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Peach (*Prunus Persica*) Fruit Response to Anoxia: Reversible Ripening Delay and Biochemical Changes

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The use of modified atmospheres has been successfully applied in different fruits to delay the ripening process and to prevent physiological disorders. In addition, during normal ripening, hypoxic areas are generated inside the fruit; moreover, anaerobic conditions may also arise during fruit post-harvest storage and handling. In consequence, the fruit is an interesting model to analyze the metabolic modifications due to changes in oxygen levels. In this work, a 72 h anoxic treatment by using an N_2 storage atmosphere was applied to peaches (*Prunus persica* L. Batsch) after harvest. Ripening was effectively delayed in treated fruits, preventing fruit softening, color changes and ethylene production. Metabolic changes induced by anoxia included induction of fermentative pathways, glycolysis and enzymes involved in both sucrose synthesis and degradation. Sucrose, fructose and glucose contents remained unchanged in treated fruit, probably due to sucrose cycling. Sorbitol was not consumed and citrate was increased, correlating with citric acid cycle impairment due to O_2 deprivation. Malate content was not affected, indicating compensation in the reactions producing and consuming malate. Changes in malic enzymes and pyruvate orthophosphate dikinase may provide pyruvate for fermentation or even act to regenerate NADP. After fruit transfer to aerobic conditions, no signs of post-anoxia injury were observed and metabolic changes were reversed, with the exception of acetaldehyde levels. The results obtained indicate that peach fruit is an organ with a high capacity for anoxic tolerance, which is in accord with the presence of hypoxic areas inside fruits and the fact that hypoxic pre-treatment improves tolerance to subsequent anoxia.

Keywords: Anoxic atmosphere • Ethylene • Metabolism • Peach • Post-harvest • *Prunus persica*.

Abbreviations: ACO1, AAC (aminocyclopropane-1-carboxylic acid) oxidase 1; ADH, alcohol dehydrogenase; AI, acid invertase; DTT, dithiothreitol; FK, fructokinase; GK, glucokinase; LDH, lactate dehydrogenase; NI, neutral invertase; NA, N_2 atmosphere; NAD-MDH, NAD-malate dehydrogenase; NAD-ME, NAD-malic enzyme; NADP-ME, NADP-malic enzyme; OAA, oxaloacetate; PDC, pyruvate decarboxylase; PEPC, phosphoenolpyruvate carboxylase; PCK, phosphoenolpyruvate carboxykinase; PEG, polyethylene glycol; PFK, ATP-dependent phosphofructokinase; PFP, PP_i-dependent phosphofructokinase; PK, pyruvate kinase; PPDK, pyruvate orthophosphate dikinase; RT–PCR, reverse transcription–PCR; SDH, sorbitol dehydrogenase; SPS, sucrose-P-synthase; SS, sucrose synthase; SSC, soluble solid content.

Introduction

During ripening, fleshy fruits are transformed into palatable products, with modifications in color, texture, levels of sugars, organic acids and volatile compounds leading to changes in nutritional quality, flavour and aroma (Giovannoni 2004). The peach (*Prunus persica* L. Batsch) is a climacteric fruit whose ripening process is controlled by the production of ethylene, although other hormones are also involved in this process (Trainotti et al. 2007). Changes in the physical characteristics, chemical composition and metabolic processes take place during ripening (Borsani et al. 2009). However, a rapid ripening in peach fruit is responsible for its short shelf-life. Fruit market life can be extended by slowing ripening by refrigeration; nevertheless, the cold storage life of peaches is limited by chilling injury (Lurie and Crisosto 2005, and references therein). In order to prevent or alleviate chilling injury symptoms, different pre-harvest and post-harvest treatments have been applied to this fruit, including salicylic acid treatments, exposure to sublethal high temperature or the use of modified atmospheres (Lurie and Crisosto 2005, Budde et al. 2006, Lurie 2006, Wang et al. 2006, Jin et al. 2009, Lara et al. 2009).

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Oxygen is closely linked to the rate of respiration of harvested products, as, if its internal concentration decreases, respiration also decreases. In addition, oxygen is required for ethylene biosynthesis and, thus, its concentration affects ethylene evolution in peaches (Lurie and Pesis 1992). Therefore, the use of a low-oxygen environment or an anoxic atmosphere is able to delay the ripening process and to prevent physiological disorders in fruits (Pesis et al. 1993, Tonutti et al. 1997, Bonghi et al. 1999). The use of modified atmospheres has been successfully applied in different fruits as a complement to refrigeration or even as an alternative (Nanos et al. 1992, El-Mir et al. 2001, Jiang et al. 2004, Polenta et al. 2005). Nevertheless, a low-oxygen environment may provoke detrimental effects such as abnormal ripening, production of off-flavors, flesh browning and a large increase in ethanol and acetaldehyde (El-Mir et al. 2001, Fallik et al. 2003).

Several studies have been performed in plants regarding the molecular and biochemical responses to anaerobic stress (hypoxia or anoxia), with flooding being the most studied environmental condition associated with hypoxia (Geigenberger 2003, Gibbs and Greenway 2003, Greenway and Gibbs 2003, Colmer and Greenway 2005, Fukao and Bailey-Serres 2007, Licausi and Perata 2009). With regards to fruit, although several studies have indicated the potential use of controlled atmospheres in delaying ripening, only a few studies have analyzed the molecular responses and metabolic changes of this organ in relation to anaerobic treatment (Pasentsis et al. 2007, Pedreschi et al. 2008, Shi et al. 2008, Pedreschi et al. 2009a). In this respect, the fruit is an interesting model to analyze the metabolic modifications due to anoxia or hypoxia, since during normal ripening metabolite gradients occur in relation to the in situ hypoxic areas generated inside the fruit (Pedreschi et al. 2009b, Biais et al. 2010). Moreover, anaerobic conditions may also arise during fruit post-harvest storage and handling, e.g. because of incorrect ventilation, due to sealing or packing, or due to the application of wax coatings. Analysis of the fruit metabolic changes that take place due to anaerobic treatment is required for the identification of factors involved in a better tolerance to anoxia and/or hypoxia. Therefore, in the present work the impact of anoxic treatment on peach was assessed through the evaluation of ethylene production, organoleptic characteristics, levels of enzymes involved in central metabolic pathways and the contents of metabolites such as sugars, sorbitol, fermentation products and organic acids. In addition, upon returning the fruits to aerobic atmospheres, peaches were tested regarding their capacity to continue normal ripening, by analyzing the biochemical changes in the fruit flesh during the post-anoxic ripening.

Results

Quality attributes of 'Dixiland' peach

Anoxic treatment effectively prevented the most relevant organoleptic changes that usually take place during normal ripening under aerobic conditions (Borsani et al. 2009). The pronounced decrease in firmness after 3 d in aerobic conditions (R3) was prevented under an N_2 atmosphere (NA), with NA peaches showing the same fruit firmness as after harvest (R0) (**Table 1**). In addition, ground color also remained unchanged with respect to R0 fruit, in contrast to fruit held in air of the same post-harvest age (R3, **Table 1**). This behavior of treated fruit (NA) is in agreement with ethylene levels, which remained similar to those at R0 (**Fig. 1**). In fruit treated with N_2, the prevention of the increase in ethylene that occurs under normal air conditions is also in agreement with the low levels of *Pp-ACO1*, encoding aminocyclopropane-1-carboxylic acid oxidase 1 (**Fig. 1**). Upon transferring treated fruit to aerobic conditions (NA + 3), *Pp-ACO1* levels increased and ethylene was also released in NA + 3 peaches, reaching similar levels to those of peaches of the same post-harvest age ripening in air (R5) (**Fig. 1**). In accordance with these results, firmess was also decreased in NA + 3 peaches with respect to NA and R0, but to a lesser extent than in fruit ripening in air (R3 and R5), and ground and pulp colors reached levels similar to R5 peaches (**Table 1**). On the other hand, soluble solid content (SSC) and titratable acidity of treated fruit were similar to those of fruit of the same post-harvest age ripening under aerobic conditions (R3) (**Table 1**). Nevertheless, after transfer to aerobic conditions, SSC and titratable acidity remained unaltered and similar to those of R5 fruit.

Table 1 Quality attributes of 'Dixiland' peach at different stages after harvest and after exposure to an N_2 atmosphere

Parameter	Sample				
	R0	R3	R5	NA	NA + 3
Firmness (N)	60.1 ± 5.9a	18.3 ± 4.2b	11.3 ± 1.7c	60.4 ± 2.7a	37.8 ± 9.5d
Ground color					
H value	101.0 ± 1.7a	96.3 ± 2.4b	92.9 ± 3.1c	99.7 ± 1.6a	95.2 ± 2.5bc
Pulp color					
H value	96.2 ± 2.1a	94.5 ± 1.5ab	90.2 ± 1.8c	94.4 ± 1.2a	92.1 ± 1.6bc
Soluble solids (°Brix)	11.3 ± 1.2a	10.7 ± 0.8ab	11.5 ± 0.9a	10.2 ± 0.9b	10.8 ± 0.8ab
Acidity [H^+] (mM)	134.3 ± 11.6a	107.3 ± 3.2b	108.7 ± 6.1b	96.3 ± 3.2b	96.3 ± 7.6b

Different letters within each parameter indicate statistically significant differences.

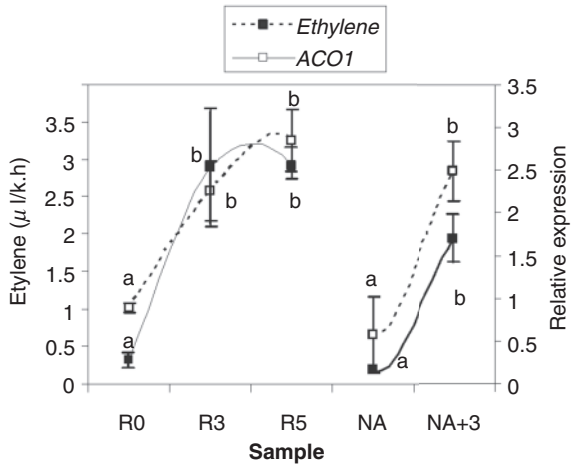

Fig. 1 Relationship between ethylene production and *Pp-ACO1* level. Fruit were collected (R0) and allowed to ripen during 3 and 5 d in air (R3 and R5) or maintained in an anoxic chamber for 3 d (NA) and then transferred to aerobic conditions for another 3 d (NA + 3). Expression analysis of *Pp-ACO1* involved in ethylene biosynthesis was determined by real-time RT–PCR. The means of the results obtained, using three independent mRNAs as template, are shown. Each reaction was normalized using the C_t values corresponding to *P. persica* elongation factor 1 mRNA. The *y*-axis refers to the fold difference in a particular transcript level relative to its amount found in peaches after harvest (R0). Standard deviations are shown. Values with the same letters are not significantly different ($P < 0.05$).

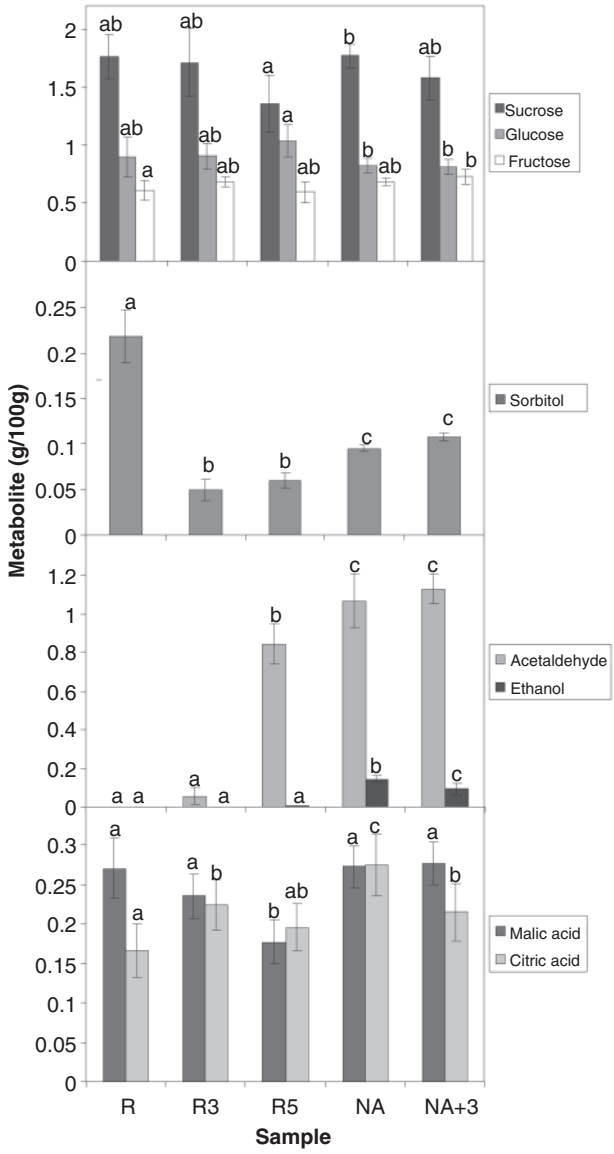

Fig. 2 Sugars, organic acid and fermentation products in peach fruit ripening at ambient temperature or exposed to an anoxic atmosphere. Samples were collected immediately after harvest (R0), after 3 and 5 d under normal air conditions (R3 and R5, respectively), after 3 d in an N_2 atmosphere (NA) and after transfer to air for 3 d following the anoxic treatment (NA + 3). Values are expressed in g per 100 g of fruit fresh weight and represent the mean of at least 14 independent determinations ± SD. For each metabolite, bars with the same letters are not significantly different ($P < 0.05$).

Regarding sugar contents, neither sucrose, glucose nor fructose levels were significantly altered during ripening under normal air conditions or after anoxic treatment (**Fig. 2**). Nevertheless, sorbitol levels, which decreased during normal ripening (R3 and R5, with respect to R0), were also decreased in NA and NA + 3 peaches but to a lesser extent than in fruit of the same post-harvest age ripening in air (R3 and R5, respectively, **Fig. 2**).

Fermentation products, ethanol and acetaldehyde, were greatly increased in treated fruits (NA) in comparison with R3 or R0 fruits (**Fig. 2**). When treated fruits were transferred to aerobic conditions for 3 d, the amount of acetaldehyde remained high, while ethanol was decreased. Nevertheless, ethanol content in NA + 3 was higher than in R5 (**Fig. 2**).

With respect to content of organic acids, an increase in the citric acid level was observed in treated fruits (NA) with respect to R0 and R3 peaches (**Fig. 2**). On the other hand, malic acid levels, which decreased in R5 peaches, remained at the same level as R0 in treated fruits (NA and NA + 3, **Fig. 2**). Upon transferring treated fruits to air conditions, a decrease in citric acid was observed, reaching values similar to those of R5 (**Fig. 2**).

Enzymes involved in sugar and organic acid metabolism

Several enzymes involved in sugar metabolism were analyzed in N_2-treated and untreated peach fruit through quantitative real-time reverse transcription–PCR (RT–PCR) or by activity measurements (**Figs. 3**, **4**).

Regarding sucrose metabolism, nitrogen treatment for 3 d notably increased the transcripts encoding sucrose synthase (SS; EC 2.4.1.13, *Pp-SS*) and sucrose-P-synthase (SPS; EC 2.3.1.14, *Pp-SPS1* and *Pp-SPS2*) (**Fig. 3A**), with a >30-fold increase in *Pp-SPS2* in NA with respect to R0 and fruits of the same post-harvest age (**Fig. 3A**). With regards to neutral

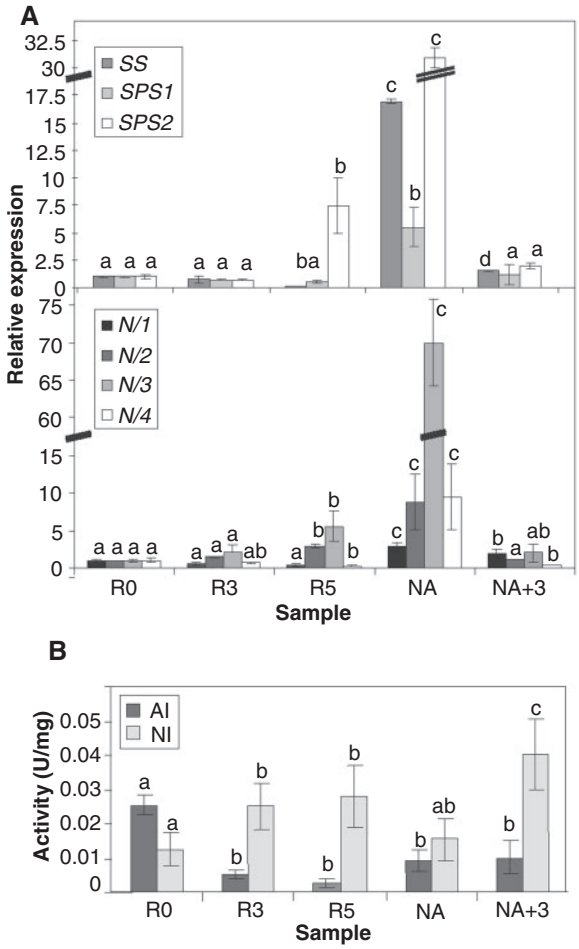

Fig. 3 Analysis of enzymes involved in sucrose metabolism. (A) Expression analysis, assessed by quantitative real-time RT–PCR, of transcripts encoding sucrose synthase (SS), sucrose-P-synthase (SPS) and neutral invertase (NI). Means of the results obtained, using three independent RNAs as template, are shown. Each reaction was normalized using the C_t values corresponding to elongation factor 1 mRNA. The *y*-axis refers to the fold difference in a particular transcript level relative to its amount found in R0. (B) Activity assays of invertases. Peach fruit were collected immediately after harvest (R0) and after 3 (R3) and 5 d (R5) in air or, alternatively, exposed to anoxic treatment for 3 d (NA) followed by 3 d in air (NA + 3). Standard deviations are shown. For each transcript or activity analyzed, bars with the same letters are not significantly different ($P < 0.05$).

invertases (NI; *Pp-NI-4*), increases were also observed after anaerobic treatment, with increases of nearly 3-fold in the case of *Pp-NI1* and up to 70-fold in the case of *Pp-NI3* with respect to R0 (**Fig. 3A**). The increase was also pronounced when untreated fruit of the same post-harvest age (R3) were compared (**Fig. 3A**). However, after transfer to aerobic conditions, transcripts levels were significantly decreased, returning to the same levels as those found after harvest (R0) or in untreated peaches of the same post-harvest age (R5) (**Fig. 3A**). In contrast to these results, NI activity was not modified in treated fruits (NA) with respect to R0 or R3 (**Fig. 3B**). However, after transfer to aerobic conditions (NA + 3), NI activity significantly increased (3.2-fold with respect to R0) (**Fig. 3B**). On the other hand, acidic invertase (AI) activity was not affected by the treatment; the decrease in AI activity observed in fruits in aerobic conditions was maintained in fruits treated with N_2 (NA and NA + 3) (**Fig. 3B**).

Regarding sorbitol usage, the transcript encoding sorbitol dehydrogenase (*Pp-SDH*; Yamada et al. 2001) was induced nearly 15-fold in NA fruits with respect to R0. Upon transfer to aerobic conditions, *Pp-SDH* returned to values similar to those found in untreated fruit (R3 or R5) (**Fig. 4A**).

With respect to the enzymes involved in the further metabolization of glucose and fructose, enzymes catalyzing key steps, such as glucokinase (GK), fructokinase (FK), ATP-dependent phosphofructokinase (PFK), PP_i-dependent phosphofructokinase (PFP) and pyruvate kinase (PK), were analyzed. The decrease in *FK* that takes place during ripening under aerobic condition (R3 and R5 vs. R0) was prevented in N_2-treated fruits (NA) (**Fig. 4A**). Nevertheless, after return to aerobic conditions, a decrease in this transcript with respect to R0 were observed, as in R5 peaches (**Fig. 4A**). No significant changes in the activity of GK, PFP or PK were observed during peach softening at ambient conditions or after N_2 treatment (**Fig. 4B**). On the other hand, PFK activity, which is constant during ripening in air, was increased after anoxic treatment (**Fig. 4B**).

In agreement with an anoxic treatment and high levels of ethanol and acetaldehyde (**Fig. 2**), the activity of enzymes involved in fermentation was induced in NA fruits (**Fig. 4B**). Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities were greatly increased (6- and 6.5-fold, respectively) with respect to untreated fruit (R3), followed by an almost 2-fold increase in lactate dehydrogenase (LDH) activity (**Fig. 4B**). When treated fruits were transferred to aerobic conditions for 3 d (NA + 3), the activity of these enzymes was decreased to the levels observed in R5 fruits (**Fig. 4B**).

Several enzymes involved in organic acid metabolism were also analyzed by either activity measurements or Western blot analysis (**Fig. 5**). The increase in phosphoenolpyruvate carboxylase (PEPC) activity during post-harvest at ambient atmosphere was prevented by N_2 treatment (**Fig. 5A**). On the other hand, no changes in phosphoenolpyruvate carboxykinase (PCK) activity were observed in N_2-treated vs. untreated fruit (NA vs. R3, and NA + 3 vs. R5) (**Fig. 5**). An increase in the immunoreactive band of pyruvate orthophosphate dikinase (PPDK) was detected in N_2-treated fruits (NA) with respect to peach ripening under an aerobic atmosphere (R3). Nevertheless, after transfer to aerobic conditions (NA + 3) the intensity of this band was practically the same as in untreated fruit (R5) (**Fig. 5B**). With regards to malate-consuming enzymes, both NAD- and NADP-malic enzyme (NAD-ME and NADP-ME, respectively) activities were increased in treated fruits (NA) with respect to R3 (**Fig. 5**). On the other hand, no significant change in NAD-malate dehydrogenase (NAD-MDH) activity was detected during peach ripening under either aerobic or anaerobic conditions (not shown).

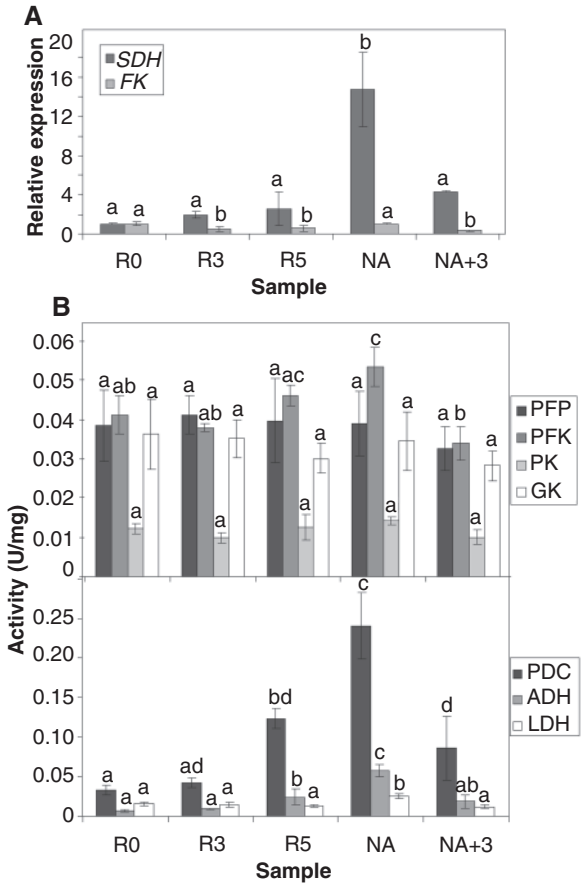

Fig. 4 Enzymes involved in peach fruit carbon metabolism. (A) Expression analysis of transcripts encoding sorbitol dehydrogenase (SDH) and fructokinase (FK). Quantitative real-time RT–PCR was carried out as described in the Materials and Methods. Means of the results obtained, using three independent RNAs as template, are shown. Each reaction was normalized using the C_t values corresponding to elongation factor 1 mRNA. The *y*-axis refers to the fold difference in a particular transcript level relative to its amount found in R0. (B) Activity measurements of enzymes involved in glycolysis (PFP, PFK, PK and GK) and in fermentation (PDC, ADH and LDH). For each enzyme, bars with the same letters are not significantly different ($P < 0.05$). Values represent the mean of at least six independent determinations using different fruits ± SD. Peach fruit were collected immediately after harvest (R0) and after 3 (R3) and 5 d (R5) in air or, alternatively, exposed to anoxic treatment for 3 d (NA) followed by 3 d in air (NA + 3).

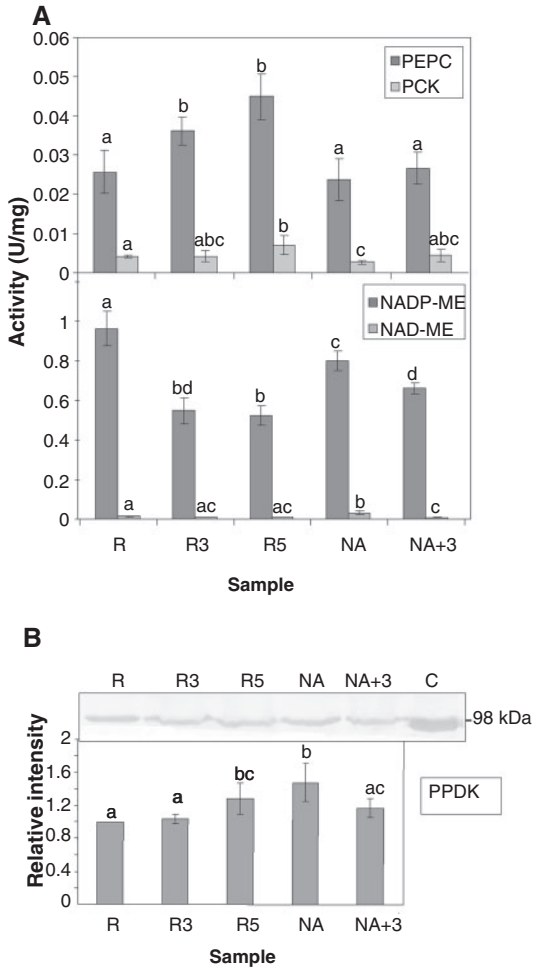

Fig. 5 Enzymes involved in organic acid metabolism in peach fruit. (A) Activity assays of enzymes involved in PEP metabolism (PCK and PEPC) and in the consumption of malate (NADP-ME and NAD-ME). Values represent the mean of at least six independent determinations in different fruits ± SD. (B) Western blot analysis of PPDK. A 25 µg aliquot of total soluble protein was added per lane; as assay control (C), maize crude extract was used. The molecular mass of the immunoreactive bands is shown on the right (kDa). The quantification of the immunoreactive bands is expressed in relation to the amount in R0 ($n = 3$). Bars with the same letters are not significantly different ($P < 0.05$).

Discussion

An anoxic atmosphere is effective in delaying fruit softening and organoleptic modifications, and preventing ethylene synthesis

Organoleptic properties, such as fruit firmness and epidermis and pulp color, were maintained in fruits exposed to an anoxic atmosphere for 3 d (NA) as in fruits after harvest (R0), while these parameters varied accompanying ripening under an oxygenic environment (**Table 1**). However, the delay in ripening observed under an anoxic atmosphere was reversible since, after transfer to normal air (NA + 3), these organoleptic properties showed the same tendency as those of untreated fruits (R5, **Table 1**). Other parameters, such as SSC and titratable acidity, were not modified in NA + 3 with respect to R5 fruits (**Table 1**). In addition, pulp color became more reddish in NA + 3 than in R5 fruit (**Table 1**), coloration that may be even more attractive for consumers. In comparison, peaches subjected to heat for 72 h delayed their softening during the treatment but, after transfer to ambient conditions, they softened more quickly than untreated fruit (Lara et al. 2009). Similar results regarding fruit color under modified atmospheres have been described for

peaches exposed to high CO_2 (Fernandez Trujilio et al. 1998) and for apricots under anoxic atmospheres (Botondi et al. 2000).

The behavior observed in fruit firmness and color in peaches exposed to anoxia may be explained in terms of ethylene production. The large increase in this hormone, which takes place in fruit ripening in aerobic conditions (**Fig. 1**), was prevented in N_2-treated fruit (NA), correlating with the low *ACO1* levels detected (**Fig. 1**). This effect is reversed when fruit are re-exposed to oxygen, as accounted for by increases in ethylene and *Pp-ACO1* to levels similar to those of untreated fruit (R5, **Fig. 1**). Ethylene production has also been modified in peaches under very low O_2 or high CO_2 concentrations (Lurie and Pesis 1992, Bonghi et al. 1999); moreover, it was found that the lower the oxygen content in the fruit environment, the lower the ethylene synthesis (Weichmann 1987). In addition, increased acetaldehyde, which is observed in treated fruit (**Fig. 2**), has also been suggested to inhibit ACO activity (Burdon et al. 1996, Mathooko 1996, Beaulieu and Saltveit 1997, Pesis, 2005) and to decrease polygalacturonase activity, reducing fruit softening (Lurie and Pesis 1992). It is interesting to note that the ethylene level in fruit under anaerobic treatment changes in an opposite manner to that in submerged plant organs, in which a rapid increase in internal ethylene concentration after submergence is accompanied by increases in ACO transcripts and activity (Fukao and Bailey-Serres 2008). Thus, the response of plant organs to low O_2 levels due to submergence, where ethylene is a key regulator of acclimation (Fukao and Bailey-Serres 2008), may follow biochemical programs different from those analyzed in fruit in the present work.

Anoxic treatment increases fermentation pathways relative to the induction under aerobic ripening

N_2 treatment for 3 d effectively induced fermentative pathways in peaches, as reflected by increased PDC, ADH and LDH activities and acetaldehyde and ethanol levels (**Figs. 2**, **4**, **6**). After transfer to aerobic conditions, the activities of fermentative enzymes returned to the levels seen in unexposed fruits (R5) (**Fig. 4B**); nevertheless, acetaldehyde content did not decrease (**Fig. 2**). In contrast, the ethanol level decreased in NA + 3 fruit (**Fig. 2**), which may be the result of a combination of reduction in the production due to a decrease in PDC and ADH activity; metabolization to acetaldehyde by ADH; and loss through evaporation (Pesis 2005). The metabolization of ethanol by ADH contributes to the acetaldehyde pool and may explain the high level of this metabolite under conditions of decreased PDC activity in NA + 3 fruit (**Fig. 2**). On the other hand, since in R5 fruit acetaldehyde is also dramatically increased in spite of the low PDC and ADH activity (similar to NA + 3), it is not surprising that NA + 3 still has slightly higher levels of acetaldehyde as compared with R5 and does not fall below these levels. Besides the effect of acetaldehyde on ethylene biosynthesis and fruit softening discussed above, the higher levels of acetaldehyde in NA + 3 than in R5 peach (**Fig. 2**) could constitute an extra advantage of the anoxic treatment, since in fruit flavor testing assays peaches with higher ethanol and acetaldehyde contents were chosen as the favorites (Pesis 2005). Although high levels of acetaldehyde and ethanol can lead to deleterious effects on fruit metabolism (Ke et al. 1995), in the present work normal ripening is re-established after treated fruits are transferred to aerobic conditions for 3 d. The increase in LDH activity under N_2 treatment (**Fig. 4B**) indicates that carbon flux from pyruvate is also diverted to lactic fermentation. In Arabidopsis, lactic production is required to stimulate ethanolic fermentation (Dolferous et al. 2008), probably adjusting the pH for PDC activity (Dennis et al. 2000). However, heat treatment in peach induced alcoholic but not lactic fermentation (Lara et al. 2009). In the present work, although a large increase in fermentation occurs in peach fruit under a 72 h anaerobiosis (**Fig. 6**), no deleterious effects are observed. This result may be explained in terms of an intrinsic capacity of the fruit organ to cope with low oxygen levels, as hypoxic areas are normally present inside fruits (Borsani et al. 2009, Pedreschi et al. 2009b, Sweetman et al. 2009, Biais et al. 2010), and studies in several species have indicated that a hypoxic pre-treatment improves tolerance to subsequent anoxia (El-Mir et al. 2001, Licausi and Perata et al. 2009).

Sucrose metabolization pathways are induced due to N_2 treatment in peach fruit without a decrease in sucrose levels

All the *Pp-SS* and *Pp-NI* transcripts analyzed in peach were highly up-regulated by anoxia (**Fig. 3A**). In many species, SS has been shown to be an anoxia-induced polypeptide (Dennis et al. 2000, Geigenberger 2003), while invertase genes were strongly repressed by low O_2 in maize (Zeng et al. 1999). Sucrose degradation to hexose-phosphate via SS or via invertase differs in terms of energy costs, i.e. while metabolization of one sucrose molecule through invertase requires two ATP molecules, breakdown via SS requires only one PPi (**Fig. 6**). Due to an energy crisis or in response to a need to decrease O_2 consumption, the sucrose degradation pathway through SS which consumes less energy would be favored over the invertase pathway under anoxia (Bologa et al. 2003). The large increase in *NI* transcripts due to anoxia in peach fruit is thus surprising (**Fig. 3A**). Nevertheless, total NI activity was not modified in NA fruit with respect to R3 but greatly increased after 3 d under ambient conditions (NA + 3; **Fig. 3B**). Thus, the increase in NI transcript level in NA fruit is reflected at the protein level later, when fruits are transferred to normoxia and the energy crisis is over and protein synthesis is probably restored. Similarly, during ripening as well as after heat treatment, modifications at the level of expression of different *Pp-NI* genes were not correlated with changes in total NI activity (Borsani et al. 2009, Lara et al. 2009). New scenarios for the relevance of invertases in the coordination of metabolic processes in different

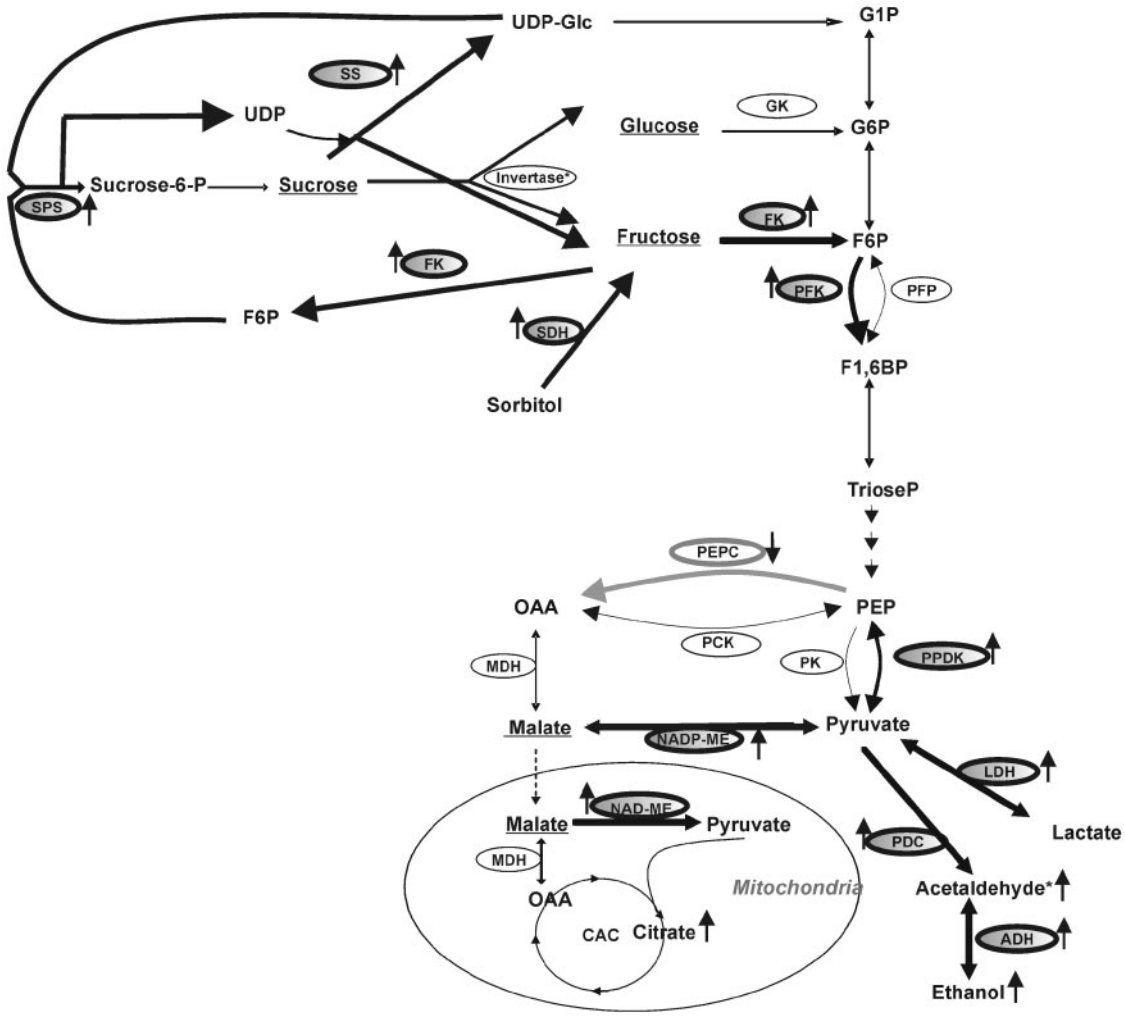

Fig. 6 Simplified metabolic scheme and changes observed after anoxic treatment in relation to typical metabolism during post-harvest ripening of peach. Reactions analyzed in the present study involving the metabolism of sugars and major organic acid are shown. Only metabolites that are used as substrates for the next enzyme reaction are shown. Enzymes and metabolites that are increased or decreased after N_2 treatment with respect to fruit of the same post-harvest age under an aerobic atmosphere (R3) are indicated by ↑ and ↓, respectively. Underlined metabolites were not modified by the anoxic treatment. Acetaldehyde is marked with * since its level in NA + 3 samples is much higher than that in R5 fruit. The enzyme invertase is particularly highlighted since specific transcripts encoding this enzyme were induced. CAC, citric acid cycle; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6BP, fructose-1,6-bisphosphate; OAA, oxaloacetate.

compartments have been described (Vargas and Salerno 2010). Therefore, an in-depth analysis of the subcellular localization of the invertases encoded by each NI transcript is essential to assign particular roles for each invertase in peach fruit.

SPS is a key enzyme involved in sucrose synthesis but not in its degradation (**Fig. 6**). It is involved in the translocation of photoassimilates from sink to source tissues and its levels increase during ripening of fruits such as banana in relation to sucrose synthesis (Huber and Huber 1996, Nascimento et al. 1997). The meaning of an increase in *Pp-SPS1-2* under the anoxic treatment, when sucrose-consuming pathways are also induced, is uncertain since a cycle of sucrose synthesis and degradation would occur (**Figs. 3**, **6**). However, many other tissues show sucrose cycling, which controls several important physiological functions (Roby et al. 2002) as well as sucrose accumulation by making the net metabolite flux more sensitive to small changes in each unidirectional reaction (van der Merwer et al. 2010). Similarly, in anoxic rice coleoptiles, a cycle of starch synthesis and degradation is apparently active, as accounted for by increases in expression of α-amylase and genes involved in starch synthesis (Lasanthi-Kudahrttige et al. 2007). In banana fruit, *SPS* expression is regulated by abiotic factors and phytohormones (Choudhury et al. 2008). It is thus reasonable that in peach fruit the *SPS* gene is regulated by stress conditions such as anoxia, probably in an ethylene-independent manner, as *SPS* genes were not modified in response to 1-methylcyclopropene treatment in peach (Ziliotto et al. 2008).

Finally, it is interesting to note that sucrose, glucose and sucrose levels were not modified either in treated fruit (NA) or after 3 d in air following the treatment (NA + 3) with respect to peaches of the same post-harvest age held in air (R3 and R5, respectively) (**Fig. 2**). Therefore, even when a stimulation of glycolysis is expected under N_2 treatment in order to maintain the energetic status of the cell due to less efficient fermentation vs respiration, these sugar levels do not decrease. It is possible that other compounds such as starch, pectins or organic acids may fuel glycolysis (**Fig. 6**). On the other hand, the increase in both the degradative and synthetic sucrose pathways may result in unchanged levels of this metabolite (**Fig. 6**).

Higher levels of sorbitol in treated fruit (NA) with respect to peaches of the same post-harvest age (R3) (**Fig. 2**) are in contrast to the higher levels of *Pp-SDH* encoding a sorbitol-metabolizing enzyme (**Figs. 4A**, **6**). Nevertheless, it is highly possible that low NAD levels under anoxia may limit SDH activity in vivo, which results in lower sorbitol metabolization.

Regarding fructose-6-phosphate phosphorylation, while PFP activity was not modified under anoxic conditions, PFK activity was increased (**Figs. 4B**, **6**). It is interesting that PFK using ATP is induced compared with PFP. Increased glycolytic flux is needed to support pyruvate demand in the fermentative pathways induced by anoxia. PFK is a major control enzyme of glycolytic flux (Plaxton 1996) and it is likely to be a rate-limiting enzyme of glycolysis under anoxia (Huang et al. 2008). PFP is usually induced under hypoxia or anoxia and its activity is many times lower than that of PFK, as is the case of citrus (Falcone Ferreira et al. 2006). In contrast, in ripening peach fruit, similar levels of PFK and PFP have been measured (Borsani et al. 2009); therefore, even though PFP in peach is not induced under anoxia, its activity is already high. In addition, the increase in PPDK immunoreactive protein in NA with respect to R0 and R3 (**Fig. 5B**) is in agreement with an increase in the glycolytic flux improving triose phosphate chaneling through pyruvate. Increased levels of PPDK and aldolase have been found in other tissues under oxygen stress, such as in rice coleoptiles (Moons et al. 1998, Huang et al. 2005). While neither PPDK expression nor activity has been identified in tomato (Bahrimi et al. 2001) and no immunoreactive protein was found in different berries (Famiani et al. 2005, Famiani and Walker 2009), it is clear that PPDK in peach fruit is an important enzyme regulated during ripening (Borsani et al. 2009) and stress conditions such as high temperature (Lara et al. 2009) and anoxia (**Fig. 5**).

Pathways of organic acid metabolism are modified under anoxia, but not by the malate level

With regards to organic acid metabolism, PEPC activity was lower in treated fruit (NA) with respect to untreated fruit (R3) or peaches recently harvested (R0) (**Fig. 5A**). In addition, PCK activity (**Fig. 5**) was similar in treated (NA) and untreated fruit (R3). Transcript profiling of rice coleptiles revealed that PEPC was highly repressed and PCK was strongly expressed under anoxia (Lasanthi-Kudahettige et al. 2007). Therefore, under anoxic conditions, there is a preference for PEP channeling to pyruvate production, which is fermented, rather than to oxaloacetate (OAA) production through PEPC and PCK, with respect to ripening under an oxygen atmosphere (**Fig. 6**).

Regarding malate–pyruvate conversion (**Fig. 6**), an increase in both NADP-ME and NAD-ME activities was observed in treated fruit (NA) with respect to fruit under aerobic conditions (R3) (**Fig. 5**). In agreement, in mandarins exposed to N_2 for 24 h, an increase in NADP-ME protein was observed by a two-dimensional difference gel electrophoresis approach (Shi et al. 2008), while in tobacco and maize roots exposed to hypoxia an increase in *NADP-ME* or NADP-ME activity was also observed (Maurino et al. 2001, Detarsio et al. 2008, Müller et al. 2008). In tobacco and Arabidopsis, NADP-ME is able to function in the direction of malate generation (Gerrard Wheeler et al. 2008, Müller et al. 2008). Thus, on the one hand, in peach under anoxic conditions, malic enzymes may provide pyruvate for fermentation by consuming malate. On the other hand, NADP-ME may synthesize malate functioning in the carboxylation direction by using CO_2 released by PDC and generating oxidized NADP in conditions of oxygen deprivation. The impairment in the citric acid cycle under anoxic environment, which limits NADH re-oxidation and thus inhibits NAD-dependent enzymes of the cycle, explains the citric acid increase in fruit exposed to anoxia with respect to fruits recently harvested (R0) and to those ripening in air (R3, **Fig. 2**). Conversely, the malate pool is not modified (**Fig. 2**), probably due to an equilibrium between reactions involved in its synthesis and degradation (**Figs. 5**, **6**).

Finally, it is important to note that when treated fruit are transferred to aerobic conditions the aerobic metabolism is restored, with enzymes affected by the treatment (PFK, NAD-ME, NADP-ME, PDC, ADH and LDH, **Figs. 3–5**) showing the same levels as in fruit ripening under aerobic conditions (R5, R3 or R0).

In conclusion, the present work contributes to a better understanding of the anoxic response of Dixiland peach fruit. N_2 treatment inhibits ethylene production and it is effective to maintain peach organoleptic properties during a period of 3 d, avoiding the irreversible changes which could ultimately produce alterations in further ripening under aerobic conditions. The fact that the major changes produced by the treatment are reversed when peach fruits are transferred to normal air indicates that peach is able to tolerate the anoxic treatment, without symptoms of post-anoxic injury.

Materials and Methods

Plant material and treatments

Assays were conducted with peach fruits [*P. persica* (L.) Batsch] cv. 'Dixiland' grown in the Estación Experimental Agropecuaria INTA, San Pedro, Argentina (Budde et al. 2006), during 2002 and harvested in 2003. The flesh firmness of the fruits at harvest (R0) was typically at 60.1 ± 5.9 N (**Table 1**), which corresponded

to approximately 93 d after bloom. Immediately after picking, the fruit were manually selected for uniformity of color, size and firmness. They were divided and treated at 20°C with humidified (90–95% relative humidity) streams (100 ml min^{-1}) of air or, alternatively, N_2. Chambers of 20 liters containing 30 fruits were used in both cases. Samples were taken immediately after harvest (R0) and after 3 and 5 d in the air chamber (R3 and R5, respectively); and after 3 d under an anoxic (N_2) atmosphere (NA). For a group of fruit under N_2 for 3 d, the anoxic atmosphere was replaced by air and after 3 d the fruit were sampled (NA + 3).

About 20–30 fruits from each group were used for color, firmness, SSC, acidity and metabolite measurements. Representative mesocarp tissue was also collected from the different sample fruits, immediately frozen in $N_{2(l)}$ and stored for further experiments at −80°C.

Fruit quality traits determination and metabolite measurements

Flesh firmness, SSC, ground color and titratable acidity were determined as previously described (Borsani et al. 2009). D-Glucose, D-fructose, sucrose, sorbitol, malic acid, citric acid, ethanol and acetaldehyde were determined as previously described (Borsani et al. 2009, Lara et al. 2009). The level of each metabolite is expressed as g per 100 g of fresh peach mesocarp.

Ethylene measurement

Ethylene synthesis was measured in a pool of three fruit enclosed in 3 liter sealed jars (Budde et al. 2006) with the following modifications: the nitrogen carrier gas flow rate was 30 ml min^{-1}, hydrogen carrier flow was 30 ml min^{-1} and air flow was 400 ml s^{-1}. Injector, oven and detector temperatures were 100, 100 and 250°C, respectively. Three replicates were conducted.

Protein extraction and quantitation

For enzyme activity measurements, total protein from peach mesocarp tissue was extracted and desalted as previously described (Borsani et al. 2009). For SDS–PAGE, proteins were extracted under denaturing conditions as in Lara et al. (2009). Protein concentration was determined in crude extracts using the Bio-Rad protein assay reagent, and bovine serum albumin as standard.

Enzyme assay

The activity of enzymes was measured spectrophotometrically in a final volume of 1 ml at 30°C and 340 nm using a UNICAM Helios β spectrophotometer (UNICAM Instruments). The reaction mixtures used for each enzyme were as follows (Borsani et al. 2009):

(i) Alcohol dehydrogenase (EC 1.1.1.1, ADH): 85 mM MES, pH 6.5; 5 mM acetaldehyde; and 0.15 mM NADH.

(ii) ATP-dependent phosphofructokinase (EC 2.7.1.11, PFK): 50 mM Tris–HCl, pH 7.5; with 5% (w/v) polyethylene glycol (PEG); 5 mM $MgCl_2$; 0.5 mM ADP; 1 mM dithiothreitol (DTT); 4 mM fructose-6-phosphate; 0.15 mM NADH; 0.2 U of aldolase; 1 U of triose phosphate isomerase; and 0.1 U of glycerol-3-phosphate dehydrogenase starting the reaction with ATP.

(iii) Glucokinase (EC 2.7.1.2, GK): 0.1 M Tris–HCl, pH 8.5; 5 mM $MgCl_2$; 0.5 mM NAD; 10 mM glucose; 2 mM ATP; and 2 U of glucose-6-phosphate dehydrogenase. The reaction was started with ATP.

(iv) Invertases (EC 3.2.1.26): neutral invertase (NI) activity was assayed in 200 mM HEPES-NaOH, pH 7.5; 200 mM sucrose; and an aliquot of the protein extract. The mixture was incubated at 30°C for different times and the progress of the reaction was followed, detecting the amount of glucose produced by using the glucose oxidase/horseradish peroxidase assay. Acid invertase (AI) was assayed in the conditions described above, although the reaction mixture contained 100 mM acetic acid/sodium acetate buffer, pH 5.0. In the case of AI, prior to glucose determination, the aliquot was neutralized.

(v) Lactate dehydrogenase (EC 1.1.1.27, LDH): 50 mM NaPi, pH 7.5; 10 mM pyruvate; 0.2 mM NADH; and 1 mM methylpyrazole, which inhibits PDC activity.

(vi) NAD-malate dehydrogenase (EC 1.1.1.37, NAD-MDH): was assayed in the OAA reduction direction using a 50 mM imidazole, pH 6.8, medium containing 1 mM OAA and 0.15 mM NADH.

(vii) NAD-malic enzyme (EC 1.1.1.39, NAD-ME): 50 mM HEPES, pH 7.3; 2 mM NAD; 2 mM L-malate; 5 mM DTT; 75 μM CoA; 5 mM $MgCl_2$; 5 mM $MnCl_2$; and 10 U of MDH.

(viii) NADP-malic enzyme (EC 1.1.1.40, NADP-ME): 50 mM Tris–HCl, pH 7.5; 0.5 mM NADP; 10 mM L-malate; and 10 mM $MgCl_2$. The reaction was started with malate.

(ix) Pyruvate decarboxylase (EC 4.1.1.1, PDC): 85 mM MES, pH 6.5; 25 mM NaCl; 1 mM $MgCl_2$; 2 mM DTT; 2 mM thiamine pyrophosphate; 0.15 mM NADH; 50 mM oxamate; 3 U of ADH; and 10 mM pyruvate, where oxamate acts as an inhibitor of LDH activity. Crude extracts were pre-incubated for 30 min in the reaction media in the absence of pyruvate, NADH and ADH.

(x) Phosphoenolpyruvate carboxylase (EC 4.1.1.31, PEPC): 100 mM Tris–HCl, pH 8.0; 20% (v/v) glycerol; 10 mM $MgCl_2$; 10 mM $NaHCO_3$; 4 mM PEP; 0.15 mM NADH; and 10 U of MDH.

(xi) Phosphoenolpyruvate carboxykinase (EC 4.1.1.49, PCK): 50 mM HEPES, pH 7.3; 4 mM PEP; 10 mM $NaHCO_3$; 2.5 mM $MgCl_2$; 2.5 mM $MnCl_2$; 0.15 mM NADH; 10 U of MDH; and 3 mM ADP.

(xii) Pyruvate kinase (EC 2.7.1.40, PK): 25 mM HEPES, pH 7.2; 20 mM KCl; 10 mM $MgCl_2$; 2 mM PEP; 0.15 mM NADH; 0.5% (w/v) PEG; 2 mM DTT; 1 mM ADP; and 0.4 U of LDH. The reaction medium was previously bubbled with helium. Enzymatic activity was corrected for interference by PEP-phosphatase activity by omitting ADP from the reaction mixture.

(xiii) PP_i-dependent phosphofructokinase (EC 2.7.1.90, PFP): 50 mM Tris–HCl, pH 7.5; with 5% (w/v) PEG; 5 mM $MgCl_2$; 0.5 mM $NaPP_i$; 1 mM DTT; 4 mM fructose-6-phosphate; 2 μM fructose-2,6-bisphosphate; 0.15 mM NADH; 0.2 U of aldolase; 1 U of triose phosphate isomerase; and 0.1 U of

glycerol-3-phosphate dehydrogenase, starting the reaction with NaPPi.

Gel electrophoresis

SDS–PAGE was performed in 10% (w/v) polyacrylamide gels. Immunoblotting was carried out as previously described (Borsani et al. 2009) using a 1 : 1,000 dilution of antibodies against *Zea mays* L. pyruvate orthophosphate dikinase (PPDK; EC 2.7.9.1; Chastain et al. 2000). Quantification of the intensity of the bands was conducted by image analysis software in at least three independent blots. The mean value of the immunoreactive bands in peaches recently harvested (R0) was arbitrarily set at 100%.

RNA isolation and RT–PCR

Total RNA was isolated from 4 g of tissue 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 (Sambrook et al. 1989). First-strand cDNA was synthesized with MoMLV-reverse transcriptase (Promega) and using 3 μg of RNA and oligo(dT).

Quantitative real-time RT–PCR

Relative expression was determined by quantitative real-time RT–PCR in an iCycler iQ detection system (Bio-Rad), using the intercalation dye SYBRGreen I (Invitrogen) as previously described (Lara et al. 2009). PCR primers (**Supplementary Table S1**) were designed based on peach fruit cDNA sequences in databases and analyzed as in Borsani et al. (2009). A 10-fold dilution of cDNA was used as template. PCR controls were performed in the absence of added reverse transcriptase. The specificity of the PCRs was confirmed by melting curve analysis as well as by agarose gel electrophoresis of the products. Each RNA sample was run in triplicate and repeated in at least two independent sets of treatments, generating a total of six replicates per gene per sample. Relative gene expression was calculated using the 'Comparative $2^{-\Delta\Delta CT}$, method (Livak and Schmittgen 2001) with elongation factor 1 (*Pp-EF1*) as the reference gene. To test whether *Pp-EF1* behaves as a housekeeping gene in the analyzed samples, the gene expression index was plotted against the sample, and linearity and low slope were verified (Brunner et al. 2004). Results were expressed in relation to the values obtained for peaches recently harvested (R0).

Statistical analysis

Data were tested using one-way analysis of variance (ANOVA). Minimum significant differences were calculated by the Bonferroni, Holm–Sidak, Dunett and Duncan tests ($\alpha = 0.05$) using the Sigma Stat Package.

Supplementary data

Supplementary data are available at PCP online.

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