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Jasmonates mediate increased levels of ginsenosides in *Panax Quinquefolius* cambial meristematic cell cultures

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Abstract

Background CMCs (cambial meristematic cells) are important resources for enhanced biotechnological production of plant secondary metabolites (SMs). However, CMC culture has not yet been established for American ginseng (*Panax quinquefolius*), an important source of ginsenosides, which are highly active SMs. Here, we investigated the molecular mechanisms involved in SMs' production in induced CMC and DDC (dedifferentiated cell) cultures through comparative biochemical, metabolomics, and transcriptome analyses.

Results Relative to dedifferentiated cells (DDCs), CMCs showed significantly higher growth rates and content of ginsenosides (Rg1, Rd1, Re, and Rd), crude protein, fat, total sugar, and endogenous jasmonates (JA, MEJA, and JA-ILE). We identified 521 differential metabolites and 12,591 DEGs (differentially expressed genes). JA-related metabolic pathways, biosynthesis of SMs, and plant hormone signal transduction were significantly induced in CMCs compared to DDCs. Notably, most differential ginsenosides and flavonoids were up-regulated in CMCs. Key highly induced DEGs in the ginsenoside biosynthesis pathway, including the terpenoid pathway-related genes, cytochrome P450 family genes, and UDP-glucuronosyl/UDP-glucosyl transferases, were uncovered. Meanwhile, phytohormone, antioxidant enzyme, and transcription factors (TFs)-related candidate genes were also identified. It was noteworthy that most differentially expressed TFs (particularly EP2/ERF) were highly induced in CMCs compared to DDCs.

Conclusions Our results highlight that high levels of endogenous jasmonates promote the growth and synthesis of ginsenosides, flavonoids, and polysaccharides in American ginseng CMCs. Moreover, they provide fundamental bases for optimizing *Panax spp.* CMC cultures for sustainable industrial-scale production of ginsenosides.

Keywords *Panax quinquefolius*, Cambial meristematic cell, Ginsenoside, Secondary metabolites, Cell culture, Candidate genes

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Background

Medicinal plant-derived drugs, foods, and dietary supplements are essential for improving or treating specific conditions of human physical, mental, and spiritual health and to ensuring sustainable human wellness [1, 2]. Among the most used medicinal plants, *Panax spp*. (including *P. quinquefolius*, American ginseng, AG), are recognized worldwide for their high therapeutic values [3]. AG is native to North America. It has been introduced to China recently and included in the homology catalogue of medicines and foods [4]. Notably, AG received approbation in 2020 from the National Health Commission of the People's Republic of China and the State Administration for Market Regulation to be used in food industries for improving the nutritional value of wine, tea, honey, and beverage products [4]. AG is a rich source of nutraceuticals, such as ginsenosides and polysaccharides [4, 5]. Ginsenosides are the major active compounds in *Panax spp*. Studies revealed that they possess various pharmaceutical abilities, including anticancer, antimetastatic, antioxidant, hypertension, anti-inflammatory, antidiabetes, hepatoprotective, and neuroprotective [6–10]. However, ginsenoside production is still low, limiting its large-scale use and exploitation in pharmaceutical industries. Therefore, it is of great interest to test and apply advanced biotechnological tools and approaches to improve ginsenoside biosynthesis in *Panax spp* and boost its industrial production.

Plant cell culture represents an efficient, controllable, eco-friendly, and sustainable biotechnological approach to producing higher-value natural products for cosmetics, pharmaceuticals, and food industries [11, 12]. Culturing DDCs (callus) from hairy roots, cell suspensions, adventitious roots, and ultimately, on a large scale (bioreactors) has been considered an excellent alternative for improving ginseng biomass and ginsenoside production [5, 13–15]. Different elicitors, such as jasmonates and vanadate, have been used to increase the biomass yield and accumulation of ginsenosides [16–18]. However, there are several obstacles to the industrial production of SMs from DDC culture, such as low yields, slow cell growth, performance instability, industrial scale-up, etc [11]. Most critical, DDCs are heterogeneous, susceptible to harmful genetic changes and epigenetic variations, and unable to divide continuously [11, 19, 20]. Unlike DDCs, CMCs can differentiate and divide indefinitely, which makes them a promising platform to overcome limitations by DDC cultures, enabling the sustainable industrial production of high-value active metabolites [11, 19, 21]. CMC culture has been established and adopted in many plant species for the enhanced production of important SMs [22–25]. For instance, CMC culture has been established to improve the production of triterpenoids in *Ocimum basilicum* [23]. It has been established in *Panax ginseng* and tested for enhanced production of ginsenoside F2 and gypenoside XVII [21]. Unfortunately, no study on CMC culture for ginsenoside production has been reported in AG. We hypothesized that AG CMCs might be excellent resources for the large-scale production of ginsenosides. Therefore, establishing an efficient CMC culture in AG will promote the production and availability of ginsenosides for diverse uses.

Ginsenosides consist of glycosylated triterpenoids that are biosynthesized from the MVA (mevalonate) and MEP (methylerythritol phosphate) pathways, with the MVA pathway playing the most important role [26–29]. Cytochrome P450 and UGTs (UDP-glucuronosyltransferases) catalyze the key steps of ginsenoside biosynthesis [26, 27, 30, 31]. TFs and phytohormones are the key players in regulating ginsenoside biosynthesis [26, 27, 30, 31]. For example, Yao et al. found that the TF *PgWRKY4X* promotes ginsenoside biosynthesis and accumulation by activating squalene epoxidase [32]. Meanwhile, Jiang et al. found that *PgERF120* positively regulates the biosynthesis of ginsenosides by modulating ethylene levels [33]. With the advancement of genomic tools and techniques [34]Exploring the metabolic and molecular divergences between DDCs and CCMs will offer valuable resources for optimizing AG CMC culture for increased production of ginsenosides.

In this study, we established AG CMC culture and investigated differences in biochemical, metabolic, and molecular processes between DDCs and CMCs through integrated biochemical, metabolomics, and transcriptomics analyses. Our objectives were to promote CMC cultures in *Panax spp*, reveal molecular mechanisms involved in improved nutraceuticals' biosynthesis in CMCs of AG, and identify potential candidate genes for genomics-assisted production of high quantities of ginsenosides. Our results may sustain enhanced industrial production of key bioactive compounds from *Panax* species.

Results

Morphology and growth of American ginseng cambium meristematic cells (CMCs) and dedifferentiation cells (DDCs)

To characterize metabolome differences and variation in molecular processes between American ginseng stem cells (cambium meristematic cells, CMCs) and callus cells (dedifferentiation cells, DDCs), we induced CMC and DDC culture (Fig. S1A-G). Microscopic observations showed that CMCs have a soft texture and appear white as single cells or small cell clusters (Fig. 1A, B). In contrast, DDCs form dense large cell clusters with a light yellow color (Fig. 1D, E). Notably, CMCs contain small and numerous vacuoles, while DDCs only contain one large vacuole (Fig. 1C, F). Both CMC and DDC growth rates

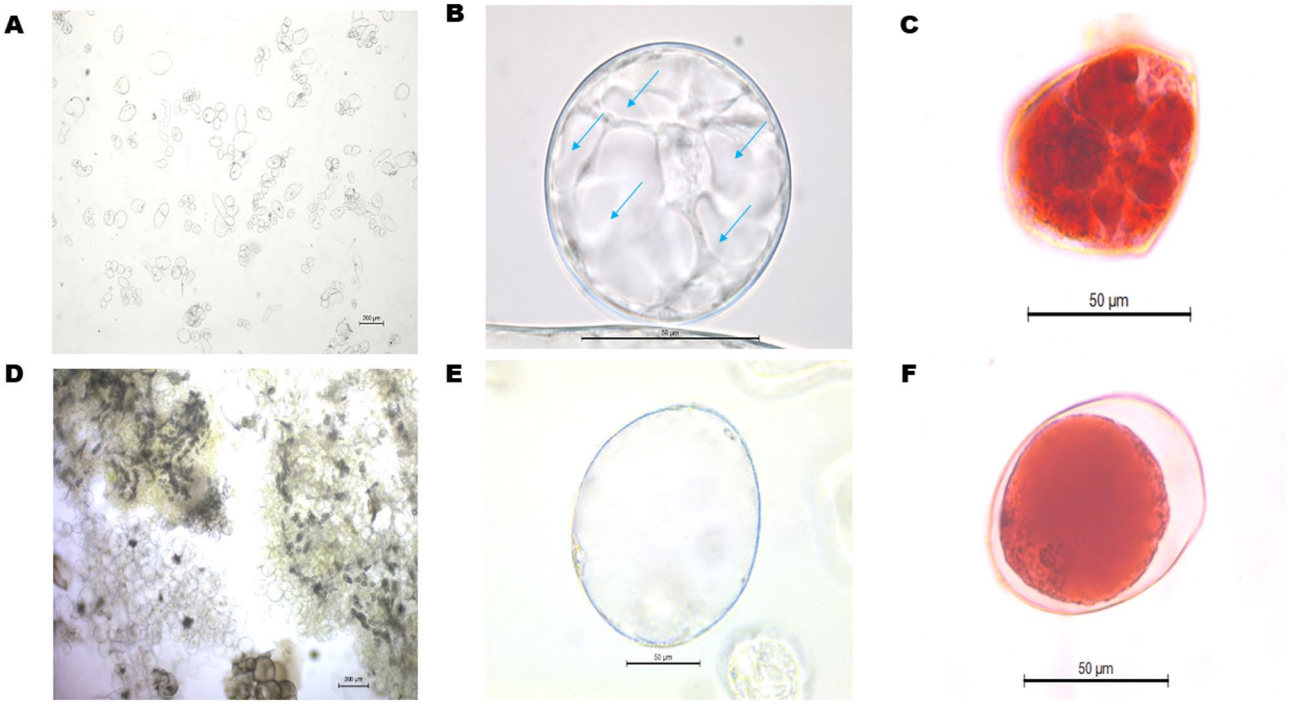

Fig. 1 Microscopic observations of stem cells (CMCs) and callus tissue (DDCs). **A** and **D**Micrographs CMCs and DDCs, respectively, under a light microscope. **B** and **C** Micrographs of CMCs showing multiple vacuoles (blue arrows) in each cell. **E** and **F** Micrographs of DDCs showing a unique big vacuole in each cell. Scale bars are shown on each micrograph

increased in the culture solution for up to 20 days, then decreased (Fig. 2A, B). However, compared to DDC, the CMC growth rate was significantly higher from the 15th day (Fig. 2A, B).

JAs are crucial for plant growth and development and are key stimulators of plant cell proliferation rate in in-vitro conditions [35, 36]. We investigated the levels of endogenous JA (jasmonic acid), MEJA (methyl jasmonate), and JA-ILE (jasmonoyl-isoleucine) in CMCs and DDCs. The results disclosed that both the levels of JA, MEJA, and JA-ILE in CMCs were significantly higher than in DDCs (Fig. 2C). Particularly, the JA-ILE level in CMCs was more than 3-times higher than that of DDCs (Fig. 2C). Zeocin is an antibiotic that binds to and cleaves DNA, leading to toxicity and cell death. The CMCs and DDCs' sensitivities to zeocin were dose-dependent, with CMCs recording the significantly highest cell death rates under 150 and 300 μg/mL zeocin, respectively (Fig. 2D, E).

Phytochemical composition and antioxidant capacity of CMCs and DDCs

Ginsenosides are characteristic bioactive compounds in American ginseng with tremendous pharmacological properties [37]. The content of ginsenosides Rg1, Rb1, Re, and Rd in CMCs was 3.18-, 2.96-, 3.58-, and 1.91-time significantly higher than in DDCs, respectively (Fig. 3A). We also evaluated the content of crude protein, fat, and total sugar in the two cell types. As shown in Fig. 3B-D, the crude protein, crude fat, and total sugar contents in dried CMCs were significantly higher than in dried DDCs.

A sophisticated antioxidant system is essential for cell survival in culture media. CMCs exhibited significantly higher levels of H_2O_2 and MDA, suggesting an induced oxidation stress (Fig. 3E, F). Interestingly, in addition to the higher content of ginsenosides, the activities of antioxidant enzymes POD, CAT, and SOD in CMCs were significantly higher than in DDCs, which might protect cells from oxidation damage (Fig. 3A, G-I).

Differential metabolites (DMs) between CMCs and DDCs

To explore the differences in secondary metabolism between CMCs and DDCs, we carried out a global metabolomics profiling. We identified and classified a total of 1,103 metabolites, including terpenoids, flavonoids, lipids, amino acids and derivatives, phenolic acids, nucleotides and derivatives, alkaloids, etc. (Table S1). QC samples exhibited extremely strong positive correlations, confirming the repeatability of the experiment (Fig. S2A). PCA, correlation, and HCA analyses revealed significant differences in the metabolite profiles of CMCs and DDCs (Fig. 4A and S2B, C). Notably, the PCA showed that the metabolite profiles of CMCs and DDCs could be discriminated by PC1 of 62.47% and by PC2 of 14.67% (Fig. 4A). As shown in Fig. S3A, the score plot of OPLS-DA

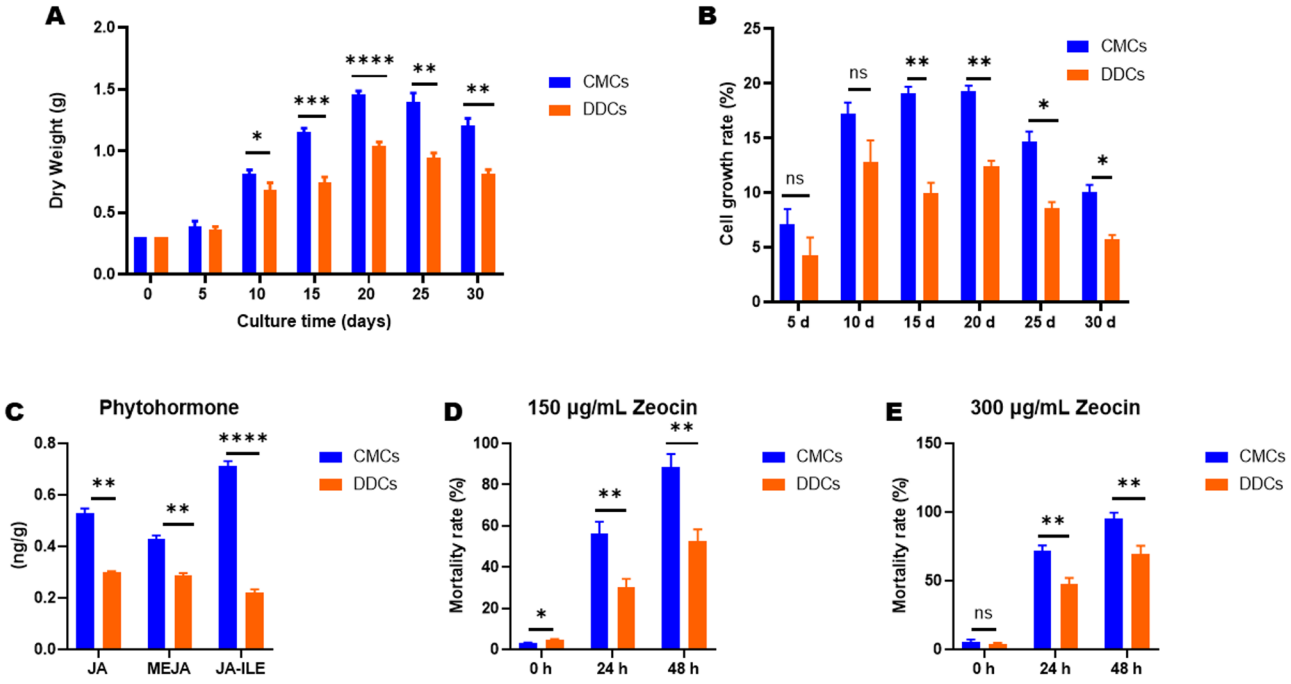

Fig. 2 Growth and levels of endogenous jasmonates (JAs) in AG CMCs and DDCs. **A** Dry biomass weight. **B** Growth rates. **C** Variation in endogenous JAs. **D** Mortality rate under 150 μg/mL zeocin. **E** Mortality rates under 300 μg/mL zeocin. *, **, ***, and **** indicate significant differences at $P < 0.05$, 0.01, 0.001, and 0.0001, respectively. ns, not significant

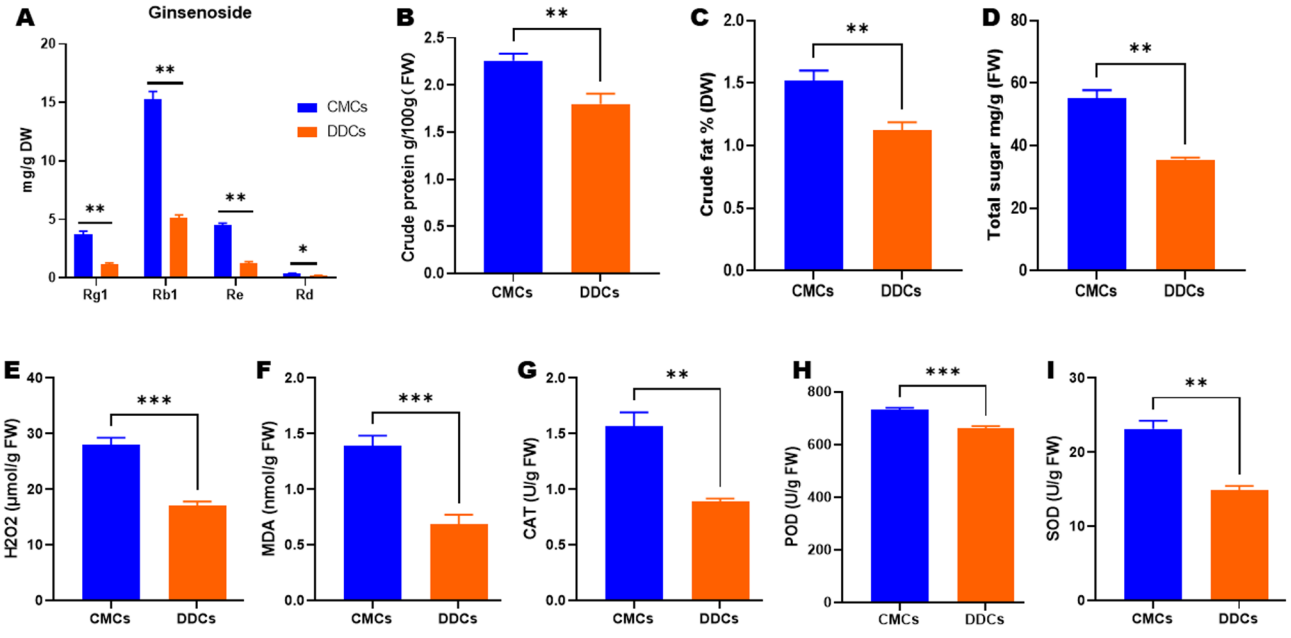

Fig. 3 Phytochemicals' content and antioxidant system of AG CMCs and DDCs. **A** Content of ginsenosides. **B**-**D** Variation in crude protein, crude fat, and total sugar, respectively. **E**)-**F** Levels of H_2O_2 and MDA, respectively. **G**-**I** Enzymatic activities of CAT, POD, and SOD, respectively. *, **, ***, and **** indicate significant differences at $P < 0.05$, 0.01, 0.001, and 0.0001, respectively

provided support for the high difference of CMCs and DDCs' metabolite profiles. The OPLS-DA model showed strong goodness of fit and reliability, with an R^2Y and Q^2 of 0.98 and 0.931, respectively (Fig. S3B).

We uncovered a total of 521 DMs (differential metabolites), including 351 down-regulated (high level in DDCs) and 170 up-regulated (high level in CMCs) between the two cell types (Fig. 4B, Table S2). Fig. S4 presents the top 20 most significantly differential metabolites between the

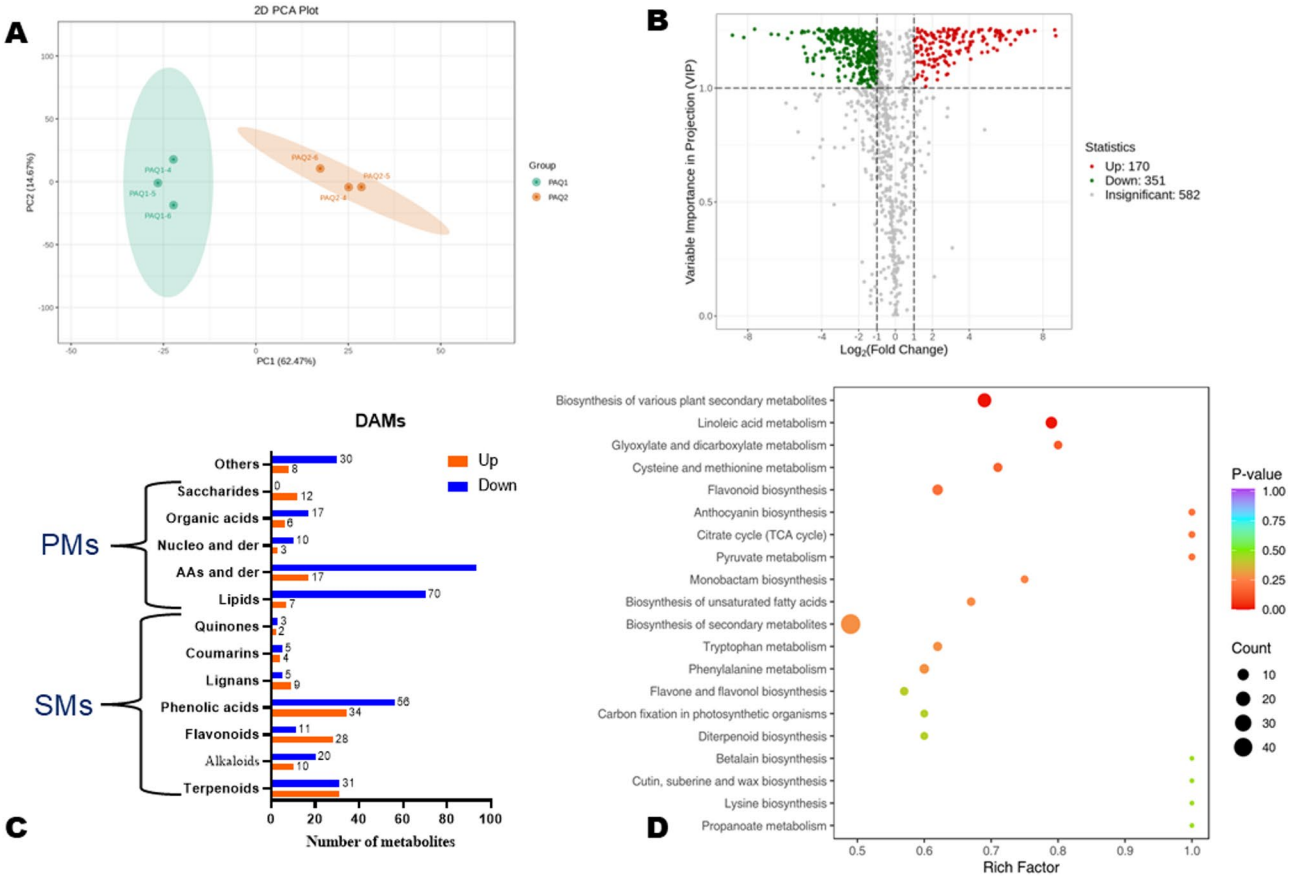

Fig. 4 Metabolite profiles and differential metabolites (DMs) between CMCs and DDCs). **A** Principal component analysis (PAQ-1, DDCs; PAQ-2, CMCs). **B** Volcano plot of DMs. **C**: Classification of DMs. **D** KEGG annotation and enrichment analysis of DMs

two cell types. The classification of DMs revealed that there were more up-regulated differential flavonoids, lignans, and saccharides in CMCs compared to DDCs (Fig. 4C). The same number of 31 differential terpenoids was detected in the two cell types (Fig. 4C). More differential lipids, phenolic acids, amino acids and derivatives, and nucleotides and derivatives accumulated greatly in DDCs than in CMCs (Fig. 4C). KEGG analysis assigned the DMs between CMCs and DDCs primarily in biosynthesis of secondary metabolites, flavonoid biosynthesis, linoleic acid metabolism, glyoxylate and dicarboxylate metabolism, and cysteine and methionine metabolism (Fig. 4D).

Variation of secondary metabolites (SMs) between CMCs and DDCs

To expose the variation in the accumulation patterns of SMs in the two cell types, we examined their relative contents. Although there was an equal number of differential terpenoids in CMCs and DDCs, most ginsenosides showed up-regulation in CMCs, except 6'-*O*-acetyl-ginsenoside Rgl, notoginsenoside R9, pseudoginsenoside RT5, and pseudoginsenoside Rt4 (Fig. 5A, Table S2). Many important differential bioactive flavonoids showed higher accumulation in CMCs in contrast to alkaloids in DDCs (Fig. 5B, C, Table S2). The flavonoids 3-*O*-acetylpinobanksin, aromadendrin, cyanidin-3-*O*-(6"-*O*-caffeoyl) glucoside, drimiopsin C, kaempferol-3,7-*O*-diglucoside, kaempferol-3-*O*-sophoroside, luteolin-7,3'-di-*O*-glucoside, and maesopsin were over 5-fold up-regulated in CMCs (Table S2). N-p-coumaroylhydroxyputrescine, N-trans-ferulic tyramine, N1,N8-bis(sinapoyl)spermidine, and nicotianamine (alkaloids) were 5.82-, 4.101-, 3.96-, and 4.14-fold up-regulated in CMCs, respectively (Table S2). The accumulation patterns of differential phenolic acids, lignans, coumarins, quinones, and tannins are presented in Fig. S5A, B.

Differentially expressed genes (DEGs) between CMCs and DDCs

To reveal differential transcriptional mechanisms that underlie the high accumulation of key SMs in CMCs, we performed a comparative transcriptome analysis. The summary of the high-quality RNA-seq data of CMCs and DDCs is presented in Table S3, with Q20 and Q30 above 98% and 94%, respectively. RT-qPCR validation of eight randomly selected DEGs is presented in Fig. S6.

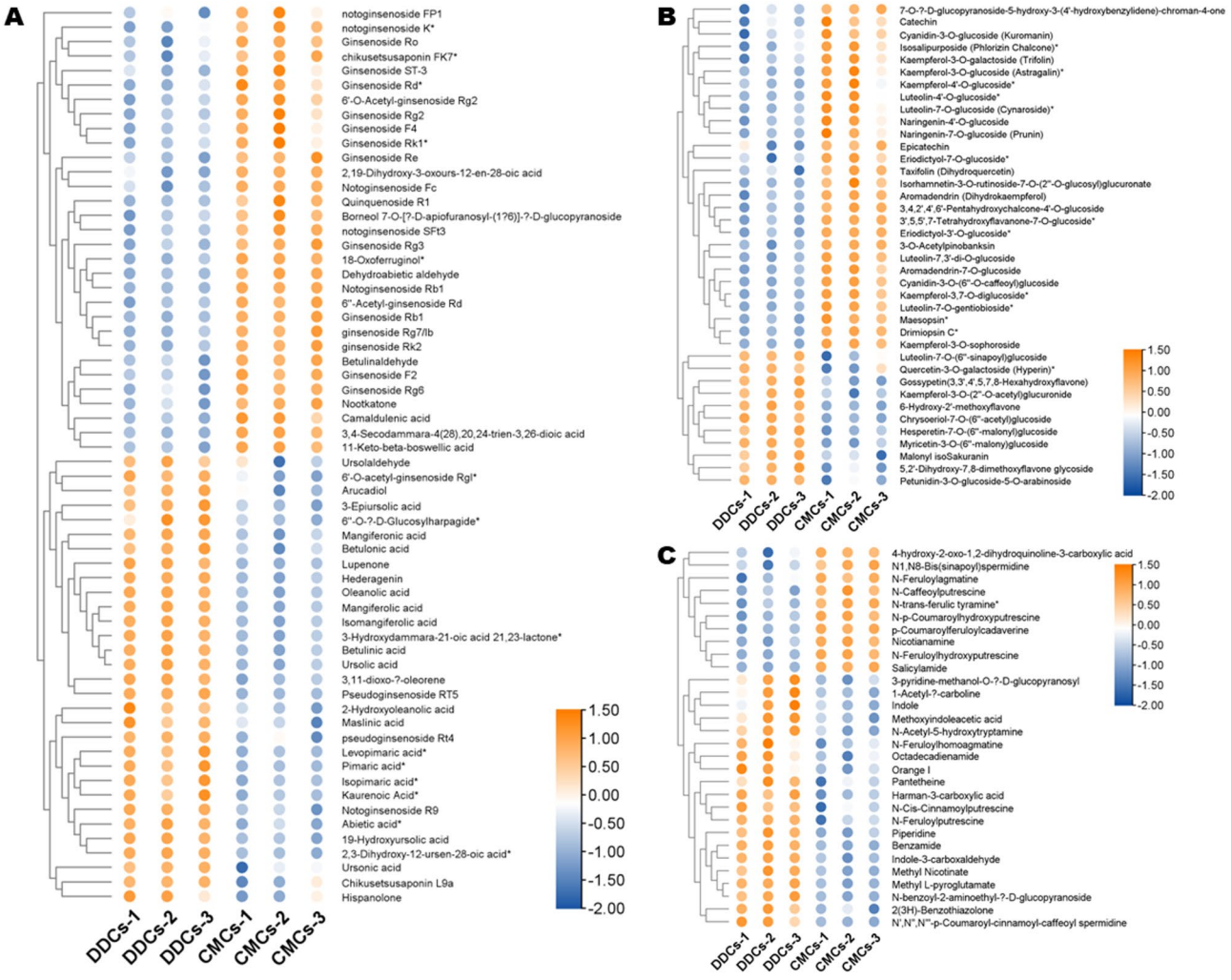

Fig. 5 Accumulation patterns of differential terpenoids **A**, flavonoids **B**, and alkaloids **C** in CMCs and DDCs

CMCs and DDCs displayed significant transcriptome differences, as revealed by PCA and correlation analyses of samples (Fig. S7A, B). Samples of CMCs and DDCs clustered separately on the PCA plot and could be differentiated by 46.4% and 18.55% of PC1 and PC2, respectively (Fig. S7A).

A total of 12,591 DEGs were identified, highlighting extensive transcriptional divergence between CMCs and DDCs (Fig. S8). 6,365 of the DEGs were significantly induced in CMCs compared to DDCs (Fig. S8). To confirm the identity of cell types, we examined the expression of PXY (Plant Receptor-like Kinase PXY) and WOX (WUSCHEL-related homeobox), which are critical for stem cell development. The results revealed that these PXY3, WOX9, and WOX8 were significantly up-regulated in CMCs compared to DDCs (Fig. S9). GO enrichment analysis assigned the DEGs mainly to "JA mediated signaling pathway", "cellular response to JA stimulus", "regulation of JA mediated signaling pathway", "cellular response to fatty acid", "cellular response to hypoxia", "cellular response to oxygen levels", "cellular response to decreased oxygen levels", "molecular adaptor activity", "cellular metal ion homeostasis", and "cellular cation homeostasis" (Fig. 6A). KEGG enrichment revealed biosynthesis of secondary metabolites, plant hormone signal transduction, protein processing in endoplasmic reticulum, ubiquitin mediated proteolysis, plant-pathogen interaction, and ribosome as the most differential pathways between the two cell types (Fig. 6B). Integrated KEGG enrichment analysis of DMs and DEGs highlighted the differential control of biosynthesis of secondary metabolites and plant hormone signal transduction between the two cell types (Fig. S10).

Mining of potential candidate genes related to ginsenoside biosynthesis

Ginsenosides are glycosylated triterpenoids known as the main bioactive compounds in the Panax genus [26, 27]. We filtered and examined the expression of DEGs related to terpenoid biosynthesis pathways (Ko00904, Ko00130,

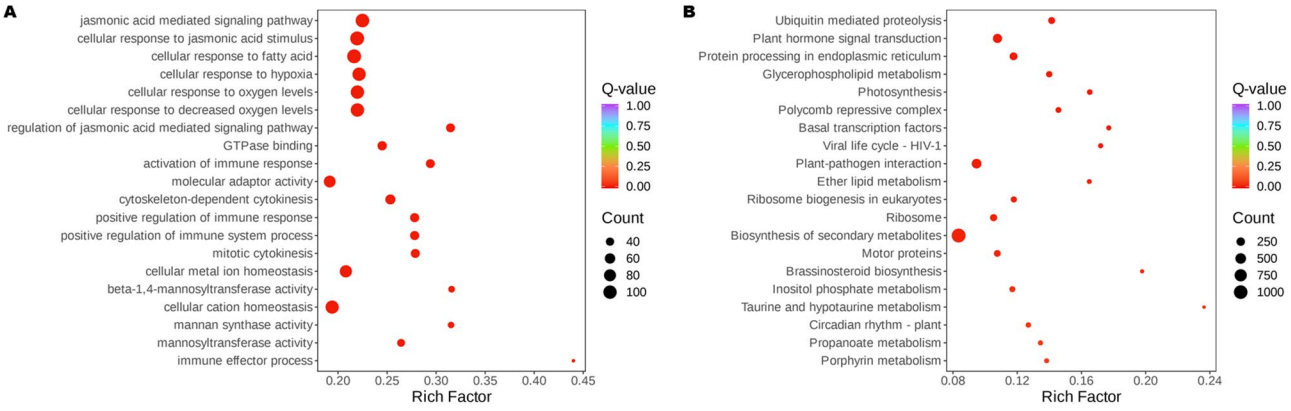

Fig. 6 Functional analysis of differentially expressed genes (DEGs) between CMCs and DDCs. **A** GO annotation and enrichment analysis of DEGs. **C** KEGG annotation and enrichment analysis of DEGs

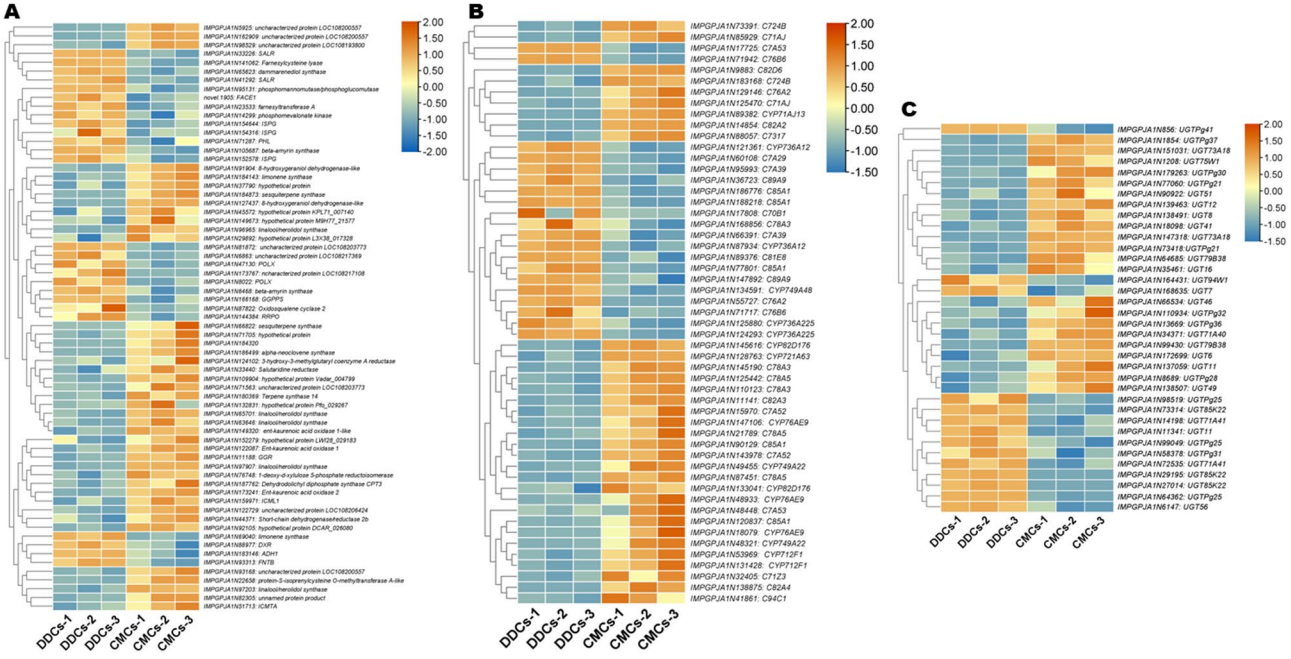

Fig. 7 xpression patterns of terpenoid biosynthesis- **A**, cytochrome P450- **B**, and UGT **C**-related DEGs in CMCs and DDCs

Ko00902, Ko00900, and Ko00909). The results showed that most DEGs (41/67) related to these pathways were significantly up-regulated in CMCs compared to DDCs (Fig. 7A). Of them, the genes *IMPGPJA1N184873* (sesquiterpene synthase), *IMPGPJA1N184320* (sesquiterpene synthase), *IMPGPJA1N66822* (alpha-neoclovene synthase), *IMPGPJA1N186499* (sesquiterpene synthase), *IMPGPJA1N127437* (8-hydroxygeraniol dehydrogenase-like), *IMPGPJA1N91904* (8-hydroxygeraniol dehydrogenase-like), and *IMPGPJA1N184143* (limonene synthase) were induced in CMCs with a Log2FC of 9.327, 8.61, 7.758, 7.508, 6.053, 4.551, and 4.083, respectively (Table S4).

Cytochrome P450 family genes and UDP-glucuronosyl/UDP-glucosyl transferases catalyze the key steps of ginsenoside biosynthesis [26, 27]. The expression patterns of CYP (|Log2FC| > 2) and UGT-related DEGs are shown in Fig. 7B, C, with most of them exhibiting up-regulation in CMCs. Twelve CYP-related DEGs, including *CYP71AJ13*, *CYP76AE9*, *C82A2*, *CYP749A22*, *C82A4*, *C82A3*, *C7A52*, *C85A1*, *C82D6*, *C7317*, *C78A3*, and *CYP76AE9* were significantly induced in CMCs with a Log2FC higher than 5 (Table S4). Meanwhile, six UGT-related DEGs, including *UGT71A40*, *UGT73A18*, *UGTPg28*, *UGT11*, *UGTPg36*, and *UGT73A18* were significantly induced in CMCs with a Log2FC higher than 2 (Table S4). As efficient ROS scavenging is essential for cell growth, we also searched for potential candidate genes related to the antioxidant system and seven highly induced (Log2FC > 5) DEGs in CMCs, including *PER51*,

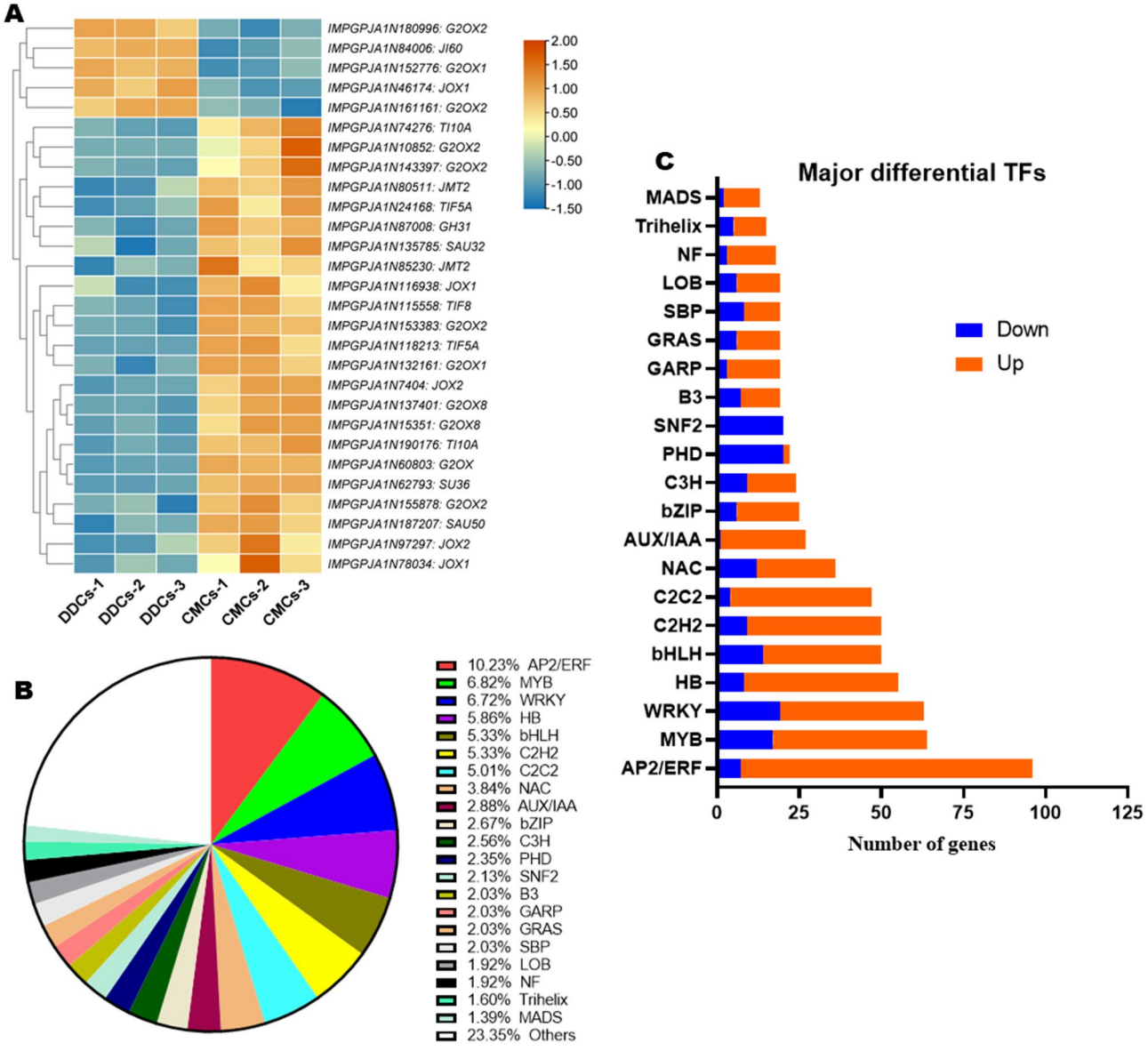

Fig. 8 Differentially expressed phytohormones and transcription factors (TFs) between CMCs and DDCs). **A** Expression patterns of major phytohormone-related DEGs.**B** Classification of differentially expressed TFs. **C** Regulation-based classification of major differentially expressed TFs

GRXS2, *GRXC3*, *PER72*, *GLR34*, *GLR28*, and *LAC11*, were identified (Fig. S11 and Table S4).

Phytohormone and transcription factor (TF)-related candidate genes

Plant hormone signal transduction was significantly differentially regulated between CMCs and DDCs. Of the 28 screened phytohormone-related DEGs, 23 were significantly up-regulated in CMCs compared to DDCs (Fig. 8A). JA-related pathways were particularly induced in CMCs (Fig. 6B). We identified four highly induced JA-related candidate genes, including *TIF5A*, *TI10A*, *TIF5A*, and *TI10A*, with Log2FC of 7.46, 4.22, 3.851, and 3.508, respectively (Table S4). In addition, five gibberellin (*G2OX2*, *two G2OX8*, *G2OX2*, and *G2OX*) and one auxin-related (*SAU36*) highly induced (Log2FC ˃ 4.4) candidate genes were identified (Table S4).

TFs interact with phytohormones to regulate metabolic processes in plant cells. We identified a total of 938 differentially expressed TFs between CMCs and DDCs (Table S5). EP2/ERF (10.23%), MYB (6.82%), WRKY (6.72%), HB (5.86%), bHLH (5.33%), C2H2 (5.33%), and C2C2 (5.01%) were the major differentially expressed TFs (Fig. 8B). The number of significant up-regulated TFs in CMCs was higher than in DDMs, except for PHD and SNF2 (Fig. 8C), indicating the induction of TF family genes was essential for enhanced SM biosynthesis and accumulation in CMCs. The top 20 highly induced TFs (Log2FC ˃ 9.09)

in CMCs included 13 EP2/ERFs (two *DRE1B*, *DREB1B*, four *DRE1D*, three *ERF17*, *ERF109*, *ERF34*, and *ERF4*), three HB-BELLs (*BHL8*), two MYBs (*DIVARICATA*), one NAC (*NAC90*), and one Tify (*TIF5A*) (Table S5).

Discussion

CMC culture is an advanced and efficient platform for the sustainable industrial production of high-value bioactive compounds [11, 19, 21]. This study established this technique in AG for the first time, which will enable the increased production of key active phytochemicals in Panax, such as ginsenosides and polysaccharides. Consistent with previous studies that reported low biomass yields and slow cell growth of DDCs compared to CMCs [11], we found that the biomass and growth rate of AG CMCs were significantly higher than those of DDCs. The higher growth rate of AG CMCs will facilitate industrial scale-up and downstream processing. The higher growth rate of CMCs could be attributed to their significantly high content of endogenous jasmonates and vacuoles. JAs are key regulators of plant growth and development and elicitors of plant cell proliferation rate in in-vitro conditions [35, 36]. The positive regulatory function of jasmonates on the biomass yield and accumulation of ginsenosides in cultured Panax cells has been reported [18, 38]. AG CMCs contained multiple vacuoles, while DDCs only had one big vacuole. Vacuoles are crucial for plant growth and development, playing diverse functions, such as maintaining cell turgor pressure and pH, controlling the transport and storage of substances, regulating intracellular environmental stability, controlling the localization and transport of key proteins, and responding to various stresses [39, 40]. CMCs exhibited higher levels of MDA and H_2O_2 than DDCs, which could be explained by limited oxygen availability due to the high growth rate. However, CMCs showed a strong antioxidant system (higher activities of antioxidant enzymes, induced expression of antioxidant-related genes, higher content of SMs), indicating they did not suffer any oxidative stress. The higher sensitivity of CMCs to zeocin than DDCs could be explained by increased DNA damage, resulting in enhanced oxidative stress and cell death. Zeocin causes important DNA damage in plant cells, leading to the down-regulation of histone genes and the cell cycle [41]. These findings show that further optimization of AG CMC culture conditions is required to minimize the impacts of stresses and guarantee a high yield and production rate.

CMC cultures are key platforms for the sustainable and improved production of important SMs [22–25]. In *Ocimum basilicum*, significantly higher production of triterpenoids has been achieved with CMC culture [23]. In line with this, AG CMCs exhibited significantly higher content of ginsenosides, crude protein, total sugar, and crude fat. The comparative metabolite profiling revealed that most differential ginsenosides, flavonoids, and saccharides were up-regulated in CMCs. Consistent, Lee et al. reported an increased accumulation of ginsenoside F2 and gypenoside XVII in CMCs of *Panax ginseng* [21]. These results show that CMC culture is a promising platform for the sustainable production of major bioactive compounds (ginsenosides and polysaccharides) in *Panax spp*. As a support, most genes that catalyze the key steps of ginsenoside biosynthesis were up-regulated in CMCs compared to DDCs. Differential lipids, amino acids and derivatives, organic acids, and other terpenoids were particularly up-regulated in DDCs compared to CMCs, suggesting that DDC culture may be more appropriate for the production of primary metabolites of interest. The higher levels of ginsenosides, flavonoids, and polysaccharides in CMCs infer that they may exhibit more pronounced pharmacological capacities than DDCs. Dose-dependent pharmacological studies are needed to clarify this statement.

Biosynthesis of SMs and plant hormone signal transduction were the most significantly differentially regulated pathways between CMCs and DDCs. Notably, JA-related metabolic pathways were induced considerably in CMCs, as well as most differentially expressed phytohormone-related DEGs. Phytohormones regulate transcriptional changes towards the induction of secondary metabolism and accumulation of SMs in plants [42, 43]. Besides its role in mediating various plant developmental processes, JAs play a versatile enhancement function of promoting the production of SMs that are second messengers in plant responses to stresses [44]. JAs also boost SMs' production in medicinal plants, therefore increasing their pharmacological values [45]. These results emphasize that jasmonates mediated the higher biosynthesis and accumulation of ginsenosides in CMCs. Previous studies have demonstrated that JA enhances signal transduction toward increased levels of ginsenosides by inducing the expression of ginsenoside biosynthesis-related genes, including squalene synthase, squalene epoxidase, 3-hydroxy-3-methylglutaryl-CoA reductase, cycloartenol synthase, β-amyrin synthase, dammarenediol-II synthase, CYP enzymes, protopanaxadiol synthase, UGTs, and TFs [16, 28]. Consistent, the expression levels of most terpenoid synthesis genes, CYPs, and UGTs were significantly up-regulated in CMCs. TFs play key regulatory roles in ginsenoside biosynthesis [26–28]. Concordantly, we found that most of the differentially regulated TFs were up-regulated in CMCs compared to DDCs. These up-regulated TFs might interact with key-induced phytohormones to regulate the biosynthesis of major active phytochemicals in AG. The crucial regulatory functions of TFs in SM biosynthesis in medicinal plants have been extensively discussed [46]. WRKY TFs

are critical regulators of important natural product biosynthesis in plants [47]. Supportively, *PgWRKY4X* was found to stimulate ginsenoside biosynthesis by activating squalene epoxidase [32]. It was noteworthy that the top 20 most induced TFs included 13 EP2/ERFs, indicating that EP2/ERF TFs are the key positive regulators of the biosynthesis of ginsenosides in AG. The TF *PgERF120* was identified as a major regulatory gene of ginsenoside bioaccumulation in ginseng [33]. These findings suggest that elicitation with ethylene may improve ginsenoside biosynthesis in *Panax* cells. We uncovered several potential highly induced candidate genes that should undergo functional characterization and validation for exploitation in biotechnological-assisted ginsenoside production. Notably, transient overexpression and/or knockout of identified TFs, jasmonate pathway inhibitors, and other phytohormone-related candidate genes is required to reveal their specific roles in AG CMC growth and metabolism.

Conclusion

In summary, this study established CMC culture to enhance the in-vitro ginsenoside production in American ginseng. For instance, biochemical and metabolomics analyses showed that CMCs accumulated significantly higher levels of ginsenosides (Rg1, Rd1, Re, Rd, etc.), flavonoids, crude protein, fat, and total sugar compared to DDCs. Increased levels of endogenous jasmonates (JA, MEJA, and JA-ILE) and expressions of phytohormone, antioxidant enzyme, and TF-related genes were essential for higher growth rates and induced biosynthesis of SMs in CMCs. Notably, JA-related metabolic pathways, biosynthesis of SMs, and plant hormone signal transduction were the most significantly differentially regulated pathways between CMCs and DDCs. 521 DMs and 12,591 DEGs between CMCs and DDCs were identified. Key potential candidate genes for stimulating CMC growth and ginsenoside biosynthesis were uncovered, including cytochrome P450, UGT, antioxidant enzyme, phytohormone, and TF family genes. It was noteworthy that EP2/ERF family genes were the most highly induced in CMCs. Our findings provide evidence for higher production of SMs in CMCs. Moreover, they represent fundamental bases for optimizing *Panax spp.* CMC cultures for sustainable in-vitro bioproduction of ginsenosides.

Materials and methods

Preparation of root explants of American ginseng

Fresh roots from 5-year-old AG were harvested from October to November of the same year (**Fig. S1A**) in Changbai Mountain, Jilin Province. The soil on the surface of the roots was cleaned, followed by rinsing with tap water for 2–3 h and absorption of the surface water using filter paper. The explants were then subsequently sterilized with 10% H_2O_2, 75% medical ethanol, and 0.1% (w/v) mercuric chloride solution for three, two, and fifteen minutes, respectively. Explants were rinsed (sterile distilled water) three times after each sterilization. Finally, sterilized explants were soaked in a 150 mg/L citric acid solution, wiped and cut into 0.5–1.5 mm circular discs (Fig. S1B, C).

Induction of stem cells (cambial meristematic cells, CMCs) and callus tissue (dedifferentiated cells, DDCs) cultures of American Ginseng

The discs were placed on a triangular flask (one explant per bottle) containing 50 mL of solid MS medium (which contained 30 g/L sucrose, 2 mg/L α - naphthole acetic acid (NAA), 2 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D), 3.8 g/L gellan gum, and pH 5.80) and then incubated for 24 h (dark cultivation at 25 ℃). After 14–18 days, transparent cell clusters can be observed (Fig. S1D). After 30 days, they were transferred to a subculture MS medium (20 g/L sucrose, 1 mg/L NAA, 1 mg/L 2,4-D, 3.8 g/L gellan gum, pH 5.80). The subculture conditions were 24 h in the dark at 25 ℃, and subcultured every 20 days to obtain stable growth of American ginseng CMCs (Fig. S1F).

For the DDCs, the discs were cultivated under the same conditions in a triangular flask containing 50 mL of solid MS medium (which contained 30 g/L sucrose, 3.8 g/L gellan gum, 4 mg/L α - naphthol acetic acid (NAA), 2 mg/L 6-Benzylinopurine (6-BA), pH 5.80). After about 14–18 days, significant thickening of the explant can be observed, with complex cell clusters growing around it (**Fig. S1E**). After 30 days, they were transferred to a subculture MS medium (20 g/L sucrose, 2 mg/L alpha naphthole acetic acid (NAA), 1 mg/L 6-Benzylinopurine (6-BA), 3.8 g/L gellan gum, pH 5.80) and subcultured every 20 days.

After continuous stable cultivation of the obtained CMCs/DCDs of American ginseng for 6 months, 6 g of cells cultured for 12 days were taken and subcultured in a 250 mL triangular flask containing 100 mL of MS medium (30 g/L sucrose, 1.0 mg/L 6-BA, and 0.5 mg/L 2,4-D, with a pH of 5.80 for suspension culture. Cells were collected after 0, 5, 10, 15, 20, and 25 days of suspension culture for freeze-drying, weighing, and evaluation of growth rate and ginsenoside content. Cell growth rate (%) = ((cultured cell dry weight - initial cell dry weight)/(initial cell dry weight * time)) * 100.

Microscopic observation

For non-staining observation, 1 mL of 0.9% physiological saline was added to 0.1 g of CMCs/DDCs, gently blown, and beaten evenly to prepare a cell suspension. Then, two drops of cell suspension were placed on a glass slide for microscopic observation (Leica DM750). The

neutral red staining observation was carried out following the method by Lee et al. [21]. Briefly, 1 mL of Ringer solution was added to 0.1 g of CMCs/DDCs and mixed evenly. One drop of cell suspension was placed on a glass slide, followed by two drops of 0.05% (w/v) neutral red Ringer solution for staining for 8 min. Then, absorption of the dye with a filter paper. Finally, one drop of Ringer solution was added to the cells, followed by microscopic observation (Leica DM750).[1, 2]

Sensitivity of American ginseng CMCs and DDCs to bleomycin

The sensitivity of CMCs and DDCs to bleomycin (zeocin) was evaluated following previously described methods [21, 48]. A 2 mg/mL zeocin stock solution was prepared by mixing 20 mg of zeocin with an appropriate amount of sterile MS medium solution. A fluorescent agent solution was also prepared by dissolving 1 mg of fluorescein diethyl ester (FDA) in 1.0 mL of acetone, followed by mixing 0.2 mL of FDA-acetone solution with 1.8 mL of sterile MS medium. The mixture was then diluted 10 times to obtain a 100 μ g/mL concentration.

6.0 g of 20-day solid cultured CMCs and DDCs were subcultured in a 250 mL conical flask containing 100 mL of MS liquid medium under continuous shaking (120 r/min) in the dark at 25 ℃. On the 20th day of suspension culture, 1.0 mL of cells was placed in a sterile 24-well plate, and an appropriate amount of zeocin solution was added to achieve final concentrations of 150 and 300 μg/mL. The treated suspension cells were cultured (dark, 120 r/min shaking, and 25 ℃) for 0, 24, and 48 h, respectively. Next, an appropriate amount of FDA solution was added to achieve 200 ng/mL (final concentration). After shaking (120 r/min) for 20 min and rinsing with sterile PBS buffer three times, the suspensions were subjected to fluorescence microscopic observations (Zeiss Axioplan 2 multifunctional fully automatic fluorescence microscope, Carl Zeiss, Germany). The excitation and emission wavelengths were set to 485 nm and 530 nm, respectively. Each sample was independently measured five times, and the maximum and minimum values were discarded to calculate the average mortality rate. Each experiment was repeated three times.

Cell death rate (%) = (Total cell count - number of live cells)/(Total cell count)*100%.

Evaluation of endogenous jasmonate levels and antioxidant system

Jasmonates (JA, MEJA, JA-ILE) were determined by UPLC (Agilent 1290; Agilent Technologies, Waldbronn, Germany), and tandem MS/MS (Tandem mass spectrometry, Applied Biosystems 6500 Quadrupole Trap, https://sciex.com.cn/) per previously described method [49]. The data were collected in the MRM (multiple reaction monitoring) mode, and the MultiQuant software was used for data analysis. Jasmonic acid and methyl jasmonate (MEJA) standards were acquired from Sigma-Aldrich (http://www.sigmaaldrich.com/united-states.html). The jasmonoyl isoleucine (JA-ILE) standard was bought from Olchemim (http://www.olchemim.cz/).

The crude protein, crude fat, and total sugar content were assessed using Chinese standard methods GB 5009.5–2016, GB5009.6–2016, and GB/T 15,672 – 2009, respectively. SOD (superoxide dismutase), POD (peroxidase), and CAT (catalase) activities, as well as MDA (malondialdehyde) and hydrogen peroxide (H_2O_2) contents, were assessed using their detection-specific kits (Suzhou Comin Biotechnology Co., LTD, Suzhou, China).

Determination of ginsenoside content in AG CMCs and DDCs

The extraction and HPLC analysis of ginsenosides in CMCs and DDCs were performed following the recently reported methods [50]. Ginsenosides Rg1, Re, and Rb1 reference substances were acquired from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). The injection volume in the HPLC was 10 μL. Chromatographic conditions: Agilent 1260 HPLC instrument; Thermo-C18 chromatographic column (250 mm × 46 mm, 5 μm); filler, octadecylsilane bonded silica gel; mobile phase A, acetonitrile; and mobile phase B, 0.1% phosphoric acid solution. The gradient elution is specified in Table S6. The detection wavelength was 203 nm, and the Column temperature was 40 ℃. The ginsenoside contents were calculated based on the peak of ginsenoside Rb1.

Metabolite profiling of CMCs and DDCs

Three replicates of freeze-dried samples (50 mg powder for each) were extracted with 70% pre-cooled (–20 °C) methanol (1.2 mL) by vortexing for 30 s once every 30 min (6 times in total), followed by centrifugation (12,000 g, 8 min, and 4 °C) and supernatant collection. After filtration of extracts (0.22 μm micropore membrane filter, SCAA-104), LC-MS (Liquid chromatography-mass spectrometry) analysis was achieved using the QTRAP® 6500 + mass spectrometer and the ExionLC™ AD system (SCIEX, Framingham, MA, USA) at Metware Biotechnology Co. Ltd., Wuhan, China, as per previously reported methods [51–53]. Metabolites with more than 20% missing values were excluded. A three-step process was then applied to remove redundant peaks, and the signal intensities were log-transformed prior to multivariate analyses in R (vs. 4.3.0). The packages pheatmap and MetaboAnalystR were used for HCA (hierarchical clustering analysis) and OPLS-DA (orthogonal partial least squares discriminant analysis). The package prcomp was used for PCA (principal component analysis). DMs (differential

metabolites) were screened out by the ggplot2 program (R software) at thresholds of p-value < 0.05, |Log2FC| > 1, and VIP ≥ 1. Induced pathways were unveiled by KEGG analysis (http://www.kegg.jp/kegg/pathway.html).

Transcriptome sequencing and analysis

Total RNA extraction from three replicates of CMC and DDC samples was achieved by ethanol precipitation and CTAB-PBIOZOL. Quality and quantity assessment of the RNA samples was carried out using a Qubit fluorescence quantifier and a Qsep400 high-throughput biofragment analyzer. The RNA sequencing was achieved at Metware Biotechnology Co., LTD., Wuhan, China, following previously described methods [54, 55]. Briefly, the enriched mRNA was fragmented into short fragments (fragmentation buffer). Following reverse transcription into cDNA using NEB, Next Ultra RNA Library Prep Kit (NEB #7530, New England Biolabs, Ipswich, MA, USA). Next, adapters were ligated to the purified double-stranded cDNA fragments, followed by purification with the AMPure XP Beads (1.0X) and size selection via PCR amplification. The obtained libraries were subjected to Illumina sequencing (Novaseq6000) after pooling them according to the effective concentration and the target sequencing output data volume, yielding 150 bp paired-end reads.

Clean reads were screened out using fastp (version 0.18.0), and alignment to the *P. quinquefolius* genome (BioProject: PRJNA752920, BioSample: SAMN20855195) [56] was performed using HISAT2 (v2.0.5) software [57, 58]. Mapped reads assemblage was done with StringTie v1.3.1 [59], and transcript normalization (calculation of FPKM values, fragments per kilobase per million mapped fragments) was achieved using Cufflinks (v2.2.1) [60]. DEGs detection was performed with the DESeq2 software [61], with criteria of FDR (false discovery rate) < 0.05 and |Log2fold change| ≥ 1. KOBAS (2.0) and GO seq software were utilized for KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/kaas) and GO (Genes Ontology, http://geneontology.org/) analysis, respectively.

RT-qPCR (real-time quantitative polymerase chain reaction)

First-strand cDNAs were synthesized using the TransScript® First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). RT-qPCR was conducted on LightCycler96 instrument (Roche, Switzerland) with SYBR Green Mix (TIANGEN, Beijing, China). The internal reference was *P. quinquefolius GAPDH* gene. The specific primers for each analyzed gene are presented in **Table S7**. Relative expression levels were computed by the $2^{-\Delta\Delta Ct}$ method [62].

Data analysis

Microsoft Excel was used to organize the data. GraphPad Prism 9 was used for statistical tests at $P < 0.05$ and for graph construction. Results are presented as mean ± SD (standard deviation). TBtools was used to generate Venn diagrams and heat maps [63].

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-07469-8.

Supplementary material 1.

Supplementary material 2.

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Not applicable.

Authors' contributions

Conceptualization, P.Z.; Data curation, P.Z.; Formal analysis, P.Z.; Funding acquisition, P.Z.; Investigation, H.L., J.L., H.Z., X.D., Z.H.and Y.Y.; Methodology, P.Z., Z.L., X.Z., T.W., Y.H. and W.G.; Project administration, P.Z. and C.Y.; Resources, Z.Z.; Software, P.Z. and C.Y.; Supervision, C.Y.; Validation, P.Z.; Visualization, H.L.; Writing – original draft, P.Z., H.L., J.L., X.D., Z.H.; Writing – review & editing, Y.Y., Z.L., X.Z., T.W., Y.H. and C.Y. All authors have read and approved the final version of the manuscript.

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Data availability

The raw RNA-seq data are available as SRAs from NCBI at [https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1189319](https:/www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1189319). All other datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The experiments did not involve endangered or protected species. This study used root samples from 5-year-old *Panax quinquefolius*. Samples were collected from Changbai Mountain, Jilin Province, China. Full permission was granted from the local government and the respective offices to collect the samples. We declare that the collection and use of plant materials in this study comply with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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