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The effect of ethylene on squalene and β-sitosterol biosynthesis and its key gene network analysis in *Torreya grandis* nuts during post-ripening process

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ABSTRACT

Squalene and β-sitosterol are health-benefit compounds due to their nutritional and medicinal properties. It has been reported that the content of these bioactive compounds is relatively high in *Torreya grandis* nuts. However, it is not yet known what changes in squalene and β-sitosterol accumulation occur during the special post-ripening process of *T. grandis* nuts and the effect of the well-known ripening hormone ethylene on the regulatory mechanism of their biosynthetic pathways. Thus, we performed transcriptome and metabolite analyses. The results showed that ethylene not only promoted the post-ripening process but also enhanced the accumulation of squalene by inducing gene expression in the mevalonate pathway. At the same time, ethylene treatment also promoted the accumulation of other sterols but inhibited gene expression in the β-sitosterol biosynthesis pathway. In addition, co-expression and correlation analysis suggested a framework for the transcriptional regulation of squalene and β-sitosterol biosynthesis genes under ethylene treatment.

1. Introduction

Squalene and phytosterol are classes of important bioactive compounds present naturally in plant oils (Ostlund et al., 2002). Both are vital to the growth and development of plants and also benefit human health (Valitova et al., 2016). Squalene is widely used by the cosmetic industry that attribute to its emollient and antioxidant properties to the skin (Huang et al., 2009). At the same time, scientific research has shown that squalene also has pharmacological functions, can prevent cardiovascular diseases, and has antitumor and anticancer effects against many kinds of cancers, including ovarian, breast, and lung cancer (Lozano-Grande et al., 2018). Additionally, β-sitosterol is one of the most important phytosterols and is considered to possess cholesterol lowering, anti-cancer, anti-inflammatory properties and immunomodulatory activity (Ambavade et al., 2014).

The biosynthesis of squalene and β-sitosterol is a complex and multistage process. Both possess common precursors that are biosynthesized via methylerythritol phosphate (MEP) and mevalonate (MVA) pathways in higher plants (Lozano-Grande et al., 2018). Several studies have suggested that major phytosterols are derived from the MVA pathway, while reports have also shown that β-sitosterol and stigmasterol accumulate equally from both the MVA and MEP pathways (Hasunuma et al., 2008; Valitova et al., 2016). Numerous reports showed that there are many genes are involved in the biosynthesis of squalene and β-sitosterol, and several of them have been reported to be the rate-limiting enzymes in the MVA and MEP pathway, such as 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), 1-deoxy-D-xylulose-5-phosphate synthase (DXS), geranylgeranyl pyrophosphate synthase (GGPS), squalene synthase (SQS), squalene epoxidase (SQE), and C24-sterol methyltransferases 1 and 2 (SMT1, SMT2) (Schaller, 2004; Schmidt et al., 2010). Transgenic tobacco plants expressing full-length *HMGS* and

Abbreviations: 1-MCP, 1-methylcyclopropene; CAS, cycloartenol synthase; DEG, differentially expressed gene; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; ETH, Ethephon; FPS, farnesyl pyrophosphate synthase; GGPS, geranylgeranyl pyrophosphate synthase; HDR, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; IPI, isopentenyl diphosphate isomerase; MEP, methylerythritol phosphate; MCS, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; MK, mevalonate kinase; MVA, mevalonate; PMK, phosphomevalonate kinase; SMT, C24-sterol methyltransferases; SQE, squalene epoxidase; SQS, squalene synthase.

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HMGR showed enhanced seed phytosterol levels compared to the wild-type (Hey et al., 2006; Liao et al., 2021). Both *SQS* and *SQE* have been reported to be functionally important in the biosynthesis of sterols in transgenic plants (Busquets et al., 2008; Laranjeira et al., 2015). Similarly, co-expression of *HMGR* and *SMT1* enhanced the carbon flux towards squalene and phytosterols (Holmberg et al., 2003).

The biosynthesis regulation of squalene and β-sitosterol is controlled both at transcriptional and posttranslational levels by diverse environmental factors and plant hormones, of which phytohormones have been proposed as an important regulator (Devarenne et al., 2002; Souter et al., 2002; Wentzinger et al., 2002). Nevertheless, systematic reports on the underlying plant hormones controlling mechanisms for squalene and phytosterol production have not been presented and remain to be further studied, although a few studies have suggested that the plant hormone ethylene and brassinosteroids can influence the content of squalene and phytosterol and gene expression, including that of HMGS, HMGR, farnesyl pyrophosphate synthase (FPS), and SMT, in their biosynthetic pathway in several plants (Lv et al., 2016; Inês et al., 2019; Ding et al., 2020).

Torreya grandis (Taxaceae) is a long-lived economic tree species; its nuts are a popular food high in nutritious and bioactive compounds (Wu et al., 2018; Lou et al., 2019; Suo et al., 2019). Our previous study found that *T. grandis* nuts contain relatively high levels of squalene and β-sitosterol and investigated the biosynthesis pathways of squalene and β-sitosterol in nuts of different *T. grandis* cultivars (Suo et al., 2019). These results suggest that *T. grandis* nuts may be a potential natural source of these precious bioactive compounds. However, unlike many other tree nuts, the ripening of *T. grandis* nuts does not occur on the tree but takes place several days after collection, and it seems that this process is key for oil biosynthesis and bioactive compound accumulation, which is a necessary stage for nut quality promotion (Zhang et al., 2020). Because *T. grandis* nuts are covered with sarcotesta, the first stage for post-ripening usually require the nuts to be stacked about 15 days to make the sarcotesta crack. Then, the sarcotesta can be removed and enter the second process of post-ripening. Therefore, it is necessary to develop techniques to accelerate the cracking of *T. grandis* sarcotesta, thus promoting the process of post-ripening. Ethylene is a well-known post-ripening hormone that can promote fruit ripening by affecting a variety of primary and secondary metabolic processes (Liu, Pirrello & Chervin, 2015). However, if the content of squalene and β-sitosterol changed during the post-harvest ripening process and if ethylene can promote the post-ripening process of *T. grandis* nuts, benefitting the biosynthesis of squalene and β-sitosterol, these interesting scientific problems are worth investigating.

Therefore, the effect of ethylene and 1-methylcyclopropene (1-MCP) on post-harvested ripening of *T. grandis* nuts was investigated in this study. The purpose was to determine whether ethylene promotes post-ripening and whether there was a change in the content and biosynthesis of squalene and β-sitosterol to reveal the complex regulation networks between the accumulation of squalene and β-sitosterol and ethylene signaling in *T. grandis* nuts. Our results will not only provide new insights into the molecular basis of the biosynthesis and regulation of squalene and β-sitosterol during the post-harvest ripening process of some nuts or fruits, but also be helpful for the establishment and application of post-ripening control technologies.

2. Material and methods

2.1. Plant materials

Torreya grandis trees used in this study were cultivated at Zhaojia Town, Zhuji City, Zhejiang Province, China (29°76′ N, 120°47′ E). The trees were grown with standard fertilization, irrigation, and pest control practices. Nine 14-year-old trees with similar growth conditions were selected for nut collection. About 50 kg non-cracking nuts of uniform size (about 31.66 ± 0.43 mm length and 21.25 ± 0.53 mm width) and similar pericarp color (green) were harvested at hand on September 8th in 2019. Then, nuts without obvious mechanical injury or damage from pests and diseases were selected and left overnight at room temperature for subsequent experiments.

2.2. Treatments

T. grandis nuts were randomly divided into three groups; three treatments were performed, and the treatment concentrations, namely 3000 μL/L ethephon (ETH; Sigma) and 0.2 μL/L 1-methylcyclopropene (1-MCP; Sigma), and the control were chosen, according to preliminary experimental results. The treatment and control groups were stored at a constant temperature of 25 °C and relative humidity of 90%, and three biological replicates were carried out. Samples were collected at 0, 3, 6, 9, and 12 days after treatment. Kernels with the sarcotesta and testa removed were sectioned and immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.3. Determination of cracking rate in T. grandis nuts during post-harvest ripening

The total number of cracked nuts were determined for each treatment after 0, 3, 6, 9, and 12 days. The cracking rate was calculated by NC/NT (NC, number of cracked nuts; NT, number of total nuts).

2.4. Determination of ethylene emission

About 40 nuts collected at 0, 3, 6, 9, and 12 days were placed in a 1 L airtight jar for 8 h at 25 °C for ethylene collection, and the gas samples were collected in an air collection bag by the use of a 1 mL gas-tight syringe. The ethylene content was analyzed according to Ding et al. (2020). A Thermo Trace 1300 gas chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a flame ionization detector was used. The carrier gas was N_2 at a flow rate of 1 mL min^{-1}, the injector and detector temperatures were 200 °C and 250 °C, respectively, and the oven temperature was 100 °C.

2.5. Determination of squalene and β-sitosterol content

Squalene and β-sitosterol were extracted from *T. grandis* nut oil and quantified by gas chromatography according to Suo et al. (2019). Briefly, 0.1 g crude oil was saponified with 2% KOH (w/v) in 2 mL ethanol at 80 °C for 15 min. Then, the unsaponifiable fraction was extracted in hexane. After centrifugation, the organic phase was dried in an oven at 37.5 °C overnight, and the residue was redissolved for determination. For gas chromatography, an Agilent 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) was used. The program and parameter settings were used as previously described (Suo et al., 2019).

2.6. Determination of soluble sugar, starch, soluble protein, and oil content

The soluble sugar, starch, soluble protein, and oil content were measured and calculated according to our previous methods (Zhang et al., 2020).

2.7. Transcriptome analysis

RNA samples from 0 and 9 day *T. grandis* nut samples after different treatment (control, 1-MCP, ETH) were extracted according to the standard protocol. Sequencing libraries were constructed and sequenced on an Illumina HiSeq™ 4000 platform (Illumina Inc., San Diego, CA, USA). After *de novo* assembly, the obtained unigenes were blasted and annotated by the NCBI non-redundant protein sequences (Nr), gene ontology (GO), Kyoto encyclopedia of genes and genome (KEGG), and

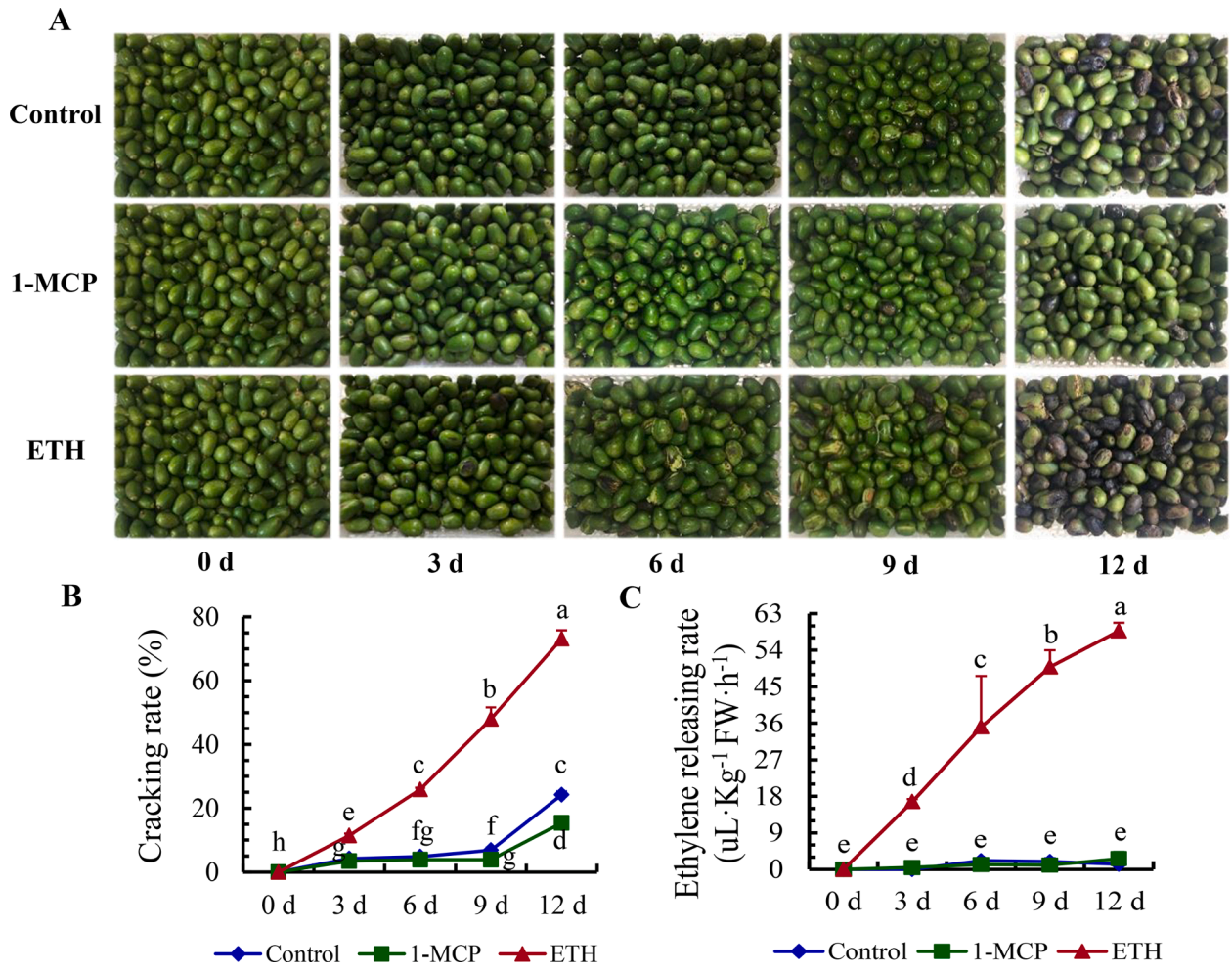

Fig. 1. The effect of various treatment on the post-ripening process of *Torreya grandis* nuts. (A) Morphology changes; (B) Cracking rate; (C) Ethylene production. 1-MCP, 1-methylcyclopropene; ETH; Ethephon.

evolutionary genealogy of genes with non-supervised orthologous groups (eggNOG) and Swiss-Prot databases. The differential expression of unigenes (DEGs) was assessed with the R 'ggplots2' package based on a threshold of $\log_2|\text{fold change}| \geq 1$, FDR < 0.001, and p-value < 0.05, and the function of the DEGs was analyzed by KEGG enrichment (Suo et al., 2019).

2.8. Phytosterol-related metabolites analysis

T. grandis nut samples were ground into powder, and 100 mg powder was extracted overnight at 4 °C with 70% aqueous methanol. Then, the extracts were analyzed using an UPLC (Shim-pack UFLC SHIMADZU CBM30A) system coupled with a triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems 6500 QTRAP). The mobile phase was solvent A: water with 0.04% acetic acid; solvent B: acetonitrile with 0.04% acetic acid. A gradient program described by Chen et al. (2020) was used for sample measurements. For MS/MS analysis, turbo spray was used as the ion source, and the operation parameters were as follows: 550 °C source temperature, 5500 V ion spray voltage (IS), and ion source gas I, gas II, and curtain gas were set at 50, 60, and 30.0 psi, respectively. Triple quadrupole (QQQ) scans were acquired as MRM experiments with collision gas (nitrogen) set to 5 psi.

2.9. qRT-PCR validation

Genes involved in the squalene and phytosterol biosynthesis pathway were selected for qRT-PCR analysis. The PCR primers used in this study are listed in Table S1. A CFX96 Real-time PCR system (Bio-Rad, Hercules, CA, USA) was used, and the program was run according to our previous studies (Suo et al., 2019).

2.10. Statistical analyses

Pearson's correlation coefficient (r) was used to calculate the correlation between experiment data. Significance was performed with one-way analysis of variance (ANOVA) and Student's t test, and a p value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Ethylene promote the post-ripening process of T. grandis nuts

Post-harvest ripening is a crucial process for some fruits, such as banana, kiwifruit, avocado, and *T. grandis,* to develop into edible ripe fruit (Prasanna et al., 2007). Ethylene is a well-known ripening hormone and is widely applied to promote the post-harvest maturation of many fruits (Liu, Pirrello et al., 2015). During ripening, the fruit undergoes many changes, including color change, biosynthesis and accumulation of nutrients, and pericarp cracking (Adams-Phillips et al., 2004; Tohge et al., 2014). For *T. grandis* nuts, the first stage of post-ripening is to make the sarcotesta crack (Zhou et al., 2019). In this study, ethylene treatment significantly promoted the cracking of *T. grandis* nuts, while 1-MCP treatment showed a significant inhibitory effect (Fig. 1A). Compared with the control group, the sarcotesta of *T. grandis* nuts were

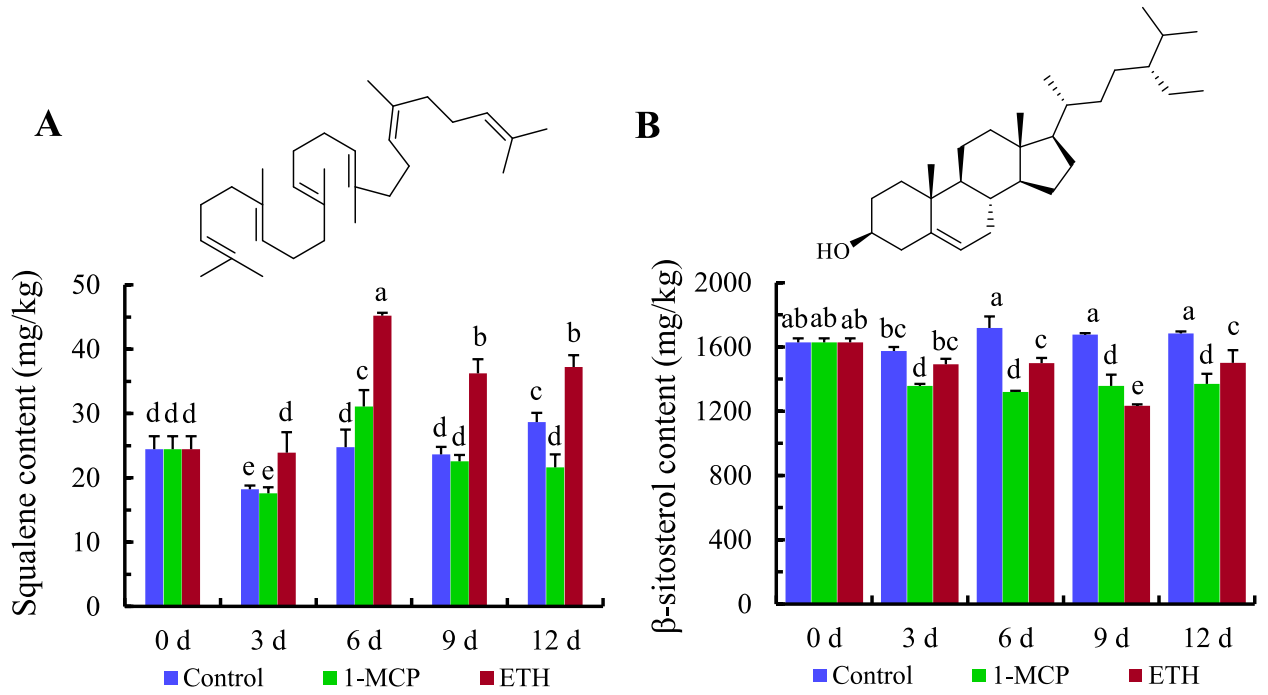

Fig. 2. The contents of squalene (A) and β-sitosterol (B) in the post-ripening *Torreya grandis* nuts under 1-MCP and ETH treatment. 1-MCP, 1-methylcyclopropene; ETH; Ethephon.

obviously cracked at 6 days, and the cracking rate reached about 50% with no obviously rotten nuts at 9 days after ethylene treatment (Fig. 1B). At the same time, ethylene production remained at a lower value during the post-harvest ripening process of the control and 1-MCP treatment conditions, while a significantly higher level of ethylene release was observed with ethylene treatment (Fig. 1C). These results

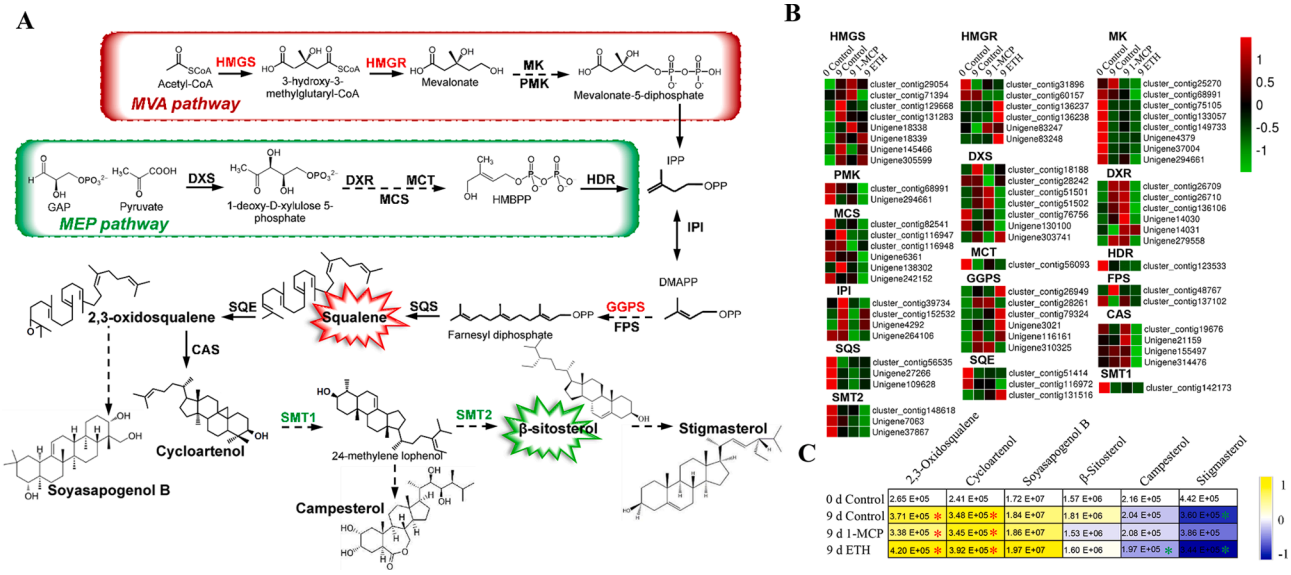

Fig. 3. Schematic presentation of squalene and phytosterol biosynthesis in *Torreya grandis* nuts. (A) Squalene and phytosterol biosynthesis pathway. Enzymes in red/green color indicate relative high gene expression in *T. grandis* nuts under ETH treatment. (B) Relative expression levels of genes in squalene and phytosterol biosynthesis pathway in *Torreya grandis* nuts under 1-MCP and ETH treatment. The scale bar indicates FPKM ratios, colors from green to red indicate the relative expression level for each gene in squalene and phytosterol biosynthesis pathway. (C) Relative contents of several phytosterol in *Torreya grandis* nuts under 1-MCP and ETH treatment. The scale bar indicates log2 transformed fold change of several phytosterol, the relative contents also shown in figures, asterisk (*) indicate statistically significant differences ($p < 0.05$). Abbreviations: 1-MCP, 1-methylcyclopropene; CAS, cycloartenol synthase; DMAPP, dimethylallyl diphosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; ETH; Ethephon; FPS, farnesyl diphosphate synthase; GAP, glyceraldehydes-3-phosphate; GGPS, geranyl diphosphate synthase; HDR, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase; HMBPP, (E)-4-hydroxy-3-methylbut-2-enyl diphosphate; HMGR, 3-hydroxy-3-methyl glutaryl coenzyme A reductase; HMGS, 3-hydroxy-3-methyl glutaryl coenzyme A synthase; IPI, isopentenyl diphosphate isomerase; IPP, isopentenyl diphosphate; MCS, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; MCT, 2-C-methyl-D-erythritol 4-phosphate cytidylytransferase; MEP, methylerythritol phosphate; MK, mevalonate kinase; MVA, mevalonate; PMK, phosphomeralonate kinase; SMTs, Δ24-sterol methyl transferases; SQE, squalene epoxidase; SQS, squalene synthase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

indicated that ethylene can effectively promote the cracking of *T. grandis* nuts during the post-harvest storage process.

3.2. Effect of ethylene on the biosynthesis and accumulation of squalene and β-sitosterol in post-ripening T. grandis nuts

As important secondary metabolites, squalene and β-sitosterol are usually extracted from kernel oils (Delgado-Zamarreno et al., 2009). Both of them accumulated to relatively high levels in *T. grandis* nuts (Suo et al., 2019). In this study, both squalene and β-sitosterol showed a slow increase during the process of post-ripening (Fig. 2). While the content of squalene increased significantly with ethylene treatment, it reached the highest level at 6 days with a concentration of 45.20 mg/kg (Fig. 2A). However, the accumulation of β-sitosterol was slightly inhibited by ethylene treatment (Fig. 2B). Similar results were also observed in other plants, ethylene treatment can induce the biosynthesis of squalene precursors in *Dioscorea zingiberensis* and papaya fruit, and the total content of phytosterol metabolites in apple peels was markedly influenced by 1-MCP treatment (Rudell et al., 2011; Diarra et al., 2013; Fabi and do Prado, 2019). These results indicated that the plant regulator ethylene not only accelerated the process of post-ripening, at the same time as benefitting squalene biosynthesis, but slightly inhibited β-sitosterol accumulation in *T. grandis* nuts.

To comprehensively understand the effects of ethylene on the biosynthesis of squalene and β-sitosterol during the post-ripening process, we performed a transcriptomic analysis of *T. grandis* nuts after 0 and 9 days of 1-MCP and ethylene treatment. A total of 10.26 to 11.69 billion clean reads were obtained and used for the next analysis. The Q30 and Q20 values were calculated to be 95.34–98.06% (Table S2). After assembled, the putative functions of the unigenes were annotated by the NR, GO, KEGG, eggNOG, and SwissProt databases (Table S3). DEG analysis found that compared with day 0, a total of 15,862 DEGs (7,126 upregulated genes and 8,736 downregulated genes) were identified in the 9-day control group, while a total of 4,216 (2,632 upregulated genes and 1,584 downregulated genes) and 5,818 DEGs (3,079 upregulated genes and 2,739 downregulated genes) were identified in the 9-day 1-MCP and ethylene treatment groups, respectively, compared with the 9-day control group (Fig. S1). Furthermore, KEGG enrichment analysis found that among the top 20 categories, terpenoid biosynthesis-related pathways were significantly enriched (Fig. S2). In addition, qRT-PCR results showed a significant and positive Pearson's correlation ($r^2 = 0.71$, $p < 0.01$) with the RNA-seq data, which indicated that the present transcriptomic data was reliable enough for further analysis (Fig. S3).

The biosynthesis of squalene and β-sitosterol is derived from the isoprenoid pathway (Nagegowda, 2010). In total, our transcriptomic profiling analysis identified 24 unigenes, including HMGS, HMGR, mevalonate kinase (MK), and phosphomevalonate kinase (PMK) involved in the MVA pathway, and 21 unigenes involved in the MEP pathway, including DXS, 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (MCT), 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase (MCS), and 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR) (Table S4). HMGS and HMGR are two rate-limiting enzymes in the MVA pathway; DXS, DXR, and HDR also control the key steps in the MEP pathway (Vranová et al., 2013). Numerous studies have shown that overexpression of them enhanced the biosynthesis and accumulation of squalene and several phytosterols (Suzuki et al., 2004; Hey et al., 2006). In the present study, DEG analysis showed that the relative expression of several unigenes of *HMGS* and *HMGR* was induced under ethylene treatment, while almost all identified unigenes in the MEP pathway showed a downregulation by ethylene treatment (Fig. 3A and 3B). Similarly, ethylene regulate the MVA pathway through the gene expression of *HMGS* in *Catharanthus roseus* (Zhang et al., 2018). The expression of *Malus domestica* (apple) *MdHMGR2* was significantly induced by ethylene in transgenic *Arabidopsis thaliana* (Lv et al., 2016). Ethylene treatment also enhanced the gene expression of *HMGR* and *cycloartenol synthase (CAS)* in *Dioscorea zingiberensis* (Diarra et al., 2013). 1-MCP treatment inhibited the expression of several *HMGRs*, *MK* and *isopentenyl diphosphate isomerase* (*IPI*) also downregulated at transcription level after 113 days of 1-MCP treatment in 'White Winter Pearmain' apple fruit (Ding et al., 2020). This suggests that instead of the MEP pathway, ethylene treatment may induce gene expression in the MVA pathway, and the relatively high expression of *TgHMGS* and *TgHMGR* may contribute to the accumulation of the initial precursors (IPP and DMAPP) of squalene and phytosterol in post-ripening *T. grandis* nuts.

Furthermore, IPP and DMAPP produced by the MVA and MEP pathways are used for the biosynthesis of squalene and phytosterol, which are catalyzed by a series of enzymes (Vranová et al., 2013). A total of 15 unigenes encoding IPI, GGPS, FPS, and SQS that participate in squalene biosynthesis were identified in the present study (Table S4). Eleven unigenes involved in downstream phytosterol biosynthesis, including *SQE*, *CAS*, and *SMTs*, were identified (Table S4). Among them, most of the transcripts of *GGPS* were upregulated by ethylene treatment, while the expression of all members of *FPS*, *SQS*, *CAS*, and *SMTs* was inhibited under ethylene treatment (Fig. 3B). 1-MCP treatment decreased the expression of *FPS* at the transcription level in apple fruit (Ding et al., 2020). Both *GGPS* and the *ethylene-responsive transcription factors* were found to have coordinated expression in Masson's pine (Liu, Zhou et al., 2015). Combined with the higher squalene content under ethylene treatment, it was suggested that, together with *TgHMGS* and *TgHMGR*, the induced expression of *TgGGPS* may play a key role in the accumulation of squalene in *T. grandis* nuts under ethylene treatment (Fig. 2A; Fig. 3B).

In addition, β-sitosterol level was slightly decreased after ethylene treatment for 6 days in the present study (Fig. 2), and this result may be due to the fact that squalene is not only a precursor of β-sitosterol, but also a common precursor for the biosynthesis of other sterols (Suo et al., 2019). Although the squalene content increased under ethylene treatment, ethylene also promoted the accumulation of other sterols, such as soyasapogenol B, which exhibited a high relative content under ethylene treatment in *T. grandis* nuts (Fig. 3C). The other important reason is that ethylene treatment inhibited the expression of *TgCAS*, *TgSMT1*, and *TgSMT2*, the key enzymes in the β-sitosterol biosynthesis pathway in *T. grandis* nuts, leading to a decrease in β-sitosterol and other sterols, including campesterol and stigmasterol, in this pathway (Fig. 3B and 3C). Studies have demonstrated that the most important stages in phytosterol biosynthesis are the steps catalyzed by SMTs. In transgenic tobacco and Arabidopsis, *SMT1* and *SMT2* mutants cause a lower content of β-sitosterol and campesterol (Diener et al., 2000; Schaeffer et al., 2001). Taken together, the results indicated that ethylene treatment slightly inhibited β-sitosterol biosynthesis and accumulation by increasing the content of other sterols to some extent and by decreasing the expression of β-sitosterol biosynthetic-related genes in post-ripening *T. grandis* nuts.

3.3. Co-expression network analysis identified key genes and regulators of squalene and β-sitosterol biosynthesis under ethylene treatment

Squalene and β-sitosterol biosynthesis is controlled by a complex network of internal and external factors, of which phytohormones function as crucial regulators (Souter et al., 2002). The plant hormone ethylene plays a key role in the post-ripening process of *T. grandis* nuts and also affects the biosynthesis of squalene and β-sitosterol (Figs. 1–3). To gain a comprehensive understanding of the underlying regulation mechanism, we analyzed ethylene signaling and its correlation with squalene and β-sitosterol biosynthesis. In total, 38 unigenes, including ethylene-responsive transcription factor (*ERFs*, *RAP2*, *AP2*), ethylene signaling components (*CTR1* and *EIN3*), ethylene receptor (*ETR1* and *ETR2*), and a regulator of ethylene signaling (*RTE1*), were identified, and most of those transcripts exhibited induced expression with

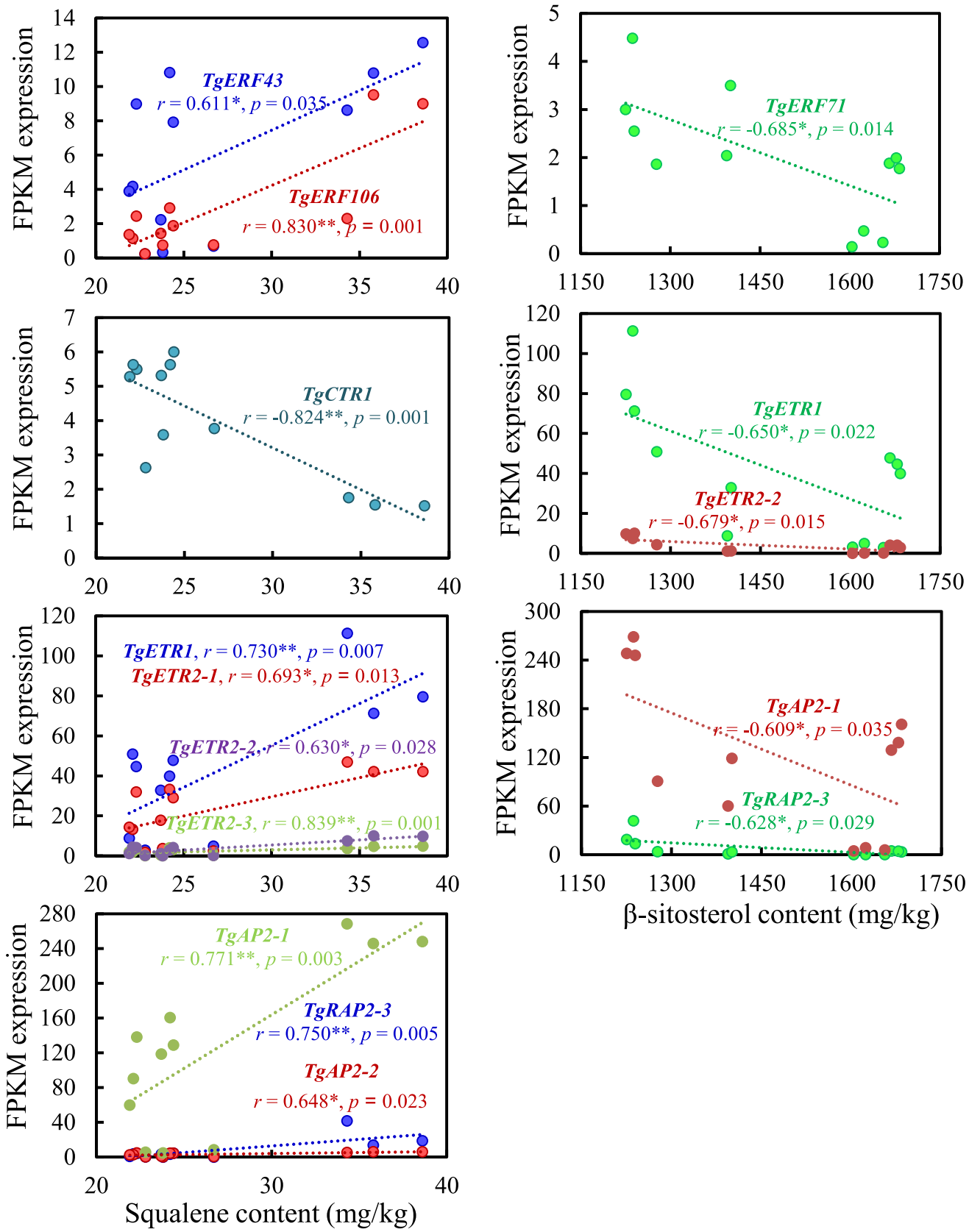

Fig. 4. Correlation analysis between squalene and β-sitosterol contents and the expression level of *transcription factors* in ethylene signaling in post-ripening *Torreya grandis* nuts under 1-MCP and ETH treatment. *TgERF43* (*cluster_contig148183*), *TgERF106* (*cluster_contig94384*), *TgERF71* (*Unigene284210*), *TgAP2-1* (*Unigene29829*), *TgAP2-2* (*cluster_contig98292*), *TgRAP2-3* (*cluster_contig49476*), *TgCTR1* (*cluster_contig131380*), *TgETR1* (*cluster_contig162923*), *TgETR2-1* (*Unigene136133*), *TgETR2-2* (*cluster_contig74668*), *TgETR2-3* (*Unigene274950*).

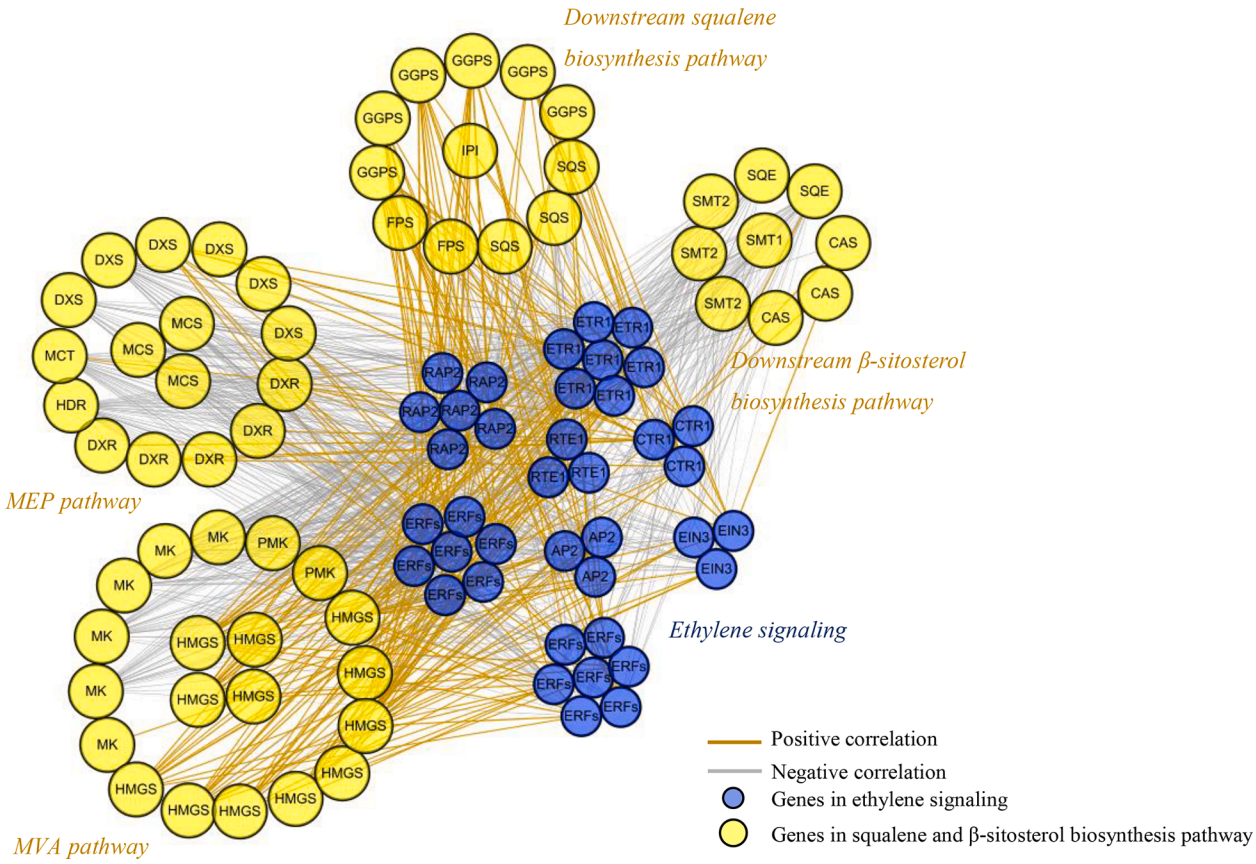

Fig. 5. Co-expression networks associated with squalene and β-sitosterol biosynthesis regulation in post-ripening *Torreya grandis* nuts under 1-MCP and ETH treatment. The network was constructed using 59 unigenes in squalene and β-sitosterol biosynthesis pathway, and 39 unigenes in ethylene signaling. The gene ID and correlation coefficients were shown in supplementary Table S6, and the dynamic network were visualized with Cytoscape (version 4.0).

ethylene treatment (Table S5). Pearson's correlation analysis (*r*) showed that the content of squalene was significantly and positively correlated with the relative expression of ethylene signaling-related genes (*TgERFs*, *TgRAP2*, *TgAP2*, *TgCTR1*, and *TgETRs*, $p < 0.01$), and the content of β-sitosterol exhibited a moderate negative correlation (Fig. 4).

At the same time, co-expression network analysis showed a strong positive correlation of ethylene signaling-related genes with *TgHMGS* and *TgHMGR* in the MVA pathway and *TgGGPS* downstream of the squalene biosynthesis pathway. By contrast, negative correlations were observed with *TgSMT1* and *TgSMT2* in the sterol biosynthesis pathway (Fig. 5; Table S6). This is consistent with several studies showing that the expression of *HMGS* and *HMGR* can be induced by ethylene treatment, and *ERF* also showed a co-expression with *GGPS* (Liu, Zhou et al., 2015; Lv et al., 2016). Based on the co-expression network, we screened and obtained the candidate key genes in ethylene signaling, including *TgERFs*, *TgAP2*, and *TgETR1* (Fig. 6). The effect of ethylene on squalene and β-sitosterol accumulation may be achieved by the transcriptional regulation of gene expression in squalene and β-sitosterol biosynthetic pathways through *TgERFs*, *TgAP2*, and *TgETR1* in post-ripening *T. grandis* nuts, while the precise regulation mechanisms underlying these effects need further study.

4. Conclusions

In the present study, the cracking rate and squalene content were increased, and β-sitosterol levels were decreased under ethylene treatment, suggesting that exogenous ethylene accelerated the cracking of *T. grandis* nuts, and effect the biosynthesis and accumulation of bioactive compounds during post-ripening process. Transcriptome analysis suggested that the biosynthesis of squalene may be attributed to the ethylene-induced gene expression of *TgHMGS* and *TgHMGR* in the MVA pathway. Metabolite analysis found that the relatively high content of other phytosterols and inhibited expression of *TgSMT1* and *TgSMT2* may be the main reasons for the decreased β-sitosterol content under ethylene treatment. Furthermore, co-expression network analysis suggested that the effect of ethylene on squalene and β-sitosterol accumulation may be achieved by the transcriptional regulation of gene expression in squalene and β-sitosterol biosynthetic pathways through several ethylene-responsive factors in post-ripening *T. grandis* nuts. The findings in our study provide new data on the role of ethylene signaling on squalene and phytosterol biosynthesis regulation during post-ripening of *T. grandis* nuts. However, further research is needed to better understand the hormonal regulation of bioactive compound biosynthesis in *T. grandis* and other tree nuts.

CRediT authorship contribution statement

Yuanyuan Hu: Methodology, Investigation, Writing - review & editing. **Jinwei Suo:** Investigation, Formal analysis, Writing – original draft. **Guoxiang Jiang:** Formal analysis, Investigation. **Jiayi Shen:** Investigation, Validation. **Hao Cheng:** Investigation, Formal analysis. **Heqiang Lou:** Visualization, Validation. **Weiwu Yu:** Data curation, Validation. **Jiasheng Wu:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing - review & editing. **Lili Song:** Conceptualization, Funding acquisition, Supervision, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

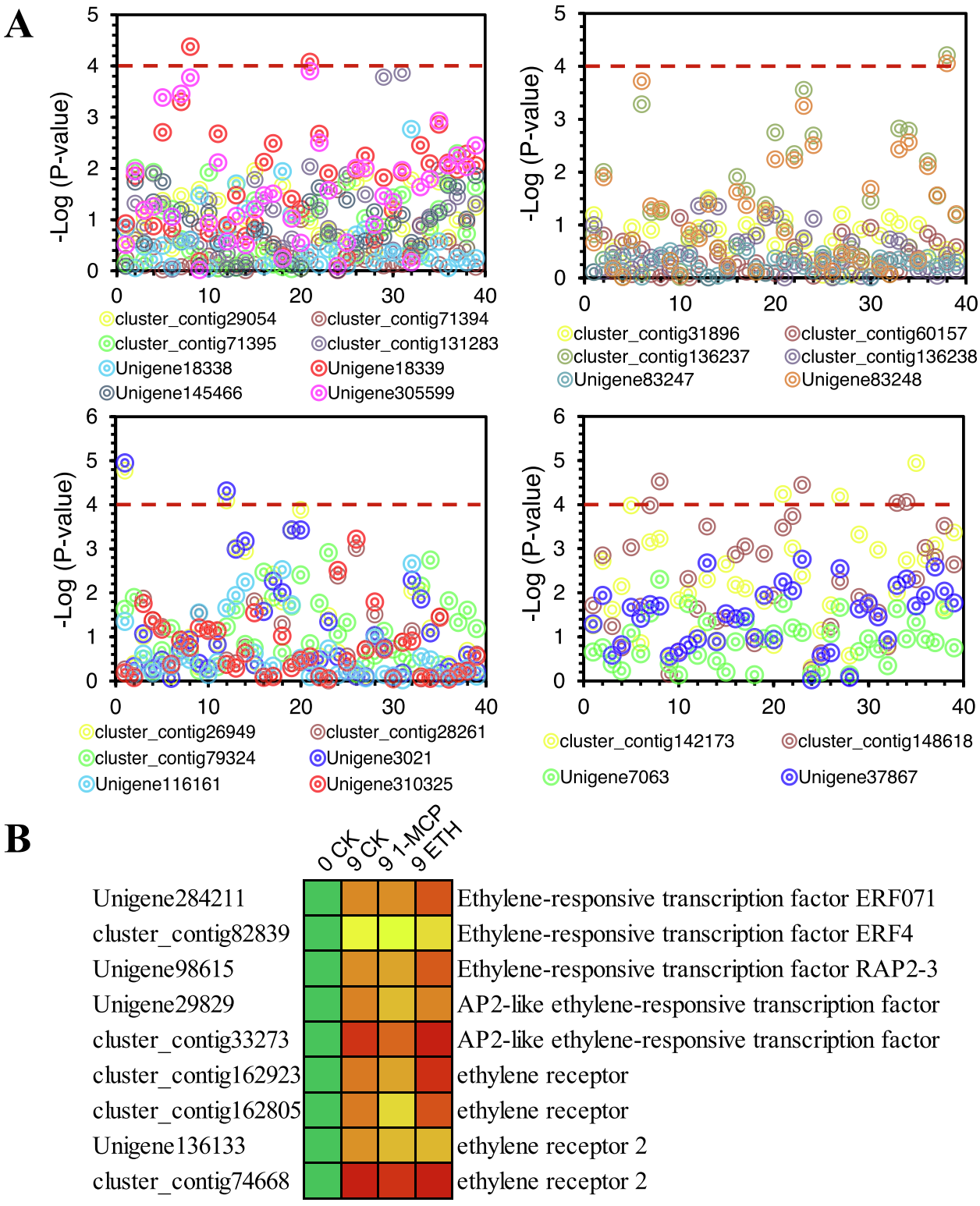

Fig. 6. Statistical analysis of correlations between *transcription factors* and *squalene and β-sitosterol biosynthesis related genes*. (A) Statistical analysis of correlations between *TFs* and *TgHMGS, TgHMGR, TgGGPS, TgSMTs*; (B) Expression level of *TFs* with a threshold level of –log10 (P-value) ≥ 4. Abbreviations: GGPS, geranyl diphosphate synthase; HMGR, 3-hydroxy-3-methyl glutaryl coenzyme A reductase; HMGS, 3-hydroxy-3-methyl glutaryl coenzyme A synthase; SMTs, Δ24-sterol methyl transferases; TFs, transcription factors.

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.2021.130819.

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