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Involvement of phospholipases and sucrose in carbon starvation-induced non-chilling peel pitting in citrus fruit

Paco Romero, Fernando Alférez[1], María T. Lafuente*

Department of Food Biotechnology, Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), Av. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

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ABSTRACT

The involvement of different isoforms of genes encoding phospholipases D (*CsPLDα, CsPLDβ, CsPLDδ, CsPLDγ* and *CsPLDζ*) and A_2 (*CsPLA$_2$α, CsPLA$_2$β* and *CsPAT1*) on starvation-induced postharvest non-chilling peel pitting (NCPP) has been compared in the inner (albedo) and outer (flavedo) parts of the peel of citrus fruit treated or not with sucrose (Suc). The study has been performed in Navelate (*Citrus sinensis* (L.) Osbeck) sweet orange, which is prone to NCPP, stored under non-stressful environmental conditions (90–95 % relative humidity (RH) and 20 °C). Transcriptional changes, as well as respiration rate and ATP content evolution during fruit storage were compared in both peel tissues. Results indicated that the albedo is more susceptible than the flavedo to starvation; and that, at early stress stage, ATP and all *CsPLD* isoforms and *CsPLA$_2$β* are good indicators of carbon starvation in the albedo, and *CsPLDβ* in the flavedo. These carbon starvation-induced signals were not activated when Suc was applied as an external energy source. In the second phase of starvation, expression of all *CsPLD*-encoding genes increased with NCPP; and *CsPLDγ* and *CsPLDζ* showed major increases in both peel tissues. The correlation of the expression of *CsPLA* isoforms with damage development was lower. In this phase, Suc may protect the fruit by providing additional energy sources to sustain respiration; and by favouring phospholipid-derived signaling messengers mediated by *CsPLDβ* and *CsPAT1* in the albedo, *CsPLDζ* in the flavedo, and *CsPLA$_2$β* in both tissues. Results from the examination of changes in gene expression point out tissue specificities in the expression of *CsPL* genes but also different susceptibility to starvation between the flavedo and the albedo in citrus fruit.

1. Introduction

Nutrient deprivation may be an important abiotic stress that fruit undergo after detachment since they have to sustain respiration and survive from their own energy reserves for prolonged periods during postharvest storage. However, little is known about the mechanisms underlying carbon starvation and energy shortage caused by fruit detachment and their putative involvement in postharvest losses (Saquet et al., 2003; Yi et al., 2008; Romero et al., 2020). Different studies report on the responses of culture cells, and of both photosynthetic tissues and non-photosynthetic tubers containing abundant carbon respiratory sources to carbon starvation (Aubert et al., 1996; Smith and Stitt, 2007; Baena-González and Sheen, 2008); but knowledge in non-photosynthetic sink organs like the peel of ripe citrus fruit, which has fewer carbon sources, is scarce. Different evidences from our group suggested the involvement of carbon starvation and energy demand to sustain respiration on the development of a postharvest physiological disorder in citrus fruit, which manifests as depressed areas affecting the inner (albedo) and outer (flavedo) part of the peel, named non-chilling peel pitting (NCPP) (Cajuste et al., 2011; Lafuente et al., 2014; Establés-Ortiz et al., 2016; Romero et al., 2020). Moreover, results from Romero et al. (2020) in citrus fruit were in concordance with others showing that cellular perception of energy shortage, determined as a decrease in adenosine triphosphate (ATP), may affect cell membrane integrity (Rawyler et al., 1999, 2002).

Phospholipids are key structural elements of biomembranes but also serve as rich sources for second messengers in plants (Wang, 2001; Meijer and Munnik, 2003). Membrane integrity can be lost because of phospholipid catabolism in plants exposed to severe stress; but activation of phospholipases (PLs) under mild stress may lead to signaling molecules triggering plant defense responses. PLD catalytic activity on phosphatidylcholine (PCho), producing choline (Cho) and the second lipid messenger phosphatidic acid (PA), initiates the downstream response cascade; in turn, PA can be degraded by phospholipases A (PLA).

* Corresponding author.
E-mail addresses: promero@iata.csic.es (P. Romero), alferez@ufl.edu (F. Alférez), mtlafuente@iata.csic.es (M.T. Lafuente).
[1] Current address: Southwest Florida Research and Education Center. University of Florida-IFAS, Immokalee, FL 34142, USA.

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Different PLD and PLA_2-encoding genes have distinguishable functions in the response to stresses in plants and citrus fruit (Wang, 2001; Ryu, 2004; Romero et al., 2013a, 2014); and a specific crosstalk between genes encoding these enzymes in the regulation of the response to different stresses has been suggested in plants and in citrus fruit (Romero et al., 2014). Alteration of membrane integrity has been related to the development of NCPP caused by sharp changes in peel water potential and in the activities of PLD and PLA_2 (Alférez et al., 2008; Cronjé et al., 2017). Moreover, the role of different PLD and PLA_2-encoding genes in response to water stress has been reported in model plants and citrus fruit and leaves (Katagiri et al., 2001; Hong et al., 2008; Romero et al., 2013a, 2014). Other data indicate that PLD may be involved in the induction of chilling injury in cucumber and citrus fruit, which in the latter manifests as peel damage but showing different symptomology to NCPP (Mao et al., 2007; Lafuente et al., 2017). However, no information exists about the involvement of PLD and PLA_2 encoding genes in starvation-induced NCPP. Moreover, transcriptional approaches to unravel their involvement on the response of plants to starvation are scarce.

As far as we know, no information exists in plants involving PLA_2 in the starvation response. On the other hand, literature about starvation and PLD in plants mainly focus in PLDζ isoform and refers to vegetative tissues and inorganic phosphate (Pi) (Cruz-Ramírez et al., 2006). In fact, this is a key gene in the biosynthesis of galactolipids, hydrolysing PCho and phosphatidylethanolamine (PE) to produce diacylglycerol and Pi. In this regard, it is noticing that treating citrus fruit with an external source of carbon (glycerol) favors alternative modes of energy in the flavedo leading to the increase of Pi, which is necessary for ATP production, and reduced NCPP development associated with fruit detachment-induced energy shortage (Romero et al., 2020). In suspension cells, it has been also shown that phospholipid catabolic pathway and, specifically, PLD were activated at the early stages of starvation (Lee, 1998).

In spite of sugars acting as external energy sources and controlling regulatory networks and interactions with multiple signaling pathways (Rolland et al., 2002), the effect of sugars on phospholipid catabolism and signaling remains unknown in plants. However, in the context of the present work, it merits attention that adding external energy sources as glycerol or pyruvate to Suc-starved cells stopped the accumulation of PCho and reduced cell autophagy (Aubert et al., 1996). Here, aiming to understand the role of PLD and PLA on starvation-induced postharvest NCPP development, we have examined transcriptional changes of 5 PLD and 3 PLA-encoding genes in the flavedo and albedo of Navelate (*Citrus sinensis* (L.) Osbeck) sweet orange, which is prone to develop NCPP. To that end, the fruit was stored under conditions that minimize environmental stress and do not alter water, osmotic and turgor potentials of flavedo and albedo tissues (constant 90–95 % relative humidity (RH) and 20 °C) (Cajuste et al., 2010). Storage conditions were also selected to avoid alteration of the storage atmosphere (unwaxed fruit held in open plastic boxes), to focus on the starvation effect. Selection of genes was done on view of previous research indicating their putative involvement in the development of NCPP in citrus fruit held under stressful storage conditions (Romero et al., 2014). Transcriptional changes, as well as respiration and ATP content, have been compared in the flavedo and albedo, because both tissues are affected by NCPP (Cajuste et al., 2011) and their morphology and ability to provide energy is different (Matas et al., 2010; Establés-Ortiz et al., 2016). Likewise, tissue-specificity in the expression of the citrus PL-encoding genes has been proven (Romero et al., 2014). Moreover, the effect of an external energy source (Suc) on these changes is examined.

2. Material and methods

2.1. Fruit material and chemical treatments

Full mature Navelate (*Citrus sinensis* (L.) Osbeck) sweet oranges (external colour index, a/b = 0.58 ± 0.03) were randomly harvested from trees grown in commercial orchards in Lliria (Valencia, Spain). The fruit with no visual defects were immediately delivered to the laboratory and divided into two groups. Fruit from the first group were dipped for 2 min in aqueous solutions containing 10 mM Suc and fruit from the second group were dipped in water and used as control. The Suc concentration was selected because its efficacy reducing NCPP (Romero et al., 2020). After drying at room temperature, fruit in both groups were sorted into two subgroups. The first subgroup was made up of three replicates of ten fruit each to estimate NCPP along fruit storage up to 10 d; and the second subgroup of three replicates of five fruit per storage period, which were used for ATP and CO_2 analyses and for determining changes in the expression levels of genes encoding the enzymes PLD and PLA_2. Two discs from the equatorial zone of each fruit were taken at each sampling period to determine immediately CO_2 production of flavedo and albedo as described below (Section 2.4). The rest of the tissue from flavedo and albedo of the fruit used for taking the discs was immediately frozen, homogenised in liquid nitrogen and kept at −80 °C for later determinations. All the fruit were stored at 20 °C and 90–95 % RH to minimize temperature and water stresses.

2.2. Fruit colour and determination of non-chilling peel pitting incidence

Fruit colour at the time of harvest was determined in 3 replicates of 10 fruit as previously described by González-Aguilar et al. (2000) using a Minolta CR-300 Chromameter (Konica Minolta Inc, U.S.A.) at three locations around the equatorial plane of the fruit. The colour index was expressed as the *a/b* Hunter ratio that is used for colour measurement in citrus fruit (Lafuente et al., 2014). This ratio is positive for orange fruit and negative for green fruit. The incidence of NCPP disorder, which manifests as collapsed surface areas that affects to the albedo and the flavedo (Supplementary Fig. S1), was estimated by calculating the percent of fruit showing damage. The same fruit were used at the various evaluations dates and the results are provided as the mean of three biological replicate samples of ten fruit each ± standard error.

2.3. RNA isolation, cDNA synthesis and RT-qPCR analysis

Total RNA isolation, cDNA synthesis and RT-qPCR analysis were performed as previously described by Romero et al. (2013b). Total RNA was extracted from the albedo and flavedo tissues and treated with Ribonuclease-free DNAse (Applied Biosystems) according to the manufacturer's instructions for removing genomic DNA contaminations. Total RNA concentration was measured spectrophotometrically, and its quality and integrity verified by agarose gel electrophoresis and GelRed staining (Biotium). The cDNAs from all samples were synthesized from 2 µg of total RNA. First-strand cDNA synthesis was performed by using SuperScript III RT (Invitrogene) and Ribonuclease Inhibitor (Invitrogene) following the manufacturer's instructions. Gene-specific primer pairs were designed for the gene expression analysis with DNAMAN 4.03 software (Lynnon BioSoft). Forward and reverse sequences for specific primers (*CsPLDα, CsPLDβ, CsPLDδ, CsPLDγ* and *CsPLDζ;* and *CsPLA2α, CsPLA$_2$β* and *CsPAT1*) are shown in Supplementary Table S1. For data normalization, two reference genes (*CsACT* and *CsTUB*) were used. A LightCycler480 System (Roche Diagnostics) was used with SYBR Green 1 Master (Roche Diagnostics) to monitor cDNA amplification at 95 °C for 10 s, 60 °C for 5 s and 72 °C for 10 s. The Relative Expression Software Tool (REST, http://rest.gene-quantification.info) was used for gene expression analysis. Expression levels for all albedo and flavedo samples were referred to that obtained in the albedo and flavedo of freshly harvested fruit, respectively. All the

values are provided as the mean of three biological replicates samples, with at least two technical replicates ± standard error.

2.4. Determination of ATP and respiration rate

ATP analysis was performed on freeze-ground albedo and flavedo samples as reported by Romero et al. (2020). Briefly, 100 mg of the frozen tissues were extracted twice with 1.2 mL of chilled 0.5 g L^{-1} trichloro acetic acid (TCA) using a Mini Beadbeater 8 Cell Disruptor (Biospec Products, Inc.). After centrifugation (14,000 x *g* at 4 °C for 6 min), an aliquot of 0.6 mL was taken from each supernatant. The combined aliquots were neutralized and then diluted with 0.1 M Tris acetate buffer (pH 7.75), so that the final sample TCA concentration was lower than 0.1 %, to determine the ATP content using a luciferin/luciferase kit from Sigma (Catalog Number FL-AA, Sigma-Aldrich, St. Louis, MO, USA) by following the manufacturer's instructions. Two independent diluted extract aliquots per biological replicate of albedo or flavedo sample were analyzed.

The respiratory rate of flavedo and albedo discs were determined at each sampling period immediately after separating the flavedo and albedo from the fruit. They were measured periodically during fruit storage in three replicate samples. Two discs of 7 mm diameter per fruit were taken from the same fruit used to determine ATP or changes in gene expression; and a total of five fruit per replicate were used. Three replicate samples of albedo or flavedo discs were incubated at 20 °C in sealed 15-mL glass tubes for 1 h for CO_2 determination. One mL of gas sample was withdrawn from the head space of each tube with a hypodermic syringe and injected into a Gas Chromatograph (Perkin Elmer Autosampler), equipped with a Chromosorb 102 column kept at 60 °C and a thermal conductivity detector. The results of ATP and respiration rate are provided as the mean of three biological replicate samples ± standard error.

2.5. Statistical analysis

The results are the means of three biological replicated samples ± standard error. A mean comparison using the Tukey's test was made to determine whether mean values differed significantly at $P \leq 0.05$. The analyses were performed with the Statgraphics Plus 4.0 Software (Manugistics, Inc.).

3. Results

3.1. Effect of Sucrose on changes in NCPP incidence

The incidence of NCPP was determined in Navelate oranges stored under conditions that minimize atmospheric or environmental stresses (unwaxed fruit held in open plastic boxes at 90–95 % RH and 20 °C) since they may favor this physiological disorder in citrus fruit (Ben-Yehoshua et al., 2001; Alférez et al., 2003; Romero et al., 2012). Under these storage conditions, weight loss was lower than 1 % (Supplementary Fig. S1A), no difference in weight loss was found between the control and the Suc-treated fruit (Supplementary Fig. S1A), and the fruit developed the NCPP syndrome (Supplementary Fig. S1B) after 2 d (Fig. 1). The incidence of the disorder increased during fruit storage. Such incidence was relevant both in treated and untreated fruit, but was significantly reduced by treating the fruit with Suc (Fig. 1). By day 5, about the 43 % of control fruit showed NCPP, while the disorder was only detected in about the 23 % of the Suc-treated fruit. Important differences were maintained until the end of the experiment between the control and the Suc-treated fruit, when the incidence of the disorder in control fruit was close to the 60 % (Fig. 1).

3.2. Changes in respiration and ATP content in the flavedo and albedo

The respiratory rate of the flavedo was much higher than that of the albedo. Both tissues showed the characteristic respiration trend in starved plants (Krotkov, 1939). It decreased in both tissues after fruit detachment and such decrease was transitory (Fig. 2). Such transient decrease occurred immediately after harvesting the fruit (1 d) and was more marked in the albedo, which showed lower ability to recover the respiratory rate of freshly harvested fruit. Treating the fruit with Suc clearly modified such trend in the flavedo, since respiration of the Suc-treated fruit remained almost constant during fruit storage (Fig. 2A). Suc had a lower effect in the albedo in terms of the initial and transient decrease in respiration although the treatment increased the ability of these fruit to recover the respiratory rate observed immediately after detachment (Fig. 2B).

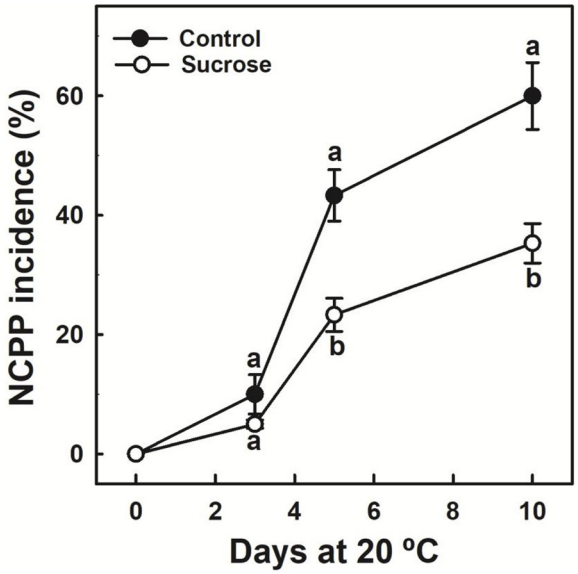

Fig. 1. Changes in NCPP incidence in Navelate sweet orange, treated (◯) or not (●) with Suc, during fruit storage at 20 °C and 90–95 % RH. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the control and the Suc-treated fruit for the same storage period.

The ATP content also decreased after fruit detachment in the inner and outer part of the peel, although a sharp but transient increase in ATP occurred in the albedo of control fruit by day 1. However, by day 3, ATP content was about 2-fold lower in the albedo (0.0021 mol kg^{-1}) (Fig. 3B) than in the flavedo (0.0043 mol kg^{-1}) (Fig. 3A). Suc reduced the decrease in ATP content in both tissues and its effect was observed first in the albedo (Fig. 3A and 3B). Thus, no relevant effect of Suc was found by day 3 in the flavedo (0.0036 mol kg^{-1} in the flavedo of Suc-treated fruits), while ATP levels were about twice in the albedo of the Suc-treated fruit (0.0045 mol kg^{-1}) respect to the control fruit (0.0021 mol kg^{-1}). This effect was observed later (7 d) in the flavedo. Keeping control fruit stored for longer periods led to an increase in ATP content in both peel tissues, which was higher in the flavedo, and such increases were reduced by applying Suc.

3.3. Effect of fruit detachment and sucrose on expression levels of PLD- and PLA_2-encoding genes in the flavedo and the albedo

Levels of expression of the different PLD and PLA_2 isoforms were always higher at harvest in the flavedo than in the albedo (Fig. 4). Among the five genes encoding PLD (*CsPLDα, CsPLDβ, CsPLDδ, CsPLDγ* and *CsPLDζ*), major differences between both tissues were found in *CsPLDβ* and *CsPLDγ* genes, whose expression was about 6- and 12-fold higher in the flavedo, respectively. Among the three genes encoding PLA_2 (*CsPLA$_2$α, CsPLA$_2$β* and *CsPAT1*), *CsPAT1* and *CsPLA$_2$α* showed major differences (14- and 8-fold higher in the flavedo, respectively) (Fig. 4).

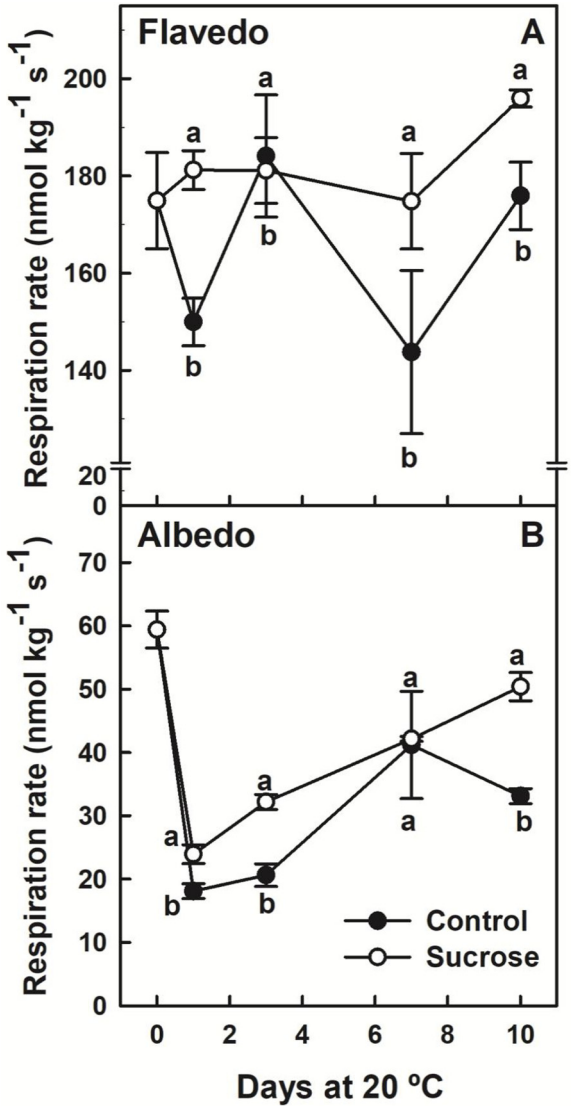

Fig. 2. Changes in respiration rate in the flavedo (A) and albedo (B) of Navelate sweet orange, treated (◯) or not (●) with Suc, during fruit storage at 20 °C and 90-95 % RH. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the control and the Suc-treated fruit for the same storage period.

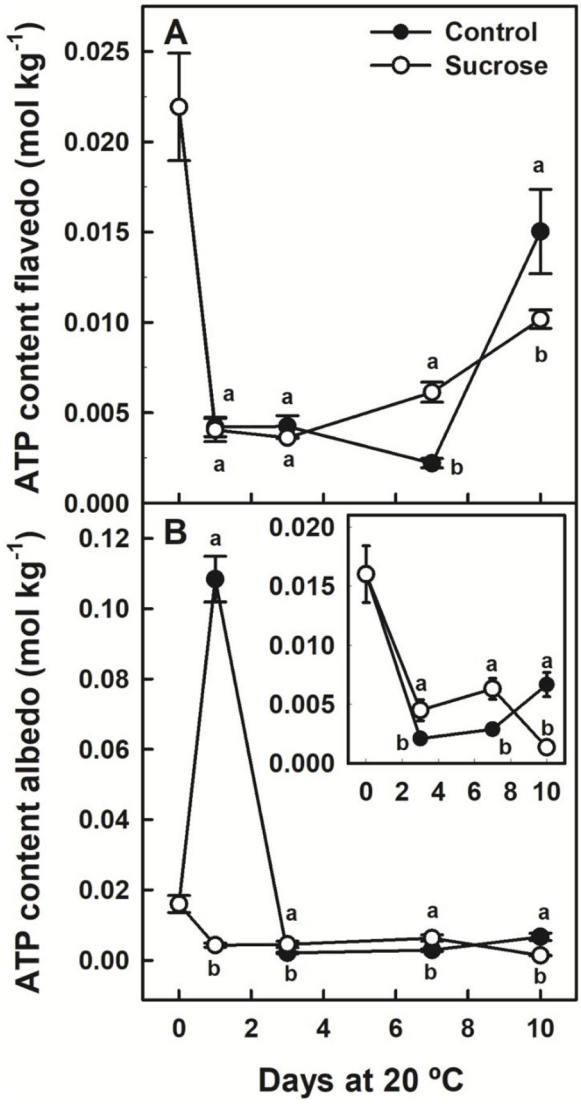

Fig. 3. Changes in ATP content in the flavedo (A) and albedo (B) of Navelate sweet orange, treated (◯) or not (●) with Suc, during fruit storage at 20 °C and 90–95 % RH. Inset panel in Fig. 3B shows ATP values in the albedo in a wider scale to better illustrate the differences between the ATP content of fruit treated or not with Suc. The legends of X and Y axis on graph of the inset panel are the same to those of the outset panel. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the control and the Suc-treated fruit for the same storage period.

The expression of PLD-encoding genes increased in the flavedo of control fruit after fruit detachment. The pattern of changes in gene expression differed during fruit storage at 20 °C and 90–95 % RH (Fig. 5). The expression of *CsPLDα, CsPLDδ, CsPLDγ* and *CsPLDζ* increased after 3 d. The *CsPLDδ*–encoding gene showed the highest increase. Such increase was transitory and reached a maximum by day 3. A faster (1 d) and transient increase was also observed in the expression of the *CsPLDβ*–encoding gene, although it was lower. In contrast, the expression of *CsPLDα, CsPLDγ*, and *CsPLDζ* increased from 3 to 10 d. Treating the fruit with Suc had a strong impact in the trend of changes in the expression of all PLD-encoding genes in the flavedo (Fig. 5). In general, Suc avoided the rise observed in the control non-treated fruit. This effect was more marked in the *CsPLDα-, CsPLDγ-* and *CsPLDδ*-encoding genes. Moreover, expression levels of the *CsPLDβ* and *CsPLDζ* isoforms in the flavedo of the Suc-treated fruit mirrored those of their respective control fruit (Fig. 5).

PLD-encoding genes were also up-regulated in the albedo after fruit detachment (Fig. 6). Except for *CsPLDβ*, expression patterns in the albedo did not parallel those observed in the flavedo (Fig. 5), although

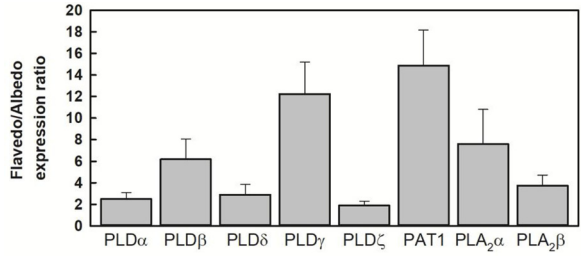

Fig. 4. Relative expression levels of *CsPLD-* and *CsPLA-* encoding genes in the flavedo respect to the albedo. The error interval indicates the standard error of the estimated mean value.

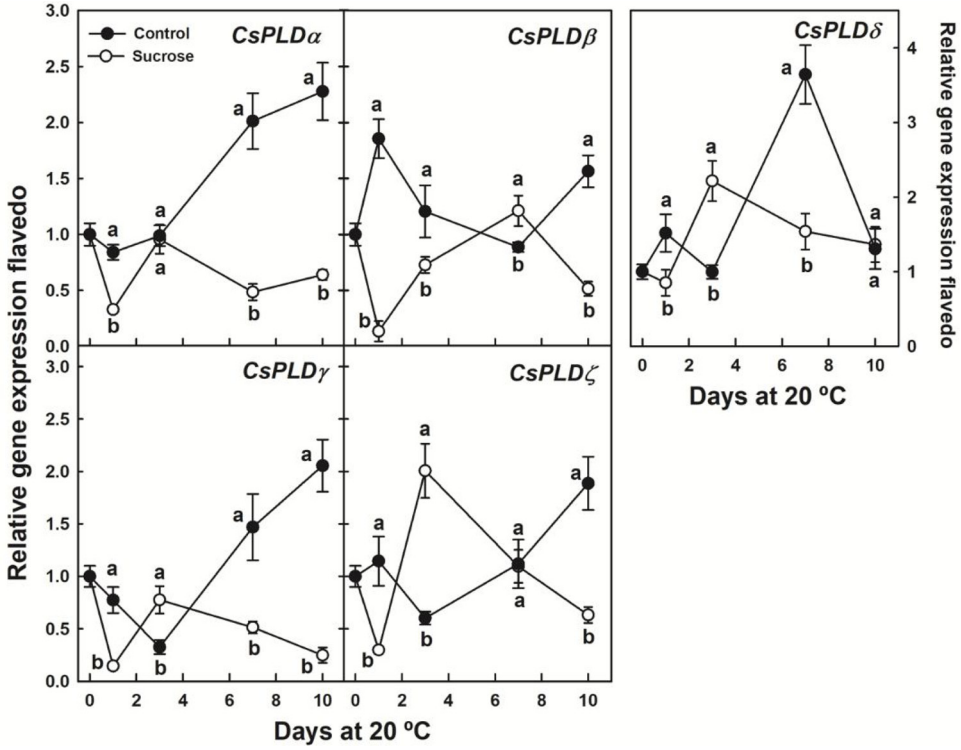

Fig. 5. Changes in the expression of genes encoding PLD in the flavedo of Navelate sweet orange, treated (◯) or not (●) with Suc, during fruit storage at 20 °C and 90-95 % RH. Values are expressed as relative gene expression, which was calculated respect to the flavedo of freshly harvested fruit. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the control and the Suc-treated fruit for the same storage period.

some similarities were found for each individual gene. A common feature for all PLD-encoding genes was the early (1d) and transient increase in their expression, which was specially marked in the *CsPLDδ*-encoding gene (7.5-fold increase) (Fig. 6). The expression of this gene also peaked by day 7, which paralleled the trend found in the flavedo. However, the relative increase in gene expression was much higher in the albedo (Figs. 5 and 6). In this tissue, the time course changes in the expression of the *CsPLDα*-encoding gene was similar to that of *CsPLDδ* although the transcript accumulation was lower. Finally, expression levels of the *CsPLDγ* and *CsPLDζ* isoforms increased, like in the flavedo, at the end of the experiment. The relative increase found in *CsPLDγ* was about 4.5-fold higher in the albedo (Figs. 5 and 6). As in the flavedo, in general, Suc had an effect reducing the up-regulation of PLD-encoding genes in the inner peel part (Fig. 6). Examination of changes in the expression of the *CsPLDβ* gene revealed that Suc repressed the early (1 d) rise in the expression of this gene in the albedo. However, it caused a marked transient up-regulation, respect to its control sample, at a later storage period (Fig. 6).

CsPLA$_2$α was down-regulated after detachment in the inner and outer peel parts, although its expression increased at long term in the flavedo (Fig. 7). Suc had a much lower effect on changes in the expression of this gene than in the expression of the PLD-encoding genes. It had also a lower effect on the expression of *CsPAT1* in the flavedo (Fig. 7A), which transiently increased after fruit detachment, reaching a maximum by day 3. The expression of this gene barely changed in the albedo of the control fruit but Suc had a marked effect favouring a transient peak by day 7 after fruit detachment (Fig. 7B). An early (1 d) transient increase in *CsPLA$_2$β* expression levels occurred as a consequence of detachment in both the flavedo and albedo of control fruit, which was repressed by applying Suc (Fig. 7). Its expression barely changed after 3 d in the albedo (Fig. 7B) and it increased in the flavedo (Fig. 7A). Such increase was reduced by Suc (Fig. 7A) although the treatment markedly increased *CsPLA$_2$β* expression in the albedo, both at the beginning and at the end of the experiment (Fig. 7B).

4. Discussion

Data obtained in control and Suc-treated fruit on respiration, ATP content, and expresion of PL-encoding genes participating in plant defense signaling but also in lipolysis in starved cells, reinforce the idea that energy shortage caused by carbon starvation is an important factor for NCPP development in detached citrus fruit stored under non-stressful environmental conditions (Cajuste et al., 2011; Establés-Ortiz et al., 2016; Romero et al., 2020). Our results also support that the albedo is more susceptible to this stress than the flavedo. In fact, respiration of the flavedo was much higher than that of the albedo, which reflects a higher metabolic activity, and agrees with the higher ability of the flavedo to provide energy (Matas et al., 2010). In addition, the trend of changes in albedo respiration better mimicked the characteristic respiratory trend associated with starvation in plants. This trend is characterized by an early transient decrease in the respiration rate, associated with the decrease in cellular carbohydrate levels, which is followed by a second survival phase (Krotktov, 1939; Brouquisse et al., 1991). This second phase involves energy reconfiguration to provide additional energy sources to sustain respiration and requires breakdown of proteins and lipids and other alternative modes of energy metabolism (Brouquisse et al., 1991). In this work, the relative decrease in respiration was more marked in the albedo than in the flavedo, and the second phase delayed in the inner tissue, (Fig. 2), which is in concordance with lower ability of the albedo to provide energy sources (Matas et al., 2010).

The results also agree with previous findings indicating that sugar starvation can be reversed and respiratory rate restored by sugar replenishment until starvation becomes irreversible (Brouquisse et al., 1991; Graham et al., 1994). This result suggest that exogenous Suc serves as an additional fuel for respiration and reduces the use of alternative energy sources like the breakdown of lipids and proteins that ends in membrane deterioration and peel damage in citrus fruit (Cajuste et al., 2011; Establés-Ortiz et al., 2016). This is in concordance with findings showing that extenal energy souces like glycerol or ATP reduce lipid and protein degradation, and NCPP in citrus fruit (Romero et al., 2020). The effect of Suc on maintaining respiration was lower in the

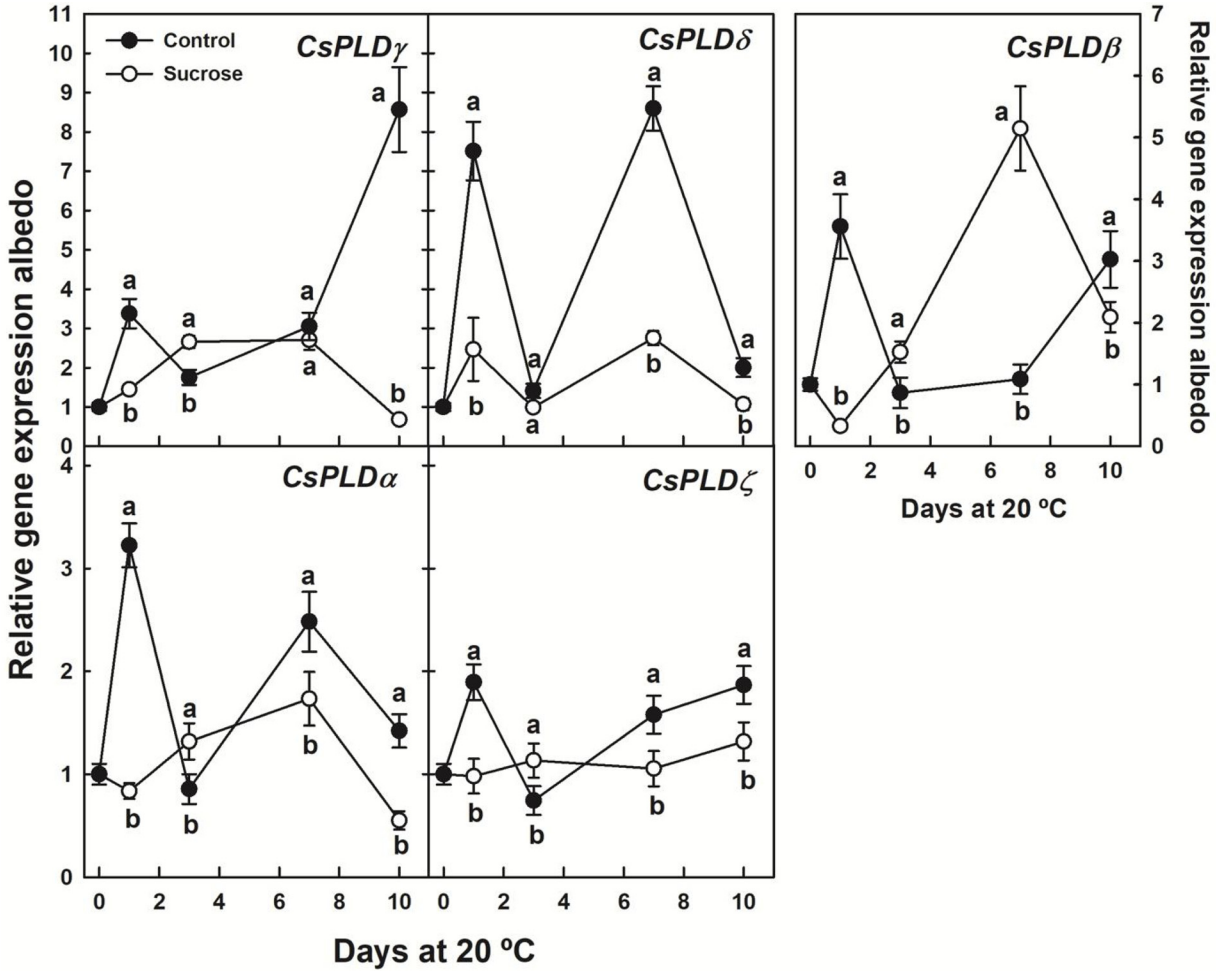

Fig. 6. Changes in the expression of genes encoding PLD in the albedo of Navelate sweet orange, treated (◯) or not (●) with Suc, during fruit storage at 20 °C and 90-95 % RH. Values are expressed as relative gene expression, which was calculated respect to the albedo of freshly harvested fruit. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the control and the Suc-treated fruit for the same storage period.

albedo than in the flavedo, However, this exogenous energy source had a marked effect on changes in ATP and on the expression of PLD and PLA_2- encoding genes in the less energetic peel tissue (albedo) (Figs. 3B,). It was surprising to find the early sharp and transient increase in ATP peaking at 1 d in the albedo of control fruit (Fig. 3B) since previous findings in citrus fruit (Romero et al., 2020) and in plant cells (Rawyler et al., 1999, 2002), indicate that cellular perception of energy shortage, manifested as a decrease in ATP, precedes to damage development associated with loss of cell membrane integrity. However, we should bear in mind that this early peak, occurring immediately after harvesting the fruit, was avoided by treating the fruit with Suc and was followed by a sharp decline in ATP at the second starvation phase (Fig. 3), which preceded NCPP development (Fig. 1). As mention above, in this phase, lipid utilization starts to cope with carbon-starvation. The situation is not simple, but an interesting idea from these results and others indicating that exogenous ATP reduces energy shortage by over-representing energy-related processes and orchestrates plant defense responses in citrus fruit aiming to content cell damage propagation (Romero et al., 2020), is that ATP is a sensitive signal of carbon starvation in the albedo both in the first and second starvation stages. The first rise would be associated with cell perception of the stress at detachment aiming to activate plant defense responses (Romero et al., 2020); and the subsequent decline, would work as the signal for lipid utilization because of a more advanced starvation stage (Rawyler et al., 1999, 2002). In line with this, Suc avoided the first increase in ATP and reduced the ATP decline by about twice at the beginning of the second phase.

The results showing the early (1 d) transient increase in gene expression in the albedo also suggest that these genes may be good indicators of carbon starvation in the inner part of citrus fruit peel, which agrees with results in suspension cells showing the activation of PLD at the early stage of starvation (Lee, 1998). At this first starvation stage (1 d), Suc avoided the up-regulation of all PLD-encoding genes (Fig. 6) and of *CsPLA$_2$β* (Fig. 7B). Therefore, these early transient increases, which mainly occurred in the albedo, agree with the role of phospholipids as rich sources for signaling messengers in plant defense (Wang, 2001; Meijer and Munnik, 2003). In this regard, it is noticing the higher expression of all genes at harvest in the outer peel part (Fig. 4), since this is the first barrier against biotic and abiotic stresses in fruit.

At the second starvation phase, among the PLD-encoding genes, it is remarkable the effect of Suc increasing the expression of *CsPLDβ* (Fig. 6); and that of *CsPLDζ* (Fig. 5). This gene has been related to Pi starvation and participates in its production (Cruz-Ramírez et al., 2006); and in citrus fruit it has been shown that treating citrus fruit with an external source of carbon (glycerol) favors alternative modes of energy leading to the increase in Pi, necessary for ATP production, and reduced NCPP caused by energy shortage after fruit detachment (Romero et al., 2020). Such Suc-induced increases were transitory and occurred after 3 d in the flavedo (*CsPLDζ*) and 7 d in the albedo (*CsPLDβ*), when NCPP was detectable. Therefore, these genes may also

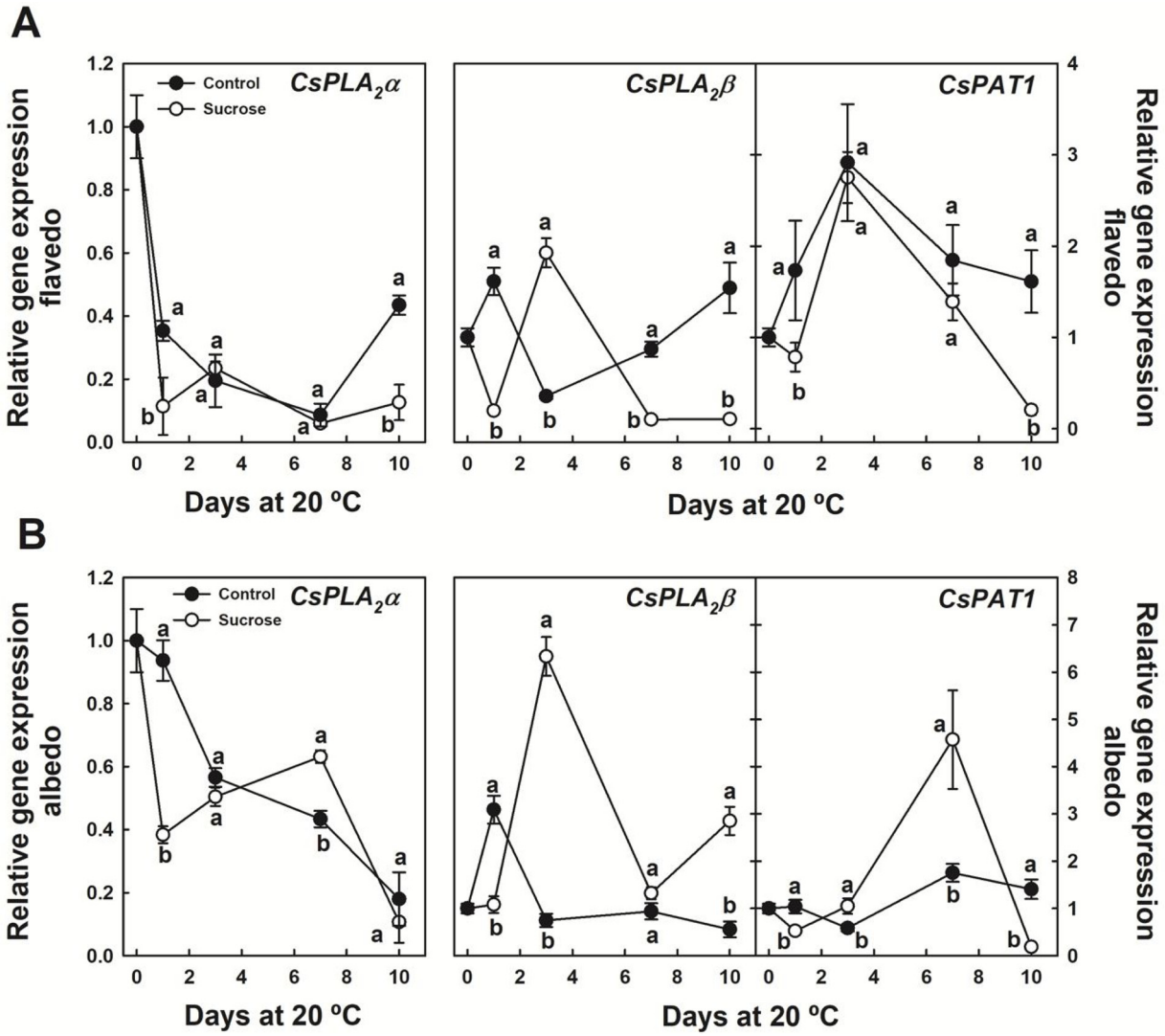

Fig. 7. Changes in the expression of genes encoding PLA in the flavedo (A) and the albedo (B) of Navelate sweet orange, treated (◯) or not (●) with Suc, during fruit storage at 20 ˚C and 90–95 % RH. Values are expressed as relative gene expression, which was calculated respect to the flavedo (A) or the albedo (B) of freshly harvested fruit. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the control and the Suc-treated fruit for the same storage period.

mediate Suc phospholipid-derived signaling at the second starvation phase in citrus fruit. Their expression increased in control fruit at long-term storage (Figs. 5 and 6) and, therefore, we cannot rule out their participation in damage development after long-term starvation because of need of lipid utilization to sustain respiration in fruit that were non-treated with Suc. This appears to be also the case of the other PLD-encoding genes, whose expression increased both in the flavedo and the albedo at long-term storage, especially that of *CsPLDδ* which showed major increases, overall in the albedo. Interestingly, the expression of *PLDδ* can be activated by reactive oxygen species (ROS) (Wang, 2005), while the *CsPLDδ* increases were transitory and ROS is an important component in starvation-induced NCPP (Cajuste and Lafuente, 2007; Establés-Ortiz et al., 2016; Romero et al., 2020). Moreover, the rises in *CsPLDδ* expression (Figs. 5 and 6) were attenuated by adding Suc, which is in concordance with previous findings indicating that treating citrus fruit with energy sources attenuates ROS-related responses in citrus fruit (Romero et al., 2020). Therefore, we cannot rule out the participation of ROS in the pattern of changes of this gene. Finally, it is remarkable the major increase in expression of *CsPLDγ* in the albedo and flavedo at long-term starvation, which was avoided by adding Suc (Figs. 5 and 6). Therefore, this gene might be a good marker of starvation-induced peel damage in citrus fruit.

Changes in expression of *CsPLA$_2$β* in the flavedo and albedo (Fig. 7) at the early starvation stage and in the Suc-treated fruit showed a similar trend to that of *CsPLDζ* and *CsPLDβ*. Therefore, this gene might be also a good indicator of energy shortage at short-term starvation and contribute to flavedo damage at long-term (Fig. 7A). In addition, comparative transcriptomic analysis of control and Suc-treated fruit indicates that Suc, besides contributing to NCPP reduction by providing additional energy sources, might favour phospholipid-derived signaling mediated by *CsPLA$_2$β* aiming to content lesion propagation. Suc also favored a transient accumulation of *CsPAT1* in the albedo peaking at day 7, which suggests that this PLA-encoding gene also contribute to the protective effect of Suc in the albedo. A similar transient increase was observed in the flavedo of Suc-treated fruit, which preceded to that found in the albedo, but this was similar in control fruit. This result points out that *CsPAT1* gene expression is differentially regulated by Suc in citrus depending on the tissue. However, it could be also related to the higher content of this sugar in the flavedo, which should be high enough to favor up-regulation of *CsPAT1*. Previous investigations indicated that *CsPLA$_2$α* is related to the development of NCPP induced in citrus fruit by severe water stress that causes very severe peel damage

(Romero el al., 2013a) but its expression remained almost unchanged during mild dehydration (Romero et al., 2014). Under our experimental conditions, the expression of this gene sharply decreases in the flavedo and albedo after fruit detachment, slightly increase only in the flavedo by the end of the experiment, and was barely affected by exogenous Suc, which brings to question the participation of this *CsPLA$_2$* isoform on starvation-induced NCPP. Therefore, data from this and from previous studies indicate that changes in the expression of this gene differently correlates with the incidence of NCPP depending on the environmental factor causing the development of this physiological disorder, which envisages a potential use for this gene as molecular marker for agronomic purposes. Likewise, while *CsPLA$_2\beta$* and *CsPAT1* isoforms appear to be more likely related to NCPP alleviation in fruit stored under non-stressful environmental conditions than to contribute to its development, *CsPLA$_2\beta$* was highly induced and *CsPAT1* down-regulated under mild dehydration process (Romero et al., 2014).

In addition to its essential role as substrate in carbon and energy metabolism, Suc has important hormone-like functions as primary messenger in signal transduction (Rolland et al., 2002). Taken together, results indicate that treating citrus fruit with Suc may reduce damage by increasing carbon sources for respiration but also by stimulating *CsPLA$_2\beta$* and *CsPAT1* isoforms in the albedo and *CsPLA$_2\beta$* in the flavedo. Moreover, Suc stimulated *CsPLDβ* in the albedo and *CsPLDζ* in the flavedo. Therefore, our results point out a crosstalk between this sugar and PLD and PLA$_2$ signaling in the peel of citrus fruit. Likewise, a crosstalk has been proposed between PLD and PLA-encoding genes (Zhao et al., 2013), and we have found a parallelism between expression profiles of *CsPLA$_2\beta$* and *CsPAT1* isoforms in the albedo of Suc-treated fruit. This indicates a putative link existing between both isoforms and Suc signaling in citrus fruit.

5. Conclusions

The albedo is more susceptible than the flavedo to carbon starvation caused by fruit detachment. ATP as well as all *CsPLD* genes and *CsPLA$_2\beta$* are good indicators of the early stage of carbon starvation in the albedo and *CsPLDβ* in the flavedo. At long-term, all *CsPLD* isoforms may participate in lipid degradation ending in peel damage, and at a major extent the isoforms *CsPLDγ* and *CsPLDζ*, whose expression highly increased in the inner and outer peel parts. The putative participation of *CsPLA* isoforms on damage was lower. Results also indicate a link existing between *CsPLA$_2\beta$* and *CsPAT1* isoforms and Suc signaling in citrus fruit. This sugar, used as an external energy source to delay starvation, reduced perception of carbon starvation stress at early stages of the process (1 d); and at later stages played a dual role on reducing NCPP damage. Thus, reduction of NCPP by Suc may be due in part to its ability to provide additional energy sources to sustain respiration after fruit detachment; and, on the other hand by favouring at long-term phospholipid-derived signaling messengers mediated by *CsPLDβ* and *CsPAT1* in the albedo, *CsPLDζ* in the flavedo, and *CsPLA$_2\beta$* in both tissues. Results also indicate that differences in gene expression between both tissues appear to be related to their different susceptibility to starvation but also to tissue specificity.

Author statement

M.T. Lafuente and F. Alférez conceived the project. M.T. Lafuente designed the experiments. P. Romero, M.T. Lafuente and F. Alférez performed the experiments. M.T. Lafuente wrote the manuscript and all the authors approved the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix B. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2020.111295.

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