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New insights into the accumulation of vitamin B_3 in *Torreya grandis* nuts via ethylene induced key gene expression

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ABSTRACT

Vitamin B_3, derived primarily from plant sources, is an essential nutrient for humans. *Torreya grandis* is rich in vitamin B_3, however, the mechanism underlying the biosynthesis and regulation of vitamin B_3 in *T. grandis* remains unclear. A systematic transcriptomic investigation was thus conducted to identify the gene expression pattern of vitamin B_3 biosynthesis in 10 *T. grandis* cultivars. The findings suggest that biosynthesis occurs mainly via the aspartate pathway. Expression and correlation analyses indicate that aspartate oxidase (AOX) and quinolinate synthase (QS) may play important roles in vitamin B_3 accumulation. Furthermore, co-expression network and ethephon treatments indicate that the ethylene response factor (ERF) may be involved in the regulation of vitamin B_3 biosynthesis in *T. grandis* nuts. Our findings not only help to elucidate the biosynthesis of vitamin B_3, but also provide valuable resource material for future genomic research and molecular-assisted breeding to develop genotypes with higher vitamin B_3 levels.

1. Introduction

B vitamins are essential human nutrients as they are involved in numerous pivotal metabolic processes (Fitzpatrick et al., 2012). Vitamin B_3, also known as niacin, has long been recognized for its role as a cofactor or coenzyme in the synthesis of NAD^+ and $NADP^+$ in the human body, and it thus contributes to numerous metabolic processes (Roje, 2007). Severe vitamin B_3 deficiency usually leads to pellagra (Fitzpatrick et al., 2012; Stea et al., 2018). Vitamin B_3 has been suggested to be effective in serious of disease, such as angiocardiopathy, reduced serum lipid and cholesterol levels, malignant tumors, and skin cancer (Miret and Munné-bosch, 2014; Liu et al., 2020; Luo et al., 2020; Ratnarajah et al., 2020). It is derived mainly from plant sources, primarily vegetables, grains, and derived products (oils) (Fitzpatrick et al., 2012). However, species, cultivars, and storage organs differ widely in their vitamin B_3 content (Lebiedzińska and Szefer, 2006; Wolak et al., 2016). It is therefore essential to examine the levels, biosynthesis, and molecular regulation of vitamin B_3 in plants.

The pathways involved in vitamin B_3 biosynthesis have previously been debated (Roje, 2007). Initially, the aspartate and tryptophan pathways were thought to be involved. However, subsequent studies have suggested that *de novo* vitamin B_3 biosynthesis in plants occurs mainly via aspartate precursors, whereas in animals, fungi, and some bacteria, it occurs via the tryptophan pathway (Katoh et al., 2006; Ashihara et al., 2015). Furthermore, genomic and bioinformatic research using relevant databases has indicated that *Arabidopsis* synthesizes vitamin B_3 from the aspartate pathway, whereas rice utilizes both aspartate and tryptophan as starting amino acids (Katoh and Hashimoto, 2004; Katoh et al., 2006). In the aspartate pathway, nicotinate mononucleotide (NaMN) is formed from L-aspartate via the catalysis of aspartate oxidase (AOX), quinolinate synthase (QS), and quinolinate phosphoribosyltransferase (QPT) (Roje, 2007). NaMN is converted first to nicotinic acid adenine dinucleotide, then to nicotinamide adenine dinucleotide (NAD), and then finally degraded to vitamin

Abbreviations: 5′-N, 5′-nucleotidase; AOX, aspartate oxidase; DEG, differentially expressed gene; ERF, ethylene response factor; JA, Jasmonic acid; NAD, nicotinamide adenine dinucleotide; NADpp, NAD pyrophosphatase; NaMN, nicotinate mononucleotide; NIC, nicotinamide deamidase; NMNN, nicotinamide mononucleotide nucleosidase; NNAT, NaMN adenyltransferase; QPT, quinolinate phosphoribosyltransferase; QS, quinolinate synthase; TF, transcription factor.

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B_3 by various enzymes (Gerdes et al., 2012). Most of the enzymes in the aspartate pathway, including AOX, QS, QPT, NAD synthase, and nicotinamide deamidase (NIC) have been isolated, although the gene for nicotinamide mononucleotide nucleosidase (NMNN) has not yet been identified (Katoh et al., 2006; Gerdes et al., 2012; Hao et al., 2018). Studies in *Arabidopsis* suggest that AOX, QS, NaMN adenyltransferase (NNAT), and NAD synthase play essential roles in the aspartate pathway (Hashida et al., 2007; Schippers et al., 2008). Mutations in *AOX*, *QS*, *QPT,* and *NNAT* not only block the biosynthetic pathway but also affect the growth and development of the plants themselves (Sinclair et al., 2000; Hashida et al., 2007; Hao et al., 2018).

In addition, several recent studies have shown the regulatory role of phytohormones in the gene expression of enzymes in the aspartate pathway (Li et al., 2019). A mutation in the *AtQS* gene influences ABA signal transduction in *Arabidopsis* and the expression of the QS gene is repressed directly by ABI4, a transcription factor (TF) in the ABA response pathway (Hong et al., 2020; Wei et al., 2020). NtMYC2, a transcription factor in jasmonic acid (JA) signaling, can recognize the promoter regions of QPT and activate the expression of QPT in transgenic tobacco (Hashimoto and Shoji, 2011). Ethylene response factor (ERF)-type transcription factors, including ERF189, have recently been identified as direct and specific regulators of tobacco *QPT2* (Shoji & Hashimoto, 2011). However, the enzymes and the transcriptional regulation of genes responsible for vitamin B_3 biosynthesis is not yet completely understood and previous work has mostly focused on the model plants *Arabidopsis* and tobacco (Roje et al., 2007). Consequently, for most plants, little is known about their biosynthesis and molecular regulation of vitamin B_3, and the genomic characteristics and differential expression patterns for the genes in the vitamin B_3 biosynthesis pathway has not yet been described. A comprehensive understanding of the steps and regulatory mechanisms in this pathway is therefore required and will help to enable the improvement of plant vitamin B_3 levels via genetic and metabolic engineering approaches.

Torreya grandis (Taxaceae) is an important economic tree species native to China, with dioecious flowers and drupe-like fruits with nut-seeds (He et al., 2016). Roasted- *T. grandis* nuts are popular and economically important, as they are highly nutritious. Furthermore, they are also a natural source of medicinal and edible foods and nutraceutical supplements, especially for the high contents of bioactive compounds and vitamins (Ni et al., 2015; He et al., 2016; Wu et al., 2018; Lou et al., 2019; Suo et al., 2019). Nowadays, although vitamin biosynthesis has been widely studied in plants, vitamin B_3 biosynthesis in *T. grandis* is not well understood. It is likely that vitamin B_3 levels vary widely with geographical distribution, development stage, and cultivar (Kim et al., 2014). However, to the best of our knowledge, these differences, and the regulatory mechanisms of vitamin B_3 biosynthesis, have not yet been examined for *T. grandis*. Most notably, knowledge is lacking about the genomic characteristics and expression patterns of genes in the vitamin B_3 biosynthetic pathway of *T. grandis*.

Therefore, our primary objective was to determine vitamin B_3 levels in the nuts from different *T. grandis* cultivars. The secondary objective was to identify candidate genes and the probable regulatory mechanisms that were involved in vitamin B_3 biosynthesis. We found that vitamin B_3 contents were highly dependent on the cultivar and differential gene expression, which suggested that the *de novo* biosynthesis of vitamin B_3 occurs mainly via the aspartate pathway in *T. grandis* nuts. Furthermore, the key genes regulating vitamin B_3 accumulation among the different *T. grandis* cultivars were identified using Pearson's correlation analysis, sequence alignment, and phylogenetic analysis. Moreover, a potential regulatory mechanism of the key genes expression was also proposed using the co-expression network and exogenous ethylene experiments. These findings will help to elucidate the key genes and regulatory steps controlling vitamin B_3 levels in *T. grandis* nuts. Our results will be valuable for future molecular research and genetic screening for vitamin B_3-rich *T. grandis* genotypes.

2. Material and methods

2.1. Plant materials

Samples from 10 *T. grandis* cultivars (Z08, S14, W03, R19, Y05, A17, A33, R14, Y44, and Z05) were collected from the cities of Zhuji, Fuyang, Huizhou, Shengzhou, Huangshan, and Shaoxing, in Zhejiang and Anhui provinces (Table S1). The trees were maintained using the standard fertilization, irrigation, and pest control practices recommended for *Torreya* cultivars (Dai et al., 2008). The nuts were collected at the mature stage (120 days after seed protrusion, DASP) from all 10 cultivars. Then the kernel samples, with the sarcotesta and testa removed, were sectioned, and immediately frozen in liquid nitrogen and stored at −80 °C, for later vitamin B_3 determination, transcriptome sequencing, and quantitative real-time (qRT)-PCR analysis.

2.2. B vitamin assay

For the extraction of the B vitamins, *T. grandis* kernel (0.5 g) samples were ground into a powder. The extraction method for vitamins B_1, B_2, and B_9 was in accordance with that described by Al-Farga et al. (2016). For the extraction, 5 mL of 30% metaphosphate was added to the samples, macerated in a glass blender, and then diluted to 25 mL. The extraction of vitamin B_3 was according to the GB 5009.89–2016. The powder was extracted in 25 mL of distilled water, followed by the addition of 5 mol/L hydrochloric acid and 5 mol/L sodium hydroxide solution, to adjust the pH to 4.5. Both mixtures were extracted using a sonicator at 50 °C for 10 min, then centrifuged at 3000×*g* for 10 min, and then the supernatant was filtered through a 0.45 μm filter membrane (Whatman Inc., Maidstone, UK) before HPLC analysis. The B vitamin content analyses were performed using an Agilent 1200 series HPLC system (Agilent, Böblingen, Germany). The injection volume was 20 μL for each sample, and a C_{18} column was used, with a column temperature of 25 °C. The mobile phase was 5 mmol/L sodium 1-hexanesulfonate with 0.7% acetic acid (v/v) and 0.2% triethylamine (v/v) (solvent A), and methanol (solvent B), and the flow rate is 1.0 mL/min. The gradient elution profile was as follows: 0–8 min, 0% B; 8–20 min, 0–25% B; 21–30 min, 25–45% B; 30–31 min, 45–0% B; and 31–45 min, 0% B.

2.3. RNA extraction, cDNA preparation, and sequencing

RNA samples were extracted from the kernels of each cultivar using an RNAprep Pure Plant Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The quantity and quality (purity and integrity) of the total RNA was then determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and agarose gel electrophoresis. The cDNA libraries were constructed from a total of 3 μg RNA samples, according to the standard protocol. The paired-end sequencing was performed in Hangzhou He Yi Gene Technology Co., Ltd, and the Illumina HiSeq™ 4000 platform (Illumina Inc., San Diego, CA, USA) was used.

2.4. Functional annotation and differentially expressed gene (DEG) analysis

De novo assembly of the mRNA-seq reads was performed using Trinity v. 6.0, and unigene functions were annotated using a BLASTX search against the following databases: Nr (http://www.ncbi.nlm.nih.gov/), Nt (http://www.ncbi.nlm.nih.gov/), SwissProt (http://www.uniprot.org/), COG (http://www.ncbi.nlm.nih.gov/COG/), KEGG (http://www.genome.jp/kegg/), and GO (http://www.geneontology.org/). The expression of unigenes were calculated using the fragments per kilobase per million fragments (FPKM) method, and the differential expression of the unigenes was judged using the absolute value of $\log_2$ (fold change) ≥ 1, false discovery rate (FDR) < 0.001, and $p < 0.05$.

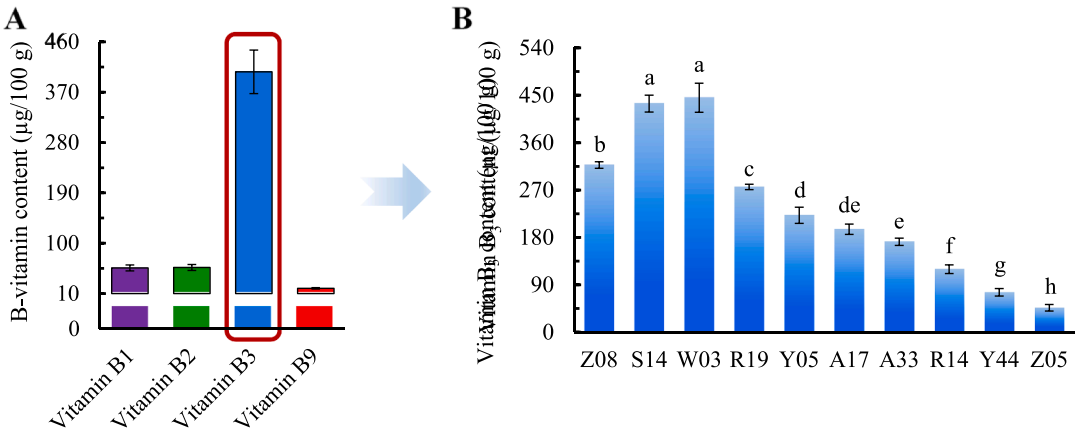

Fig. 1. B vitamin contents in nuts of 10 *Torreya grandis* cultivars. (A) Contents of vitamin B_1, B_2, B_3, and B_9 in nuts of *Torreya grandis*; (B) Contents of vitamin B_3 in nuts of different *Torreya grandis* cultivars.

After that, the function of the DEGs was analyzed by GO and KEGG pathway enrichment (Suo et al., 2019).

2.5. Phylogenetic tree and sequence analysis

Phylogenetic tree analysis of selected genes was constructed using MEGA 6.0, and using the neighbor-joining method with 1000 bootstrap replicates. Amino-acid sequence alignment was performed using DNAMAN 6.0.

2.6. Gene co-expression network analysis

Pearson's correlation coefficients (*r*) were calculated to select the positive and negative correlations between vitamin B_3 contents and the TFs expression in the nuts of different *T. grandis* cultivars. On this basis, we further analyzed the correlations between candidate transcription factors and the FPKM value of genes in the B3 biosynthesis pathway, then the correlations were used to construct the dynamic network and visualized with Cytoscape (version 4.0).

2.7. RNA isolation and qRT-PCR analysis

A subset of the unigenes involved in vitamin B_3 biosynthesis were assessed using qRT-PCR analysis on a CFX96 Real-time PCR system (Bio-Rad, Hercules, CA, USA). The PCR primers used in this study are listed in Table S2 and Table S3, and the program was as follows: 95 °C for 10 min, followed by 40 cycles of 10 s at 95 °C, 10 s at 57 °C, and 20 s at 72 °C. Then a melting curve analysis was added that starting at 60 °C and increasing to 95 °C. The relative expression level of each gene was determined using the *18S* gene as an endogenous reference and was calculated according to Livak and Schmittgen (2001) using the $2^{-\Delta\Delta Ct}$ method. The experiments were repeated three times.

2.8. Effects of ethylene treatment on vitamin B_3 biosynthesis

To verify the effects of ethylene on vitamin B_3 biosynthesis regulation, *T. grandis* nuts were harvested in September 2019. Those with no infection or physical damage were selected for the ethylene treatment (ethephon solutions, Sigma) with the treatment concentration of 4000 mg/kg according to preliminary experimental results. The treatment and control group were stored at a constant temperature of 25 ℃ and relative humidity of 90%, and three biological replicates were carried out. During the treatments, nut samples were collected after 0, 3, 6, and 9 days for vitamin B_3 assay and gene expression analysis.

For the determination of the vitamins and their derivatives after ethylene treatment, we performed metabolomic profiling analysis. After freeze-drying, the samples were ground into powders and kernel metabolite extracts were prepared using the method described by Chen et al. (2020). Metabolite screening was performed using an Ultra Performance Liquid Chromatography (Shim-pack UFLC SHIMADZU CBM30A) system coupled with tandem mass spectrometry (Applied Biosystems 6500 QTRAP). The flow phase and mass spectrometry conditions were in accordance with those described by Chen et al. (2020).

2.9. Statistical analysis

SPSS 18.0 (SPSS Inc., Chicago, IL) was used to carry out one-way ANOVA and Student's *t* test analyses and a $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Vitamin B_3 levels vary with the T. grandis cultivars

The vitamin B complex has important functions in energy metabolism and as it cannot be synthesized in the human body it must be replenished in the daily diet (Strohm et al., 2016). Plants are one of the main sources of B vitamins in the human diet (Fitzpatrick et al., 2012). However, most vitamins are concentrated in the outer layers of the grain, and certain kinds of processing may greatly reduce their levels (Slavin, 2015). Various legumes, seeds, and nuts, such as sesame, sunflower, and pumpkin seeds, also the B vitamins-rich diet sources (Lebiedzińska and Szefer, 2006).

We previously measured B vitamin (B_1, B_2, B_3, and B_9) levels in *T. grandis* nuts, and among them, the B_3 levels tended to be much higher than those of the other water-soluble B vitamins (Fig. 1A). Hence, we focused on vitamin B_3 in this study. The results showed that vitamin B_3 levels differed among the *T. grandis* cultivars, and the highest vitamin B_3 levels were in cultivars S14 and W03, at 0.43 mg/100 g and 0.45 mg/100 g, respectively (Fig. 1B). Similarly, it has been found in other plants that the vitamin B_3 levels are highly dependent on the variety and cultivar (Kim et al., 2014). Niacin levels ranged from 2.2 – 4.0 mg/100 g in the seeds of black and yellow soybean varieties (Kim et al., 2014), while vitamin B_3 levels ranged from 0.55 to 0.94 mg/100 g in walnut kernels from three varieties (Jentsch and Morgan, 1949). Overall, these results suggest that the content of vitamin B_3 varied with the different *T. grandis* cultivars, and that cultivars S14 and W03 may be potential natural sources of vitamin B_3 and thus crucial for future research and functional breeding.

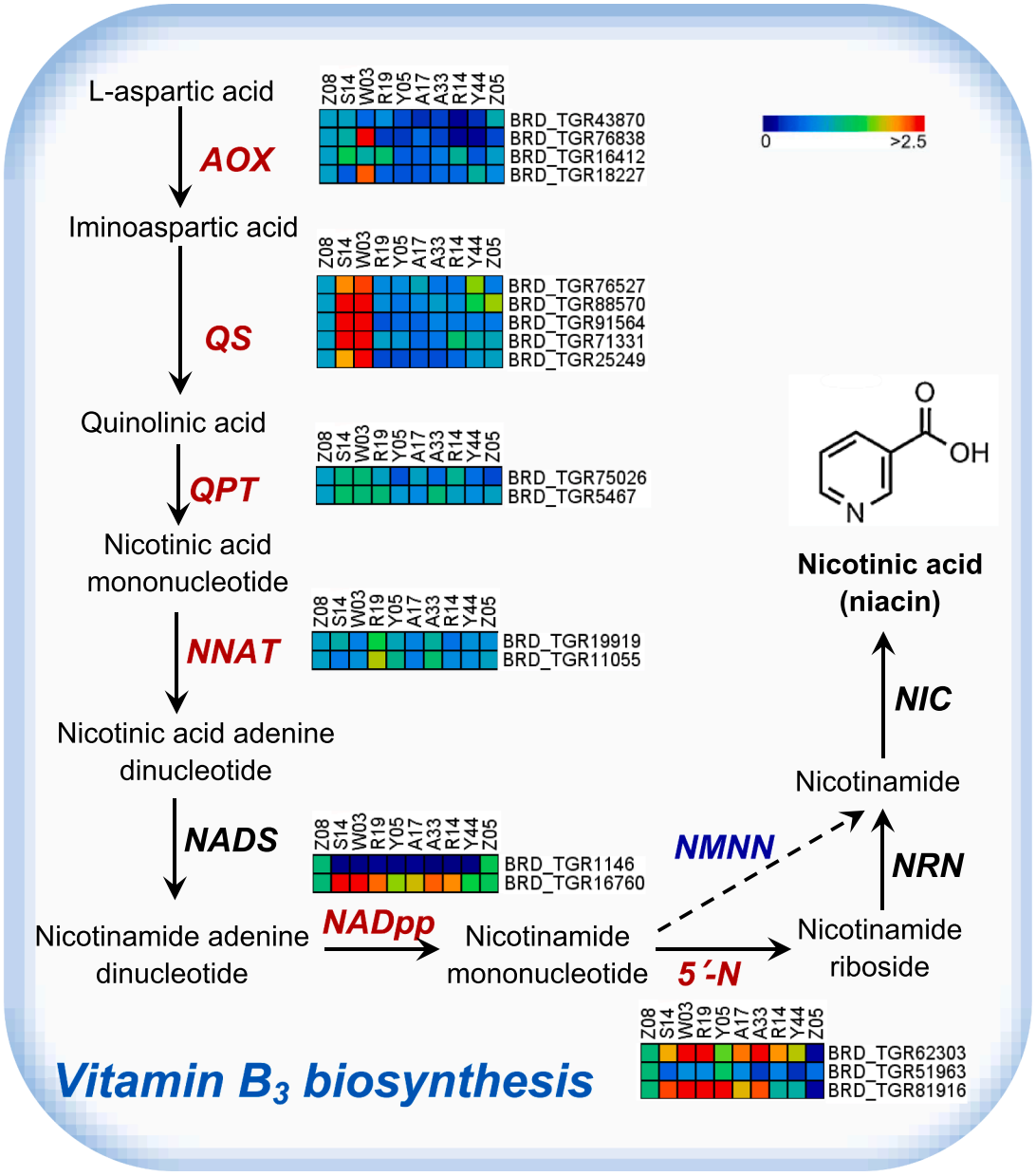

Fig. 2. Schematic presentation of vitamin B_3 biosynthesis in *Torreya grandis* nuts. The scale bar indicates FPKM ratios, colors from blue to red indicate the relative expression level for each gene in vitamin B_3 biosynthesis pathway. Enzymes in red/black color indicate genes identified/ do not identified in *T. grandis* cultivars, enzyme in blue color indicate gene missing in all plants. Abbreviations: 5′-N, 5′-nucleotidase; AOX, aspartate oxidase; NADpp, NAD pyrophosphatase; NADS, NAD synthase; NIC, nicotinamide deamidase; NMNN, nicotinamide mononucleotide nucleosidase; NNAT, NaMN adenyltransferase; NRN, nicotinamide riboside nucleosidase; QPT, quinolinate phosphoribosyltransferase; QS, quinolinate synthase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. The biosynthesis and key enzymes controlling vitamin B_3 accumulation in different cultivars

As vitamin B_3 is crucial for human health, so is our understanding of its biosynthesis mechanisms (Roje, 2007; Fitzpatrick et al., 2012). In the study, the transcription profiles of nuts in 10 *T. grandis* cultivars were sequenced. At least 54,343,810 paired-end clean reads were obtained from each cultivar, with average Q20 and Q30 values of 97.44% and 93.32%, respectively, and an average GC content of 46.23% (Table S4). The putative functions of the assembled unigenes were annotated by aligning them with several public protein databases with an E-value $<$ 0.00001. The overall functional annotation is described in Table S5 and Table S6. DEG analysis found that at least 2045 upregulated and 5411 downregulated unigenes were differentially expressed in the cultivars, compared with their expression in cultivar Z08 (Fig. S1). Subsequently, gene ontology (GO) and KEGG enrichment was applied to classify the functions of the DEGs (Fig. S2). Among the top categories, the biosynthesis of secondary metabolites was significantly enriched (Fig. S3). Furthermore, we performed quantitative RT-PCR analysis to confirm the expression levels of the genes identified by RNA-seq analysis. A significant positive Pearson's correlation ($r^2 = 0.751$, $p < 0.01$) was found between the RNA-seq data and the qRT-PCR results (Fig. S4).

Although there is some controversy regarding vitamin B_3 biosynthesis in plants, our transcriptome sequencing results have shown that consistent with the findings for *Arabidopsis*, *de novo* vitamin B_3 biosynthesis in *T. grandis* nuts occurs mainly through the aspartate pathway, and not the tryptophan pathway (Katoh et al., 2006; Noctor et al., 2006; Roje, 2007). Our transcriptome profiling analysis identified almost all

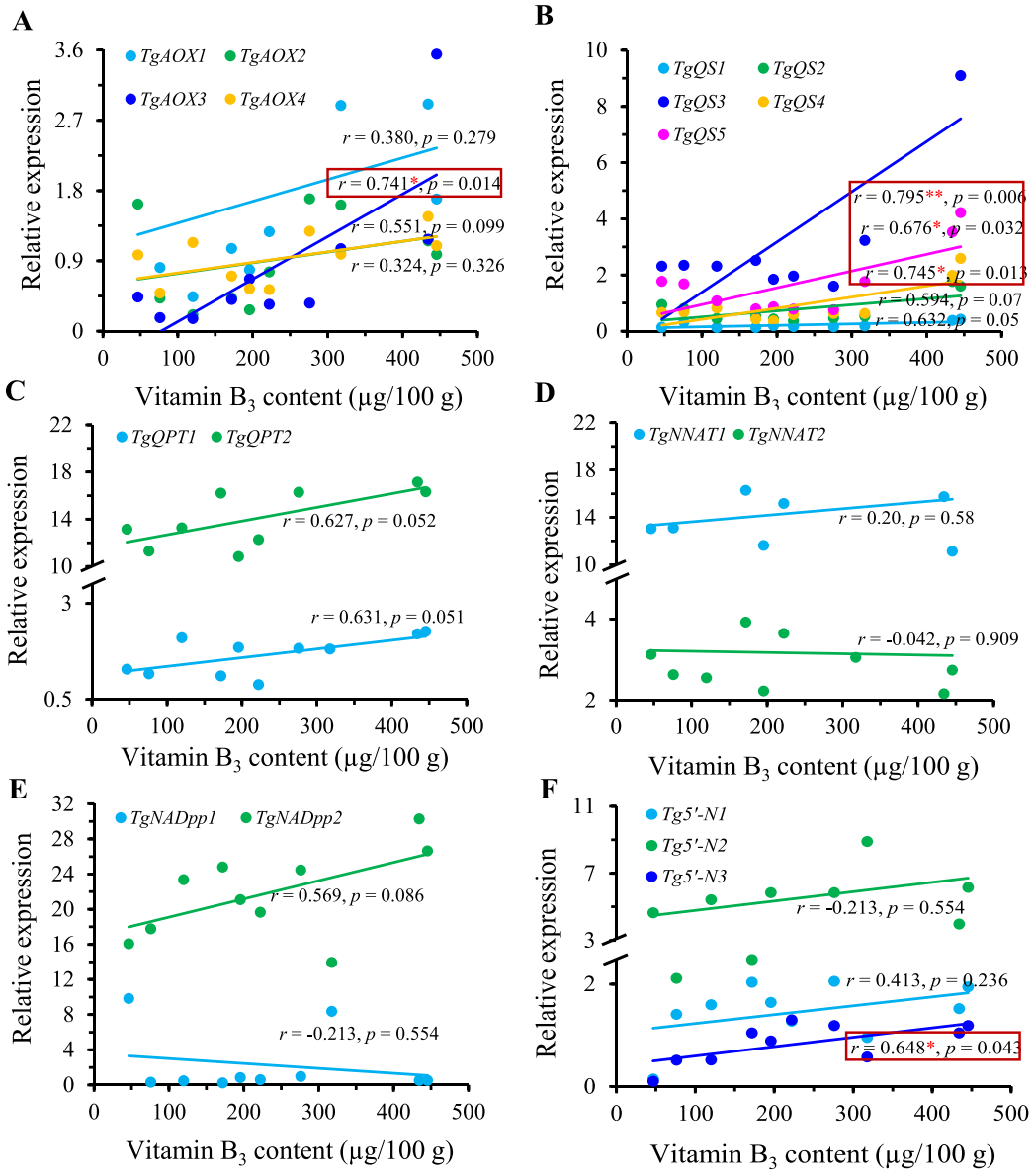

Fig. 3. Correlation analysis between vitamin B_3 content and the expression level of candidate genes involved in the vitamin B_3 biosynthesis pathway in nuts of different *Torreya grandis* cultivars. TgAOX1 (BRD_TGR43870), TgAOX2 (BRD_TGR18227), TgAOX3 (BRD_TGR76838), TgAOX4 (BRD_TGR16412), TgQS1 (BRD_TGR76527), TgQS2 (BRD_TGR88570), TgQS3 (BRD_TGR91564), TgQS4 (BRD_TGR71331), TgQS5 (BRD_TGR25249), TgQPT1 (BRD_TGR75026), TgQPT2 (BRD_TGR5467), TgNNAT1 (BRD_TGR19919), TgNNAT2 (BRD_TGR11055), TgNADpp1 (BRD_TGR1146), TgNADpp2 (BRD_TGR16760), Tg5′-N1 (BRD_TGR62303), Tg5′-N2 (BRD_TGR51963), Tg5′-N 3(BRD_TGR81916).

the unigenes that participate in the aspartate pathway, including AOX, QS, and QPT, and seven of the unigenes encoding three of the enzymes—NNAT, NADpp, and 5′-nucleotidase (5′-N) (Table S7). When assessing homologues of the genes specific to the tryptophan pathway, only kynurenine 3-monooxygenase was identified (Table S6). These results suggest that the main pathway responsible for vitamin B_3 accumulation in *T. grandis* is the aspartate pathway.

Furthermore, the expression of genes in the *de novo* aspartate pathway differed substantially among the *T. grandis* cultivars (Fig. 2). Two of the *AOX* unigenes were downregulated in most of the cultivars, while the other two were upregulated in S14 and W03 and downregulated in the other cultivars. The expression of *QS* and *QPT* was highest in S14 and W03 (Fig. 2, Table S7). The expression of *NNAT* declined, while the one transcript of the *NADpp* unigenes was induced. Similarly, two of the transcripts of *5′-N* were highly expressed in most cultivars (Fig. 2, Table S7). Therefore, most of the genes (*TgAOX*, *TgQS*, *TgNADpp*, and *Tg5′-N*) involved in the vitamin B_3 biosynthesis pathways were upregulated in S14 and W03, in which a marked accumulation of vitamin B_3 was also observed (Figs. 1 and 2, Table S7). Moreover, there were positive correlations between *TgAOX*, *TgQS*, and *Tg5′-N* transcription levels and vitamin B_3 contents, and the correlation coefficients for TgAOX3, TgQS3, and TgQS5 were greater than 0.74 ($p < 0.05$) (Fig. 3), which strongly suggested key roles for TgAOX and TgQS in the accumulation of vitamin B_3 in the nuts of *T. grandis*.

Combined with the phylogenetic and sequence analysis, TgAOX and TgQS were shown to have a close relationship between the proteins from higher plants, such as *Arabidopsis* and *Oryza sativa*, and they contained conserved functional domains (Fig. S5). Transgenic *Arabidopsis* studies found that AOX and QS were the rate limiting enzymes for vitamin B_3 precursor biosynthesis (Katoh et al., 2006; Hao et al., 2018). *Arabidopsis OLD5* encodes a QS protein, and the *AtOLD5* mutant showed reduced overall QS protein activity and disturbed the *de novo* pathway for NAD

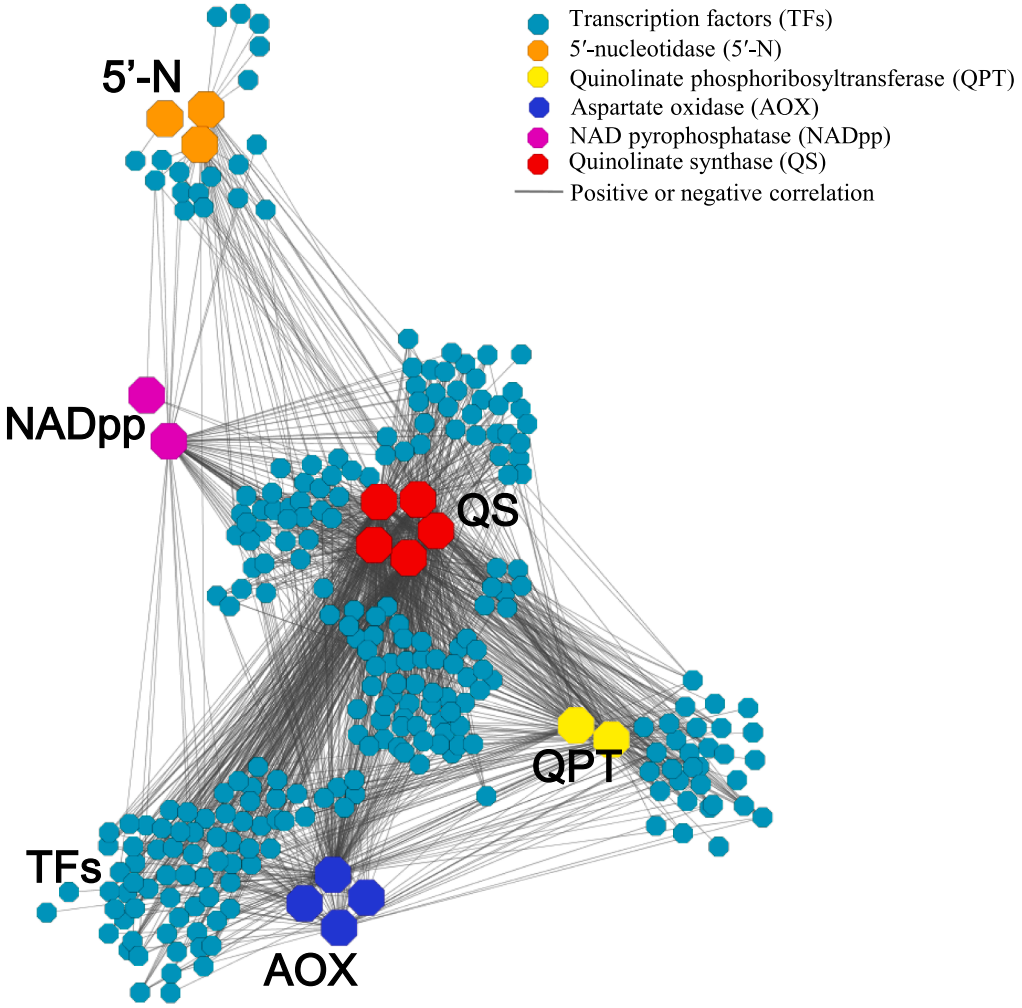

Fig. 4. Coexpression networks associated with Vitamin B_3 biosynthesis regulation in nuts of *Torreya grandis*. The Gene ID of transcription factors and related correlation coefficients were shown in supplementary Table S9.

synthesis (Schippers et al., 2008). Therefore, it is speculated that TgAOX and TgQS are the key enzymes in the aspartate pathway in *T. grandis* nuts, and the upregulation of *TgAOX* and *TgQS* may be responsible for the relatively high accumulation of vitamin B_3 in cultivars S14 and W03.

3.3. The accumulation of vitamin B3 after ethylene treatment may be achieved by the upregulation of TgAOX and TgQS through ERF

It has been reported that the transcriptional regulation of several genes, including *QS* and *QPT*, in the aspartate pathway can be induced by hormone signaling, such as ABA, JA, and ethylene, and several transcription factors have been identified (Hashimoto and Shoji, 2011; Hong et al., 2020; Wei et al., 2020). In this study, a total of 270 transcripts of TFs were found correlated with vitamin B_3 contents in the nuts of different *T. grandis* cultivars (Table S8). Among them, 251 TFs, including those of WRKY, MYB, ERF, VOZ, and TCP etc., were found to exhibit positive or negative correlations with the expression of genes involved in vitamin B_3 biosynthesis (Table S9). Based on the network analysis, we found that most of the TFs showed their highest network degrees with the *AOX* and *QS* genes, which suggests that these TFs mainly participate in the regulation of the expression of these two enzymes in the vitamin B_3 synthesis pathway of *T. grandis* nuts (Fig. 4).

Based on the correlation analysis between the expression level of genes involved in vitamin B_3 biosynthesis pathway and the candidate TFs, we selected the strongest correlated TFs with $-\log_{10}(P) \geq 4$ (Fig. 5A and B). Among them, the ERF gene family exhibited extremely significant correlations with *TgAOX* and *TgQS*, which suggested that TgERFs may be the key transcription factors involved in vitamin B_3 biosynthesis (Fig. 5C, Table S10). ERF is the ethylene-responsive transcription factor and is reportedly involved in the regulation of diverse plant developmental and biosynthetic pathways (Xu et al. 2019; Binder, 2020; Feng et al., 2020). Studies have found that ERF189 can bind to the promoter region of tobacco *QPT2*, the other key enzymes in the vitamin B_3 biosynthesis pathway and is essential for the expression of *QPT2* (Shoji & Hashimoto, 2011). However, whether ERF can regulate the expression of *AOX* or *QS* has not previously been reported. Thus, we performed the exogenous ethephon treatment for further verification. Real time PCR showed that ethylene treatment induced the expression of *TgERFs* (Fig. 6A). At the same time, *TgAOX* and *TgQS* were also upregulated after the ethephon treatment, and the relative contents of vitamin B_3 were also increased in the nuts of *T. grandis* (Fig. 6A and B, Table S11). These results suggest that the ethylene treatment can promote the biosynthesis of vitamin B_3, which is likely to be achieved through the regulation of the expression of *TgAOX* and *TgQS* by the ERF TFs, but this specific regulatory mechanism requires further investigation.

4. Conclusions

Vitamin B_3 contents in the nuts of *T. grandis* were highly dependent on the cultivar, suggesting that vitamin B_3 accumulation and

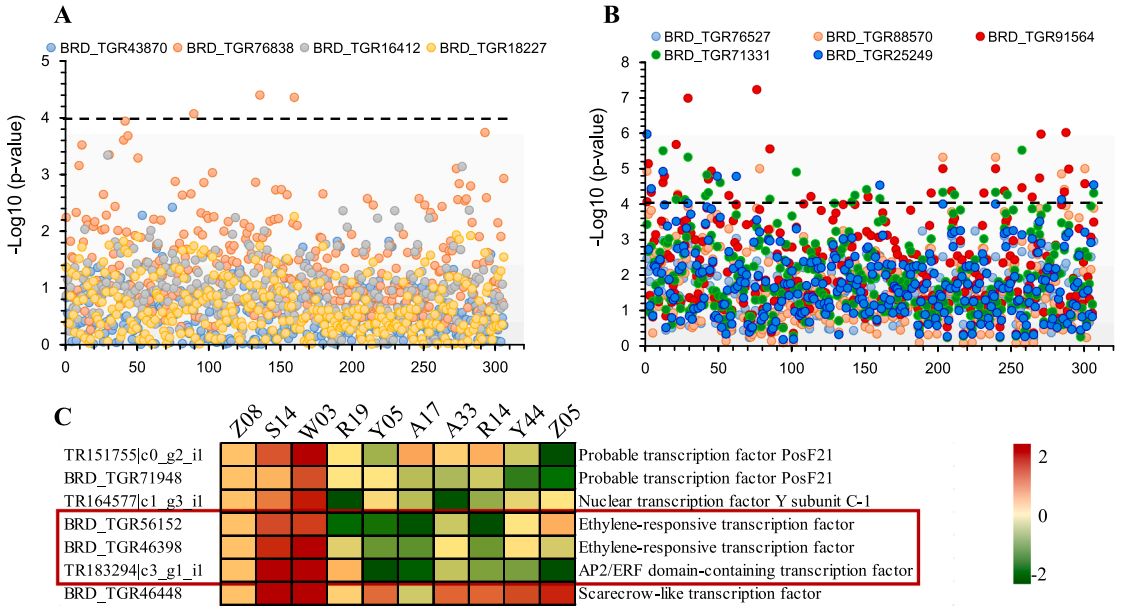

Fig. 5. Statistical analysis of correlations between *transcription factors* and *vitamin B_3 biosynthesis related genes*. (A) Statistical analysis of correlations between *TFs* and *TgAOXs*; (B) Statistical analysis of correlations between *TFs* and *TgQSs*; (C) Expression level of *TFs* with a threshold level of –log10 (P-value) ≥ 4. AOX, aspartate oxidase; QS, quinolinate synthase; *TFs,* transcription factors.

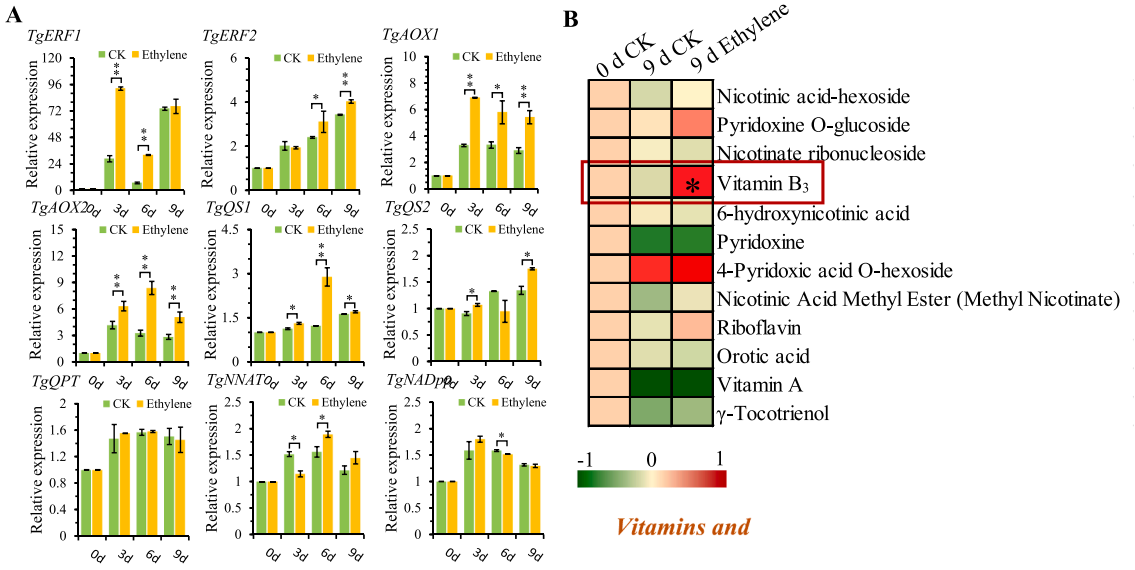

Fig. 6. Gene expression levels and vitamin B_3 contents in *Torreya grandis* nuts after ethylene treatment. (A) Relative expression of *ethylene-responsive factors* and *vitamin B_3 biosynthesis related genes*. (B) Contents of nicotinic acid niacinamide. AOX, aspartate oxidase (AOX1: BRD_TGR43870, AOX2: BRD_TGR16412); ERF, ethylene-responsive factor (ERF1: BRD_TGR56152, ERF2: BRD_TGR46398); NADpp, NAD pyrophosphatase (NADpp: BRD_TGR1146); NNAT, NaMN adenyltransferase (BRD_TGR11055); QPT, quinolinate phosphoribosyltransferase (QPT: BRD_TGR76527); QS, quinolinate synthase (QS1: BRD_TGR76527, QS2: BRD_TGR25249).

biosynthesis is regulated by several key enzymes or genes. To explore this, we conducted systematic transcriptome analysis of vitamin B_3 biosynthesis among different *T. grandis* cultivars. The findings suggest that *de novo* vitamin B_3 biosynthesis occurs mainly via the aspartate pathway in *T. grandis* nuts. The candidate genes (*TgAOX3* and *TgQS3*) showed significant correlations with the vitamin B_3 contents among the different *T. grandis* cultivars. Moreover, network analysis suggested that the ERF gene family may be the regulatory factors in the B_3 biosynthesis pathway. The ethylene treatment consistently promoted the biosynthesis of vitamin B_3, which strongly indicates that this is likely to be achieved through the regulation of the expression of *TgAOX* and *TgQS* by ERF. Our results not only elucidate the key genes in B_3 accumulation but may also identify a framework for the transcriptional regulation of vitamin B_3 biosynthesis genes. At the same time, they provide a precious genetic resource for the study of the molecular mechanisms underlying cultivar-specific regulations of vitamin B_3 biosynthesis in plants.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.131050.

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