Characterization of low temperature-induced plasma membrane lipidome remodeling combined with gene expression analysis reveals mechanisms that regulate membrane lipid desaturation in *Carica papaya*

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ABSTRACT *Carica papaya* is a cold-sensitive horticultural crop, the mechanisms underlying its cold sensitivity remains unclear which limit its genetic improvements in cold tolerance. In this study, aqueous dextran-polyethylene glycol two-phase partition systems were applied to purify plasma membrane from papaya leaves grown under 28 °C and 10 °C, the plasma membrane total lipids were analyzed by

lipidomic approach; meanwhile, the transcription levels of putative fatty acid desaturases were quantified by RT-qPCR. We found that cerebrosides are dominate plasma membrane lipid species, followed by phospholipids and free sterols. Cold treatment increased the mono-unsaturated and di-unsaturated phospholipids, and decreased di-saturated phospholipids, the proportion of total phospholipids was not affected. Cold stress enhanced the transcription of *CpFAD4*, *CpFAD6* and *CpFAB2-2*. Pathway analysis suggested that the cold induction of *CpFAB2-2* transcription and CpFAD2 enzyme activities play important roles for plasma membrane lipidome remodeling under cold stress. Our findings uncovered potential targets to improve papaya cold tolerance.

Keywords: Carica papaya, plasma membrane, cold stress, lipidomic, fatty acid desaturase, phospholipids

1. Introduction

Plasma membrane (PM) is a lipid bilayer with integral proteins and peripheral proteins (Singer and Nicolson, 1972). Based on chemical properties PM lipids can be fractioned into phospholipids (PLs), neutral lipids (NLs), and glycolipids (GLs). PLs include phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), and

phosphatidylglycerol (PG); NLs are constituted by various free sterols (FSs), while GLs include steryl glucosides (SG), acylated steryl glucosides (ASG), and cerebrosides (CER) (Lodish et al., 2000). The lipid composition of the plasma membrane and tonoplast of higher plants is substantially different from that of other cellular membranes in that there is a high proportion of sterols (both free and glucosylated forms) and cerebrosides, neither of which are present in other cellular membranes (Douce and Joyard, 1990; Larsson et al., 1990). The plasma membrane from oat, rye, spinach, potato, and barley has beencharacterized, the proportion of PLs, CERs, and sterols showed considerable variations among them (Palta et al., 1993; Rochester et al., 1987; Uemura and Steponkus, 1994).

The plasma membrane is of primary importance to maintain its structural integrity during a freeze-thaw cycle. Chilling injury includes two processes: an early reversible process and a later irreversible process (Yoshida, 1991). The lipid components of membranes determine the physical and chemical properties of cell membranes and are considered the most important factors for membrane function under low temperature (Lyons, 1973; Nishida & Murata, 1996). Characterizing response of membrane lipids to low temperature can identify early causal factors contributing to chilling injury. The establishment of aqueous dextran-polyethylene glycol two-phase partition systems facilitates the isolation of highly purified plasma membrane, cold-induced PM lipid changes have been characterized from several plant species. These studies found that the increase in the proportion of PLs and a decrease in proportion of glucocerebroside is commonly observed following cold treatment, while a decrease in glycosylated sterols SGs and ASGs and an increase in FSs also have been noted (Lynch and Steponkus 1987; Martz et al., 2006; Uemura et al. 1995; Uemura and Steponkus 1994; Yoshida and Uemura 1986; Zhou et al., 2014;). Besides these lipid compositional changes, increasing unsaturation of membrane lipids is positively correlated with plant chilling tolerance (Mikami & Murata 2003). Chilling sensitive plants grown at lower hardening temperature have also been shown to increase unsaturated fatty acids (Kaniuga et al. 1999; Kasamo et al. 1992; Kojima et al. 1998). Cold acclimating potato (*Solanum*

commersonii) accumulated linoleic acid (C18:2) in the membrane glycerolipids of leaves, whereas commercial, non-acclimating potato (*Solanum tuberosum*) did not (Vega et al. 2004).

Tropical and subtropical plant species generally are cold-sensitive, their growth and development are strongly affected by low but above-freezing temperature. Papaya (*Carica papaya*) is one of the major tropical fruits with optimum growth temperature between 26-32 °C; its growth is suppressed when average daily temperature below 10 °C (Crane 1994). With changing climate, the frequency is raising that cold air invade deep into papaya production regions during winter or early spring, which cause yield loss and quality deterioration. Despite its economic importance, little is known why papaya is sensitive to cold temperature, which hinder the efforts to improve its cold tolerance. PM lipids has been recognized to associate with plants' cold sensitivity, to our best knowledge no work has been done to investigate papaya PM lipidome remodeling and the underlying mechanisms in response to cold stress. Recent advancements in lipidomic technologies enable lipidome characterization at molecular resolution. The objectives of the present study are: (i) characterize, in detail, the lipid composition of papaya leaf plasma membrane; (ii) investigate plasma membrane remodeling under cold stress; (iii) identify the mechanisms for plasma membrane remodeling by cold stress. This work provided qualitative and quantitative evidence on cold-induced PM lipidome remodeling for the adaption to low temperature and the underlying molecular mechanisms, these knowledges could offer new avenue for papaya genetic improvements with enhanced cold tolerance.

2. Materials and methods

2.1 Plant materials

Seeds of *Carica papaya* cv '*Zhongbai*' were immersed in tap water and floating seeds discarded, remaining seeds were incubated in 28 °C incubator for 2 d at speed of 200 rpm min^{-1} (ZQZY-CF, Zhichu, Shanghai, China), then rinsed with tap water and transferred into petri dish filled with wet paper towel, incubated in 30 °C plant growth chamber (LGX-400B-LED, Prandtl, Ningbo, Zhejiang, China) under continuous light (160 μmol photon m^{-2} s^{-1}). Keeping paper towel wet during seed germination. Right after root emergence from seed coat, seeds were transplanted into composite soil of peat soil: vermiculite: perlite (3:1:1, v/v/v) in plastic soil pot (10 cm x 10 cm x 8.5 cm) , then moved to 28 °C growth chamber under 16 h light/ 8 h dark for further growth.

2.2 Cold treatment

Seedlings at stage of 7-9 true leaves were used for cold treatment. Seedlings were divided into control and cold treatment groups,grown in two growth chambers under continuous light (160 μmol photon m^{-2} s^{-1}), respectively. To initiate cold treatment, growth chamber temperature was programmed to decrease at 0.25 °C.h^{-1}, three days later the chamber temperature reached to 10 °C, and was held there for additional 2 days, then the leaves were harvested. The control plants were kept in 28 °C growth chamber for 5 days, and the leaves were harvested at same time as cold treatment group.

2.3 Transmission electron microscopy

The second leaf from control and cold treatment plants were harvested, and cut into small pieces (2 mm x 4 mm) from the central part of leaves, then fixed in 5% (v/v) glutaraldehyde solution overnight in 4 °C. Samples were rinsed three times with 0.1 M PBS buffer (pH7.2), post fixed with 1% (w/v) osmium tetroxide in 100 mM PBS buffer at 4 °C for 2-2.5 h, rinsed 3 times with PBS buffer, then dehydrated through 30% (v/v) and 50% (v/v) ethanol for 15 min. Samples were stained with saturated uranyl acetate in 70% (v/v) ethanol overnight, rinsed with 70% (v/v) ethanol several times, then dehydrated in 90% (v/v) and 100% ethanol for 17 min, the dehydration step was repeated in 100% ethanol. Samples were

treated sequentially with acetone: ethanol (1:3, v/v), acetone: ethanol (1:1, v/v), acetone: ethanol (3:1, v/v) and 100% acetone for 17 min each. The dehydration step was repeated in 100% acetone. Samples were infiltrated through a graded acetone/Epon/Spurr's epoxy resin and polymerized at 70 °C for 24 h. Samples were sectioned at 70 nm thickness using an ultra 35° diatome diamond knife, the thin sections were collected on 200 mesh copper thin bar grids, and observed under transmission electron microscope (HT7700, Hitachi, Japan).

2.4 Plasma membrane isolation and H^+-ATPase activity assay

The plasma membrane isolation follows the method as Larsson et al. (1987) with minor modifications. Papaya leaves (20 g) were homogenized in 80 mL of buffer (330 mM sucrose, 50 mM Tris-MES (pH7.8), 5 mM EDTA, 5 mM DTT, 0.5 mM PMSF, 0.2% (w/v) BSA, 0.6% (w/v) PVP, 5 mM ascorbic acid) for 1 min by using a kitchen homogenizer (JYL-C91T, Jiuyang, Jinan, Shandong, China). The homogenate was filtered through 240 μm nylon cloth and centrifuged at 10,000 g for 10 min (5418, Eppendorf, Germany) to pellet most of the chloroplast and mitochondria. The supernatant was centrifuged at 50,000 g for 30 min to get microsomal pellet (LYNX 6000, Thermo Fisher, USA). The pellet was suspended in buffer containing 330 mM sucrose, 5 mM potassium phosphate (pH7.8), 5 mM KCl, 1 mM DTT, and 0.1 mM EDTA, 9.0 g of the suspension was loaded into a pre-prepared 27.0 g phase mixture to give a 36.0 g phase system with a final composition of 6.2% (w/w) Dextran T500, 6.2% (w/w) polyethylene glycol 3350, 0.33 M sucrose, 5 mM KCl, 5 mM potassium phosphate (pH7.8), 1 mM DTT, 0.1 mM EDTA. The phase system was thoroughly mixed by ~40 inversions of the tube, then centrifuged at 1500 g for 10 min for phase separation. The above phase separation was repeated three times. The final phases were diluted at least 2 folds with buffer containing 0.33 M sucrose, 5 mM KCl, 5 mM potassium phosphate (pH7.8), 1 mM DTT, and 0.1 mM EDTA, then centrifuged at 100,000 g for 40 min (Optima XPN-100, Beckman, USA), the plasma membrane was recovered from the lower phase, and

immediately used for lipid isolation or suspend in buffer containing 0.33 M sucrose, 5 mM KCl, 5 mM Tris-MES (pH6.5), 1 mM DTT, and 0.1 mM EDTA for H^+-ATPase measurement. Membrane protein concentration was measured by using Pierce®660 nm Protein Assay Reagent (22660， Thermo Fisher, USA) by following the manual instruction, calibration curve was made simultaneously by using 0-, 50-, 125-, 250-, 500-, 750-, and 1000- $\mu g.mL^{-1}$ BSA. Absorbance was measured at 660 nm in Multiskan GO (Thermo Fisher, USA). The ATPase activity was measured by following the methods of Sandstrom (1987) with small modification. H^+-ATPase was present from both plasma membrane and tonoplast with distinct characteristics: the plasma membrane isoform is inhibited by vanadate but insensitive to KNO_3 (Campillo and Steponkus, 1984); in contrast, the tonoplast isoform was inhibited by KNO_3 but insensitive to vanadate (Sze, 1985). Thus, the activity difference before and after vanadate or KNO_3 addition can be used to estimate the plasma membrane purity. The reaction buffer contained 30 mM Tris-MES (pH6.5), 5 mM $MgSO_4$, 50 mM KCl, 0.02% (w/v) Triton X-100, 5 mM Na_2-ATP. Tubes containing 480 µL reaction buffer were divided into four groups-negative control, positive control, vanadate treatment, and nitrate treatment. 20 µL of deactivated plasma membrane fraction, native plasma membrane fraction (containing 10 µg membrane protein), 0.1 mM Na_3VO_4, and 100 mM KNO_3 were added into above labeled tube in triplicates. The tubes were incubated in 30 °C water bath for 30 min, the reaction was terminated by adding 2.5 mL stop buffer (0.5 mM PVP, 86 mM $(NH_2OH)_2$:H_2SO_4, 5.3 mM EDTA-Na_2, 0.2% (v/v) H_2SO_4, 1.3% (w/v) $(NH_4)_6Mo_7O_{24}$). 0.25 mL of color developing agent (50 mM Na_2CO_3， 6.47 M NaOH) was immediately added into each tube, the absorbance was measured at 720 nm within 10-40 min. Phosphate calibration curve was made by using Na_2HPO_4 at concentration gradient of 0-, 1.5-, 3.0-, 4.5-. 6.0-, and 7.5-mM, the plasma membrane fraction was replaced by 20 µL buffer containing 0.33 M sucrose, 5 mM KCl, 5 mM Tris-MES (pH6.5), 1 mM DTT, and 0.1 mM EDTA; the stop solution, color developing agent and absorbance measurement were performed as above. Phosphate released from ATP was determined by using phosphate calibration curve.

155 *2.5 The isolation of neutral lipid, glycolipid, and phospholipid*

156 Total lipids were isolated from purified plasma membrane by following the method of Bligh and
157 Dyer (1959). 1 mL chloroform and 2 mL methanol were added into 0.8 mL of purified plasma membrane,
158 vortexed for 2 min; 1 mL of chloroform was added, vortexed 30 s; 1 mL of water was added, vortexed 30
159 s. The organic phase (lower phase) was transferred into a new tube, dried down under gentle nitrogen
160 stream to get total PM lipids.

161 The PGs, NLs and GLs were isolated from total lipids as the method described by Lynch and
162 Steponkus (1987). SPE column (CNWBOND Si SPE cartridge, Anpel, Shanghai, China) was activated by
163 rinse with 5 mL chloroform. Total lipids were dissolved in 2 mL of chloroform:acetic acid (100:1, v/v),
164 loaded into activated SPE column. The column was washed with 20 mL of chloroform:acetic acid (100:1,
165 v/v), the elution was collected which contain NLs. GLs fraction was eluted by the sequential addition of
166 10 mL acetone and 10 mL acetone:acetic acid (100:1, v/v); PLs fraction was eluted by 7.5 mL
167 methanol:chloroform:water (100:50:40, v/v/v). 2.25 mL chloroform and 3 mL water were added to the
168 PLs containing eluent, vortexed for 30 s, then centrifuged at 1500 g for 2 min, the lower organic phase
169 was recovered and dried down by CentriVap (LABCONCO，Kansas, US) to obtain PLs.

170 *2.6 Derivatization*

171 Total leaf lipids or PLs were digested by adding 0.25 mL of 1.25 M HCl (diluted in methanol), 0.4 mL
172 of chloroform and 25 μg of heptadecanoic acid as internal standard (17:0, Sigma, St. Louis, MO, USA), ,
173 incubated at 50 °C for 5 h, then dried down under gentle nitrogen stream. For derivatization, 50 μL of
174 MSTFA containing 1% TMCS and 0.2 mL pyridine was added into digested mixture, and heated at 50 °C
175 for 1 h, then the samples were ready for GC injection.

The SG and ASG were analyzed as the method described by Wewer et al. (2011). The samples were digested by adding 1 mL HCl (diluted with methanol) and 25 μg dihydrocholesterol (Solarbio, Beijing, China), heated at 90 °C for 30 min. 1 mL of 0.9% NaCl and 1 mL hexane were added, vortexed, then centrifuged at 1500 g for 3 min, the upper phase was recovered and dried. For derivatization, 50 μL of MSTFA containing 1% TMCS was added into dried sample and heated at 80 °C for 30 min. The derivatization solution was dried down by CentriVap, then dissolved in 200 μL hexane before GC analysis. Free sterols were derivatized in the same way by omitting the acid lysis step.

2.7 GC-MS and GC-FID analysis

Each sample was analyzed by GC-MS (QP2010 Ultra, Shimadzu, Japan) and GC-FID (GC-2010 plus, Shimadzu, Japan), respectively. The MS data was used for compound identification by comparing their mass spectra with National Institute of Standard database (NIST 14), the FID data was used for compound quantitation by normalizing individual peak area to that of internal standard.

The fatty acid methyl esters were analyzed in DB-23 column (0.25 mm × 30 m × 0.25 μm, Agilent, USA). The oven temperature was initiated at 150 °C, then raised to 200 °C at a speed of 2 °C.min^{-1}, held for 5 min before back to 150 °C for next sample injection.

The derivatized sterols were analyzed in DB-1 column (0.25 mm × 30 m × 0.25 μm, Agilent, USA). The oven temperature was initiated at 150 °C, then raised to 280 °C at a speed of 10 °C.min^{-1}, held for 10.5 min before back to 150 °C. Helium was used as carrier gas at speed of 1 mL.min^{-1}, the split ratio was 20:1.

2.8 Lipidomic analysis

Total lipids isolated from purified plasma membrane (equivalent to 0.3-0.4 mg total proteins) were dissolved in chloroform, and introduced into the ESI source on a triple quadrupole MS by continuous

198 infusion (XEVO-TQS, Waters, US), quantification were performed as described previously (Shiva et al.,
199 2018; Kansas Lipidomic Research Center, https://www.k-state.edu/lipid/). Scan modes for SG, ASG-16:0,
200 ASG-18:0, ASG-18:1, ASG-18:2, ASG-18:3, and HexCer were NL197.1, NL435.3, NL463.35, NL461.35,
201 NL459.35, 457.3, and NL162.0, respectively. Three biological replicates were used.

202 *2.9 RNA isolation and real time PCR*

203 Total RNA was isolated from papaya leaves by using RNAprep Pure Plant Kit (polysaccharides &
204 polyphenolics-rich) (Tiangen, Beijing, China). Before reverse transcription reaction total RNA was treated
205 with DNase I (Tiangen, Beijing, China) to remove potential genomic DNA contamination. One microgram
206 of total RNA was reverse transcribed using PrimeScriptTM RT Reagent Kit (TaKaRa, Japan) and a mix of
207 oligo $(dT)_{12\text{-}18}$ primer plus random hexamers in a total volume of 20 µL. The RT products were diluted
208 three folds before serving as qPCR template. qPCR was performed in a total volume of 20 µL using a
209 SYBR Premix Ex TaqTMII (TliRNaseH Plus) kit (TaKaRa, Dalian, China) and CFX ConnectTM Optics Module
210 (Bio-Rad, USA) according to the manufacturer's instructions. Each reaction was performed in triplicate
211 along with an internal control reaction. Relative gene expression levels were calculated according to the
212 $2^{-\Delta\Delta Ct}$ comparative CT method (Livak and Schmittgen, 2001). Papaya fatty acid desaturases were
213 identified through literature search, the proteins or cDNA sequences from related plant species were
214 used to query papaya genome database (https://phytozome.jgi.doe.gov/pz/portal.html). Based on
215 sequence similarity papaya orthologs were identified. Gene-specific primer pairs were designed by using
216 Primer 5.0 software (Table S1).

217 *2.10 Statistical Analysis*

218 Student t-test was performed in Excel package, three biological replicates were used, $p<0.05$ was
219 set as statistically significant.

3. Results

3.1 Papaya cell membrane was sensitive to cold stress

When observed under transmission electron microscope, the plasma membrane from cold-treated plants showed pronounced invagination (Fig. 1A and 1B). The chloroplast and mitochondria structures also were obviously disrupted by cold treatment. Under 28 °C chloroplasts exhibited oval shape with regular grana stacks and thylakoid membranes, transit starch granules were in the size of 5 μm (Fig. 1C); after 10 °C treatment chloroplast became swollen with markedly reduced grana stacks and unstacked stroma thylakoid membranes; starch granules became two folds size of control (Fig. 1D). Potato and tobacco chloroplasts showed similar damage after cold hardening (Popov et al., 2016; Tishel and Maxelis, 1966). In potato, the membrane-bound Pi-triose translocators responsible for removing the osmotically active degradation products could be deactivated by low which is, while the starch degrading enzymes could remain active, this would lower the water potential of the chloroplast stroma and result in an influx of water from the cytoplasm, thus cause chloroplast swelling (Heldt and Rapley, 1970). However, papaya leaf transit starch was accumulated to higher levels after cold treatment (Fig. 1C and 1D). This raise the possibility that cold stress induce papaya leaf starch accumulation through different mechanisms from potato: papaya leaf starch degrading enzymes may be deactivated by cold stress , or the respiration rate was reduced to larger degree compared to photosynthesis, meanwhile the phloem transport could be inhibited by cold, sugar accumulate in chloroplast, this will enhance starch synthesis and chloroplast swollen(Fig. 1C and 1D). For mitochondria, under 28 °C they showed round shape with dense matrix and tubular cristae; after cold treatment mitochondria became swollen, dense particles aggregated in stroma (Fig. 1E and 1F). These structural changes suggested that papaya cell membranes are sensitive to cold stress. After the chilling-treated plants moved back to 28 °C all of

them successfully recovered without obvious leaf lesion, suggesting that these structural changes are reversible.

3.2 Total leaf fatty acid composition was slightly altered by cold stress

Seven major fatty acids (FAs) were identified from total leaf lipids, including palmitic acid (16:0), palmitoleic acid (16:1), hexadecatrienoic acid (16:3), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3). The proportion of 18:3 accounted for ~60 mol% of total fatty acid, followed by 16:0 and 18:2, 16:3 was detected at trace amounts (Fig. S1 A). Thus, papaya is a typical 18:3 plant species. Compared to 28 °C growth temperature the proportion of 18:3 showed a small but significant reduction by cold treatment, the proportions for other fatty acids were not significantly affected (Fig. S1 A). As a result, following cold treatment the fatty acid double bond index (DBI) was only slightly reduced from 2.16 to 2.08 (Fig. S1 B).

3.3 Plasma membrane was highly purified from leaf tissue by phase partitioning

The bulk leaf fatty acid changes above may not exactly reflect what is happened from plasma membrane (Fig. S1.). To measure the plasma membrane lipid compositional changes, plasma membrane was first purified from papaya leaves by using aqueous dextran-polyethylene glycol two-phase partition systems. H^+-ATPase was used as marker to evaluate the purity of plasma membrane. H^+-ATPase activities from purified plasma membrane and microsomal fraction was measured before and after vanadate or KNO_3 addition, the data was used to calculate the purity of the isolated plasma membrane. We demonstrated that about 75% plasma membrane was successfully recovered from microsomal fraction; the purity of the prepared plasma membrane reached 90%, with 8% contaminant from vacuole (Table 1).

3.4 *The compositions of polar lipids, neutral lipids and glycolipids were affected differently by cold stress*

264 The total lipids isolated from purified plasma membrane were fractioned into PLs, NLs, and GLs and
265 analyzed individually. Under 28 °C growth temperature the fatty acids from PLs were constituted of 16:0
266 (40.0 mol%), 18:0 (8.5 mol%), 18:1 (6.4 mol%), 18:2 (28.9 mol%) and 18:3 (16.4 mol%). The saturated
267 fatty acids (16:0 and 18:0) accounted for almost half of the total fatty acids, unsaturated fatty acids
268 (18:1+18:2+18:3) accounted for the rest half (Fig. 2A). In contrast, papaya total leaf lipids were
269 dominated by polyunsaturated 18:3 (60 mol%) followed by saturated 16:0 (18 mol%) (Fig. S1 A, Fig. 2A).
270 These discrepancies suggest that other subcellular compartments are dominated by polyunsaturated
271 fatty acid 18:3. The proportions of 16:0 and 18:0 FAs significantly reduced by cold treatment, while the
272 proportion of 18:2 increased at the expense of 18:3 reduction (Fig. 2A). As a result, following cold
273 treatment the double bond index of PLs increased from 1.13 to 1.25 (Fig. 2B). Pineapple, another cold
274 sensitive crop, Interestingly, following cold treatment the proportion of 16:0 and 18:3 from its fruit
275 membrane showed similar decline trends as papaya leave, while the proportion of 18:2 showed
276 opposite changes between these two plants. This likely due to the facts that the proportion of
277 unsaturated fatty acids account for more than 90 mol% total fatty acids on the pineapple PM (Zhou et
278 al., 2014).

279 The NLs fraction contains three major free sterols (FS)- campesterol, stigmasterol, and β-sitosterol.
280 Under 28 °C growth temperature, the proportion of β-sitosterol accounted for 60 mol% of total FS,
281 while campesterol and stigmasterol attributed about 20%, respectively. Following cold treatment, the
282 proportion of β-sitosterol slightly increased at the expense of stigmasterol reduction, while the
283 proportion of campesterol was not affected (Fig. 2C). However, the absolute contents of FS were
284 significantly increased by cold treatment (Fig. 2D).

285 β-sitosterol was the principal steryl moieties of SG and ASG and accounted for more than 70 mol%
286 of total sterols combined. Following cold treatment, the proportion of campesterol-containing SG and

ASG increased at the expense of β-sitosterol-containing SG and ASG reduction, the proportion of stigmasterol-containing SG and ASG was not affected (Fig. 2E).

3.5 Lipidomic analysis identified PM lipid species that were responsive to cold stress

The data above only offered PM lipid changes at group levels, to examine cold-induced PM remodeling at molecular resolution total lipids isolated from the purified PM were also analyzed by TQS-based lipidomic platform. In total, 156 PLs, 31 SG and ASG, and 20 cerebrosides (CER) were detected (Table S2-S3).The PLs detected from purified PM included MGDG, DGDG, PG, lysoPG, PC, lysoPC, PE, lysoPE, PI, PS, and PA (Table S2-S3). MGDG and DGDG are established plastid lipids and were also reported from purified PM fraction (Andersson et al., 2003; Mongrand et al., 2004). Although eukaryotic PC(32, 14/18) and PE(32,14/18) were reported from oat and rye PM (Uemura and Steponkus, 1994), 14C fatty acid was not detected from papaya PM phospholipid fraction (Fig. 2), suggesting that such eukaryotic PC32 and PE32 were not present from papaya leaf. PG(32, 16/16), PC(32, 16/16), PE(32, 16/16), PI(32, 16/16) and PA(32, 16/16) were synthesized through prokaryotic pathway, their presence from PM likely represent contaminations from other subcellular compartments during PM purification. Collectively these lipids accounted for 8.7 mol% and 6.9 mol% of total PM polar lipids from plants grown at 28 °C and 10 °C, respectively (Table S2-S3). These data were in accordance with the PM purity assessment by H^+-ATPase (Table 1). One interesting feature about papaya plasma membrane is its high lipid to protein ratio (9.7 μmol mg^{-1} protein at 28 °C growth temperature); cold stress did not affect this ratio (9.6 μmol mg^{-1} protein).

At 28 °C, GLs accounted for 85.0 mol% of the total PM lipids followed by PLs (12.9 mol%) and FS (2.1 mol%) (Fig. 3A and 3B). Cold stress did not significantly affect the overall PM lipid composition (Fig. 3B). However, the absolute contents of FS increased more than 15 folds by cold stress (Fig. 3A).

For PM phospholipids, PC and PE accounted for 43 mol% and 40 mol%, followed by PA (6.3 mol%), PI (5.0 mol%), PS (3.1 mol%), and PG (1.0 mol%). In addition, trace amounts of LysoPG, LysoPC, and LysoPE also were detected (Fig. 4A and 4B). Following cold treatment, the proportion of PC significantly decreased and compensated by the increase of PG, PE, and PA. The proportions of other PLs were not significantly affected (Fig. 4B). For the PM of pineapple fruit, cold storage resulted in the increase of PA and decrease of PI while other PLs unaffected (Zhou et al., 2014), suggesting that PMs from different cold sensitive plants could leverage similar and unique strategies to cope with cold stress.

The lipid species responsible for these changes were further examined at individual molecular level. The decrease in total PC was contributed exclusively by the reduction of mono-unsaturated species PC(34:2, 16:0/18:2) and PC(34:3, 16:0/18:3). Meanwhile, mono-unsaturated PC(34:1, 16:0/18:1) and di-unsaturated PC(36:6, 18:3/18:3) were significantly increased by cold stress (Table S2-S3). These data suggested that cold stress reduce the desaturation of PC(34:1) to PC(34:2). Following cold stress the total proportion increase of PG was attributed to the higher mono-unsaturated PG(34:2, 16:0/18:2) and PG(34:3, 16:0/18:3). For PE, the proportion increase was ascribed to higher mono-unsaturated PE(34:3, 16:0/18:3) and PE(36:3, 18:0/18:3) as well as di-unsaturated PE(36:6, 18:3/18:3) (Table S2). For PA, the proportion increase was attributed to higher mono-unsaturated PA(34:2, 16:0/18:2) and PA(34:3, 16:0/18:3); in addition, di-unsaturated PA(36:4, 18:1/18:3), PA(36:5, 18:2/18:3), and PA(36:6, 118:3/18:3) also made additional contribution (Table S2-S3). Since the increase of PA(34:2) and PA(34:3) was accompanied by the decrease of PC(34:2) and PC(34:3) (Fig. 5A and 5B), raise the possibility that PA could be synthesized through PC hydrolysis during cold stress.

CER was the dominant GLs and accounted for 91mol% of total GLs while SG and ASG contribute the rest 9 mol% (Fig. 4C and 4D). Most CERs contain hydroxyl fatty acids. The major acyl chains of ASG (esterified at the 6-O position of glucose) were 16:0 (51.4 mol%), 18:2 (22.7 mol%), 18:3 (12.1 mol%),

18:1(7.0 mol%), and 18:0 (6.6 mol%). The pairing of acyl chains and sterol moieties demonstrated that β-sitosterol was dominant in all ASG subspecies (Table S2-S3). Cold stress did not significantly affect GLs content or composition (Fig. 4C and 4D), although three CERs (42:0, 42:1, and 42:2) were significantly reduced by cold stress (Table S2).

3.6 The expressions of papaya putative fatty acid desaturases were differentially affected by cold stress

To understand the molecular base underlying cold induced PM lipidome remodeling, papaya putative fatty acid desaturases, including *CpFAD2*, *CpFAD4*, *CpFAD5*, *CpFAD6*, *CpFAD8*, *CpFAB2-1*, and *CpFAB2-2* were identified from papaya genome, their transcriptional levels at 28 °C and 10 °C growth temperature were quantified by RT-qPCR. We found that the transcriptional levels of *CpFAD4*, *CpFAD6*, and *CpFAB2-2* were significantly up-regulated by cold stress, while the expressions of the rest desaturases were not affected (Fig. 5). FAD4 and FAD6 are plastid-localized desaturases (Falcone et al., 1994; Gao et al., 2009), their up-regulation suggested potential increase of the desaturation levels from plastid membrane by cold stress although this is out of the focus for this study.

4. Discussion

4.1 Papaya leaf PM lipid is different from other characterized plant species

Compared cold-hardy plant such as *Arabidopsis* and rye, papaya leaf PM was characterized by its high levels of hexacerebrosides (78 mol% from normal grow sample) and low levels of free sterols (2.2 mol%) (Fig. 3; Fig. 4C and 4D); in contrast, rye PM was enriched with medium level of hexacerebrosides (16 mol%) and high level of free sterols (32 mol%) (Lynch and Steponkus, 1987); Arabidopsis PM was dominated by PLs (46.8 mol%) and FS (37.7 mol%) (Uemura et al., 1995). The acyl chain of ASG in papaya PM was dominated by saturated 16:0 (Table S2). Although there are substantial differences in the proportion of free (FS) and glucosylated forms of sterols (SG and ASG) in the plasma membrane of plant

species (Palta et al., 1993; Uemura and Steponkus, 1994; Yoshida and Uemura, 1984), their proportion is rather low in papaya PM compared to orchard, Arabidopsis, or potato (Fig. 3; Fig. 4C and 4D).

Another feature regarding papaya PM is its relative low level of PLs (12.9 mol%) compared to rye (32 mol%) or Arabidopsis (46.8 mol%), which may associate with its cold sensitivity. The calculation of 34 C to 36C ratios from various PLs indicated that papaya plasma membrane PGs are dominated by PG34, followed by PI; in contrast, PC, PE, PS and PA contain similar proportion of 34C and 36C (Fig. S2). In addition, papaya leaf PM phospholipids were highly enriched with saturated fatty acids compared to other subcellular compartments (Fig. 2, Fig. S1). The proportions of mono-unsaturated, di-unsaturated, and di-saturated lipid within individual PLs were calculated, mono-unsaturated PLs dominate each PM phospholipid (Fig. 6). The lower PL levels and the higher proportion of mono-unsaturated lipid species of papaya PM would make it more sensitive to cold stress.

4.2 Low temperature-induced papaya plasma membrane lipidome remodeling reveal its inherent sensitivity to cold stress

Following cold acclimation free sterols in rye PM increased and compensated by the reduction of SG plus ASG (Lynch and Steponkus, 1987), suggesting cold may inhibit UDP-glucose:sterol glucosyltransferase and acyl transferase activities in rye. In contrast, cold stress significantly increases the FS content without affecting the SG and ASG (Fig.2D; Fig. 4C and 4D). This suggested that papaya leverage different mechanisms as rye to regulate its FS contents. One possibility is that cold stress could activates papaya MVA pathway, thus more carbon was channeled into sterol synthesis; meanwhile, cold stress did not affect UDP-glucose:sterol glucosyltransferase and acyl transferase activities which catalyze the synthesis of SG and ASG from FS.

Plasma membrane CER content have been associated with cold sensitivity of plants. To support this notion, rye PM contains intermediate high level of CER, and is progressively decreased during cold

acclimation (Uemura and Steponkus, 1994). Arabidopsis PM contains low proportion of CER, and reduced by cold treatment (Uemura et al., 1995). These data suggested that reducing plasma membrane CER level could be beneficial for plant adaptation to cold. Papaya and oat are cold sensitive, their plasma membrane contains high proportion of CER, which were not reduced by cold(Fig. 4C and 4D), thus, their fitness under cold may not be improved through a period of cold exposure.

Higher PL contents are regarded as an important contributor to cold tolerance in plants, and the capacity of PL synthesis under low temperature is an important aspect to acquire cold tolerance (Ishikawa and Yoshida, 1985; Uemura and Yoshida, 1984; Uemura and Steponkus, 1994; Yoshida and Uemura, 1986). In papaya, the proportion increase of PG, PE, and PA were compensated with the proportion of PC decrease (Fig. 3, Fig. 4A and 4B),the total proportion of PLs from papaya PM kept unchanged before after cold stress treatment (Fig. 3). Since each PLs class show different physicochemical features, individual PL compositional change could show diverse effects on PM stability. The decrease in the proportion of PC increases the intrinsic curvature of the constituent monolayers and the propensity for freezing-induced the H_{II} phase formation. PE in the plasma membrane is most prone to undergo the lamellar-to-H_{II} phase transition, its proportion increase will facilitate the H_{II} phase formation. PA has been shown to promote negative curvature in bi-layer membranes and increase membrane permeability in membrane vesicles *in vitro* (Berglund et al., 2001; Kooijman et al., 2003). High content of PA in PM was found to destabilize membranes, resulting in loss of both membrane integrity and functionality of membrane proteins during wounding, senescence, freezing and drought (Testerink & Munnik, 2011). Thus, these cold-induced compositional remodeling of PLs in papaya PM unlikely improve its cold and freezing tolerance.

The increase of unsaturation of PLs also play important roles for cold tolerance. In rye and oat, the increase in PL during cold acclimation is associated with an increase in the proportion of di-unsaturated

species of PC and PE and a corresponding decrease in mono-unsaturated species (Uemura and Steponkus, 1994). For papaya PM, cold stress increased not only the proportions of di-unsaturated PC, PI and PA but also the proportions of mono-unsaturated PG, PE, PI and PS, and compensated by the decrease of di-saturated PG, PC, PI and PA (Fig. 6). These changes in the unsaturation levels of PLs would be beneficial for papaya cold adaptation.

CER and FS are poorly hydrated lipid classes, and PLs are highly hydrated lipid classes. The high abundance of CER and low proportion of PLs make papaya plasma membrane with lower bilayer hydration, thus lower spatial separation between bilayers at given osmotic pressure. Following cold treatment, the proportion of PLs and CER kept unchanged, and FS contents were significant increased (Fig. 3A), these characteristics would facilitate the H_{II} phase formation, and make papaya membrane vulnerable to cold and freezing stress. These lipid features may also restrict their interactions with membrane proteins especially for those water-soluble proteins, this could explain why papaya plasma membrane showed such high lipid to protein ratio.

4.3 The mechanisms of PM phospholipid desaturation under cold stress

PM phospholipids are synthesized on endoplasmic reticulum through eukaryotic pathway (Li-Beisson et al., 2013), and the fatty acid precursors are synthesized and exported from plastid. The lipidomic data suggested *de novo* lipid biosynthesis play critical roles for the remodeling of PLs on papaya plasma membrane under cold stress (Fig. 7). *CpFAB2-2* encodes a plastid stearoyl-ACP Δ^9 desaturase that convert stearic acid (18:0) into oleic acid (18:1Δ^9) (Shanklin and Somerville, 1991), its up-regulation would increase the level of 18:1-CoA, and likely compensated by the levels of 16:0-CoA and 18:0-CoA reduction (Fig. 7). The re-distribution of fatty acid precursors for lipid biosynthesis in the ER could reasonably explain the observed PM lipidome remodeling by cold stress: (1) why the mono-unsaturated lipids increased compensated with di-saturated lipid species reduction (Fig. 6); (2) why the

proportion of PC(36), PE(36), PA(36), PS(36) and PI(36) increased following cold treatment (Table S2; Fig. 7); (3) why the proportion of 16:0 and 18:0 fatty acids from PL fraction reduced (Fig. 2A).

Microsomal oleic acid desaturase (FAD2) catalyzes the first extra-plastidial desaturation in plants, converting oleic acid (18:1) to linoleic acid (18:2) (Okuley et al., 1994). Following cold treatment, the transcriptional level of FAD2 was unaffected (Fig. 5); however, the proportion of 18:2 from PLs fraction increased significantly (Fig. 2A). Thus, the posttranslational rather than transcriptional regulations of FAD2 should be accountable for the higher contents of 18:2. In *Arabidopsis*, FAD2 catalytic activities are regulated by different isoforms of cytochrome b5 (Cb5) or aminophospholipid ATPase (ALA10) (Botella et al., 2016; Kumar et al., 2012). The activities of CpFAD2 could be regulated by similar mechanisms under cold temperature. Interestingly, the higher proportion of 18:2 from plasma membrane PL fraction did not led to a higher level of 18:3. On contrary, the proportion of 18:3 was reduced(Fig. 2A). These data suggested that an unidentified CpFAD3 isoform, which convert 18:2 to 18:3, was reduced by cold stress.

5. Conclusions

In this study, we isolated highly purified plasma membrane from papaya seedlings grown under normal temperature and cold temperature and performed lipidomic analysis. We uncovered papaya's characteristics plasma membrane lipid composition and its lipidome remodeling under cold stress. By combining the simultaneous gene expression and pathway analysis, we found that the transcriptional regulation of *CpFAB2-2* and the posttranslational regulation of CpFAD2 activities could play critical roles for the plasma membrane remodeling of PLs by cold stress. This work offered us with much needed knowledge base for the genetic improvement of cold tolerance in papaya.

Author contributions

M.C., RL, X.H. and Z.D. carried out experiments; R.L., M.C., S.H. and W.Z. analyzed data; M.C. designed the experiments; M.C. and R.L. wrote the manuscript; W.Z. helped with manuscript editing. All authors contributed to manuscript revision, read and approved the submitted version. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Supplementary data

Figure S1. Papaya leaf fatty acid compositional changes under cold stress. (A) and (B) Papaya leaf fatty acid composition and double bond index under 28 °C and 10 °C growth temperature. Data were expressed as mean ± stand deviation (n = 3). Asterisk indicate statistically significant ($p<0.05$).

Figure S2. The ratio of 34C to 36C for each phospholipid class under 28 °C and 10 °C growth temperature. Data were expressed as mean ± stand deviation (n = 3). Asterisk indicate statistically significant ($p<0.05$).

Table S1. Primer sequence for papaya fatty acid desaturases and control gene

Table S2. The mol % of papaya plasma membrane lipids grown under 28 °C and 10 °C

Table S3. The Plasma membrane lipid contents grown under 28 °C and 10 °C (nmol per mg protein)

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598

599 **Figure captions**

600 **Fig. 1.** Cold stress affect ultrastructure of papaya leaves. pm: plasma membrane; c: chloroplast; m:
601 mitochondria; s: starch granule. Bar = 10 μm (A-D), bar = 4 μm (E-F).

602 **Fig. 2.** The fatty acid composition and double bond index of phospholipids (A, B), free sterols
603 composition and contents (C, D) and glycolipid composition (E) of papaya leaf plasma membrane under
604 normal and low growth temperature. FS, free sterol; GL, glycolipids. Data were expressed as mean ±
605 stand deviation (n = 3). Asterisk indicate statistically significant ($p<0.05$).

606 **Fig. 3.** Phospholipid, glycolipid and free sterol contents (A) and composition (B) from papaya leaf plasma
607 membrane. Data were expressed as mean ± stand deviation (n = 3). Asterisk indicate statistically
608 significant ($p<0.05$).

Fig. 4. The compositional changes of phospholipids and glycolipids fractions from plasma membrane under normal and chilling temperature. Data were expressed as mean ± stand deviation (n = 3). Asterisk indicate statistically significant ($p<0.05$).

Fig. 5. Cold induced the expression of CpFAD4, CpFAD6 and CpFAB2-2. The gene transcriptional levels were quantified by RT-qPCR. Data were expressed as mean ± stand deviation (n = 3). Asterisk indicate statistically significant ($p<0.05$).

Fig. 6. Cold stress affected the proportion of mono-unsatureated, di-unsatureated and di-saturated lipid species from each phospholipid class. Data were expressed as mean ± stand deviation (n=3). Asterisk indicate statistically significant ($p<0.05$).

Fig. 7. A proposed regulation model of papaya plasma membrane phosopholipid biosynthesis under low temperature. Red arrow indicates higher levels at 10 °C than that of 28 °C; blue arrow indicates lower levels at 10 °C than that of 28 °C; dashed line represents projected metabolite changes.

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632 **Table 1.** H^+-ATPase activities in microsomal and plasma membrane fractions

	subcellular compartment	microsomal	plasma membrane
total activities	-	466.8 ± 1.6	349.5 ± 3.2
the activities after VO_4^{3-} addition	plasma membrane	202.1 ± 8.8	35.7 ± 9.6
the activities after NO_3^- addition	vacuole	395.3 ± 5.5	321.6 ± 26.4
the activities from plasma membrane		264.7 ± 7.2	313.8 ± 10.4
the activities from vacuole		71.5 ± 6.9	27.9 ± 23.3

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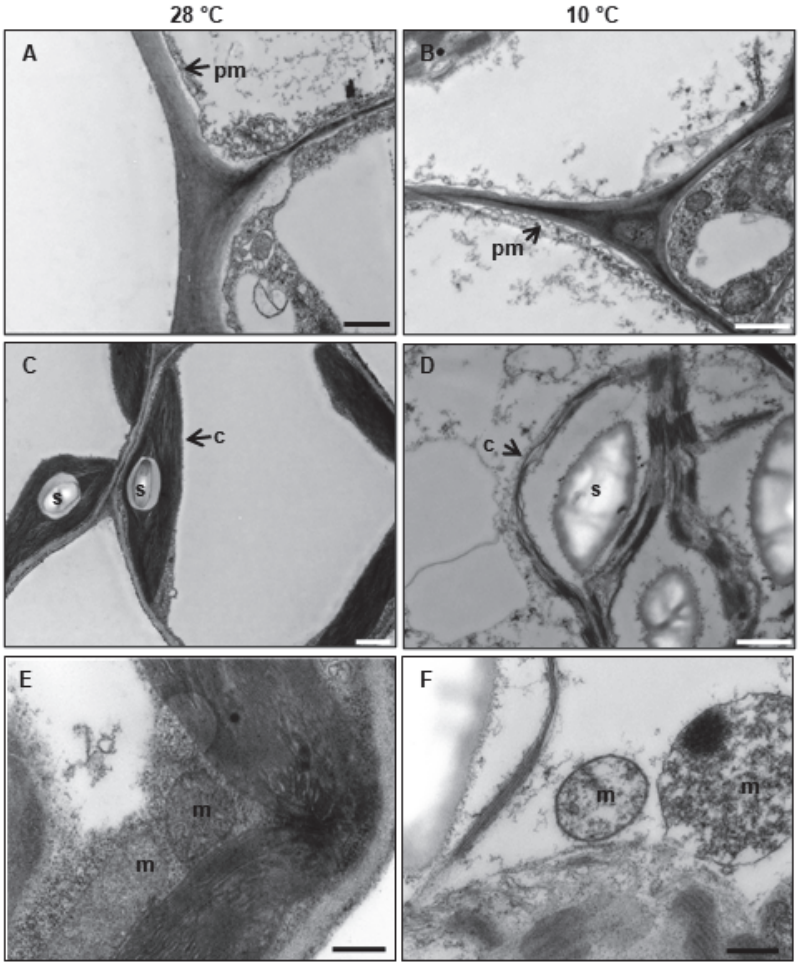

Fig. 1. Cold stress affect ultrastructure of papaya leaves. pm: plasma membrane; c: chloroplast; m: mitochondria; s: starch granule. Bar = 10 µm (A-D), bar = 4 µm (E-F).

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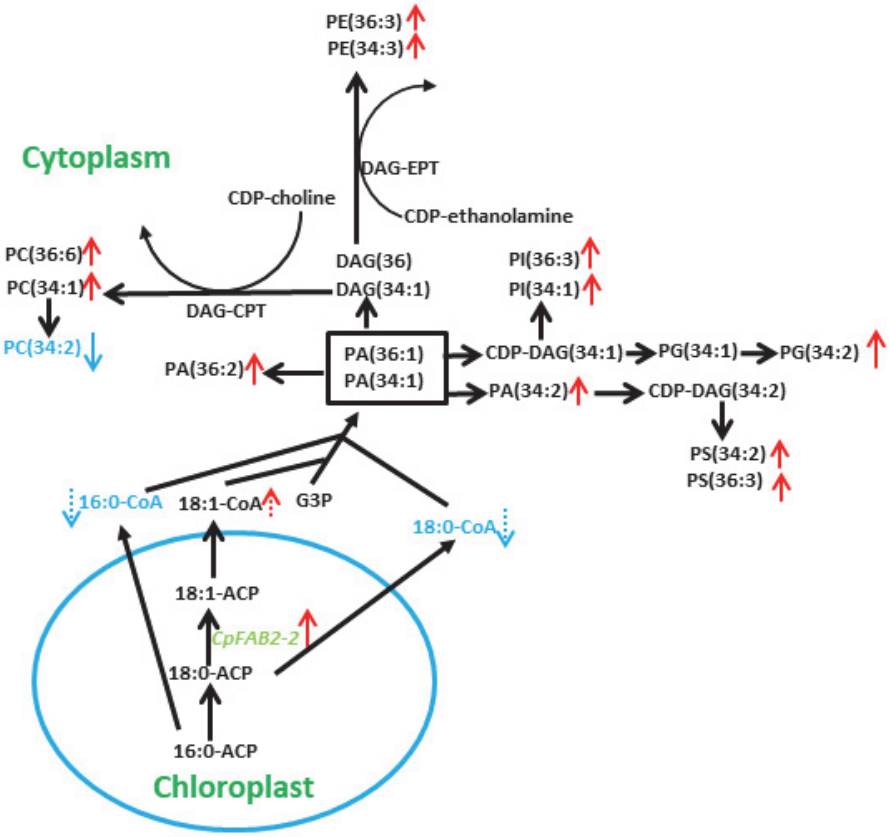

Fig. 7. A proposed regulation model of papaya plasma membrane phosopholipid biosynthesis under low temperature. Red arrow indicates higher levels at 10 °C than that of 28 °C; blue arrow indicates lower levels at 10 °C than that of 28 °C; dashed line represents projected metabolite changes.

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