

# Paclobutrazol suppresses the synthesis of key secondary metabolites by antagonizing gibberellin signaling in *Pseudostellaria heterophylla*

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**Keywords:** Paclobutrazol, *Pseudostellaria heterophylla*, Gibberellin, saponins, heterophyllin B

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# Abstract

**Background** *Pseudostellaria heterophylla* is one of the most well-known traditional Chinese medicines, and its secondary metabolites play an important medicinal role. Paclobutrazol (PBZ), an antagonist of the plant hormone gibberellin, is widely used to increase the yield of *P. heterophylla*. Whether PBZ affects the synthesis and accumulation of the plant's secondary metabolites is unclear. Here we investigated the effects of PBZ on the production of polysaccharides, saponins and heterophyllin B in the root tubers of *P. heterophylla*, and further explored its molecular mechanisms.

**Results** We found that PBZ promoted the yield of *P. heterophylla*, but reduced the accumulation of polysaccharides, saponins and heterophyllin B. PBZ dramatically downregulated genes associated with gibberellin synthesis and reduced concentration of the hormone in root tubers. Inhibition of gibberellin signaling by PBZ altered the expression of genes involved in synthesis and metabolism of polysaccharides, saponins and heterophyllin B. Exogenous gibberellin reversed these effects.

**Conclusion** These results suggest that PBZ suppresses the synthesis of saponins, polysaccharides and heterophyllin B by antagonizing gibberellin signaling in *P. heterophylla*, providing important guidance for its cultivation.

## Background

*Pseudostellaria heterophylla* belongs to the Caryophyllaceae family of flowering plants [1, 2]. Its root tubers, Radix Pseudostellariae, are highly valued in traditional Chinese medicine and provide several health benefits due to high concentrations of certain secondary metabolites. Polysaccharides in the tubers exert anti-oxidant effects; saponins, anti-inflammatory effects; and cyclic peptides, antibacterial and antitumor effects [3–6]. To promote root tuber growth, *P. heterophylla* is often cultivated in the presence of paclobutrazol (PBZ), a triazole fungicide that retards internodal plant growth [7]. Whether PBZ affects the synthesis and accumulation of secondary metabolites in root tubers of *P. heterophylla* is unclear, and answering this question is important in order to optimize the medicinal efficacy of the plant material.

PBZ is an antagonist of the plant hormone gibberellin [8], which is a key regulator of plant growth and development [9, 10]. PBZ inhibits gibberellin biosynthesis, reducing internodal growth such that stems become thicker and roots grow more [11]. Since gibberellin affects the synthesis and accumulation of secondary metabolites [12], we speculated that PBZ might affect the synthesis and/or metabolism of polysaccharides, saponins and heterophyllin B in *P. heterophylla* root tubers, in turn affecting their medicinal efficacy.

To test this hypothesis, we systematically investigated the effects of PBZ on the growth of *P. heterophylla* as well as on the synthesis and metabolism of secondary metabolites in root tubers.

## Results

## **PBZ stimulates growth of *P. heterophylla* root tubers but decreases their secondary metabolite content**

Root tubers of *P. heterophylla* were collected at different time points, and the abundance of starch granules, polysaccharides, saponins and heterophyllin B were assessed. The root tubers grew rapidly between 100 and 180 days after seeding, a period known as tuberous root enlargement (Fig. 1A). The total abundance of polysaccharides, saponins and heterophyllin B in the root tubers of *P. heterophylla* increased with time (Fig. 1B-F).

We next investigated the effects of PBZ treatment on root tuber growth and the content of secondary metabolites in tubers. PBZ significantly promoted root tuber growth (Fig. 2A-D) and significantly reduced their content of polysaccharides, saponins and heterophyllin B (Fig. 3A-F).

## **Pbz Decreases Secondary Metabolite Levels In Root Tubers By Downregulating Gibberellin Synthesis**

To investigate how PBZ may affect secondary metabolite content in root tubers, we examined the expression of gibberellin synthase by quantitative qRT-PCR (Tables 1 and 2). PBZ dramatically downregulated *GA20ox1*, *GID1* and *GID2* (Fig. 4A). It also reduced the concentration of gibberellin in root tubers (Fig. 4B), which correlated with a reduction in abundance of polysaccharides, saponins and heterophyllin B (Fig. 4C). Indeed, we found that PBZ significantly downregulated genes encoding the subunits of saponin synthase, the precursor peptide of heterophyllin B, and  $\beta$ -1,3-glucan synthase. At the same time, PBZ upregulated the genes encoding subunits of cellulose synthase (Fig. 4D-F).

Table 1  
Primers for quantitative PCR

| <b>Gene</b>    | <b>Forward(5'→3')</b>         | <b>Reverse(5'→3')</b>    |
|----------------|-------------------------------|--------------------------|
| <i>PhACT2</i>  | CTCCATACCGATAAATGAAGGC        | CACTGTTCCAATCTATGAGGGTTA |
| <i>HMGR</i>    | ATGTCATTGGCATCTCTGGA          | CTTCCTTGATGACAGCCTCA     |
| <i>ACTIN</i>   | CTTCCGATTCTGCTCAAAG           | GGGATCTCACCGATCATCTC     |
| <i>CsID1</i>   | AAACCCTGGAGGCCTCTTAC          | CACAGCCAGACTGCATCTTC     |
| <i>CsID2</i>   | ATGGAATGGTTGGAGACGGT          | CCCTAGAACAACCAGCCTGA     |
| <i>CsI</i>     | GTGCGTATGAGTGGGTTGTC          | CCCTCCGATGTATTGGCTCT     |
| <i>CsIG</i>    | TCGTATACGTTGCTCGGGAA          | GATTGACGAGCTGACGATGG     |
| <i>CsIE</i>    | ACGAAGACGAGTTGCCAAAG          | GCCTCCCAAAGAGCATGAAA     |
| <i>GS</i>      | TTGCTGGGTGGTGTTCCTTG          | GACGCCCTTCCTAAGTGTCT     |
| <i>PMK</i>     | TTGGCAGTGGATTTGATGTT          | ACAATGGCTTCCCGAAGTAG     |
| <i>MDD</i>     | CAAGATGCTGGAGTGAAGGA          | GCTTTCATCAGAGAGCACGA     |
| <i>IPPI</i>    | GAGGAATGCTGCACAAAGAA          | CAGCATACGACCAAGAGGAG     |
| <i>SS</i>      | AGGCAATCCAGAAAGTTTGC          | GGACATGATGATCGCCAGTA     |
| <i>SE</i>      | AACTGCTACAGCCTGGAGGT          | TGCAGTAAATTGCTCCAAGG     |
| <i>β-A280</i>  | ATACAACAACACGTGCCCAT          | CTTAGCCAACTCGTGGACCT     |
| <i>prePhHB</i> | AGCCGAGTATTTTTGGGGGT<br>CTTCC | CATAAAGGCCCAACGGTGACGGAC |

Table 2  
Gene screening from transcriptome databases

| Encoded enzyme                                            | Gene name                      | Minigene number | Length (bp) |
|-----------------------------------------------------------|--------------------------------|-----------------|-------------|
| 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA) | <i>HMGR</i>                    | c50562_g1       | 2697        |
| Phosphodioxy mevalerate kinase (PMK)                      | <i>PMK</i>                     | c47313_g1       | 1937        |
| Mevalerate pyrophosphate decarboxylase (MVD)              | <i>MDD</i>                     | c53051_g1       | 1728        |
| Isoprenyl pyrophosphate isomerase (IPPI)                  | <i>IPPI</i>                    | c1497_g1        | 1170        |
| Squalene synthase (SQS)                                   | <i>SS</i>                      | c65449_g4       | 928         |
| Squalene epoxidase (SQLE)                                 | <i>SE</i>                      | c59462_g1       | 2245        |
| Hederagenin 28-O-beta-D-glycosidase                       | <i><math>\beta</math>-A28O</i> | c56046_g1       | 1626        |
| Cellulinoid synthase                                      | <i>CsID1</i>                   | c55631_g1       | 4933        |
|                                                           | <i>CsID2</i>                   | c65503_g1       | 4061        |
|                                                           | <i>Csl</i>                     | c58017_g2       | 2909        |
|                                                           | <i>CslG</i>                    | c52048_g1       | 2603        |
|                                                           | <i>CslE</i>                    | c50652_g1       | 2545        |
| $\beta$ -1,3-glucan synthase                              | <i>GS</i>                      | c28029_g1       | 1104        |

## Exogenous Gibberellin Restores Secondary Metabolite Levels In Pbz-treated Root Tubers

To confirm that PBZ influences levels of polysaccharides, saponins and heterophyllin B in root tubers *via* gibberellin pathways, we treated *P. heterophylla* with different concentrations of the hormone in the absence of PBZ (Fig. 5A). Exogenous gibberellin acted in a concentration-dependent manner to upregulate genes encoding enzymes of secondary metabolite synthesis in root tubers, as well as the abundance of polysaccharides, saponins and heterophyllin B (Fig. 5B-G). Next, we treated plants with gibberellin in the presence of PBZ. The hormone reversed PBZ-induced downregulation of secondary metabolite-associated enzymes (Fig. 6A-C) and the PBZ-induced decrease in starch granules, polysaccharides, saponins and heterophyllin B (Fig. 6D-H).

## Discussion

By regulating the synthesis and transport of auxin, the hormone gibberellin helps plants respond to environmental stimuli and develop properly [9, 17]. Gibberellin has been shown to influence germination, elongation, growth, flowering, organ formation and gravitropism. Here we expand that list of functions to include the synthesis of polysaccharides, saponins and heterophyllin B in root tubers of *P. heterophylla*.

*P. heterophylla* is used to produce numerous traditional Chinese medicines [13]. The plant exerts its medicinal effects in large part through the heterophyllin B, saponins, and polysaccharides in its root tubers [18]. Here we show that despite its benefits in promoting *P. heterophylla* yield [19, 20, 21], it reduces the accumulation of saponins and heterophyllin B, suggesting that PBZ may be incompatible with sustainable cultivation of *P. heterophylla* for medicinal use.

We found that PBZ upregulated genes involved in polysaccharide synthesis but downregulated genes involved in synthesis of saponin and heterophyllin B in root tubers of *P. heterophylla*. These results may explain how PBZ can promote the growth of root tubers on one hand, i.e. by increasing polysaccharide content, while reducing their content of saponin and heterophyllin B.

We found that PBZ downregulated gibberellin synthesis in *P. heterophylla*, consistent with a previous study in *Eucalyptus grandis* [22]. In addition, we found that exogenous gibberellin reversed the PBZ-induced decreases in gene expression and secondary metabolite levels in root tubers. Our results imply that gibberellin increases levels of saponins and heterophyllin B in *P. heterophylla* by upregulating the enzymes that synthesize those compounds. This is in line with observations that plant hormones affect the production of secondary metabolites [23, 24]. The positive actions of gibberellin observed in our study contrast with its inhibition of genes that encode enzymes to synthesize phenylpropane and flavonoids, which allows the hormone to inhibit fruit rust [25, 26].

Our study suggests that PBZ may not be suitable for medicinal cultivation of *P. heterophylla*, and that it may be possible to optimize levels of medicinally active secondary metabolites in the plant, especially saponins and cyclic peptides, by fine-tuning the level of gibberellin. This study provides guidance for cultivation of *P. heterophylla* and potentially other plants important in traditional Chinese medicine.

## Conclusions

Our study suggests that PBZ may not be suitable for medicinal cultivation of *P. heterophylla*, and that it may be possible to optimize levels of medicinally active secondary metabolites in the plant, especially saponins and cyclic peptides, by fine-tuning the level of gibberellin. This study provides guidance for cultivation of *P. heterophylla* and potentially other plants important in traditional Chinese medicine.

## Methods

### Plant material

Seeds of *P. heterophylla* were purchased from Shibing Seed Company (Kaili, China) and sown in a greenhouse at 18–25°C at a relative humidity of 60–88% and an average illumination of 17.06–31.42 KLX. Seedlings (60 days old) were individually transplanted into flowerpots with a diameter of 20 cm and height of 30 cm, and cultured under the same conditions.

## Treatments With Pbz Or Gibberellin

PBZ (Solarbio, China) was diluted with water to 20 mg/L. Gibberellin (Takara, Japan) in DMSO was diluted with water to 25, 75 and 150 mg/L. *P. heterophylla* plants (100 days old) were watered once every 10 days with 200 mL PBZ or gibberellin solution. Control plants were watered with 200 mL water.

## Microscopy Of Root Tubers

Root tubers of *P. heterophylla* were fixed with Formalin-Aceto-Alcohol, dehydrated, embedded in paraffin, then sliced on an RM2245 microtome (Leica, Weztlar, Germany) into sections 8–10 µm thick. Sections were dewaxed using xylene and ethanol, rinsed for 2 min with tap water (continuous drip), then rinsed with distilled water for 1 min. Iodine-potassium iodide reagent was applied dropwise for 30 s. Sections were imaged by phase-contrast and dark-field on an BX41 microscopy (Olympus, Tokyo, Japan). Positively stained areas were quantified as a proportion of the total area using Image J software (version 1.45J; National Institutes of Health, Bethesda, MD, USA). In all experiments, at least three samples from each of at least eight plants were analyzed for each condition.

## Analysis Of Secondary Metabolites

High-performance liquid chromatography was used to quantify heterophyllin B in *P. heterophylla* root tubers according to the standard procedure in the Pharmacopoeia of the People's Republic of China. The extract was separated on an LC-20AD Prominence HPLC system (Shimadzu, Kyoto, Japan). Solvent A was water-trifluoroacetic acid (TFA; 0.25%, v/v), and solvent B was acetonitrile-TFA (0.25%, v/v). The gradient of solvent B (v/v) was as follows: 0–5 min, 5%; 5–25 min, 5%–65%; 25–35 min, 65%; 30–33 min, 65%–100%; 33–35 min, 100%; and 35–36 min, 5%.

Heterophyllin B was identified based on comparison with the retention time and spectral data of heterophyllin B chromatogram. The content of heterophyllin B in the test solution was calculated using the one-point external standard method. Standard heterophyllin B (content of 98%) was purchased from Alfa Biotechnology (Chengdu, China).

The polysaccharide content of *P. heterophylla* root tubers was determined with the phenol-sulfuric acid method [13]. A standard curve was obtained with glucose. Total saponins were extracted from root tubers by the ultrasonic method. Briefly, 100 g of root tuber powder was weighed and soaked in distilled water for 2 hours. After ultrasonic extraction for 1 hour, 70% ethanol was added, the extract was allowed to

stand overnight, then it was drained and compressed, after which it was extracted with n-butanol. The various extracts were then combined and concentrated to dryness on a Rotavap. The residue was dissolved in 10 mL methanol, and a sample of 0.05 mL was dried, redissolved in 0.2 mL of 5% vanillin and 0.8 mL perchloric acid added, heated for 15 min in a 60 °C water bath, mixed with 5 mL of glacial acetic acid, and shaken well. The absorbance was measured at 560 nm, and the concentration of total saponins in the test solution was obtained from the standard curve.

## Rna Extraction And Quantitative Qrt-pcr

Total RNA was extracted from each sample with the RNAiso Plus Reagent (TaKaRa, Japan) according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA using a reverse transcriptase kit (TaKaRa, Japan). Quantitative qRT-PCR was performed on a 7500 real time PCR system (Applied Biosystems, California, USA). with 10 µL of reaction solution containing 1 µL of cDNA, 0.3 µM of each primer, and 5 µL of iQ SYBR Green Supermix by using. Sequences of primer pairs are listed in Table 1. The target genes (Table 2) were selected because of their known roles in the synthesis of polysaccharides, saponins and heterophyllin B [14–16]. Gene expression was quantified using the  $-\Delta\Delta C_t$  method relative to *PhACT2* as the internal reference.

## Statistical analysis

Results were expressed as mean  $\pm$  SD. Data were plotted and statistically analyzed using GraphPad Prism8.0 (Graphpad, Chicago, USA). Differences between mean values were evaluated using the independent-samples *t* test and one- or two-way ANOVA, followed by Tukey's multiple comparison test for *post hoc* comparisons when equal variances were assumed. Differences associated with  $P < 0.05$  were considered significant.

## Abbreviations

### PBZ

Paclobutrazol

### KLX

kilolux

### HPLC

High performance Liquid Chromatography

### qRT-PCR

Quantitative Real-time PCR

### GA20ox1

2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein

### GID1

Gibberellin-insensitive dwarf1

GID2

Gibberellin-insensitive dwarf2

**DMSO**

Dimethyl sulfoxide.

## Declarations

### Ethics approval and consent to participate

In this study, seeds of *P. heterophylla* were purchased from Shibing Seed Company (Kaili, China). The seeds were collected with permission in accordance with institutional and national guidelines. All methods were performed in accordance with the relevant guidelines and regulations.

### Consent for publication

Not applicable.

### Availability of data and materials

All data generated or analyzed in this study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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### Authors' contributions

CHX designed the experiments, analyzed the data, and contributed to the final version of the manuscript. TZ designed the experiments, supervised the study, and contributed to the final version of the manuscript. JQZ analyzed the data and contributed to the final version of the manuscript. CZ performed experiments, analyzed the data, and wrote the first draft of the manuscript. ZOY, CGY, STW, QSY, JX and LYL contributed to the final version of the manuscript. All authors reviewed and approved the manuscript before submission.

### Acknowledgements

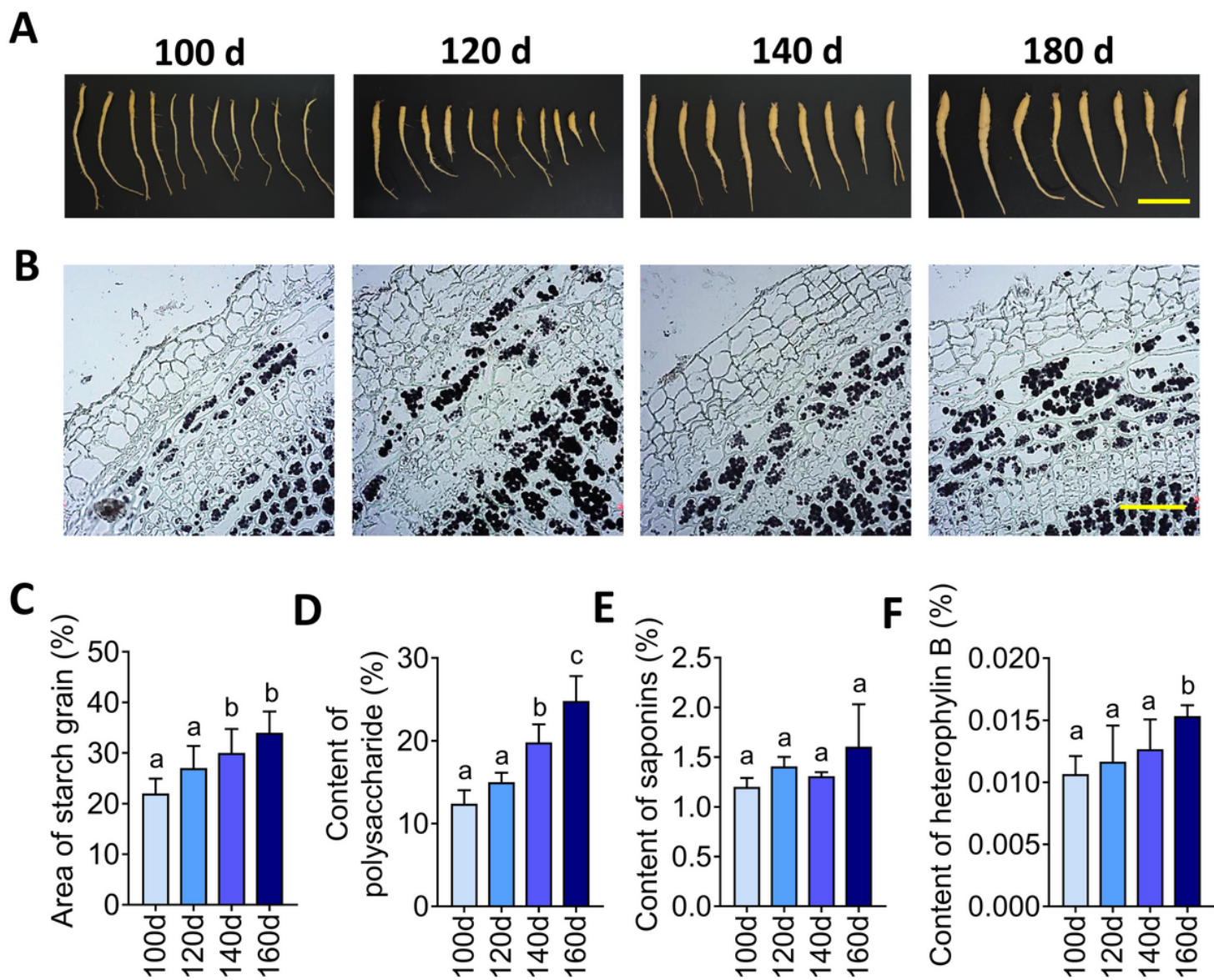
Not applicable.

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## Figures



**Figure 1**

**Content of polysaccharides, saponins and heterophyllin B in root tubers of *P. heterophylla***

(A) Photographs of root tubers at different time points (days) after seeding. Scale bar, 4 cm.

(B) Representative micrographs of starch granules in root tubers. Scale bar, 25  $\mu$ m.

(C-F) Total starch grains and content of heterophyllin B, saponins and polysaccharides in root tubers.

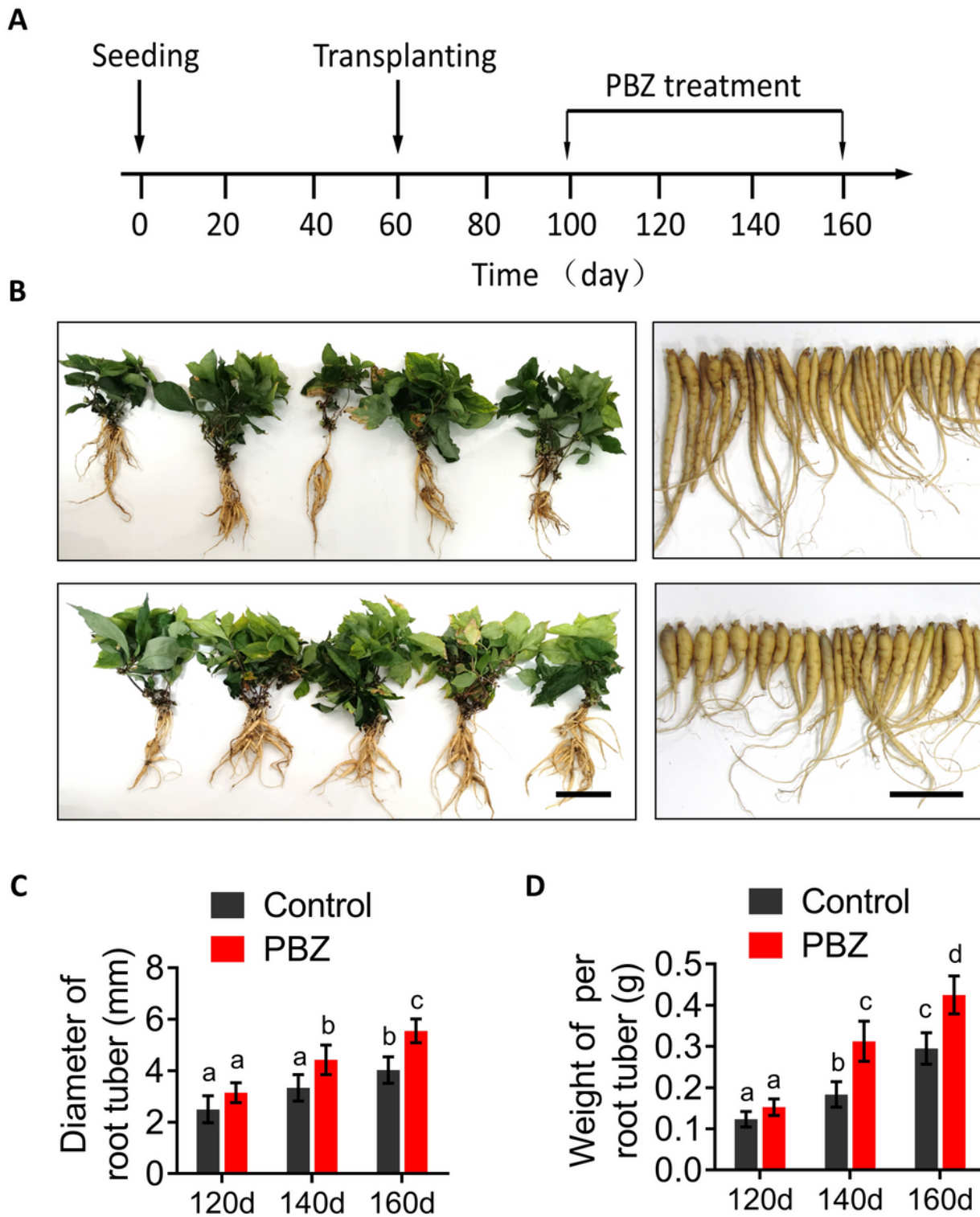


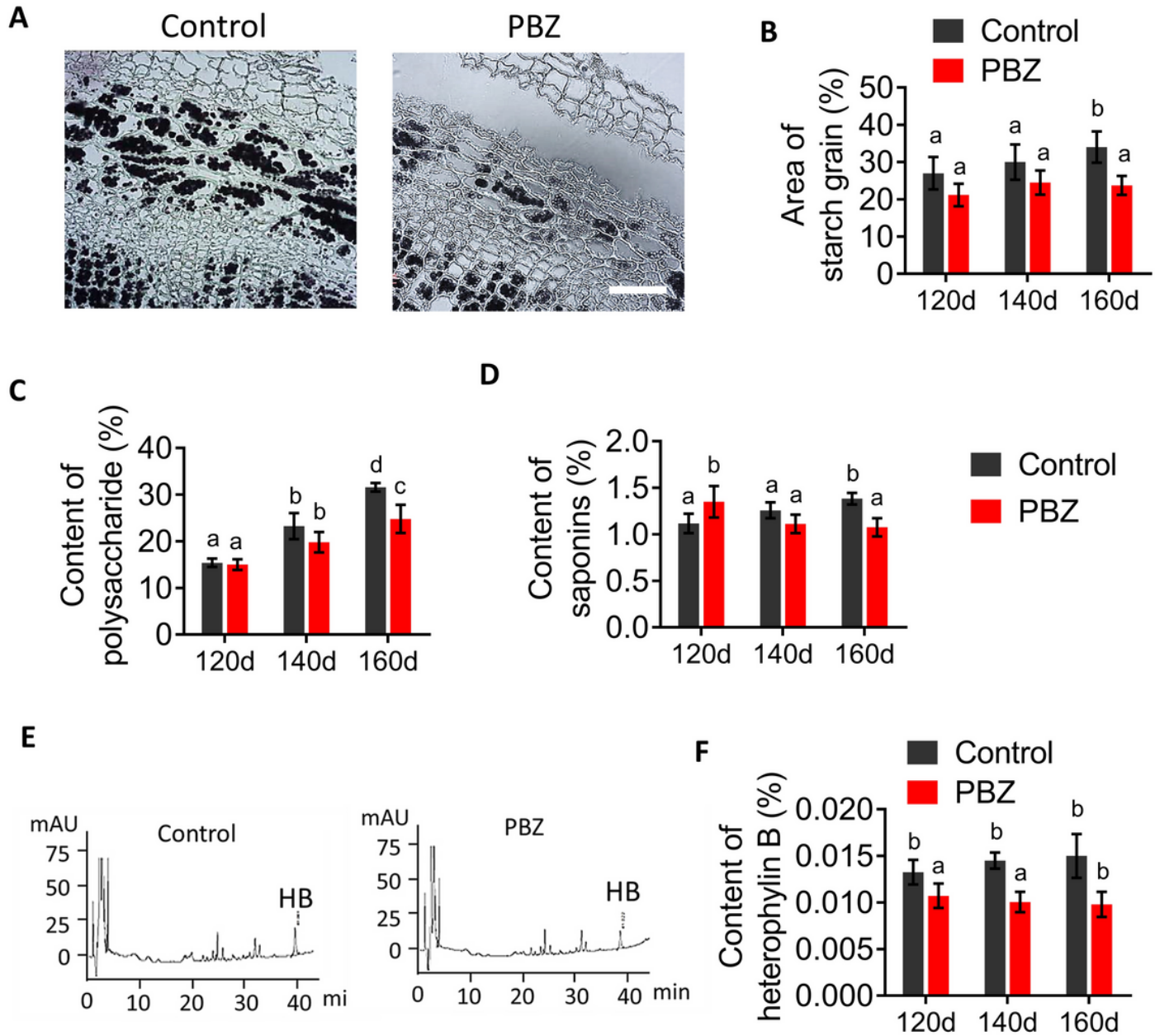
Figure 2

PBZ stimulates growth of *P. heterophylla* root tubers

(A) Study design and timeline of PBZ treatments.

(B) Photographs of root tubers after PBZ treatment on day 160. Scale bar, 4 cm.

(C-D) Average diameter and weight of root tubers after PBZ treatment at the indicated time points.

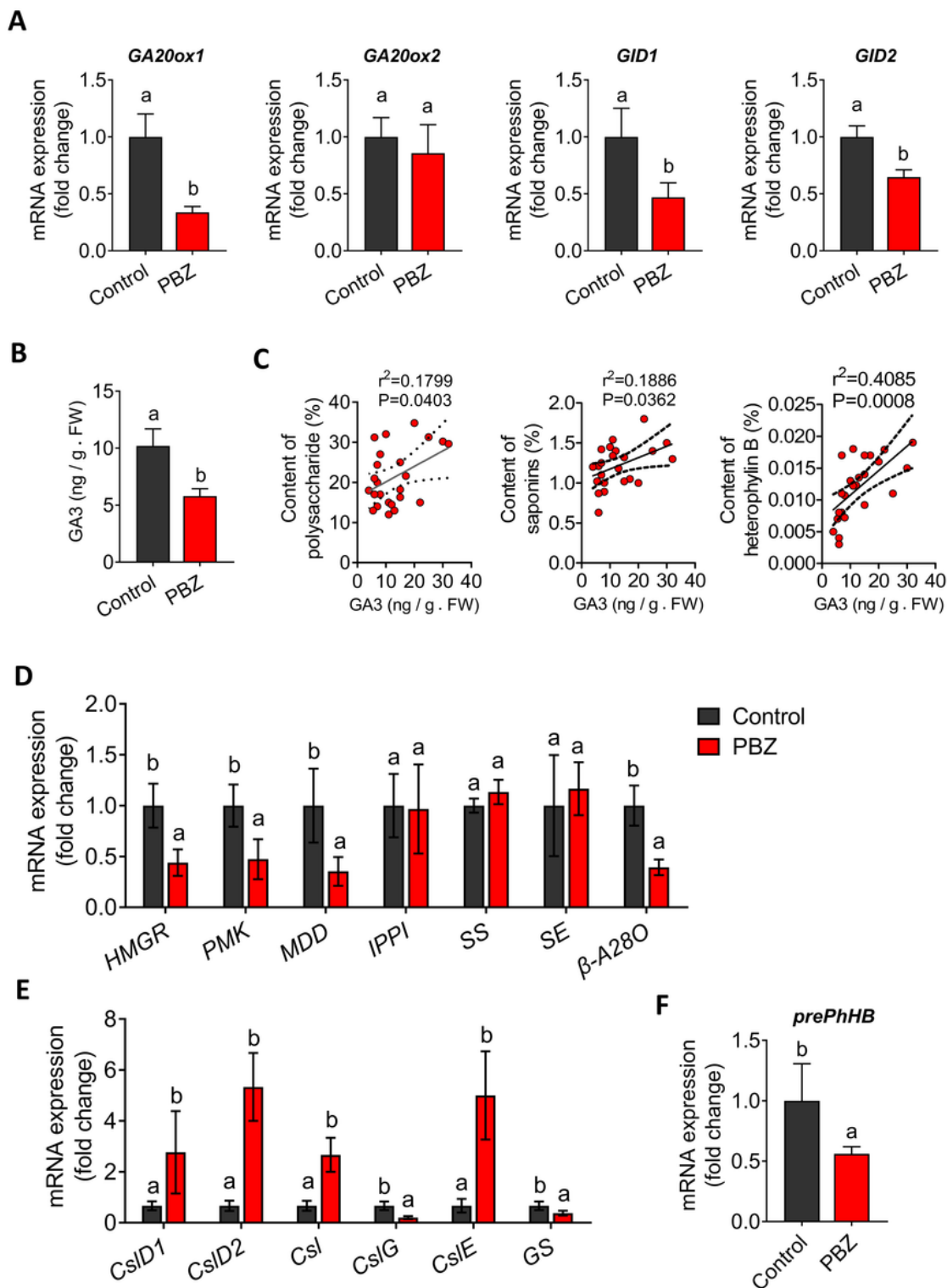


**Figure 3**

### PBZ decreases secondary metabolites in the root tubers of *P. heterophylla*

(A) Representative micrographs of starch grains in root tubers. Scale bar, 25  $\mu$ m.

(B-F) Total starch grains and content of heterophyllin B, saponins and polysaccharides in root tubers after the indicated number of days.



**Figure 4**

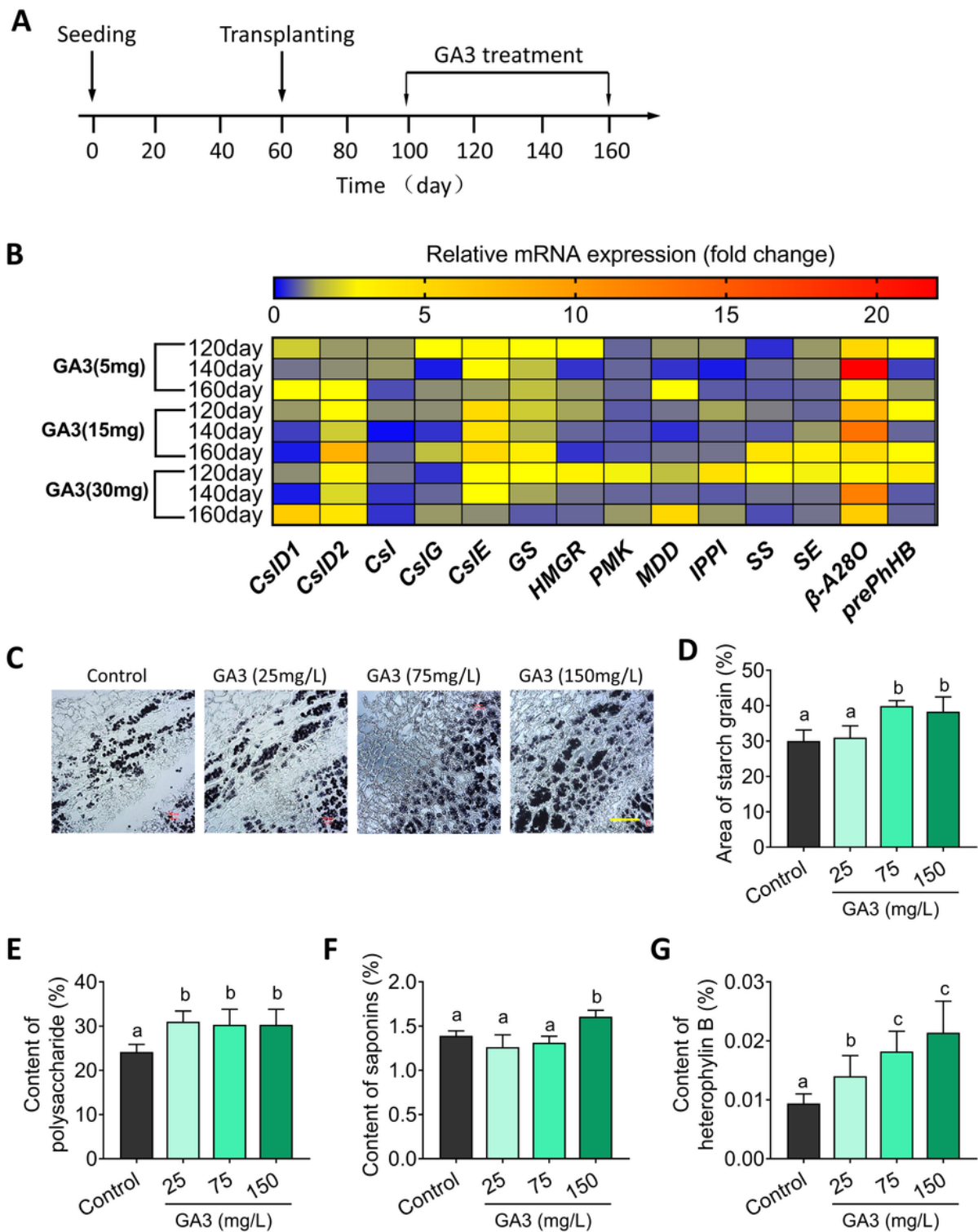
**PBZ alters secondary metabolite abundance by inhibiting gibberellin synthesis in the root tubers of *P. heterophylla***

(A) Levels of mRNAs encoding gibberellin synthase in root tubers after PBZ treatment. Genes are defined in Table 2.

(B) Amount of gibberellin in root tubers after PBZ treatment.

(C) Correlation of gibberellin levels with levels of saponins, polysaccharides, and heterophyllin B in root tubers.

(D-F) Levels of mRNAs encoding enzymes that synthesize saponins, polysaccharides, or heterophyllin B in root tubers after PBZ treatment. Genes are defined in Table 2.



## Figure 5

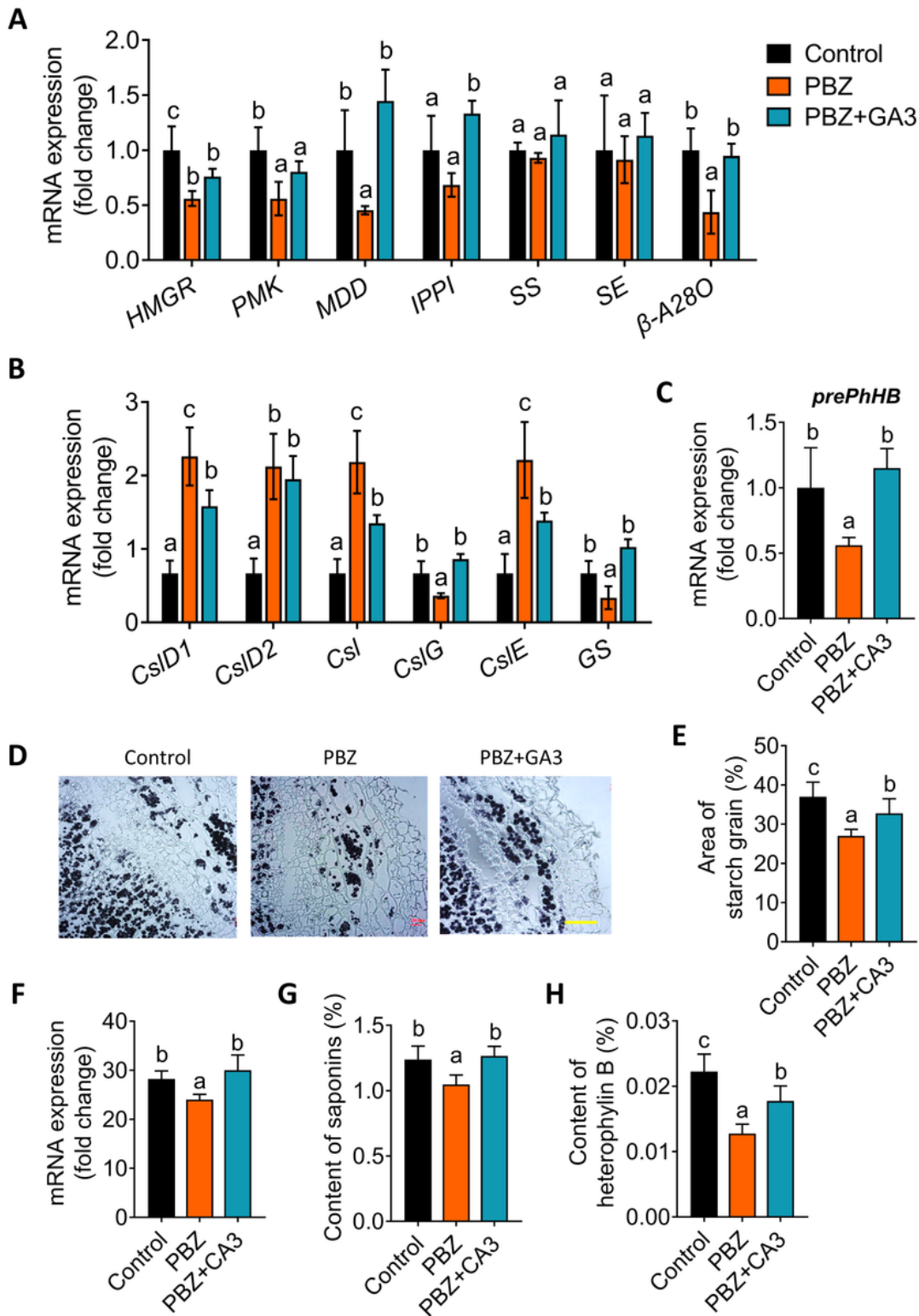
### Exogenous gibberellin increases secondary metabolite levels in root tubers of *P. heterophylla*

(A) Study design and timeline of exogenous gibberellin treatments.

(B) Levels of mRNAs encoding enzymes that synthesize saponins, polysaccharides, and heterophyllin B in root tubers. Genes are defined in Table 2.

(C) Representative micrographs of starch grains in root tubers. Scale bar, 25  $\mu\text{m}$ .

(D-G) Total starch grains and content of heterophyllin B, saponins and polysaccharides in root tubers.



**Figure 6**

**Exogenous gibberellin restores secondary metabolite levels in root tubers of PBZ-treated *P. heterophylla***

(A-C) Levels of mRNAs encoding enzymes that synthesize saponins, polysaccharides, and heterophyllin B in root tubers. Genes are defined in Table 2.

(D) Representative micrographs of starch grains in root tubers. Scale bar, 25  $\mu\text{m}$ .

(E-H) Total starch grains and content of heterophyllin B, saponins and polysaccharides in root tubers.