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RESEARCH ARTICLE

Effects of tree age on flavonoids and antioxidant activity in *Torreya grandis* nuts via integrated metabolome and transcriptome analyses

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Abstract

The nuts of *Torreya grandis* are abundant in flavonoids, providing potential antioxidant activity. Considering that *T. grandis* can yield nuts for hundreds or thousands of years, effects of tree age on flavonoids and antioxidant activity of *T. grandis* nuts were targeted in this study for the first time. Results revealed that antioxidant activity and total flavonoid contents in 100- and 1000-years-old *T. grandis* nuts were much higher compared to 10-years-old *T. grandis*. The content of 19 flavonoids showed significant correlations with antioxidant activity in 100- and 1000-years-old trees using metabolites profiling. Transcriptome and coexpression analysis indicated that seven genes were associated with aged-related flavonoids biosynthesis. Further analysis showed that abscisic acid and gibberellin might regulate the age-related differential flavonoids accumulation. Our study provides a novel viewpoint that aged *T. grandis* is likely to present a better source of natural antioxidants and has yet to be examined in other tree models.

KEYWORDS
flavonoids, Metabolomics, nuts, Torreya grandis, transcriptome, tree age

*Jingwei Yan, Hao Zeng and Weijie Chen contributed equally to this work.

1 | INTRODUCTION

Torreya grandis, belonging to the Taxaceae family, is a large evergreen coniferous tree mainly found in Asia and North America (He et al., 2016; L. Shi et al., 2018; L. K. Shi et al., 2018; Yan et al., 2022a). It is widely cultivated in subtropical hilly areas of China. Its drupe-like fruits with nut seeds encompass a variety of bioactive compounds, which not only provide an attractive flavor and crispy taste, but also exhibit nutritional value and multiple health effects such as antioxidant, and so on (Ding et al., 2020; Lou et al., 2019; Lou et al., 2022; Song et al., 2021; Suo et al., 2019, 2022). Among *T. grandis* bioactives, flavonoids account for many of its effects in addition to its excellent antioxidant activity (Zhang et al., 2022).

Flavonoids are a family of secondary metabolites found ubiquitously in food plants. To date, more than 6000 members of flavonoids have been identified (Manzoor et al., 2020; Sagar et al., 2022). The basic flavonoid structure is a C6-C3-C6 system, and have three rings labeled as A, C, and B. Based on ring oxidation level and substitution form in C ring, flavonoids are further subdivided into six categories that are flavonols, flavanones, flavones, proanthocyanidins, isoflavones, and anthocyanins. The substitution forms in A and B rings could determine individual members within a category (L. Wen et al., 2017). As efficient bioactive antioxidants, a series of flavonoids can prevent damage caused by oxidative stress or injury, which could improve body immunity and reduce cancer occurrence acting as chemo preventive agent (Gimenez-Bastida & Zielinski, 2015; Hu et al., 2016; Nijveldt et al., 2001; Peng et al., 2020).

The biosynthetic pathway of flavonoids has long been established derived from phenylpropanoids (Yonekura-Sakakibara et al., 2019). It starts with the condensation of three molecules of malonyl-CoA with one molecule of 4-coumaroyl-CoA to form naringcnin chalcone via chalcone synthase (CHS) enzyme. Then, chalcone isomerase (CHI) isomerizes chalcones to yield flavanones. Flavanone presents an intermediate that is converted via isoflavone synthase (IFS) and flavone synthase (FNS) to generate isoflavones and flavones, respectively. Flavanone is likewise catalyzed by flavanone 3-hydroxylase (F3H) and flavonol synthase (FLS) to produce flavonol, or via dihydroflavonol 4-reductase (DFR) to generate leucoanthocyanidins leading to anthocyanidins via anthocyanidin synthase (ANS) (Nabavi et al., 2020; Shirley, 1996; W. Wen et al., 2020).

Our previous study in young *T. grandis* nuts revealed that *TgCHS*, *TgDFR1*, and *TgANS* were involved in flavonoid biosynthesis (10-years-old tree) based on comparative transcriptome and metabolomics analyses (Zhang et al., 2022). As a perennial plant species, *T. grandis* has an extremely long lifespan and can produce fruits for hundreds or even thousands of years (B. Wang et al., 2022). Hernández et al. (2011) found that the older *Cistus clusii* had higher levels of proanthocyanidins (Hernández et al., 2011). In *Cistus ladanifer*, young leaves show obviously greater synthesis of flavonoids than mature leaves (Valares Masa et al., 2016). In *Ginkgo biloba*, the flavonoid content in leaves decreases with age (Q. Wang et al., 2022; Yao et al., 2012). These studies reveal that the effect of age on the flavonoids synthesis might differ in several plant species. However, flavonoid compositions of *T. grandis* nuts at different tree ages is completely unknown especially in such a long life span tree.

In this study, total flavonoid content and antioxidant activity in *T. grandis* nuts were assessed at different tree ages. Furthermore, flavonoids compositions of *T. grandis* nuts at different tree ages via large-scale metabolome analysis to identify changes in individual flavonoids. Finally, mechanism of age-related flavonoid biosynthesis was revealed via transcriptome analyses. Results from this study aid to provide valuable information for the identification of most efficacious sources of natural antioxidants as exemplified in *T. grandis* nut tree.

2 | MATERIAL AND METHODS

2.1 | Plant material

T. grandis cv. Merrillii grafting trees at Zhaojia Town (120° 52′ E, 29° 70′ N), Zhuji City, China, were used in this study. The seeds were collected from three different tree ages of *T. grandis* determined by using an increment borer, including young (10-years-old), adult (100-years-old), and old (1000-years-old). All the samples in each tree age were collected from six trees in September of 2022, which was the mature stage of *T. grandis* nuts (Yan et al., 2022b). Samples were immediately frozen in liquid nitrogen, and stored at −80°C till further analysis.

2.2 | Analysis of total flavonoids, carotenoids, and vitamin E

The total flavonoid content was measured as described in Zhang et al. (2022). In brief, 1 mL extract solution of nuts (about 10 mg) was mixed with 0.2 mL aluminum chloride (2%), 3 mL methanol, 0.2 mL potassium acetate (1 mol/L), and 5.6 mL distilled water. After reaction for 10 min, the absorbance at 415 nm was measured and total flavonoid content was calculated.

The total contents of carotenoids and vitamin E were measured according to the protocol of carotenoid detection kit (Leagene, China) and vitamin E test kit (Comin, China).

2.3 | Analysis of DPPH radical scavenging and FRAP antioxidant assays

DPPH free radical scavenging activity was analyzed as described in Zhang et al. (2022). Briefly, 0.1 mL extracted solution (methanol) of nuts was mixed with 7.9 mL 2, 2-diphenyl-1-picrylhydrazyl (DPPH, 0.03 g/L) at 25°C for 30 min. The absorbance at 517 nm was measured. Then the DPPH radical scavenging activity was calculated.

For determination of Ferric Reducing Ability of Plasma (FRAP) assay, 4.5 mL of FRAP reagent including 1 mmol/L 2,4,6-tripyridyl-2-triazine (TPTZ) dissolved in HCl (40 mmol/L) and 2 mmol/L ferric chloride dissolved in sodium acetate (0.25 mol/L, pH 3.6) was mixed with 0.1 mL

extracted solution of *T. grandis* nuts. After reaction at 25°C for 4 min, the absorbance at 593 nm was determined. FRAP was calculated following the exact method of Benzie and Strain (1996).

2.4 | RNA extraction and transcriptome analysis

Total mRNA from nut samples was extracted and purified according to the protocol of Aprep Pure Plant Kit (Tiangen, China). The double-stranded cDNA was generated using the random hexamer priming, and the blunt ends were added with an A base for the ligation of sequencing adapters. The fragment of ligated cDNA after size screening with AMPure XP beads was enhanced using adapter-specific primers. The insertional fragments of the library with the concentration of 1.5 ng/μL were detected by Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The high-quality reads were obtained and the transcriptome of the species was obtained by splicing (Trinity, V2.6.6) after filtering the original sequencing data. Then, the RSEM (V1.2.28), DESeq2 (V1.20.0), and clusterProfiler (V3.8.1) were used to calculate for gene expression, and then screen and cluster the differentially expressed genes, respectively. The false discovery rate (FDR) was obtained by using the Benjamin–Hochberg method to correct the hypothesis test probability (*p* value). The screening criteria for differential gene were $|\log2FC| \geq 1$ and FDR < 0.05.

2.5 | Metabolites extraction and analysis

The nuts of *T. grandis* (about 100 mg) were grounded after freezing with liquid nitrogen, and then resuspended with 2 mL cold 80% methanol. The samples were incubated on ice for 5 min followed by centrifugation at 15,000 *g*, 4°C for 20 min. After dilution with 53% methanol, the supernatant was subsequently transferred to a new Eppendorf tube for centrifugation at 15,000 *g*, 4°C for 20 min. Finally, the supernatant was injected into the liquid chromatography-mass spectrometry (LC-MS)/MS system. The ExionLC™ AD system (SCIEX) coupled with a QTRAP® 6500+ mass spectrometer (SCIEX) in Novogene Co., Ltd. (Beijing, China) was used to perform the LC-MS/MS analyses in positive/negative polarity modes. The supernatant was injected into a Xselect HSS T3 (2.1 × 150 mm, 2.5 μm) using a 20-min linear gradient at a flow rate of 0.4 mL/min for. Eluent A is 0.1% formic acid in water and eluent B is 0.1% formic acid in acetonitrile solution using the following gradient: 2% B, 2 min; 2–100% B, 15.0 min; 100% B, 17.0 min; 100–2% B, 17.1 min; 2% B, 20 min. The detection of metabolites using Multiple Reaction Monitoring was based on Novogene in-house database. The Q3 was used for the metabolite quantification. The Q1, Q3, RT (retention time), DP (declustering potential), and CE (collision energy) were used to the metabolite identification. The data files generated by High Performance Liquid Chromatography HPLC-MS/MS were processed using the SCIEX OS Version 1.4 to integrate and correct the peak. The main parameters were: minimum peak height, 500; signal/noise ratio, 5; Gaussian smooth width, 1. The area of each peak represents the relative content of the corresponding substance.

2.6 | Flavonoids coexpression network

The R package WGCNA V1.71 was used for constructing a coexpression network of flavonoids (Langfelder & Horvath, 2008). The differentially expressed genes from 10-, 100-, and 1000-years-old were used for analysis. The matrix was raised to a soft-threshold power. Modules are defined as clusters of highly interconnected genes, and genes within the same cluster have high-correlation coefficients with one another. For the present analysis, the minimum module size was set to 30, and modules with highly correlated eigengenes (based on a threshold of 0.25) were merged.

3 | RESULTS

3.1 | Analysis of antioxidant activity, total flavonoids, carotenoids, and vitamin E in *T. grandis* nuts at different tree ages

To compare antioxidant activity of *T. grandis* nuts collected from different tree age, samples from three tree ages of *T. grandis*, including young (10-years-old), middle (100-years-old), and old (1000-years-old) were collected (Figure 1a). The DPPH radical scavenging activity and FRAP of nuts in 100- and 1000-years-old *T. grandis* tree were both higher compared to those in 10-years-old *T. grandis* tree, whereas there were no obvious differences among 100- and 1000-years-old *T. grandis* trees for the two parameters (Figures 1b and 1c). These results suggest that age might affect antioxidant efficacy in *T. grandis* nuts.

The nuts of *T. grandis* are abundant in several bioactive classes such as flavonoids, carotenoids, and vitamin E to mediate for the antioxidant activity (Lou et al., 2019; Zhang et al., 2022). To explore which bioactive substances might be associated with the increased antioxidant activity in aged *T. grandis* tree, total flavonoid contents, carotenoid contents, and vitamin E levels were measured in nuts at different *T. grandis* tree age. Among monitored parameters, total flavonoid content but not that of total carotenoid and vitamin E in nuts of 100- and 1000-years-old *T. grandis* tree that appeared at much higher levels than in 10-years-old *T. grandis* tree. No obvious differences were observed between 100- and 1000-years-old *T. grandis* tree in the above bioactive compounds (Figures 1d–1f). These results suggest that differences in total flavonoid contents might contribute for differences of antioxidant activity in nuts at different *T. grandis* tree age.

3.2 | Flavonoids profiling in *T. grandis* nuts at different tree ages

To further pinpoint differential flavonoid composition in nuts of *T. grandis* at different tree ages, LC-MS was performed for flavonoids profiling coupled to multivariate data analysis for specimens classification. A total of 107 flavonoid metabolites were identified in our study (Table S1), including flavones (36, 34%), flavanones (18, 17%), flavonoid glycoside (16, 15%), flavonols (15, 14%), isoflavones (8, 7%), anthocyanins (8, 7%), and chalcones and dihydrochalcones (6, 6%) (Figure 2).

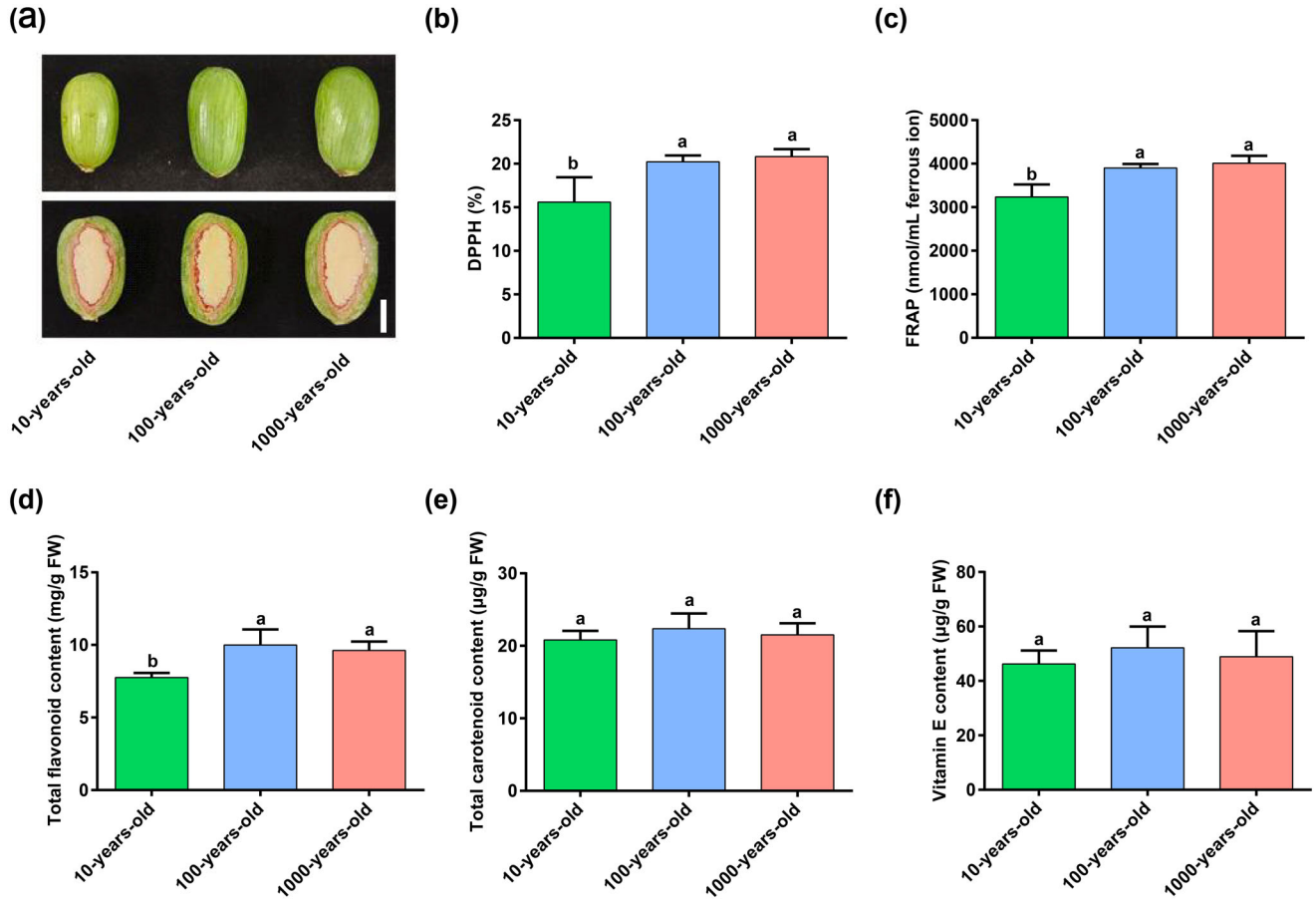

FIGURE 1 The effect of tree age on the antioxidant activity, total flavonoids, carotenoids, and vitamin E content in *T. grandis* nuts. (a) The nuts of *T. grandis* at different tree ages. Scale bar represents 1 cm. (b) The DPPH radical scavenging activity and (c) FRAP of *T. grandis* at different tree ages. Total contents of flavonoid (d), carotenoid (e), and vitamin E (f) in nuts at different tree ages. Error bars in (b–f) represent SD ($n = 3$). Different letters in (b–f) indicate a significant difference compared to the control (10-years-old) as determined by one-way ANOVA for $p < 0.05$

3.3 | Identification of the differential flavonoids in *T. grandis* nuts at different tree ages

To comprehensively determine the dynamic accumulation of 107 flavonoid metabolites in nuts of *T. grandis* at different tree ages, the K-means clustering analysis was performed to show nine distinct subclasses (Figure 3a and Table S2). Subclasses 2, 3, and 9 exhibited obvious decreases with the increases of tree age. Subclasses 4, 5, and 8 displayed the significant increases with the increases of tree age. Subclasses 1 and 7 showed different change trend with the increases of tree age. Notably, subclass 6, which is the highest proportion (17.8%) of flavonoid metabolites, is composed of four flavonols (narcissoside, quercetin 7-O-rutinoside, methylQuercetin O-hexoside, and gallocatechin-gallocatechin), four flavanones (naringerin, naringenin, butin, and pinobanksin), four flavones (8-C-hexosyl-apigenin O-hexosyl-O-hexoside, selgin 5-O-hexoside, isoquercitrin, and di-C,C-hexosyl-apigenin), three flavonoid glycoside (selgin C-hexoside, apigenin C-glucoside, and eriodictiol 6-C-hexoside 8-C-hexoside-O-hexoside), two chalcones and dihydrochalcones (phloretin and naringenin chalcone), one isoflavones (ononin), and one anthocyanins (petunidin 3-O-glucoside) (Figure 3b). This subclass showed significant increase of flavonoids from nuts of 10-years-old tree to nuts of 100- and 1000-years-old tree, which was similar to the changes of total flavonoids and antioxidant activity.

3.4 | Transcriptome profiling of *T. grandis* nuts at different tree ages

To explore the molecular mechanism underlying differences in flavonoid metabolites at different tree ages, nine cDNA libraries for *T. grandis* nuts at different tree ages were constructed using RNA-sequencing. An average of 6.72 G of clean bases (Q20 > 97.83%, Q30 > 93.82%) were obtained for each sample (Table S3). Principal component analysis showed nine samples that were dispersed between groups and relatively concentrated within groups (Figure 4a). Differential expressed genes (DEGs) were screened by gene Fragments per Kilobase Million (FPKM) values, and a total of 3815 DEGs (including up- and downregulation) were revealed from heatmap (Figure 4b and Table S4).

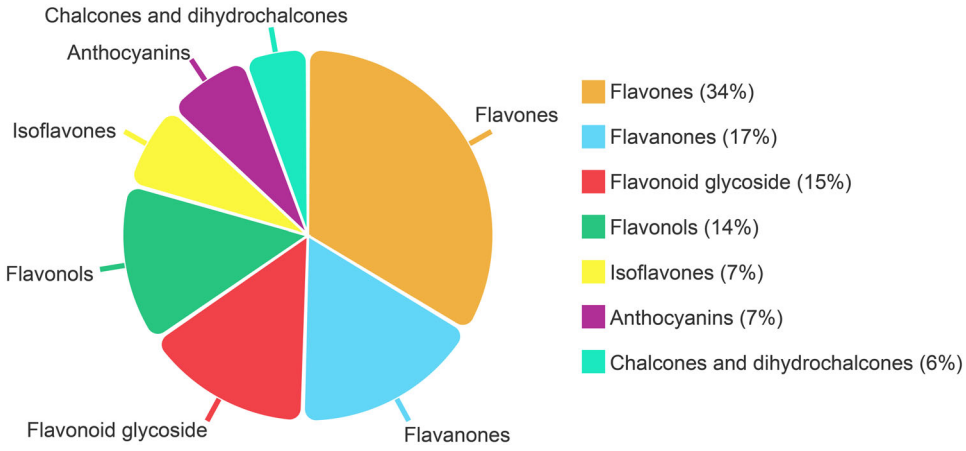

FIGURE 2 Metabolite profiling of flavonoid in *T. grandis* nuts at different tree ages. The proportion of different category of flavonoid subclasses in *T. grandis* nuts based on detected flavonoids using LC-MS

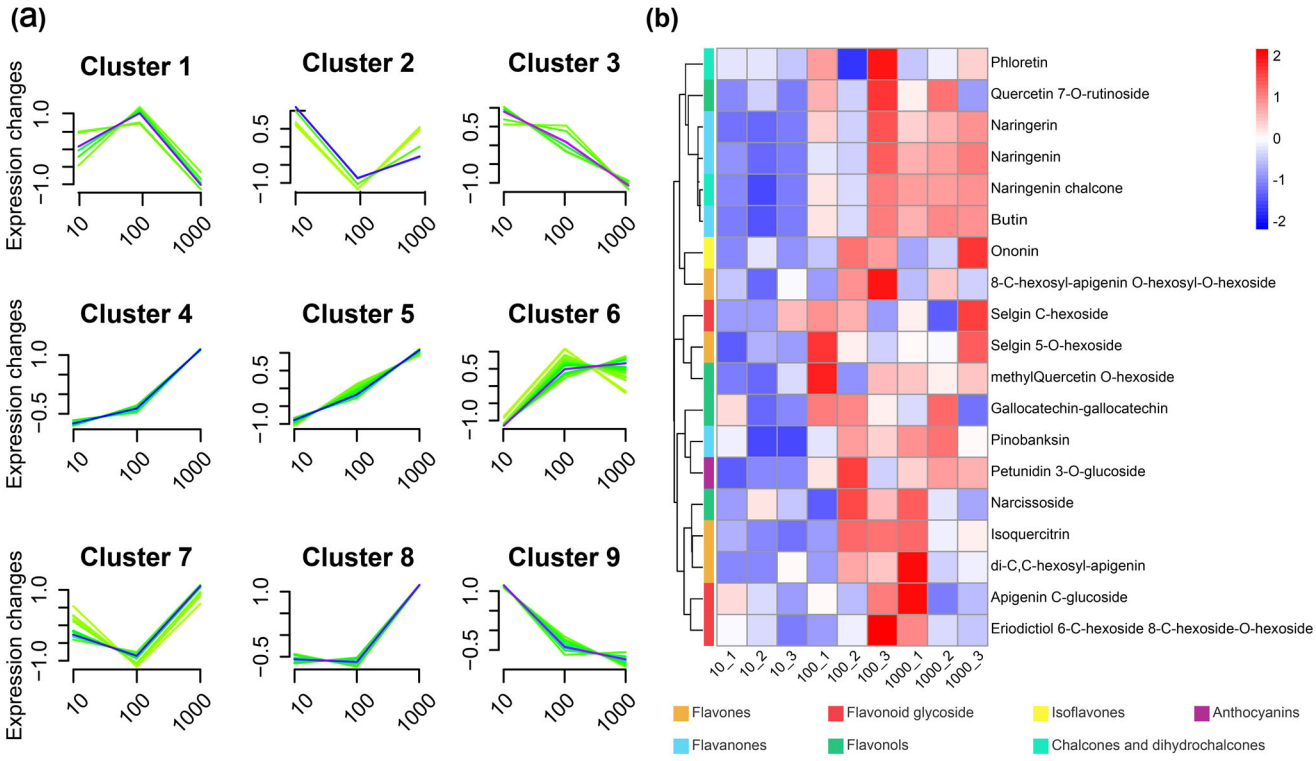

FIGURE 3 Identification of the differential flavonoid metabolites of *T. grandis* nuts at different tree ages. (a) K-means clustering of the metabolites from the nuts of different *T. grandis* nuts. Nine clusters (subclasses 1–9) of flavonoid metabolites were divided based on the dynamic changes of metabolites in nuts at different tree ages. (b) The cluster of different flavonoids metabolites in subclass 6

3.5 | Identification of coexpressed gene networks by weighted gene coexpression network analysis

Weighted gene coexpression network analysis (WGCNA) is a system biology approach to investigate network of strongly linked genes rather than individual genes, which has been successfully used in multiple genomics studies (S. Liu et al., 2021). To identify gene coexpression modules, the obtained 3815 DEGs were assessed using the R-package WGCNA (Langfelder & Horvath, 2008). In this investigation, the matrix was increased to a soft-thresholding power of 18. The minimum module size was set to 30, and modules with highly connected eigengenes (based on a criterion of 0.25) were combined. There were nine different modules revealed (Figure S1), with each tree branch representing a module, and each leaf representing a gene in a hierarchical clustering dendrogram that displayed the modules in color (Figure 5a). As shown in Figure 5(b), a correlation analysis between the 19 flavonoids

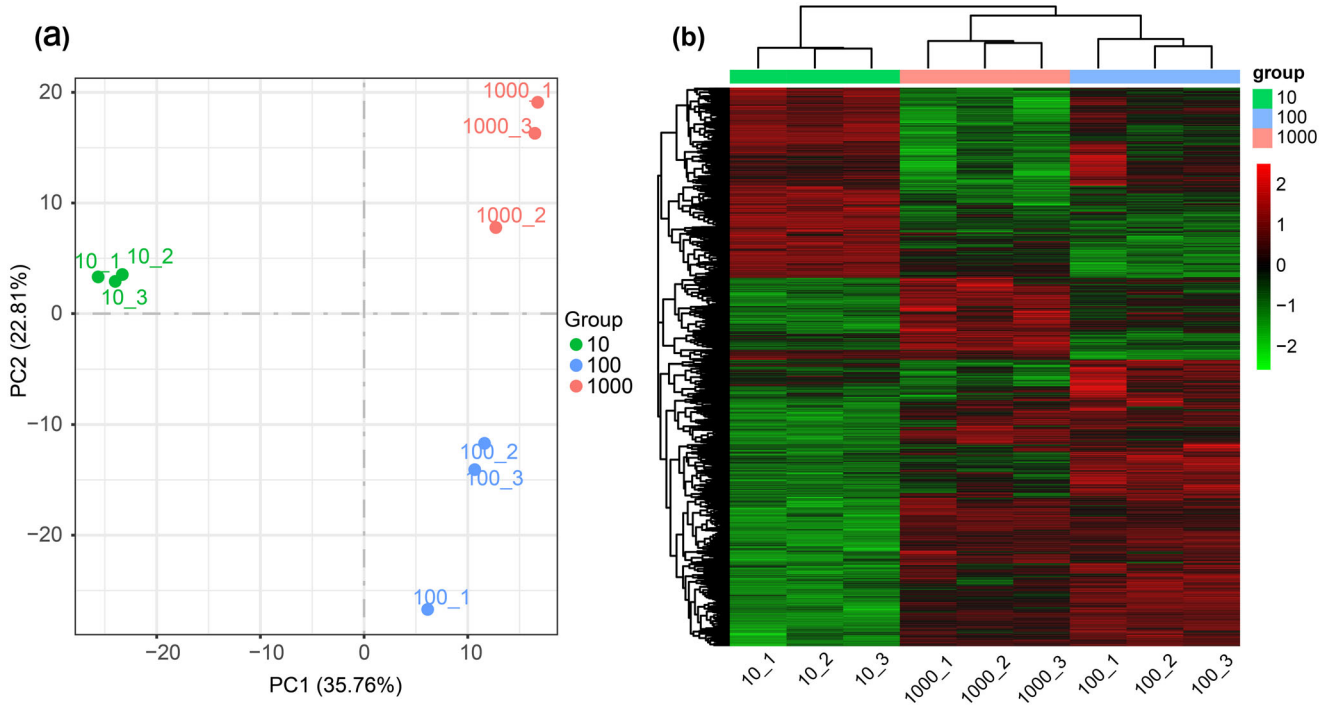

FIGURE 4 Transcriptome analysis of differential expressed genes of *T. grandis* nuts at different tree ages. (a) Principal component analysis (PCA) of monitored gene expression data in nuts at different tree ages. (b) Heat map of differential expressed genes in nuts at different tree ages

and 9 distinct modules was conducted. The colorful shading in the chart indicated significant correlations between the mentioned modules and metabolites (red and blue corresponding to positive and negative correlation, respectively). Application of module-trait association analysis, revealed turquoise, and brown modules, consisting of 1637 genes, were probable modules involved in the biosynthesis of flavonoids owing to their highly substantial link with flavonoids level. Notably, seven flavonoid biosynthesis-related genes, such as three *TgDFRs* (evm008600L.14, evm007621L.26, and evm007621L.19), one *TgFLS* (evm002478L.5), one *TgLAR* (evm026838L.13), and two *TgANRs* (evm005783L.42 and evm009902L.67) in the turquoise and brown modules, were substantially highly expressed in the 100- and 1000-years-old tree than in 10-years-old tree, implying that these genes largely contributed to the downstream accumulation of flavonoids in aged *T. grandis* tree (Figure 5c and Table S5). In addition, some transcription factors (*TgERFs*, *TgbHLHs*, etc.) with these key genes were associated in that module. Based on the linkage degree by WGCNA, a regulatory network of core genes and transcription factors in age-related flavonoids biosynthesis was constructed (Figure 5d).

3.6 | Gene related to hormone signaling and biosynthesis

DEGs related to hormones signaling and biosynthesis were screened from the RNA-seq data (Figure 6 and Table S6). Among which, biosynthesis and signaling pathway of abscisic acid (ABA) and gibberellin (GA) occupied a higher proportion. In the ABA signaling pathway, the expression of 1 *TgPYR/PYL* (evm025334L.1) was higher in nuts of aged tree (100- and 1000-years-old), whereas expression of three *TgPP2Cs* (evm003573L.10, evm010397L.3, and evm005641L.59) was lower in nuts of aged tree. There were eight genes involved in ABA biosynthesis pathway, such as *TgNCEDs* (evm028102L.1 and evm017754L.18), and the expression levels of which were found higher in nuts of aged *T. grandis* tree. In GA signaling pathway, expression of two *TgGID1s* (evm021562L.4 and evm018822L.3), four *TgGID2s* (evm018299L.6, evm003811L.23, evm002620L.5, and evm001922L.38), and three *TgPIF3s* (evm002678L.7, evm002609L.37, and evm002451L.26) were higher in nuts of aged tree, whereas expression of two *TgDELLAs* (evm010956L.2 and evm005523L.9) were lower in nuts of aged tree. There were five genes involved in GA biosynthesis pathway, such as *TgGA3ox* (evm011425L.22), and the expressions of which were higher in nuts of aged *T. grandis* tree. These results imply that ABA and GA might affect age-related flavonoid accumulation via analysis of the expression of hormone-related genes.

4 | DISCUSSION

T. grandis, cultivated in China for more than 1500 years, is a rare and unique species (Chen & Jin, 2019). *T. grandis* cv. Merrillii is the only grafted and thoroughbred species of *T. grandis*, to yield drupe like fruits with nut seeds that are suitable for human consumption (Chen & Jin, 2019; Cui et al., 2018). *T. grandis* nuts are rich in flavonoids with multiple biological effects such as antioxidative, anti-inflammatory, antiviral, antifungal, antitumor, and antihelminthic activities (Ding et al., 2020;

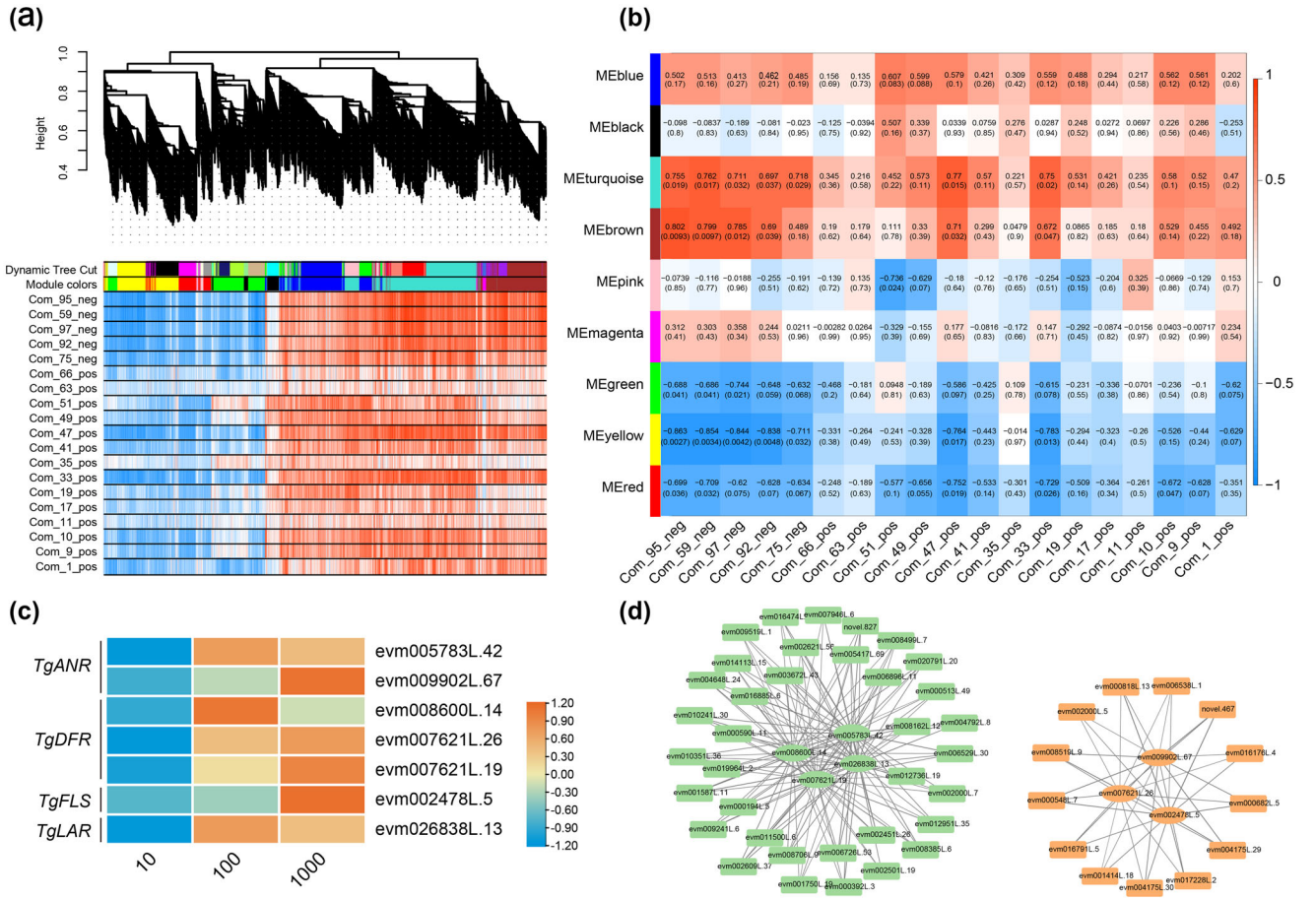

FIGURE 5 Gene coexpression modules associated with flavonoids. (a) Clustering dendrogram presenting nine modules of coexpressed genes and their correlation with flavonoids. (b) Correlations coefficient and significance between modules and 19 flavonoids contained the correlation and *p*-value. (c) The DEGs related to flavonoids. (d) Regulatory network of core genes and transcription factors in age-related flavonoids biosynthesis via cytoscape_3.9.0 software. Ovals represent the key genes related to flavonoids biosynthesis, and squares represent transcription factors.

Lou et al., 2019; Song et al., 2021; Suo et al., 2019, 2022). As a perennial plant species, it has an extremely long lifespan and can survive for hundreds or thousands of years. In Kuiji mountain region in Zhejiang province, the area with largest production of *T. grandis* nut, there have more than 70,000 *T. grandis* trees over 100 years and 4000 *T. grandis* trees over 1000 years (Chen & Jin, 2019). However, differences in chemical composition and antioxidant properties of *T. grandis* nuts at different tree ages are still unknown.

This study revealed that *T. grandis* nuts from young tree (10-years-old) exhibited weak antioxidant activity compared to other old aged tree (100- and 1000-years-old) (Figure 1b and 1c), indicating that nuts of aged trees might present better source of extracts used for antioxidant activity. Previous studies have shown that flavonoids alongside with carotenoids and vitamin E are the key antioxidant chemicals in *T. grandis* nuts (Lou et al., 2019; Zhang et al., 2022). Our study indicated that the strong antioxidant activity of nuts from aged *T. grandis* might be associated with elevated flavonoid content (Figure 2). This is the first time age effect on *T. grandis* nut flavonoid composition and antioxidant activity is reported in literature.

A recent study have identified nine major flavonoids including chrysoeriol, naringenin, and butin, which accounted for the antioxidant activity of nuts in young *T. grandis* (Zhang et al., 2022). Herein, and based on large-scale metabolites profiling, 19 flavonoids, such as naringenin, naringenin chalcone, pinobanksin, isoquercitrin, narcissoside, and butin, showed significant correlations with antioxidant activity in 100- and 1000-years-old trees (Figure 4b). Numerous studies have shown that some of the aforementioned flavonoids exert potential antioxidant properties, which could be used for medicinal purposes. For example, naringenin and naringenin chalcone have anti-inflammatory and antiallergic activities in mice with therapeutic potential for inflammation-induced sepsis, fulminant hepatitis, fibrosis, and cancer (Escribano-Ferrer et al., 2019; Salehi et al., 2019; Tutunchi et al., 2020). Narcissoside and rutin exert antiacute myeloid leukemia activities and the ability to relieve mitochondrial-induced oxidative stress (Dubey & Dubey, 2020; T. Liu et al., 2020). These studies provide valuable information for development of better natural antioxidants using aged *T. grandis* and for potential incorporation in nutraceuticals used as antioxidants.

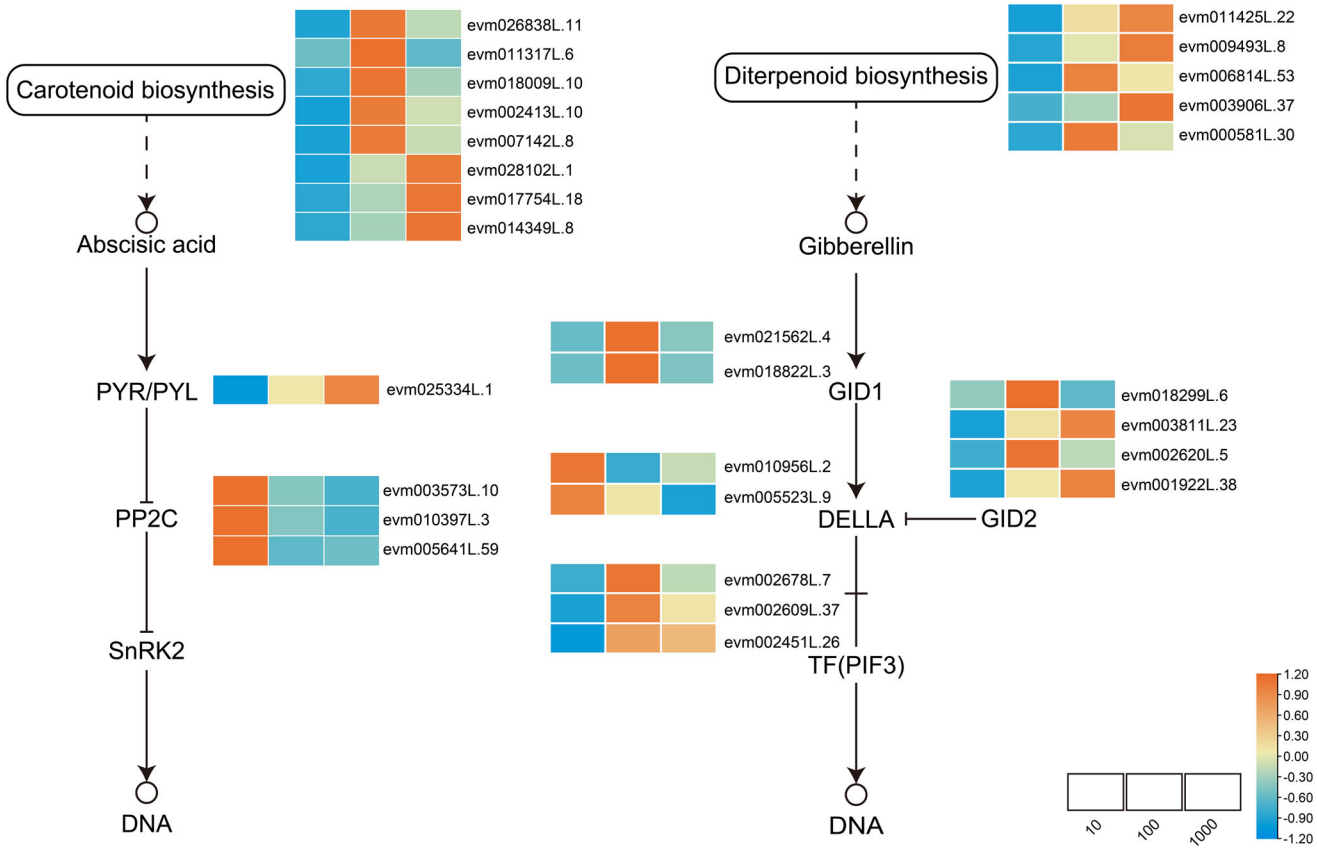

FIGURE 6 Gene-related to hormone signaling and biosynthesis in nuts at different tree ages. Diagrams of biosynthesis pathway and signal transduction of abscisic acid and gibberellin

In *G. biloba*, GbCHS, GbF3H, and GbFLS, which are the key structural genes in flavonoids biosynthesis, are believed to be involved in the age-related decrease of flavonoid (Q. Wang et al., 2022). A recent study found that the leaves of rejuvenated *G. biloba* had a marked increase in the accumulation of flavonol glycosides and revealed that flavonoids biosynthesis genes (GbCHS, GbFLS, GbF3'H, GbDFR, and GbLAR) might play vital roles in this process (Lu et al., 2022). This means that mining key genes involved in age-related flavonoid accumulation of *T. grandis* is very important. Here, 7 DEGs (*TgDFRs*, *TgFLS*, *TgLAR*, and *TgANRs*) were revealed to be related to the age-related flavonoid accumulation of *T. grandis* based on WGCNA of flavonoids and gene expressions. Furthermore, several transcription factors were found to be closely correlated to the expression of these genes, that is,, *TgDFRs*, *TgFLS*, *TgLAR*, and *TgANRs*, implying that the above transcription factors might act as key regulators for age-related flavonoids accumulation.

The accumulation of flavonoid in plant is typically regulated by phytohormones (Brunetti et al., 2019; Gupta et al., 2018; Jan et al., 2021). For example, GA upregulates flavonoids accumulation in *Gingko biilba* (Lu et al., 2022). Likewise in tea plants, treatment with ABA obviously increased flavones, anthocyanins, flavonols, and isoflavones (Gai et al., 2020). In this study, the expressions of genes related to ABA and GA biosynthesis and signaling pathway were elevated in aged *T. grandis*, implying that ABA and GA might regulate the age-related accumulation of flavonoid. Aged *T. grandis* tree is highly vulnerable to more adverse conditions (B. Wang et al., 2022) and to account for the increased production of flavonoids to improve tree fitness. Considering the roles of ABA and GA in defense, and we propose that the high ABA and GA levels might upregulate flavonoids biosynthesis concurrent with improved antioxidant actions and resistance to stress conditions. Gai et al. (2020) showed that ABA upregulated the expressions of *CsCHI*, *CsDRF*, *CsF3'H*, and *CsFLS* to increase the accumulation of flavonoids (Gai et al., 2020). Treatment with ABA results in a higher accumulation of total anthocyanin via increasing the expression of *VvCHI* and *VvF3H* in grape (Koyama et al., 2018). In *Medicago truncatula*, GA inhibits the biosynthesis of flavonoid and isoflavonoid via regulating the transcripts of flavonoid biosynthesis genes, such as *MtCHI*, *MtCHS*, and *MtDFR* (Sun et al., 2021). Future work needs to unravel the mechanism by which ABA and GA affect the age-related flavonoid accumulation.

5 | CONCLUSION

In this study, effects of tree age on the flavonoids and antioxidant activity of *T. grandis* nuts were analyzed. Results showed that antioxidant activity and the total flavonoid contents in 10-years-old *T. grandis* were lower compared to those in 100- and 1000-years-old

T. grandis. The contents of 19 flavonoids showed significant correlations with antioxidant activity in 100- and 1000-years-old trees. Coexpression analysis indicated that seven key genes (*TgDFRs*, *TgFLS*, *TgLAR*, and *TgANRs*) were associated with aged-related flavonoids biosynthesis in *T. grandis* nuts. Further analysis indicated that genes related to ABA and GA biosynthesis (*TgNCEDs* and *TgGA3ox*) and signaling pathway (*TgPYR/PYL*, *TgPP2Cs*, *TgGID1s*, *TgGID2s*, *TgPIF3s*, and *TgDELLAs*) were correlated with the accumulation of flavonoids in 100- and 1000-years-old trees, implying that ABA and GA might regulate age-related flavonoid accumulation and identification of such action mechanism is the next logical step to be explored. These results can help to identify targeted flavonoids at different tree ages of *T. grandis* known to exert strong antioxidant activity and health benefits, and provides valuable information for ontogeny in *T. grandis* and its chemical makeup. Similar platform can also be used to assess age-related secondary metabolites accumulation pattern in other long life span trees such as olive tree of notable nutraceutical value.

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CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest to declare for this publication

ETHICS STATEMENT

None declared.

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SUPPORTING INFORMATION

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