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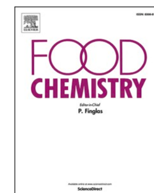

# Ethylene treatment promotes umami taste-active amino acids accumulation of *Torreya grandis* nuts post-harvest by comparative chemical and transcript analyses

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ABSTRACT

Amino acids play critical roles in physiological processes and also contribute significantly to fruit quality. In this study, the effect of exogenous ethylene on amino acids metabolism and related genes expression in *Torreya grandis* were investigated. The results revealed that ethylene treatment (3000 μL $L^{-1}$ for 24 h) significantly increased amino acids level. Umami amino acids were distinctly upregulated in ethylene-treated versus control nuts, with glutamic and aspartic acids to demonstrate 1.9-fold and 2.1-fold increase. Transcriptome analysis revealed that deferentially expressed genes were mainly enriched in alanine aspartate and glutamate metabolism. RT-qPCR confirmed that ethylene treatment up-regulated expression of their biosynthesis genes (*TgGOGAT1*, *TgAATC1*, *TgAATC4*) concurrent with suppression of their degradation enzymes (*TgGS2*, *TgGAD1*, *TgGAD3*, *TgASNS1*). Ethylene treatment appears to promote umami taste-active amino acids and improve *T. grandis* nut quality post-harvest.

## 1. Introduction

*Torreya grandis* (*T. grandis*), as a potential plant within the genus *Torreya* in the Taxaceae family, is one of the rare and precious economic tree species unique to southeastern China (Li & Dai, 2007). *T. grandis* nuts are rich in oil, protein and multiple bioactive compounds, such as sciadonic acid, squalene, β-sitosterol, and tocopherol (Suo et al., 2019; Song et al., 2021). Its seeds are one of the world's rarest dry nuts and are quite popular in China owing to their rich nutritional value, biological effects, unique aroma and attractive flavor, among which the composition of the different amino acids is one of the major contributor to nuts pleasant flavor. Unlike other nuts, *T. grandis* nuts require a highly coordinated and sophisticated postharvest ripening stage for the biosynthesis of its various nutrients (Zhang et al., 2020). However, the changes of amino acids during the postharvest ripening stage and their influencing factors remain unclear. Consequently, monitoring for changes in nutrients and flavor imparting classes and their regulation can help future attempts to improve nuts quality.

Amino acids, as the building blocks of proteins, play pivotal roles in participating in various physiological processes (Pratelli and Pilot, 2014). According to whether they can be synthesized by the human body, free amino acids (FAAs) can be divided into essential amino acids (EAAs) and non-essential amino acids (NEAAs) (Wu, 2009). Meanwhile, FAAs are involved in the overall taste of many foods, and their content

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and composition are important indicators for evaluating food quality and sensory attributes (Wu et al., 2018). Owing to their sensory attributes, FAAs are also a class of taste active compounds, which could be divided into umami, sweet, bitter and aromatic amino acids (Chen & Zhang, 2007). The nature of delicacy in food sensory perception is often related to the umami flavor of certain food (Mouritsen et al., 2019). Umami, which originates from the Japanese word for savory and delicious, is considered as a basic taste typified by the amino acid glutamic acid (Glu) and its salt monosodium glutamate (MSG) with a brothy, savory, rich or meaty taste sensation (Yamaguchi, 1991). In addition, aspartic acid (Asp) also contributes a lot to umami substances (Gao et al., 2021).

The biosynthesis and degradation of umami acids are tightly linked to the intermediates or precursors of both tricarboxylic acid (TCA) cycle and Calvin cycles *via* transamination (Pratelli & Pilot, 2014). Glu occupies a central position in amino acids metabolism in plants (Forde & Lea, 2007). Glu synthase (GOGAT, E.C. 1.4.7.1) catalyzes the conversion of glutamine and 2-oxoglutarate into two Glu molecules, thereby providing Glu for ammonium assimilation. Glutamine synthetase (GS, E.C. 6.3.1.2) plays a major role in fixing ammonium ($NH4^+$) to form the amino acid glutamine. The net outcome of the GS–GOGAT cycle lies in the production of Glu which can then be incorporated into other amino acids such as proline (Pro), Asp, asparagine (Asn), methionine (Met), lysine (Lys), and threonine (Thr) by the action of a wide range of multi-specific aminotransferases (Forde & Lea, 2007). Aspartate aminotransferase (AATC, E.C. 2.6.1.1) is important for the metabolism of both Asp and Asp-derived amino acids, as well as for TCA cycle-associated organic acids (Li et al., 2020). Asparagine synthetase (ASNS, EC 6.3.5.4) catalyzes the transfer of the amide group in glutamine to Asp to form asparagine and Glu (Gaufichon et al., 2016). The amino acids metabolism is one of the most important biochemical adaptations to many environmental stresses, and it also provides precursors for many secondary metabolites *via* the shikimic acid pathway. For many postharvest crops, the study of changes in amino acids profile and their regulation also provides insights into the physiological status of plants (Shulaev et al., 2008).

Ethylene, as an important plant endogenous hormone, regulates a wide range of physiological processes and is also involved in fruit ripening (Giovannoni, 2007). Both exogenous and endogenous ethylene can affect many fruit quality traits, such as cell wall disassembly and fruit softening (Zhang et al., 2018), an increase in specific flavor volatiles (Shen et al., 2016), and increase in functional components (Suo et al., 2022). For example, ethylene treatment on apple fruit up-regulated the expression of 17 genes including branched-chain amino acid aminotransferase (BCAT), aromatic amino acid aminotransferase (ArAT), amino acid decarboxylase (AADC), and regulated the production of major volatiles (Yang et al., 2016). Our previous study revealed that exogenous application of ethylene on *T. grandis* nuts during post-harvest ripening stage increased squalene content concurrent with induced gene expression of the mevalonate pathway (Hu et al., 2022b). Besides, ethylene can promote tocopherol accumulation *via* Asp pathway in *T. grandis* nuts (Suo et al., 2022). However, it remains unknown whether and how amino acids pool change during the post-harvest ripening stage, and whether or how it is regulated by ethylene as main regulator of such physiological process.

Consequently, the effect of ethylene on amino acids accumulation in *T. grandis* nuts during the postharvest ripening stage was investigated in this study and results are reported herein for the first time. The overall objectives of this study are (i) to reveal dynamic changes in amino acids after postharvest ethylene treatment and (ii) to determine changes in the transcript pattern though RNA sequencing and the differential expression analysis associated with amino acids metabolism in ethylene-treated nuts of *T. grandis* post ripening stage. Our results provide important reference value for improving the postharvest quality of *T. grandis* nuts and add to the role of ethylene in improving fruits and seeds nutritive value and flavor quality post harvesting.

## 2. Material and methods

### 2.1. Plant material

Nine 14-year-old *Torreya grandis* trees planted in Zhaojia Town, Zhuji City, Zhejiang Province, China (29°76′ N, 120°47′ E) were picked as the experimental material for this study. A total of 50 kg of nuts with no obvious pests and diseases, and similar in size, shape and maturity were transported to the laboratory within 4 h after harvest. After removing the sarcotesta (arils), nuts were cleaned with purified water and left to air-dry overnight at room temperature before being used for subsequent experimental treatment.

### 2.2. Ethylene treatments

The *T. grandis* nuts were divided into two lots,ch comprised of approximately 20 kg nuts further subjected to ethylene treatment or left untreated to serve as control, respectively. According to preliminary experimental results (Hu et al., 2022a), a treatment dose of 3000 uL $L^{-1}$ ethephon (Sigma, St. Louis, MO, USA) was chosen in this study found effective to elicit a response in *T. grandis* nuts. The treated nuts were totally sprayed with 20 mL ethephon solution, mixed well to ensure that each nut was evenly spayed, then sealed and placed in a sealed storage box (*ca.* 80 cm in length, 40 cm in width, 15 cm in height) for 24 h. The ethylene treatment (ETH) and control treatment (CK) were kept under dark conditions at 25℃ constant temperature and 90 % relative humidity. Samples with three biological replicates were collected at 0, 5, 10, 15, 20 and 25 days after the different treatments. After manual removal of the seed husks and coats, the kernels were minced and quickly frozen in liquid nitrogen and stored at –80 °C for further analysis.

### 2.3. Measurement of free amino acids

The content of free amino acids was determined using the method of Kawai et al. (2009) with slight modification. The ground nuts (5.0 g) were placed in 60 mL deionized water and mixed well. The mixture was then placed in a boiling water bath for 1 h and vortexed every 15 min. After cooling to room temperature, extract was centrifuged at 10,000 *g* for 15 min. The procedure was repeated twice, then supernatant was collected and made up to 100 mL with deionized water. Next, 5 mL of the supernatant was aliquoted and placed in 5 mL 10 % sulfonic acid salicylic acid solution and mixed well. The mixture was centrifuged at 12,000 *g* for 15 min at 4℃. The supernatant was collected and passed through a 0.22 μm filter membrane (Millipore, Massachusetts, USA). The free amino acids were determined using an automatic amino acid analyzer (L-8900, Hitachi High Technologies, Tokyo, Japan). The injection volume was 20 μL, separation column temperature and the reaction column temperature were set at 57℃ and 135℃, respectively. The flow rate of buffer solution was at 0.35 mL $min^{-1}$, and the flow rate of ninhydrin was at 0.35 mL $min^{-1}$. The detection wavelength of channel 1 and channel 2 were set at 570 nm and 440 nm, respectively. The standard amino acid solutions, type B and type ANII, were obtained from Wako (Wako-shi, Japan).

### 2.4. RNA extraction and transcriptome analysis

RNA samples from *T. grandis* nuts at 0 and 10 days under different treatments (CK and ETH) were extracted following the CTAB method (Chang et al., 1993). The quantity and quality of the total RNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and agarose gel electrophoresis on 1.5 % (w/v) agarose gel. Sequencing libraries were constructed and sequenced using 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

**Table 1**
Levels of free amino acids (μg $g^{-1}$ FW) in *T. grandis* nuts under different treatments during postharvest ripening stage. The different letters indicate significant differences ($P < 0.05$) under control treatment and ethylene treatment.

| Amino acids | Day 0 | CK | | | | | ETH | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NEAA | | Day 5 | Day 10 | Day 15 | Day 20 | Day 25 | Day 5 | Day 10 | Day 15 | Day 20 | Day 25 |
| Asp | 83.34 ± 7.50e | 129.07 ± 7.20 cd | 125.51 ± 16.01 cd | 132.64 ± 22.06 cd | 114.72 ± 11.81d | 126.06 ± 9.81 cd | 150.84 ± 3.43c | 214.00 ± 26.10b | 198.80 ± 11.18b | 238.71 ± 4.98a | 202.76 ± 16.54b |
| Ser | 46.34 ± 1.86b | 42.64 ± 4.16bc | 30.49 ± 2.01ef | 23.19 ± 3.45 h | 24.40 ± 4.51gh | 31.87 ± 1.45ef | 55.66 ± 1.07a | 40.33 ± 0.82 cd | 29.51 ± 1.62 fg | 32.52 ± 3.51ef | 35.54 ± 5.30de |
| Glu | 140.31 ± 42.60 cd | 154.48 ± 15.24bcd | 150.25 ± 18.36bcd | 138.39 ± 56.23 cd | 107.43 ± 23.34d | 138.62 ± 13.66 cd | 206.32 ± 36.86ab | 225.57 ± 9.61a | 185.06 ± 33.88abc | 199.66 ± 43.39abc | 179.52 ± 30.62abc |
| Pro | 44.01 ± 7.91a | 23.82 ± 3.82b | 14.03 ± 4.24b | 15.23 ± 0.97b | 18.30 ± 9.10b | 29.67 ± 15.79ab | 44.44 ± 12.75a | 25.70 ± 9.23b | 23.15 ± 3.13b | 26.32 ± 8.19b | 28.02 ± 11.22b |
| Gly | 8.56 ± 0.39d | 11.27 ± 2.53 cd | 9.84 ± 2.57 cd | 10.39 ± 2.18 cd | 11.73 ± 5.15bcd | 8.99 ± 2.86d | 17.22 ± 0.58a | 13.02 ± 2.27abcd | 14.37 ± 0.64abc | 16.39 ± 2.15ab | 14.26 ± 2.43abc |
| Ala | 27.78 ± 4.72abc | 30.36 ± 7.13ab | 18.90 ± 4.60c | 23.03 ± 3.71bc | 26.20 ± 2.63abc | 21.58 ± 0.25bc | 35.48 ± 5.08a | 23.66 ± 1.53bc | 29.24 ± 2.02ab | 34.30 ± 9.20a | 33.68 ± 8.00a |
| Tyr | 19.12 ± 3.70de | 34.25 ± 10.05bc | 19.72 ± 2.14de | 10.57 ± 6.37efg | 5.78 ± 0.25 g | 7.46 ± 3.57 fg | 65.36 ± 10.48a | 39.07 ± 6.24b | 16.67 ± 4.04def | 22.47 ± 1.86d | 25.27 ± 2.73 cd |
| GABA | 61.92 ± 13.69c | 93.81 ± 19.71bc | 70.40 ± 7.21c | 66.56 ± 24.16c | 80.26 ± 12.70bc | 91.24 ± 3.29bc | 154.93 ± 49.67a | 79.03 ± 3.11bc | 81.61 ± 6.17bc | 79.73 ± 27.07bc | 120.32 ± 29.48ab |
| Total | 431.37 ± 14.01ef | 519.7 ± 21.64d | 439.14 ± 14.70ef | 420.01 ± 36.32ef | 388.83 ± 22.94f | 455.48 ± 5.33e | 730.25 ± 23.38a | 660.37 ± 13.09b | 578.42 ± 13.64c | 650.08 ± 18.50b | 639.38 ± 8.07b |
| **EAA** | | | | | | | | | | | |
| Thr | 25.57 ± 2.33bc | 28.41 ± 3.15b | 18.56 ± 1.51def | 11.16 ± 1.47 h | 12.33 ± 1.31gh | 16.31 ± 2.60efg | 32.8 5 ± 0.78a | 21.67 ± 4.59 cd | 14.20 ± 0.96fgh | 17.37 ± 3.15def | 19.62 ± 2.25de |
| Val | 30.85 ± 6.15abcd | 35.66 ± 4.01abc | 23.92 ± 8.80cde | 10.71 ± 2.30e | 11.85 ± 1.24e | 43.43 ± 13.48ab | 45.41 ± 8.35ab | 44.76 ± 13.07ab | 15.41 ± 0.76de | 23.13 ± 7.24cde | 28.41 ± 15.11bcd |
| Met | 19.51 ± 3.92a | 19.3 ± 3.58ab | 12.60 ± 0.81abc | 10.92 ± 3.59bc | 10.92 ± 5.77bc | 9.22 ± 0.83c | 16.85 ± 2.34abc | 14.25 ± 5.53abc | 11.13 ± 5.80abc | 13.5 ± 3.43abc | 19.45 ± 7.07a |
| Ile | 21.11 ± 0.45bc | 22.85 ± 2.13b | 17.51 ± 0.21cde | 15.14 ± 2.72ef | 12.74 ± 2.69f | 17.50 ± 1.36cde | 28.41 ± 1.14a | 19.28 ± 4.04bcd | 16.83 ± 0.95de | 19.89 ± 2.09bcd | 21.73 ± 2.47b |
| Leu | 36.66 ± 4.26ab | 33.71 ± 2.45bc | 22.98 ± 4.56e | 24.68 ± 8.38de | 21.93 ± 3.19e | 27.70 ± 1.07cde | 41.47 ± 2.69a | 27.82 ± 3.24cde | 23.93 ± 3.50e | 31.43 ± 1.72bcd | 32.44 ± 2.41bc |
| Phe | 31.17 ± 8.08b | 32.94 ± 6.78b | 23.17 ± 7.31bcd | 17.48 ± 7.58bcd | 12.46 ± 3.86 cd | 9.16 ± 1.68d | 47.63 ± 13.11a | 21.95 ± 2.83bcd | 12.00 ± 3.66 cd | 25.86 ± 17.10bc | 13.17 ± 6.49 cd |
| Lys | 42.25 ± 3.64c | 45.25 ± 3.92bc | 31.65 ± 3.91d | 30.43 ± 6.45d | 28.29 ± 3.16d | 32.25 ± 8.26d | 68.27 ± 9.13a | 53.20 ± 4.11b | 46.41 ± 3.28bc | 50.65 ± 3.15bc | 52.02 ± 4.85bc |
| His | 8.44 ± 1.43de | 2.98 ± 0.59e | 3.46 ± 2.93e | 2.35 ± 1.50e | 12.93 ± 5.20 cd | 2.41 ± 1.83e | 13.99 ± 9.39 cd | 19.72 ± 1.99bc | 10.69 ± 6.36de | 30.66 ± 6.11a | 23.62 ± 2.16ab |
| Arg | 20.07 ± 3.07c | 34.73 ± 11.43b | 20.64 ± 3.30c | 18.58 ± 7.02c | 8.97 ± 7.66c | 11.46 ± 9.52c | 44.46 ± 5.42ab | 45.53 ± 7.67ab | 34.38 ± 3.37b | 46.25 ± 3.19ab | 53.53 ± 7.57a |
| Total | 235.62 ± 5.28c | 255.82 ± 4.73bc | 174.5 ± 11.27d | 141.43 ± 15.21e | 132.44 ± 6.49e | 165.62 ± 5.96d | 339.35 ± 7.03a | 268.2 ± 6.50b | 184.97 ± 4.40d | 258.73 ± 7.17bc | 263.99 ± 3.84b |
| **Total FAA** | 667.00 ± 18.01d | 775.52 ± 44.12c | 613.64 ± 24.32de | 561.44 ± 88.54ef | 521.27 ± 40.38f | 621.10 ± 17.77de | 1069.60 ± 40.33a | 928.57 ± 32.63b | 763.39 ± 28.90c | 908.82 ± 43.21b | 903.37 ± 20.57b |

Note: NEAA, non-essential amino acid. EAA, essential amino acid. FAA, free amino acid. All values are the mean ± SD in triplicate.

sequences (Nr), Kyoto encyclopedia of genes and genome (KEGG), Swiss-Prot databases, evolutionary genealogy of genes with non-supervised orthologous groups (eggNOG) and gene ontology (GO). The differential expression of unigenes (DEGs) was judged using the absolute value of log2(Fold change) ≥ 1, false discovery rate (FDR) < 0.001, and $P < 0.05$, and the function of the DEGs was analyzed by KEGG and GO enrichment (Suo et al., 2019).

### 2.5. Quantitative real-time PCR (RT-qPCR)

The extracted total RNA was purified from contaminated genome DNA by gDNA eraser, and then 1 μg RNA was used for the first-strand cDNA synthesis using PrimeScript™ RT regent Kit following the manufacturer's protocol (Takara, Dalian, China) and then diluted with water (1: 5). Real time PCR was carried out using a CFX96 instrument with Ssofast Eva Green Supermix Kit (Bio-Rad, California, USA). The specificity of primers was confirmed by both melting curves and product sequencing before use. The PCR reaction mixture (20 μL total volume) consisted of 10 μL 2 × real-time PCR mix (Bio-Rad, California, USA), 6 μL DEPC $H_2O$, 2 μL diluted cDNA, and 1 μL of each primer (10 μM). The PCR program was initiated for 3 min at 95℃, then followed by 45 cycles of 95℃ for 10 s, 60℃ for 30 s, and completed with a melting curve analysis program. Relative expression level was calculated using the $2^{(-\triangle\triangle Ct)}$ method and using *TgActin* gene as the internal control. Three biological replicates were used for RNA extraction at each sampling point. Primers for RT-qPCR analysis are listed in Supplementary Table S1.

### 2.6. Statistical analysis

The figures were created with Origin 8.0 (MicroCal Software) and SigmaPlot 12.5. Least-significant differences (LSD) at the 5 % level were estimated using SPSS Statistics 20.0 (SPSS Inc., Chicago, IL, USA). The data were presented as mean ± standard deviation (SD). Principal component analysis (PCA) was applied to comprehensively evaluate the impact of ethylene treatment on amino acids pool of *T. grandis* nuts.

## 3. Results and discussion

### 3.1. Analysis of amino acids pool of T. Grandis nuts with postharvest ethylene treatment

Previous studies revealed that *T. grandis* nuts are rich in 17 kinds of amino acids, and FAAs content was as high as 118.1 g/kg, of which EAAs accounted for 38.61 % of total FAAs presenting a potential source of EAAs (Li et al., 2005). Another study illustrated that *T. grandis* nuts at

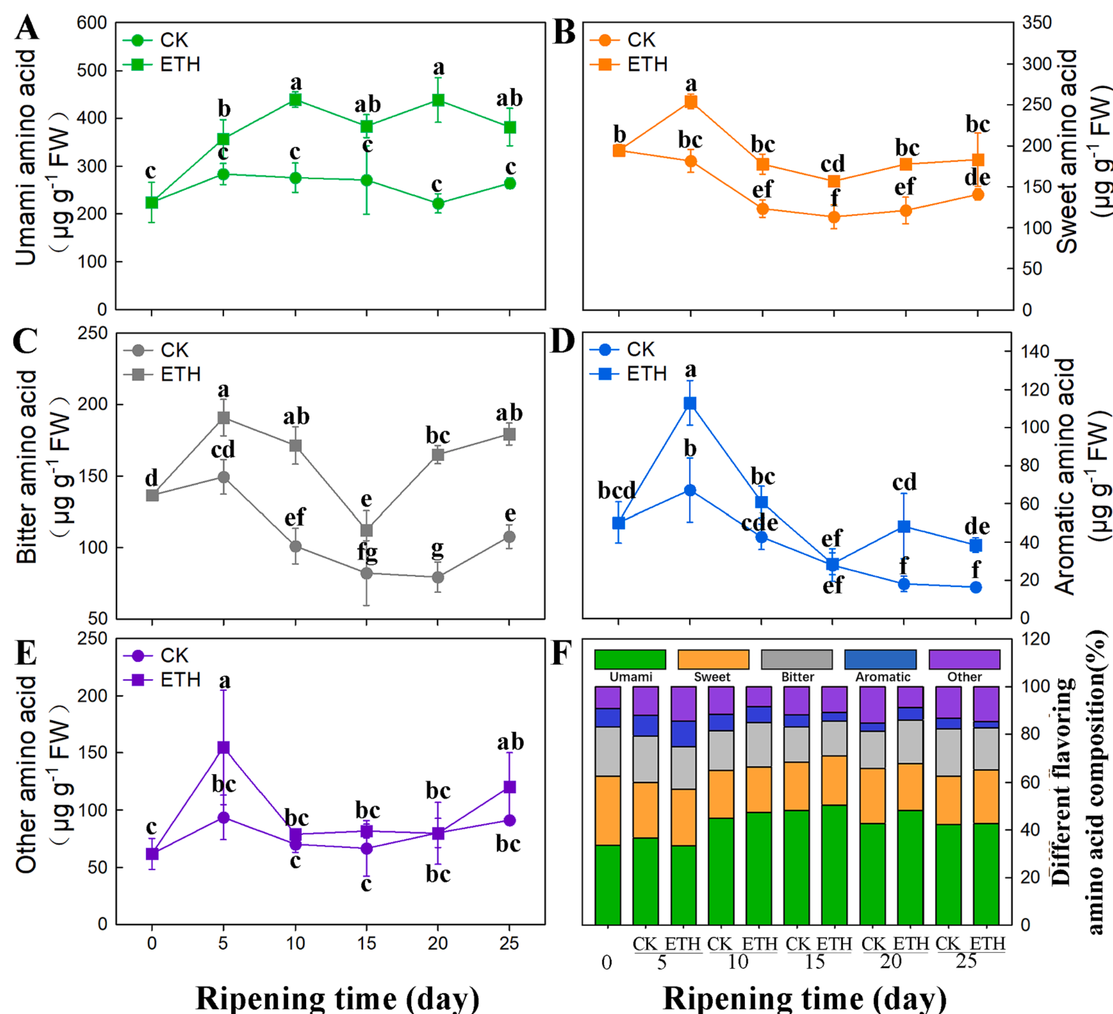


**Fig. 1.** Level changes of the different amino acids in *T. grandis* nuts with different treatment during the postharvest ripening stage. A, changes in umami amino acids; B, changes in sweet amino acids; C, changes in bitter amino acids; D, changes in aromatic amino acids; E, changes in other amino acids; F, bar chart showing composition of different flavor amino acids in *T. grandis* nuts with different treatment during postharvest ripening stage. Error bars indicate SE from three replicates. Different letters indicate the significant differences ($P < 0.05$) in *T. grandis* nuts with different treatment during the postharvest ripening stage.

different developmental stages showed significant variation in their amino acids content using a widely targeted metabolomics approach (Lou et al., 2022). In this study, changes in amino acids during the postharvest ripening stage and the effect of ethylene on their levels were investigated. The amino acids composition and content (μg $g^{-1}$ of sample) of the untreated *T. grandis* nuts and ethylene-treated nuts are shown in Table 1. A total of 17 different kinds of amino acids were detected in *T. grandis* nuts at different postharvest ripening periods (Day 0, 5, 10, 15, 20, 25) under control and ethylene treatment, including Asp, Thr, serine (Ser), Glu, glycine (Gly), alanine (Ala), valine (Val), Met, isoleucine (Ile), leucine (Leu), Tyr, phenylalanine (Phe), Lys, histidine (His), arginine (Arg), Pro and GABA (Table1). Throughout the postharvest ripening stage, FAAs level increased from day 0 to day 5 reaching highest level of 775.52 μg $g^{-1}$ at day 5 followed by a decline. It is worth noting that compared with the control group, the FAAs content of *T. grandis* treated with ethylene showed significant increase by 35.97 % – 74.35 % (Table 1). The content of EAAs increased by 16.09 % – 64.48 % as a whole, and the accumulation pattern of EAAs was consistent with that of FAAs, reaching maximum value of 218.11 μg $g^{-1}$ on day 5, accounting for 28.12 % of the total FAAs. Chang detected 17 different amino acids in 37 different pecan species, of which FAAs content ranged from 41.22 mg $g^{-1}$ – 87.6 mg $g^{-1}$ and EAAs content was at 13.88 mg $g^{-1}$ – 26.1 mg $g^{-1}$ (Chang et al., 2020). A total of 29 walnut varieties from six main walnut producing areas such as Xinjiang and Yunnan were selected to determine their protein composition. The FAAs content of walnut ranged from 343.22 mg $g^{-1}$ – 985.99 mg $g^{-1}$, among which EAAs accounted for 30 % – 41 % of total FAAs (Yang et al., 2017).

The representative chromatograms of amino acids profile obtained at different postharvest stage and ethylene treatment are presented in Fig. S1, which showed significant discrepancy between ethylene treated and untreated nuts. Studies showed that ethylene can promote or inhibit amino acids accumulation. For example, exogenous application of ethylene on soybean leaves increased EAAs and NEAAs levels by five and six times, respectively (Ban et al., 2020). In green papaya, ethylene stimulated ripening concurrent with increase in some amino and fatty acids (Der Agopian et al., 2020). Likewise, in 'Huangguan' pears, exogenous ethylene promoted Pro accumulation and antioxidant activity to alleviate the chilling injury symptoms (Wei et al., 2019). However, high levels of ethylene impeded the accumulation of FAAs, EAAs and NESSs in rice grains (Xu et al., 2022) suggestive for a dose effect. Our study showed that *T. grandis* nuts treated with ethylene during the postharvest ripening stage showed much higher FAAs, EAAs and NESSs levels, indicating that ethylene significantly promoted amino acids biosynthesis in *T. grandis*.

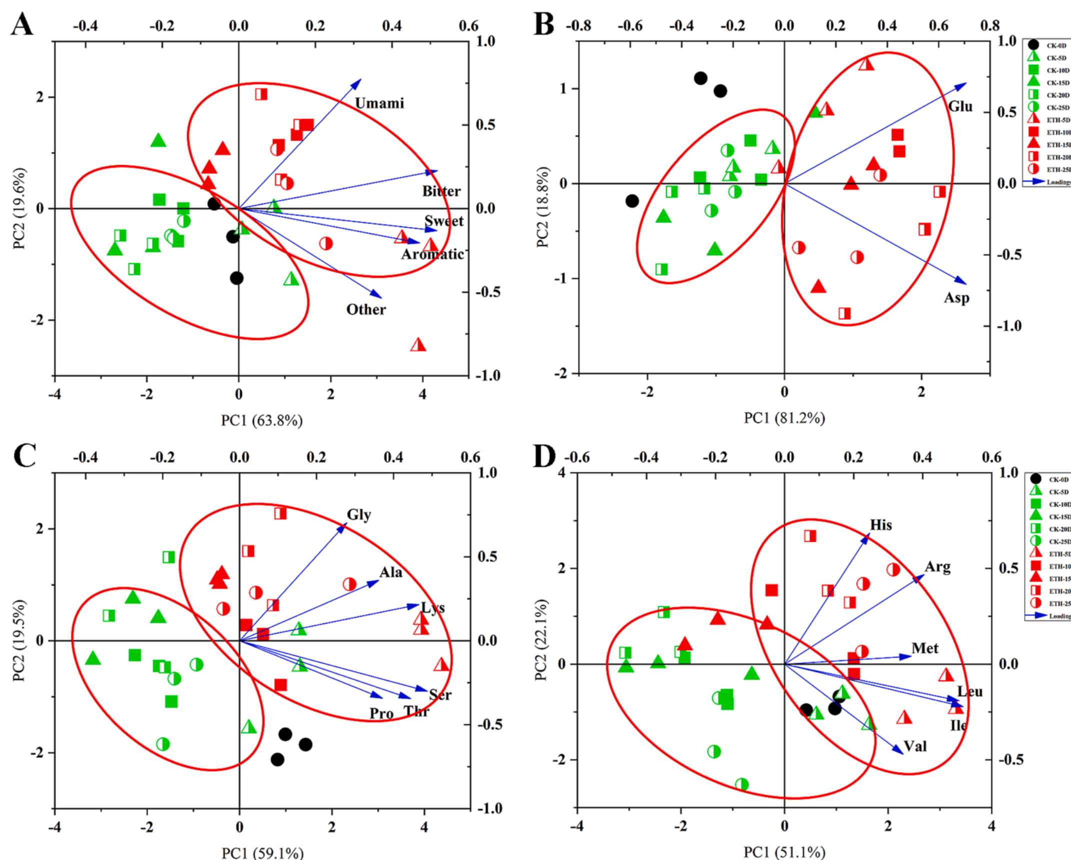


**Fig. 2.** PCA of the different kinds of amino acids in *T. grandis* nuts with different treatments during the postharvest ripening stage. A, PCA of the different flavor amino acids; B, PCA of the umami amino acids; C, PCA of the sweet amino acids; D, PCA of the bitter amino acids. The red ellipses reflect the distinguishability of nuts with different treatments.

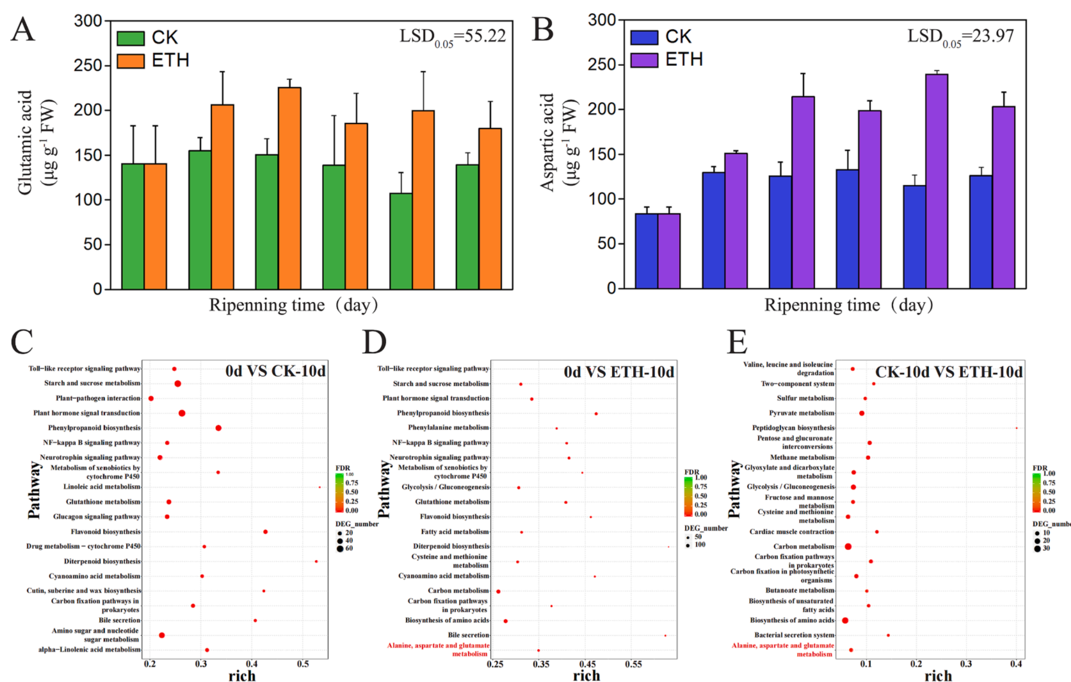


**Fig. 3.** The changes of aspartic acid and glutamic acid levels and KEGG analysis of DEGs between ethylene-treated nuts and control nuts. A, changes in glutamic acid level; B, changes in aspartic acid level; C, KEGG classification of DEGs from day 0 vs CK-day 10; D, KEGG classification of DEGs from day 0 vs ETH-day 10; E, KEGG classification of DEGs from CK vs ETH-day 10. Error bars represent standard error based on three biological replicates. LSD values represent LSD at $P = 0.05$.

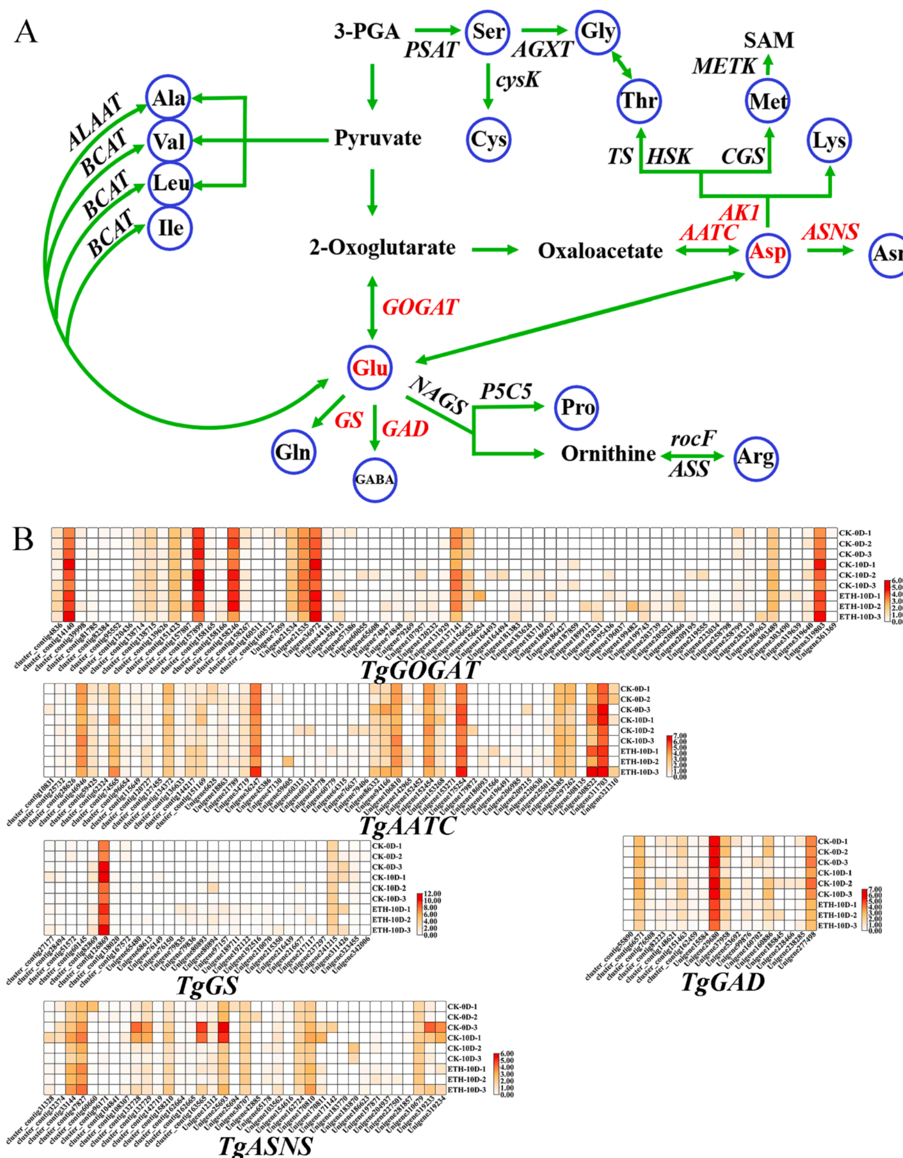


**Fig. 4.** The metabolic pathway of amino acids (A) and changes in transcripts of several key metabolic genes involved in the production of aspartic acid and glutamic acid (B) under different treatments during the postharvest ripening stage in *T. grandis* nuts.

### *3.2. Effect of ethylene on accumulation of umami taste-active amino acids in T. Grandis nuts during postharvest ripening stage*

Amino acids serve as precursors for various secondary metabolites, such as flavonoids, alkaloids and lignins, and to affect fruit quality such as aroma or taste in many horticultural crops i.e., green tea and mushroom (Li et al., 2020; Matsumoto and Ikoma, 2020; Zhang et al., 2013). Umami amino acids typically include: Glu and Asp, sweet amino acid include: Thr, Gly, Ser, Ala, Pro and Lys, whereas bitter amino acids include: Ile, Leu, Met, Val, His and Arg. Aromatic amino acids mainly include Tyr and Phe (Chen & Zhang, 2007). Our results in *T. grandis* nuts revealed that the proportion of umami amino acids under different treatments accounted for the major part of the flavor amino acids, up to 33 %–50 %, followed by sweet amino acids (19 %–29 %) and bitter amino acids (15 %–20 %), while aromatic amino acids were the least (3 %–11 %) (Fig. 1). During the postharvest ripening process, the overall change trend of umami amino acids under control was not significant, while the overall trend of umami amino acids increased dramatically after ethylene treatment, and to reach its maximal level on day 10 detected at 439.56 μg $g^{-1}$ (Fig. 1A). The content of sweet amino acids initially increased, then decreased and tended to be stable, reaching highest level on day 5 at 253.92 μg $g^{-1}$ (Fig. 1B). The overall change trend of bitter amino acids was consistent with that of sweet amino acids except that lowest level was detected on day 15 (Fig. 1C). The content of aromatic amino acids and other amino acids was found similar, showing a trend of a first increase followed by a decline and tending to be stable with maximum level observed on day 5 (Fig. 1D-E).

To determine whether there were differences in amino acids composition with ethylene treatment during the postharvest process, a principle component analysis (PCA) was carried out based on the five amino acid categories as input dataset (Fig. 2) with a total variance coverage of 83.4 %. The umami, bitter and sweet amino acids showed a distinct separation between ethylene-treated and control nuts, revealing that ethylene treatment significantly promoted their biosynthesis, especially umami amino acids that appeared most affected (Fig. 2A). Further analysis of umami amino acids showed that both Glu and Asp were positively regulated by ethylene treatment, and their contents varied considerably accordingly (Fig. 2B; Fig. 3A-B). The most apparent umami flavor differences between ethylene-treated and control samples was observed on day 10 (Fig. 2B). Among sweet amino acids, Gly was

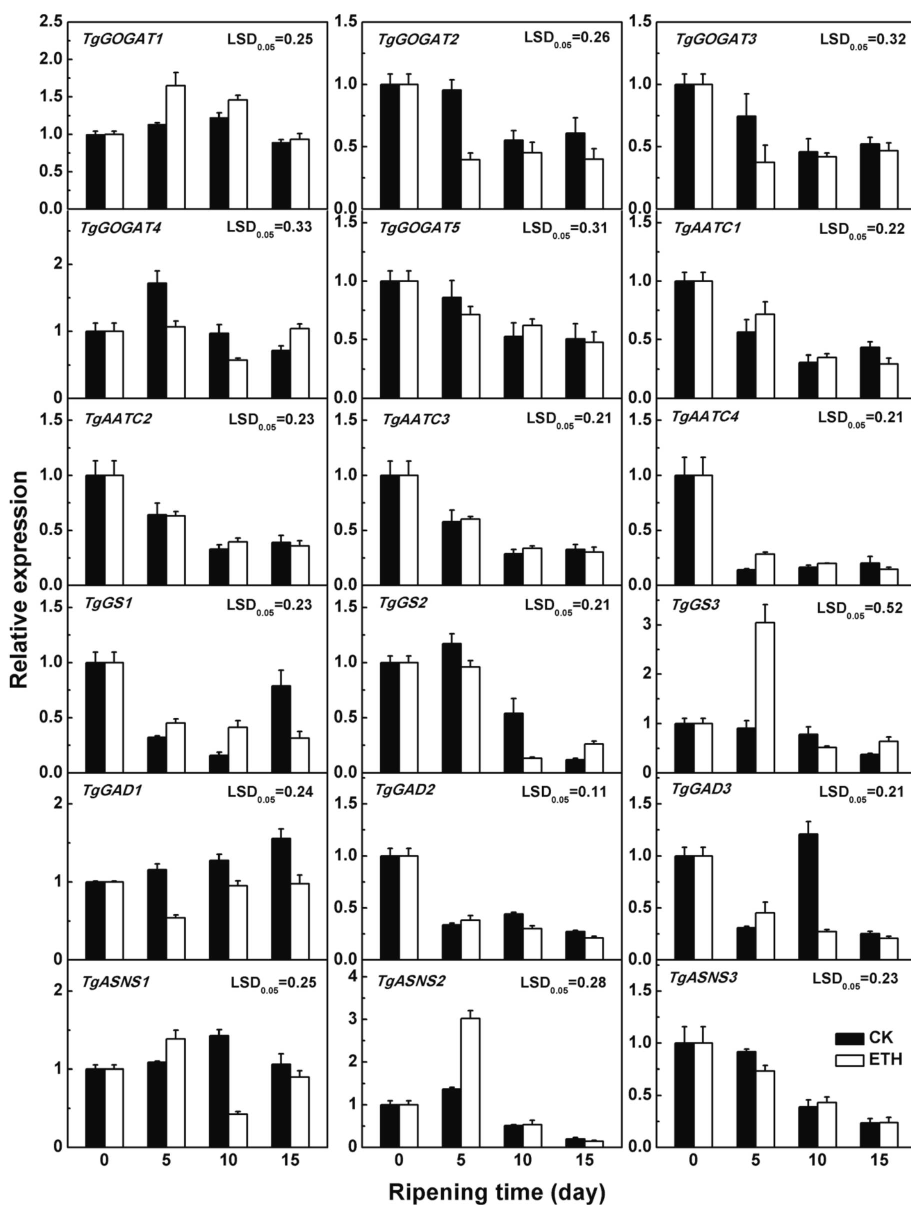


**Fig. 5.** Relative gene expression level of Glu and Asp metabolic pathways in response to ethylene treatment in *T. grandis* nuts during the postharvest ripening stage. Nuts were treated with 3000 μL L-1 ethephon for 24 h at 25℃ and 90 % relative humidity. Gene expression was analyzed using RT-qPCR and expression levels were calculated relative to corresponding values on day 0. The black bars indicate the control samples, and the white bars indicate the ethylene treated samples. Error bars represent standard error based on three biological replicates. LSD values represent LSD at $P = 0.05$.

the most affected by ethylene treatment (Fig. 2C). His and Arg levels in the bitter amino acid were significantly found at higher levels than that of control post ethylene treatment (Fig. 2C; Table 1). Umami is a pleasant savory taste mainly attributed to Glu and Asp, the contents of which are known to be affected by various factors (Zhang et al., 2013). For example, amino acid analysis of fermented foods revealed that Glu had a pronounced umami taste, being the most abundant amino acid and chief active taste component (Kawai et al., 2009). Besides, Glu and Asp provided the characteristic umami flavor to tomatoes and their content increased significantly during ripening stage (Boggio et al., 2000). Our study indicated that the content of Glu and Asp in *T. grandis* nuts increased significantly post ethylene treatment during the postharvest ripening stage (Fig. 3A-B).

### *3.3. Deferentially expressed genes (DEGs) responsible for umami amino acids variations in T. Grandis nuts under postharvest ethylene treatment*

Ethylene affects plant growth and development mainly by regulating key enzymes and the expression of genes involved in metabolism (Li et al., 2019). To further investigate the potential molecular mechanism of umami amino acids biosynthesis in *T. grandis* nuts under postharvest ethylene treatment, nine samples (day 0 and day 10) with different treatments were used for RNA-seq analysis. After obtaining high-quality transcriptome data, DESeq was employed to perform differential analysis of gene expression. The conditions for identification of differential genes were as such: expression difference fold $|\log2FoldChange| > 1$, significant $P$-value $< 0.05$. A total of 11,345 DEGs, including 5401 up- and 5944 down-regulated genes, were identified on day 0 compared with day 10 under control treatment (Fig. S2A). In day 0 vs ETH day 10, 12,442 genes were up-regulated versus 10,292 genes that showed down-regulation (Fig. S2B). In the CK-day 10 vs ETH- day 10, 2898 genes were up-regulated versus 1229 down-regulated genes (Fig. S2C). DEGs were grouped into various KEGG metabolic pathways (Fig. 3). Interestingly, "alanine aspartate and glutamate metabolism" was remarkably enriched at day 0 vs ETH-day 10 and CK-day 10 vs ETH-day 10 comparison groups (Fig. 3D-E). After mapping these gene changes onto metabolic pathways using KEGG (Fig. 4A), a total of 67 candidate genes for GOGAT, 53 candidate genes for AATC, 30 candidate genes for GS, 18 candidate genes for GAD and 37 candidate genes for ASNS were revealed (Fig. 4B). Interestingly, phenylpropane metabolic and flavonoid metabolic

pathways were significantly enriched in ethylene treated samples, indicating that ethylene treatment can promote increase of flavonoids in *T. grandis* nuts, which needs further research (Fig. 3). Studies on the relationship between ethylene and flavonoids are mostly concentrated in fruits, such as apples (An et al., 2018) and pears (Ni et al., 2020).

The candidate genes with higher expression level were further analyzed using real time RT-qPCR for validation (Fig. 5). Five candidate genes encoding *TgGOGAT* were identified, and RT-qPCR analysis indicated that expression of *TgGOGAT1* was positively regulated by ethylene treatment, to reach highest level on day 5, which contributed to the accumulation of Glu at higher levels. In addition, although expression level of *TgAATC1* and *TgAATC4* showed downregulation, their expression levels were still significantly higher than that of the control group on day 5, which were regulated by ethylene and promoted the increase in Asp level. Among genes involved in degradation of Glu and Asp, expression levels of *TgGAD1* was found likewise suppressed and significantly lower than those of control through the whole postharvest ripening stage. *TgGAD3* and *TgGS2* appeared to be also responsive to ethylene treatment, and their expression levels were significantly lower than control group on day 10, which allowed for less Glu to be metabolized and accounting for its increased level. Moreover, associated with Asp metabolism, only the expression level of *TgASNS1* responded to ethylene treatment found significantly lower than that of the control group on day 10. In soybean, exogenous application of ethylene significantly increased the free amino acid content in its leaves and stems, while in creeping bentgrass, exogenous application of AVG reduced amino acids in its leaves, albeit the molecular mechanism remains unresolved (Ban et al., 2021; Jespersen et al., 2015). However, there are further recent studies in rice, but contrary to previous findings, high ethylene level negatively affected amino acids biosynthesis and inhibited the activities of GOGAT, AST, and ALT (Xu et al., 2022). Our results suggested that ethylene treatment induced the biosynthesis of Glu and Asp in *T. grandis* nuts after harvest, which is likely to be mediated via the up-regulated expression of the biosynthetic genes (*TgGOGAT1*, *TgAATC1*, *TgAATC4*) concurrent with the down-regulated expression of degradation genes (*TgGS2*, *TgGAD1, TgGAD3*, *TgASNS1*).

## 4. Conclusions

This study provides the first comprehensive profile of amino acids compositional changes in *T. grandis* nut during the postharvest ripening stage and further the effect of ethylene on its amino acids metabolism. The results suggested that application of ethylene on *T. grandis* nuts resulted in a distinctive increase in total FAAs, EAAs and NEAAs, indicating that application of ethylene could improve the nutritional quality of *T. grandis* nut and its flavor quality. Furthermore, PCA indicated that umami amino acids including Glu and Asp were distinctly separated between ethylene-treated and control nuts. The transcriptomic KEGG analysis and the combined gene expression levels indicated that the biosynthesis genes of Glu and Asp (*TgGOGAT1*, *TgAATC1*, *TgAATC4*) were up-regulated, whereas degradation mediated genes (*TgGS2*, *TgGAD1, TgGAD3*, *TgASNS1*) showed down-regulation with ethylene treatment, revealing their important roles in upregulation of Glu and Asp biosynthesis. To sum up, these results provide a theoretical basis and scientific guidance for improving the postharvest quality of *T. grandis* nuts.

## CRediT authorship contribution statement

**Zuying Zhang:** Conceptualization, Investigation, Writing – review & editing. **Wenchao Chen:** Methodology, Validation, Investigation, Formal analysis. **Liu Tao:** Validation, Investigation, Formal analysis. **Xixing Wei:** Methodology, Validation. **Lingling Gao:** Investigation, Formal analysis. **Yadi Gao:** Investigation, Formal analysis. **Jinwei Suo:** Formal analysis. **Weiwu Yu:** Data curation. **Yuanyuan Hu:** Formal analysis, Data curation. **Baoru Yang:** Writing – review & editing. **Huifeng Jiang:** Writing – review & editing. **Mohamed A. Farag:** Writing – review & editing. **Jiasheng Wu:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Lili Song:** Methodology, Investigation, Writing – original draft, Writing – review & editing.

## 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.

## Data availability

Data will be made available on request.

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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.2022.135214.

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