3 for 20d, 40d or 60d. All tuberous roots of per strain were measured, and their means were used for statistical chart. Data represents mean ± sem. n = 11 strain/group. Statistical analysis was performed using one way ANOVA followed by Tukey's multiple comparison test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. (L) Photos represented the changes of tissue structure in the expanded portion of root tuber of 20 mg/L, 75 mg/L or 150 mg/L GA3-treated *P. heterophylla.* Scale bar, 100 μm. (M -Q) The changes of the radius of xylem, the cellular layers of xylem, the width of secondaryphloem, cellular layers of secondaryphloem and vescular cambium fter treating 20mg/L, 75mg/L or 150mg/L GA3 for 60d. 8 tuberous roots of per strain were measured, and their means were used for statistical chart. Data represents mean ± sem. n = 6 strain/group. Statistical analysis was performed using one way ANOVA followed by Tukey's multiple comparison test; $^{*}p < 0.05$, $^{**}p < 0.01$.

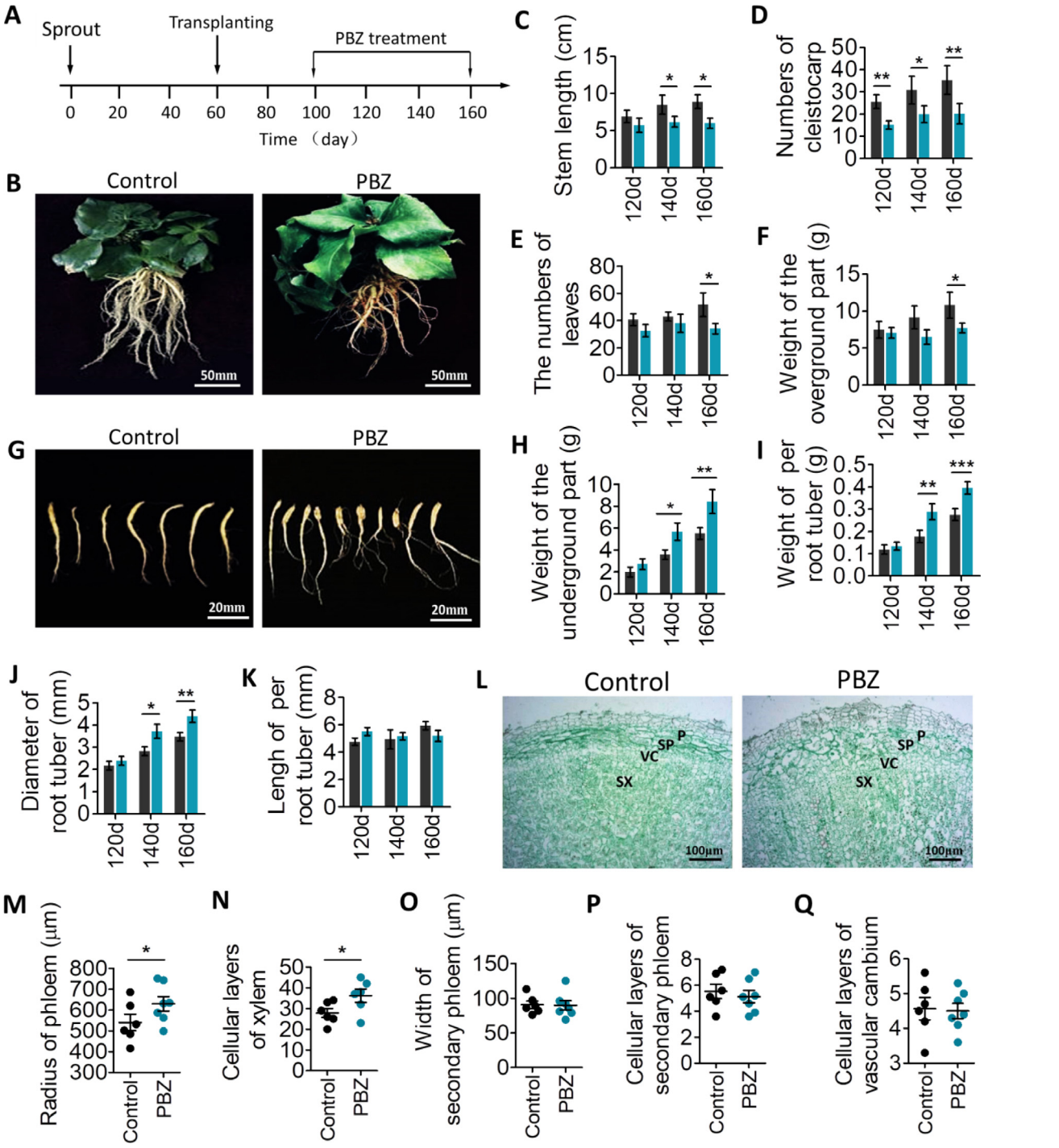


Fig. 3. Blocking the biosynthesis pathway of GA3 accelerated root tuber expanding. (A) Time-line for PBZ treating to *P. heterophylla*. (B) Photos represented the changes of appearance of *P. heterophylla* after treating PBZ for 60d. Scale bar, 50 mm. (C -F) The changes of stem length, number of cleistocarp, number of leaves and the weight of overground part after treating PBZ for 20d, 40d or 60d. Data represents mean ± sem. n = 10 strain/group. Statistical analysis was performed using Independent-Samples T test; * $p < 0.05$, ** $p < 0.01$. (G) Photos represented the changes of root tuber of *P. heterophylla* after treating PBZ for 60d. Scale bar, 20 mm. (H -K) The changes of the diameter of per root tuber, weight of the underground part, weight of per root tuber, the length of per root tuber after treating PBZ for 20d, 40d or 60d. All tuberous roots of per strain were measured, and their means were used for statistical chart. Data represents mean ± sem. n = 10 strain/group. Statistical analysis was performed using Independent-Samples T test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$. (L) Photos represented the changes of tissue structure in the expanded portion of root tuber of PBZ-treated *P. heterophylla.* Scale bar, 100 μm. (M -Q) The changes of the radius of xylem, the cellular layers of xylem, the width of secondaryphloem, cellular layers of secondaryphloem and vescular cambium after treating PBZ for 60d. 8 tuberous roots of per strain were measured, and their means were used for statistical chart. Data represents mean ± sem. n = 6 strain/group. Statistical analysis was performed using Independent-Samples T test; *$p < 0.05$.

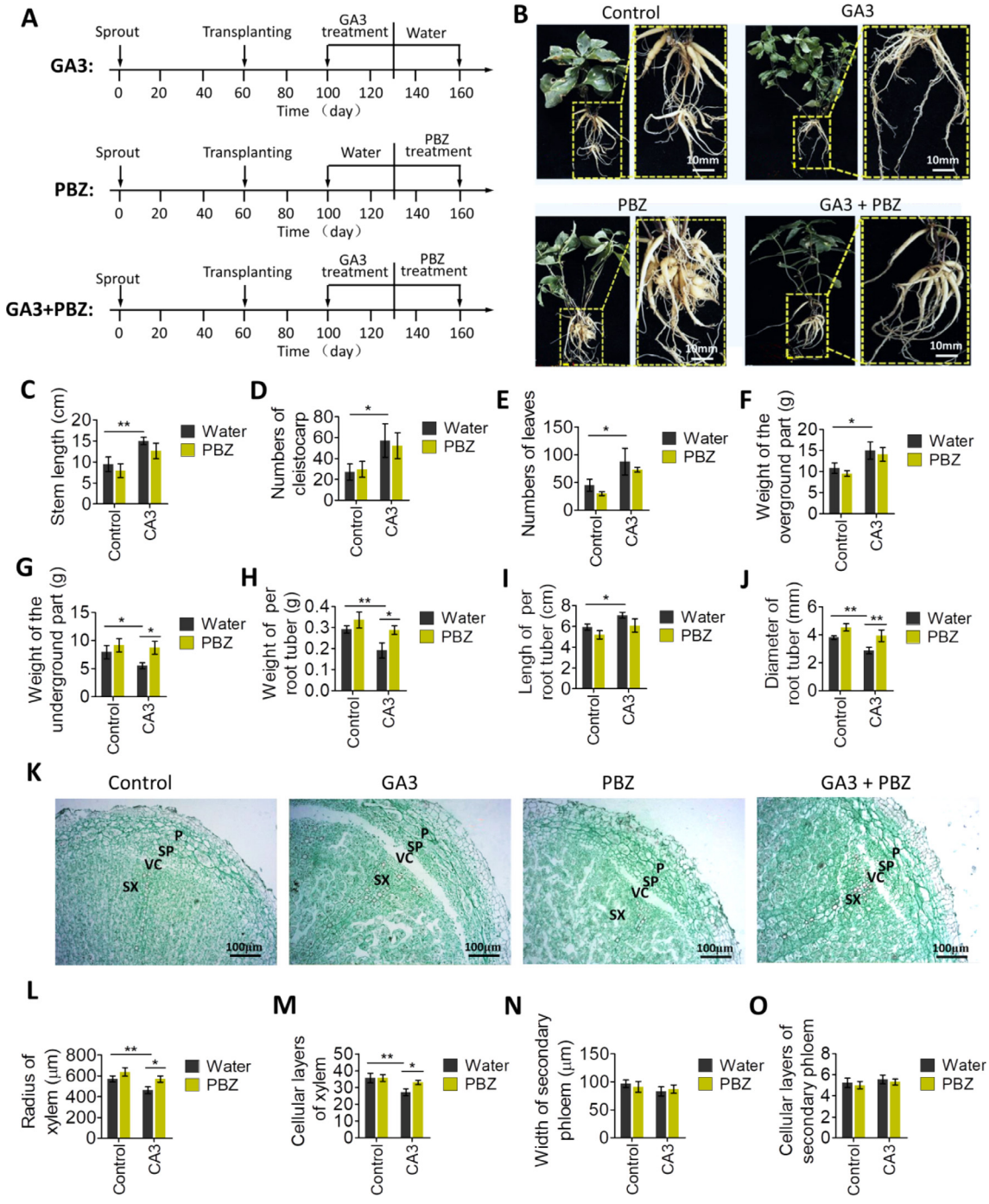


Fig. 4. Blocking the synthesis of gibberellin after exogenous gibberellin treatment could get tuberous roots expanding back on track again. (A) Experiment design for blocking the synthesis of gibberellin after exogenous gibberellin treatment. The GA3 group treated 150 mg/L GA3 between 100 and 130 days, the PBZ group treated PBZ between 130 and 160 days, and the GA3 + PBZ group treated 30 mg GA3 between 100 and 130 days following treated PBZ between 130 and 160 days. (B) Photos represented the changes of appearance of *P. heterophylla* after treating GA3 or/and PBZ. Scale bar, 10 mm. (C -F) The changes of stem length, number of cleistocarp, number of leaves and the weight of overground part after treating GA3 or/and PBZ. Data represents mean ± sem. n = 10 strain/group. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test; * $p < 0.05$, ** $p < 0.01$. (G -J) The changes of the diameter of per root tuber, weight of the underground part, weight of per root tuber, the length of per root tuber after treating GA3 or/and PBZ. All tuberous roots of per strain were measured, and their means were used for statistical chart. Data represents mean ± sem. n = 10 strain/group. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test; * $p < 0.05$, ** $p < 0.01$. (K) Photos represented the changes of tissue structure in the expanded portion of root tuber after treating GA3 or/and PBZ. Scale bar, 100 μm. (L -O) The changes of the radius of xylem, the cellular layers of xylem, the width of secondaryphloem, cellular layers of secondaryphloem and vescular cambium after treating GA3 or/and PBZ. 8 tuberous roots of per strain were measured, and their means were used for statistical chart. Data represents mean ± sem. n = 6 strain/group. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test; * $p < 0.05$.

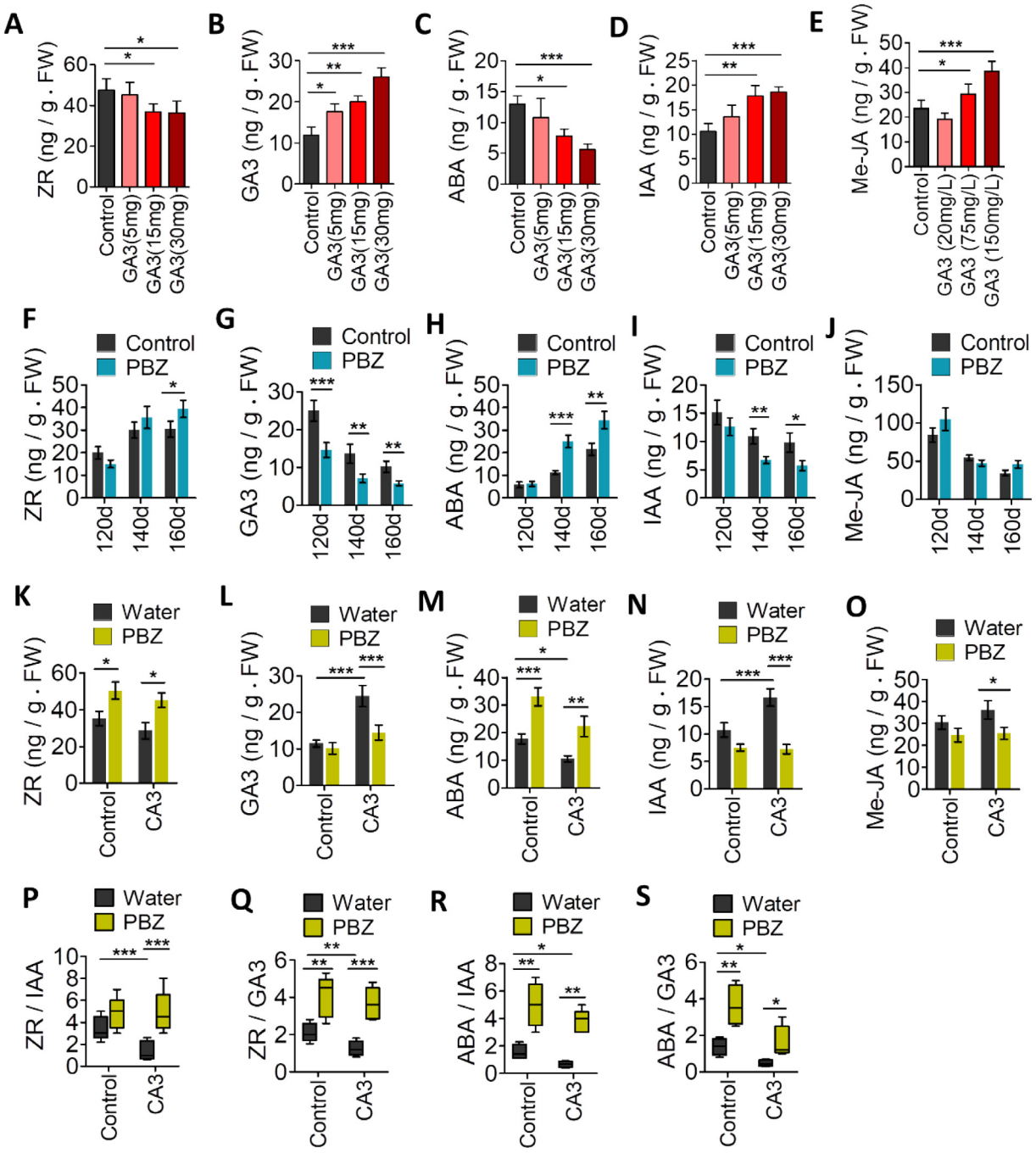


Fig. 5. GA3 disturbs the balance of endogenesis hormones in xylem of tuberous roots. (A -E) The changes of the content of zeatin-riboside (ZR), gibberellic acid (GA3), indoleacetic acid (IAA), abscisic acid (ABA) and methyl ester-jasmonic acid (Me-JA) in xylem of root tuber after treating 20 mg/L, 75 mg/L or 150 mg/L GA3 GA3 for 60d. Data represents mean ± sem; n = 8 strain/group. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test; * $p < 0.05$, ** $p < 0.01$, ***$p < 0.005$. (F -J) The changes of the content of ZR, GA3, IAA, ABA and Me-JA in xylem of root tuber after treating PBZ for 20d, 40d or 60d. Data represents mean ± sem; n = 8 strain/group. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. (K -O) The changes of the content of ZR, GA3, IAA, ABA and Me-JA in xylem of root tuber after treating GA3 or/and PBZ. Data represents mean ± sem; n = 8–9 strain/group. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test; * $p < 0.05$, *** $p < 0.005$. (P -S) The changes of the ratio of ZR/GA3, ZR/IAA, ABA/GA3 and ABA/IAA in xylem of root tuber after treating GA3 or/and PBZ. Data represents mean ± sem; n = 8–9 strain/group. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test; * $p < 0.05$, *** $p < 0.005$.

3.6. GA3 affected endogenesis hormones by regulating their synthesis-related genes in xylem of tuberous roots

To investigate the molecular mechanism of gibberellin affecting plant hormone balance, we examined the changes of hormone synthesis-related genes by qPCR. The results showed that GA3 or PBZ treatment dramatically changed the expression of IAA synthesis-related genes (*GH3.1* and *TMC*), CTK synthesis-related genes (*A-IPT2* and *CKX1*), GAs synthesis-related genes (*GA20ox1* and *GID2*), ethylene synthesis-related genes (*ACO2, ASC2* and *SAMS*), ABA synthesis-related genes (*AOO* and *CYP707A1*) in xylem of root tuber of *P. heterophylla* (Fig. 6A). PBZ treatment reversed the GA3-induced increase in *GH3.1*, *TMC, A-IPT2, GA20ox1* expression in xylem of root tuber of *P. heterophylla* (Fig. 6B–G). Moreover, PBZ

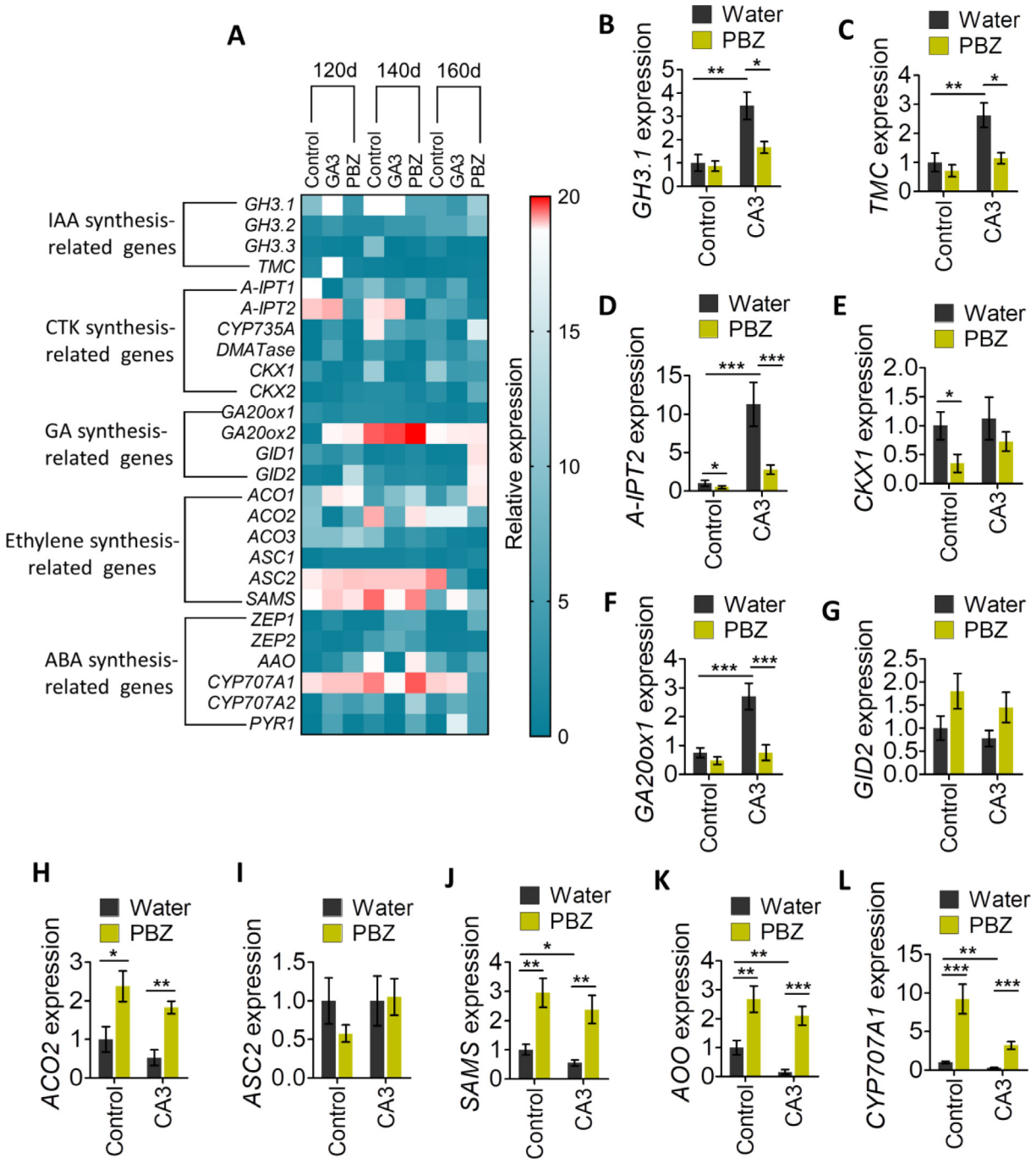


Fig. 6. GA3 affected endogenesis hormones by regulating their synthesis-related genes in xylem of tuberous roots. (A) Hot-map revealed the changes of plant hormones-related genes expression in xylem of root tuber after treating GA3 or PBZ for 20d, 40d or 60d in *P. heterophylla*. Test results from q-PCR were standardized to pre-treatment (100d) levels. Data represents mean; n = 4 strain/group, per sample performed three repeats. (B -L) The changes of IAA-related genes (*GH3.1* and *TMC*), CTK-related genes (*A-IPT2* and *CKX1*), GAs-related genes (*GA20ox1* and *GID2*), ethylene-related genes (*ACO2, ASC2* and *SAMS*), ABA-related genes (*AOO* and *CYP707A1*) expressions were examined by qPCR in xylem of root tuber of *P. heterophylla* after treating GA3 or/and PBZ. Data represents mean ± sem; n = 4 strain/group, per sample performed three repeats. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test; * $p < 0.05$, ** $p < 0.001$.

treatment reversed the GA3-induced decrease in ethylene synthesis-related genes (*ACO2* and *SAMS*), ABA synthesis-related genes (*AOO* and *CYP707A1*) in xylem of root tuber of *P. heterophylla* (Fig. 6H–L).

4. Discussion

GA3 plays a crucial role in plant development and in response to environmental stimuli. GA3 regulated organ formation and gravitropism by modulating the plant hormone auxin synthesis and transporters. However, the effect of gibberellin on tuberous roots expanding is still unclear. Here we reported novel properties of gibberellin that disturbs the balance of endogenesis hormones and stunts tuberous roots expanding in *Pseudostellaria heterophylla* (see Fig. 7).

As one of the main Chinese medicinal materials, *Pseudostellaria heterophylla* possessed multiple functions. The main part of medicine of *P. heterophylla* is its root tuber. The properties of root tuber

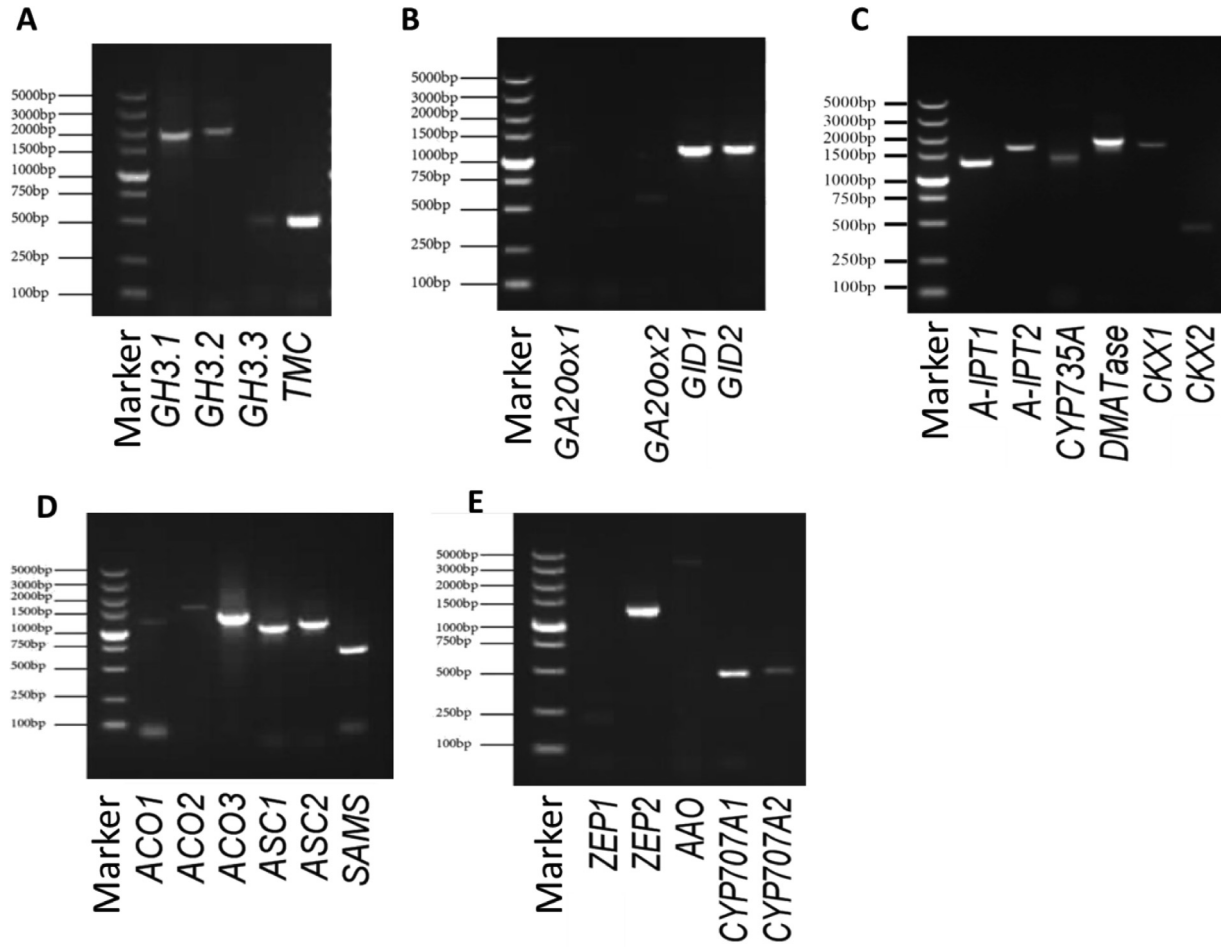


Fig. 7. S1 Identifying the molecular weight of endogenesis hormones synthesis-related genes in *P. heterophylla*. The molecular weight of IAA-related genes (A), CTK-related genes (B), GAs-related genes (C), ethylene-related genes (D), ABA-related genes (E) expressions were identified by PCR in *P. heterophylla*.

directly affect the quality and yield of *P. heterophylla* (Wang and Ng, 2001). However, its development and root tuber formation are rarely reported. Thus, we first made a complete time-axis of *P. heterophylla* from seedling (0d) to harvest (180d). We found overground part of *P. heterophylla* stop growing, and their underground part set out accelerated growth at 120d. About root tuber formation, *P. heterophylla* generates haustorial roots at about 60d, and these haustorial roots keep expand during 80d−160d. After 160d, root tuber of *P. heterophylla* cease to grow. These finding suggested that the expanding period of root tuber of *P. heterophylla* is mainly between 80 days and 160 days.

We examined the plant hormone-related genes from transcriptome database obtained previously to explore the mechanism of root tuber expanding of *P. heterophylla* (Li et al., 2016). We found major of these genes were hyperactive in phloem and xylem of root tuber in *P. heterophylla*. The result suggested that root tuber expanding associated with plant hormone synthesis, transport and bioactivation in root tuber in *P. heterophylla* (Suttle et al., 2013). We further measured the content variation of Me-JA, GA, ZR, ABA and IAA during the development of root tuber of *P. heterophylla* with ELISA. The results showed that the content of ZR and ABA increased gradually following root tuber expanding of *P. heterophylla*. In contrast, the content of GA3, IAA and Methyl ester-jasmonic acid (Me-JA) were decreased gradually following root tuber expanding of *P. heterophylla*. These results suggested that the root tuber expanding was positively associated with the ABA, but negatively associated with GA3 in root tuber of *P. heterophylla* (Debast et al., 2011).

The *P. heterophylla* were treated with different concentrations of GA3 during root tuber expanding. We found GA3 treatment decreased the diameter of root tuber, the weight of the underground part and the weight of per root tuber in a dose-dependent and time-dependent manner, suggesting taht GA3 suppressed the root tuber expanding. It is noteworthy that CA3 treatment increased stem length, the number of cleistocarp, leaf numbers and the weight of the overground part of *P. heterophylla*. These results suggest GA3 could awaken the plant's meristem and stimulate growth. Numerous studies have also shown that gibberellin treatment promotes stem growth in various plants (Qin et al., 2011). Other studies have shown that gibberellin can promote the activity of apical meristem and increase the geotropism of plants (Nugroho et al., 2012). Our data also showed that GA3 reduces root tuber diameter, but it increases the length of root tuber of *P. heterophylla*. These results suggest GA3 mainly activates the apical meristem in stem and root of plants. But expanding of root tuber needs to activate the lateral meristem. GA3 treatment broke the established distribution of nutrients, which results in suppressing root tuber expanding of *P. heterophylla* (Brock and Kaufman, 1988).

As an auxiliary verification, the *P. heterophylla* were treated with PBZ during root tuber expanding to blocking the biosynthesis of GA3. We found blocking the biosynthesis of GA3 suppressed stem growth and promoted the root tuber expanding. These results suggest that blocking the biosynthesis of GA3 during root tuber expanding can be one of the possible ways to increase the yield of *P. heterophylla* (Pal et al., 2016). It was shown that the

development of adventitious root of *P. heterophylla* included four developmental stages, including promeristem, primary meristem, primary structure and secondary structure stages, and was similar to that of other herbaceous dicotyledons. Different activities of vascular cambium from top to tail of the root led the different diameters of it, so that the root became the spindle-shaped root tuber (Chiatante et al., 2018). GA3 treatment decreased the radius of xylem and the cellular layers of xylem, but had no effect on the secondaryphloem and vescular cambium. In contrast, PBZ treatment decreased the radius of xylem and the cellular layers of xylem. These results suggest that the structure foundation of root tuber expanding of *P. heterophylla* focus on the xylem (De Zio et al., 2018). Exogenous or endogenous gibberellin delayed root tuber expanding by inhibiting the formation of secondary xylem (Liu et al., 2018). After treatment with exogenous gibberellin, *P. heterophylla* were treated with PBZ to inhibit endogenous gibberellin synthesis. We found PBZ treatment reversed GA3-induced decrease in diameter and weight of root tuber, but it did not reverse GA3-induced increase in length of stem and root. These results suggest that inhibiting the synthesis of endogenous gibberellin in late period could not save the effect of exogenous gibberellin on the overground parts of *P. heterophylla.*

The growth of plant tissues mainly depends on the hormones regulation (Martins et al., 2018). We next examined the changes of various plant endogenous hormones in xylem of root tuber of GA3- or/and PBZ-treated *P. heterophylla.* We found GA3 treatment increased ZR, GA3 and IAA, and decreased ABA and Me-JA in in xylem of root tuber of *P. heterophylla* (Gao et al., 2017a, Gao et al., 2017b; Liang et al., 2018; Wang et al., 2017). In contrast, inhibiting gibberellin synthesis could increase the content of ZR and ABA, and decrease the content of GA3 and IAA in xylem of root tuber. These data suggest that GA3 disturbs the balance of endogenesis hormones to stunt tuberous roots expanding[8].

Regulating endogenesis hormones may involve synthesis, transport and biological activation. Numerous studies have shown that gibberellin regulates the transport and biological activation of plant hormones. Our study found GA3 regulated synthesis of endogenesis hormones by modulating their genes expression. These endogenesis hormones contain IAA, endogenesis GA3, ABA, Me-JA, ZR and ethylene.

5. Conclusion

GA3 promotes the activity of apical meristem and the geotropism of plants. Our results demonstrated GA3 promote stem tip and root tip growth but suppress the root tuber expanding. These results suggest GA3 broke the established distribution of hormones by regulating the hormones' synthesis, transport and biological activation to activate the apical meristem and suppress lateral meristem. Regulating GA3 signaling during root tuber expanding could be one of the possible ways to increase the yield of *P. heterophylla*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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