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Differential lipidome remodeling during postharvest of peach varieties with different susceptibility to chilling injury

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Peaches ripen and deteriorate rapidly at room temperature. Therefore, refrigeration is used to slow these processes and to extend fruit market life; however, many fruits develop chilling injury (CI) during storage at low temperature. Given that cell membranes are likely sites of the primary effects of chilling, the lipidome of six peach varieties with different susceptibility to CI was analyzed under different postharvest conditions. By using liquid chromatography coupled to mass spectrometry (LC–MS), 59 lipid species were detected, including diacyl- and triacylglycerides. The decreases in fruit firmness during postharvest ripening were accompanied by changes in the relative amount of several plastidic glycerolipid and triacylglyceride species, which may indicate their use as fuels prior to fruit senescence. In addition, levels of galactolipids were also modified in fruits stored at 0°C for short and long periods, reflecting the stabilization of plastidic membranes at low temperature. When comparing susceptible and resistant varieties, the relative abundance of certain species of the lipid classes phosphatidylethanolamine, phosphatidylcholine and digalactosyldiacylglycerol correlated with the tolerance to CI, reflecting the importance of the plasma membrane in the development of CI symptoms and allowing the identification of possible lipid markers for chilling resistance. Finally, transcriptional analysis of genes involved in galactolipid metabolism revealed candidate genes responsible for the observed changes after cold exposure. When taken together, our results highlight the importance of plastids in the postharvest physiology of fruits and provide evidence that lipid composition and metabolism have a profound influence on the cold response.

Introduction

Peach (*Prunus persica* L. Batsch) is a climacteric fruit that ripens after harvest, prior to human consumption. While ripening prepares fruit for human consumption, marketing and shipping require lengthy storage periods. Refrigeration is used to slow ripening and to extend fruit market life; however, several fruits can develop chilling injury (CI) during storage at low temperature. CI is genetically influenced and triggered by a combination of the

Abbreviations – CI, chilling injury; CS, cold-stored fruits; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; EL, Elegant Lady; FD, Flordaking; H, harvested fruits; LM, Limón Marelli; MGDG, monogalactosyldiacylglycerol; R2, Rojo 2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; RG, Red Globe; SL, Spring Lady; RS, room temperature-stored fruits; TAG, triacylglycerol.

temperature and length of storage (Lurie and Crisosto 2005). In peach, CI includes internal and external browning, flesh breakdown, woolliness, reddish discoloration, loss of ability to ripen and increased incidence of decay when stored for more than 2–3 weeks at temperatures below 8°C (Lurie and Crisosto 2005).

The comprehension of the complex molecular events that underlie the response to cold treatment leading to CI in peach fruit has dramatically increased since the application of the so-called -omics strategies. For example, transcriptomic studies following cold storage using single or contrasting genotypes with differential susceptibility to cold have allowed the identification of part of the molecular mechanisms involved in peach cold tolerance (González-Agüero et al. 2008, Ogundiwin et al. 2008, Vizoso et al. 2009, Dagar et al. 2013, Pons et al. 2015). In addition, proteomic studies have also identified key proteins involved in the protection to cold or in the development of CI symptoms (Lara et al. 2009, Nilo et al. 2010, Almeida et al. 2016). Recently, an analysis of primary metabolism using gas chromatography–mass spectrometry (GC–MS) indicated that differential metabolic rearrangements due to cold in different peach varieties are related with CI resistance, while candidate biomarkers of resistant/susceptibility to this disorder were also identified (Bustamante et al. 2016). To date, however, there are no reports using large-scale lipidomics approaches to investigate the influence of low temperatures on peach fruit lipid composition.

Dysfunction of cell membranes at low temperature is considered to be one of the major molecular events leading to the development of CI symptoms. Normal functioning of integral cell membrane depends on the fluidity of the membrane, which is strongly influenced by its lipid composition. A higher proportion of unsaturated fatty acids in lipids generally lead to a lower incidence of CI (Marangoni et al. 1996). In peach fruit, it appears that a higher unsaturation degree of membrane lipid and *N*-acylphosphatidylethanolamine accumulation are beneficial for maintaining membrane fluidity, leading to an enhanced tolerance of peach fruit to chilling stress (Zhang and Tian 2010). In the present work, the differential remodeling of the lipidome during the postharvest of peach varieties with contrasting susceptibility to CI was investigated. These varieties have been previously characterized in detail (Bustamante et al. 2016, Genero et al. 2016, Monti et al. 2016), allowing the question of how variation in chilling tolerance between these fruits is reflected in their lipid composition. In addition, the common changes in the lipidome of the six varieties were also analyzed after storage of fruits at ambient or low temperature, providing information about the general responses to normal ripening and low temperature stress, respectively. Finally, the relative expression levels of transcripts encoding enzymes involved in the metabolism of key lipids revealed part of the regulatory mechanisms involved in the response of fruits to cold.

Materials and methods

Fruit material and postharvest treatments

Assays were conducted with peach (*P. persica* L. Batsch) fruit of six different varieties (Flordaking, Rojo 2, Spring Lady, Red Globe, Elegant Lady and Limón Marelli) grown in the Estación Experimental Agropecuaria INTA, San Pedro, Argentina. The principal agronomic characteristics of each variety and the fruit quality parameters are described in Monti et al. (2016), Bustamante et al. (2016), Tables S1 and S2. Fruits were collected at S4 stage (Lombardo et al. 2011) and manually selected for uniformity of color, size and firmness, and divided into four groups according to Bustamante et al. (2016) (Fig. S1). Representative mesocarp tissue was collected from at least 20 fruits from the different groups, immediately frozen in liquid nitrogen and stored at −80°C for further experiments. The results shown in the present work correspond to fruits collected during the 2009/2010 season, although similar results were obtained for some peach varieties grown during 2011/2012.

Lipid extraction and LC–MS analysis

The lipid extraction method was performed as described by Giavalisco et al. (2011). Briefly, mesocarp tissue of peach fruits (n = 5) was ground using ceramic mortar and pestle pre-cooled with liquid nitrogen, and 25 mg of the powder was used for lipid extraction using 1 ml of methanol: methyl tert-butyl ether: water (1:3:1) mixture. After incubation in 4°C and sonication for 10 min in an ice-cooled sonic bath, 500 ml of water: methanol (3:1) mixture was added. This led to the formation of two phases: a lipophilic phase and a polar phase. The lipophilic phase was collected, vacuum-dried and stored at −80°C until used.

The dried lipid extracts were re-suspended in 500 µl buffer B (see below) and transferred to a glass vial. Two microliters of this sample were injected on a C8 reversed phase column (100 mm × 2.1 mm × 1.7 µm particles waters), using a Acquity UPLC system (Waters, Milford, MA). The two mobile phases were water with 1% 1 *M* NH_4Ac and 0.1% acetic acid (Buffer A), and acetonitrile:isopropanol (7:3) containing 1% 1 *M* NH_4Ac

and 0.1% acetic acid (Buffer B). The gradient separation, which was performed at a flow rate of 400 µl min^{-1}, was: 1 min 45% A, 3 min linear gradient from 45 to 35% A, 8 min linear gradient from 35 to 11% A, 3 min linear gradient from 11 to 1% A. After washing the column for 3 min with 1% A the buffer was set back to 45% A and the column was re-equilibrated for 4 min. Mass spectra were acquired, in positive and negative ionization modes, using an Exactive mass spectrometer (Thermo-Fisher, Carlsbad, CA) using altering full scan and all-ion-fragmentation scan mode, covering a mass range from 100 to 1500 m/z. The spectra were recorded from 1 to 17 min of the UPLC gradients. Processing of chromatograms, peak detection and integration were performed using REFINER MSH 10 (GeneData, Basel, Switzerland). Processing of mass spectrometry data included the removal of the fragmentation information, isotopic peaks and chemical noise. Selected features were annotated using an in-house lipid database.

Fatty acid extraction and GC-FID analysis

For GC-FID (gas chromatography flame ionization detection) analysis, mesocarp tissue was ground using ceramic mortar and pestle pre-cooled with liquid nitrogen, and 500 mg of the powder was resuspended in 1 ml of 1 N HCl in methanol. An internal standard (100 µl of FA15:0, pentadecanoic acid) was added to each sample before incubation at 80 °C in a water bath for 30 min. After cooling to room temperature, 1 ml of 0.9% NaCl and 1 ml of 100% hexane were added to each vial. Vials were shaken for 5 s and centrifuged for 4 min at 21000*g*. The upper FAME-containing hexane phase was transferred to a new glass vial, where it was concentrated in a stream of N_2. Finally, FAMEs were dissolved in hexane and filled into GC glass vials. The details of the GC-FID method are as follows: injector temperature of 250 °C; helium carrier gas; head pressure 25 cm s^{-1} (11.8 psi); GC column, J&W DB23 (Agilent, Santa Clara, CA), 30 m × 0.25 mm × 0.25 µm; detector temperature 250 °C; detector gas H_2 40 ml min^{-1}, air 450 ml min^{-1}, He make-up gas 30 ml min^{-1}.

The double bond index (DBI), a measure of the membrane lipid unsaturation, was calculated according to Skoczowski et al. (1994) as follows:

$$DBI = \frac{[\sum(\text{percentage of fatty acid content} \times \text{number of double bond})]}{100}$$

RNA isolation and reverse transcription

Total RNA was isolated from 4 g of peach mesocarp using the method described by Meisel et al. (2005). The integrity of the RNA was verified by agarose electrophoresis. The quantity and purity of RNA were determined spectrophotometrically. First-strand cDNA was synthesized from 3 µg of RNA with MoMLV-reverse transcriptase following the manufacturer's instructions (Promega, Madison, WI) using oligo(dT) primer.

Quantitative real-time PCR

Relative expression was determined by performing quantitative real-time PCR (qRT-PCR) in an iCycler iQ detection system with the Optical System Software version 3.0a (Bio-Rad, Hercules, CA), using the intercalation dye SYBRGreen I (Invitrogen, Carlsbad, CA) as a fluorescent reporter, with 2.5 m*M* $MgCl_2$; 0.5 µ*M* of each primer and 0.04 U μl^{-1} of GoTaq Polymerase (Promega, Madison, WI). PCR primers were designed based on peach fruit cDNA sequences published in GDR Genome Database for Rosaceae (http://www.rosaceae.org) and Phytozome (https://phytozome.jgi.doe.gov), using "primer3" software (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) in order to produce amplicons of 141–241 bp in size (Table S3). A 10-fold dilution of cDNA obtained as described above was used as template. PCR controls were performed in the absence of added reverse transcriptase to ensure RNA samples were free of DNA contamination. Cycling parameters were as follows: initial denaturation at 94 °C for 2 min; 40 cycles of 96°C for 10 s, 58°C for 15 s and 72°C for 1 min; and 72°C for 10 min. Melting curves for each PCR were determined by measuring the decrease of fluorescence with increasing temperature (from 65 to 98°C). The specificity of the PCRs was confirmed by melting curve analysis as well as by agarose gel electrophoresis of the products. Relative gene expression was calculated using the 'Comparative $2^{-\Delta\Delta CT}$' method (Livak and Schmittgen 2001) and DNA-repair enzyme (*PpDNArep*) as reference gene (Genero et al. 2016). Each RNA sample was run in triplicate and repeated at least with two independent sets of treatments generating a total of 6 replicates per gene per sample.

Statistical analysis

Levels of individual lipid species were normalized to the total ion count (TIC) of each sample and also across the day of measurement to reduce batch effect (Bromke et al. 2015); the resulting data matrix was used for further analysis. Principal component and hierarchical clustering analysis of log_2-transformed data and correlation analysis between lipids based on Pearson correlation were conducted using the software package XLSTAT (Microsoft Excel). Data presented were analyzed using two way

analysis of variance (ANOVA) with storage temperature and genotype as factors.

Results

Peach varieties characteristics and postharvest treatments

Six peach varieties with different agronomic characteristics (Table S1, Monti et al. 2016, Bustamante et al. 2016) were selected in the present work: Flordaking (FD), Rojo 2 (R2), Spring Lady (SL), Red Globe (RG), Elegant Lady (EL) and Limón Marelli (LM). Fruits from each variety were collected at commercial maturity and flesh firmness between 40 to 70 N, which allowed the ending of the ripening process to take place after harvest (Monti et al. 2016). Harvested fruits (H) were stored in chambers at 20°C for 3–5 days depending on the variety, until reaching firmness and organoleptic characteristics suitable for consumption (RS fruits, Fig. S1). Another group of fruits were stored at 0°C for short time (3–5 days depending on the variety, CS fruits); or for 21 days (CS21 fruits, Fig. S1). The cold storage treatment at 0°C for 21 days was selected because it successfully discriminates peach varieties according to their different degrees of resistance/susceptibility to chilling injuries (CI) (Bustamante et al. 2016, Genero et al. 2016). After 21 days at 0°C, fruits were stored at 20°C for ripening (CS21 + RS fruits, Fig. S1). Firmness and soluble solid content were measured in fruits from all postharvest conditions (H, RS, CS, CS21 and CS21 + RS) and described in Table S2 and Bustamante et al. (2016). The amount of expressible juice measured in RS and CS21 + RS fruits (Table S2) allowed us to classify the varieties in: susceptible (FD and R2) and resistant (EL, LM, RG and SL) to CI as reported in Genero et al. (2016) and Bustamante et al. (2016). Other CI symptoms, such as flesh browning or internal reddening, were not detected in the varieties selected and the postharvest conditions applied in the present work.

Lipidomics reveals a diversity of lipids accumulated in peach fruit

Changes in peach fruit lipid composition during the postharvest were assessed by LC–MS, and the relative abundance of 59 lipid species was determined, including: phosphatidylcholine (PC) (13 species), phosphatidylethanolamine (PE) (10 species), phosphatidylglycerol (PG) (3 species), phosphatidylinositol (PI) (1 species), lyso phosphatidylcholine (lysoPC) (2 species), monogalactosyldiacylglycerol (MGDG) (3 species), digalactosyldiacylglycerol (DGDG) (6 species) and triacylglycerol (TAG) (21 species). In Fig. S2, the lipids detected reproducibly at harvest (H) in each peach variety and the peak intensities of each lipid molecular species within each class are shown.

Principal component (PCA), hierarchical clustering (HCA) and lipid-lipid correlation analysis

The changes in all lipids analyzed as a function of postharvest conditions and varieties selected suggest a fairly complex picture (Fig. 1, Table S4). The data set obtained was examined by principal component analysis (Fig. 2, Table S5), with three principal components (PC1-3) explaining 68.8% of the overall variance of the lipid profiles (34.7, 21.9 and 12.2% for PC1, PC2 and PC3, respectively). PCA separates the samples in two main groups along PC1, which divide the peach fruits depending on the postharvest condition: one group is composed by ripened fruits of each variety (RS and CS21 + RS), independently to whether the fruits were subjected to 21 days of cold storage or not; and the other is composed by H and fruits that were subjected to either short or long cold storage treatment (CS and CS21 fruits). Varieties resistant to CI (LM, EL, RG and SL) can be found in different parts of the PCA graphs. Among the lipids that most contribute to PC1 separation, higher levels of PC 34:1, PC 36:1, TAG 52:3, TAG 54:4 and TAG 54:5 are found in H and cold-stored (CS and CS21) samples; while higher levels of PC 36:3 (2) are found in ripened fruits (RS and CS21 + RS) (Fig. 2, Table S5). PC2 and all higher-order components do not contribute to separate the samples in any biologically meaningful group. The contribution of individual variables to each principal component and the compounds that contribute the most to PC1, PC2 and PC3 are shown in Table S5 and Fig. 2.

Applying HCA to the full data set also revealed interesting results (Fig. 3). The samples were separated into four main clusters, which divide the peach fruits depending on the variety and the postharvest condition. Similar to PCA, samples were separated depending on the postharvest treatment: ripened fruits (RS and CS21 + RS) group together, meanwhile H and cold-stored fruits (CS and CS21) form another group. However, in this case, SL and LM fruits cluster together and separate from FD, RG, EL and R2 varieties (Fig. 3). In addition, the HCA map obtained indicates that the peach varieties susceptible to CI (FD and R2) cluster together among the different subgroups, except in the case of CS samples (Fig. 3).

Correlation analysis performed on the entire data set of lipids of each of the six different varieties at the different postharvest treatments was also performed in order to identify associations of lipid molecular species (Fig. 4). Out of 1711 total pairs analyzed, 797 resulted in significant correlation coefficients ($P < 0.05$). Of these correlating pairs 472 were positive and 325 negative (Fig. 4).

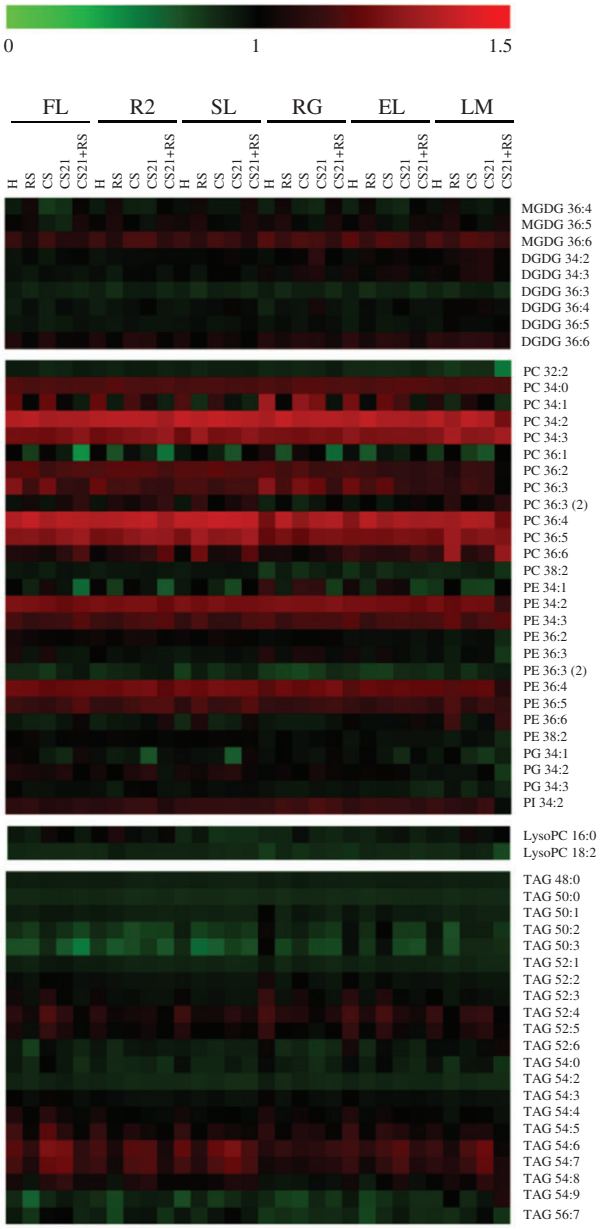

Fig. 1. Distribution of lipid species analyzed by LC–MS in peach fruits during ripening and after cold storage. The graph shows the relative level of each lipid species in the six varieties (FD, R2, SL, RG, EL and LM) subjected to different postharvest treatments (H, RS, CS, CS21 and CS21 + RS). Normalized values are shown on a color scale (shown at the top of the figure), which is proportional to the content of each lipid. Mean values of 5 independent determinations for each sample were expressed as log2 using the MultiExperiment Viewer software (MeV v4.4.1, Saeed et al. 2003). Every lipid is annotated using C x:y where x is the total carbon number and y is the total number of bound. For example, PC/PE 36:3 and PC/PE 36:3 (2) are isomers of the same lipid, they have the same sum formula and are the same lipids but the position of the double bound is different.

The TAGs, especially TAG 50:1, TAG 52:2, TAG 52:3, TAG 54:4 and TAG 54:5, showed the highest number of positive correlations within a sub-class. Negative correlations were detected between the group of TAGs and some membrane lipids, such as MGDG 36:4, MGDG 36:5, DGDG 36:4, DGDG 36:5, PC 36:3 (2) and PC 36:5. In addition, highly positive correlations between PC 36:6 and PE 36:6, PC 32:2 and PE 36:4, PC 36:3 and PE 36:3, and PC 36:1 and PE 34:1 were also found. By contrast, PC 36:3 (2) correlated negatively with PC 34:1 and TAG 52:3, and PC 36:6 displayed negative associations with PE 34:1 and PC 36:1 (Fig. 4).

Common changes in lipid composition during the postharvest ripening of the six varieties

During the postharvest ripening, some lipids showed statistically significant changes ($P < 0.05$) when comparing RS and H samples of the six peach varieties. We can find lipids that increase during ripening (ratios higher than 1); lipids that decrease during postharvest ripening (ratios lower than 1); and lipids with no significant changes when comparing H and RS samples and thus, they are not modified during fruit softening (Table S6). In all the six peach varieties, the only lipid that consistently increases is MGDG 36:4, with rises of up to 5.0-fold in RG; while MGDG 36:6, DGDG 36:3, DGDG 36:6, TAG 52:5 and TAG 54:4 significantly decrease during ripening (Table S6). In addition, the content of TAG species decreases during ripening of at least one variety, with the exception of TAG 54:9, which increases in LM (Table S6).

Common and differential changes in lipid composition after cold exposure of the six peach varieties

In order to analyze the extent to which cold treatment modifies the lipid composition and identify lipid markers that could be correlated with CI resistance in peach fruit, the relative lipid content of CS, CS21 and CS21 + RS samples was compared with that detected at harvest (H) and RS fruits in each peach variety. Table S6 shows the lipids that are significantly modified, either increased or decreased, in CS vs H, CS21 vs H, CS vs RS and CS21 + RS vs RS, for each peach fruit variety.

A general overview of the lipid composition rearrangements of peach fruits after each cold treatment shows that the lipid content alterations are dependent on the time of exposure to cold. When comparing cold-stored fruits vs H, it is evident that after 21 days at 0°C (CS21 vs H), the number of lipids that are modified is higher than after short cold treatment (CS vs H). However, if peach fruits

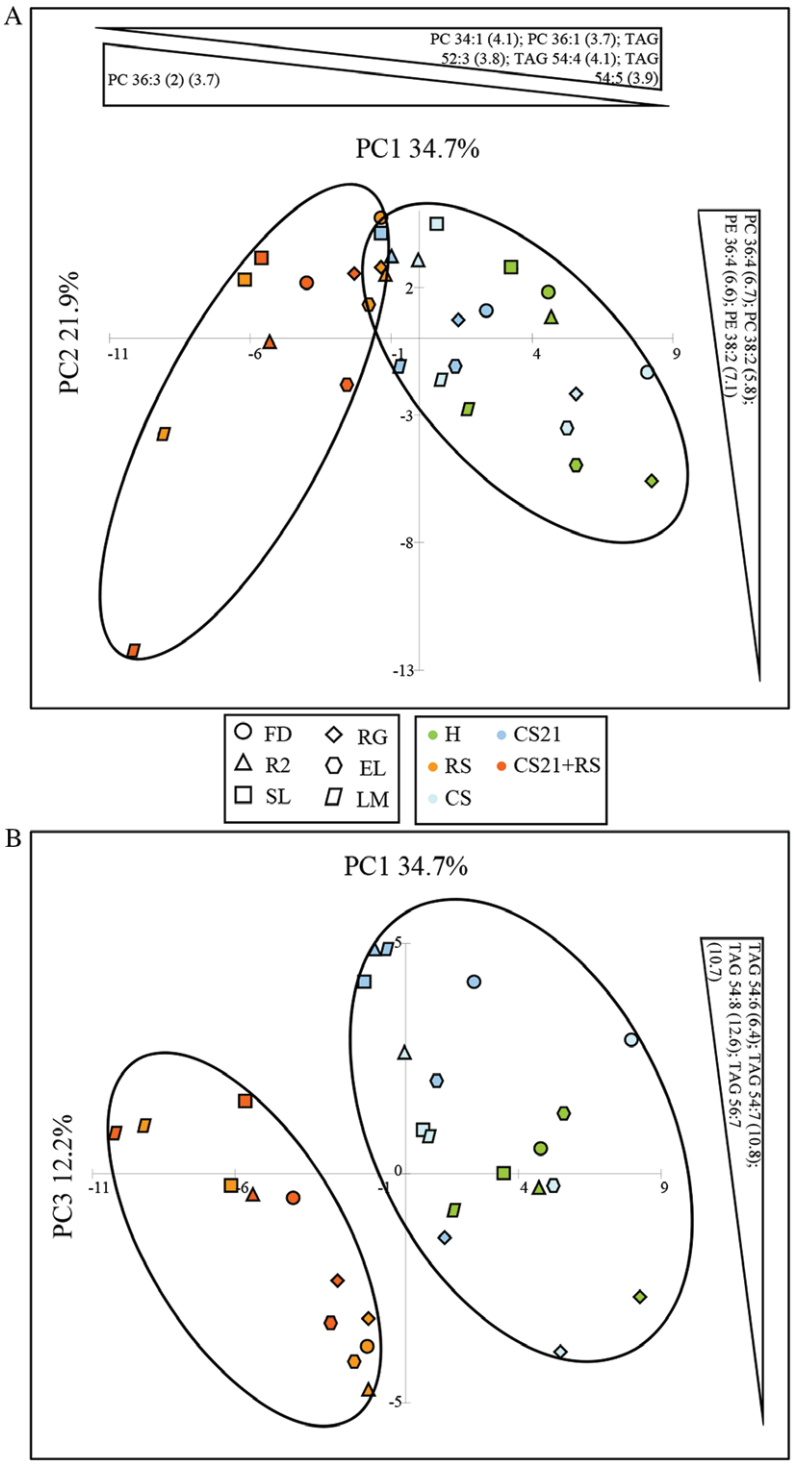

Fig. 2. PCA of the lipid composition of peach fruit varieties subjected to different postharvest treatments. Lipid composition of peach fruit samples from six varieties (FD, R2, SL, RG, EL and LM) subjected to different postharvest treatments (H, RS, CS, CS21 and CS21 + RS) was analyzed by PCA. The first principal component (PC1) is shown on the *x*-axis vs the second principal component (PC2) on the *y*-axis in (A); and vs the third principal component (PC3) on the *y*-axis in (B). The lipids that contribute the most to each component separations are indicated on the top (PC1) and on the right (PC2 in A and PC3 in B). The variance explained by each component (%) is indicated in parentheses. Samples from each variety are indicated by geometric shapes and postharvest conditions are color coded: H, in green; RS, in light orange; CS, in light blue; CS21, in dark blue; CS21 + RS, in dark orange.

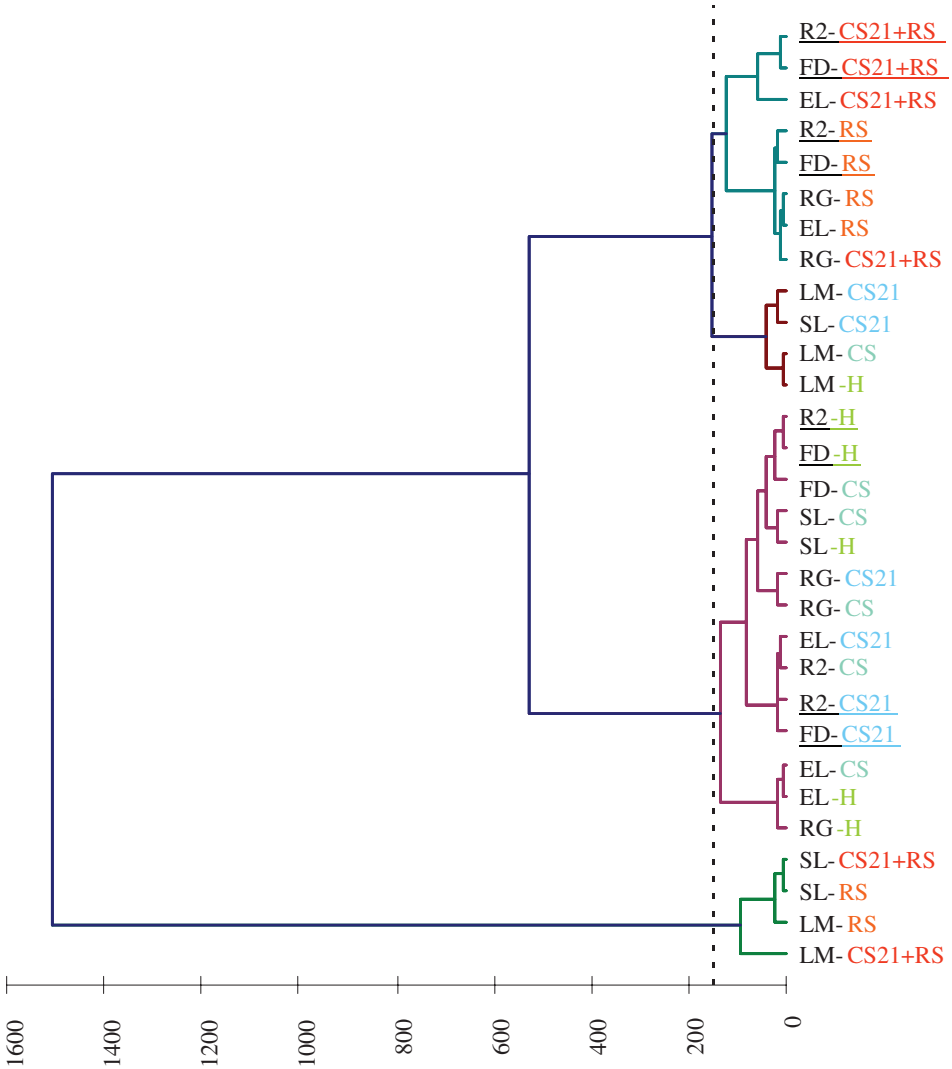

Fig. 3. HCA of peach fruits from six varieties subjected to different postharvest treatments based on their lipid composition. Peach fruit samples from six varieties (FD, R2, SL, RG, EL and LM) subjected to different postharvest treatments (H, RS, CS, CS21 and CS21 + RS) are divided in four principal clusters depending on the variety and the postharvest condition. Samples from susceptible varieties (FD and R2) that clustered together are underlined and postharvest treatments are color-coded: H, in green; RS, in light orange; CS, in light blue; CS21, in dark blue; CS21 + RS, in dark orange.

stored at 0°C are compared with fruits stored at 20°C at the same postharvest time (CS vs RS), the number of lipids that changes is even larger (Table S6), indicating that the lipid composition of CS fruits is more similar to H samples than to ripened fruits (RS). Among the modifications observed in the lipidome of all the six peach varieties, higher levels of DGDG 36:4 and PC 38:2 are found in CS21 fruits when compared with H samples, with rises of up to 7.0- and 3.3-fold in RG, respectively. In contrast, MGDG 36:5 content decreases after 21 days at 0°C (Table S6). In addition, the content of several plastidic glycerolipids are modified in fruits of all the varieties stored at 0°C for a short period in relation to fruits of the same postharvest age under 20°C ripening conditions (DGDG 36:3, DGDG 36:6 and MGDG 36:6 increase; MGDG 36:4 and MGDG 36:5 decrease).

One of the goals of the present work was to identify lipid markers that could be correlated with CI resistance in peach fruits. In this way, the lipid composition of susceptible (R2 and FD) and resistant (EL, RG, LM and SL) to CI varieties was compared in the different postharvest conditions (Table S6). After short cold storage (CS), PC 36:3 content decreases in susceptible varieties, while it does not change in resistant ones, in relation to H. When compared CS21 vs H, levels of DGDG 34:3 increase in resistant fruits, from 1.6 (SL) to 4.1 (RG), meanwhile it

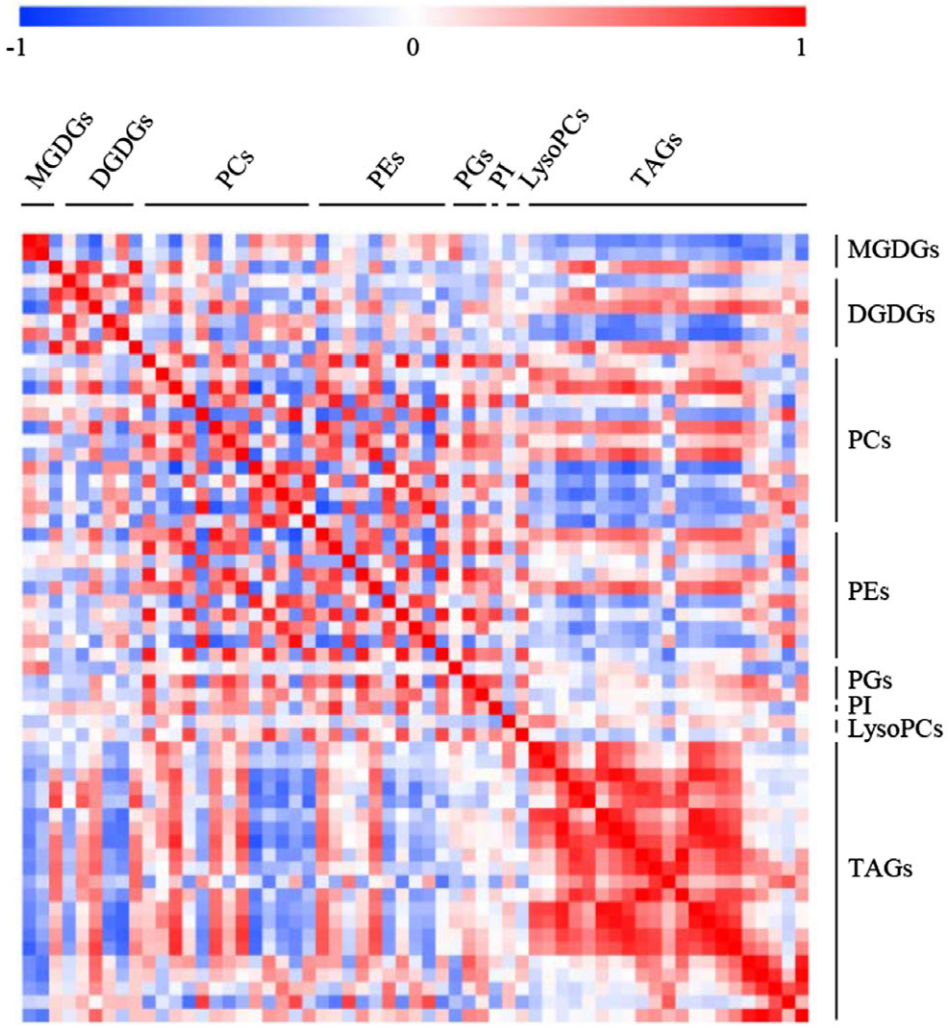

Fig. 4. Visualization of lipid-lipid correlations of LC–MS data of each peach variety. Correlation coefficients were calculated by applying Pearson correlation. Each square represents the correlation between each lipid species heading the column and the lipid species heading the row with a color scale (color scale key at the top of the figure).

remains the same in R2 and FD. Finally, CS21 + RS fruits of susceptible varieties showed higher levels of several membrane lipids (PC 34:3, PC 36:3 (2), PC 36:5, PC 36:6, PE 34:3, PE 36:3 (2) and PE 36:6) in relation to RS, while in resistant varieties, the content of these lipids does not change or even decreases (Table S6).

Analysis of transcripts encoding enzymes involved in galactolipid metabolism in varieties with contrasting susceptibility to CI

In view of the fact that galactolipids were the lipids that changed most during the postharvest ripening and after cold storage of the six varieties tested, the relative level of transcripts encoding enzymes involved in galactolipid metabolism was studied by qRT-PCR in Springlady (SL, resistant to CI) and Flordaking (FD, susceptible to CI) varieties (Genero et al. 2016). Galactolipids contain one or two galactose molecules attached to the *sn*-3 position of a glycerol backbone, respectively MGDG and DGDG, and are unique to plastid membranes from which they represent up to 80% of the total lipids. The final step in MGDG biosynthesis occurs in the envelope of chloroplasts and non-green plastids and is catalyzed by MGDG synthase (MGD, UDP-galactose: 1,2-diacylglycerol 3-β-D-galactosyltransferase), and DGDG is subsequently synthesized by galactosylation of MGDG, via DGDG synthase (DGD, UDP-galactose:3-(β-D-galactosyl)-1,2-diacylglycerol 6-α-galactosyltransferase) (Kelly and Dörmann 2004).

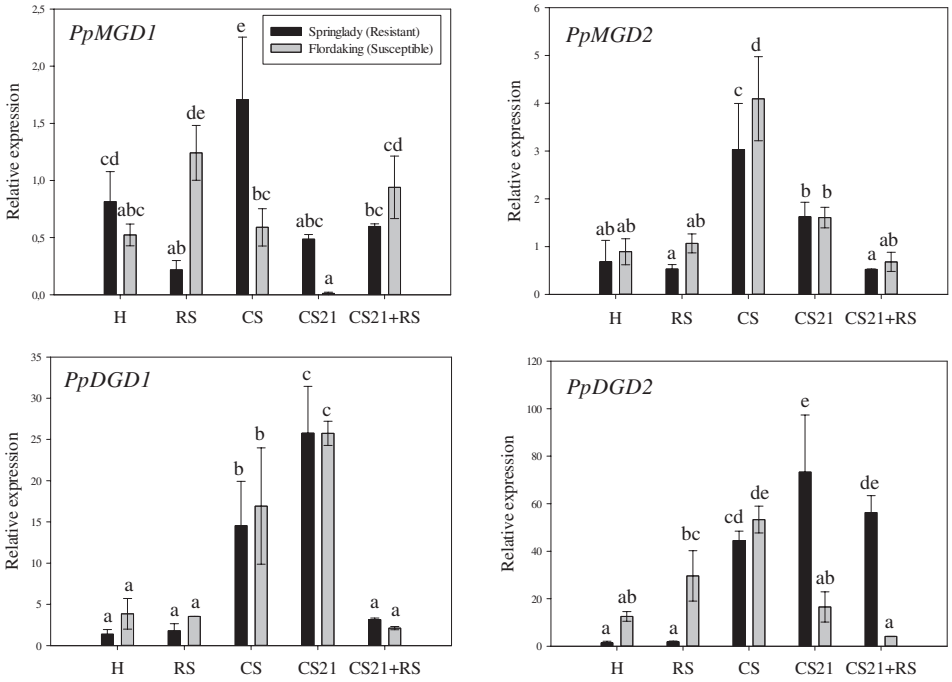

Fig. 5. Expression analysis of transcripts encoding enzymes involved in galactolipid metabolism in peach fruits from SL and FD varieties during the post-harvest at different temperatures. For each sample, means of the results obtained, using three independent RNA biological replicates as templates, are shown. Each reaction was normalized using the C_t values corresponding to *Prunus persica* DNA-repair enzyme mRNA (Table S3). Y axis refers to the fold difference in a particular transcript level relative to its amount found in peaches analyzed after harvest (H). Standard deviations are shown. For each transcript analyzed, bars with the same letters are not significant different ($P < 0.05$).

An in silico analysis of *P. persica* genome, based on literature of Arabidopsis galactolipid biosynthesis genes (Awai et al. 2001, Kelly et al. 2003, 2016), allowed the identification of two transcripts encoding putative MGDs (called *PpMGD1* and -*2*) and two peach sequences encoding putative DGDs (called *PpDGD1* and -*2*) (Table S7). Although only PpMGD1 and PpDGD1 are predicted to be plastidic (Table S7), localization of MGD2 and DGD2 proteins in plastids has been confirmed in Arabidopsis (Awai et al. 2001, Kelly et al. 2003). Therefore, the relative expression levels of *PpMGD1*, *PpMGD2*, *PpDGD1* and *PpDGD2* were investigated in SL and FD varieties during the postharvest at different temperatures.

During ripening (RS vs H), *PpMGD2*, *PpDGD1* and *PpDGD2* were not modified in the varieties tested, while *PpMGD1* transcript decreased and increased during fruit softening of SL and FD, respectively (Fig. 5). When SL and FD fruits were stored at low temperature, *PpMGD1* and *PpMGD2* transcripts were temporally induced after short cold storage in comparison to H, except in the case of *PpMGD1* in FD variety, and reverted to their initial levels when fruits were stored for a longer period of time (CS21 vs H) (Fig. 5). In relation to *PpDGD1* and *PpDGD2* genes, levels of transcripts increased when fruits were stored at low temperature for a short period of time (CS vs H), and reached even higher levels when they were stored for 21 days (CS21 vs H), with the exception of *PpDGD2* in the susceptible variety. Finally, *PpDGD2* was the only transcript that changed when comparing RS and CS21 + RS fruits: the transcript increased and decreased in SL and FD varieties, respectively (Fig. 5).

Changes of fatty acid composition in total lipids

To investigate the relationship between degree of lipid unsaturation and cold tolerance, fatty acid composition and DBI of total lipids from the six peach varieties subjected to different postharvest treatments were determined. Linoleic acid (C18:2) and palmitic acid (C16:0) were the major fatty acids in mesocarp tissue (Table S8). As shown in Table S8, the patterns of changes in the fatty acids detected did not show correlation with the storage temperature or genotype analyzed. However, when the DBI was calculated (Table S9), a significant increase of this value was observed after cold exposure of susceptible cultivars (Flordaking: in CS21 vs H, Rojo 2: in CS vs H, CS vs RS and CS21 vs H).

Discussion

Lipid profiling has been successfully applied to investigate the effects of temperature stress on cellular lipid composition in Arabidopsis, *Atriplex lentiformis*, wheat and tomato leaves (Burgos et al. 2011, Degenkolbe et al. 2012, Higashi et al. 2015, Li et al. 2015, Spicher et al. 2016). However, until this work, there were no reports on the effects of temperature on lipid composition of fruit tissue. In this study, 59 lipid species were reproducibly detected in mesocarp tissue of peach fruit by using LC–MS. Even when the number of lipids identified in peach fruit was lower than that found in leaves from other plant species (about 200 different lipid species), our results provide information about the dynamics of different lipid species under different postharvest storage conditions and allow the identification of possible lipid markers for chilling resistance.

Postharvest ripening of peach fruits is accompanied by plastidic lipid remodeling and a reduction in the storage lipid content

In the present work, postharvest ripening of the six varieties selected was accompanied by a decrease in fruit firmness, modifications in the galactolipid composition and a decrease in the content of TAGs (Fig. 6A).

MGDG and DGDG seem to be important in the ripening process of peach fruit. In all the varieties, the increase of MGDG 36:4 (non-bilayer lipid) and the decrease of some DGDG species (DGDG 36:3 and DGDG 36:6, bilayer lipids) could destabilize the chromoplast membranes (Hincha et al. 1998), in a process that would be characteristic of the normal ripening (Table S6). In addition, even though some MGDG and DGDG species change during ripening (RS vs H, Table S6), and the total DGDG content decrease in SL variety (Table S10), no correlation was found between the galactolipid content and the transcript levels of *PpMGD1*, *PpMGD2*, *PpDGD1* and *PpDGD2* genes (Table S6, Fig. 5), which may be due to translational or posttranslational regulation of these enzymatic activities during fruit ripening, or the participation of putative galactolipases (Troncoso-Ponce et al. 2013). Particularly, MGD1 activity has been found to be posttranslationally regulated by acidic lipids such as phosphatidic acid, sulfoquinovosyldiacylglycerol and PG in cucumber seedlings (Ohta et al. 1995). In photosynthetic tissues, the abundance of galactolipids in the thylakoid membranes suggests they have not only typical bilayer functions but also specific roles, such as stabilization of photosynthetic complexes, membrane architecture and thylakoid stack formation (Dörmann 2013). In Arabidopsis, it was demonstrated that galactolipids are essential for proper thylakoid biogenesis, photosynthesis and embryo development (Kobayashi et al. 2007). During fruit ripening, chloroplasts differentiate into photosynthetically inactive chromoplasts in a process characterized by the degradation of the thylakoid membranes, and by the active synthesis and accumulation of carotenoids (Cheung et al. 1993). In tomato, isolated fruit chromoplasts have the capacity to synthesize ATP through the operation of an ATP synthase complex, driven by a membrane proton gradient generated by an electron transport process in which NADPH acts as an electron donor (Pateraki et al. 2013). Even though the components of the chromorespiratory pathway are still unclear, a preliminary model suggests that the electron transport chain is probably located in the inner membranes of chromoplasts, which form elongated sacs or convoluted compartments (Renato et al. 2014). However, if the modifications in the galactolipid composition observed in this work conduce to change the stability of the plastidic membranes during normal ripening, and if these modifications could be associated with the regulation of respiratory processes in the mesocarp of peach fruit, is still unknown.

In relation to storage lipids, 21 molecular species of TAGs were identified. Most of these TAG species decrease during ripening of at least one variety (Table S6) according to correlation results (Fig. 4), resulting in a reduction of the total TAG content in all the varieties tested (Table S10). In the Dixiland variety, a decrease in amino acid levels, coupled to an induction of transcripts encoding amino acid and organic acid catabolic enzymes, indicated that amino acids were used as respiratory substrates during the ripening process (Lombardo et al. 2011). Thus, a similar role for TAGs is proposed in the present work, which, along with amino acids, would help to maintain the cellular status prior to fruit senescence.

Major modulation of galactolipids during cold

Chloroplasts and photosynthetic membranes respond to environmental changes. They acclimate to intensity and quality of light, and also to temperature changes (Kanervo et al. 1997, Lichtenthaler 2010, Burgos et al. 2011, Rochaix et al. 2012, Spicher et al. 2016). In non-green tissues, such as fruits, the effect of temperature on non-photosynthetic plastids and plastidic membranes has not been explored yet. By using an untargeted lipidomic approach, the changes in the lipidome of six peach varieties subjected to cold treatment were investigated. At low temperature, most of the modifications were linked to changes in the content

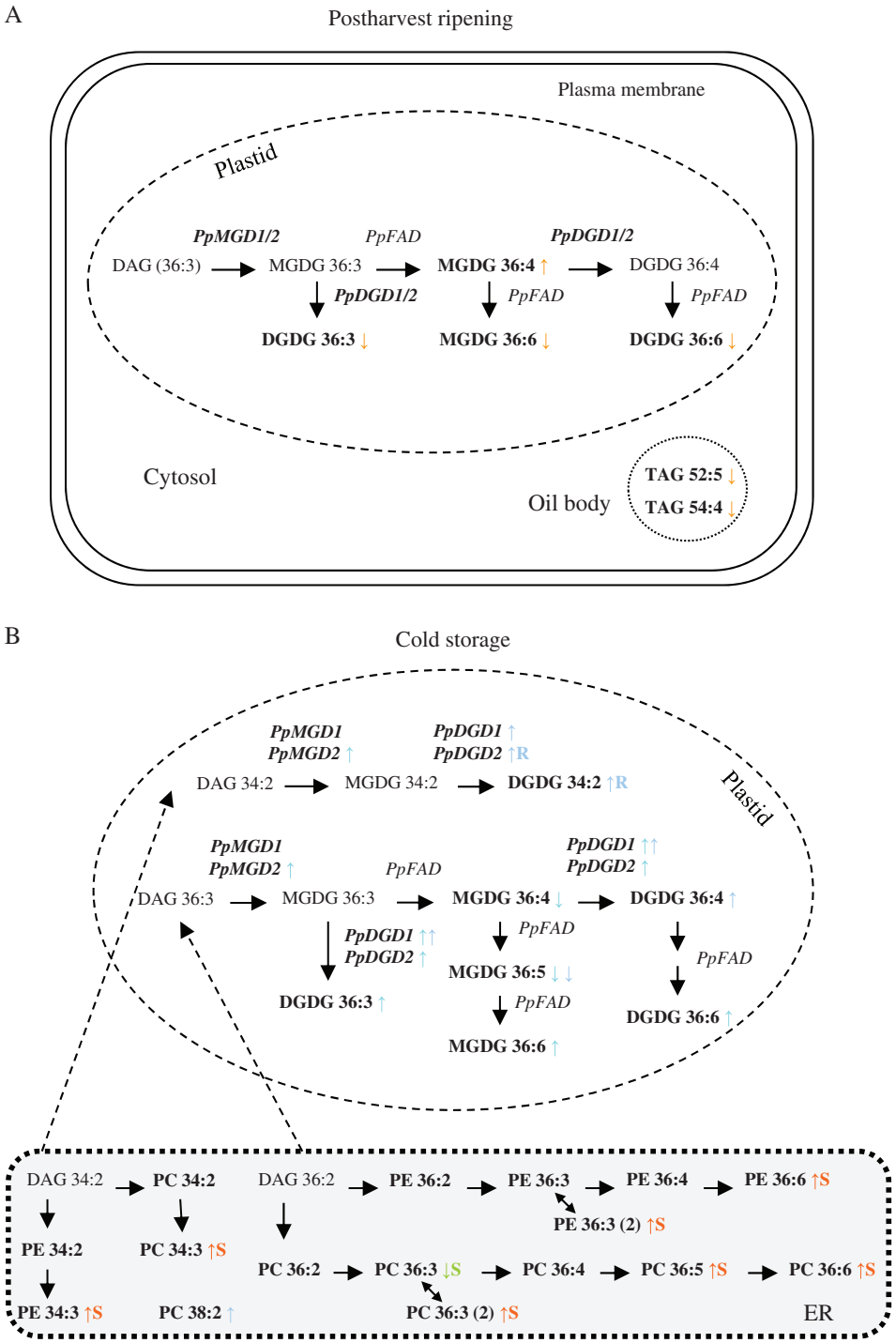

Fig. 6. Representative scheme of key processes occurring during postharvest ripening (A) and after cold storage (B) of peach fruits**.** The scheme summarizes the most relevant lipid compositional changes and shows possible related processes occurring during postharvest ripening and after cold storage of peach fruits. Lipids and transcripts measured in this work are shown in bold. ↑ and ↓ represent increase and decrease, respectively, in lipid or transcript levels. Different colors indicate changes in lipid composition or transcript levels when comparing different postharvest conditions: light orange, RS vs H; green, CS vs H; dark blue, CS21 vs H; light blue, CS vs RS; and dark orange, CS21 + RS vs RS. *PpFAD*, *PpDGD* and *PpMGD* transcripts encode putative desaturases, DGDG synthases and MGDG synthases from peach, respectively. R, resistant; S, susceptible; ER, endoplasmic reticulum.

of galactolipids, indicating that plastidic membranes would be the main target of cold in mesocarp tissue of peach fruit. The results obtained indicate that while the total DGDG content increased significantly in SL, R2, RG and LM varieties after cold storage (in CS vs H, CS21 vs H and CS vs RS), the relative amount of some specific species of DGDG (DGDG 36:4, in CS21 vs H; DGDG 36:3 and DGDG 36:6, in CS vs RS) increased in all the varieties tested (Tables S6 and S10). On the contrary, the total MGDG content decreased in SL, FD, EL, RG and LM varieties after cold treatment (especially in CS21 vs H), meanwhile the relative amount of some specific species of MGDG (MGDG 36:4, in CS vs RS; MGDG 36:5 in CS21 vs H and in CS vs RS) decreased in all the six varieties (Tables S6 and S10). Similar results have been described in Arabidopsis and *Thellungiella salsuginea* leaves during cold acclimation (Moellering et al. 2010, Degenkolbe et al. 2012, Zhang et al. 2013). Low temperature produced a reduction in MGDG and a concomitant increase in DGDG content, as well as the appearance of tri and tetragalactolipids (not detected in this work; Moellering et al. 2010, Degenkolbe et al. 2012, Zhang et al. 2013). As MGDG is a non-bilayer lipid that can severely destabilize membranes, whereas DGDG is a bilayer lipid that may increase membrane stability, it is proposed that an increase in the ratio of bilayer- to non-bilayer-forming membrane lipids results in the stabilization of membranes during freezing (Hincha et al. 1998). In wheat leaves, total DGDG and MGDG content increases and decreases, respectively, after cold treatment. Additionally, changes in the relative amount of some specific species of DGDG are also detected when plants are grown at low temperature, in relation to plants grown at room temperature: DGDG 36:6, the most abundant DGDG, increases; DGDG 34:3, DGDG 36:5 and DGDG 36:4 decrease (Li et al. 2015). Unexpectedly, MGDG 36:6, the most unsaturated and abundant MGDG detected in this work, increases in all six cultivars when CS fruits are compared with RS samples (Fig. S2, Table S6). However, the decrease in the content of this galactolipid species during ripening and the unchanged levels observed in CS fruits in relation to H (Table S6) suggest that the decrease in MGDG 36:6 content during ripening is inhibited by cold treatment in CS samples. Thus, it can be considered that in CS fruits, where ripening processes would be stopped, cold treatment itself does not increase the MGDG 36:6 content to avoid CI, but merely maintains it constant.

As MGDGs and DGDGs are the main lipids modified by cold, the enzymes involved in the metabolism of these plastidic lipids were analyzed by qRT-PCR in SL and FD varieties (Fig. 5). When compared CS vs RS fruits, the increase and decrease of some DGDGs and MGDGs species (Table S6), respectively, along with the increase in the total DGDG content in SL variety (Table S10), seem to be more related with rises in *PpDGD1* and *PpDGD2* transcripts. However, the synthesis of MGDG from diacylglycerol (DAG) via PpMGD2 can not be discarded due to transcript levels of *PpMGD2* gene increase significantly after short cold storage of both varieties, in relation to RS fruits, which may result in maintaining a constant level of total MGDG (Table S10). In addition, the increase and decrease in DGDG 36:4 and MGDG 36:5 in all the varieties tested (Table S6), respectively, and the changes observed in the total DGDG and MGDG contents after 21 days at 0°C in relation to H fruits (Table S10), could be the result of rises in the level of the transcript encoding *PpDGD1*. The results obtained also indicate that *PpDGD2* could be responsible for the differential accumulation of DGDG 34:2 in the resistant varieties, as this transcript increases in SL and remains constant in FD when compared CS21 vs H samples (Fig. 5). Overall, our results suggest that the modifications in the relative content of galactolipids at low temperature are more related to the turnover of MGDG into DGDG by PpDGD1 and PpDGD2 than to the MGDG biosynthesis via PpMGD1 or PpMGD2. In Arabidopsis, MGDG is synthesized from DAG by a multigenic family of MGDG synthases, AtMGD1, AtMGD2 and AtMGD3. Subsequently, MGDG is transformed to DGDG via AtDGD1 and AtDGD2. MGDs and DGDs enzymes are localized in the chloroplasts of Arabidopsis leaves and only *AtMGD1* and *AtDGD1* are regulated positively by cold (Li et al. 2015). Based on changes in glycerolipid content and transcripts dynamics under suboptimal temperature conditions, Li et al. (2015) proposed a model that emphasizes the glycerolipid pathway coordination in response to growth temperature in plants, suggesting that glycerolipid composition and the degree of fatty acid desaturation are two major factors underpinning membrane performance during temperature stress. However, when the degree of unsaturation of total lipids was analyzed in all the samples, it was found that the DBI increased after cold storage of susceptible varieties (Table S9), indicating that lipid unsaturation would not be important for maintaining membrane fluidity in resistant peach fruits stored at low temperature. Only MGDGs seem to increase the degree of unsaturation in cold-stored fruits, as the contents of MGDG 36:4 and MGDG 36:5 decrease, while MGDG 36:6 increases in the six varieties when compared CS and RS samples (Table S6).

In addition to the observed changes in the galactolipid levels after cold storage, an increase in the relative content of PC 38:2 was also detected in all the varieties

after 21 days at 0°C (CS21 vs H) (Table S6). It has been shown that an increase in the content of diunsaturated species of PC in rye protoplasts leads to an increased tolerance of the plasma membrane against freezing and osmotic stresses (Steponkus et al. 1988). In Arabidopsis leaves, the relative contents of PC 38:2 and PC 38:4 (not detected in this work) correlated with freezing tolerance in acclimated plants (Degenkolbe et al. 2012). Thus, the increase in PC 38:2 levels observed in all the varieties could be part of an adaptive response of fruits to long cold treatment. Given its low abundance (Fig. S2), the treatment applied (21 days at 0°C) may not lead to major reorganization of the plasma membrane; however, additional work is required to support this observation. Fig. 6B summarizes part of the regulatory mechanisms in response to cold in peach fruit. In the scheme, the action of putative desaturases, which are involved in mediating the formation of double bonds in fatty acyl moieties, was included in order to explain some of the changes associated with the different postharvest conditions.

Correlation between relative abundance of lipids and CI tolerance

The results presented here indicate a major restructuration of plastidic lipids following exposure to cold; however, these changes may represent a consequence of cold stress without any functional role in peach fruit protection against CI. In order to discriminate the processes that are critical for CI tolerance from those that are merely responsive to low temperature, the lipid reconfiguration in susceptible and resistant varieties was compared. Though PCA analysis does not discriminate between resistant and susceptible varieties (Fig. 2), FD and R2 cluster together in the HCA map, except after short cold storage (Fig. 3), indicating that susceptibility to CI is characteristic of a particular lipid group in peach fruit.

Interestingly, the lipids that most correlated with the tolerance to CI were those associated with the plasma membrane. When compared CS21 + RS and RS fruits, susceptible varieties showed higher levels of several species of PC and PE: PC 34:3, PC 36:3 (2), PC 36:5, PC 36:6, PE 34:3, PE 36:3 (2) and PE 36:6, while in resistant varieties, the content of these lipids does not change or even decreases (Table S6). According to this, some of these membrane lipids showed positive correlation among them (PC 34:3 and PE 34:3, PC 36:3 (2) and PE 36:3 (2), PC 36:6 and PE 36:6; Fig. 4). In peach fruit, CI symptoms mainly develop during fruit ripening after cold storage. Here, the visual evaluation of CS21 + RS fruits indicated no apparent evidence of CI symptoms in any variety (Bustamante et al. 2016). However, although not externally visible, it is well known that these symptoms may be present. Flesh browning is generally related to tissue deterioration or senescence, which leads to changes in membrane permeability and the interaction between phenols and polyphenol oxidase, which are generally found in separate compartments in the cell (Lurie and Crisosto 2005). In this work, even though flesh browning was not apparent in FD and R2 varieties, results obtained suggest that increases in the relative contents of certain phospholipids species (in CS21 + RS vs RS) may destabilize the plasma membrane when fruits ripen after a long cold treatment. In Arabidopsis leaves, the relative amounts of PC and PE increase after 2 weeks at 4°C (Degenkolbe et al. 2012). During cold acclimation, increases in phospholipid content have been repeatedly observed in plants, and it is frequently assumed that this is of adaptive value (Steponkus 1984). However, only the relative content of PC 36:3 seems to follow a similar trend: it decreases and does not change in susceptible and resistant varieties after short cold treatment with respect to H, respectively (Table S6). Therefore, the study of the physical properties of membrane lipids in susceptible and resistant varieties is a future challenge, since it will reveal the relationship between lipid composition and membrane integrity under cold stress.

Finally, the only plastidic lipid that showed correlation with the tolerance to CI was DGDG 34:2, which increased significantly (up to 4.1-fold in RG variety) in resistant varieties and remained constant in susceptible ones after long cold storage, in relation to H fruits (Table S6). Fig. 6B outlines the metabolic pathway involved in the differential accumulation of this bilayer lipid in SL and FD varieties, as discussed earlier.

Author contributions

Conception and design of the work: C. A. B., M. V. L., M. F. D.; Acquisition of data for the work: C. A. B., Y. B., L. L. M., J. G., C. O. B.; Analysis of data for the work: C. A. B., Y. B., L. L. M., M. V. L., M. F. D.; Interpretation of data for the work: C. A. B., Y. B., A. R. F., M. F. D.; Manuscript revision and approval: C. A. B., Y. B., L. L. M., J. G., C. O. B., M. V. L., A. R. F., M. F. D.; Accountability: C. A. B., Y. B., L. L. M., J. G., C. O. B., M. V. L., A. R. F., M. F. D.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Relevant agronomic characteristics of the six varieties.

Table S2. Firmness, soluble solids and expressible juice of fruits from the six peach varieties.

Table S3. Sequences of the oligonucleotide primers used for qRT-PCR.

Table S4. Numerical data for the content of the 59 lipid species analyzed by LC–MS.

Table S5. Variable contribution to the principal components (%) shown in Fig. 2.

Table S6. Lipid fold change between RS and H, CS and H, CS21 and H, CS and RS, and CS21 + RS and RS.

Table S7. Putative genes involved in galactolipid metabolism.

Table S8. Fatty acids (%) quantified by GC-FID.

Table S9. DBI of total lipids.

Table S10. Fold changes for different lipid classes between RS and H, CS and H, CS21 and H, CS and RS, and CS21 + RS and RS.

Fig. S1. Schematic representation of peach fruit treatments after harvest.

Fig. S2. Relative abundance of lipid species analyzed by LC–MS in peach fruits.

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