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New sights into lipid metabolism regulation by low temperature in harvested *Torreya grandis* nuts

Minying Zhou,[†] Lili Song,[a,b†] Shan Ye,[a] Wei Zeng,[a,b] Heikki Hännien,[a] Weiwu Yu,[a] Jinwei Suo,[a] Yuanyuan Hu[a] and Jiasheng Wu[a*]

Abstract

BACKGROUND: *Torreya grandis*, a large evergreen coniferous tree with oil-rich nuts, undergoes a crucial ripening stage after harvest that results in oil accumulation, finally giving rise to the nut that is edible in roasted form. To understand lipid metabolism in *T. grandis* nuts during the post-harvest ripening period, the effects of low temperature on lipid content, fatty acid composition, lipid biosynthesis and degradation were investigated.

RESULTS: The lipid content increased during ripening at room temperature and a low temperature slowed down this increase. Linoleic acid content increased at low temperature, which was accompanied by an increase in the microsomal oleate desaturase (FAD2) activity and FAD2 expression. Furthermore, a low temperature attenuated lipid peroxidation as indicated by lower contents of malondialdehyde, hydroperoxide and total free fatty acid in *T. grandis* nuts during the ripening stage, as well as the down-regulation of gene expression of lipid degradation-related enzymes such as phospholipase D and lipoxygenases.

CONCLUSION: The findings of the present study indicate that a low temperature increased polyunsaturated fatty acid contents by increasing FAD2 biosynthesis and decreasing lipid peroxidation, thereby improving the oil yield in *T. grandis* nuts during the post-harvest ripening period.

Supporting information may be found in the online version of this article.

Keywords: *Torreya grandis* nuts; post-harvest ripening; low temperature; fatty acid biosynthesis; lipid peroxidation

INTRODUCTION

Post-harvest ripening is a time-dependent and environmentally regulated process occurring commonly in seeds and fruits of plants. It affects several critical seed traits, such as fiber content and composition, lipid metabolism, and the levels of vitamins and various anti-oxidants.[1] There is strong evidence suggesting that the ripening in seeds of oil and crop plants, such as almonds, walnuts and olive, can alter lipid metabolism, resulting in increasing contents of unsaturated fatty acids (UFAs).[2,3] This development process that takes place during the post-harvest ripening stage is essential for meeting the increasing demand for healthy dietary oils in the seeds of oil and crop plants.

It has been proposed that a successful strategy to increase lipid unsaturation is to increase the activities of fatty acyl desaturases. These desaturases, such as stearoyl-ACP desaturase (SAD), oleate desaturase (FAD2) and linoleci desaturase (FAD3), comprise a group of enzymes that increase the degree of unsaturation by converting a single bond between two carbon atoms into a double bond.[4,5]

A low temperature can increase UFA content in plants by altering the activities of the desaturases.[4] The activity of FAD2, which is encoded by a single *FAD2* gene, was found to be influenced by temperature in soybean and safflower. *Arabidopsis fad2* mutants with decreased levels of polyunsaturated fatty acids (PUFAs) exhibit withering when exposed to a temperature of 12 °C.[6] Horiguchi *et al.*[7] reported that the enhanced trienoic fatty acid content in wheat root tips caused by exposure to low temperatures is accompanied by an increase in FAD3 protein expression. Furthermore, the UFA level in oilseeds or fruits subjected to a low temperature depends on the membrane fluidity, which is influenced by the hydrolysis of membrane phospholipids and the peroxidation of the constituent PUFAs by phospholipid-degrading enzymes.[8,9] Phospholipase D (PLD) and lipoxygenases (LOX) are phospholipid-degrading enzymes that cause the degradation of UFAs, resulting in a reduced cell membrane fluidity.[10] Increases of

* *Correspondence to: J Wu, State Key Laboratory of Subtropical Silviculture, Zhejiang A&F University, Zhejiang, People's Republic of China. E-mail: wujs@zafu.edu.cn*

† *These authors contributed equally to this work.*

a *State Key Laboratory of Subtropical Silviculture, Zhejiang A&F University, Zhejiang, People's Republic of China*

b *Sino-Australia Plant Cell Wall Research Centre, School of Forestry and Biotechnology, Zhejiang A&F University, Zhejiang, People's Republic of China*

membrane associated PLD and LOX activities have been observed in response to exposure to chilling stress in maize.[10] However, Liu *et al.*[11] reported that litchi fruit stored for 10 days at 3–5 °C had lower activities of PLD and LOX and also lower levels of membrane permeability compared to the controls. These findings suggest that the regulation of the final polyunsaturated acids related to lipid metabolism in plants during the ripening stage still needs to be clarified.

Torreya grandis is a large, evergreen coniferous tree with significant economic value in China and has been listed as a national key protected wild plant species.[12,13] The consumption of *T. grandis* nuts is increasingly popular in Eastern China because of their medicinal properties resulting from their anthelmintic, antitussive, carminative, laxative, antifungal, antibacterial and antitumor activities. *Torreya grandis* nuts contain approximately 42.61–54.39% oil, and 80% of this comprises UFAs, mainly oleic acid and linoleic acid.[14] Previous studies have suggested that these high UFA levels contribute to a lowering of the risk for coronary heart disease.[12,15]

Post-harvest ripening is a crucial stage in *T. grandis* nuts because it results in oil accumulation and an alteration of the fatty acid composition.[16] However, the effects of low temperature on *T. grandis* nuts during the post-harvest ripening stage, particularly its effects on the UFA contents and the changes in the lipid metabolism, remain unclear. We hypothesize that the increased contents of UFAs in *T. grandis* nuts after harvest are caused by two effects of low temperature: (i) enhanced lipid biosynthesis via increased desaturase activities resulting from up-regulation of their gene expression levels and (ii) decreased lipid peroxidation via reduced activities of PLD and LOX enzymes resulting from down-regulation their gene expression levels, thereby maintaining membrane integrity. Accordingly, the present study aimed to determine the effects of low temperature on two properties of *T. grandis* nuts during the post-harvest ripening stage: (i) the oil content and the fatty acid composition and (ii) the molecular regulation underlying the biosynthesis and peroxidation of lipids. The findings of the present study may improve our understanding of the responses of this species to low temperature, as well as provide a theoretical foundation for maximizing the contents of unsaturated lipids in this important medicinal plant.

MATERIALS AND METHODS

Plant materials and experimental treatments

In the present study, nut samples were collected from the Merrillii cultivar of *T. grandis* at a commercial orchard in Zhuji, Zhejiang Province, China, in September 2015. When the sarcotesta, comprising the fleshy covering of the nuts, turned yellow and dissilient, the nuts were harvested at 8.00–10.00 h (mature stage, 520 ± 5 days after blooming) and transported to laboratory within 6 h. Nuts with a similar shape, color and no blemishes were selected, and sarcotesta were removed manually.

The experimental treatments were conducted in an incubator (MIR-554; Panasonic, Kadoma, Japan) using two ripening treatments: 4 ± 1 °C (low temperature) and 25 ± 1 °C (room temperature) and the relative humidity (RH) was kept above 90%, where the air temperature can be manipulated independently. The experiment was a completely randomized design with three technical replications (chambers). Approximately 5 kg of nuts (1800 nuts) was used in each ripening treatment experiment. In both experimental temperatures, the nuts were incubated in the chambers until the coat colour changed to black after 28 and 42 days for the treatments at room temperature and low temperature, respectively. The samples were manually stirred and flushed with fresh air for 10 min each day to avoid creating anoxic conditions during treatment. Nut samples were collected every 7 days for the treatment at room temperature and every 14 days at low temperature. Three duplicate samples are available at each treatment time point.

Measurements of lipids and fatty acid composition

Kernel tissue from 30 nuts was ground in an Osterizer blender (Galaxie Model Number 869-18R; Jarden Consumer Solutions, Boca Raton, FL, USA) into homogeneous flour (40-mesh). Then, the 10 g sample was extracted in a Soxhlet apparatus with petroleum ether solvent (boiling point range: 38.7–54.8 °C) for 8 h. The sample was defatted and dried overnight (10–12 h) and weighed to calculate lipid content.

Fatty acid composition was measured by gas chromatography (TRACE-1300; Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 0.5 g nut oil was methyl esterified with 10 mL of ethyl ether and *n*-hexane mixture (1:1, v:v) solution and 10 mL of methanol consisting of 2 mol L^{-1} KOH. The upper layer of the solution was collected and subjected to gas chromatography coupled with a silica capillary column (DB wax; Agilent Technologies Inc., Santa Clara, CA, USA; 30 m × 0.25 mm × 0.25 μm). Helium was used as the carrier gas at a flow rate of 0.8 mL min^{-1}. The oven temperature was initially held at 170 °C for 0.5 min, then increased to 230 °C at a rate of 5 °C min^{-1} and maintained at 230 °C for 13 min. Injection port and detector temperature were set to 260 and 250 °C, respectively. In total, 37 different types of methyl-esterified fatty acids were mixed as the standard for determination of fatty acid composition (97.8–99.9%; Supelco, Bellefonte, PA, USA). Additionally, 5(*Z*),11(*Z*),14(*Z*)-eicosatrienoic acid (> 98%; Larodan, Monroe Michigan, USA) was methyl-esterified and used as the standard for the determination of sciadonic acid content. The relative percentage of fatty acids in the kernel oil was calculated based on each fatty acid peak area relative to the total area of all the fatty acid peaks.

Enzyme activities of lipid biosynthesis

Approximately 5 g of frozen nuts tissue was ground into powder and homogenized with 25 mL of phosphate-buffered saline (PBS) (pH 7.4). After being centrifuged at 2500 × *g* for 20 min, supernatant was collected for enzyme activity measurements. Plant acetyl-CoA carboxylase (ACCase), FAD2, FAD3 and FAS enzyme-linked immunosorbent assay (ELISA) kits (Beijing Fangchengbaijin Technology, Beijing, China) were used for the determination of enzyme activities using a microplate spectrophotometer (MS 352 Multiskan; Labsystems Diagnostics, Oy, Vantaa, Finland). Enzyme activities were calculated in terms of units based on the standard curve of the ELISA kit and expressed as nmol g^{-1} fresh weight.

Lipid peroxidation measurements

Total free fatty acid (FFA) content

FFA was extracted with isopropanol and hexane (7.5 mL) in accordance with a previous method.[17] Briefly, 10 g of frozen nuts were ground into powder and homogenized with isopropanol (37.5 mL) and hexane (7.5 mL). Then, the mixture was filtered through a filter membrane (no. 4) and titrated with 0.05 mol L^{-1} NaOH to obtain the oil content as described by The American Oil Chemists' Society.[18] Next, 5 g oil was extracted with isopropanol: hexane (75:15) and titrated with 0.05 mol L^{-1} NaOH to obtain the FAA content. FFA (%) was expressed as a percentage of oil content.

Lipid hydroperoxide content

Production of lipid hydroperoxides, a primary product of lipid oxidation, was measured as describeed by to Shantha and Decker.[19] Briefly, oil sample (0.3 mL) was mixed three times with 1.5 mL isooctanol + isopropanol (3:1, v:v) solution. After centrifugation for 2 min at 3400 × *g*, 0.2 mL of the organic layer was mixed with a methanol + butanol solution (2:1, v/v). Then, 15 μL of ammonium thiocyanate and ferrous iron solution were added. The absorbance was read at 510 nm using a spectrophotometer (UV-2500 Spectrophotometer; Shimadzu, Kyoto, Japan). Hydroperoxide content was determined based on a cumene hydroperoxide standard curve.

Malondialdehyde (MDA) content

MDA content was determined by Wang *et al.*[20] Approximately 5 g of frozen powdered nuts tissue were homogenized with 25 mL of PBS (pH 7.8), then centrifuged for 15 min at 5000 × *g*. Supernatant (3 mL) was mixed with 3 mL of 10% trichloroacetic acid and 1 mL of 0.6% thiobarbituric acid. The mixture was heated at 95 °C for 15 min and then rapidly ice-cooled. After centrifugation at 5000 × *g* for 20 min, the absorbance was recorded at 532, 600 and 450 nm. MDA content, expressed as nmol g^{-1}, was calculated as 6.45 ($A_{532} - A_{600}$) − 0.56A_{450}.

Lipid peroxidation-related enzyme activities

Frozen nuts tissue (5 g) was ground into powder, homogenized with 25 mL of Tris-HCl buffer and centrifuged at 14000 × *g* for 10 min at 4 °C. The reaction substrate was prepared by adding 0.4 g of phosphatidylcholine, then evaporated at 35 °C and redissolved in 100 mmol L^{-1} acetate acid buffer (pH 5.5, containing 5 mmol L^{-1} dithiothreitol and 25 mmol L^{-1} $CaCl_2$). For the PLD assay, 1 mL of crude enzyme extracts and 3 mL of reaction substrate were shaken for 1 h at 28 °C. The water layer was collected and 2 g of Reinecke salt was added. After centrifugation at 16000 × *g*, the precipitate was redissolved in 3 mL of acetone. A standard curve for choline was made by diluting 20 mg of choline chloride in 100 mmol L^{-1} acetate buffer (pH 5.6). PLD activity was expressed as μmol of choline min^{-1}.

LOX activity was assayed at 25 °C by monitoring the formation of conjugated dienes from linoleic acid at 234 nm.[21] The reaction mixture (3 mL) contained 50 mmol L^{-1} sodium phosphate (pH 7), 10 mmol L^{-1} sodium linoleic acid and 0.1 mL of enzyme solution. LOX activity was determined as the change in absorbance (min^{-1}) at 234 nm and expressed as U mg^{-1} protein.

Expression analysis of oil metabolism-related genes

To determine the molecular mechanisms underlying the effects of low temperature on harvested nuts storage, 20 nuts from each sample were collected for RNA extraction using the 74 904 RNeasy Plant Mini Kit (Qiagen, Valancia, CA, USA). Reverse transcriptase-polymerase chain reaction (RT-PCR) measurements were conducted on genes related to the lipid metabolism. First-strand cDNA synthesis was conducted with 3 μg of total RNA using a PrimeScript RT reagent Kit (Takara Biotech, Otsu, Japan). Specific primers were designed using Primer Quest (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and *ACTIN* was used as a control (see Supporting information, Table S1). PCR amplifications were carried out using the SYBR Premix Ex Tap II Tli Rnase H Plus (Takara Biotech) on a Roche LightCycler 480 System (Roche, Basel, Switzerland). The program was set as: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 55 °C for 10 s, 72 °C for 20 s, and 72 °C for 2 min. All experiments were performed at least three times.

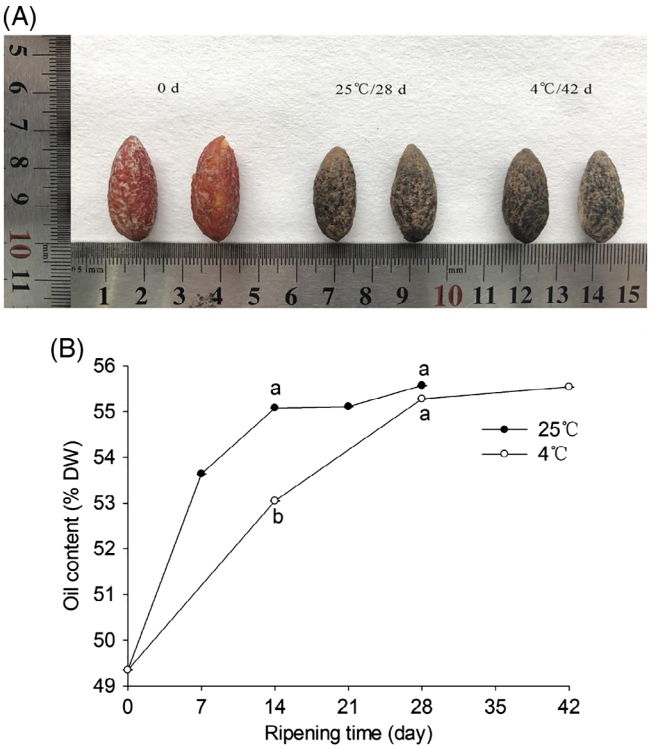

Figure 1. External changes (A) and changes in oil contents (B) in dehulled *Torreya grandis* nuts during post-harvest ripening at 25 °C and 4 °C in 90–95% RH. The data indicate the mean ± SD ($n = 3$). Different lowercase letters indicate statistically significant differences between the two ripening temperatures ($P < 0.05$ in the LSD test).

Statistical analysis

Data are presented as the mean ± SD. Statistical analysis was performed by one-way analysis of variance using SPSS, version 16.0 (SPSS, Chicago, IL, USA). A least significance difference (LSD) was used and $P < 0.05$ was considered statistically significant.

RESULTS

Oil content, fatty acid composition and enzymatic activities related to lipid biosynthesis

The seed coat of *T. grandis* nuts is reddish brown at harvest and becomes black during the post-harvest ripening stages (Fig. 1A). The oil content displayed an increasing pattern during the ripening process (Fig. 1B). The oil content of nuts exposed to a low temperature was significantly lower than that of nuts exposed to room temperature at the same ripening time (Fig. 1B). Interestingly, the oil content of nuts exposed to low temperature for 42 days did reach the same level as that of nuts exposed to room temperature for 28 days. Overall, the fully ripening time of nuts was delayed by treatment at low temperature (on day 42 of ripening time) compared to treatment at room temperature (on day 28 of ripening time).

The composition and contents of the individual fatty acids were assessed in the total oil extracted from nut during the ripening time from each treatment (Table 1). Linoleic, oleic acid and sciadonic acid were found to be the major UFAs (PUFAs) in *T. grandis* nuts (Fig. 2). The oleic acid content in nuts displayed a decreasing pattern, whereas linoleic acid (C18:2) and linolenic acid (C18:3) contents exhibited an increasing trend during the ripening process (Table 1). Compared with nut samples before ripening, the

linoleic acid PUFA contents of nuts at room temperature for 28 days of ripening time increased by 4.4% and 3.4%, respectively. The linoleic acid and PUFA contents of nuts at a low temperature for 42 days of ripening time were significantly higher than those of nuts at room temperature for 28 days of ripening time.

During the ripening stage, the activities of ACCase, FAS and FAD3 exhibited a decreasing/increasing trend. The lowest ACCase activity was observed on day 14 of ripening time both at room temperature and low temperature (Fig. 3A). The FAS and FAD3 activities decreased and showed the lowest levels on days 7 and 14 at room temperature and low temperature, respectively (Fig. 3B,D). The ACCase, FAS and FAD3 activities were significantly higher in nuts treated at room temperature than in those at low temperature for the same ripening time (Fig. 3A,B,D). FAD2 activity increased over time, with a maximum value on days 21 and 28 at room temperature and low temperature, respectively. Nuts treated at a low temperature maintained a higher level of FAD2 activity than those at room temperature treated for the same ripening time ($P < 0.05$) (Fig. 3C).

FFA content, lipid peroxidation products and enzymatic activities related to lipid peroxidation

MDA and lipid hydroperoxide exhibited an increasing trend in nuts treated at room temperature and low temperature during the ripening process (Fig. 4A,B). In addition, MDA and lipid hydroperoxide were significantly higher in nuts treated at room temperature than at low temperature for the same ripening time. FFA in nuts treated at room temperature displayed a decreasing/increasing/decreasing pattern, whereas there was a decreasing trend in nuts treated at low temperature during the ripening stages (Fig. 4C). FFA was significantly higher in nuts treated at room temperature compared to a low temperature for the same ripening time.

PLD activity in nuts treated at room temperature maintained a stable value during the ripening stage, whereas it showed a decreasing trend in nuts treated at low temperature (Fig. 5A). LOX activity exhibited an increasing trend in nuts treated at room temperature and low temperature during the ripening process (Fig. 5B). Both PLD and LOX activities were significantly higher in nuts treated at room temperature compared to those at low temperature for the same ripening time.

The expression of genes related to lipid metablism

The expression of seven genes related to lipid metablism during ripening is shown in Fig. 6. In treatments at room temperature treatment, *TgACCase* and *TgLACS* expression decreased in the first 14 days and dramatically increased subsequently (Fig. 6A,B). Interestingly, *TgSAD*, *TgFAD2* and *TgFAD3* showed a similar increasing expression pattern during the ripening process (Fig. 6C–E). The expression of *TgACCase*, *TgLACS* and *TgFAD* was at a relatively higher level for treatment at room temperature compared to low temperature. The expression of *TgFAD2* was significantly higher at day 42 for treatment at low temperature than that at day 28 at room temperature ($P < 0.05$) (Fig. 6C,D).

TgPLD for treatment at room temperature remained constant during the ripening stage, whereas it significantly decreased at low temperature (Fig. 6F). During the ripening process, there was a significant increase in *TgLOX* levels for both treatment at room temperature and treatment at low temperature. Overall, the increase rate of *TgLOX* expression was significantly higher in nuts treated at room temperature than in those at low temperature.

Table 1. Fatty acid composition of Torreya *grandis* nut oil under room (25 °C) and low temperature (4 °C) ripening treatments

Fatty acid	Post-harvest ripening time at 25 °C (day)					Post-harvest ripening time at 4 °C (day)				
	0	7	14	21	28	0	14	28	42
Palmitic acid (C:16:0)	7.34 ± 0.04 a	7.10 ± 0.03 b	7.02 ± 0.03 b	6.91 ± 0.02 c	6.91 ± 0.03 c	7.34 ± 0.04 a	6.90 ± 0.10 c	6.90 ± 0.06 c	6.91 ± 0.09 c
Stearic acid (C18:0)	2.90 ± 0.05 a	2.71 ± 0.06 b	2.75 ± 0.04 b	2.74 ± 0.05 b	2.60 ± 0.03 c	2.90 ± 0.05 a	2.62 ± 0.06 c	2.61 ± 0.05 c	2.60 ± 0.04c
Oleic acid (18:1)	32.84 ± 0.21 a	32.60 ± 0.25 ab	32.43 ± 0.19 bc	32.32 ± 0.23 bc	31.72 ± 0.18 c	32.84 ± 0.21 a	32.19 ± 0.25 c	31.71 ± 0.16 c	31.20 ± 0.18 d
Linoleic acid (18:2)	41.68 ± 0.22 e	42.22 ± 0.19 d	42.56 ± 0.14 cd	42.89 ± 0.17 c	43.50 ± 0.25 b	41.68 ± 0.2 2e	42.55 ± 0.19 cd	43.37 ± 0.24 b	43.91 ± 0.16 a
Linolenic acid (C18:3)	0.40 ± 0.02 d	0.41 ± 0.01 d	0.42 ± 0.00 d	0.48 ± 0.02 ab	0.49 ± 0.02 a	0.40 ± 0.02 d	0.45 ± 0.01 c	0.46 ± 0.02 bc	0.45 ± 0.01 c
Arachidic acid (C20:0)	0.79 ± 0.03 b	0.69 ± 0.02 c	0.70 ± 0.04 c	0.72 ± 0.04 c	0.71 ± 0.03 c	0.79 ± 0.03 b	0.90 ± 0.02 a	0.69 ± 0.04 c	0.68 ± 0.04 c
Eicosenoic acid (C:20:1)	0.62 ± 0.02 b	0.63 ± 0.03 ab	0.64 ± 0.02 ab	0.64 ± 0.01 ab	0.66 ± 0.00 a	0.62 ± 0.02 b	0.65 ± 0.02 ab	0.63 ± 0.01 ab	0.65 ± 0.03 ab
Eicosadienoic acid (C20:2)	2.62 ± 0.05 e	2.86 ± 0.05 bc	2.76 ± 0.04 d	2.74 ± 0.06 d	2.82 ± 0.04 cd	2.62 ± 0.05 e	2.86 ± 0.04 bc	2.91 ± 0.03 b	3.01 ± 0.05 a
Eicosatrienoic acid (C20:3)	10.81 ± 0.11 a	10.78 ± 0.13 a	10.72 ± 0.07 ab	10.56 ± 0.06 b	10.59 ± 0.08 b	10.81 ± 0.11 a	10.88 ± 0.06 a	10.72 ± 0.07 ab	10.59 ± 0.08 b
SFA	11.03 ± 0.05 a	10.50 ± 0.06 b	10.47 ± 0.06 bc	10.37 ± 0.07 c	10.22 ± 0.06 d	11.03 ± 0.05 a	10.42 ± 0.07 bc	10.20 ± 0.05 d	10.19 ± 0.06 d
UFA	88.79 ± 0.10 c	89.50 ± 0.08 b	89.53 ± 0.03 b	89.63 ± 0.06 b	89.78 ± 0.07 a	88.79 ± 0.10 c	89.58 ± 0.06 b	89.80 ± 0.07 a	89.81 ± 0.03 a
MUFA	33.46 ± 0.06 a	33.23 ± 0.04 b	33.07 ± 0.07 c	32.96 ± 0.03 cd	32.38 ± 0.09 e	33.46 ± 0.06 a	32.84 ± 0.14 d	32.34 ± 0.11 e	31.85 ± 0.07 f
PUFA	55.51 ± 0.08 f	56.27 ± 0.07 e	56.46 ± 0.05 d	56.67 ± 0.08 c	57.40 ± 0.11 b	55.51 ± 0.08 f	56.74 ± 0.11 c	57.46 ± 0.09 b	57.96 ± 0.12 a
UFA/SFA	8.07 ± 0.05 d	8.52 ± 0.04 c	8.55 ± 0.02 c	8.64 ± 0.06 b	8.78 ± 0.05 a	8.07 ± 0.05 d	8.60 ± 0.06 bc	8.80 ± 0.02 a	8.81 ± 0.04 a

The nuts were harvested approximately at the time when they are shed in natural conditions and then they were ripening at the two temperatures for the indicated number of days. The proportion of each fatty acid is indicated as percentage of all fatty acids; and the contents of total saturated fatty acids (SFA), total unsaturated fatty acids (UFA), total monounsaturated fatty acids (MUFA) and total polyunsaturated fatty acids (PUFA) is given as percentage of lipid contents. All data are expressed as the mean ± SD ($n = 3$). Different lowercase letters in the same row indicate statistically significant differences between the ripening times ($P < 0.05$).

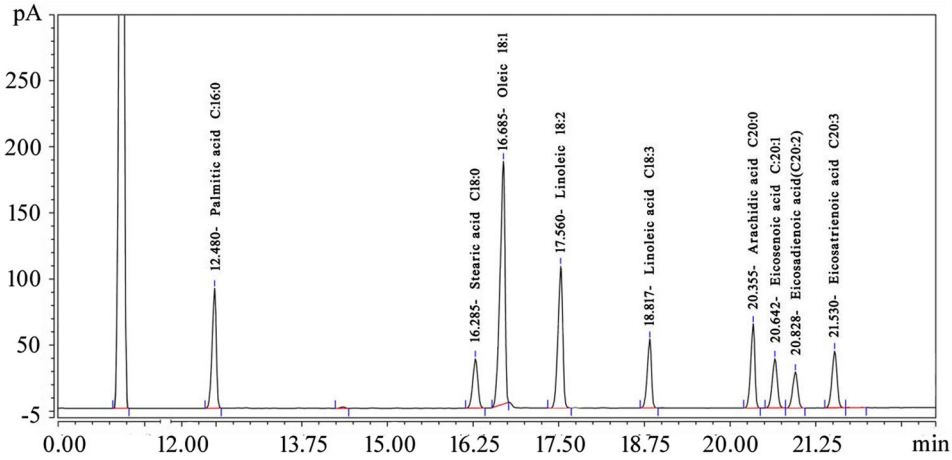

Figure 2. The standard gas chromatogram of the nine main fatty acids in *Torreya grandis* nuts.

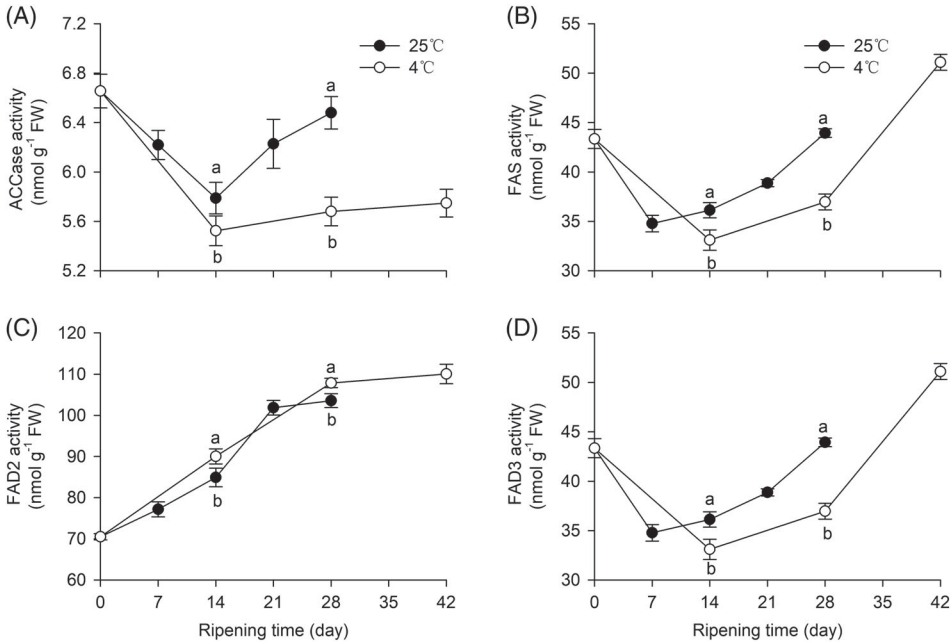

Figure 3. The activities of ACCase (A), FAS (B), FAD2 (C) and FAD3 (D) of *Torreya grandis* nuts during post-harvest ripening at 25 °C and 4 °C in 90–95% RH. The data indicate the mean ± SD ($n = 3$). Different lowercase letters indicate statistically significant differences between the two ripening temperatures ($P < 0.05$ in the LSD test).

DISCUSSION

Fats have been considered as a part of the basic food supply in the human diet over a long period of time, which has attracted increasing attention because they are not only the main energy and nutrition source, but also they have a close relationship with human health and disease.[22–24] There is a dose-dependent relationship between the intake of saturated fatty acids (SFAs) and blood low-density lipoprotein (LDL) cholesterol concentrations, and a dietary increase in UFAs showed a lower serum cholesterol, thus indirectly reducing the risk of cardiovascular disease and possibly the incidence of cancers, asthma and diabetes.[25–28] For this reason, it is been recommended that dietary plant oils are increased aiming to improve the ratio of UFAs to SFAs. *Torreya grandis* seeds are one of the world popular nuts with a high amount of oil, especially UFA. It was reported that the amount of UFAs in oilseeds is largely determined during the ripening stage after harvest. This is because, during that stage, biochemical and physiological changes contribute to the increase in the contents of fatty acid contents in the oil.[1] Furthermore, ripening aggravates lipid peroxidation via the activities of PLD and/or LOX, resulting in the release of free fatty acids and subsequent lipid peroxidation. Previous studies suggest that temperature is one of the main environmental factors influencing oil concentration during nut ripening.[29] In the present study, we investigated fatty acid composition, lipid peroxidation products, and lipid metabolism related to biosynthesis and peroxidation in *T. grandis* nuts during the post-harvest ripening stage and found that a low temperature increases PUFA contents by increasing FAD2 biosynthesis and decreasing lipid peroxidation.

Effect of low temperature on fatty acid composition in *T. grandis* nuts during the post-harvest ripening stage

In the present study, lipid content in the nuts increased continuously under room temperature conditions for 28 days during

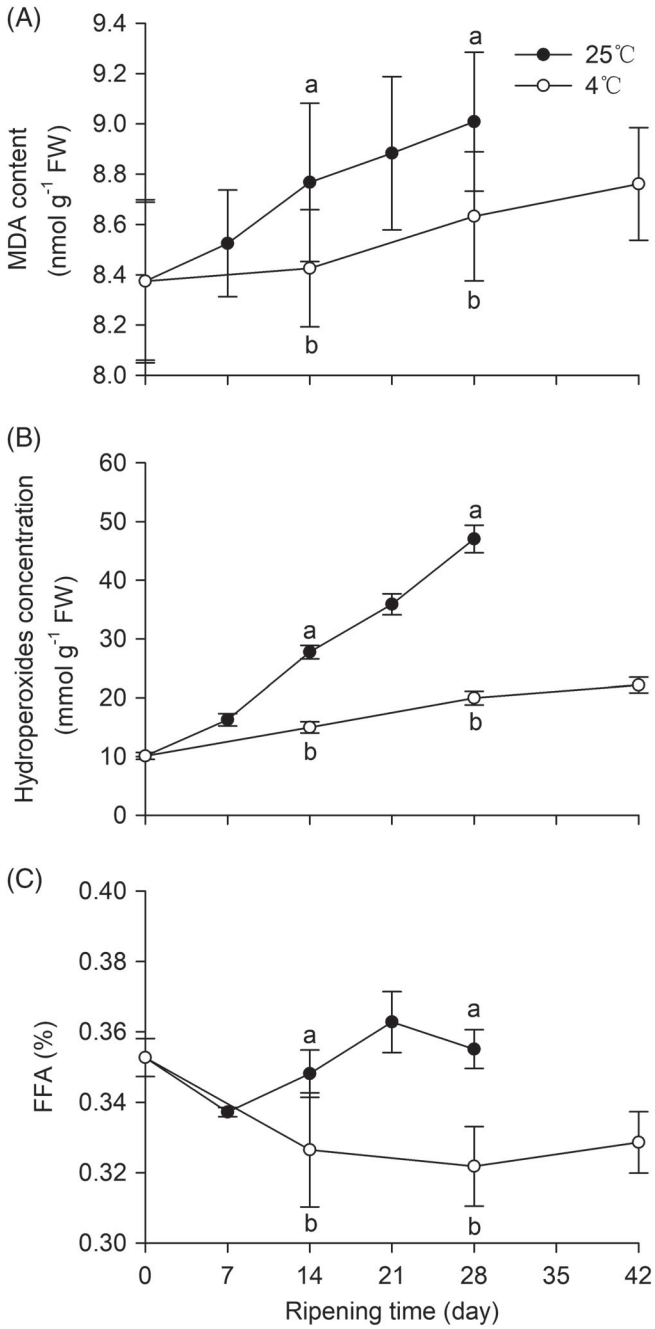

Figure 4. Contents of MDA (A), hydroperoxides (B) and FFA (C) of *Torreya grandis* nuts during post-harvest ripening at 25 °C and 4 °C in 90–95% RH. The data indicate the mean ± SD ($n = 3$). Different lowercase letters indicate statistically significant differences between the two ripening temperatures ($P < 0.05$ in the LSD test).

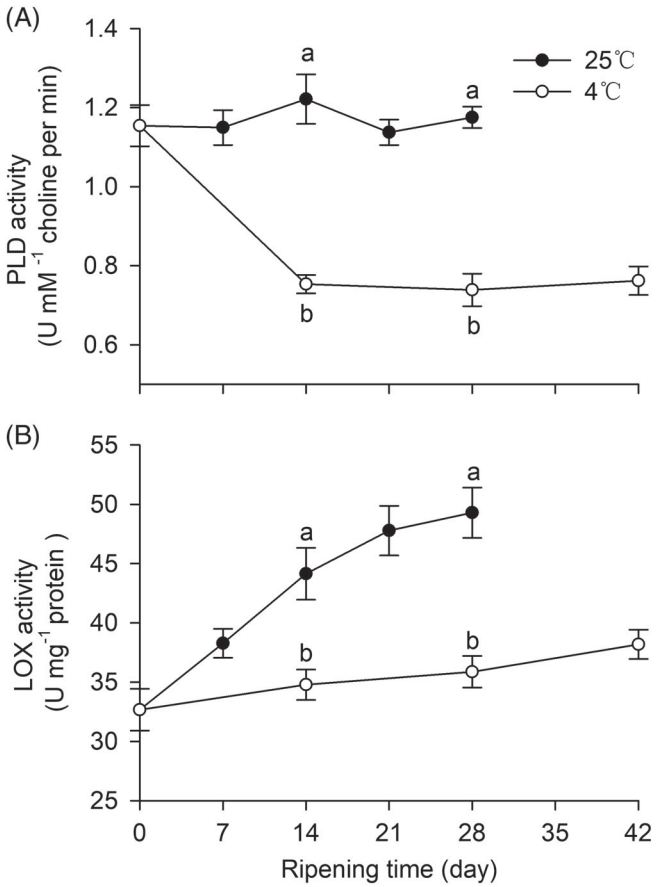

Figure 5. Activities of PLD (A) and LOX (B) of *Torreya grandis* nuts during post-harvest ripening at 25 °C and 4 °C in 90–95% RH. The data indicate the mean ± SD ($n = 3$). Different lowercase letters indicate statistically significant differences between the two ripening temperatures ($P < 0.05$ in the LSD test).

the ripening period (Fig. 1B). At the end of that period, the colour of the coat outside the kernel changed from reddish–brown to black (Fig. 1A), indicating an improved lipid synthesis in *T. grandis* nuts during the ripening stage. This is in agreement with the findings of Ye *et al.*[16] Interestingly, lipid content in the nuts under low temperature conditions reached the same level as that in the nuts at room temperature, although the ripening time was extended from 28 to 42 days under low temperature conditions (Fig. 1B).

In the present study, post-harvest ripening increased the UFA content under room temperature conditions, as indicated by an increase in UFAs, especially PUFAs, and a significant decrease in SFA over time (Table 1). These changes indicate a conversion from SFAs to PUFAs during the ripening stage. Coinciding with the increased lipid content in the nut oil under room temperature conditions (Fig. 1B), linoleic acid content significantly increased over time, whereas oleic acid content markedly decreased (Table 1), suggesting that an increase of linoleic acid synthesis increases lipid contents during ripening. The results of the present study are in agreement with those of Conde *et al.*[3] who found that the ratio of oleic acid to linoleic acid markedly decreases as a result of an increase in linoleic acid synthesis during the over ripening of olive seeds. In the present study, a further slight increase in linoleic acid and PUFA contents was observed on day 42 under low temperature conditions compared to those on day 28 under room temperature conditions, indicating the role of low temperature with respect to determining PUFA levels (Table 1). This has been observed earlier in various tissues of several plant species exposed to low temperatures, such as *Arabidopsis* leaves, *Olea europaea* fruits and safflower seeds.[30,31]

Effect of low temperature on lipid biosynthesis in *T. grandis* nuts during the post-harvest ripening stage

The skeletal pathway of *de novo* fatty acid synthesis is well characterized in plant tissues.[32,33] As the first committed step in the pathway, the carboxylation of acetyl-CoA to malonyl-CoA by ACCase

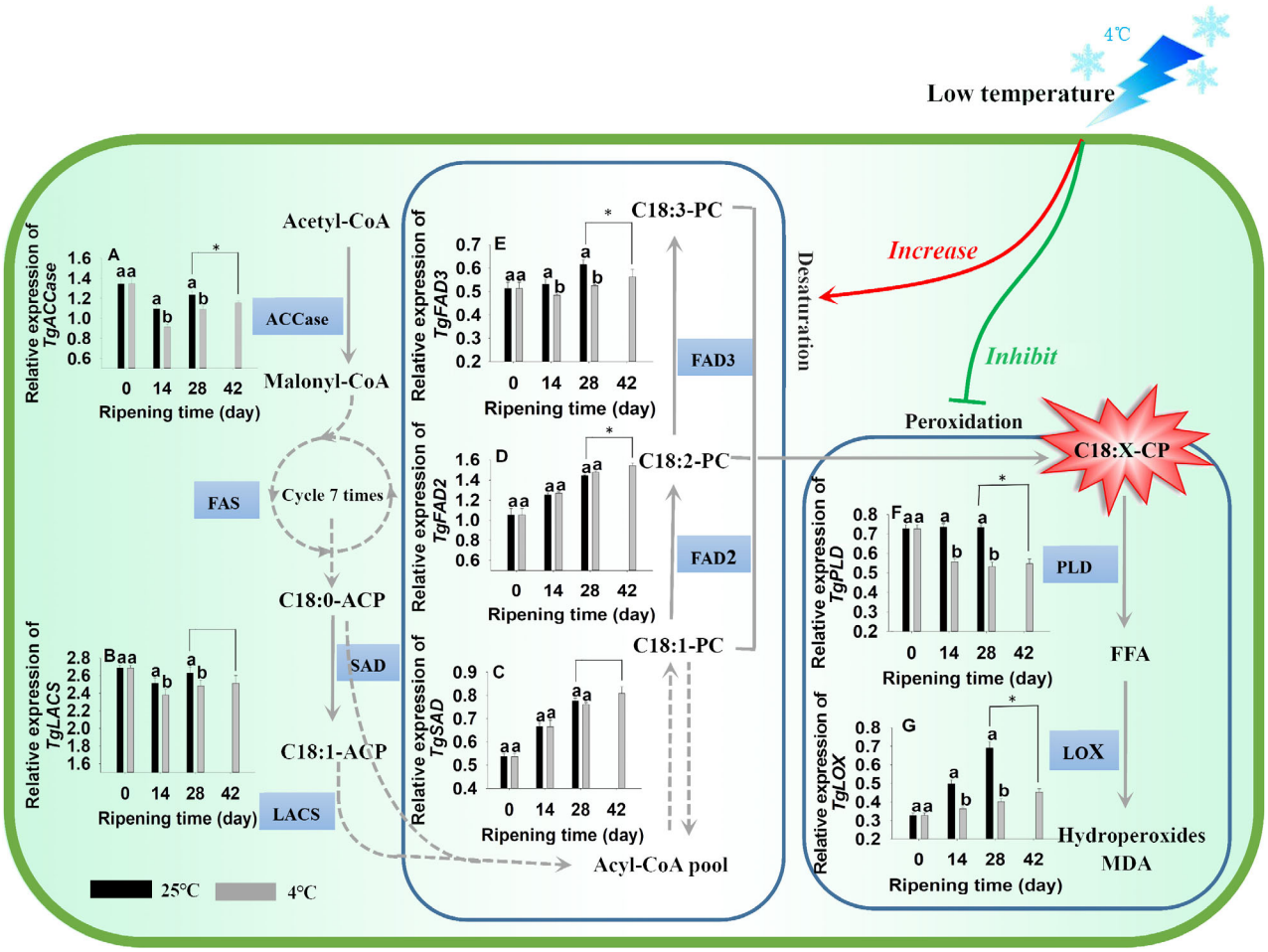

Figure 6. Expression profiles of genes related to lipid biosynthesis and peroxidation of *Torreya grandis* nuts during post-harvest ripening at 25 °C and 4 °C in 90–95% RH: *TgACCase* (A), *TgLACS* (B), *TgSAD* (C), *TgFAD2* (D), *TgFAD3* (E), *TgPLD* (F) and *TgLOX* (G). The data indicate the mean ± SD ($n = 3$). Different lowercase letters indicate statistically significant differences between the two ripening temperatures at the same sampling time and an asterisk (*) indicates a statistically significant difference between the two ripening treatments on their respective ending days of the ripening treatment (both at $P < 0.05$ in the LSD test). ACCase, acetyl-CoA carboxylase; FAS, fatty acid synthase; SAD, stearoyl-acyl carrier protein desaturase; LACS, long-chain acyl-CoA synthetase; DAG, diacyl-glycerol; TAG, triacylglycerol; FAD2, oleoyl desaturase; FAD3, linoleoyl desaturase; PLD, phospholipases D; FAA, non-esterified fatty acid; LOX, lipoxygenase; MDA, malondialdehyde. The metabolic pathways related to the expression of the examined genes are also shown.

controls the rate of fatty acid synthesis. As an easily dissociable multisubunit complex, FAS forms 16- or 18-carbon fatty acids.[34] Furthermore, FAD2 and FAD3 determine oleic acid and linolenic acid contents in plants. In the present study, coinciding with the changes in SFA and UFA during the ripening process at room temperature, and especially with the increase of linoleic acid content, ACCase and FAS activity decreased, whereas FAD2 activity increased in the *T. grandis* nuts over time (Fig. 3), possibly contributing to the increased oil content and the increased contents of PUFAs.[31,35]

Although several studies on *SAD*, *FAD2* and *FAD3* genes have been conducted in crops, such as soybean and sunflower,[36,37] the present study is the first to report on the isolation and characterization of SAD (two *SAD* unigenes, *TgSAD_20454* and *TgSAD_20048*), FAD2 (three *FAD2* unigenes, *TgFAD2_25920*, *TgFAD2_17606* and *TgFAD2_21048*) and FAD3 (two *FAD3* unigenes, *TgFAD3_18460* and *TgFAD3_23658*) in *T. grandis* nuts. Investigations into the mechanisms of temperature-dependent alterations of fatty acid composition in plant membrane lipids have provided evidence for control at both the transcriptional and post-transcriptional levels for genes encoding the fatty acid desaturase.[38] In the present study, we have shown that low temperature induces an enhanced expression of the *FAD2* unigene in *T. grandis* nuts (Fig. 6D). Similarly, increased transcript levels of FAD2 genes under cold stress have been earlier found with purslane and cotton.[39,40] Combined with the increased activity of FAD2, we suggest that a low temperature might improve the lipid biosynthesis in *T. grandis* nuts during the ripening stage.

Effect of low temperature on lipid peroxidation in *T. grandis* nuts during the post-harvest ripening stage

The production of unsaturated fatty acids is closely associated with lipid peroxidation, which involves enzymes such as PLD and LOX, thereby leading to the release of FFAs and creation of a range of oxidized lipid products.[41] Conde *et al.*[3] reported that a major challenge to maximizing oil yield from ripe olive fruit mesocarp is the strong tendency of storage triacylglycerols to be partially broken down by PLD and/or LOX, especially after the fruit has been separated from the parent plant. Slocombe *et al.*[42] also reported that oil content in senescing leaves can be significantly increased by blocking fatty acid breakdown. In the present study, lipid peroxidation occurred in *T. grandis* nuts during the post-harvest ripening stage, as indicated by an increase

in the contents of MDA, lipid hydroperoxide and FFA (Fig. 4). The peroxidation of unsaturated fatty acids was a result of the enhancement of PLD and LOX activity, which directly leads to the deterioration of grain nutritional quality.[41] Gayen *et al.*[43] showed that attenuating LOX activity using RNAi technology improves oil stability and the viability of transgenic rice seeds because, in comparison, FFA contents were lower in non-transgenic rice seeds during storage. In support of our second hypothesis, we found that the activities of the lipid peroxidation-related PLD and LOX increased during the ripening stage at room temperature, coinciding with changes in the contents of MDA, hydroperoxide and FFA (Figs 4 and 5). The increasing expression levels of *TgPLD* and *TgLOX* during the ripening period (Fig. 6F,G) further confirmed this. However, in comparison with room temperature, treatment at low temperature attenuated lipid peroxidation in *T. grandis* nuts during the ripening stage by down-regulating the transcriptional expression and reducing the enzyme activity of lipid peroxidation-related enzymes, such as PLD and LOX, in agreement with the lower contents of MDA, hydroperoxide and FFA (Figs 4–6). Although this potential feedback requires further investigation, our results suggest, that by up-regulating the transcriptional expression of lipid biosynthesis-related enzymes and preventing lipid peroxidation, low temperature application in *T. grandis* nuts during the post-harvest ripening improves oil yield by increasing the contents of PUFAs, particularly linoleic acid content.

CONCLUSIONS

The lipid content in *T. grandis* nuts stored at room temperature increases during the post-harvest ripening period. Treatment at low temperature increases the contents of PUFAs, particularly linoleic acid. This may be caused by the up-regulation of FAD2 biosynthesis and the down-regulation of lipid peroxidation. The results of the present study improve our understanding of the mechanisms of the low temperature regulation of fatty acid contents and the composition in *T. grandis* nuts during the post-harvest ripening and, in this way, facilitate an improvement in the quality of nuts of this important medicinal plant.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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