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Adjustments of both phospholipids and sphingolipids contribute to cold tolerance in stony hard peach fruit by continuous ethylene

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ABSTRACT

Horticultural products, including peaches, are susceptible to chilling injury (CI), on which the effect of ethylene is still controversial, and the underlying mechanism remains elusive. Here, changes in biochemical and molecular mechanisms involved in phenolic and lipid metabolism were compared between stony hard peaches with continuous ethylene (CETH) and controls. CETH effectively compromised internal browning incidence, accompanied by the inhibited activity of peroxidase but enhanced phenolic content, and less membrane leakage with reduced H_2O_2 and malondialdehyde contents. Intriguingly, CETH elevated levels of phospholipids and unsaturation of their acyl chains, coincident with the lower transcript levels of *phospholipase Dα1* but higher *fatty acid desaturase2/8.1*, and the enhanced sphingolipid contents and biosynthesis concomitant with higher transcript levels of *glucosylceramide synthase* but lower *inositol phosphorylceramide synthase3*. Therefore, CETH can ameliorate fruit CI through adjusting phenolic and lipid metabolism, especially comprehensive remodeling of phospholipids and sphingolipids to contribute to the membrane stability.

1. Introduction

Cold storage is a normal economic strategy to prolong shelf life of horticultural products and maintain product quality. However, long-term storage under low temperature conditions will cause a series of physiological disorders, collectively referred to as chilling injury (CI). A plethora of approaches can be applied to alleviate CI, and ever-growing evidence supports the involvement of phytohormones in conferring chilling tolerance, including ethylene (Sevillano et al., 2009).

Ethylene is a gaseous phytohormone involved in plant and fruit development, fruit ripening and abiotic stresses, including chilling stress. However, there is a controversy on the effect of ethylene on chilling tolerance or CI. It has been reported that ethylene has a negative effect on diverse species of fruit such as avocado (Pesis et al., 2002) and zucchini (Megias et al., 2014), and reverse cases were observed in banana (Li et al., 2015b) and peach (Zhu et al., 2019; Wang et al., 2017). Moreover, accumulating studies have shown that ethylene and its signaling elements are indispensable to chilling tolerance. In plants, genetic evidence of ethylene response factors (ERFs) such as C-repeat/dehydration-responsive element binding factors (CBFs) demonstrated they played a positive effect on chilling tolerance (Cook et al., 2004; Mizoi et al., 2012). In fruit, such as peach and pear, low temperature conditioning (LTC) induced endogenous ethylene production, signaling, as well as alleviated CI (Yao et al., 2018; Wang et al., 2017).

Peach is a climacteric fruit with a short shelf life of often less than

Abbreviations: AAPT, aminoalcohol aminophosphotransferase; CBF, C-repeat binding factor; Cer, ceramide; CETH, continuous ethylene; CI, chilling injury; CerP, Cer phosphate; DG, diacylglycerol; ERF, ethylene response factor; DGDG, digalactosyl diacylglycerol; FAD, fatty acid desaturase; GlcCer or CerG, glucosylceramides; GCS, GlcCer synthase; GPAT, glycerol-3-phosphate acyltransferase; IB, internal browning; LCB, long chain bases; LOX, lipoxygenase; LTC, low temperature conditioning; MDA, malondialdehyde; OPLS-DA, orthogonal projections to latent structures discriminant analysis; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PLD, phospholipase D; POD, peroxidase; PPO, phenol oxidase; PS, phosphatidylserine; ROS, reactive oxygen species; SLD, sphingolipid desaturase; TG, triacylglycerol.

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one week. It is also sensitive to chilling and prone to CI, with symptoms such as mealy texture, lignification, failure of softening, loss of aroma, and internal browning (IB), which usually seem to be a typical sign of CI (Wang et al., 2017; Sevillano et al., 2009). The IB is mainly influenced by content of phenolics in the vacuole, enzymes of polyphenol oxidase (PPO) and peroxidase (POD), as well as compartmentalization of membrane system that separates IB enzymes in cytosol from phenolics in the vacuole (Toivonen and Brummell, 2008).

The function and integrity of membrane system depend on the adjustment of diverse species of lipids, including glycerolipids and sphingolipids, which are enriched in the membrane system in plant cells, such as plasma membrane and tonoplast (Hou et al., 2016). Recent lipidome results suggested that changes of comprehensive glycerolipid molecular species including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidic acid and digalactosyldiacylglycerol (DGDG), as well as unsaturation state of their acyl chains were closely associated with fruit CI (Zhu et al., 2019; Chen et al., 2019; Bustamante et al., 2018; Kong et al., 2018; Wang et al., 2017).

The glycerolipid adjustments are regulated by a list of related enzymes. For instance, phospholipase D (PLD) and lipoxygenase (LOX) have long been considered as key detrimental factors for cold tolerance through degrading phospholipids and peroxidizing unsaturated fatty acid, respectively, to impair the membrane integrity and functionality (Mao et al., 2007; Jiao et al., 2019; Jannatizadeh et al., 2019). In addition, fatty acid desaturase (FAD) is known for the desaturation of fatty acids in acyl chains of glycerolipids and has been demonstrated to be positively associated with cold tolerance (Zhang and Tian, 2009; Jannatizadeh et al., 2019).

Sphingolipids are involved in development and abiotic stresses, including cold stress, and their structural and signaling roles were documented in pioneering reports. For example, the silencing of Δ*8SLD* in tomato plants led to membrane damage as indicated by the increase of relative electrolyte leakage rate (Zhou et al., 2016). It has been observed that CerPs were transiently and considerably induced in Arabidopsis when exposed to chilling conditions (Cantrel et al., 2011).

Accumulating reports have suggested that there were associations between ethylene and lipids. For instance, the levels of glycerolipids were dramatically adjusted in apple fruit with the alleviation of CI by 1-Methylcyclopropene (1-MCP), an inhibitor of ethylene signal perception (Busatto et al., 2018). In our previous study, phospholipid levels and metabolism was greatly influenced by LTC treatment exhibiting less CI and the induction of ethylene production (Wang et al., 2017). It has been demonstrated that the sphingolipid signaling participated in the modulation of phytohormone signaling for coping with abiotic stresses. For instance, Cer (24:1) directly interacted with constitutive triple response 1 *in vitro*, a negative regulator of ethylene response, to inhibit its kinase activity and is assumed to enhance ethylene response to abiotic stresses (Xie et al., 2015). However, the lipid molecular species regulated by ethylene remain elusive.

Recently, we have performed the exogenous ethylene treatment on a melting peach cultivar 'Hujingmilu' with high level of ethylene emission, and investigated the profiles of lipidome and transcriptome at the end of cold storage and shelf life (Zhu et al., 2019). However, the effects of ethylene on lipidome and phenolic metabolism regarding IB in peaches with low level of ethylene emission are not clear.

2. Materials and methods

2.1. Materials and treatments

A clingstone stony hard peach (*Prunus persica* L. Batsch cv. Xiazhimeng) was chosen as study material. The fruit with commercial maturity (135 days after full bloom) were obtained from a commercial orchard in Hefei City, Anhui Province, China, and then transported to the laboratory in Anhui Agricultural University once harvested. The fruit were divided into two groups. One group fruit were fumigated at a 64 L jar with continuous 14 μL L^{-1} ethylene in cycles of 6 days of fumigation plus one hour of ventilation, after pre-storage for 15 days, all at 0 °C and 90 % ~95 % relative humidity, termed as continuous ethylene (CETH) group; the control fruit were undergone with the same treatment in the absence of ethylene. The fruit were transferred to 20 °C for 2 d to mimic the shelf life after storage at 0 °C for 59 days.

The fruit mesocarp at opposite sides of suture line was sampled regardless of IB at the harvest day and the end of shelf life (59 + 2 for convenience), and frozen in liquid nitrogen and preserved at −80 °C for further use. Fifteen fruit of three biological replicates were designed for each treatment, with five fruit in each replicate.

2.2. Internal browning (IB) index, relative electrolyte leakage rate and firmness

Based on the IB area of peach mesocarp, the IB index was measured according to the standard described by Wang et al. (2017), and relative electrolyte leakage rate was detected as described by Wang et al. (2013). The fruit firmness was determined by a TA-XTplus texture analyzer (Stable Micro System Ltd., UK) with a 5 mm diameter probe. The tests were performed at a speed 1 mm s^{-1} with a 5 mm puncture depth on opposite sides of fruit without exocarp.

2.3. Determination of total phenolics

The frozen fruit were grounded to powders with liquid nitrogen, and 0.5 g powders were weighed to extract total phenolics. Two and a half milliliter 70 % ethanol (v/v) was mixed with the samples and placed under ultrasonic conditions for 20 min. After centrifugation at 4000 × g min^{-1} for 10 min, 250 μL supernatant was mixed with 250 μL forsyl (1 M), 500 μL sodium carbonate (20 %, v/v) and 4 mL distilled water. The reaction mixtures were incubated at room temperature for 25 min, before the detection of absorbance at 730 nm. Gallic acid (1 g L^{-1}) was serially diluted and used to calculate the standard curve in the reaction. The results were expressed as g kg^{-1} fresh weight (FW).

2.4. Analysis of H_2O_2 and malondialdehyde (MDA)

The content of H_2O_2 in the samples was extracted with acetone precooled at −20 °C and determined by using a commercial kit (Nanjing Jiancheng, Jiangsu, China) following the manufacturer's instructions.

The determination of MDA was performed followed the method of Dhindsa et al. (1981) with minor modifications. Five hundred milligrams of fruit frozen powder was mixed with 2.5 mL trichloroacetic acid (5 %, v/v). After centrifugation at 12,000 × g min^{-1} for 10 min, 1 mL supernatant was mixed with 1 mL thiobarbituric acid (0.67 % in trichloroacetic acid (5 %, v/v)). The samples were incubated in boiling water for 30 min, cooled to room temperature, and then the samples' absorbance at 450 nm, 532 nm and 600 nm were detected sequentially. The contents of MDA were expressed as mmol kg^{-1} FW.

2.5. Enzymatic activity analysis of polyphenol oxidase (PPO) and peroxidase (POD)

The enzymatic activities of PPO and POD were detected as described by Jin et al. (2011) with minor modifications. Weighed 0.5 g of frozen fruit powders and was mixed with phosphate buffer (0.2 M, pH 6.4, 3 % PVPP (v/v)). After centrifugation at 12,000 × g min^{-1} for 20 min, the supernatant was collected as crude enzyme samples.

The reaction was triggered by adding 100 μL of crude samples to the mixture, including 2 mL phosphate buffer and 2 mL catechol solution (65 mmol L^{-1}). The absorbance at 398 nm was recorded within 2 min, and the enzymatic activity of PPO was expressed as U mg^{-1} protein.

To detect POD enzymatic activity, 1 mL crude solution was mixed with phosphate buffer, 1 mL guaiacol (0.25 %, v/v) and 0.1 mL H_2O_2

(0.75 %, v/v). The absorbance was recorded immediately at 460 nm for 3 min, and the enzymatic activity of POD is expressed as U mg^{-1} protein.

The soluble proteins in crude samples were determined by the method of Bradford (1976).

2.6. Lipidome analysis and real-time quantitative polymerase chain reaction (RT-QPCR)

The lipidome analysis and RT-QPCR were carried out according to our published work (Chen et al., 2019). The primers' information is listed in **Table S1** of supplementary materials.

2.7. Statistics

The experiment was designed according to the random principle. Variance analyses were performed using GraphPad Prism Software (v8.0, GraphPad, USA), according to nonparametric Kruskal-Wallis test and One-way Analysis of Variance followed by Fisher's least significant difference (LSD) for multiple comparisons between samples, as well as Student's *t*-test for lipid molecular species, at the significance level of $p < 0.05$. The orthogonal projections to latent structures discriminant analysis (OPLS-DA) were conducted with 'ropls' package on R.

3. Results

3.1. Effects of continuous ethylene (CETH) on IB index, relative electrolyte rate, and firmness

Fruit IB was first observed on the edge of mesocarp at the end of shelf life for two days after cold storage for 59 d at 0 °C (59 + 2, Figs. 1A-B) and CETH fruit displayed a significantly ($p = 0.0036$) lower level of IB index compared with controls (Fig. 1C). Relative to fruit at the harvest day, the relative electrolyte leakage rate of shelf life fruit increased by an average 10 % (Fig. 1D), with that of CETH fruit significantly ($p = 0.047$) lower relative to controls. There was no significant difference in the firmness between all fruit (Fig. 1E).

3.2. Changes of phenolic metabolism and membrane lipid peroxidation

Regarding the substrates and enzymes involved in IB, the content of phenolics and enzymatic activities of PPO and POD were probed. A significant ($p = 0.0008$) 1.3-fold increase in phenolic content was observed in CETH fruit (Fig. 2A). There was no significant difference in PPO activity between samples (Fig. 2B). POD activity was enhanced after harvest day, and with significantly ($p = 0.003$) lower levels in CETH fruit than controls (Fig. 2C).

Lipid peroxidation level was evaluated in fruit. Compared with fruit at the harvest day, the levels of H_2O_2 and MDA were controlled and decreased in CETH fruit, respectively (Fig. 3); those were significantly ($p < 0.0001$ and $p = 0.0053$, respectively) lower than that in controls at the end of shelf life. The results indicated that CETH fruit displayed reduced peroxidation of membrane lipids.

3.3. Characterizations of differentially accumulated glycerolipids and sphingolipids, and potential lipid biomarkers of CI

The lipidome of samples was investigated to explore the effect of CETH on membrane lipids. The forty lipid molecular species were differentially accumulated including storage lipids (13), phospholipids (19), galactolipid (1) and sphingolipids (7) (Fig. 4A). Relative to other lipids, the levels of triacylglycerols (TGs) among storage lipids were influenced, with two TGs (19:4/17:1/17:2, 26:0/8:0/16:2) in CETH fruit being approximately 4.5-fold higher levels than those in controls, and two TGs (4:0/11:0/16:0, 4:0/13:1/18:3) approximately 2.0-fold lower. Besides, phospholipids in CETH fruit exhibited consistently higher levels compared with those in controls, including PCs (7) and PEs (10) molecular species, which were characterized by enrichment of polyunsaturated fatty acids (Fig. 4A, Fig. S1). The great differences in carbon number and acyl-chain unsaturation were observed among PEs, such as PE (26:3/8:0) and PE (25:3/9:0). Two PGs with reversed changes were also identified, with PG (16:0/18:1) and PG (18:2/18:2) being approximately 1.7-fold higher and 5.0-fold lower in CETH fruit, respectively (Fig. 4A).

Besides, three subclasses of sphingolipids, ceramides (Cer) and its derivatives glucosylceramide (GlcCer or CerG) and Cer phosphates (CerP), were identified through lipidome analysis; those levels in CETH fruit were significantly higher than that in controls (Fig. 4A). The general number of carbons ranged from 14 to 36 with dihydroxy long chain bases (LCB), including three (Cers,d18:1/17:0+O, d32:0, d34:0), one CerG3 (d16:0) and three CerPs including one CerP (d12:0/24:5), which is composed of a short dihydroxy LCB and long acyl chain with five double bonds (Fig. 4A).

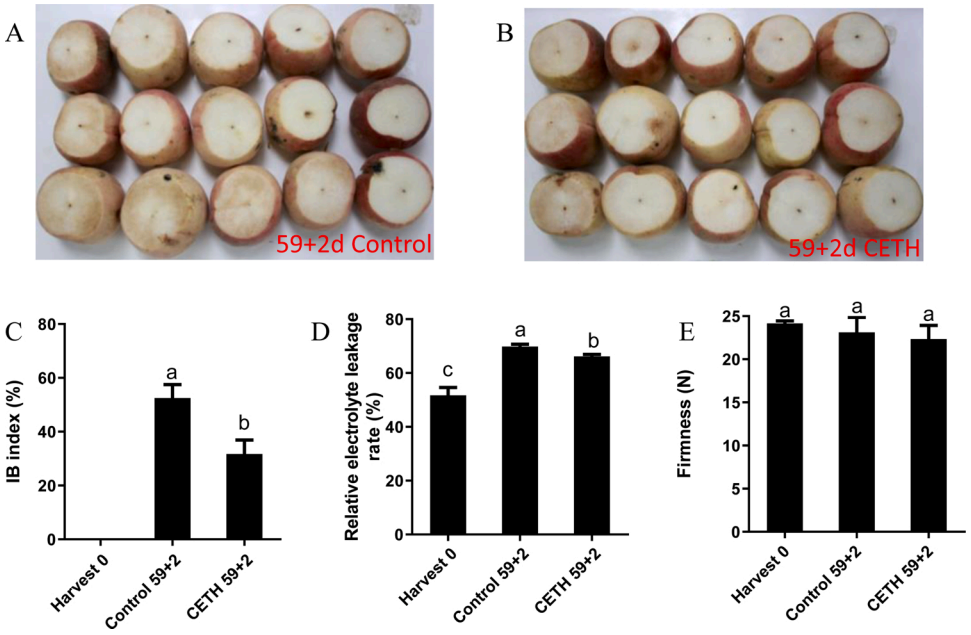

Fig. 1. The attributes of peach mesocarp at the harvest day and the end of shelf life at 20 °C. A and B, the IB pictures of Control and CETH fruit, respectively, at the end of shelf life; C, IB index; D, relative electrolyte leakage rate; E, firmness. The data in the figures represent means ± SD (n = 3). The different letters denote the significant difference between samples based on the results of multiple comparisons through nonparametric Kruskal-Wallis test for IB and the Fisher's least significant difference (LSD) for firmness and relative electrolyte leakage rate, at the significance level of $p < 0.05$. CETH, continuous ethylene.

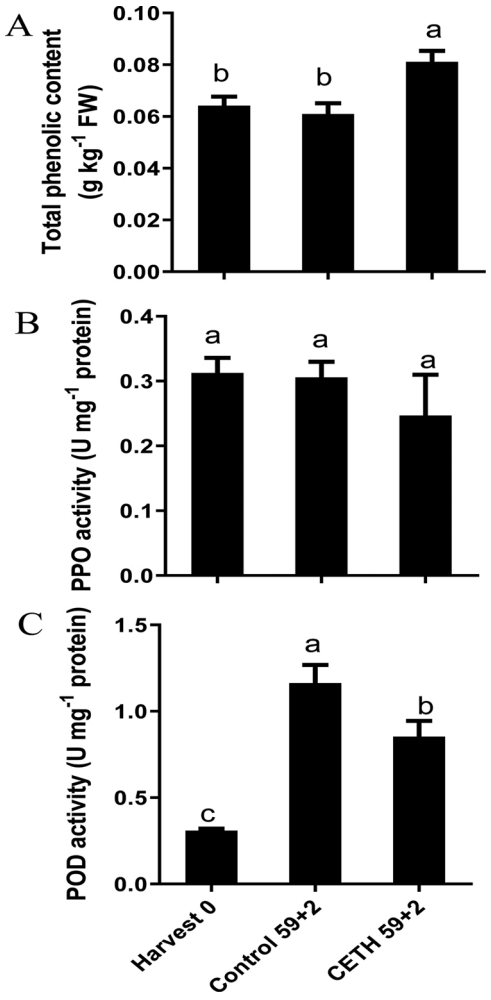

Fig. 2. The phenolic content (A) and enzymatic activities related to IB in peach fruit. B, enzymatic activity of PPO; C, enzymatic activity of POD. The data in the figures represent means ± SD (n = 3). The different letters denote the significant difference between samples based on the results of multiple comparisons through the Fisher's least significant difference (LSD) method at the significance level of $p < 0.05$. CETH, continuous ethylene.

An OPLS-DA model was established to screen out the potential lipid markers that can distinguish the effect of ethylene from control. It is well fitted and predictive as indicated by high R^2X (0.782) and Q^2Y (0.948), and clear divergence between CETH fruit and controls (Fig. 4B). In the model, the variable importance of projection (VIP) represents a summary vector to explain the total importance of variances. There is a clear negative correlation between VIP and P values from Student's *t*-test indicating the difference between CETH fruit and controls (Fig. 4C). All significantly different lipids ($p < 0.05$) presented the high VIP values (VIP > 1), potentially acting as biomarkers including 1 DG (17:4/19:4), 2 PCs (18:3/18:3, 16:0/18:3), 4 PEs (18:3/21:1, 18:3/22:1, 16:0/16:0, 22:1/22:6) and 1 Cer (32:0), of which levels were significantly higher in CETH fruit relative to controls (Fig. 4A).

3.4. Transcriptional changes of genes related to phospholipid and sphingolipid biosynthesis and metabolism

In the aspect of gene expression of glycerolipid biosynthesis, the transcript levels of *glycerol-3-phosphate acyltransferase (GPAT)* and *aminoalcohol aminophosphotransferase (AAPT)* at the end of shelf life were significantly lower than those at the harvest day, but there was no significant difference between CETH fruit and controls (Fig. 5A-B). Regarding glycerolipid degradation and peroxidation, CETH fruit showed an approximately 5.0-fold reduction in transcript levels of *PpPLDα1* in comparison with controls at 59 + 2 d and fruit at the harvest day, between which no significant difference was observed (Fig. 5C). There were no significant changes in transcript levels of *PpLOX3.1* between CETH fruit and controls at 59 + 2 d (Fig. 5D). For fatty acid desaturation, transcript levels of *PpFAD2* and *PpFAD8.1* were significantly ($p = 0.0003$ and $p = 0.012$) induced after harvest, by an average 8.0-fold and 150.0-fold, respectively, with those in CETH fruit being approximately 1.3-fold and 2.7-fold higher than that in controls (Fig. 5E-F).

Cers could further be derived to CerG (or GlcCer) and IPC through several genes encoding Δ8 sphingolipid desaturase (△8SLD) and GlcCer synthase (GCS, GlcCer route), and △4SLD and inositol phosphorylceramide synthase (IPCS, IPC route), respectively. The transcript levels of all genes involved in Cer derivation were significantly inhibited after harvest (Fig. 6). In the GlcCer route, transcript levels of *PpGCS* were 2.0-fold higher in CETH fruit, relative to controls, and there was no significant difference in transcript level of *Pp△8SLD* between samples at the end of shelf life (Fig. 6A-B). On the other hand, CETH fruit showed an inhibited derivation of IPC as indicated by a significantly and approximately 2.0-fold lower transcript level of *PpIPCS3* (Fig. 6C-D). No

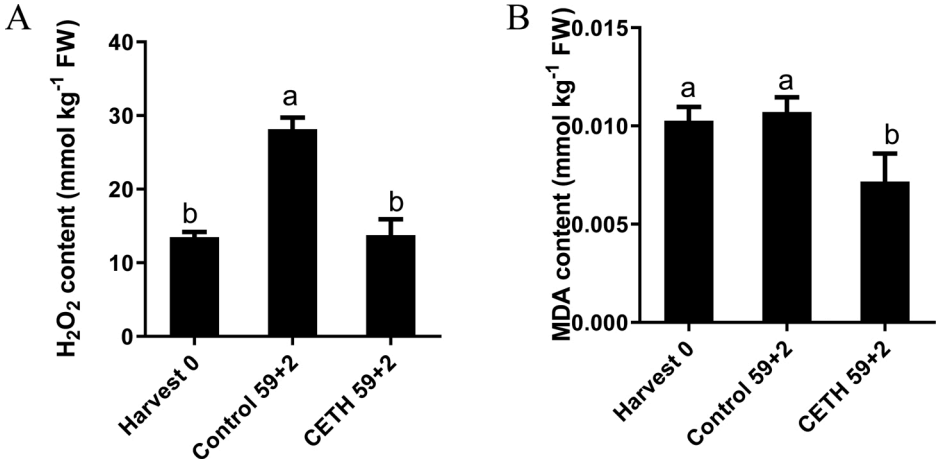

Fig. 3. The contents of H_2O_2 (A) and MDA (B) in peach fruit at the harvest day and the end of shelf life at 20 °C. The data in the figures represent means ± SD (n = 3). The different letters denote the significant difference between samples based on the results of multiple comparisons through the Fisher's least significant difference (LSD) method at the significance level of $p < 0.05$. CETH, continuous ethylene.

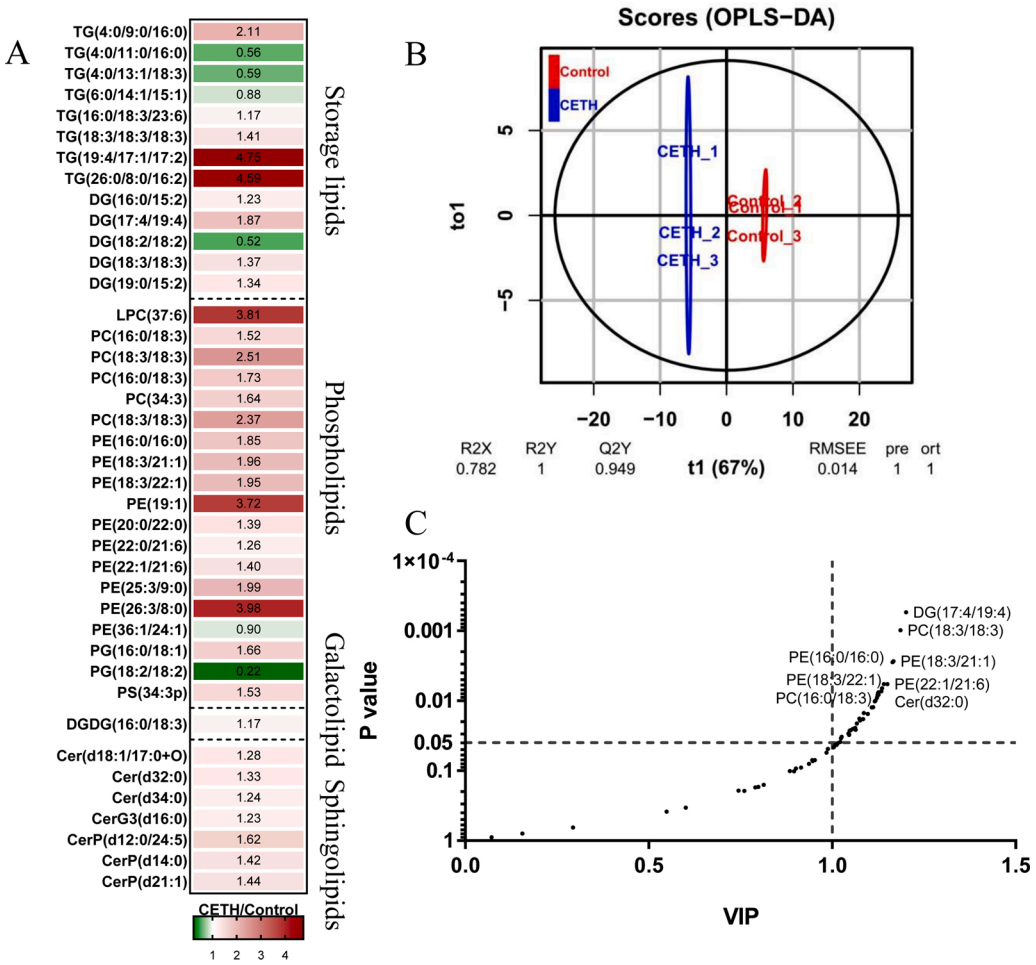

Fig. 4. Differences of glycerolipids and sphingolipids in the CETH fruit compared with controls at 59 + 2 d. A, heatmap of differentially changed lipid species in CETH fruit relative to controls. B, scatter plot between VIP and P value from Student' s *t*-test analysis for identification of differential accumulated lipid species between CETH and control fruit; C, OPLS-DA score plot. Cer, ceramide; CerG3, triglycosylceramide; CerP, ceramide phosphate; DG, diacylglycerol; DGDG, digalactosyl diacylglycerol; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; TG, triacylglycerol. 'd' in the CerPs/Cers/CerGs means two hydroxyls in the LCB moiety. '+O' in the Cer (d18:1/17:0+O) represents the addition of a hydroxyl group to the acyl chain. 'p' in the 'PS (34:3p)' represents a 1-O-(1-alkenyl)-2-acyl (plasmalogen) species.

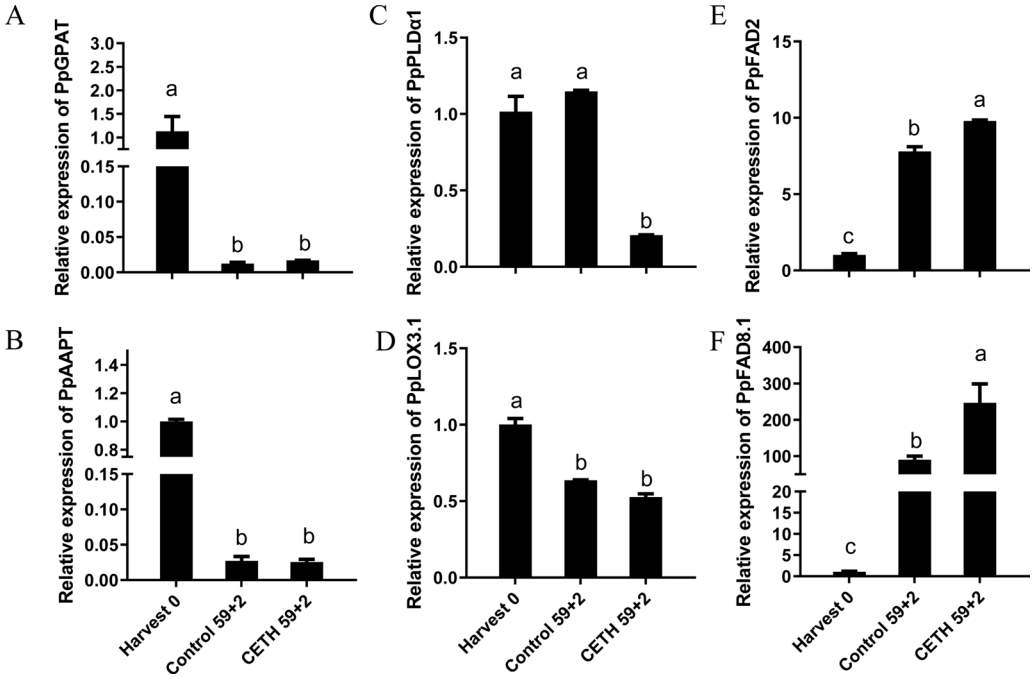

Fig. 5. The expression of genes related to phospholipids and fatty acid desaturation. A, *PpGPAT*; B, *PpAAPT*; C, *PpPLDα1*; D, *PpLOX3.1*; E, *PpFAD2*; F, *PpFAD8.1*. The data in the figures represent means ± SD (n = 3). The different letters denote the significant difference between samples based on the results of multiple comparisons through the Fisher's least significant difference (LSD) method at the significance level of $p < 0.05$. The abbreviations are list as follows: AAPT, amino alcohol aminophos-photransferase; CETH, continuous ethylene; FAD, fatty acid desaturase; GPAT, glycerol-3-phosphate acyltransferase; LOX, lipoxygenase; PLD, phospholipase D.

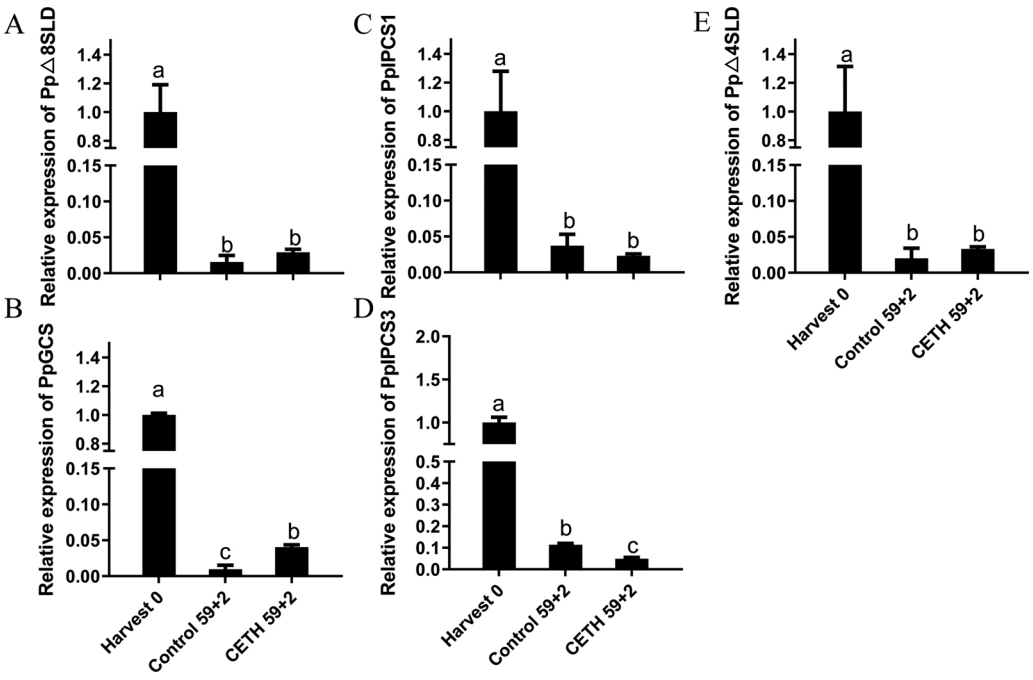

Fig. 6. The expression of genes related to sphingolipid metabolism. A, *Pp*△8*SLD*; B, *PpGCS*; C, *PpIPCS1*; D, *PpIPCS3*; E, *Pp*△*4SLD*. The data in the figures represent means ± SD (n = 3). The different letters denote the significant difference between samples based on the results of multiple comparisons through the Fisher's least significant difference (LSD) method at the significance level of $p < 0.05$. The abbreviations are list as follows: CETH, continuous ethylene; GCS, glucosylceramide synthase; IPCS, inositol phosphorylceramide synthase; SLD, sphingolipid desaturase.

significant difference of *Pp*△*4SLD* expression was observed between CETH and controls (Fig. 6E).The data suggested that CETH fruit promoted level of GlcCer route and inhibited level of IPC route for Cer derivations.

4. Discussion

4.1. Ethylene is essential for chilling tolerance in fruit

The ethylene has long been considered as a negative factor to aggravate CI in diverse species of fruit, including peach. For example, exogenous ethylene caused more severe flesh mealiness of CI in peach fruit stored at different low temperatures for 28 d, compared with controls (Jin et al., 2011); as an inhibitor of ethylene perception, 1-MCP (0.5 μL L^{-1}) fumigation significantly ameliorated the IB among CI symptoms (Jin et al., 2011), which is different from the observation from Fan et al. (2002) that 500 μL L^{-1} 1-MCP fumigation aggravated the IB in peach fruit during shelf life after cold storage. These data suggest that appropriate levels of endogenous ethylene may have a positive effect in defense of cold stress for a specific species or cultivar. Different from traditional transient fumigation of ethylene (100 μl L^{-1} ETH for 24 h) before cold storage or extra repeated fumigation of ethylene every 24 h during cold storage in our recent report (Zhu et al., 2019), this study, based on the preliminary screening results, was carried out with continuous ethylene fumigation at a low level of 14 μL L^{-1} after 15 d of cold storage at 0 °C. The significantly lower levels of IB were observed in CETH fruit relative to controls (Fig. 1A), consistent with the result of our recent publication (Zhu et al., 2019). The browning spots of CI in pear fruit was reduced by transient ethylene fumigation (100 μL L^{-1} for 24 h) (Wei et al., 2019). Deficiency of *SlACS2* in tomato fruit showed a decreased ethylene production and an increased extent of CI, suggesting a positive role of ethylene in coping with cold stress (Yu et al., 2019).

According to our preliminary study, exogenous ethylene could induce the ripening processes of the stony hard 'Xiazhimeng' peaches and exhibit flesh softening phenotype. Therefore, here the firmness was evaluated to indicate the effects of ethylene on fruit ripening. The fact of no significant softening difference between CETH and control at 59 + 2 d indicated that CETH did not remarkably accelerate ripening processes. These results suggest that CETH is an effective strategy to ameliorate CI in peach fruit. The different extent to IB inhibition between transient and CETH treatment in peach fruit need to be further evaluated.

4.2. Ethylene promotes phenolic production and inhibits POD enzymatic activity

Phenolic substrate and related enzymatic activities are the direct factors influencing IB occurrence; the phenolics could be depleted by POD with the cooperation of PPO to induce IB. With the increase in phenolic content, the decline in POD enzymatic activity was observed in CETH fruit (Fig. 2). The POD activity was restrained by diverse treatments, such as melatonin (Gao et al., 2016), glycine betaine (Wang et al., 2019), MeJA (Chen et al., 2019), which compromised the occurrence of the IB in peach fruit. Moreover, the decreased ethylene production but increased POD activity was observed in the repression of peach ripening by 1-MCP (Liu et al., 2005).

In addition to acting as substrates for tissue browning, phenolics play protective roles against oxidation resulting from reactive oxygen species (ROS) and conferring cold tolerance in peach fruit (Gao et al., 2016; Wang et al., 2019). Here, CETH treatment compromised relative electrolyte leakage rate and inhibited the contents of H_2O_2 and MDA (Figs. 1B and 3), which is in accordance with the report in pear fruit that ethylene-treated fruit showed lower levels of H_2O_2 and MDA, accompanied by higher activity of antioxidant system (Wei et al., 2019). Further investigation is needed to be performed to test whether the activity of antioxidant system is induced by exogenous ethylene in peach fruit under cold stress.

4.3. Ethylene retards phospholipid degradation and enhances desaturation to protect membrane integrity and functionality

Another factor influencing IB is the barrier of the membrane compartmentation from the contact between phenolics in the vacuole and browning enzymes in the cytosol. The membrane is prone to the occurrence of ion leakage by peroxidation damage from ROS such as the primary H_2O_2, to produce MDA (Gill and Tuteja, 2010). In addition to membrane lipid peroxidation, the barriers of the membrane closely depend on membrane integrity and functionality comprising the unsaturation level of acyl chains and the characteristics of head groups in

lipids, such as the size and hydration state of polar head groups (Li et al., 2006). PCs and PEs are the dominant structural membrane lipids of multiple plants and fruit species, including peach fruit, where several PC and PE molecular species were potential biomarkers for mealy symptom and IB of CI through comparisons between chilling susceptible and resistant population or treatment and control, at the end of shelf life after long term cold storage (Bustamante et al., 2018; Zhu et al., 2019). Recently, the effects of ethylene on contents of phospholipid molecular species and metabolism in CI of melting peach cultivar with high endogenous ethylene emission (Zhu et al., 2019), have been evaluated, however, that in stony hard peach cultivar with trace ethylene emission are unclear.

GPAT is the first step in the *de novo* synthesis of glycerolipids in acyl-CoA pool, and AAPT is a difunctional enzyme for production of both PCs and PEs from diacylglycerol; in the degradation of glycerolipids, PLD and LOX catalyzes the degradation of phospholipids and peroxidation of polyunsaturated fatty acids in membrane systems, respectively. Here, enhanced levels of PCs and PEs in CETH fruit were in parallel with the maintenance of phospholipid biosynthesis and decline of phospholipid degradation, indicated by the unchanged transcript levels of *PpGPAT* and *PpAAPT*, but the down-regulated transcript levels of *PpPLDα1,* respectively (Fig. 5A-C). It has been shown that PCs and PEs were the main substrates of PLDα, which was negatively correlated with CI in diverse plants and fruit, including peach fruit (Zhang and Tian, 2010; Wang et al., 2017). Additionally, the content of PCs and PEs were higher in 0 °C fruit with less CI than 5 °C fruit, accompanied by lower transcript level of *PpPLDα*, among which *PLDα1* was the most abundant family member in peach fruit (Zhang and Tian, 2010; Wang et al., 2017), and PLDα1 deficiency in Arabidopsis resulted in increased cold tolerance (Rajashekar et al., 2006). Moreover, the inhibited expression of *PpPLDα1* was also observed in peach fruit with less CI by LTC, which remarkably induced ethylene accumulation and biosynthesis (Wang et al., 2017). Therefore, the results suggest that ethylene restraints PC and PE degradation through the inhibition of the degradation pathway such as PLDα1 rather than regulation on the biosynthesis pathway.

Compared with the results published by Bustamante et al. (2018), the chilling related galactolipids except DGDG 34:3 in the report were not significantly changed in CETH fruit in relation to controls in this study, whereas phospholipids PC 34:3, PC 36:6 and PE 34:3 were repeatedly found to be correlated with chilling tolerance. Intriguingly, two PC molecular species, PC 34:3 (16:0/18:3) and PC 36:6 (18:3/18:3) were characterized by both the differential accumulation in CETH fruit and potential biomarkers for ethylene effect (Fig. 4C). It is consistent with the results in our previous study regarding peach fruit CI alleviated by LTC, which also elevated these two PCs and endogenous ethylene production (Wang et al., 2017). In our recent studies, the increase of PC 36:3 (18:0/18:3) and PC 38:3, were observed in ethylene-treated peach (a melting cultivar 'Hujingmilu'), showing less extent to CI compared with controls at the end of shelf life (Zhu et al., 2019). In another cultivar 'Xiazhimeng', the levels of PC 34:2 (16:0/18:2), PC 36:4 (18:2/18:2) were induced by MeJA treatment, showing less extent to CI after cold removal (Chen et al., 2019) and it has been demonstrated that the ethylene production was promoted by the exogenous MeJA in tomato (Yu et al., 2009) and apple (Li et al., 2017) fruit during postharvest ripening. The results suggest that these two molecular species associated with cold tolerance may have a specific response to ethylene signals, with variations in the number of carbon and double bonds among different cultivars.

Besides, there were also remarkable differences in the number of carbon and double bond between two acyl chains of PEs that were significantly enhanced in CETH fruit, such as PE 34:3 (26:3/8:0) and PE 34:3 (25:3/9:0) (Fig. 4A). The acyl compositions of sn-1 and sn-2 show a dramatically different effect on physical structures and function of the membrane, as a fatty-acid-chain in position sn-1 inserts more deeply into bilayer membrane than that in position sn-2 (Fajardo et al., 2011). The high unsaturation state in PCs and PEs could be due to the enhanced transcript levels of *PpFAD2* and *PpFAD8.1*, which are the first two predominant transcripts of *PpFADs* closely related to CI in peach fruit in our previous studies (Chen et al., 2019; Wang et al., 2017).

PG is usually located in the plastid, and its positive effect on cold tolerance was reported in photosynthesis of plants (Xue et al., 2019). However, there are few reports regarding its correlation with CI in fruit. Here, CETH fruit exhibited distinct adjustment for individual PG molecular species, including 1.66-fold increase in the level of PG 34:1 (16:0/18:1) **(**Fig. 4A**),** different from the absent detection in our recent report (Zhu et al., 2019). Intriguingly, the increase in PG 34:1 (16:0/18:1) level was repeatedly observed in fruit treated by MeJA fumigation, which showed the CI alleviation in peach fruit at the end of shelf life (Chen et al., 2019). A pioneer report in wheat suggested that PG 34:1 and PG 34:2 were associated with cold tolerance (Cheong et al., 2019). These results support the notion of potential positive effect of PG 34:1 (16:0/18:1) in protection of postharvest fruit from chilling conditions. Notwithstanding PG 34:1 (16:0/18:1) maybe not equal to PG 34:1, PG 34:1 presented an evident correlation with cold tolerance in chilling sensitive and resistant peach varieties (Bustamante et al., 2018).

4.4. Ethylene promotes accumulation of Cers and its derivatives to facilitate cold response

According to the structural diversity, sphingolipids can be classified into four groups in plants, Cers, LCBs, GlcCers (or CerGs) and glycosylinositolphosphoryl Cers. They are shared with a LCB, which binds to a fatty acid acyl chain to form Cers. The Cers can be further derived into a variety of molecular species by adding diverse polar head groups, such as CerP, and glucose (GlcCer or CerG). Besides, the characteristics of fatty acid acyl chains and LCBs in Cers also contribute to the structural complexity. The Cers are synthesized by condensation of palmitic CoA and serine, and can be phosphorylated and dephosphorylated by Cer kinases and Cer phosphatase, respectively. Besides, according to the results in Arabidopsis, Cers can be further modified into GlcCer and GIPC, by the GlcCer route (Δ8SLD and GCS), and the IPC route (Δ4SLD and IPCS), respectively (Huby et al., 2019).

It has been documented that Cer content was accumulated in response to cold stress (Nagano et al., 2014). CETH fruit showed enhanced accumulation of Cers, especially with high level of Cer (d32:0) identified as one of potential biomarkers of IB (Fig. 4A). The over-accumulation of Cers induced by overexpression of ceramidase increased the oxidation tolerance induced by the production of ROS (Li et al., 2015a). However, the peroxidation levels are inhibited by CETH, as indicated by lower levels of H_2O_2 and MDA (Fig. 3). Zhao et al. (2019) reported that the application of plant-derived Cers in strawberry fruit triggered the increased levels of superoxide dismutase activity and total phenolic content, which is consistent with the improvement of phenolic content in CETH fruit (Fig. 2A).

As one of the important Cer derivatives, CerGs is a key constituent in lipid raft of the membrane, which plays a role in signal transduction and affects membrane stability (Lynch, 2012). The accumulation of CerG1 (d18:1/c24:0) (also named GlcCer, Cer binds with one glucose residue) was correlated with freezing tolerance in Arabidopsis (Degenkolbe et al., 2012). Here, CerG3 (d16:0) is a glycosylated Cer of three glucose residues, and its levels were enhanced by CETH, concomitant with induced transcript level of *PpGCS* and inhibited that of *PpIPCS3* (Figs. 4 and 6). The results are in accordance with our previous observation that peach fruit treated by LTC induced endogenous ethylene production and retarded CI; GlcCer route was promoted through strengthening its biosynthesis with higher transcript levels of *PpΔ4/8SLD* and *PpGCS*, and weakening the IPC route with lower that of *PpIPCS1* (Wang et al., 2017). The results indicated that the enhanced accumulation of CerG via strengthening its biosynthesis and repressing IPC biosynthesis may be a conservative strategy in the defense of cold stress, which is likely linked with ethylene responses.

The higher levels of three CerPs were observed in CETH fruit

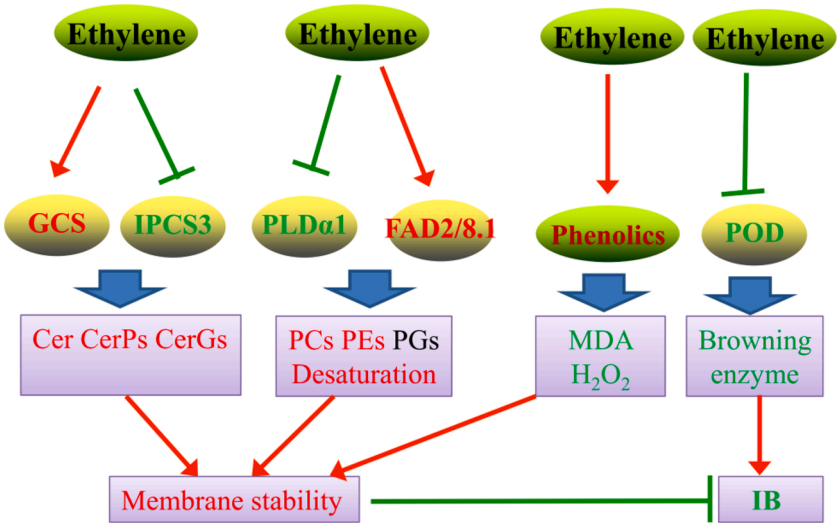

Fig. 7. A scheme for effects of CETH on phenolic metabolism and lipid metabolism in the postharvest CI of 'Xiazhimeng' peach fruit. Red arrows mean the enhancement effect; green lines represent inhibitory effect; the red fonts mean higher level in the comparison between CETH fruit and controls; the green fonts denote lower level.

(Fig. 4A). CerP was rapidly and transiently induced by cold stimuli in wide type Arabidopsis plants and cultured cells (Dutilleul et al., 2015). In that report, the impairment of CerP production in the *AtACD5* mutant could not affect the cold response including unchanged expression of CBFs, which was considered as the main cold response factors (Cook et al., 2004). The protective role of CerP from programmed cell death was demonstrated by genetic evidence in Arabidopsis (Liang et al., 2003).

5. Conclusions

In this study, changes in metabolites, enzymatic activities, and transcripts of genes associated with phenolic metabolism and lipid remodeling were explored to unravel the CI alleviation by continuous ethylene treatment in peach fruit after cold removal. We found that ethylene not only inhibited POD activity and membrane peroxidation, partially through promoted phenolics accumulation, but also triggered comprehensive reprogramming of lipids, particularly including enhanced accumulations of PCs and PEs and unsaturation state through compromising lipid degradation by PLDα1 and inducing desaturation by FAD2/8.1. Additionally, a list of sphingolipids including Cers, CerGs and CerPs were elevated via promoting GlcCer route and repressing IPC route by GCS and IPCS3, respectively (Fig. 7). The ethylene concentration and treatment manner, storage temperature, and cultivar difference should be considered in order to identify the interactions between ethylene and IB in terms of lipidomic adjustments. The results will pave the way for further practical application of ethylene in IB attenuation and provides insights into the cold tolerance conferred by ethylene.

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CRediT authorship contribution statement

Shuqi Chen: Writing - original draft, Methodology, Investigation, Software. **Mengshuang Chen:** Data curation, Investigation, Software. **Yali Li:** Validation. **Xin Huang:** Validation. **Dequan Niu:** Investigation. **Arif Rashid:** Writing - review & editing. **Changjie Xu:** Resources, Methodology. **Ke Wang:** Conceptualization, Supervision, Writing - review & editing, Visualization.

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2020.111332.

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