Food Chemistry 115 (2009) 405–411

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Crucial contribution of membrane lipids' unsaturation to acquisition of chilling-tolerance in peach fruit stored at 0 °C

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ARTICLE INFO

Article history:
Received 2 September 2008
Received in revised form 20 October 2008
Accepted 3 December 2008

Keywords:
Chilling injury
Membrane lipids unsaturation
Membrane fluidity
Omega-3 fatty acid desaturase
Peach fruit

ABSTRACT

Peach fruits (*Prunus persica* L.) were less prone to chilling injury (CI) when stored at 0 °C than at 5 °C for 30 days. In order to make known the mechanism involved, the relationship between CI and membrane lipid unsaturation was investigated in this study. The results demonstrated that peach fruit stored at 0 °C manifested higher membrane lipid fluidity and higher membrane lipid unsaturation than at 5 °C. In addition, a higher omega-3 fatty acid desaturase gene (*FAD*) mRNA level and a higher level of linolenic acid (C18:3) were found when peach fruits were stored at 0 °C. The findings indicated that the higher membrane lipid unsaturation in peach fruit stored at 0 °C was beneficial in maintaining membrane lipid fluidity and enhancing tolerance of peach fruit to low temperature stress, and the C18:3 level could be regulated by omega-3 *FAD*.

1. Introduction

Chilling injury (CI) is manifested by certain plants, especially those of a temperate origin, when exposed to low but non-freezing temperature for a period of time (Saltveit & Morris, 1990). Like many tropical and subtropical plants, peach fruit (*Prunus persica* L.) is sensitive to chilling and CI occurs easily when peaches are refrigerated prior to marketing. The visual evidence of CI, which can be readily observed at unfavourable low temperatures, is manifested by internal browning in peach fruit flesh or flesh browning (Lurie & Crisosto, 2005). This physiological disorder is frequently related to an increase in membrane permeability and disruption of membrane integrity and cell compartmentalisation under stress conditions (Wang, Tian, & Xu, 2005). Peaches are one of the most popular fruits in the world because of their high nutritional value and pleasant flavour. Low temperature has been widely used in commercial storage, in order to maintain fruit quality and to extend marketing time. Therefore, it is valuable to explore the mechanism underlying the CI-induced changes in fruit physiology, thus enhancing resistance of peach fruit to low temperature stress.

In previous experiments, we found that a controlled atmosphere (CA, 5% O_2 + 5% CO_2) at 0 °C significantly reduced CI of peach fruit, because the fruit stored in CA conditions showed higher activities of superoxide dismutase and catalase and maintained greater membrane integrity compared to the control (Wang et al., 2005). Chilling-sensitive mango fruit treated with 5 mM oxalic acid or 2 mM salicylic acid showed higher tolerance to low temperature storage (Ding, Tian, Zheng, Zhou, & Xu, 2007). In addition, treatment with exogenous methyl salicylate increased resistance of tomato (Fung et al., 2006), sweet pepper (Fung, Wang, Smith, Gross, & Tian, 2004), and peach fruit (Han, Tian, Meng, & Ding, 2006) to low temperature stress. The improved tolerance affected by these treatments may be mainly attributed to enhanced activities of anti-oxidant enzymes or to stimulating alternative oxidase gene expression, which protects fruit cell membrane from dysfunction caused by lipid peroxidative injury (Fung et al., 2004; Han et al., 2006).

Dysfunction of cell membrane at low temperature is considered to be the primary molecular event ultimately leading to the development of CI symptoms (Parkin, Marangoni, Jackman, Yada, & Stanley, 1989). Normal functioning of integral cell membrane depends on the fluidity of the membrane, which is strongly influenced by its lipid composition (Squier, Bigelow, & Thomas, 1988). In recent years, when comparing the fatty acids in the cellular membranes of chilling-resistant *versus* chilling-sensitive plants, some investigators have found that chilling-resistant plants have a greater abundance of unsaturated fatty acids (UFAs) (Sakamoto & Murata, 2002). Mikami and Murata (2003) considered that there was a very close relationship between the UFAs level in membrane lipid and the incidence and severity of CI in chilling-sensitive plants. Lee et al. (2005) indicated that membrane lipids unsaturation was positively correlated with chilling-tolerance of fig leaf gourd roots. In addition, Ishizaki-Nishizawa et al. (1996) reported

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doi:10.1016/j.foodchem.2008.12.021

that genetic manipulation of the level of UFAs led to the eventual modification of the cold sensitivity of tobacco plants, and they proved that the UFAs level was greatly increased in transgenic tobacco plants expressing a chloroplast omega-3 fatty acid desaturase gene (*FAD*), resulting in a significant increase in chilling-resistance.

The recent breakthroughs have greatly increased our knowledge on the role of membrane lipids' unsaturation in chilling-tolerance, the molecular characterisation and expression pattern of *FAD* in seed germination, growth and development of reproductive organs of plant. However, little is known about the functions of membrane lipids' unsaturation in harvested fruits under low temperature stress, particularly in peach fruit, in which CI usually appears at 5 °C, but not at 0 °C after 20 days storage. In order to explore the possibility of an involvement of membrane lipid properties in this unexplained phenomenon, in this experiment we mainly investigated the physical properties and composition of membrane lipids, and the expression pattern of omega-3 *FAD* in peach fruit stored at 5 °C and 0 °C, and proved for the first time the function of membrane lipids' unsaturation in enhancing resistance to CI in harvested fruit.

2. Materials and methods

2.1. Fruit and treatments

Peach fruits (*P. persica* L. cv. Beijing 33) were harvested at commercial maturity from an orchard in Beijing, China, and transported to the laboratory within 2 h of harvest. Fruits were selected for uniformity (250 ± 20 g) without any damage and randomly divided into two groups. There were 270 fruits in each group. All fruits were placed in plastic boxes (40 × 30 × 25 cm) wrapped in polyethylene film (0.04 mm thickness, with 5 holes of 20 mm in diameter on upper and side surfaces) to maintain approximately 95% relative humidity. One group was stored at 5 °C and served as control; the other was stored at 0 °C. At selected intervals, CI incidence, CI index and electrolyte leakage of fruit were measured on the fresh sample consisting of 30 fruits per replicate (30 × 3) for each analysis. Then these samples were cut into small pieces and frozen in liquid nitrogen, and stored at −80 °C for other assays.

2.2. Evaluation of CI

The CI incidence and CI index were estimated on flesh browning according to the method of Wang et al. (2005). The score of CI was assessed by measuring the browning area in each fruit based on the following scale: 0 = no browning; 1 = less than 1/4 browning; 2 = 1/4–1/2 browning; 3 = 1/2–3/4 browning area; 4 = more than 3/4 browning. The CI index was calculated from the following formula:

$$(1 \times N_1) + (2 \times N_2) + (3 \times N_3) + (4 \times N_4) \times 100/(4 \times N),$$

where N = total number of fruit measured and N_1, N_2, N_3 and N_4 were the number of fruit showing the different degree of browning.

2.3. Measurement of electrolyte leakage

Electrolyte leakage was assayed according to the method described by Wang et al. (2005) with minor modifications. Briefly, plugs of flesh tissue (about 2–20 mm away from pericarp) were excised from fruit with a 9 mm diameter stainless steel cork borer. Flesh disks (4 mm thick) were cut from each plug with a stainless steel razor blade. Each treatment contained 12 disks with 3 replicates (12 × 3 disks were measured per treatment). The disks were washed three times in 0.4 mM mannitol for about 1 min each time, dried with filter paper, and put together into 50 ml conical flasks containing 40 ml of 0.4 mM mannitol. The initial electrical conductivity (*ECI*) of the solution was determined using a conductivity meter, following incubation of the flask at 25 °C for 3 h. The solution was then heated at 95 °C in a water bath for 30 min and readjusted to a volume of 40 ml before the total electrical conductivity (*ECT*) of the solution was measured. The relative electric conductivity was calculated as:

$$(ECI/ECT) \times 100.$$

2.4. Extraction and separation of lipid

Lipid extraction and separation procedure were based on the method of Veerkamp and Broekhuyse (1976) with modifications. About 10 g of frozen fruit was ground in liquid nitrogen, and then supplied with 9 ml of chloroform: methanol (2:1, v/v) and vortexed vigorously for 10 min. In order to improve layer separation, a volume of 5.4 ml of 1 mM KCl and 3 ml of chloroform were added with stirring for 5 min. Then, the mixture was centrifuged at 10,000*g* for 10 min at 4 °C and the chloroform layer was collected. The hydroalcoholic layer was subjected to a second extraction with 6 ml of chloroform. After shaking for 10 min, the mixture was centrifuged as before. The chloroform obtained in the two extractions was combined and washed 3 times with 3 ml aliquots of 0.1 mM KCl saturated with chloroform. The lower phase was dried under a stream of nitrogen and total lipids were collected. Total lipids were dissolved in 3 ml of petroleum ether (boiling range 90–120 °C) pre-equilibrated with 95% methanol. The solution was extracted three times with equal volumes of 95% methanol pre-equilibrated with petroleum ether; thereafter the three methanol extracts were combined and back-extracted with 1 ml of petroleum ether. The polar lipids were obtained by evaporation of the methanol phase under a stream of nitrogen and dissolved in an appropriate volume of chloroform: methanol (2:1, v/v) containing 0.005% butylated hydroxytoluene. The sample was stored at −80 °C until analysed.

2.5. Lipid spin labelling and measurement of electron paramagnetic resonance (EPR)

Membrane lipid fluidity was determined with EPR, according to the method of Berglund, Quartacci, Calucci, Navari-Izzo, Pinzino and Liljenberg (2002) with a slight modification. A stock solution of 5-doxylstearic acid (DSA) or 16-DSA spin probe (Sigma–Aldrich, St. Louis, MO) was added to polar lipids dissolved in chloroform at a label to lipid ratio of 1:100 (w/w). The solvent from the lipid preparation was removed under a stream of nitrogen. The dry residues were resuspended in 50 μl of an aqueous buffer (10 mM HEPES-NaOH (pH 7.6), 1 mM Na_2EDTA and 50 mM KCl) by sonication. For EPR analysis, the polar lipid suspension was transferred to a 100 μl glass capillary tube, which was then sealed at one end and inserted into a quartz sample holder for placement in the microwave cavity of the spectrometer. EPR measurements were performed on an ER-200D spectrometer (Bruker BioSpin, Rheinstetten, Germany). Spectra were obtained at X-band (9.80 GHz) with microwave power of 20 mW, modulation frequency 100 kHz and amplitude 1 G. The sweep time was 100 s and magnetic field scan 200 G. In general, the fluidity of the lipid chain can be estimated from the order parameter *S*, determined using the equation according to Griffith and Jost (1976):

$$S = 0.568 \times (T_{\parallel} - T_{\perp})/a', a' = 1/3(T_{\parallel} + 2T_{\perp}),$$

where $T_{\parallel}$ and $T_{\perp}$ are the apparent parallel and perpendicular hyperfine splitting parameters of the spectrum, respectively (Fig. 3A). In

the more rapid motion regime, as is the case of 16-DSA, the rotational correlation time (τ_c) was determined using the formulae of Simon (1979):

$$\tau_c = 6.5 \times 10^{-10} \times W_0[(h_0/h_{-1})^{1/2} - 1],$$

where W_0, the peak-to-peak line width of the central line of the spectrum, and h_0 and h_{-1}, the intensities of the central and high field lines, respectively, were measured graphically (Fig. 3B).

2.6. Individual polar lipid separation and fatty acid analysis

Individual polar lipid separation and fatty acid analysis were based on the method of Yang et al. (2004), with modifications. Briefly, the polar lipid was separated into individual phospho- and glycolipid components by two-dimensional thin-layer chromatography (TLC) on activated silica gel plates (silica gel 60, 0.25 mm thickness; Qingdao Dacon Trading Co. Ltd., Qingdao, China), using chloroform: methanol: 7 M ammonia (65: 30: 4, v/v) in the first direction and chloroform: methanol: acetic acid: water (170: 25: 25: 6, v/v) in the second direction. The chromatogram was viewed under UV light after spraying with a 0.2% solution of 2′,7′-dichlorofluorescein in 95% ethanol: 2 M KOH (1: 1, v/v). Individual polar lipids were identified by co-chromatography with authentic standards (Sigma).

For the fatty acid analysis, each fluorescent lipid area was scraped off the plate and collected in a saponification tube containing 1.5 ml sulfuric acid: methanol (5: 95, v/v) dissolved 30 μg heptadecanoic acid (C17:0, Sigma) serving as an internal standard. The sealed tube purged with nitrogen was incubated for 1 h at 85 °C. After the tube had cooled, 1 ml distilled water and 2 ml redistilled pentane were added and then the tube was centrifuged at 3000*g* for 10 min. The upper pentane layer was collected and evaporated to dryness in a steam of nitrogen, and the residue was resuspended in 50 μl of methylene chloride. Samples were analysed on a Varian CP-3800 gas chromatograph fitted with a 25 m × 0.32 mm CP-Wax 58 (FFAP)-CB capillary column connected to a flame ionisation detector. Methylated fatty acids were separated using a temperature programme (5 min at 170 °C, rising at 4 °C min^{-1}; 17.5 min at 240 °C). Authentic methylated fatty acids (Sigma) were used as external standards to identify and quantify peaks; corrections were made at this stage for losses, using the C17: 0. The double bond index (*DBI*), a measure of the membrane lipid unsaturation, was calculated according to Wismer, Worthing, Yada, and Marangoni (1998), as follows:

$$DBI = [(3 \times \text{mol\% C18 : 3}) + (2 \times \text{mol\% C18 : 2})]/[(\text{mol\% C16 : 0}) + (\text{mol\% C18 : 0}) + (\text{mol\% C18 : 1})].$$

2.7. RNA isolation and Northern blot analysis

Total RNA was isolated from the sample using the hot-phenol protocol described by Chan, Qin, Xu, Li, and Tian (2007). Briefly, 10 g of flesh was ground in liquid nitrogen and transferred to a tube containing 20 ml of prewarmed (65 °C) buffer with 7 ml of phenol and 13 ml of extraction buffer (50 mM Tris-HCl pH 9.0, 100 mM NaCl and 1% SDS). The tube was vortexed for 10 min to thoroughly mix the contents before 8 ml of chloroform was added. After a final 30 s vortexing, the tube was incubated for 10 min and centrifuged at 5000*g* for 15 min at room temperature. The supernatant was then transferred to a fresh tube, and total RNA was re-extracted with an equal volume of chloroform, followed by centrifugation at 5000*g* for 10 min at room temperature. The final supernatant was transferred to a fresh tube, and a one-third volume of isopropanol was added. The RNA was allowed to precipitate for 1 h at 4 °C, and centrifuged at 12,000*g* for 30 min at 4 °C. The pellet was washed with 1 ml of 75% ethanol, centrifuged at 12,000*g* for 10 min at 4 °C, and allowed to air-dry. Total RNA was resuspended in DEPC – treated water, quantified, and stored at −80 °C. For cDNA synthesis, forward (5′-ATGGAAGCTTCTCAGACTCTCG-3′) and reverse (5′-GGTACCAAGGGAGTTTCGTCTC-3′) primers for omega-3 *FAD* were used, according to the sequence published in GenBank (DY652204). Aliquots of 20 μg of total RNA per lane were separated on a 1.2% formaldehyde agarose gel and blotted on to a Hybond-N^+ membrane (GE Healthcare, Chalfont St Giles, UK). Afterwards, the blot was hybridised with the cDNA probe, labelled with [^{32}P] dCTP to high specific activity by random priming (Prime-a-gene® labelling system U110, Promega Corporation, Madison, WI) according to the manufacturer's instructions. After a 16 h hybridisation period, the blot was washed once for 20 min in 2 × SSC buffer, 0.1% SDS at 65 °C and twice for 10 min in 0.1 × SSC, 0.1% SDS at 65 °C, and exposed to an autoradiography film at −80 °C for 48 h. Equal loading of samples of total RNA was identified by visualisation of rRNA that had been stained with ethidium bromide.

2.8. Data analysis

All statistical analyses were performed by SPSS 11.0 (SPSS Inc., Chicago, IL). One-way analysis of variance was used to compare means. Mean separations were performed by Duncan's multiple range test. Differences at $p \leqslant 0.05$ were considered significant.

3. Results

3.1. Development of chilling injury and changes of electrolyte leakage in peach fruit

The visual symptoms of CI such as internal browning or flesh browning in peach fruit was noticed after storage at 5 °C for 20 days (Fig. 1A). After 30 days of storage at 5 °C, CI incidence reached 100% with a CI index of 91.7% (Fig. 1B, C). By contrast, no visible symptoms of CI were observed in the fruit stored at 0 °C for the same period (Fig. 1A). In addition, electrolyte leakage from the fruits stored at 5 °C was significantly higher than that from fruits stored at 0 °C during the whole storage period ($p < 0.05$) (Fig. 2).

3.2. Changes of membrane lipid fluidity

EPR spectra of lipid-incorporated 5- and 16-DSA spin, which labels at two different positions along the hydrocarbon chain, were shown in Fig. 3A, B, respectively. 5-DSA spin reported on the mobility of the double bilayer regions close to the polar lipid head groups and 16-DSA spin reported on the lipid mobility close to the core of the bilayer.

Spectra of 5-DSA showed high anisotropy having resolved extrema both in the wings and in the central region, as typical for limited motions of the chain segments close to polar head groups (Fig. 3A). Such spectra could be further analysed by determining the order parameter (*S*), which is inversely related to lipid mobility. It was calculated with the observed values of the outer ($2T_{//}$) and the inner ($2T_{\perp}$) hyperfine splitting (Fig. 3A). In comparison with that at the start of storage, *S* values were lower in all fruit samples (Fig. 3C), suggesting that the mobility of membrane lipid is enhanced in the early stage of storage, and that *S* values increase with prolonged storage time in all treatments. However, the mobility of membrane lipid in the fruit stored at 5 °C was significantly lower than that at 0 °C throughout the storage periods ($p < 0.05$) (Fig. 3C).

Spectra of 16-DSA incorporated into the bilayer showed three-line spectra with line widths sensitive to motions (Fig. 3B). Such spectra could be characterised by rotational correlation time (τ_c) for the spin probe motion by assuming that the motion was in

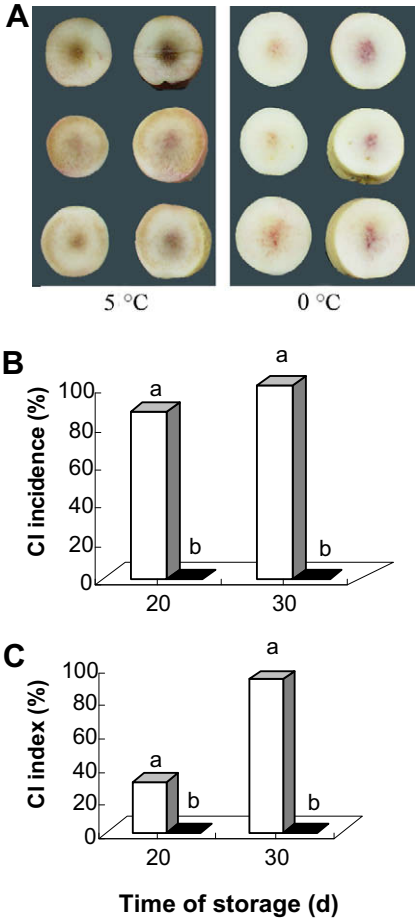

Fig. 1. Chilling symptom, CI incidence and CI index of peach fruit stored under varying conditions. Symptoms of CI (A) were assessed after 30 days of storage at 5 °C or 0 °C. CI incidence (B) and CI index (C) of peach fruit were measured after 20 and 30 days of storage at 5 °C (□) or 0 ° C (■). Data were means of three replicates per treatment containing 30 fruits. Different lower case letters indicate significant differences at $p < 0.05$.

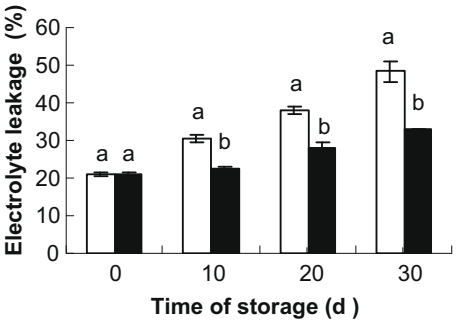

Fig. 2. Electrolyte leakage of peach fruit stored at 5 °C (open bars) and 0 °C (closed bars) for 0, 10, 20 and 30 days, respectively. Data were means of three replications per treatment. Different lower case letters indicate significant differences at $p < 0.05$.

the fast regime and isotropic. The τ_c value, which is inversely related to lipid mobility, was significantly longer in the fruits stored at 5 °C than those stored at 0 °C ($p < 0.05$) (Fig. 3D), indicating much less fluidity of the lipid bilayer of peach fruit occurs at 5 °C condition during the storage periods.

3.3. Changes of membrane lipid composition

To investigate the relationship between membrane fluidity and membrane lipid composition, fatty acid compositions of total polar lipid, and the *DBI* of cell membrane in the peach fruits stored at 5 °C or 0 °C were shown in Table 1. The fatty acids in the polar lipid were mainly palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3). Among these polar lipids, the change in C18:3 level was predominant. Compared to membrane lipid at zero-time (19.5 mol%), the level of C18:3 of membrane lipid in all peach fruits obviously increased after 10 days of storage, and reached a maximum of 26.6 and 42.0 mol%, at 5 °C and 0 °C, respectively, with a corresponding increase of DBI of cell membrane from 1.45 to 3.2 and 4.1, respectively (Table 1). In addition, both the C18:3 level and DBI of cell membrane obviously decreased with prolonged storage time in all treatments. The C18:3 level and DBI of membrane lipid in the fruit stored at 0 °C was significantly higher, but the level of C18:2 was lower than that at 5 °C ($p < 0.05$) (Table 1). The results indicate that the C18:3 levels are mainly responsible for changes in DBI.

Major glycolipids [monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG)] and phospholipids [(phosphatidyl choline (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylinositols (PI)] were isolated from the extractable polar lipids by TLC (Fig. 4A), and the amount of the individual polar lipid did not seem to be affected by storage conditions (data not shown). The fatty acid compositions (C16:0, C18:0, C18:1, C18:2 and C18:3) of the total polar lipid are present in Table 1, only the changes in the C18:3 level in each glycerolipid are shown in Fig. 4B. The difference in the C18:3 level of membrane lipid was mainly attributed to difference in the C18:3 level of the phospholipids, especially PC (Fig. 4B).

3.4. Characterisation of omega-3 fatty acid desaturase gene (FAD) by Northern blot

The gene for omega-3 *FAD*, which is a membrane-bound enzyme catalysing the conversion of C18:2 to C18:3 in lipids, was isolated from peach fruit. As shown in Fig. 5, although the expressions of omega-3 *FAD* were stimulated in all fruit after storage for 10 days, the level of omega-3 *FAD* mRNA in the fruit stored at 0 °C was significantly higher than that at 5 °C. Furthermore, the level of omega-3 *FAD* mRNA in the fruit stored at 5 °C decreased markedly with storage time, whereas the fruit stored at 0 °C had a relatively high level of omega-3 *FAD* mRNA.

4. Discussion

CI as a physiological disorder often occurs in chilling-sensitive plants when they are stressed by low temperature conditions. For most of the chilling-susceptible commodities, fruits tend to suffer severer CI problems when exposed to lower temperatures and longer periods (Saltveit & Morris, 1990). However, peach fruits are more tolerant to CI when stored at 0 °C than that at 5 °C (Fig. 1). Further evidence of this phenomenon was supported by electrolyte leakage, which can reflect membrane damage (Campos, Quartin, Ramalho, & Nunes, 2003), because electrolyte leakage of the fruits stored at 0 °C was significantly lower than that stored at 5 °C throughout the storage periods (Fig. 2). Previous studies indicated that CI of mango fruit resulted in changes in antioxidants, anti-oxidant enzymatic systems and membrane permeability (Ding et al., 2007; Han et al., 2006). The damage to the cell membrane initiates a cascade of secondary reactions, including ethylene production, increased respiration, interference in energy production, accumu-

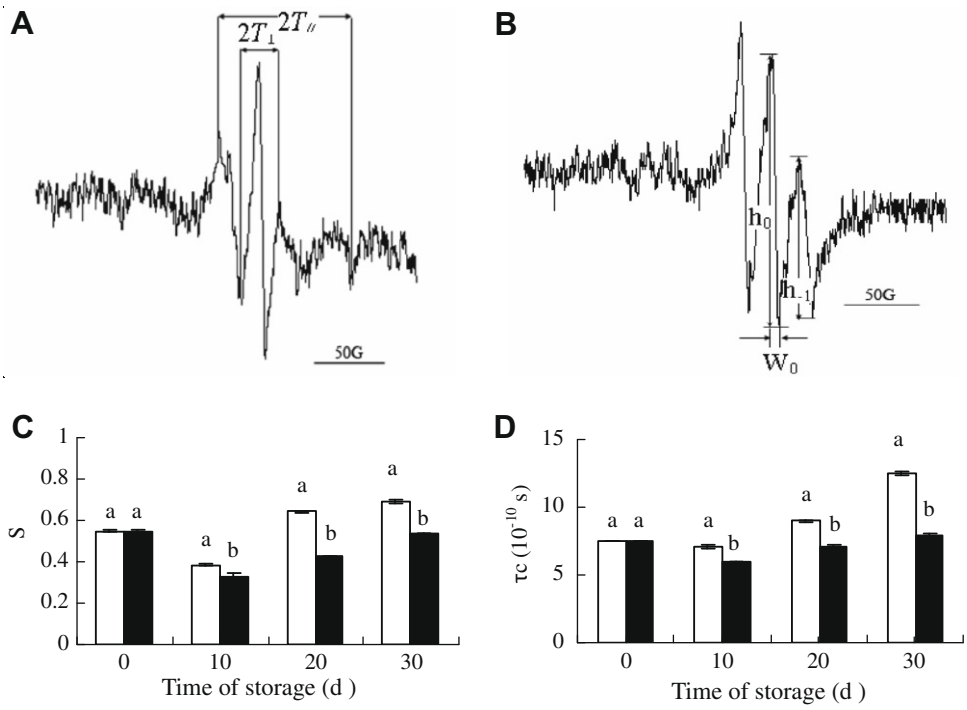

Fig. 3. Examples of EPR spectra of (A) the membrane lipid-incorporated 5-DSA and (B) 16-DSA spin, and changes of (C) the order parameter *S* and (D) the rotational time τ_c of the membrane lipid of peach fruit stored at 5 °C (open bars) or 0 °C (closed bars) for 0, 10, 20 and 30 days, respectively. Data were means of three replications per treatment. Different lower case letters indicate significant differences at $p < 0.05$.

Table 1
Fatty acid composition of total polar lipids in cell membranes isolated from peach fruit stored at 5 °C or 0 °C for 0, 10, 20 and 30 days, respectively.

Storage time (days)	Treatment	Fatty acid composition (mol.%)					DBI
		C16:0	C18:0	C18:1	C18:2	C18:3	
0		24.17 ± 0.45	23.99 ± 0.31	15.43 ± 0.19	16.96 ± 0.07	19.45 ± 0.15	1.45 ± 0.01
10	5°C	20.06 ± 0.53a	13.44 ± 0.24a	10.06 ± 0.15b	29.87 ± 0.21a	26.56 ± 0.23b	3.20 ± 0.05b
	0°C	18.99 ± 0.38b	7.35 ± 0.07b	13.19 ± 0.37a	18.54 ± 0.07b	42.01 ± 0.37a	4.13 ± 0.05a
20	5°C	36.86 ± 0.75a	20.30 ± 0.33a	9.72 ± 0.09b	22.58 ± 0.12a	10.66 ± 0.25b	1.15 ± 0.01b
	0°C	22.18 ± 0.55b	16.29 ± 0.33b	10.76 ± 0.17a	10.93 ± 0.18b	40.01 ± 0.37a	2.88 ± 0.04a
30	5°C	37.08 ± 0.76a	25.30 ± 0.37a	6.82 ± 0.11b	24.76 ± 0.02a	6.04 ± 0.17b	0.98 ± 0.01b
	0°C	36.29 ± 0.74a	16.37 ± 0.30b	9.68 ± 0.16a	12.25 ± 0.05b	25.15 ± 0.26a	1.60 ± 0.02a

The data are the average and standard error of values from three replicates per treatment. For each treatment, values in columns followed by different lower case letters indicate significant differences at $p < 0.05$.

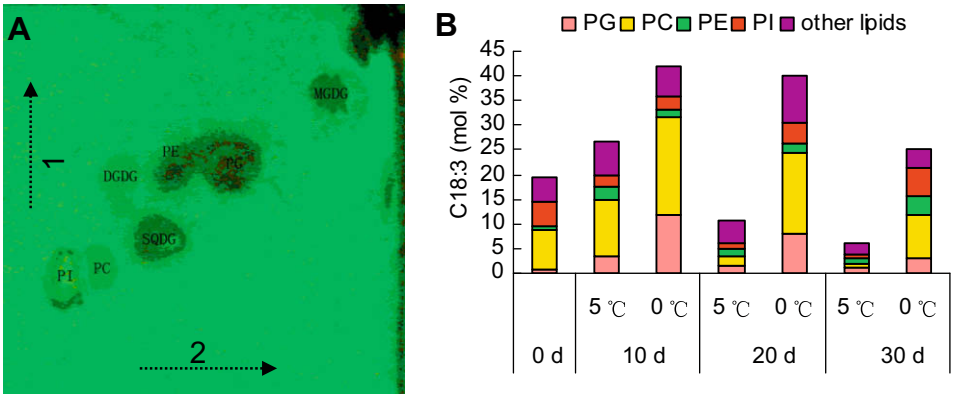

Fig. 4. Examples of chromatograms of the membrane lipid classes separated from each other by two-dimensional TLC (A) and the percentage of C18:3 accounted for by phospholipids (PC, PE, PG and PI) and other lipids (MGDG, DGDG and SQDG) (B) in the membrane lipid of peach fruit stored at 5 °C or 0 °C for 0, 10, 20 and 30 days, respectively. Data were means of three replications per treatment. Different lower case letters indicate significant differences at $p < 0.05$. '1' denotes the first dimension and '2' denotes the second dimension in TLC.

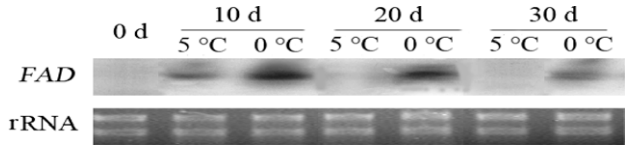

Fig. 5. The level of omega-3 fatty acid desaturase (*FAD*) mRNA in peach fruit stored at 5 or 0 °C for 0, 10, 20, 30 days, respectively.

lation of toxic compounds such as ethanol and acetaldehyde, and disruption of cellular and subcellular structures (Biolatto, Vazquez, Sancho, Carduza, & Pensel, 2005; Pennycooke, Cox, & Stushnoff, 2005).

In this study, we found that there was a relationship between membrane lipid composition and CI in peach fruit (Table 1). This finding is a novel observation that the higher membrane lipids unsaturation was attributed to the higher level of C18:3 and related to the higher chilling-tolerance of peach fruit stored at 0 °C. In addition, malondialdehyde, which was one of the final products of polyunsaturated fatty acids (PUFA) peroxidation (Alonso, Queiroz, & Magalhães, 1997), in the fruits stored at 0 °C remained at a significantly lower level than that at 5 °C (data not shown). This suggests that PUFA, especially C18:3 of peach fruit may be less prone to peroxidation when stored at 0 °C than at 5 °C. In recent years, a rapidly growing literature illustrated the benefits of UFA in human diet in alleviating cardiovascular, heart diseases, atherosclerosis, autoimmune disorder, diabetes and other diseases (Finley & Shahidi, 2001). Therefore, the high level of C18:3 induced at 0 °C may increase the importance of peach fruit for human health. In addition, we found that the higher membrane lipids' unsaturation was attributed to the higher level of C18:3 in the phospholipids, especially PC (Fig. 4B). However, the amounts of individual polar lipids separated by TLC (Fig. 4A) were not affected by storage temperature (data not shown). It has been suggested that the level of glycolipids played a crucial role in tolerance of plants to chilling (Williams, Khan, Mitchell, & Johnson, 1988). Campos et al. (2003) pointed out that chilling-tolerance of the leaves of coffee plant was due to the lower ratio of MGDG/DGDG as a result of enhanced DGDG synthesis. Our data revealed the changes of membrane lipid composition in response to low temperature stress were related to total cellular membranes (Fig. 3 and 4; Table 1). Bohn, Lüthje, Sperling, Heinz, and Dörffling (2007) suggested that the alterations in plasma membrane lipid were closely associated with the frost resistance of winter wheat seedlings.

Membrane fluidity is an important parameter that regulates membrane function by its effect on the orientation of integral membrane proteins and membrane permeability, and by its modulation of transmembrane transport processes (Los & Murata, 2004). Electron paramagnetic resonance (EPR) spectroscopy has been widely used for the quantitative assessment of the rotational mobility of lipid molecules and as a measure of molecular order within membranes (Berglund et al., 2002). An increased extent of membrane lipids' unsaturation was thought to enhance membrane fluidity, which can reduce the propensity of cellular membranes to undergo lipid phase changes, including the liquid crystalline-to-gel transitions, thus enhancing membrane integrity and cellular functions (Los & Murata, 2004). Some results have shown the dependence of membrane lipid fluidity on the level of unsaturated lipids in cyanobacteria and plant chloroplasts (Szalontai, Kota, Nonaka, & Murata, 2003; Szalontai, Nishiyama, Gombos, & Murata, 2000). Our study further proved that membrane lipid fluidity was an important signal of CI in fruit, because it was influenced by membrane composition (Table 1), and indicated that the lower unsaturation degree of membrane lipid resulted in lower membrane lipid fluidity (Fig. 3). A decrease in membrane fluidity was observed in tobacco plants transformed with glycerol-3-phosphate acyltransferase cDNA from chilling-sensitive squash by Murata et al. (1992). They considered that the transgenic tobacco plants with decreased level of UFAs in membrane lipid were sensitive to chilling. Working with coffee seedling microsomal membranes, Alonso et al. (1997) also demonstrated that membrane functions were influenced by decreased membrane fluidity, and explained the loss of membrane lipid unsaturation was due to lipid peroxidation.

Peach fruit stored at 0 °C showed a higher C18:3 level alongside a lower C18:2 level as compared to fruit stored at 5 °C (Table 1), indicating that an increase in C18:3 is largely responsible for the increase in DBI of peach fruit at 0 °C, and that omega-3 desaturase is related to the conversion of C18:2 to C18:3. The result obtained from Northern blot revealed that the cold-induced accumulation of C18:3 in the membrane of peach fruit were due to enhanced mRNA level of the omega-3 fatty acid desaturase (Fig. 5). This finding is likely to account for the higher C18:3 level of membrane lipid in the fruit stored at 0 °C than that at 5 °C (Table 1). However, the mechanism by which the cold induces an enhancement of mRNA level of the omega-3 *FAD* still remains to be characterised. Sakamoto and Murata (2002) postulated that a specific mechanism must exist for perception of a change in temperature and transduction of the signal, with subsequent enhancement of the expression of desaturase genes.

In summary, the phenomenon, that peach fruits do not suffer CI at 0 °C, but do at 5 °C after 20 d of storage, indicates that peach fruit stored at 0 °C has a greater tolerance to low temperature stress than that at 5 °C. The mechanism involved may be explained in that (i) there is a higher level of C18:3 and higher membrane lipids' unsaturation in peach fruit stored at 0 °C than that at 5 °C, which is beneficial to maintaining membrane integrity and enhancing tolerance of peach fruit to low temperature stress, and (ii) omega-3 *FAD* can regulate the C18:3 level and membrane lipid unsaturation in peach fruit.

Acknowledgment

This study was supported by the Ministry of Science and Technology of China (2008GR1300; 2006BAD22B02) and by the National Natural Science Foundation of China (U0631004).

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