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ORIGINAL ARTICLE

Brassinolide enhances cold stress tolerance of fruit by regulating plasma membrane proteins and lipids

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Abstract How to enhance fruit tolerance to cold stress is an important biological interest. In this paper, we found that mango (*Mangifera indica* L.) fruit treated with 10 μM brassinolide (BL) showed a higher tolerance to cold temperature of 5 °C. Further, we compared the changes in expression profiles of plasma membrane (PM) proteins and the corresponding gene expressions between BL-treated and control fruit. Fourteen differential proteins were positively identified by mass spectrometry, and were categorized into four groups, including transport, cellular biogenesis, defense and stress response, and unknown function. Among them, four proteins (remorin, abscisic stress ripening-like protein, type II SK2 dehydrin, and temperature-induced lipocalin) and genes encoding these proteins were up-regulated in BL treatment under cold stress. Moreover, we found that PM lipids in BL-treated fruit showed lower phase transition temperature and higher unsaturation degree, leading to higher fluidity under low temperature. These findings ascertain that PM proteins and lipids are involved in BL-mediated responses to cold stress in mango fruit, and provide novel evidence that BL plays an important role in regulating cold stress tolerance in fruit.

Keywords Brassinosteroids · Chilling injury · Lipids · Mango fruit · Membrane proteins

Abbreviations

2-DE	Two-dimensional gel electrophoresis
BL	Brassinolide
BRs	Brassinosteroids
CI	Chilling injury
CBB	Coomassie brilliant blue
DBI	Double bond index
DSC	Differential scanning calorimetry
EPR	Electron paramagnetic resonance
FAs	Fatty acids
IEF	Isoelectric focusing
PM	Plasma membrane
UFAs	Unsaturated fatty acids

Introduction

As sessile organisms, plants are frequently exposed to non-optimal growth conditions, thus they have to suffer from various environmental stresses such as low temperature, drought and high salinity. To cope with these abiotic stresses, plants have evolved sophisticated mechanisms by altering numerous processes at both physiological and molecular levels (Yamaguchi-Shinozaki and Shinozaki 2005). Cold stress prevents the expression of full genetic potential of plants owing to its direct inhibition of metabolic reactions and, indirectly, through cold-induced osmotic, oxidative and other stresses (Chinnusamy et al. 2007). It is well known that cold acclimation is a complex process involved in transcriptional and metabolic changes that result in multiple mechanisms protecting plants from

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chilling injury (CI) (Valluru et al. 2008). Recent technical advances in molecular biology, proteomics, and metabolomics have made it possible to dissect the complex processes involved in cold acclimation (Valluru et al. 2008). With cellular membrane damage in mind, alterations in lipid composition during cold acclimation, such as increased fatty acid unsaturation and phospholipid content, have been known to be associated with increase in tolerance of plants to cold stress (Moellering et al. 2010). As an important organ of plant, fruit is usually kept under low-temperature conditions after harvest to maintain quality and extend marketing time. Mango (*Mangifera indica* L.) fruit is very sensitive to cold stress because it is grown in subtropical or tropical areas. CI in mango fruits usually occurs when they are exposed to temperatures below 10 °C, depending upon ripeness and variety (Tian et al. 2010). Some plant hormones have been found to effectively reduce CI of mango fruit, and several mechanisms have been proposed. These include regulation of the solubilization of pectic substances in cell wall by methyl jasmonate (MeJA) (Han et al. 2006), suppression of reactive oxygen species (ROS) accumulation, and increases in ascorbate and glutathione levels by salicylic acid (SA) (Ding et al. 2007).

Several hormones are involved in modulating response and adaptation to a changing environment in plants (Belkhadir and Chory 2006). Brassinosteroids (BRs) are a group of plant steroidal hormones throughout the plant kingdom, and brassinolide (BL) is the first isolated BRs species (Bajguz and Tretyn 2003). BRs are perceived at the plasma membrane by a leucine-rich repeat receptor-like kinase (Wang et al. 2001). BRs have been reported to be involved in a range of developmental processes, such as stem and root growth, floral initiation, and the development of flowers and fruits (Clouse and Sasse 1998; Sasse 2003). Recent studies also revealed that BRs can confer resistance of plants to various abiotic and biotic stresses (Krishna 2003; Bajguz and Hayat 2009). Divi et al. (2010) reported that BRs promote thermotolerance and salt tolerance in *Arabidopsis thaliana* through interactions with other hormones, including abscisic acid, ethylene, and salicylic acid. Zhu et al. (2010) found that application of BL reduces postharvest decay caused by *Penicillium expansum* in jujube fruit, and that the mechanism by which BL-induced resistance against fungal pathogens is due to stimulation of defense-related enzymes. So far, physiological effects of BRs on fruit were less documented. Recently, Zaharah et al. (2012) found that level of endogenous BRs in pulp of mango fruit is very low without any pronounced changes during fruit ripening, whereas, application of epibrassinolide can increase the peak value of ethylene production, and accelerates fruit softening, which suggests that interactions between BRs and other hormones may occur in mango fruit.

The plasma membrane (PM) is considered as the primary site of injury and the lipids seem to be responsible for the PM fate when plant is subjected to low temperature stress (Uemura et al. 2006). Tolerance of PM to chilling stress is related to the enrichment of unsaturated fatty acids (UFAs) (Steponkus et al. 1988). Wada et al. (1990) found that UFAs levels are increased in lipids when cyanobacteria were exposed to low temperature, confirming the role of lipid desaturation in cold adaptation. Recently, we investigated the vital roles of C18:3 level and accumulation of *N*-acylphosphatidylethanolamines (NAPEs) in PM lipids of peach fruit under chilling temperature. The results revealed that NAPEs accumulation was related to high lipid unsaturation degree and beneficial for maintaining membrane fluidity (Zhang and Tian 2009, 2010). Although processes associated with cold acclimation in plant have been shown to be relevant to changes in lipid composition and cryobehavior of PM (Uemura et al. 2006), there have been few detailed studies on the function of PM proteins and BRs in fruit tolerance to chilling temperature. In the present study, we investigated the role BL played in the regulation of tolerance of mango fruit to chilling temperature (5 °C). We further characterized the phase behavior and fluidity of PM lipids, and determined the expressions of PM proteins and related genes in response to the chilling stress. The results indicate that exogenous application of BL improved tolerance of mango fruit to cold stress by regulating PM proteins and lipids. These findings may shed novel insights on the mechanisms by which BL mediates tolerance of plants to abiotic stress in general and cold stress in particular.

Materials and methods

Fruit and treatments

Mango fruit (*Mangifera indica* L. cv. 'Zill') at 157 N firmness and 8.2°Brix total soluble solids were harvested from Panzhihua, Sichuan Province, China, and transported to the laboratory immediately. Harvested fruit were selected based on the uniformity of size and appearance. The harvested fruit were disinfected with 1 % sodium hypochlorite (v/v) for 2 min, washed and dried in air. One part of the fruit was immersed in aqueous solution containing 10 μM brassinolide (Kanto Chemical Co., Inc. Japan) for 10 min according to our preliminary experiments. Others were immersed in distilled water for 10 min and used as control. All fruit were enclosed in plastic boxes with polyethylene film bags (0.04 mm thickness, with five holes of 20 mm in diameter on upper and side surfaces) to maintain the relative humidity at about 95 %, and stored at 5 °C. There were three replicates for each treatment.

Evaluation of chilling injury (CI)

The symptoms of CI include skin browning, pitting or sunken lesions on the peel, and pulp discoloration. The CI index was determined following protocols described by Han et al. (2006). Grade 0 represents that the fruit flesh is unaffected; grade 1 represents that there are light sunken lesions in the peel, and grade 2 represents that no less than 15 % area of the fruit shows sunken lesions. The CI index is calculated using the following formula: index of CI = $\sum$ (CI scale × percentage of corresponding fruit within each class)/3. Three replicates for each treatment were performed, and each replicate contained 30 fruits.

The relative electrolyte leakage

The electrolyte leakage was determined by the method of Zhang and Tian (2010). At indicated time, nine discs (10 mm in diameter and 4 mm in thick) from equatorial regions of nine peeled fruits from each replicate were rinsed and incubated in 40 mL of 0.4 M mannitol solutions at 25 °C for 3 h, and then the initial electrolyte leakages were monitored with a conductivity meter (HANNA, Italy). The solutions were boiled at 95 °C for 30 min and cooled quickly before the total electrolyte leakages were measured. The relative electrolyte leakage was defined as percentage of the initial electrolyte leakage.

Preparation of PM

The method of Quartacci et al. (2001) was modified for isolation of PM from mango fruit. Briefly, 160 g of frozen flesh were homogenized in 450 mL of a cold extraction buffer containing 0.33 M sucrose, 80 mM Tris, 5 mM Bis–Tris Propane (BTP)-Mes, pH 8.9, 10 mM ascorbic acid, 5 mM dithiothreitol (DTT), 5 mM Na_2-EDTA, 10 % glycerol, 0.4 % BSA, 0.4 % casein, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.15 % (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was filtered and centrifuged at 10,000*g* for 30 min. The supernatant was further ultracentrifuged at 100,000*g* for 60 min to collect the microsomal pellet. Then, the microsomal pellet was resuspended in 5 mL of suspension buffer containing 0.33 M sucrose, 5 mM KCl and 5 mM potassium phosphate, pH 7.8. The PMs were purified from the microsomal pellet in an aqueous polymer two-phase system containing 6.2 % (w/w) dextran T500, 6.2 % (w/w) polyethylene glycol 3350, 0.33 M sucrose, 3 mM KCl and 5 mM potassium phosphate (pH 7.8). After being partitioned by centrifugation at 1,500*g* for 10 min, which was repeated three times, the upper phases were diluted in 50 mM Tris–HCl (pH 7.5) and 0.25 M sucrose to a final suspension of 26.5 mL, and centrifuged for 30 min at 120,000*g*. Pellets were collected, which represented the purified PM. PM was kept at −80 °C for extraction of polar lipids and proteins. For each treatment, the preparation of PM was performed three times by sampling from different replicates. Each replicate contained nine fruits at each sampling time point.

Extraction of PM polar lipids

Total lipid extraction from the pellet membranes was performed by adding two volumes of boiling isopropanol followed by two volumes of $CHCl_3$: MeOH (2:1, v/v) containing butylhydroxytoluol (50 μg mL^{-1}). The sample was vigorously agitated for 10 min and a two-phase system was induced by adding 0.2 volume of 0.88 % KCl. After vortex and centrifugation, the upper H_2O phase was discarded, and the organic lower phase was evaporated under nitrogen stream. Polar lipids were separated from total lipids according to the procedure of our previous study (Zhang and Tian 2009). Briefly, total lipids were dissolved in 3 mL of petroleum ether (b.r. 90–120 °C) that is 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 pooled and evaporated under nitrogen stream to obtain the polar lipids. The polar lipids were dissolved in an appropriate volume of chloroform: methanol (2:1, v/v) containing 0.005 % butylated hydroxytoluene (BHT) and kept at −80 °C until analysis.

Fatty acids (FAs) analysis

The FAs methyl ester derivates from polar lipids were separated by LECO Pegasus® IV GC-TOFMS fitted with a 30 m × 0.25 mm × 0.25 μm SUPELCO waxTM10 capillary columns as previously described (Zhang and Tian 2010). The temperature program is that the initial column temperature of 140 °C was held for 5 min, increased by 4 °C per min to 240 °C and held for 0 min, and then increased by 50 °C per min to a final temperature of 255 °C for 5 min. Authentic methylated FAs (Sigma-Aldrich) were used as external standards to identify and quantify peaks; corrections were made at this stage for losses using the C17:0 internal standard. The double bond index (DBI) was calculated as follows: DBI = [(mol% trienes × 3) + (mol% dienes × 2) + (mol% monoenes)]/ $\sum$ (mol% saturated FAs).

Polar lipids of PM phase transition analysis

The phase behavior of PM polar lipids from fruit was investigated by differential scanning calorimetry (DSC) and electron paramagnetic resonance (EPR). The phase transition temperature was determined according to the

method of Raison and Wright (1983) with minor modifications. Polar lipids of 3–7 mg in chloroform were transferred with a pipette to an aluminum pan, and the solvent was removed under vacuum. Then, 20 mM Tris–acetate buffer (pH 7.2) containing 2 mM EDTA was added to the lipid to give a 300 % (w/w) excess. The pans were sealed and the lipids were hydrated for at least 16 h at 36 °C. The samples were scanned using a Perkin-Elmer DSC-Q100 DSC at 5 °C min^{-1} from −30 to 80 °C.

PM polar lipids fluidity was investigated by EPR. Prior to spin labeling, polar lipids were resuspended in 0.02 M Tris–acetate (pH7.2) containing 2 mM EDTA by brief sonication to give a final concentration of 10 to 12 mg mL^{-1}. The spin label, 5- or 16-doxylstearic acid (5-DSA or 16-DSA, Sigma-Aldrich), was added to give a molar ratio of label to lipid of 1:150. The sample was transferred to a capillary tube and its EPR spectrum was recorded using an ER-200D Bruker spectrometer at X-band (9.80 GHz) with microwave power of 20 mW. Modulation frequency of 100 kHz, which combined modulation amplitude of 1 Gauss (G) and a sweep time of 100 s gave the best results. Values for order parameter (S) and the rotational correlation time (τ_c) were estimated based on the EPR spectra of polar lipids labeled with 5-DSA or 16-DSA as described previously (Zhang and Tian 2009).

2-DE and image analysis

For preparation of PM proteins, the pellet membrane was resuspended in the Cellular and Organelle Membrane Solubilizing Reagent (ProteoPrep Membrane Extraction kit, Sigma-Aldrich) by brief sonication. The suspension was centrifuged at 14,200g for 50 min to remove the insoluble debris. The membrane protein concentration was quantified as described by Bradford (1976) with bovine serum albumin (BSA) as the standard, and 2-DE was carried out following the protocols described by Zhang et al. (2010). Membrane protein (500 μg) dissolved in 250 μL thiourea/urea lysis buffer containing 2 M thiourea, 7 M urea, 4 % (w/v) CHAPS, 1 % (w/v) DTT and 2 % (v/v) carrier ampholytes mixture (pH 5–8:pH 3.5–10, 1:1) was loaded onto 13-cm Immobiline DryStrip (pH 4–7) (GE Healthcare) by active overnight rehydration. Membrane proteins were separated by the first dimension using isoelectric focusing (IEF) and the second dimension using SDS-PAGE. The gels were stained by Coomassie brilliant blue (CBB) and scanned using a flatbed scanner (Amersham Biosciences, Uppsala, Sweden) at 600 dpi. The Image Master 2D Platinum software (Amersham Biosciences, Uppsala, Sweden) was used to analyze gel images, as described in the user's manual. For each treatment, triplicate gels resulting from three biological replicates were selected. Protein spots were selected for quantitative analysis if they were consistently visible in three replicates. The volume of each spot from three replicate gels was normalized as a percentage of the total volume and subjected to analysis of variance by a two-tailed nonpaired Student's t test with SPSS software (SPSS Inc., Chicago, IL, USA). Proteins whose expression showed statistically significant differences ($P < 0.05$) were considered for further analysis.

In-gel digestion and mass spectrometry

Protein digestion was performed as our previous reports with some modifications (Chan et al. 2007). Briefly, protein spots excised from the CBB stained gels were destained with 50 mM NH_4HCO_3 in 50 % (v/v) methanol for 1 h at 40 °C. After complete destaining and drying in a vacuum centrifuge, the gel pieces were digested at 37 °C for 16 h with 5 ng μL^{-1} trypsin. Digested peptides were extracted by three changes of 0.1 % trifluoroacetic acid (TFA) in 50 % acetonitrile, lyophilized, and used for mass spectrometric (MS) analysis by a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Framingham, MA, USA). Before spotting onto the MALDI target plates, the peptides were resuspended in 5 mg mL^{-1} matrix solution (α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile containing 0.1 % TFA). The instrument was externally calibrated with the tryptic peptide mixtures of myoglobin (Sigma-Aldrich) and operated in the positive reflection mode. MS spectra were obtained with 1,600 laser shots per spectrum and MS/MS spectra were acquired with 2,500 laser shots per fragmentation spectrum. The ten strongest peaks of each MS spectra were selected as precursor ions for the acquirement of the MS/MS fragmentation spectra. For spectra analyses and generation of peak list files, the 4000 Series Explorer™ software (Applied Biosystems) was used and the parameters were set as a signal-to-noise threshold of 10 and a minimum area of 100.

The generated peak lists were searched in NCBInr protein databases with Mascot MS/MS Ions Search program on the Matrix Science public web site (http://www.matrixscience.com). Search parameters were set as taxonomy, Viridiplantae (Green Plants); proteolytic enzyme, trypsin; max missed cleavages, 1; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); peptide mass tolerance, 0.5 Da; fragment mass tolerance, 0.6 Da. Only significant hits as defined by Mascot probability analysis were considered. Mascot uses a probability-based "Mowse score" to evaluate data obtained from tandem mass spectra. Mowse scores were reported as $-10 \times \text{Log } 10\ (P)$ where P is the probability that the observed match between the experimental data and the database sequence is a random event. Meanwhile, a Mowse score threshold is also returned by the Mascot program for each individual search. Scores greater than the threshold

were considered significant, and the best match is the one with the highest score in general.

RNA isolation and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

For total RNA extraction, 10 g of flesh was ground in liquid nitrogen and extracted with phenol as described by Moore et al. (2005). The residual DNA was digested by RQ1 RNase-Free DNase (Promega, USA), and 2 μg of RNA was used to synthesize the first-strand cDNA with M-MLV Reverse Transcriptase (Promega, USA) according to the manual protocol. Semi-quantitative RT-PCR procedure was performed as previously described by Spencer and Christensen (1999). The tubulin from mango fruit was used as internal control to normalize the cDNA stock of each sample. As gene sequences of detected proteins are unknown, degenerate primers were designed according to the homologous sequences from other plants, such as rice, potato, tomato, and grape. All the primer pairs used for PCR were listed as follows: remorin family protein (*REM*) sense (5′-ATggC (A/T) gA(g/A) gAgg Ag ACAAAgAAgg-3′) and antisense (5′-gCCTTCTTCTT(T/C)TCCA(g/A)TTgCTC CT-3′); abscisic stress ripening-like protein (*ASR*) sense (5′-AAgAAggAggAgAA (A/g)CA (C/T)CACAA-3′) and antisense (5′-AACCgCCACCgCTgCAgC(A/g)AT(C/g)TC C-3′); temperature-induced lipocalin protein (*TIL*) sense (5′-gA(A/g)gTggTgA A(g/A)ggCCT(g/C)gA-3′) and antisense (5′-(A/C)ggAgTCTTgTg(g/A) AgTTTgCT-3′); typeII Sk2 dehydrin (*TSD*) sense (5′-AACgT(C/T)(C/TA)ATgA ACACgAg(g/T)C-3′) and antisense (5′-AgACTC(C/T) TT(C/T)TC(C/T) TT (g/C)TTCT-3′). PCR conditions for amplification were 94 °C for 5 min, followed by 25 (for *REM*, *TSD*); or 30 (for *ASR* and *TIL*) cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 45 s. PCR products were separated on 1.2 % agarose gels.

Statistical analysis

All statistical analyses were performed with SPSS version 11.5 (SPSS Inc., Chicago, IL, USA) and analyzed by one-way analysis of variance (ANOVA). Mean separations were performed by a two-tailed nonpaired Student's *t* test. Differences at $P < 0.05$ were considered as significant.

Results

Development of CI in mango fruit

The most visual CI symptoms in mango fruit were the sunken lesions of pericarp (Fig. 1a, 1) and pulp darkening companying with tissue necrosis (Fig. 1a, 3). No visible symptoms of CI were manifested in all fruit during the 14-day storage period (Fig. 1a, 2, 4). After the 14-day storage, the CI incidence and CI index increased gradually with storage time, but these values were significantly lower in fruit treated with BL than in those of the control ($P < 0.05$, Fig. 1c, d). The relative electrolyte leakage, which has been widely used as an indicator of cell membrane damage, increased slightly in all fruit within storage time of 7 days. After that, there was a remarkable increase in the relative electrolyte leakage in the control fruit (Fig. 1b). In contrast, BL-treated mango fruit showed a marked slow-down of relative electrolyte leakage when stored at 5 °C (Fig. 1b). These results indicate that treatment with BL can enhance the tolerance of mango fruit to cold stress.

PM polar lipids phase behavior and fluidity

It is well known that PM is a primary site of injury under low temperature stress, and that PM lipids play an important role in maintaining the integrity of PM. To explore the mechanism by which BL reduced the occurrence of CI, firstly, PM from mango fruit treated with or without BL was prepared and purified. Then polar lipids in PM were extracted and the phase behavior and fluidity were characterized. The phase transition temperature for PM polar lipids was determined by DSC. As shown in Fig. 2, the phase transition temperatures for PM polar lipids in untreated mango fruit were relatively constant at approximately 6.5 °C during the whole storage duration, while the temperatures were markedly reduced when the fruit were stored under same conditions and duration, but treated with BL. The differences of phase transition temperature between BL treatment and the control reached 9.05 and 9.23 °C after 14 and 21 days of storage at 5 °C, respectively.

The effect of BL on the PM polar lipid fluidity of mango fruit is shown in Fig. 3. Figure 3a is a representative spectrum of 5-DSA spin-labeled lipids, which reflects the mobility of lipid hydrophobic core, and that the spectrum of 16-DSA spin-labeled lipids reflects the mobility of lipid hydrophobic regions nearer to hydrophilic face (Fig. 3b). Afterwards, order parameter (*S*) and rotational correlation time (τ_c), which are inversely related to lipid fluidity, were calculated based on the EPR spectra of polar lipids labeled with 5-DSA or 16-DSA, respectively. Both *S* and τ_c values gradually increased during the storage, while these values in fruit treated with BL were significantly lower than in those of the control ($P < 0.05$), respectively (Fig. 3c, d).

Changes of FAs in PM polar lipids

The FAs of PM polar lipids in mango fruit mainly consisted of palmitic acid (C16:0), Plamitoleic acid (C16:1),

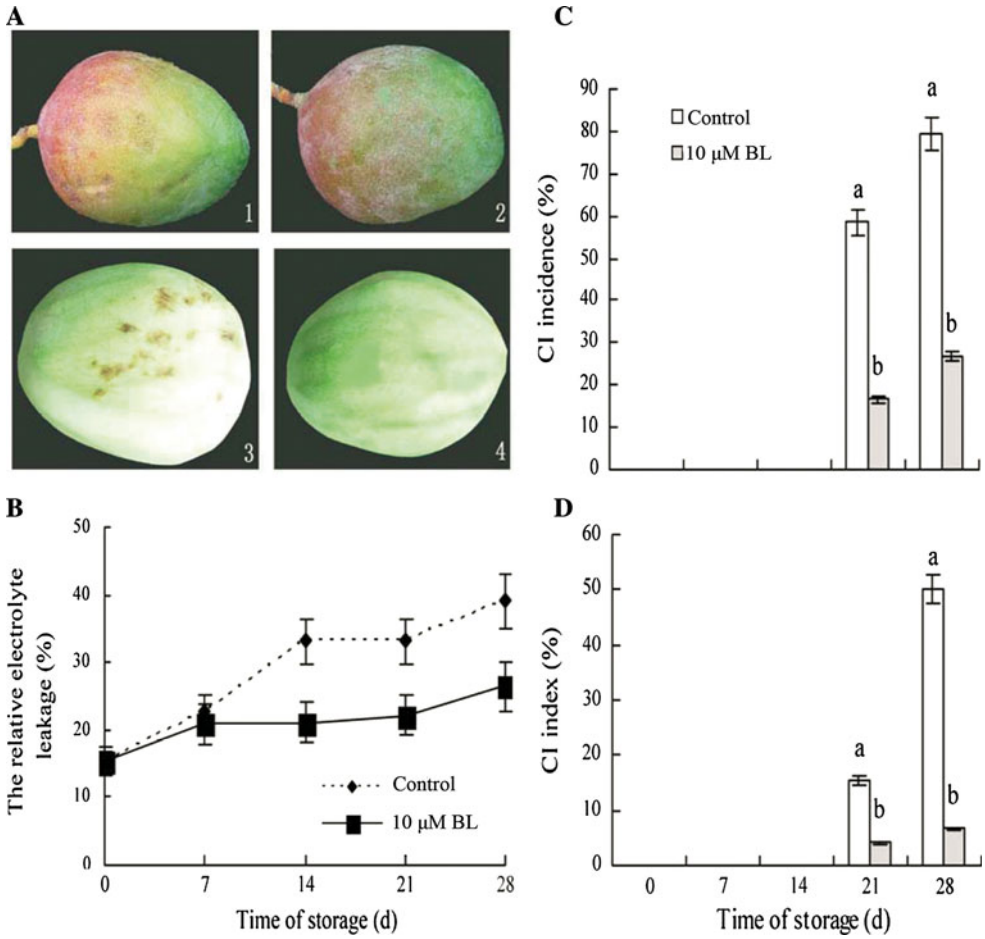

Fig. 1 Effects of BL on chilling injury (CI) symptom (**a**), relative electrolyte leakage (**b**), CI incidence (**c**) and CI index (**d**) in mango fruit stored at 5 °C. **a** *1* and *2*, unpeeled fruit; **a** *3* and *4*, peeled fruit. The fruit without CI showed smooth surface (**a** *2*) and fresh flesh (**a** *4*). But there were sunken lesions (**a** *1*) on the surface with pulp discoloration (**a** *3*) in CI fruit after 21 days at 5 °C. Data are the mean ± SE of three replicates. *Bars* represent the standard errors of the means. Columns with *different letters* are significantly different from each other according to Student's *t* test ($P < 0.05$)

stearic acid (C18:0), oleate aid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). Compared with FAs in the fruit without treatment with BL, the levels of C18:2 and C18:3, and DBI of PM polar lipids in the fruit treated with BL were significantly higher ($P < 0.05$; Table 1). In general, higher content of UFAs in membrane lipids is beneficial for decreasing the phase transition temperature (Fig. 2), thus facilitating maintenance of normal membrane fluidity.

Differently expressed proteins and gene in response to BL

With the ProteoPrep Membrane Extraction kit, highly enriched membrane proteins were prepared from the purified PM. After 2-DE, more than 700 protein spots were detected in each gel after ignoring spots with undefined shapes and areas using Image Master 2D Elite software (Fig. 4a, b). Comparative analyses of proteome were performed between fruit treated with and without BL at each sampling time point. Protein spots, which showed statistically significant changes and more than twofold changes in relative abundance, were selected as differentially expressed spots. A total of 28 spots with relatively higher abundance were analyzed by MALDI-TOF/TOF (Fig. 4a, b). Due to the lack of genome sequence information for this species, only half of these differential protein spots were positively identified by NCBInr database searching with Mowse scores significantly higher than the threshold ($P < 0.05$) (Table 2). The matched peptide sequences of these identified proteins are listed in Supplemental Table 1. Compared with control, most of these identified proteins were up-regulated in fruit treated with BL under low temperature conditions in general and in fruit displaying CI symptoms (13 up-regulated proteins for 21 and 28 days) in particular. These identified proteins were categorized into four groups, including transport (4 spots), cellular biogenesis (1 spot), defense and stress response (7 spots), and proteins with unknown function (2 spots) (Table 2). Among these 14 protein spots, four spots (spot 5, 7, 13, and 21), which were identified as membrane-associated protein and up-regulated in response to BL treatment at 21 or 28 days of storage, were noteworthy (Fig. 4c). Among them, three spots were classified as proteins associated with defense and stress response, including spot 5 (remorin family protein, REM), spot 7 (abscisic stress ripening-like

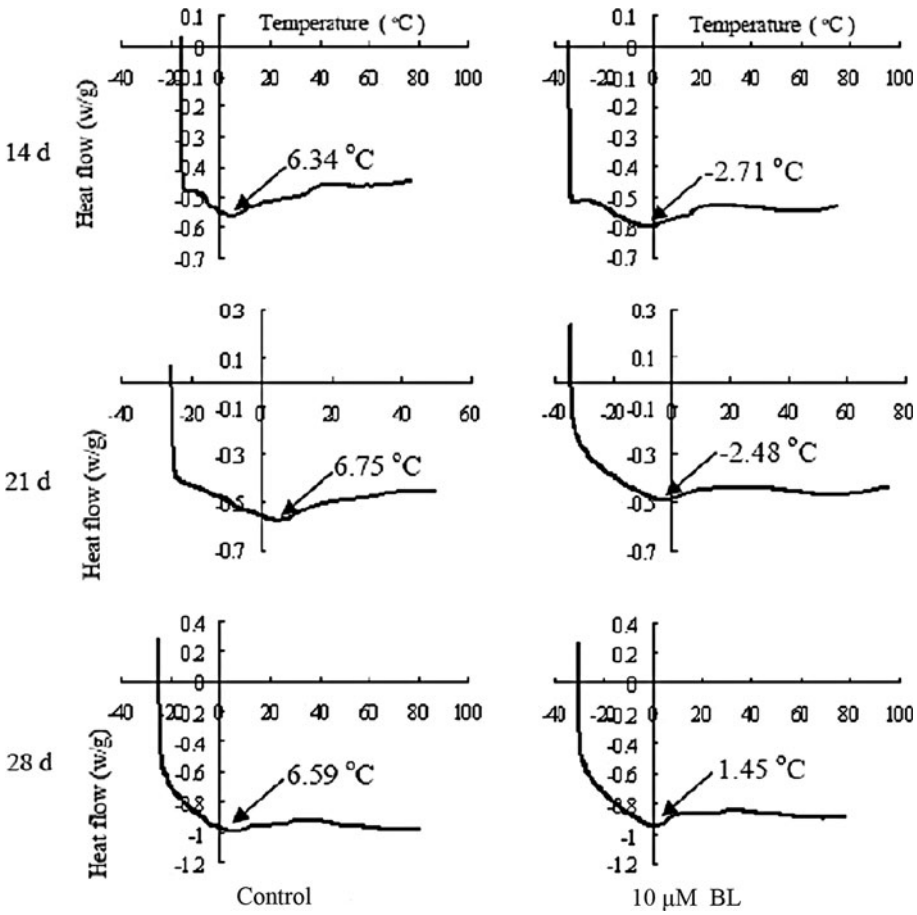

Fig. 2 Effect of BL on the phase transition temperature (calorimeter traces) of PM polar lipid in mango fruit stored at 5 °C. *Arrows* with temperature value indicate the phase transition temperature

protein, ASR), and spot 21 (type II SK2 dehydrin, TSD). Spot 13 (temperature-induced lipocalin, TIL) was established early as transport protein, but it became increasingly clear that the protein may also have many other important functions, such as membrane biogenesis and repair (Charron et al. 2002). The expression patterns of these proteins were also analyzed at the transcriptional level by RT-PCR. In general, expression of these genes was up-regulated by the treatment with BL (Fig. 5). Expression of *TIL* peaked at 21 days of storage under low-temperature conditions, while expression of *TSD* and *ASR* was highest at 14 days; and expression of *REM* reached the maximum level at 28 days of storage (Fig. 5).

Discussion

Fruit are usually kept under low-temperature conditions to maintain their quality and extend marketing time after harvest (Tian et al. 2010). CI is a serious physiological disorder in tropical and subtropical fruit when they are stored in unsuitable low-temperature conditions (Han et al. 2006). How fruit responds to cold temperature stress is biologically interesting, and how to enhance the tolerance of fruit to chilling injury is an economically important problem. Recently, some exogenous chemical compounds have been proved to be effective at enhancing resistance of fruit to cold temperature stress. For instance, Ding et al. (2007) reported that exogenous oxalic acid and salicylic acid effectively alleviate CI of mango fruit stored under low-temperature conditions. Zaharah and Singh (2011) found that nitric oxide fumigation reduces chilling injury, and maintains quality in cold-stored 'Kensington Pride' mango fruit. Meng et al. (2009) demonstrated that peach fruit exhibit higher tolerance to cold temperature after treatment with methyl jasmonate. In the present study, we found that exogenous BL markedly decreased CI incidence of mango fruit stored at 5 °C (Fig. 1c, d), suggesting that BL has the important capability in enhancing fruit tolerance to cold temperature stress.

To understand the mechanisms by which BL regulates the tolerance of mango fruit to cold temperature stress, we analyzed the effects of BL on the phase behavior and composition of PM lipid in mango fruit treated with and without BL during storage under low temperature. The temperature of the phase transition of membrane lipids

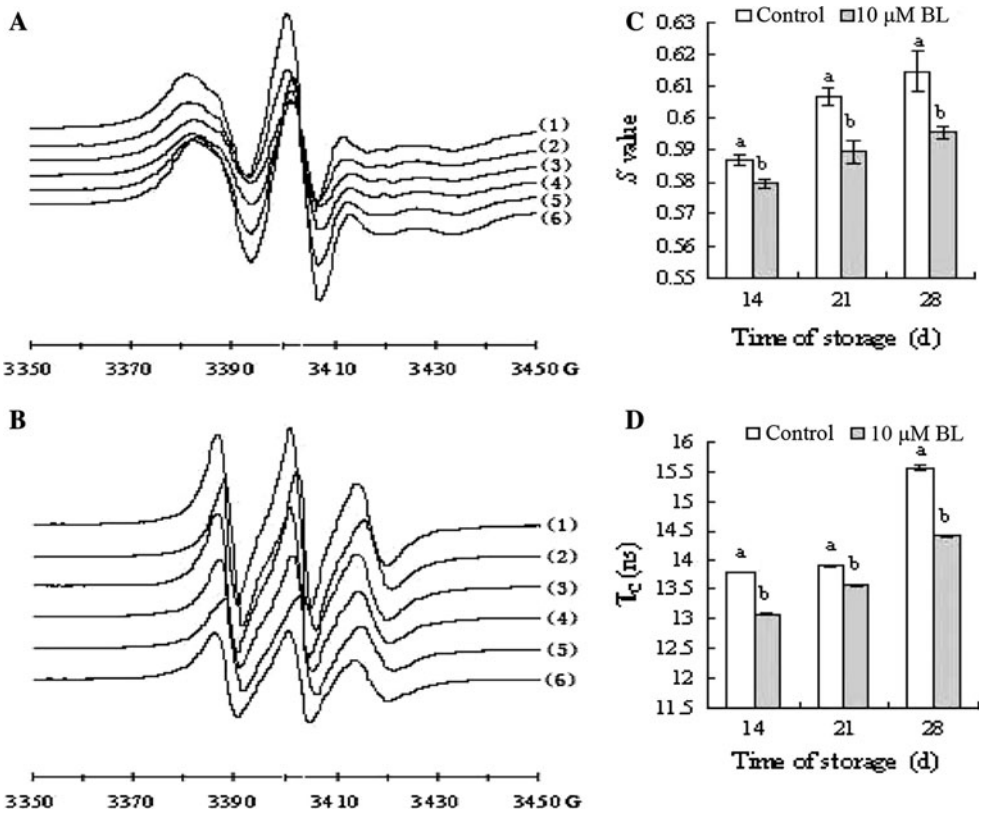

Fig. 3 Effect of BL on the fluidity of PM polar lipids in mango fruit stored at 5 °C. **a**, **b** Examples of EPR spectra of the double bilayer regions close to the polar lipid head groups with 5-DSA spin (**a**) and the lipid close to the core of bilayer with 16-DSA spin (**b**) of PM polar lipids, respectively; **c**, **d** changes in the *S* (C) and τ_c (D) of PM polar lipids in the control (*white column*) and fruit treated with BL (*gray column*) during storage at 5 °C. Data are the mean ± SE of three replicates. *Bars* represent the standard errors of the means. Columns with *different letters* are significantly different from each other according to Student's *t* test ($P < 0.05$)

Table 1 Effects of BL on the FA profile from PM polar lipids in mango fruit stored at 5 °C

Storage time (days)	Treatment	Fatty acids profile of polar lipids (mol%)						
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	DBI
14	Control	35.17 ± 1.76 a[A]	1.14 ± 0.06 a	2.08 ± 0.10 a	23.81 ± 1.19 a	26.03 ± 1.40 b	11.77 ± 0.59 b	2.90 ± 0.15 b
	10 μM BL	33.34 ± 1.67 a	0.96 ± 0.05 a	1.94 ± 0.01 b	20.11 ± 1.01 b	30.03 ± 1.50 a	13.62 ± 0.68 a	3.34 ± 0.11 a
21	Control	35.84 ± 1.79 a	1.49 ± 0.07 a	1.71 ± 0.09 a	24.65 ± 1.23 a	24.35 ± 1.22 b	11.96 ± 0.60 b	2.80 ± 0.04 b
	10 μM BL	36.30 ± 1.82 a	0.98 ± 0.05 b	1.38 ± 0.07 b	21.75 ± 1.09 b	26.41 ± 1.32 a	13.18 ± 0.66 a	2.95 ± 0.05 a
28	Control	34.49 ± 1.72 a	1.85 ± 0.09 a	1.37 ± 0.07 a	26.00 ± 1.30 a	24 .00 ± 1.20 b	12.29 ± 0.61 a	2.94 ± 0.04 b
	10 μM BL	33.51 ± 1.68 a	1.23 ± 0.06 b	1.55 ± 0.08 a	22.63 ± 1.13 b	28.54 ± 1.43 a	12.55 ± 0.63 a	3.23 ± 0.06 a

Values of each fatty acid species or DBI in the same storage time with different letters are significantly different from each other according to Student's *t* test ($P < 0.05$)

[A] Data are the mean ± SE of three replicates

positively correlates with the chilling injury temperature in plant tissue (Raison and Orr 1986). In the present study, we found that exogenous BL significantly decreased the phase transition temperature of PM lipids in mango fruit (Fig. 2), resulting in higher PM lipid fluidity (Fig. 3). It is essential for plants to maintain an intact PM with appropriate structures and properties. In addition, plants sometimes have to change their PM properties to efficiently adapt to extreme environments (Uemura et al. 2006). Membrane fluidity, which reflects the ordering and dynamics of the phospholipid alkyl chains in the membrane (Hazel and Williams 1990), has been proved to be crucially related to the tolerance of fruit to cold stress (Zhang and Tian 2010). It was also found that BL treatment induced a significant increase in UFAs of lipids (Table 1), confirming our previous premise that membrane fluidity is positively correlated with the level of UFAs (Zhang and Tian 2009). Thomashow (1999) suggested that cold-tolerant plants often contain high proportions of UFAs, which could contribute to maintain the membrane phase transition temperature below the applied chilling temperature.

Sub-proteomics approaches dealing with membrane systems can bring clues on both the membrane compartment where proteins are working and their putative cellular functions (Ephritikhine et al. 2004). In the present study, 14 BL-responsive proteins were identified using spectrometry-

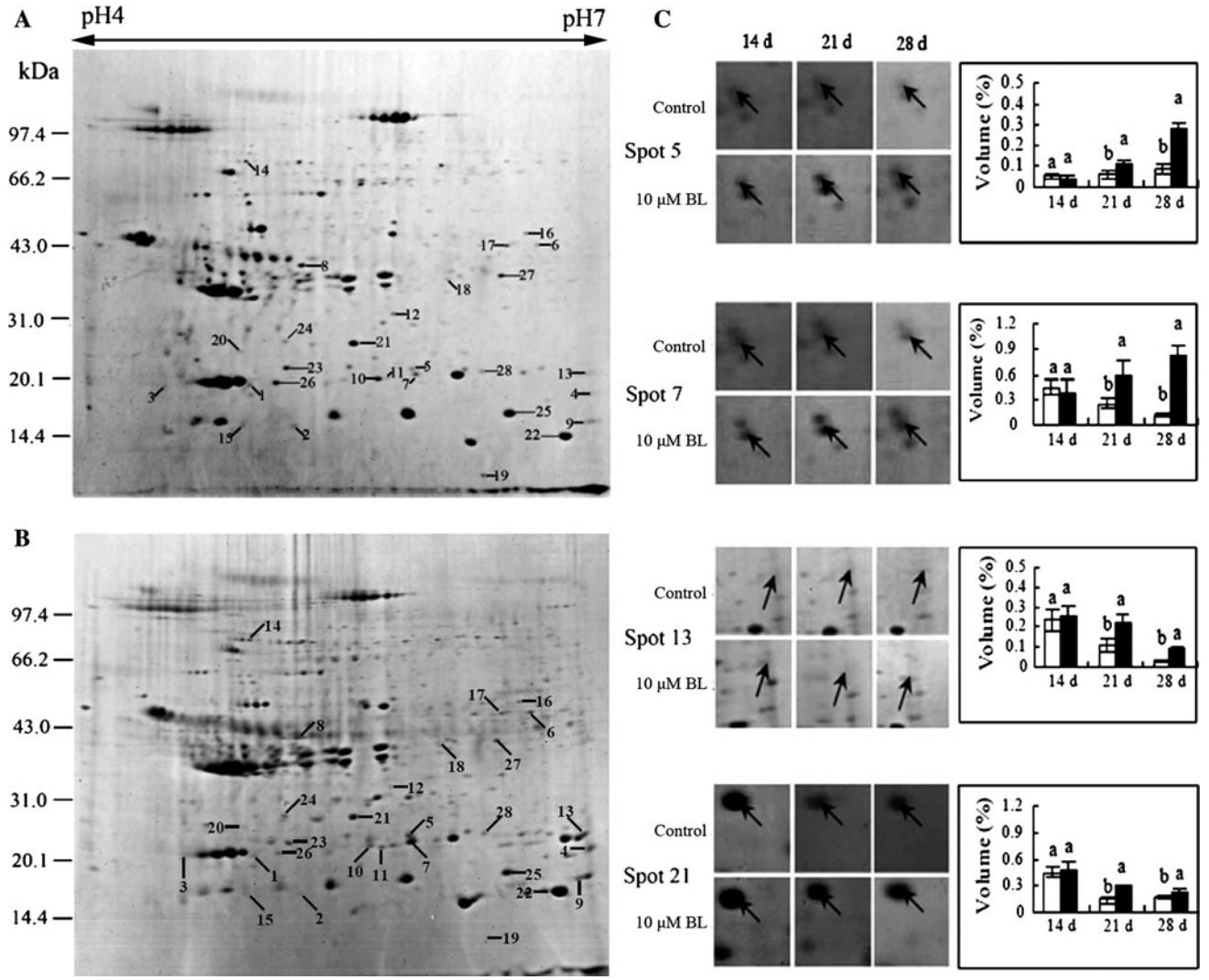

Fig. 4 Effect of BL on the expression of PM proteins in mango fruit stored at 5 °C. **a**, **b** Images of the representative 2D gels of PM proteins in the control (**a**) or fruit treated with BL (**b**); **c** close-up views (*left*) and abundance variance (*right*) of transport related membrane proteins or membrane proteins involved in defense and stress response in the control (*white column*) or fruit treated with BL (*gray column*) during storage at 5 °C for 14, 21 and 28 days, respectively. The protein spots with number were corresponded to those in Table 2

based proteomics (Table 2). Most of the proteins were plasma membrane-associated proteins. Among these proteins, some BL-responsive proteins were identified for the first time, such as remorin, an important membrane skeleton protein. The changes in abundance of the protein were greatly correlated to the changes in transcriptional levels (Figs. 4c, 5). It is well known that remorin proteins are usually responsive to biotic and abiotic stimuli (Laloi et al. 2007), and involved in signal transduction (Lefebvre et al. 2007). Bariola et al. (2004) considered that remorins might be associated with the membrane skeleton and determined the membrane integrity of plants.

Our results also revealed that BL induced up-regulation of abscisic acid stress ripening (ASR)-like protein and lipocalins (Figs. 4c, 5), which play essential roles in signal transduction under cold stress. A recent study of subcellular fractionation and Western blot analyses by Kalifa et al. (2004) indicated that ASR is localized in both cytoplasmic and nuclear chromatin compartments, and may have a distinct role as chromatin protective protein or in signal pathway in tomato (*Lycopersicon esculentum*) under stressful environments. Lipocalins generally bind to small hydrophobic ligands such as retinoids, fatty acids, steroids, odorants, and pheromones, and interact with cell surface receptors (Charron et al. 2002). The localization studies showed that the temperature-induced lipocalins (TIL) are localized at the PM, and that they act as scavengers of potentially harmful molecules known to be induced by cold stress (Charron et al. 2005). Clouse and Sasse (1998) reported that BRs increases the tolerance of plants to cold

Table 2 Membrane proteins identified in mango fruit treated with or without BL during storage at 5 °C for 14, 21, and 28 days

Spot[A]	Protein name	NCBI accession	Theo. Mr (kDa)/p*I*[B]	Species[C]	Mascot score/ threshold[D]	NP[E]	SC (%)[F]	Fruits treated with BL versus the control[G]		
								14 days	21 days	28 days
Transport										
3	Charged multivesicular body protein, putative	gi|255555154	26.4/4.71	*Ricinus communis*	97/44	2	15	0.93	4.14	3.68
23	Endomembrane-associated protein	gi|255583930	22.8/5.10	*Ricinus communis*	63/44	2	11	2.19	2.59	2.27
13	Temperature-induced lipocalin	gi|77744891	21.5/5.60	*Prunus persica*	372/45	5	33	1.08	2.09	2.44
20	Os06g0608500 SNF7 protein-like	gi|115468882	24.3/4.84	*Oryza sativa* (*japonica cultivar-group*)	178/45	2	12	0.92	2.02	2.52
Cellular biogenesis										
8	Actin	gi|32186890	41.9/5.31	*Gossypium hirsutum*	491/44	8	37	0.89	2.28	2.93
Defense and stress response										
1	Mitochondrial F0 ATP synthase D chain	gi|192910736	19.7/5.13	*Elaeis guineensis*	78/44	3	25	0.91	2.03	2.60
5	Remorin family protein	gi|34925093	21.8/6.15	*Solanum tuberosum*	37/29[H]	1	3	0.92	2.04	3.18
7	Abscisic stress ripening-like protein	gi|16588758	20.7/5.68	*Prunus persica*	503/43	7	77	0.90	2.32	6.80
10	Small heat shock protein	gi|41059801	17.4/5.98	*Prunus persica*	223/45	4	33	0.87	2.98	2.35
11	Major allergen Pru p 1	gi|82492265	17.6/5.79	*Prunus persica*	851/44	6	50	0.94	2.53	2.06
15	Putative allergen Pru du 1.06B	gi|190613891	17.4/5.10	*Prunus dulcis* × *Prunus persica*	492/45	5	43	0.84	3.07	2.53
21	Type II SK2 dehydrin	gi|73762178	28.5/5.37	*Prunus persica*	208/43	4	27	1.06	2.03	2.27
Unknown function										
22	Hypothetical protein	gi|147780708	141.8/ 8.92	*Vitis vinifera*	57/45	2	1	0.93	1.22	3.39
24	Hypothetical protein	gi|302784184	26.0/5.42	*Selaginella moellendorffii*	47/44	1	5	0.84	0.45	0.43

[A] The spot number as given in Fig. 4a, b

[B] Theo. Mr (kDa)/p*I*, theoretical molecular mass and isoelectric point based on amino acid sequence of the identified protein

[C] The species of the matched protein

[D] The score obtained from Mascot for each match and Mascot scores greater than threshold are statistically significant ($P < 0.05$)

[E] NP, the number of matched peptides

[F] SC, amino acid sequence coverage for the identified proteins

[G] Average-fold change of specific protein levels in fruit treated with BL versus the control

[H] Mascot score and threshold value obtained by Mascot search within database of SwissProt

stress because BRs function as signaling molecules to elicit the expression of steroid binding proteins such as lipocalin. Dehydrins, as one of the groups of the late embryogenesis abundant (LEA) protein family, are involved in response of plants to drought, salinity, and dehydration (Allagulova et al. 2003; Close 1996). Tunnacliffe and Wise (2007) presented several possible functions of the LEA proteins, including as antioxidants and as membrane and protein stabilizers during water stress, either by direct interaction or by acting as molecular shields. Yin et al. (2006) reported that some transgenic plants, where dehydrin genes have been introduced, showed better chilling tolerance at low temperature, and that others had only slight or no tolerance to cold stress. In a recent report, Zhang et al. (2010) demonstrated that dehydrins contribute to increased tolerance of peach fruit to cold stress. In this study, we ascertained that BL could induce the expression of dehydrins and enhance tolerance of mango fruit to cold stress.

Taken together, our results provide novel insight into the downstream targets of BL that potentially mediate cellular and physiological response. On one hand, BL treatment can up-regulate the expression of membrane proteins, such as

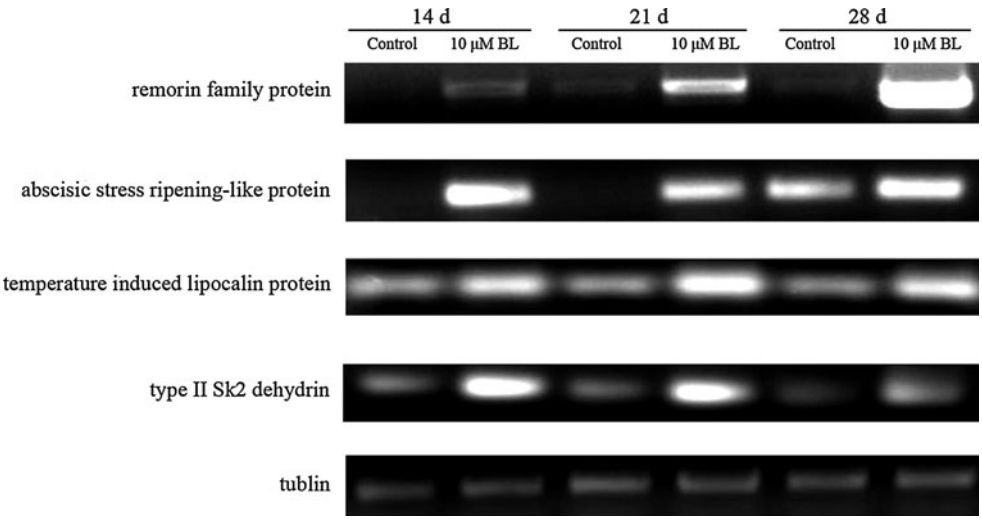

Fig. 5 Effect of BL on the expression of some relative genes on transcriptional level in mango fruit stored at 5 °C. These genes encode PM proteins, which were transport-related membrane proteins or membrane proteins involved in defense and stress response. Semi-quantitative RT-PCR was conducted using fruit stored for 14, 21, and 28 days, respectively

REM, ASR, TIL, and TSD, which is beneficial for maintaining the membrane integrity. On the other hand, BL can also regulate the membrane fluidity at low temperature by increasing C18:2 and C18:3 levels, as well as DBI to affect UFAs in membrane lipids. Among the identified membrane proteins, some may be important for membrane biogenesis and repair, scavenging harmful molecules, and stabilizing membrane structures, the others may be involved in transduction of cold signal in order to activate expression of cold acclimation-dependent genes. These proteins may be direct downstream targets of BRs signaling pathway, or indirectly regulated by other pathways interacted with BRs signaling pathway under cold stress. The studies on BL-mediated signal transducing pathway for cold stress tolerance and cross talk of BRs with other plant hormones should be carried out in future.

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Conflict of interest The authors have declared that no competing interests exist.

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