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REVIEW PAPER

What controls fleshy fruit acidity? A review of malate and citrate accumulation in fruit cells

A. Etienne[1], M. Génard[2], P. Lobit[3], D. Mbeguié-A-Mbéguié[4] and C. Bugaud[1,*]

[1] Centre de Coopération International en Recherche Agronomique pour le Développement (CIRAD), UMR QUALISUD, Pôle de Recherche Agronomique de Martinique, BP 214, 97 285 Lamentin Cedex 2, France
[2] INRA, UR 1115 Plantes et Systèmes de Cultures Horticoles, F-84914 Avignon, France
[3] Instituo de investigaciones Agropecuarias y Forestales, Universidad Michoacana de San Nicolas de Hidalgo, Tarimbaro, Michoacan, CP 58880, Mexico
[4] Centre de Coopération International en Recherche Agronomique pour le Développement (CIRAD), UMR QUALISUD, Station de Neufchâteau, Sainte-Marie, 97130 Capesterre Belle-Eau, Guadeloupe

*To whom correspondence should be addressed. E-mail: christophe.bugaud@cirad.fr

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Abstract

Fleshy fruit acidity is an important component of fruit organoleptic quality and is mainly due to the presence of malic and citric acids, the main organic acids found in most ripe fruits. The accumulation of these two acids in fruit cells is the result of several interlinked processes that take place in different compartments of the cell and appear to be under the control of many factors. This review combines analyses of transcriptomic, metabolomic, and proteomic data, and fruit process-based simulation models of the accumulation of citric and malic acids, to further our understanding of the physiological mechanisms likely to control the accumulation of these two acids during fruit development. The effects of agro-environmental factors, such as the source:sink ratio, water supply, mineral nutrition, and temperature, on citric and malic acid accumulation in fruit cells have been reported in several agronomic studies. This review sheds light on the interactions between these factors and the metabolism and storage of organic acids in the cell.

Key words: Environment, metabolism, mitochondria, organic acid, proton pump, respiration, TCA cycle, tonoplast, transport, vacuole.

Introduction

Fleshy fruit acidity, as measured by titratable acidity and/or pH, is an important component of fruit organoleptic quality (Esti *et al.*, 2002; Harker *et al.*, 2002; Bugaud *et al.*, 2011). Fruit acidity is due to the presence of organic acids, and malic and citric acids are the main acids found in most ripe fruits (Seymour *et al.*, 1993). Understanding the factors that influence the concentration of these acids in fruit cells is thus of primary importance for fruit quality improvement.

The predominant organic acid in ripe fruit varies among species. Malic acid is dominant in apple (Yamaki, 1984), loquat (Chen *et al.*, 2009), and pear (Lu *et al.*, 2011), whereas citric acid is dominant in citrus fruits (Yamaki, 1989). In many fruit species, differences in total acidity or in the balance of organic acids among cultivars are also observed, for example in loquat (Yang *et al.*, 2011), peach (Etienne *et al.*, 2002), pear (Lu *et al.*, 2011), citrus (Albertini *et al.*, 2006), pineapple (Saradhuldhat and Paull, 2007), apricot (Gurrieri *et al.*, 2001), and banana (Bugaud *et al.*, 2011).

The processes involved in the metabolism and accumulation of malic and citric acid in mesocarp cells are under both genetic and environmental control. Transcriptomics (Etienne *et al.*, 2002; Cercos *et al.*, 2006; Deluc *et al.*, 2007), metabolomics (Deluc *et al.*, 2007; Katz *et al.*, 2011), proteomics

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(Famiani *et al*., 2005; Katz *et al*., 2007), and quantitative trait loci (QTLs) (Schauer *et al*., 2006; Lerceteau-Köhler *et al*., 2012; Xu *et al*., 2012) studies have helped decipher some of the mechanisms that control acidity, and intervene at the cellular level. Many agronomic studies have shown the impacts of cultural practices, including irrigation (Wu *et al*., 2002; Thakur and Singh, 2012), mineral fertilization (Cummings and Reeves, 1971; Spironello *et al*., 2004; Ramesh Kumar and Kumar, 2007), thinning (Souty *et al*., 1999; Wu *et al*., 2002; Léchaudel *et al*., 2005), and environmental factors such as temperature (Wang and Camp, 2000; Gautier *et al*., 2005; Burdon *et al*., 2007), on fruit acidity, but how they affect malic and citric acid accumulation in the cell is still not clear.

In the last few years, process-based simulation models (PBSMs) of fruit have been increasingly used to simulate the metabolic and biophysical aspects of cell behaviour (Martre *et al*., 2011) and appear to be a powerful tool to study genotype×environment interactions (Bertin *et al*., 2010). Fruit PBSMs of the accumulation of citric acid (Lobit *et al*., 2003; Wu *et al*., 2007) and malic acid (Lobit *et al*., 2006) have been developed to predict citric and malic acid concentrations in the whole fruit during development in peach.

The aim of this review is to elucidate the physiological mechanisms that probably control citric and malic acid accumulation during fruit development and their possible regulation by genetic and agro-environmental factors. To this end, the review combines analyses of transcriptomic, metabolomic, and proteomic data related to malic and citric acid metabolism, and also the PBSMs of citric and malic acids. The three first sections describe the cell mechanisms involved in malic and citric acid accumulation and their regulation. The last section deals with the effects of agro-environmental factors (source:sink ratio, mineral fertilization, water supply, and temperature) on citric and malic acid accumulation and the related cell mechanisms they may affect.

In this review, the terms ‘malate’ and ‘citrate’, which usually describe the conjugate base of malic and citric acids, refer to all physiological forms of each compound.

Several pathways exist for malate and citrate metabolism in the mesocarp cells of fleshy fruits

Even though some organic acids are supplied by the sap, variations in the acidity of fleshy fruits are mainly due to the metabolism of malate and citrate in the fruit itself (Bollard, 1970; Ulrich, 1970; Sweetman *et al*., 2009). This section presents the metabolic pathways involved in the metabolism of the dicarboxylate malate and the tricarboxylate citrate. We first describe the pathways responsible for the initial formation of organic acids [carboxylation of phosphoenolpyruvate (PEP) in the cytosol], then the pathways responsible for the degradation of organic acids [decarboxylation of malate and oxaloacetate (OAA) in the cytosol], and finally those that allow conversion between tri- and dicarboxylates [the tricarboxylic acid (TCA) cycle in the mitochondria, the glyoxylate cycle in the glyoxysome, and citrate catabolism in the cytosol] (Fig. 1).

First step in synthesis of organic acids: PEP carboxylation in the cytosol

Formation of acidity involves the synthesis of organic acids, mostly malate and citrate, which can be stored in the vacuole in large amounts. As citrate is produced from dicarboxylates (mostly malate; see following section), the first step in the development of acidity is the synthesis of dicarboxylates, namely malate and OAA. These require fixation of CO_2 on a carbon skeleton derived from hexose catabolism (Hardy, 1968; Young and Biale, 1968), which is achieved by the carboxylation of PEP, catalysed by the phosphoenolpyruvate carboxylase (PEPC). This reaction takes place in the cytosol, since PEP is an intermediate of the glycolysis pathway, and produces OAA, which can then be reduced to malate by the cytosolic NAD-dependent malate dehydrogenase (NAD-cytMDH) (Givan, 1999) or supplied to the TCA cycle if replenishment is necessary (Leegood and Walker, 2003) (Fig. 1).

Multiple PEPC isoforms have been detected in fruits and are possibly the result of transcriptomic (Sweetman *et al*., 2009; Yao *et al*., 2009) and/or post-translational regulations (Sweetman *et al*., 2009). PEPC is controlled by both cytosolic pH and malate concentration (Lakso and Kliewer, 1975*a*; Possner *et al*., 1981; Davies, 1986) in a way that stabilizes the cytosolic pH (Smith and Raven, 1979). In grape berries, transcriptomic analysis (Or *et al*., 2000; Terrier *et al*., 2005) and measurement of enzymatic activity (Hawker, 1969; Ruffner *et al*., 1976; Diakou *et al*., 2000) pointed to a role for PEPC in malate accumulation throughout fruit development. Several studies based on analyses of transcriptomic and enzymatic activity suggest that PEPC is not responsible for the difference in malate content between low and high acid peach cultivars (Moing *et al*., 2000), apple (Yao *et al*., 2009), and loquat (Chen *et al*., 2009).

NAD-cytMDH catalyses the reversible conversion of malate into OAA, the most likely direction being the synthesis of malate (Sweetman *et al*., 2009; Yao *et al*., 2011). Even if a mitochondrial form is also present in fruit cells (see the following section), it has been shown in several fruits that NAD-cytMDH represents 70– 80% of total NAD-dependent MDH (Abou-Zamzama and Wallace, 1970; Taureilles-Saurel *et al*., 1995), explaining why total NAD-dependent MDH activity is generally related to malate synthesis in fruits (Zhao *et al*., 2007; Chen *et al*., 2009; Martınez-Esteso *et al*., 2011). Yao *et al*. (2011) showed that overexpression of the apple MdcyMDH gene encoding NAD-cytMDH resulted in an increase in malate, fructose, and sucrose content, suggesting its direct involvement in malate synthesis. Overexpression of MdcyMDH also resulted in the up-regulation of several malate-related genes/enzymes, suggesting an indirect role in malate accumulation.

Organic acid degradation: malate and OAA decarboxylation in the cytosol

Loss of acidity implies decarboxylation of carboxylates, which can occur through the conversion of tricarboxylates

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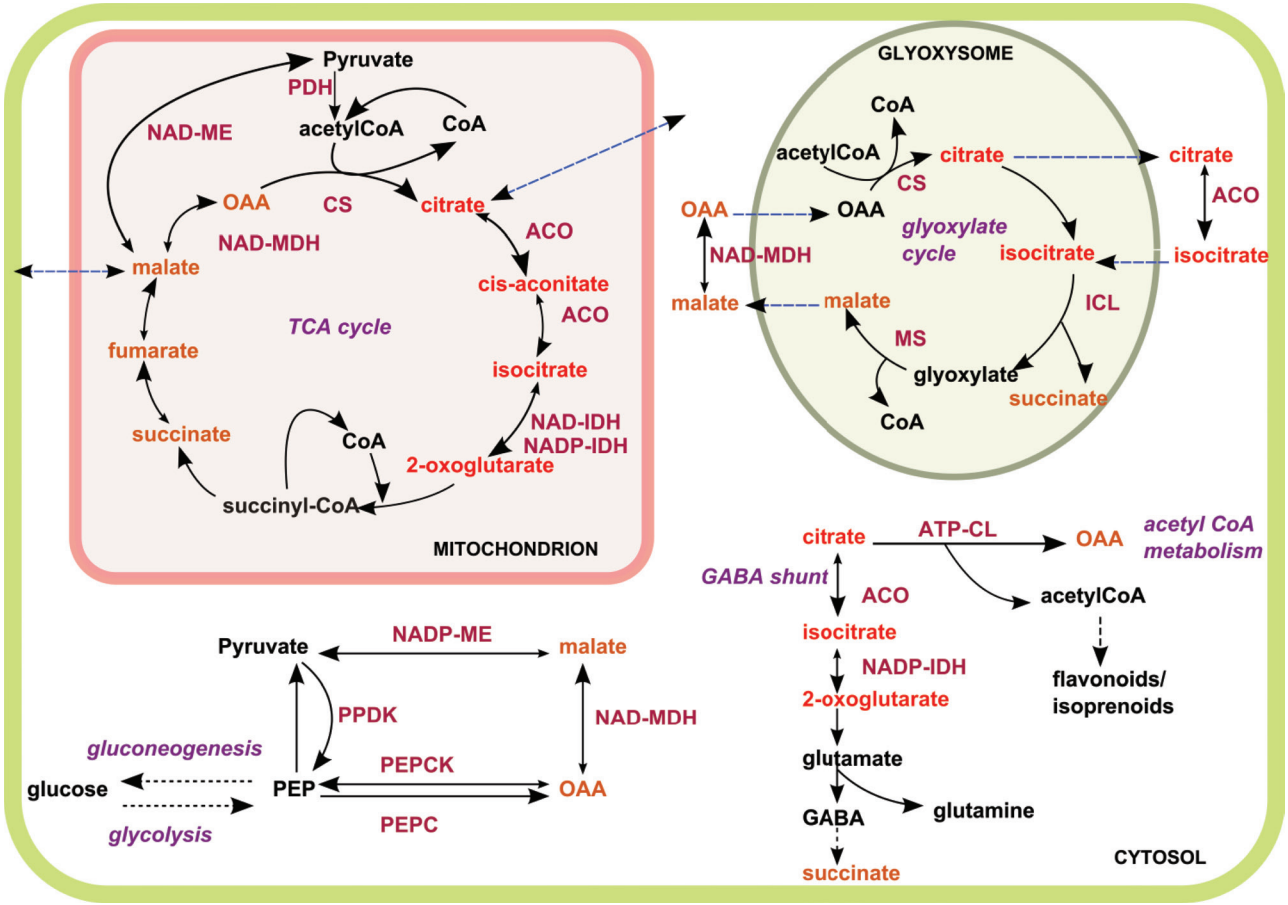

Fig. 1. Citrate and malate metabolic pathways in fruit mesocarp cells. Only the enzymes described in the paper are shown. ACO, aconitase; ATP-CL, ATP-citrate lyase; CS, citrate synthase; ICL, isocitrate lyase; MS, malate synthase; NAD-MDH, NAD-malate dehydrogenase; NAD-ME, NAD-malic enzyme; NAD-IDH, NAD-isocitrate dehydrogenase; NADP-ME, NADP-malic enzyme; NADP-IDH, NADP-isocitrate dehydrogenase; PDH, pyruvate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; PPDK, pyruvate orthophosphate dikinase. The probable direction of reversible reactions is indicated by the large arrow. Dashed blue arrows indicate malate and citrate transport. Names in orange are dicarboxylates and names in red are tricarboxylates.

into dicarboxylates (described later in the review), but also through decarboxylation of the dicarboxylates malate and OAA, leading to the degradation of organic acids (Fig. 1). Decarboxylation of OAA and malate allows the production of PEP and is linked to the activation of gluconeogenesis (Sweetman *et al.*, 2009). Gluconeogenesis is a metabolic pathway that results in the generation of glucose from PEP. It occurs mostly during fruit ripening when sugars accumulate rapidly (Sweetman *et al.*, 2009). In the past few years, proteomics (Katz *et al.*, 2011), transcriptomics, and metabolite (Carrari *et al.*, 2006; Deluc *et al.*, 2007; Fait *et al.*, 2008) analyses have provided evidence for a shift from the accumulation of organic acids to sugar synthesis during the final stage of development in several fruit species.

PEP can originate from OAA through the activity of phosphoenol carboxykinase (PEPCK) which catalyses the reversible reaction, the most likely direction being the synthesis of PEP (Leegood and Walker, 2003). This reaction requires a source of OAA that could be supplied by the oxidation of malate by NAD-cytMDH. This hypothesis is supported by the fact that PEPCK is involved in the dissimilation of malate in the flesh of several fruits (Famiani *et al.*, 2005) and possibly in the lack of malate in low acid apple cultivars (Berüter, 2004).

PEP can also originate from the conversion of pyruvate through pyruvate orthophosphate dikinase (PPDK) activity (Sweetman *et al.*, 2009). The pyruvate required for PPDK may be supplied through the carboxylation of malate by cytosolic NADP-dependent malic enzyme (NADP-cytME), which catalyses a reversible conversion, the most likely direction being the decarboxylation of malate (Sweetman *et al.*, 2009). NADP-cytME appears to be involved in the decrease in malate content during the ripening of several fruit species (Dilley, 1962; Goodenough *et al.*, 1985; Chen *et al.*, 2009; Sweetman *et al.*, 2009). Involvement of NADP-cytME during the early stage of fruit growth differs between species. Thus, in young tomato and apple fruits, NADP-cytME does not appear to play an important role in malate accumulation (Dilley, 1962; Goodenough *et al.*, 1985), whereas in young grape berries the use of a proteomics approach suggested the

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opposite (Martınez-Esteso *et al*., 2011). The contribution of NADP-cytME to the lack of malate in ripe pulp of low acid cultivars has been demonstrated in apple (Yao *et al*., 2009) and loquat (Chen *et al*., 2009). Studies of different fruit species suggest that NADP-cytME is regulated at the post-translational level (Famiani *et al*., 2000; Bahrami *et al*., 2001; Yao *et al*., 2009; Yang *et al*., 2011) by cytosolic pH and malate concentration, among others (Lakso and Kliewer, 1975*a*; Possner *et al*., 1981; Davies, 1986).

Decarboxylation of malate and OAA may also be linked to fermentative metabolism as it can occur in ripening fruit if the cytosol becomes too acidic (for a review, see Sweetman *et al*., 2009).

Conversions between di- and tricarboxylic acids: multiple compartments, multiple pathways

Once malate and OAA have been synthesized in the cytosol, they can be converted into tricarboxylates, mostly citrate, or other dicarboxylates through two metabolic pathways, the TCA cycle and the glyoxylate cycle. In its turn, citrate can be converted into dicarboxylates via several pathways [TCA cycle, glyoxylate cycle, γ-aminobutyrate (GABA) shunt, and acetyl-CoA catabolism]. All these conversion reactions can modify the acidity of fruit cells.

The TCA cycle in the mitochondria: conversions between di- and tricarboxylates

The TCA cycle results in the oxidation of pyruvate into CO_2 and a reduction in co-enzymes through a series of conversions between organic acids including malate and citrate (Fig. 1). The cycle begins with the condensation of OAA and acetyl-CoA, the latter provided by the action of pyruvate dehydrogenase on mitochondrial pyruvate. The input of acetyl-CoA allows the TCA cycle to maintain a cyclic flux mode under which it is not able to catalyse net synthesis of cycle intermediates. Therefore, export of intermediates implies non-cyclic flux modes that are known to occur in plants and have been evidenced in citrus fruit (Katz *et al*., 2011), and are likely to be controlled by ATP demand (Sweetlove *et al*., 2010). The maintenance of the pools of TCA cycle intermediates implies that for each metabolite exported, one is imported, and vice versa. These exchanges are achieved by a variety of mechanisms mediated by mitochondrial carrier proteins (for reviews, see Laloi, 1999; Haferkamp and Schmitz-Esser, 2012) that obey the general principles behind the transport of ionic species across a biological membrane (Figs 2, 3). In fruits, mitochondrial dicarboxylate/tricarboxylate transporters have been characterized at the gene level in citrus (Deng *et al*., 2008) and grape berry (Regalado *et al*., 2012), and at the protein level in citrus (Katz *et al*., 2007).

Non-cyclic flux modes allow conversion of di- and tricarboxylates (Steuer *et al*., 2007; Sweetlove *et al*., 2010) and are sustained by the activities of the TCA cycle enzymes. The enzymes that directly control citrate synthesis are the mitochondrial citrate synthase (mtCS), and citrate degradation, the mitochondrial aconitase (mtACO) and the mitochondrial NAD-dependant isocitrate dehydrogenase (NAD-mtIDH) (Fig. 1). mtCS activity is positively correlated with citrate accumulation in citrus (Sadka *et al*., 2001; Wen *et al*., 2001) and strawberry (Iannetta *et al*., 2004), but transcriptomics and protein studies suggested that this enzyme is not responsible for the difference in citrate content between low and high acid cultivars of several fruit species (Canel *et al*., 1996; Sadka *et al*., 2001; Etienne *et al*., 2002; Saradhuldhat and Paull, 2007; Tang *et al*., 2010). The involvement of mtACO, which catalyses the conversion of citrate into isocitrate (the most likely direction in mitochondria due to the way the cycle functions), in citrate accumulation has been described by Sadka *et al.* (2000a). They showed that in sour lemon, mtACO activity decreases in the early stage of fruit growth and thus could be responsible for the increase in citrate concentration observed during fruit growth. Two forms of isocitrate dehydrogenase, an NADP-dependent form (NADP-IDH) and an NAD-dependent form (NAD-IDH), can catalyse the conversion of isocitrate into 2-oxoglutarate (the most likely direction in mitochondria like for mtACO). NAD-IDH is only found in mitochondria but has rarely been characterized in fruits, and no links with citrate accumulation have been found (Sha *et al*., 2011). NADP-IDH is mainly localized in the cytosol (NADP-cytIDH), but is also found in mitochondria (NADP-mtIDH) and peroxisomes (Gálvez and Gadal, 1995; Chen, 1998). In sour lemon, Sadka *et al.* observed that NADP-mtIDH activity decreased in the early stage of fruit growth in parallel with a decrease in mtACO activity (Sadka *et al*., 2000*a*, *b*). This could reflect a general reduction in citrate metabolism in the mitochondria. Malate can be oxidized in fruit mitochondria either into OAA by mitochondrial NAD-dependent malate dehydrogenase (NAD-mtMDH) (the most likely direction in mitochondria; Sweetman *et al*., 2009), which feeds the cycle, or into pyruvate by mitochondrial NAD-dependent malic enzyme (NAD-mtME), which interrupts the cycle (Macrae and Moorhouse, 1970) (Fig. 1). These two competing metabolic pathways affect fruit acidity in different ways. While malate oxidation by NAD-mtMDH leads mainly to citrate production (Steuer *et al*., 2007; Sweetlove *et al*., 2010), hence affecting the malate:citrate ratio of fruit cells, malate oxidation by NAD-mtME leads to the degradation of acidity since organic acids must be imported into the mitochondria to compensate for the loss of malate. Malate metabolism in the mitochondria therefore depends on NAD-mtMDH and NAD-mtME activity, both of which are regulated by the concentration of NADH and the pH (Palmer *et al*., 1982; Day *et al*., 1984; Douce, 1985). In young tomato fruit, the majority of malate degradation could be due to NAD-mtME (Bahrami, 2001). Transcriptomic and proteomic analyses suggest that NAD-mtMDH is involved in malate degradation during grape berry ripening (Sweetman *et al*., 2009; Martınez-Esteso *et al*., 2011).

Catabolism of citrate in the cytosol: conversion of citrate into dicarboxylates

Once citrate has been produced by the TCA cycle, it can be degraded in the cytosol through two metabolic pathways. One is the GABA synthesis pathway, also called the GABA shunt, which leads to succinate synthesis, and the other is

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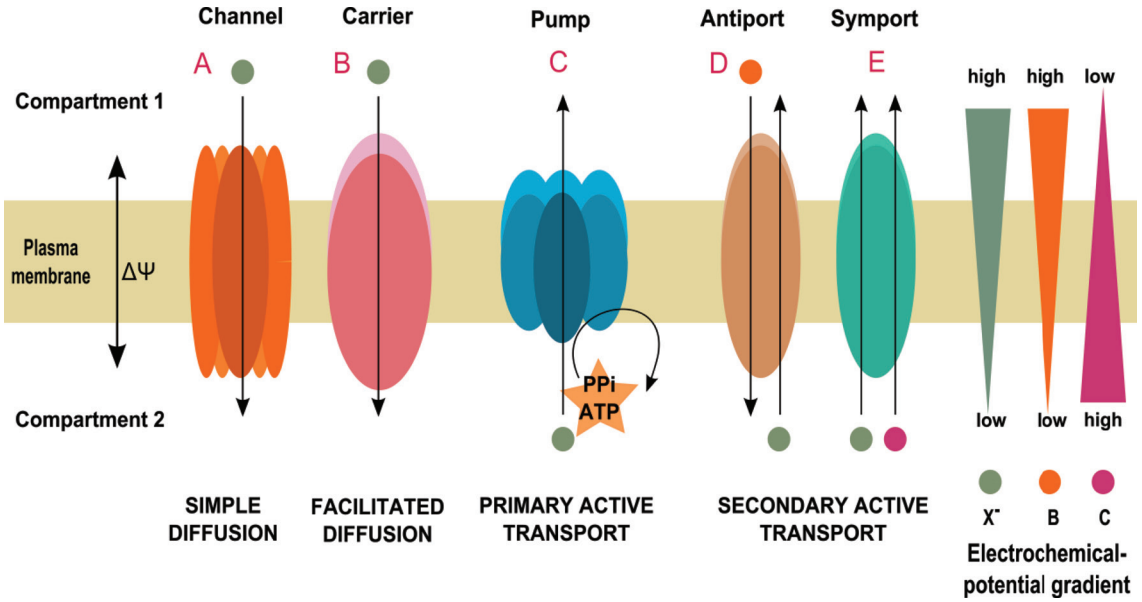

Fig. 2. Mechanisms of transport of ionic species across a biological membrane. Membrane transport is mediated by three types of membrane proteins: channels, carriers, and pumps. Channels function as selective pores through which molecules or ions can diffuse across the membrane. Carriers catalyse either the transport of a single solute, or the coupled transport of two solutes. Pumps catalyse the coupled transport of a solute with a chemical reaction. Three mechanisms allow the transport of an ionic species (X^-) across a biological membrane and are governed by a general principle of thermodynamics stating that the variation in free energy of the transport reaction ($\Delta G_{1\text{-}2}$) has to be negative. (i) Diffusion (simple or facilitated) is mediated by channels (A) in the case of simple diffusion, or by carriers (B) in the case of facilitated diffusion. This kind of transport allows the spontaneous movement of X^- down its electrochemical potential gradient [$\Delta G(X^-)_{1-2} <0$], which depends on the electric potential gradient of the membrane ($\Delta\psi$) and on the gradient of concentrations of X^- on the two sides of the membrane. $\Delta G_{1-2}=\Delta G(X^-)_{1-2}=zF\Delta\psi+RT\ln([X^-]_2/[X^-]_1) <0$ where z is the electric charge of the ionic species transported; F is Faraday's constant; R is the gas constant; and T is temperature. (ii) Primary active transport is mediated by a specific class of proteins called pumps (D). This kind of transport allows the movement of X^- against its electrochemical-potential gradient [$\Delta G(X^-)_{2-1} >0$] using the energy released from the hydrolysis of ATP or PPi ($\Delta G_{ATP\ (or\ PPi)} <0$). $\Delta G_{2-1}=\Delta G_{ATP\ (or\ PPi)}+\Delta G(X^-)_{2-1} <0$. (iii) Secondary active transport is mediated by two types of carrier proteins: antiports (E) and symports (F). This kind of transport allows the movement of X^- against its electrochemical-potential gradient [$\Delta G(X^-)_{2-1} >0$] using the energy dissipated by the downhill movement of a molecule across the membrane [$\Delta G(B)_{1-2} <0$ in the case of antiport, $\Delta G(C)_{2-1} <0$ in the case of symport]. Antiport: $\Delta G_{2-1}=\Delta G(X^-)_{2-1}+\Delta G(B)_{1-2} <0$. Symport: $\Delta G_{2-1}=\Delta G(X^-)_{2-1}+\Delta G(C)_{2-1}<0$.

cleavage into OAA and acetyl-CoA (Fig. 1). As these two pathways produce dicarboxylic acids, they are responsible for a decrease in fruit acidity.

The GABA synthesis pathway is a part of amino acid metabolism since it produces two amino acids (glutamate and GABA). This pathway also leads to the production of succinate that can then enter the TCA cycle. Two major enzymes are involved in the catabolism of citrate through the GABA shunt: cytosolic aconitase (cytACO), which catalyses the reversible conversion of citrate into isocitrate, and cytosolic NADP-dependent isocitrate dehydrogenase (NADP-cytIDH), which catalyses the reversible conversion of isocitrate into 2-oxoglutarate (Fig. 1). The involvement of the GABA shunt in citrate degradation during the ripening of citrus fruits was evidenced by proteomic and metabolite analyses (Katz *et al.*, 2011), gene expression analyses (Sadka *et al.*, 2000*b*; Cercos *et al.*, 2006), and enzymatic activity analysis (Sadka *et al.*, 2000*b*; Degu *et al.*, 2011). Activation of the GABA shunt could partially account for the lack of citrate in sweet lemon since activation of the genes involved in the degradation of 2-oxoglutarate, the precursor for GABA synthesis, was observed (Aprile *et al.*, 2011). Activation of the GABA shunt also appears to occur during post-harvest ripening of banana since an increase in 2-oxoglutarate content, NADP-IDH activity, mainly attributable to the cytosolic form (Chen and Gadal, 1990), and total ACO gene expression was observed (Medina-Suárez *et al.*, 1997; Liu *et al.*, 2004). It is likely that the rate of citrate degradation through the GABA shunt is mainly controlled by cytACO and NADP-cytIDH activities. In several genotypes of citrus, the pattern of expression of two genes encoding cytACO was associated with the timing of acid content reduction in fruits (Terol *et al.*, 2010). In tomato fruit, genetic and transgenic approaches demonstrated the key role of cytACO in the control of citrate content in ripe fruit (Morgan *et al.*, 2013). In sour lemon, NADP-cytIDH gene expression and NADP-cytIDH activity increase during fruit development and could thus be involved in the decrease in citrate content (Sadka *et al.*, 2000*a*).

The alternative citrate breakdown pathway cleaves citrate into OAA and acetyl-CoA through the activity of the ATP-citrate lyase (ATP-CL) and leads to the synthesis of

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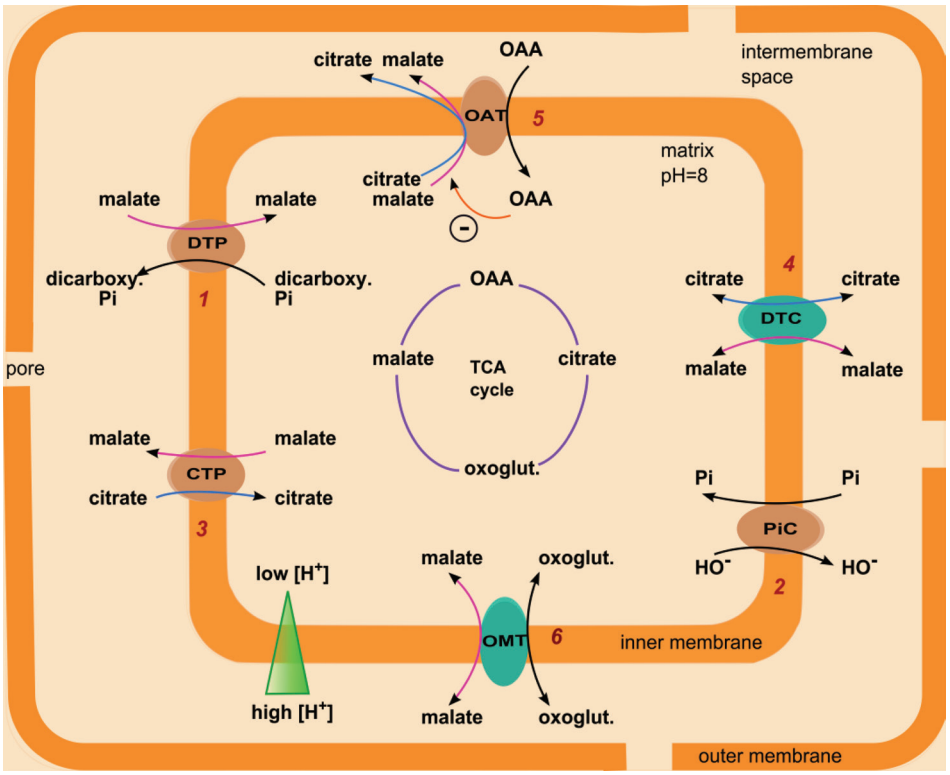

Fig. 3. Mitochondrial carriers involved in citrate and malate transport. CTP, citrate transport protein; DTC, dicarboxylate-tricarboxylate carrier; DT, dicarboxylate transport protein; OAT, oxaloacetate-malate transporter; OMT, oxoglutarate-malate translocator; PiC, phosphate carrier. The orange arrow represents the inhibition of malate efflux through the OAT by OAA. The electrochemical potential gradient of protons (green triangle) generates an electric potential gradient (negative inside) and a pH gradient (alkaline inside) that both play a role in the transport of organic acids between the cytosol and the mitochondria.

flavonoids and isoprenoids (Fig. 1). During ripening, these compounds accumulate in the fruit (Giovannoni, 2004), so it is likely that citrate catabolism through this pathway is activated during this phase. Evidence for such activation was found in mango fruit. Indeed, ATP-CL activity increased considerably during ripening, while there was a decrease in citrate content (Mattoo and Modi, 1970). Proteomic analysis identified ATP-CL in mature citrus fruit (Katz *et al.*, 2007). However, this result is in contradiction to the decrease in the levels of mRNA in this gene during ripening of citrus fruits observed by Cercos (Cercos *et al.*, 2006). Thus, the role of this pathway in the decrease in acid in citrus fruit requires further investigation.

The glyoxylate cycle: conversion of succinate and malate

The function of the glyoxylate cycle is to convert the acetyl-CoA produced in the peroxisomes by β-oxidation of fatty acids into succinate via a series of reactions involving malate and citrate (Fig. 1). Succinate is then converted into malate through the TCA cycle (Pracharoenwattana and Smith, 2008). Malate can then enter the gluconeogenesis pathway to produce glucose. In this way, the glyoxylate cycle decreases fruit acidity since it leads to the consumption of malate.

The five key enzymes involved in this metabolic pathway are located in either the glyoxysome [citrate synthase, isocitrate lyase (ICL), and malate synthase (MS)] or the cytosol (cytACO and NAD-cytMDH) (Pracharoenwattana and Smith, 2008) (Fig. 1). The location of the enzymes requires several intermediates of the cycle to cross the glyoxysomial membrane, but which transport systems are involved is still not clear (Rottensteiner and Theodoulou, 2006).

The glyoxylate cycle is possibly involved in malate accumulation in young grape berry and ripening banana fruit (Pua *et al.*, 2003; Terrier *et al.*, 2005). Activation of the glyoxylate cycle during post-harvest ripening of banana fruit could be a way to provide substrates for gluconeogenesis during a period when sugar accumulation is high (Surendranathan and Nair, 1976; Liu *et al.*, 2004). However, the involvement of the glyoxylate cycle in organic acid accumulation during fruit development could be specific to certain fruit species since no ICL proteins were detected in the flesh of several berry fruits at any stage of development (Famiani *et al.*, 2005).

The complex mechanism of vacuolar storage of organic acids

Most of the citrate and malate content of fruit is found in the vacuole (Moskowitz and Hrazdina, 1981; Yamaki, 1984), which occupies 90% of most mature fruit cells (Fontes *et al.*, 2011; Etxeberria *et al.*, 2012). This section is devoted to the mechanisms allowing their transport into and out of the vacuole.

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The 'acid trap' mechanism

The mechanism that allows the accumulation of citrate and malate in the vacuole has been described as the 'acid trap', and is enabled by the fact that these two weak acids can dissociate (Martinoia *et al*., 2007). In the cytosol, at neutral or slightly alkaline pH, almost all malate is in the form of dianion and almost all citrate in the form of trianion. In the vacuole, where the pH is acidic, the dominant species is either the protonated form or the monoanion (a significant proportion of the acids may remain in the dianion form, or even in the trianion form in the case of citrate, only in fruits with high vacuolar pH). Only dianion malate and trianion citrate can be transported into the vacuole (Lüttge and Ball, 1979; Oleski *et al*., 1987; Rentsch and Martinoia, 1991) because the transport systems involved are specific to these chemical forms (Brune *et al*., 1998; Martinoia *et al*., 2007). Once they have crossed the tonoplast and reached the acidic vacuole, they are immediately protonated, which maintains their electrochemical potential gradient and allows their continuous transport into the vacuole (Fig. 4A). It should be pointed out that trapping efficiency depends on both vacuolar pH and the electric potential gradient across the tonoplast ($\Delta\psi$). On one hand, the lower the pH, the more effective the protonation and trapping mechanism, on the other hand, the $\Delta\psi$ contributes strongly to the electrochemical potential gradient of the di- and trianion. Efflux of the protonated forms of malate and citrate probably occurs through specific carriers, but little is known on this subject (see the following sections).

The sustained transport of organic anions must be accompanied by a simultaneous influx of the equivalent amount of cations to maintain the electroneutral state of the vacuole. This is achieved by the transport of either mineral cations (mostly potassium) or protons (released from the dissociation of weak acids in the cytosol), only the latter being responsible for the acidification of the vacuole.

Malate crosses the tonoplast by facilitated diffusion

Vacuolar dianion malate uptake occurs by facilitated diffusion (Rea and Sanders, 1987; Maeshima, 2001) (Fig. 2B). In *Arabidopsis*, vacuolar malate transport is mediated at least by a tonoplast malate transporter (AttDT) (Emmerlich *et al*., 2003) (Fig. 4A, no. 1) and two members of the aluminium-activated malate transporter (ALMT) family, the AtALMT9 and AtALMT6 channels (Kovermann *et al*., 2007; Meyer *et al*., 2011) (Fig. 4A, no. 2). An AttDT homologue has been identified in grape berries and could play a role in malate transport (Terrier *et al*., 1998). ALMTs may be responsible for vacuolar malate transport in fruits since four candidate genes homologous to AtALMT9 have been identified in grape berry (Rongala, 2008), and two ALMT-like genes have been discovered in apple (Bai *et al*., 2012).

Malate currents through AtALMT9 and AtALMT6 are strongly inward rectifying; that is, malate transport occurs only in the presence of a $\Delta\psi$ (positive inside the vacuole) (Hafke *et al*., 2003; Epimashko *et al*., 2004; Hurth *et al*., 2005; Meyer *et al*., 2011). As $\Delta\psi$ is expected to decrease with a decrease in vacuolar pH (see the following section), these channels may close at low vacuolar pH when the acid trap mechanism would be most effective, perhaps as a mechanism to prevent overacidification of very acidic vacuoles. AttDT appears to play a role in the import and export of malate (Hurth *et al*., 2005), consequently this transporter could be less rectifying than the malate channel. AttDT also appears to be involved in the regulation of cytosolic pH homeostasis (Hurth *et al*., 2005).

Citrate crosses the tonoplast by facilitated diffusion and secondary active transport

In most species of fleshy fruit, vacuolar trianion citrate uptake occurs by facilitated diffusion (Fig. 2B), possibly through the malate channel (Oleski *et al*., 1987; Rentsch and Martinoia, 1991) (Fig. 4A, no. 2).The thermodynamic conditions are more favourable for the uptake of citrate than of malate at any vacuolar pH and $\Delta\psi$ (Fig. 4B, C). Thus, citrate appears to be easily transported into the vacuole as soon as its cytosolic concentration increases sufficiently (Gout *et al*., 1993). AttDT could also play a role in the transport of citrate into the vacuole, but, according to Hurth *et al*. (2005), it is not the main tonoplast citrate carrier since AttDT knock-out vacuoles contain much more citrate than wild-type vacuoles, and the transport rate of citrate was higher in AttDT knock-out plants. In citrus, several authors proposed that an ATP-dependent citrate pump may operate in addition to the malate channel. However, further investigation is needed to provide complete evidence that citrate transport is coupled to ATP hydrolysis though a single transporter and not through the tonoplastic pH gradient (ΔpH) and $\Delta\psi$ setting up by the V-ATPase (see the following section) (Canel *et al*., 1995; Brune *et al*., 1998; Ratajczak *et al*., 2003).

Citrate content generally decreases during fruit ripening (Léchaudel *et al*., 2005; Wu *et al*., 2005; Shimada *et al*., 2006; Saradhuldhat and Paull, 2007), meaning that citrate is exported from the vacuole. The existence of a symporter involved in citrate efflux has been evidenced in citrus (Fig. 2E). This carrier (CsCit1) is able to mediate the electroneutral co-transport of H^+ and $CitH^{2-}$ outside the vacuole of juice cells (Shimada *et al*., 2006) (Fig. 4A, no. 3).

Setting up the electric potential and pH gradient across the tonoplast

The main determinants of malate and citrate accumulation in the vacuole are vacuolar pH (always acidic) and the inside-positive $\Delta\psi$, with values commonly ranging between 20 mV and 30 mV (Taiz and Zeiger, 2010). Proton pumping into the vacuole contributes to the generation of both acid vacuolar pH and positive $\Delta\psi$ (Fig. 4A, nos 4 and 5). Two types of proton pumps are present in fruit vacuoles: the H^+-ATPase (V-ATPase) (Ratajczak, 2000), characterized in several fruit species (Müller *et al*., 1996, 1997; Terrier *et al*., 1998; Suzuki *et al*., 2000), and the H^+-PPiase (V-PPase) (Maeshima, 2000), also characterized in several fruit species (Terrier *et al*., 1998;

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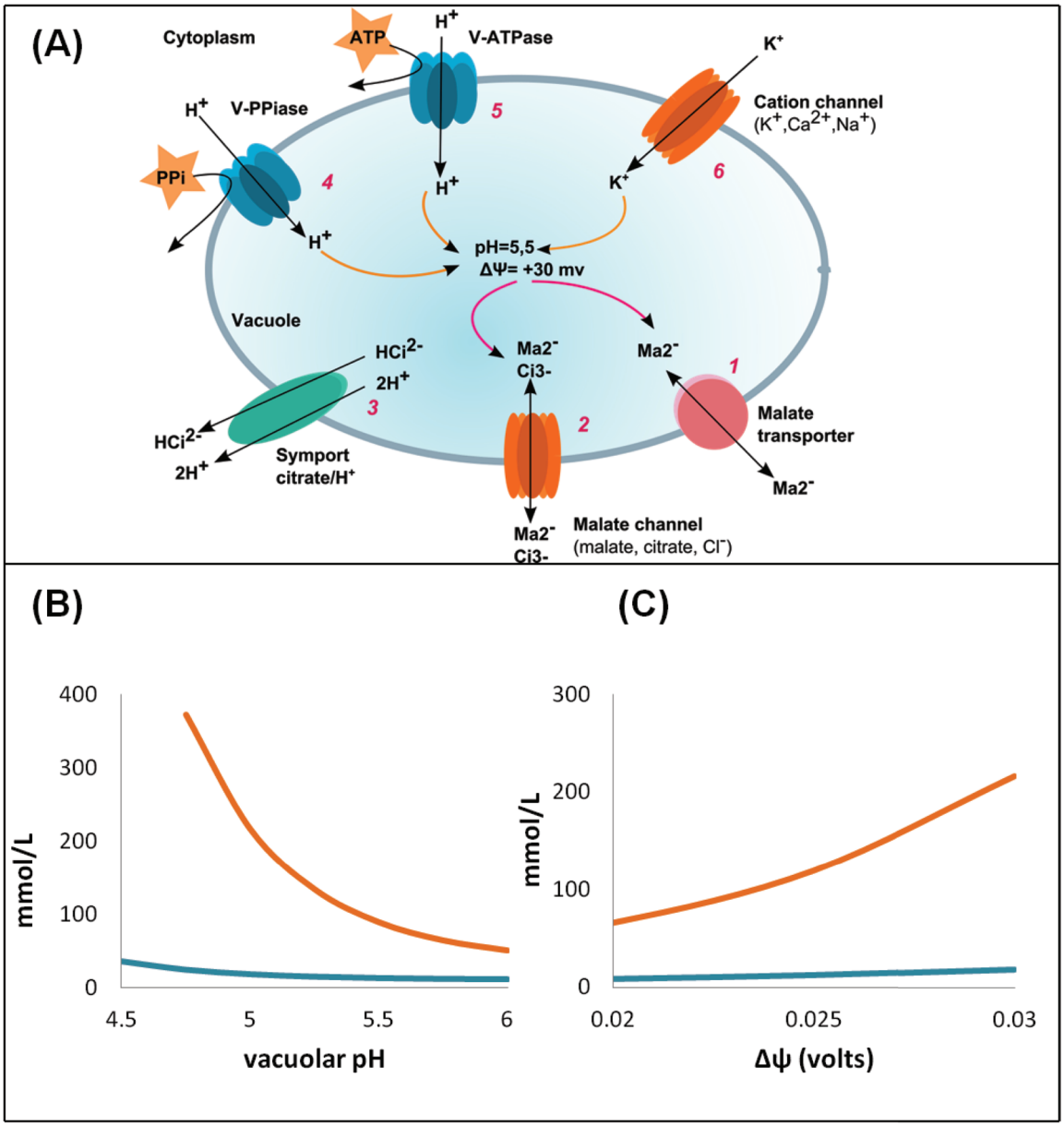

Fig. 4. (A) 'Acid trap' mechanism of vacuolar organic acid storage in fruit cells. Several tonoplastic carriers are involved in the transport of malate and citrate across the tonoplast. Once the dianions or trianions have crossed the tonoplast, they are immediately protonated due to the acid pH of the vacuole according to the following equations: Malate: $H_2Mal \leftrightarrow HMal^- + H^+ \leftrightarrow Mal^{2-} + 2H^+$, (p*K*a1 ~3.40, p*K*a2 ~5.10). Citrate: $H_3Cit \leftrightarrow H_2Cit + H^+ \leftrightarrow HCit^{2-} + 2H^+ \leftrightarrow Cit^{3-} + 3H^+$, (p*K*a1 ~3.10, p*K*a2 ~4.70, p*K*a3 ~6.40). The two vacuolar proton pumps are responsible for the acid pH of the vacuole and for the electric potential gradient across the tonoplast (Δψ). The cation channel is also involved in the regulation of the Δψ. (B) Theoretical changes in citrate (orange line) and malate (blue line) concentrations in the vacuole as a function of the pH of the vacuole. The concentrations were calculated using the Nernst equation (i.e. $\Delta G_{Mal}{}^{2-}$ and $\Delta G_{Cit}{}^{3-}$ are equal to zero, assuming that the dianion malate and trianion citrate are in thermodynamic equilibrium across the tonoplast) and the dissociation equations of the two organic acids with a vacuolar pH ranging from 4.5 to 6, and a Δψ equal to 30 mV (Martinoia *et al*., 2007). We did not consider any limitation by tonoplastic carriers.

Nernst equations:

$$\left(Mal^{2-}\right)_{vac} = \left(Mal^{2-}\right)_{cyt} * exp\frac{2 * F * \Delta\psi}{R * T}$$

$$\left(Cit^{3-}\right)_{vac} = \left(Cit^{3-}\right)_{cyt} * exp\frac{3 * F * \Delta\psi}{R * T}$$

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Suzuki *et al*., 2000). These enzymes catalyse chemiosmotic coupling between the hydrolysis of a high energy phosphate bond [ATP or pyrophosphate (PPi)] and proton transport into the vacuole. The thermodynamic conditions of these reactions are determinant for the activity of the pumps. Protons can be pumped into the lumen only if the variation in free energy of the chemiosmotic coupling (ΔG) is negative (Fig. 2C).

$$\Delta G = \Delta G_{\text{ATP or PPi}} + \Delta G_{H^+} \leq 0. \quad (1)$$

where ΔG_{ATP} and ΔG_{PPi} are the free energy of the substrate hydrolysis, and $\Delta G_{\text{H+}}$ is the free energy of proton transport. $\Delta G_{\text{H+}}$ can be written (derived from the diffusion equation of ionic species, see Fig. 2) as:

$$\Delta G_{H^+} = n\left(\text{F}\Delta\psi + 2.3\text{RT}\,\Delta pH\right) \quad (2)$$

where $\Delta\psi=\psi_{vac}-\psi_{cyt}$, $\Delta\text{pH}=\text{pH}_{cyt}-\text{pH}_{vac}$, and n is the coupling ratio (i.e. the number of protons transported during the hydrolysis of one phosphate bond).

The thermodynamic constraints impose a limit on the $\Delta\psi$ that can be achieved at a given ΔpH, as shown by combining Equations 1 and 2:

$$\Delta\psi \leq \frac{-\Delta G_{\text{ATP or PPi}}}{n\text{F}} - \frac{2.3\text{RT}}{\text{F}}\Delta pH \quad (3)$$

Dissociation equations:

$$[\text{Mal}^{2-}] = [(K'_{m1} * K'_{m2}) / (h^2 + (h * K'_{m1}) + (K'_{m1} * K'_{m2}))] * [\text{Mal}]$$

$$[\text{Cit}^{3-}] = [(K'_{c1} * K'_{c2} * K'_{c3}) / (h^3 + (h^2 * K'_{c1}) + (h * K'_{c1} * K'_{c2}) + (K'_{c1} * K'_{c2} * K'_{c3}))] * [\text{Cit}]$$

where $\Delta\psi$ is the tonoplastic electric potential gradient; $(\text{Mal}^{2-})_{cyt}$ is the cytosolic activity of the dianion malate that is equal to the product of the cytosolic concentration and activity coefficient of the dianion malate; $(\text{Cit}^{3-})_{cyt}$ is the cytosolic activity of the trianion citrate; $[\text{Mal}^{2-}]_{vac}$ is the vacuolar concentration of the dianion malate; $[\text{Cit}^{3-}]_{vac}$ is the vacuolar concentration of the trianion citrate; F is Faraday's constant; R is the gas constant; T is temperature; K'_{m1}, K'_{m2}, are apparent acidity constants of malate; K'_{c1}, K'_{c2}, K'_{c3}, are apparent acidity constants of citrate; and $h=10^{-\text{pH}}$ (Lobit *et al*., 2002). Since malate and citrate are stored in the vacuole, their cytosolic concentrations are low and were set at 1 mM (Gout *et al*., 1993; Lobit *et al*., 2006). (C) Theoretical changes in citrate (orange line) and malate (blue line) concentrations in the vacuole as a function of the $\Delta\psi$. The concentrations were calculated using the same equations as in (B), with the vacuolar pH set at 5, and the $\Delta\psi$ ranged from 20 to 30 mV.

The free energy of the substrate hydrolysis (ΔG_{ATP} and ΔG_{PPi}) is negative, but may fluctuate with the cytosolic concentrations of their substrates (Davies *et al*., 1993). The coupling ratio (determined by electrophysiology experiments) is 1 for the V-PPase (Maeshima *et al*., 1994). For the V-ATPase, the coupling ratio is variable and decreases with an increase in ΔpH (Davies *et al*., 1994; Rienmüller *et al*., 2012). In lemon fruits, Müller and Taiz (2002) also reported for the V-ATPase a coupling ratio that decreases from 2 to 1 with an increase in ΔpH. Assuming a model cytosol with a composition assumed to be representative of a plant cell, Davies *et al*. (1993) modelled the ΔpH obtained as a function of $\Delta\psi$ and showed that both pumps are able to sustain vacuolar pH as low as in the most acidic fruits, but that $\Delta\psi$ dropped to zero.

Apart from these thermodynamic limitations, various mechanisms are involved in regulating the proton pumps, including gene expression and substrate availability. Several studies of organic acid-related genes and enzymes suggested that the difference in organic acid content between species and between cultivars of fruits could be linked to differences in their proton pumps (Echeverria *et al*., 1997; Etienne *et al*., 2002; Lu *et al*., 2011; Yang *et al*., 2011). The contribution of the V-ATPase and V-PPase to proton pumping also varies during fruit development. In the grape berry and in pear, the V-PPase is most active in young tissues, but subsequently decreases, and the V-ATPase dominates during fruit ripening (Shiratake *et al*., 1997; Suzuki *et al*., 2000; Terrier *et al*., 2001). The high V-PPase activity in young fruits may be explained by the need to scavenge the PPi, a by-product and inhibitor of several polymerization reactions (synthesis of RNA, proteins, cellulose, and starch) (Maeshima, 2000). In mature tissues, PPi production may decrease as these syntheses slow down, while ATP is constantly supplied by cell respiration.

The transport of potassium (K^+) across the tonoplast also plays a role in the regulation of the $\Delta\psi$ and of the vacuolar pH. Since the concentration of cytosolic K^+ is controlled homeostatically (Leigh, 2001) and because of the small size of the cytosol, most of the K^+ supplied to the fruit cell has to be transported to the vacuole. Facilitated diffusion through vacuolar cation channels is the most likely mechanism (Isayenkov *et al*., 2010). However, in fruit with a high K^+ content such as banana, it can be calculated using the Nernst equation (Fig. 4B); with a cytosolic concentration of K^+ of ~100 mM (Leigh, 2001), and a $\Delta\psi$ of 30 mV (Martinoia *et al*., 2007), passive transport accounts for accumulation of up to 30 mM K^+ in the vacuole. This is very far from the 80 mM found in ripe banana (Chandler, 1995). Thus, in such fruits, active transport is required. The most likely mechanism is a K^+/H^+ antiport, as identified in the tonoplast of tomato plants (Leidi *et al*., 2010). Cation channels help reach a positive $\Delta\psi$ (Isayenkov *et al*., 2010), since the passive influx of K^+ hyperpolarizes the tonoplast (Fig. 4A, no. 6). In contrast, the K^+/H^+ antiport, which mediates an electroneutral exchange, has no effect on $\Delta\psi$. Concerning acidity, transporting K^+ as the balancing charge for organic anions is equivalent to storing not the acid, but its conjugated base, which leads to an increase in pH. In the case of the K^+/H^+ antiport, there is an additional effect on pH due to protons leaving the vacuole.

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Citrate accumulation could be driven by metabolism and malate accumulation by vacuolar storage

In the previous sections, we showed that both metabolism and vacuolar storage play a role in the accumulation of malate and citrate in fruit cells. A relevant question is whether their accumulation in fruit cells is primarily controlled by metabolism or vacuolar storage.

Concerning malate, we showed that the thermodynamic conditions of its transport into the vacuole may limit its accumulation. Therefore, one can hypothesize that malate accumulation in fruit cells is mainly controlled at the level of vacuolar storage, and that metabolism responds appropriately to regulate the cytosolic concentration of malate since it plays a fundamental role in the regulation of cytosolic pH (Smith and Raven, 1979). Several authors agree with this hypothesis. When comparing two apple cultivars with different acidity, Berüter *et al*. (2004) reported higher vacuolar accumulation of ^{14}C-labelled malate in the high-acid cultivar. The higher rate of malate degradation in the low-acid cultivar may only be a consequence of its impaired capacity to store malate. In interspecific introgression lines of tomato, Schauer *et al*. (2006) showed that the V-PPase gene co-localized with the QTL for malate content. In apple, Bai *et al*. (2012) suggested that one of the two ALMT-like genes discovered, *Ma1*, could be the major determinant of malate content in fruit. The relationship between malate accumulation and vacuolar functioning has been modelled in peach by Lobit *et al*. (2006). The model predicts malate accumulation in peach based on the calculation of the thermodynamic constraints on both proton and malate transports, and model results were in good agreement with experimental data, thus reinforcing the hypothesis of control by tonoplastic transports.

Concerning citrate, we showed that its accumulation in the vacuole is unlikely to be limited by thermodynamic conditions. However, the rate of citrate transport into the vacuole may be limited by the activity of its transport system, given that the malate channel transports citrate much more slowly than malate (Hafke *et al*., 2003). Thus, it is likely that citrate accumulation in the vacuole is controlled by its cytosolic concentration and consequently by its metabolism. Among several possible pathways related to citrate metabolism, the TCA cycle is the only one that allows citrate synthesis, so that citrate accumulation is probably controlled by respiration. A fruit PBSM based on a representation of the TCA cycle reactions and their responses to temperature and respiration (Lobit, 1999; Wu *et al*., 2007) led to predictions that were in agreement with observed data. In particular, this model reproduced the increase in citrate content during the early stage of fruit development and the subsequent decrease during the later stage (Léchaudel *et al*., 2005; Wu *et al*., 2005; Albertini *et al*., 2006; Saradhuldhat and Paull, 2007). The fact that citrate synthesis is positively linked to fruit respiration during the green stage, and negatively during ripening may reflect a change in the respiratory substrates used by the TCA cycle from malate (or other intermediates) to citrate. It should be noted that dilution due to pulp growth is required to explain the variations in the concentration of organic acids (Wu *et al*., 2007).

Influence of agro-environmental factors on malate and citrate accumulation in the mesocarp cells of fleshy fruits

The literature shows that the plant source:sink ratio, mineral fertilization, water supply, and temperature are the agro-environmental factors that have the most impact on fruit acidity. This section focuses on their effects on malate and citrate accumulation in fruits considering the mechanisms described above.

The source:sink ratio influences fruit acidity by modifying the supply of sugars

Orchard management practices such as fruit thinning, plant pruning, or defoliation affect the source:sink ratio of the plant, which usually results in altered sugar supply and fruit growth. These practices also affect fruit acidity (Table 1). In peach and mango, it has been observed that an increase in the source:sink ratio increases citrate content early in fruit development, and decreases it near maturity (Souty *et al*., 1999; Wu *et al*., 2002; Léchaudel *et al*., 2005). The opposite effects have been reported for malate, with a decrease during early stages followed by an increase near maturity (Wu *et al*., 2002; Léchaudel *et al*., 2005).

It can be hypothesized that during the green stages, large amounts of sugars imported from the leaves are available for the production of malate via glycolysis and its conversion to citrate via the TCA cycle. It is well known that fruits grown with a high sugar supply, due to a high source:sink ratio, are bigger and consequently have a higher respiration rate. Therefore, in these stages, an increase in fruit respiration due to a high supply of sugars may stimulate glycolysis and conversion of malate into citrate. In contrast, during ripening, sugars may no longer be available for respiration since they are stored in the vacuole (Coombe, 1976), causing a shift from sugars to organic acids (in particular citrate) as respiratory substrate. During this stage, an increase in respiration (due to bigger fruit in response to the high source:sink ratio) may stimulate the conversion of citrate into malate to maintain the pool of TCA cycle intermediates constant. This behaviour has been represented in the PBSM of citrate accumulation (Lobit *et al*., 2003; Wu *et al*., 2007), the results of which are in agreement with observations made in field trials (Génard *et al*., 1991, 1994, 1999; Génard and Bruchou, 1993).

Different but strong effects of mineral fertilization on fruit acidity

Potassium fertilization has an impact on fruit acidity, but agronomic observations are contradictory (Table 1). Some authors reported that potassium fertilization increased fruit titratable acidity (TA) (which is the amount of weakly bound hydrogen ions that can be released from the acids by NaOH

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Table 1. Impact of agro-environmental factors (source:sink ratio, mineral fertilization, water supply, and temperature) on malate and citrate concentrations, and titratable acidity (TA) in the ripe fruits of several species. Citrate content, malate content, and TA are expressed in meq 100 g^{-1} FW. The protocol of each study is summarized as two contrasted treatments applied (A and B). Differences between treatment A and B are either significant at $P < 0.05$ (*) or non-significant (NS). Concerning mineral fertilization, the total quantity of K, Mg, N, or P applied during the experimental period is given.

	Protocol	Malate content		Citrate content		TA		References
		A	B	A	B	A	B	
Source:sink ratio								
Peach (cv. Suncrest)	A: 30 leaves/fruit, B: 10 leaves/fruit	8.8*	7.8*	1.5*	3.5*			Wu *et al.* (2002; data from 1997)
	A: 30 leaves/fruit, B: 6 leaves/fruit	9.0*	8.4*	1.2*	4.9*	9.5*	7.4*	Souty *et al.* (1999)
Mango (cv. Lirfa)	A: 100 leaves/fruit, B: 10 leaves/fruit	0.04 (NS)	0.04 (NS)	0.08*	0.12*			Léchaudel *et al.* (2005)
Potassium fertilization								
Pineapple (cv. Smooth Cayenne)	A: 0 g of K/plant, B: 19.1 g of K/plant					9.6*	13.8*	Spironello *et al.* (2004)
Peach (cv. Elberta)	A: 90 g of K/tree, B: 600 g of K/tree					6.1 (NS)	6.9 (NS)	Cummings and Reeves (1971)
Banana (cv. Ney Poovan)	A: 0% SOK spray, B: 1.5% SOK spray					6.0*	3.4*	Ramesh Kumar *et al.* (2007)
Banana (cv. Robusta)	A: 0 g of K/plant, B: 274 g of K/plant					6.1*	3.4*	Vadivel *et al.* (1978)
Citrus	A: 65 kg of K/ha, B: 230 kg of K/ha					10.9*	11.7*	Alva *et al.* (2006)
Magnesium fertilization								
Peach (cv. Elberta)	A: 14 g of Mg/tree, B: 220 g of Mg/tree					6.7 (NS)	6.3 (NS)	Cummings and Reeves (1971)
Nitrogen fertilization								
Peach (cv. Redhaven)	A:150 g of N/tree, B: 610 g of N/tree					7.2 (NS)	6.3 (NS)	Cummings and Reeves (1971)
Apricot (cv. Canino)	A: 213 g of N/tree, B: 400 g of N/tree					26.0*	31.4*	Radi *et al.* (2003)
Orange (cv. Valencia)	A: 540 g of N/tree, B: 1000 g of N/tree					13.7*	14.7*	Reitz *et al.* (1957)
Pineapple (cv. Smooth Cayenne)	A: 0 g of N/plant, B: 23 g of N/plant					14.5*	11.4*	Spironello *et al.* 2004
Phosphorus fertilization								
Peach (cv. Loring)	A: 0 kg of P/ha, B: 141 kg of P/ha					8.2*	8.4*	Cummings and Reeves (1971)
Pineapple (cv. Smooth Cayenne)	A: 0 g of P/plant, B: 10 g of P/plant					13.0 (NS)	12.5 (NS)	Spironello *et al.* (2004)
Water supply								
Peach (cv. Suncrest)	A: irrigated B: non-irrigated	7.9 (NS)	8.9 (NS)	3.4 (NS)	3.1 (NS)			Wu *et al.* (2002)
Pear (cv. Williams)	A: no water stress, B: early water stress	0.26 (NS)	0.1 (NS)	2.2 (NS)	3.4 (NS)	3.3 (NS)	3.6 (NS)	Hudina *et al.* (2000)
Tomato (cv. Vanessa)	A: watered to 70% maximum water-holding capacity, B: Watered to 50% maximum water-holding capacity					5.1*	6.0*	Veit Khöler *et al.* (1999)
Apple (cv. Braeburn)	A: irrigated, B: non-irrigated					6.7*	7.5*	Mills *et al.* (1996)
Mandarin (cv. Satsuma)	A: well watered, B: severely drought stressed					29.6*	46.9*	Yakushiji *et al.* (1998)
Nectarine (cv. Spring Bright)	A: irrigated, B: deficit irrigation (33% of irrigation in 'A')	4.6*	3.1*	4.7 (NS)	4.7 (NS)			Thakur *et al.* (2012)
Clementine (cv. de Nules)	A: irrigated, B: deficit irrigation (reduced to 25% of crop evapotranspiration)					13.1*	17.1*	Gonzales-Altozano *et al.* (1999; data from 1995)
Orange (cv. Navel)	A: irrigated, B: late water stress					17.2*	21.9*	Kallsen *et al.* (2011; data from 2007)

(*Continued*)

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Table 1. Continued

	Protocol	Malate content		Citrate content		TA		References
		A	B	A	B	A	B	
Grape (cv. Monastrell)	A: irrigated, B: non-irrigated					7.3*	8.9*	De La Hera Orts *et al.* (2005)
Grape (cv. Sauvignon Blanc)	A: no water stress; B: water stress	2.2*	3.4*			7.0*	9.2*	Des Gachons *et al.* (2005; data from 1998)
Temperature								
Tomato (cv. Cervil)	A: not heated, B: heated (fruit temperature: +1,1 °C during the day, +1,3 °C during the night)					11.7*	10.5*	Gautier *et al.* (2005)
Strawberry (cv. Kent)	A: 18/12 (°C) day/night temperature, B: 25/22 (°C) day/night temperature					14.0*	12.5*	Wang *et al.* (2000)

Abbreviations: ACO, aconitase; ATP-CL, ATP citrate lyase; cytACO, cytosolic aconitase; GABA, γ-aminobutyrate; ICL, isocitrate lyase; MS, malate synthase; mtCS, mitochondrial citrate synthase; mtACO, mitochondrial aconitase; NAD-cytMDH, cytosolic NAD-dependent malate dehydrogenase; NAD-IDH, NAD-dependent isocitrate dehydrogenase; NAD-mtMDH, mitochondrial NAD-dependent malate dehydrogenase; NAD-mtME, mitochondrial NAD-dependent malic enzyme; NADP-cytIDH, cytosolic NADP-dependent isocitrate dehydrogenase; NADP-cytME, cytosolic NADP-dependent malic enzyme; NADP-IDH, NADP-dependent isocitrate dehydrogenase; NADP-mtIDH, mitochondrial NADP-dependent isocitrate dehydrogenase; OAA, oxaloacetate; PBSMs, process-based simulation models; PDH, pyruvate dehydrogenase; PPDK, pyruvate orthophosphate dikinase; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase.

titration) (Embleton *et al.*, 1978; Du Preez, 1985; Spironello *et al.*, 2004; Alva *et al.*, 2006), others that potassium fertilization decreased fruit acidity (Vadivel and Shanmugavelu, 1978; Ramesh Kumar and Kumar, 2007), and still others that it had no significant effect (Cummings and Reeves, 1971). At the cellular level, different mechanisms allow K^+ to affect the metabolism and storage of organic acids. Organic anions are synthesized in the vegetative parts to buffer the excess of organic cations absorbed from the soil (Lopez-Bucio *et al.*, 2000). As a result, the K^+ supplied to the fruit by the sap is necessarily accompanied by an equivalent amount of organic anions, mostly malate, and to a lesser extent citrate (Burström, 1945). Without any further metabolism in the fruit, this would amount to adding conjugated bases to the fruit, increasing pH, which is consistent with the positive correlation found between K^+ content and pH of grape berry juice (Mpelasoka *et al.*, 2003). However, in this case, TA would not be affected, since no protonated forms would be added to the fruit. Thus, a modification in fruit TA in response to the supply of K^+ implies that K^+ affects the synthesis or the vacuolar storage of organic acids within the fruit itself. The regulation of tonoplastic transport may be an essential contributor to the effect of K^+. In fruits that contain little K^+, K^+ transport is probably passive and thus contributes to the $\Delta\psi$ (Allen and Sanders, 1997), which in turns stimulates the transport of organic anions into the vacuole. In fruits with a high concentration of K^+, K^+ transport is probably mediated by an electroneutral K^+/H^+ antiport (Leidi *et al.*, 2010). In this case, increasing K^+ accumulation would no longer increase the $\Delta\psi$ but instead would increase the vacuolar pH, reducing the transport of organic anions. Finally, K^+ is known to be involved in the regulation of various enzymes (including the tonoplastic proton pumps), either directly (Wyn Jones and Pollard, 1983; Maeshima, 2000) or by modifying cytosolic pH (Wyn Jones and Pollard, 1983). However, this is unlikely to play an important role, because of the homeostasis of cytosolic K^+ (Leigh, 2001). The PBSM proposed by Lobit *et al.* (2006), which is based on the assumption that malate accumulation is controlled by vacuolar pH, only takes the contribution of K^+ to the acid–basic reactions in the vacuole into account. The model predicted that at low vacuolar pH (in the early stages of fruit growth), an increase in K^+ content would reduce malate accumulation, while at higher pH (during fruit ripening) it would stimulate it.

Contradictory effects of nitrogen nutrition on fruit acidity have also been reported (Table 1). Some authors found a negative correlation between nitrogen nutrition and TA (Spironello *et al.*, 2004), others found a positive correlation between nitrogen nutrition and both TA (Reitz and Koo, 1960) and organic acid content (Ruhl, 1989; Jia *et al.*, 1999; Radi *et al.*, 2003), and still others that it had no significant effect (Cummings and Reeves, 1971). Nitrogen fertilization may have an indirect impact on fruit acidity by stimulating the vegetative growth of plants. Increased vegetative growth may affect the fruit in various ways: by shading them (which would lower their temperature and reduce transpiration), or by diverting assimilates towards vegetative growth (which

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would reduce the supply of assimilates to the fruits). The effect of nitrogen on fruit acidity may also depend on the form of nitrogen applied (NO_3^- or NH_4^+). NO_3^- fertilization is likely to have a positive impact on the concentration of organic anions in the phloem sap since nitrate assimilation in the leaves requires the coordinated synthesis of organic acids (Benzioni *et al*., 1971; Smith and Raven, 1979; Scheible *et al*., 1997), which are then transported in the phloem sap together with K^+. Conversely, NH_4^+ fertilization does not cause the synthesis of organic anions, and may affect cation uptake by roots, like K^+, as observed in banana (Sathiamoorthy and Jeyabaskaran, 2001).

Very few studies have been conducted on the effects of other mineral elements on fruit acidity (Table 1). However, magnesium has been shown to have no significant effect on fruit acidity (Cummings and Reeves, 1971), and phosphorus nutrition appears to have little effect on fruit acidity (Cummings and Reeves, 1971; Spironello *et al*., 2004).

Water supply influences fruit acidity probably due to modifications in fruit water content and osmotic adjustment

The impact of water supply on fruit acidity has been widely studied (Table 1). In most cases, water supply was shown to be negatively correlated with TA and organic acid content in ripe fruits (Mills *et al*., 1996; Yakushiji *et al*., 1998; Gonzales-Altozano and Castel, 1999; Veit-Köhler *et al*., 1999; Hudina and Stampar, 2000; Wu *et al*., 2002; Kallsen *et al*., 2011). However, some authors reported a positive relationship between water supply and both TA and organic acid content in ripe fruits (Esteban *et al*., 1999; des Gachons *et al*., 2004; De la Hera-Orts *et al*., 2005; Thakur and Singh, 2012). Even if water supply modifies fruit acidity, there is apparently no change in the seasonal patterns of the accumulation of organic acids (Wu *et al*., 2002; De la Hera-Orts *et al*., 2005; Thakur and Singh, 2012). Taken together, these data suggest that water stress tends to increase organic acid content and TA in ripe fruits through a simple dilution/dehydration effect (Gonzales-Altozano and Castel, 1999). Another mechanism through which the plant water status may interfere with fruit acidity is osmotic adjustment: under water stress, all plant tissues accumulate solutes, mainly sugars and organic acids (Hummel *et al*., 2010), to lower their osmotic potential and prevent a drop in cell turgor pressure. As water stress increases the accumulation of organic acids in the leaves and xylem fluid (Andersen, 1995; Hummel, 2010), it may also increase imports of organic acids to the fruit.

Temperature influences fruit acidity by affecting both metabolism and vacuolar storage of organic acids

Increasing the temperature during fruit growth or storage decreases fruit TA (Kliewer, 1973; Rufner, 1982; Wang and Camp, 2000; Gautier *et al*., 2005) (Table 1) as well as malate and citrate concentrations, as shown in the grape berry (Buttrose *et al*., 1971; Kliewer, 1973; Rufner, 1982) and in banana (Bugaud *et al*., 2009). Nevertheless, all organic acids do not appear to be equally sensitive to temperature (Rufner, 1982; Wang and Camp, 2000).

Modifications in organic acid metabolism in response to temperature probably result from the impact of temperature on the reaction rates of glycolysis and of the TCA cycle (Araujo *et al*., 2012) by modifying enzyme activities (Lakso and Kliewer, 1975*b*), and also on the kinetic properties of the mitochondrial transport systems involved (Halestrap, 1975). The main effect of increasing temperature would be to stimulate respiration, with the above-mentioned effects on citrate metabolism (increasing citrate production during green stages and decreasing citrate production during ripening) (see previous section). Results of the fruit PBSM developed by Lobit *et al*. (2003), which models net citrate production as a function of temperature, fruit mesocarp weight, and respiration, were in good agreement with experimental data. Further simulations showed that temperature can affect fruit acidity in different ways depending on the fruit cultivar or species (Wu *et al*., 2007).

Temperature probably affects vacuolar storage of organic acids via several mechanisms. Temperature is a key variable in the thermodynamic equations that limit the operation of the proton pumps and the diffusion of organic anions through the tonoplast. In the PBSM of malate accumulation in fruit developed by Lobit *et al*. (2006), increasing the temperature reduced the ability of the fruit to accumulate malate, which is in accordance with observations made in agronomic studies. Temperature also affects membrane fluidity by modifying lipid properties (Murata and Los, 1997). Thus, high temperatures may change the tonoplastic permeability of fruit cells, which could increase leakage of solutes such as protons or protonated forms of organic acids. The increase in tonoplastic permeability could explain the increased activity of vacuolar proton pumps observed in grape berry cells in response to an increase in temperature (Terrier *et al*., 1998). The increase in proton pump transport activity may compensate for the leakage of solutes, which is known to occur during grape berry ripening (Terrier, 2002), but only partially, resulting in a net efflux of malic and citric acid to the cytosol and their further degradation (because of the cytosolic pH homeostasis), leading to a decrease in fruit acidity.

Conclusions

This review showed that accumulation of malate and citrate is the result of interactions between metabolism and vacuolar storage, and identified the main mechanisms likely to drive them. It also showed that agro-environmental factors affect the acidity of fleshy fruit by acting on various cellular mechanisms. To increase our understanding of the development of acidity in fleshy fruit, we believe that integrative approaches would be particularly appropriate (Struik *et al*., 2005; Génard *et al*., 2010). The combination of PBSMs and molecular data, as a tool for model parameterization, could advance our understanding of the response of citrate and malate accumulation to environmental fluctuations and genetic control.

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