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ORIGINAL ARTICLE

# Inhibition of aconitase in citrus fruit callus results in a metabolic shift towards amino acid biosynthesis

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**Abstract** Citrate, a major determinant of citrus fruit quality, accumulates early in fruit development and declines towards maturation. The isomerization of citrate to isocitrate, catalyzed by aconitase is a key step in acid metabolism. Inhibition of mitochondrial aconitase activity early in fruit development contributes to acid accumulation, whereas increased cytosolic activity of aconitase causes citrate decline. It was previously hypothesized that the block in mitochondrial aconitase activity, inducing acid accumulation, is caused by citramalate. Here, we investigated the effect of citramalate and of another aconitase inhibitor, oxalomalate, on aconitase activity and regulation in callus originated from juice sacs. These compounds significantly increased citrate content and reduced the enzyme's activity, while slightly inducing its protein level. Citramalate inhibited the mitochondrial, but not cytosolic form of the enzyme. Its external application to mandarin fruits resulted in inhibition of aconitase activity, with a transient increase in fruit acidity detected a few weeks later. The endogenous level of citramalate was analyzed in five citrus varieties: its pattern of accumulation challenged the notion of its action as an endogenous inhibitor of mitochondrial aconitase. Metabolite profiling of oxalomalate-treated cells showed significant increases in a few amino acids and organic acids. The activities of alanine transaminase, aspartate transaminase and aspartate kinase, as well as these of two $\gamma$-aminobutyrate (GABA)-shunt enzymes, succinic semialdehyde reductase (SSAR) and succinic semialdehyde dehydrogenase (SSAD) were significantly induced in oxalomalate-treated cells. It is suggested that the increase in citrate, caused by aconitase inhibition, induces amino acid synthesis and the GABA shunt, in accordance with the suggested fate of citrate during the acid decline stage in citrus fruit.

**Keywords** Acidity · Aconitase · Citramalate · Citrate · Citrus · Fruit development · GABA shunt · Oxalomalate

**Abbreviations**

| | |
|---|---|
| GABA | $\gamma$-Aminobutyrate |
| ICDH | Isocitrate dehydrogenase |
| 2OG | 2-Oxoglutarate |
| SSAR | Succinic semialdehyde reductase |
| SSAD | Succinic semialdehyde dehydrogenase |
| TA | Total titratable acid |
| TCA | Tricarboxylic acid |

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## Introduction

As in most commercial fruits, pulp acidity in citrus is a major component of taste quality. Citrus fruit acidity is predominantly determined by the concentration of citrate (Sinclair 1984). Citrate begins to accumulate during the second phase of fruit development, when the fruit and its juice-vesicle cells undergo rapid enlargement (Erickson

1968). Citrate accumulation continues for a few weeks, peaking when the fruit volume is about 50% of its final value, and then gradually decreasing as the fruit matures (Sinclair 1984; Shimada et al. 2006). The synthesis of citrate begins with the condensation of the two-carbon acetyl-CoA and the four-carbon oxaloacetate to yield the six-carbon citrate. The reaction is catalyzed by citrate synthase in the mitochondria, as part of the TCA cycle (Sadka et al. 2001). Regulation of the isomerization of citrate to isocitrate by aconitase, in the following metabolic step, plays a major role in citrate homeostasis (Bogin and Wallace 1966). A reduction in mitochondrial aconitase activity early in fruit development most likely generates a local increase in citrate level (Sadka et al. 2000b). The additional citrate is removed from the mitochondria to the cytosol and stored in the vacuole. During the second half of fruit development and towards maturation, citrate is removed from the vacuole via CsCit1, a $H^+$/citrate symporter (Shimada et al. 2006), and is catabolized by a cytosolic aconitase, which is induced in the pulp during the stage at which acid declines (Sadka et al. 2000b). The two aconitase isoforms, mitochondrial and cytosolic, are distinguishable by isozyme gel electrophoresis, and they display distinct sensitivities to iron availability: while the mitochondrial enzyme is resistant to iron limitation, the cytosolic enzyme loses its activity under iron depletion, resulting in higher acid levels, during acid decline stage (Shlizerman et al. 2007). Following citrate removal from the vacuole and its isomerization by the cytosolic aconitase into isocitrate, the latter is converted to 2OG by NADP-ICDH in the cytosol (Sadka et al. 2000b). It is assumed that the catabolized citrate is converted into amino acids (Sadka et al. 2000c; Cercos et al. 2006), although other metabolic pathways, such as gluconeogenesis or acetyl-CoA metabolism, may also serve as metabolic "sinks" for the carbon skeletons generated during citrate catabolism.

In addition to its function in organic acid metabolism, aconitase plays a role in other processes. Bacterial, yeast and animal aconitases act as RNA-binding proteins, which allows them to modulate the expression of genes playing a role in iron metabolism (Volz 2008). Similarly, the *Arabidopsis* enzyme binds to the 5′-untranslated region of the chloroplastic CuZn superoxide dismutase mRNA, and is suggested to play a role in regulating resistance to oxidative stress and cell death (Moeder et al. 2007). Aconitase may also regulate the carbon flow between organic acids metabolism and that of sucrose (Carrari et al. 2003). The yeast aconitase has additionally been suggested to be important for mitochondrial DNA packing (Chen et al. 2005). The activity of aconitase is subjected to multiple levels of regulation. For instance, because the enzyme contains an iron–sulfur cluster, the availability of iron triggers the switch between RNA binding and aconitase activity (Volz 2008). Similarly, NO and reactive oxygen species inactivate both the plant and animal enzymes and induce RNA-binding activity in animal cells (Drapier et al. 1994; Navarre et al. 2000). The assembly of the iron–sulfur cluster required for cytosolic aconitase activity is dependent on the activity of the mitochondrial ATP-binding cassette transporter, ATM3 (Bernard et al. 2009). Regulation of the animal enzyme's activity by phosphorylation has also been demonstrated (Deck et al. 2009). To date, three inhibitors of aconitase have been identified—fluorocitrate, oxalomalate and citramalate (Bogin and Wallace 1966; Guarrier and Buffa 1969; Adinolfi et al. 1971; Lauble et al. 1996). The inhibition by fluorocitrate and its derivative compounds is well characterized, and the crystal structure of the enzyme inhibitor complex has additionally been described (Lauble et al. 1996, and references therein). Oxalomalate, which has been shown to inhibit the animal and plant enzyme in a competitive manner, reduces the RNA-binding activity of aconitase, and also inhibits NADP-ICDH in animal cells (Adinolfi et al. 1971; Ingebretsen 1976; Eprintsev et al. 1995; Festa et al. 2000; Kim and Park 2005). Citramalate, an endogenous compound of fruit pulp, is a competitive inhibitor of citrus aconitase (Bogin and Wallace 1966). In fact, it has been suggested that inhibition of the mitochondrial aconitase by citramalate plays a role in citrate accumulation early in fruit development (Bogin and Wallace 1966). This hypothesis is based on a comparison between acidless lime and sour lemon revealing that the citramalate level is considerably higher in the acid variety than in the acidless one (Bogin and Wallace 1966). Moreover, the acidless lime does not show a reduction in mitochondrial aconitase activity, suggesting that carbon flow through the TCA cycle continues without the accumulation of citrate (Sadka et al. 2000b). The biosynthesis of citramalate in plants could result from the non-enzymatic oxidation of 4-hydroxy-4-methyl-2-oxoglutarate (parapyruvate) by $H_2O_2$ (Shannon and Marcus 1962). Alternatively, it could result from the condensation of acetyl-CoA and pyruvate by 2-isopropylmalate synthase (IPS) (de Kraker et al. 2007). IPS catalyzes the first step of valine, leucine and isoleucine biosynthesis, and it shows a remarkable homology with citramalate synthase from yeast and bacteria (Howell et al. 1999; Xu et al. 2004). The biosynthesis of the other inhibitor of aconitase, fluorocitrate, has been only poorly studied, and oxalomalate results from the condensation of oxaloacetate and glyoxylate by the enzyme oxalomalate lyase (Sekizawa et al. 1966).

In this article, the effects of citramalate and oxalomalate on aconitase expression and activity were investigated. These inhibitors reduced aconitase activity and induced citrate accumulation; however, the pattern of citramalate accumulation during fruit development questioned whether it could acts as an endogenous inhibitor of the

mitochondrial aconitase. Given that the use of these inhibitors resulted in elevated levels of citrate, we also followed changes in the metabolite profile of oxalomalate-treated cells. The results revealed a metabolic shift towards amino acid metabolism and the GABA shunt, which was in agreement with the suggested fate of citrate during the acid decline stage of citrus fruit. These data are discussed in the context of general models of carboxylic acid metabolism in fruit tissues.

## Materials and methods

### Plant material

Fruits of sour lemon (*Citrus limon*) were collected from orchards in the central coastal region of Israel. Tissue cultures originated from the juice sacs were prepared essentially as described previously (Sadka et al. 2000a). At least two cycles of growth were performed before calli were used in experiments. For organic acid analyses, fruits of 'Shamouti' orange (*C. sinensis*), sour lemon (*C. limon*), Limetta lime (*C. limettiodes*), Minneola tangelo (*C. paradise* × *C. reticulate*) and grapefruit (*C. paradisi*) were collected from orchards in the central coastal region of Israel. For citramalate field experiments, branches of Ori mandarin trees (*C. reticulata*) grafted on Troyer rootstock (*Citrus sinensis* × *Poncirus trifoliate*), and grown in the central coastal region of Israel were sprayed with a solution containing 10 mM citramalate and 0.025% (*w*/*v*) Triton ×100 as surfactant or with 0.025% (*w*/*v*) Triton ×100 (control). Sprayings were carried out on 9 June 2008 (fruit diameter of 20–25 mm), 15 August 2008 (fruit diameter of 35–40 mm) and 29 December 2008 (fruit diameter of 65–70 mm). Branches were sprayed either once, twice (first and second dates) or three times (on the three dates). The experiment was conducted in four replicates in four randomized blocks.

### Total titratable acid (TA) and organic acid analyses

Total TA content of the fruit was determined by the titration of 2 ml of juice extract with 0.1 M NaOH, with phenolphthalein as an indicator. Because citrate comprised about 90% of the total organic acids, TA was calculated as if citrate was the only organic acid in the juice, and was presented as percentage (*v*/*v*) in the juice. Citrate and citramalate were assayed in callus and fruit with a 330 Gas Chromatograph (Varian, Walnut Creek, CA, USA), essentially as described previously (Sadka et al. 2000a). We used a Megabore DB17 column (J & W Scientific, Folsom, CA, USA) with the following conditions: $N_2$ flow through, 10 ml/min; $N_2$ makeup, 20 ml/min; $H_2$, 30 ml/min; air, 400 ml/min. Detection was performed with a 4290 Integrator (Varian).

### Protein extraction, aconitase assay and analysis with antibodies

Total proteins were extracted from calli and juice sacs using essentially the same procedure, but at different tissue to buffer ratios depending on the tissue used. Frozen callus was ground in liquid $N_2$ using a mortar and pestle. About 1.2 g of the powder was transferred into a tube containing 2 ml of extraction buffer composed of 100 mM Tris–HCl pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, 4% (*w*/*v*) polyvinylpyrrolidone (MW 40,000), 14.3 mM $\beta$-mercaptoethanol and 1 mM EDTA, and homogenized with a Polytron (Kinematica, Basel, Switzerland) at setting 3–4 for 2–5 s on ice. When fruits were used, about 3 g of tissue was ground in 3 ml of 2× extraction buffer. Following centrifugation at 10,000*g* for 10 min at 4°C, the supernatant was collected and the protein contents were determined using Bradford reagent (BioRad, Hercules, CA, USA) according to the manufacturer's instructions. For aconitase isozyme analyses, about 100 μg of the protein extracts were resolved by non-denaturing polyacrylamide gel electrophoresis (PAGE). Following protein separation, the gel was stained for aconitase activity, as described previously (Sadka et al. 2000b). Spectroscopic assay of aconitase was performed by coupling it to that of NADP-ICDH, essentially as described previously (Cooper and Beevers 1960). However, as the commercial ICDH from porcine heart used in the assay is inhibited by oxalomalate (Ingebretsen 1976), the assays described in Fig. 1a and b were performed by following the formation of *cis*-aconitate, essentially as described previously (Navarre et al. 2000). For Western blot analyses, protein extracts (10–20 μg) were resolved by SDS-PAGE in a 7.5% acrylamide gel (Laemmli et al. 1970) and the gels were electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Antibodies used for aconitase detection were prepared against the short synthetic peptide YLLERGVDRKDFNSYGSR as described by Shlizerman et al. (2007), and the proteins were detected with antibodies diluted 1:1500.

### Callus fractionation

Callus was fractionated into soluble and organellar fractions, and the mitochondria were purified as described by Shlizerman et al. (2007). Usually, the activity of the mitochondrial NAD-ICDH could not be detected in the soluble fraction, but was restricted only to the mitochondrial fraction (data not shown). Similarly, the activity of glucose-6-phosphate dehydrogenase could not be detected in the purified mitochondria (data not shown).

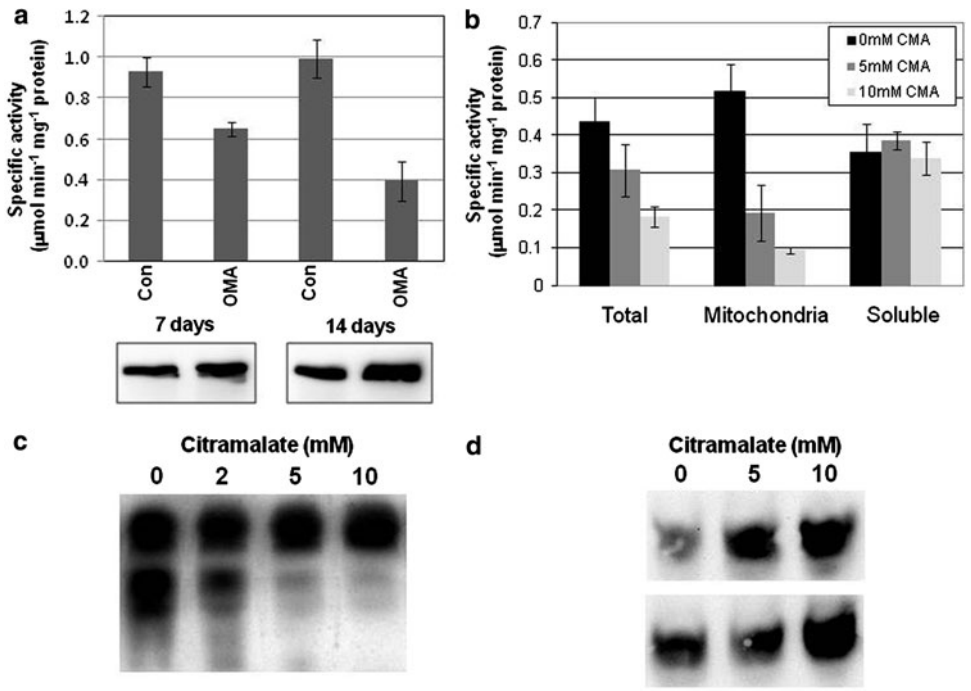



**Fig. 1** Citramalate and oxalomalate inhibit the activity of citrus aconitase. Calli were split and grown in the absence (Con) or presence of 10 mM oxalomalate (OMA) for 7 and 14 days, followed by total protein extraction and assay for aconitase specific activity (**a** *upper panel*), or western analysis using antibodies against aconitase (**a** *lower panel*). Calli were split and grown in the presence of the indicated citramalate concentrations for 3 weeks, followed by their extraction and fractionation into soluble and mitochondrial fractions, and aconitase assays (**b**). Protein extracts of callus cells treated with the indicated citramalate concentrations for 3 weeks were separated by non-denaturing PAGE and assayed, in gel, for aconitase (**c** *upper panel*). Extracts of two separate experiments treated with the indicated concentrations of citramalate were separated by denaturing PAGE, and analyzed with anti-aconitase antibodies (**d** experiment 1 *upper panel*, experiment 2 *lower panel*)

### Metabolite profiling

Metabolites were extracted and analyzed essentially as described previously (Lisec et al. 2006), except that in the primary step, 2 g of callus was ground using a mortar and pestle in the presence of liquid $N_2$. Metabolites were identified by comparison with database entries of authentic standards (Kopka et al. 2005; Schauer et al. 2005).

### Assays of amino acid-metabolizing enzymes

For alanine transaminase and aspartate transaminase, proteins were extracted essentially as described previously (de Sousa and Sodek 2003). Callus (1 g) was ground with 5-ml extraction buffer containing 50 mM Tris–HCl, pH 7.5 and 1 mM DTT with a pinch of sand and 1% polyvinylpyrrolidone (MW 40,000) using a mortar and pestle at 4°C. The homogenate was filtered through Miracloth, and centrifuged at 10,000*g* for 15 min at 4°C. A 2-ml aliquot of the supernatant was desalted by passing it through a Sephadex G-25 column with 10-ml bed volume, and the void volume was collected. Alanine transaminase, aspartate transaminase and succinic semialdehyde dehydrogenase were assayed essentially as described previously (Good and Muench 1992; de Sousa and Sodek 2003; Popov et al. 2007). For aspartate kinase and succinic semialdehyde reductase, extraction, purification and enzymatic assay were performed essentially as described by Wang et al. (2001) and Hoover et al. (2007), respectively, using 2 g of callus as the starting material.

## Results

### Inhibition of aconitase activity induced the protein level of the enzyme and increased citrate levels

The effect of aconitase inhibitors citramalate, a natural compound of citrus fruit, and oxalomalate was investigated in callus originated from lemon juice sacs. Calli were split and grown in the presence of the inhibitors, at concentrations which did not result in any growth inhibition effect during the incubation period (data not shown), and aconitase activity was assayed in total protein extracts. After 1 week of incubation, the activity of aconitase in total extracts of oxalomalate-treated cells was about 70% of that in control cells, whereas after 2 weeks of incubation, the activity in the treated cells was about 40% of that in control

cells (Fig. 1a, upper panel). Interestingly, as a result of the treatment, a slight induction in aconitase protein level was observed after 1 week, and this increase became more pronounced after 2 weeks (Fig. 1a, lower panel). In contrast, no effect of the inhibitor was detected on the mRNA levels of aconitase (data not shown). As citramalate is a natural compound of the citrus fruit pulp (see below), its effect on the extractable activities of the mitochondrial and cytosolic aconitases was investigated (Fig. 1b). The addition of 5 mM of the inhibitor resulted in marginal inhibition, while addition of 10 mM resulted in about 50% inhibition in the total extract. Higher inhibitory effect was detected in the extractable activity of purified mitochondria: about 60 and 80% inhibition by 5 and 10 mM citramalate, respectively. Surprisingly, no inhibitory effects were detected on the extractable activity in the soluble fraction. These results were confirmed by isozyme gel electrophoresis, which distinguished between the two aconitase isoforms (Fig. 1c): there was a clear reduction in the fast-migrating form, previously identified as the mitochondrial enzyme, while no reduction was evident in the slow-migrating form, previously identified as the cytosolic aconitase (Sadka et al. 2000b). Similar to oxalomalate, addition of citramalate resulted in inhibition of aconitase activity, but in an increase in the protein level, as evident from two independent experiments (Fig. 1d). In the first experiment (Fig. 1d, upper panel) there was a an increase in aconitase protein level in 5 mM-treated cells with a further, yet slighter, increase in 10 mM-treated cells. In the second experiment (Fig. 1d, lower panel), the increase was detected only upon application of 10 mM of the inhibitor most likely as a consequence of biological variation. As in the case of oxalomalate, there was no detectable effect on the mRNA levels (data not shown). As would be anticipated, following 2 and 3 weeks of incubation, 10 mM oxalomalate resulted in a circa threefold increase in citrate levels (Fig. 2a), while 2–10 mM citramalate resulted in a two- to three-fold elevation in the citrate level (Fig. 2b).

To analyze the effects of citramalate on intact fruits, it was sprayed on fruit-bearing branches of 'Ori' mandarin trees during the stages of acid accumulation (June), acid peak (mid-August) and acid decline (end of December). 2 and 7 days after the first spraying, a 40–50% increase in citramalate levels was evident in sprayed fruits as compared to non-sprayed fruits (Fig. 3a, inset), but following an additional 38 days, its level was similar in both fruits. Aconitase activity was assayed by isozyme gel electrophoresis 48 h after the first spray. As expected, one major fast-migrating isoform, corresponding to the mitochondrial aconitase, and a faint smear were detected in control fruits, and both were reduced in treated ones (Fig. 3b, inset). The faint smear did not correspond to cytosolic aconitase which normally appear as a distinct band. Similar to observations in the callus, this mitochondrial isoform was reduced by the treatment. Within 45 days of the treatment, a 40% increase in citrate content was detected in the sprayed fruits relative to controls (Fig. 3a). As expected, the increase in citrate level following 45 days was accompanied with an increase in TA from about 6.7% in control fruits to about 7.7% in treated ones (Fig. 3b). However, on the subsequent sampling dates, no significant difference was detected in the citrate or acidity between treated and non-treated fruits. Neither sprays during other stages of acid accumulation nor additional sprays had any effect (data not shown).

## Aconitase inhibition resulted in the accumulation of amino acids

GC–MS was used to analyze control and 10 mM oxalomalate-treated calli for their metabolite content—45 different metabolites were identified. The treatment resulted in a ca.

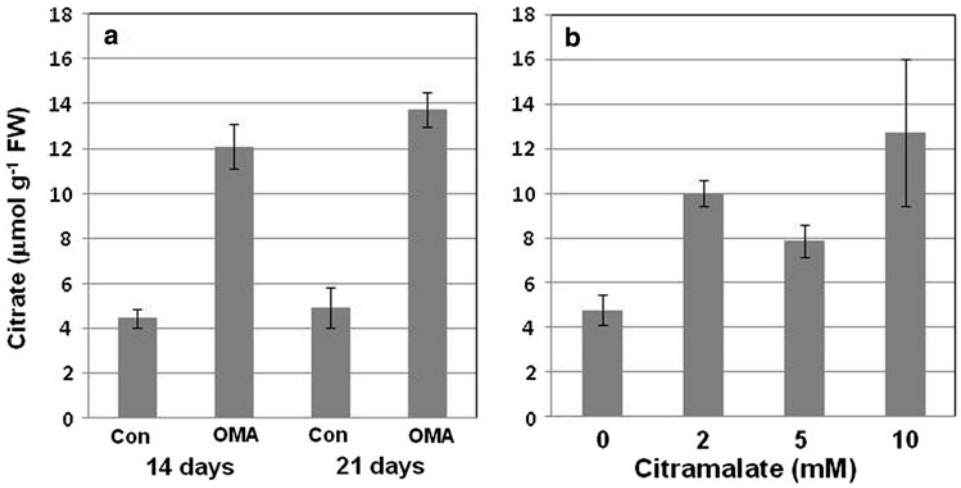



**Fig. 2** Citrate level is induced in citramalate- and oxalomalate-treated cells. Calli were split and grown in the absence (*Con*) or presence of 10 mM oxalomalate (*OMA*) for the indicated times and their citrate level was analyzed by GC (**a**). Similarly, citrate was analyzed in callus grown in the presence of the indicated citramalate concentrations for 3 weeks (**b**)

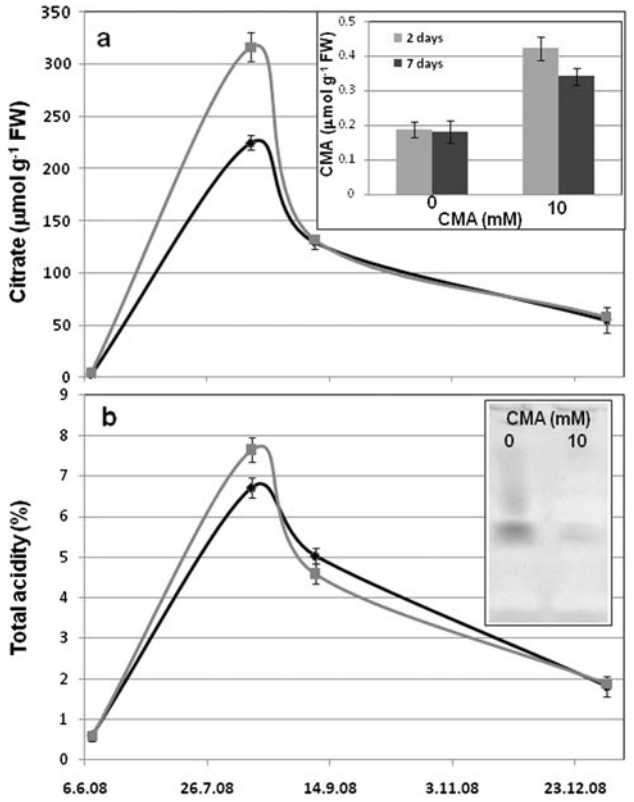



**Fig. 3** Citramalate induces fruit acidity in mandarin fruits. Fruit-bearing branches of 'Ori' mandarin were sprayed with 10 mM citramalate or water at the beginning of June, and their pulp citrate level (**a**) and total titratable acidity (**b**) were assayed at the indicated time points, representing acid incline, acid peak and acid decline stages of fruit development. Citramalate concentration in the pulp was determined 1 week after spraying (**a** *inset*), and total protein of the pulp, extracted 48 h after spraying, was analyzed for aconitase activity by isozyme gel electrophoresis (**b** *inset*)

60% increase in citrate levels, which, possibly due to biological variation, is lower than the increase detected in the previous experiment. A pronounced effect was the induction in amino acid levels; out of the 17 amino acids detected, 6 of them-$\beta$-alanine, glutamine, glycine, isoleucine, threonine and valine-displayed significant increases. Asparagine, aspartate, lysine, and methionine displayed minor, non-statistically significant, induction. In addition to citrate, the increase of three additional organic acids of the TCA cycle, fumarate, malate and succinate, were detected. An increase in the level of 4-hydroxybutyric acid, a side product of the GABA shunt, was also noted. It should additionally be commented that glutamate was below the level of detection in both control and treated calli, a result which was confirmed in a separate amino acid analysis, performed as a commercial service (Aminolab, LTD, Nez-Ziona, Israel). There was, furthermore, a significant reduction in the levels of the following sugars and sugar derivatives: sucrose, glucose, glucose-6-P, raffinose and xylose, while a non-significant reduction was observed in the level of fructose. Glycerol and glycerol-3-P were also reduced by aconitase inhibition.

Given the increase in several amino and organic acids, we next examined changes in the extractable specific activities of alanine transaminase and aspartate transaminase, known to link the organic and amino acid metabolism (Hodges 2002; Rocha et al. 2010), and aspartate kinase which plays a role in the metabolism of threonine, lysine, methionine, isoleucine and other amino acids (Azevedo et al. 2006). Callus from lemon juice vesicles was grown for a few weeks in the presence of 5 or 10 mM oxalomalate, and, as expected, the level of citric acid was induced by the treatment by two- to threefolds. The aforementioned enzyme activities were assayed. In general, all three enzymes revealed elevated maximal catalytic activities upon aconitase inhibition (Fig. 4). Maximal induction in aspartate kinase extractable specific activity was detected by following 3 weeks exposure to 10 mM oxalomalate (Fig. 4a). Most probably due to biological variation, the effect of 5 mM of the inhibitor seemed to be higher than that of 10 mM following 2 weeks of treatment. In the case of alanine transaminase, a maximal effect of four- to six-fold induction was detected with 5 mM of the inhibitor (Fig. 4b). After 2 weeks, the treatment with 5 or 10 mM inhibitor resulted in a twofold induction in the maximal catalytic activity of aspartate transaminase (Fig. 4c). As an increase in 4-hydroxybutyric acid and succinic acid was detected (Table 1), the extractable specific activities of SSAR and SSAD were also investigated. Treatment with either 5 or 10 mM of oxalomalate resulted in about twofold increase in the maximal catalytic activity of SSAR following 2 or 3 weeks (Fig. 4d). The overall effect of the inhibitor on the maximal catalytic activity of SSAD was up to a 20–30% increase following either 2 or 3 weeks of treatment (Fig. 4e).

## The correlation between citrate and citramalate content during fruit development

Citramalate has been postulated to be an endogenous inhibitor of aconitase, playing a key role in citrate accumulation. We examined the relationship between citramalate and citrate levels across a few citrus cultivars differing in their acid accumulation characteristics. Sour lemon (Fig. 5a) accumulated high levels of citrate (up to 420 µmol/g FW; ca 0.3 M), which remained relatively high-throughout fruit development. The acid level of the other four varieties was induced to peak levels thereafter and declined gradually towards fruit maturation. The varieties differed in their maximal citrate level and/or in the kinetics of its accumulation. Acidless lime (Limetta, Fig. 5b) was characterized by a very low citrate level, about two orders of magnitude lower than in sour lemon.

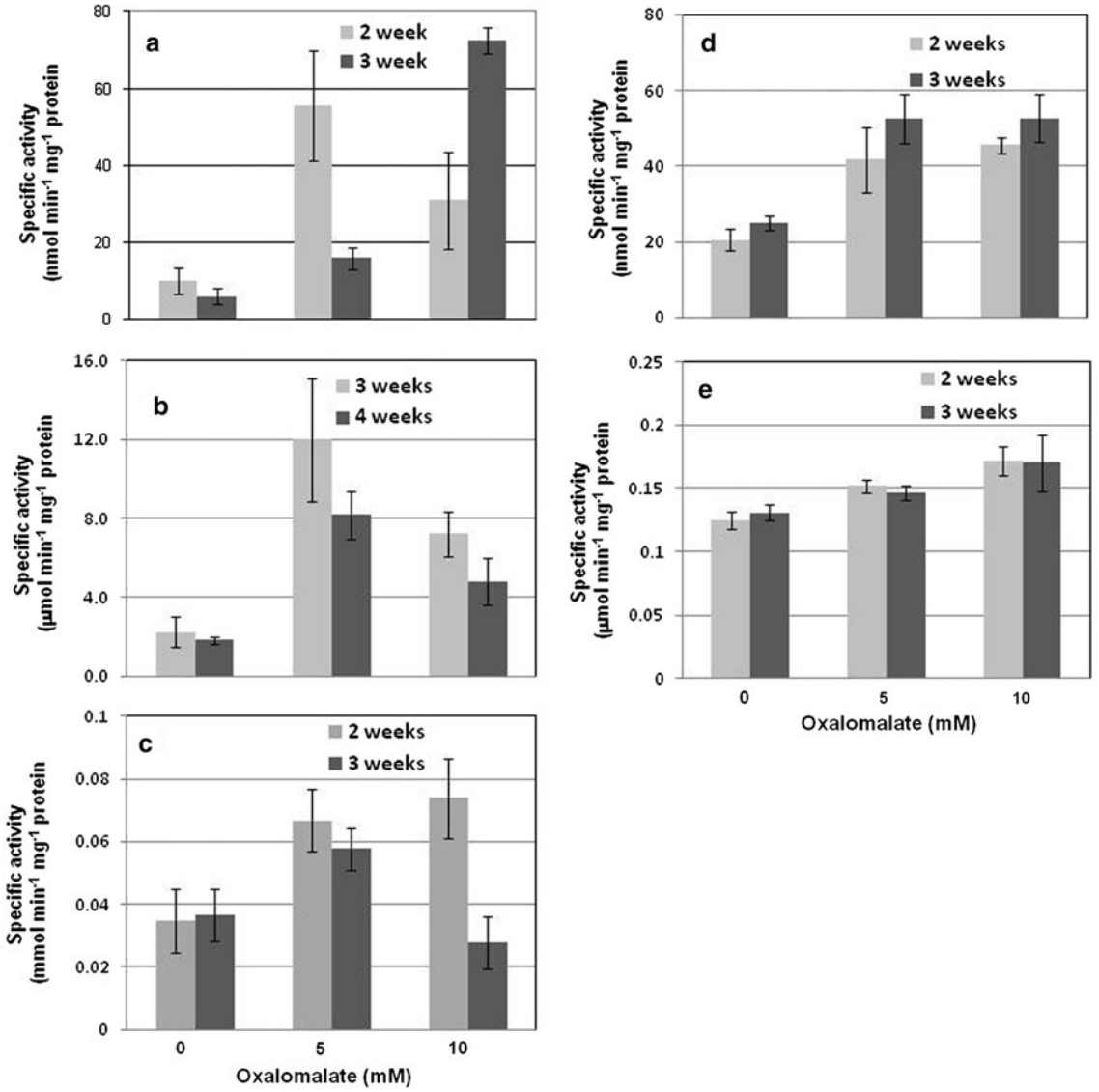



**Fig. 4** The specific activities of amino acid-metabolizing enzymes are induced in oxalomalate-treated cells. Callus, originated from juice vesicles, was grown in the presence of the indicated oxalomalate concentrations for the indicated times, and the activities of aspartate kinase (**a**), alanine transaminase (**b**), aspartate transaminase (**c**), succinic semi-aldehyde reductase (**d**) and succinic semi-aldehyde dehydrogenase (**e**) in total protein extracts were determined

Minneola tangelo (Fig. 5c) accumulated citrate to a relatively higher level, about 300 µmol/g FW, while the other two varieties, Shamouti orange (Fig. 5d) and grapefruit (Fig. 5e), accumulated it to about 170 µmol FW. In all tested varieties, the pattern of citramalate accumulation was quite similar: it was relatively high early in fruit development when citrate was low, and then gradually declined to levels similar to citrate levels. In addition, there was another peak in citramalate concentration during the second half of fruit development. In acidless lime (Fig. 5b) and grapefruit (Fig. 5e), an additional increase was evident towards harvest.

## Discussion

### Inhibition of aconitase as a tool to control fruit acidity

The development of practical tools to control fruit acidity is of crucial importance for the citrus industry. Because of its paramount role in citrate metabolism, the manipulation of aconitase could provide an important application for the control of fruit acidity. As the fruit grown in the field provides a relatively complex system to study citrate metabolism and aconitase activity, callus originated from citrus fruit juice sacs might be used to perform some of the

**Table 1** Metabolite profiling of calli treated with 10 mM of oxalomalate for 3 weeks

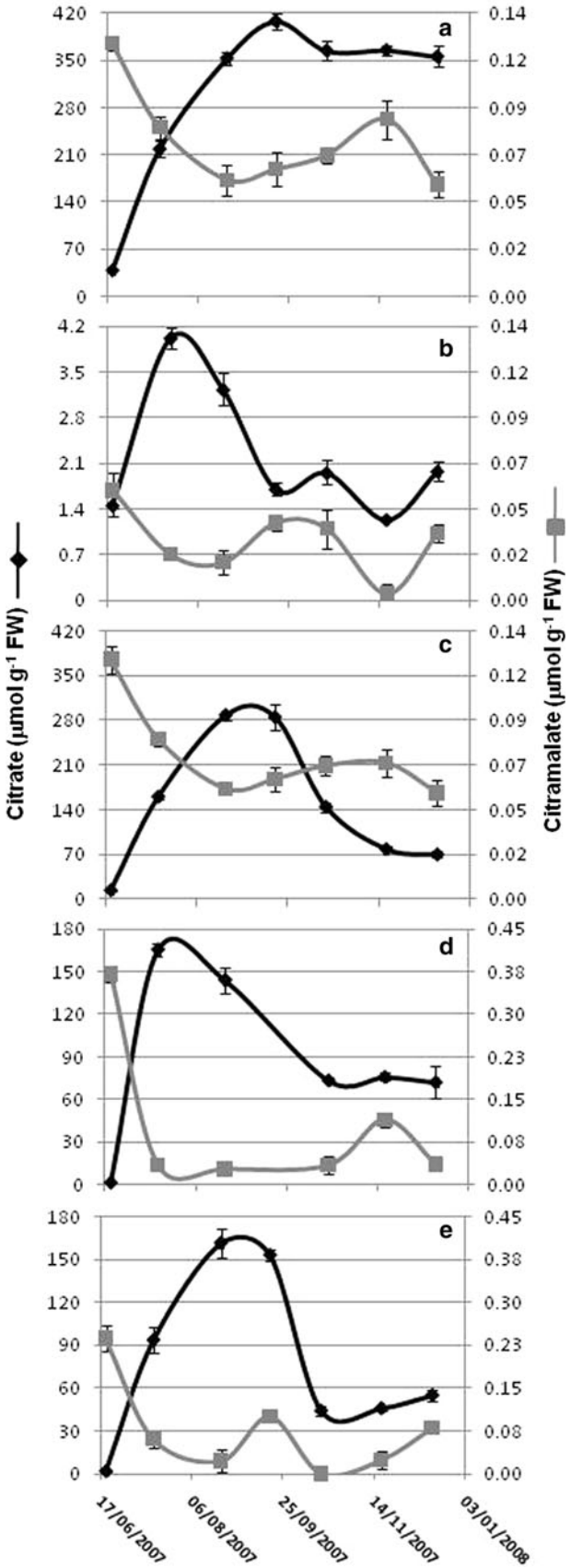
| Metabolite name | Control | Oxalomalate | *t* test |
|---|---|---|---|
| *β*-Alanine | 1 ± 0.045 | 1.48 ± 0.04 | 1.24E−05 |
| Alanine | 1 ± 0.054 | 1.13 ± 0.055 | 0.112103 |
| Arginine | 1 ± 0.107 | 1.06 ± 0.078 | 0.673774 |
| Asparagine | 1 ± 0.239 | 1.02 ± 0.096 | 0.931877 |
| Aspartic acid | 1 ± 0.127 | 1.13 ± 0.037 | 0.354341 |
| Benzoic acid | 1 ± 0.122 | 0.67 ± 0.026 | 0.023621 |
| GABA | 1 ± 0.098 | 0.76 ± 0.12 | 0.145828 |
| Butyric acid, 4-hydroxy | 1 ± 0.116 | 1.5 ± 0.054 | 0.003012 |
| Citric acid | 1 ± 0.141 | 1.68 ± 0.077 | 0.001669 |
| Docosanoic acid | 1 ± 0.033 | 0.89 ± 0.042 | 0.071263 |
| Fructose | 1 ± 0.032 | 0.92 ± 0.028 | 0.076416 |
| Fructose-6-phosphate | 1 ± 0.048 | 0.79 ± 0.063 | 0.023207 |
| Fumaric acid | 1 ± 0.132 | 1.83 ± 0.079 | 0.000296 |
| Galactinol | 1 ± 0.099 | 0.89 ± 0.036 | 0.337962 |
| Glucose | 1 ± 0.036 | 0.88 ± 0.019 | 0.013116 |
| Glucose-6-phosphate | 1 ± 0.040 | 0.8 ± 0.073 | 0.037198 |
| Glutamine | 1 ± 0.170 | 1.62 ± 0.12 | 0.013819 |
| Glycerol | 1 ± 0.018 | 0.81 ± 0.023 | 8.30E−05 |
| Glycerol-3-phosphate | 1 ± 0.036 | 0.69 ± 0.072 | 0.003399 |
| Glycine | 1 ± 0.057 | 1.16 ± 0.023 | 0.022957 |
| Heptadecanoic acid | 1 ± 0.046 | 1.04 ± 0.092 | 0.713048 |
| Hexanoic acid, 2-ethyl | 1 ± 0.084 | 0.89 ± 0.049 | 0.285207 |
| Myo-inositol | 1 ± 0.036 | 0.91 ± 0.025 | 0.077393 |
| Isoleucine | 1 ± 0.068 | 1.33 ± 0.097 | 0.019054 |
| Lactic acid | 1 ± 0.058 | 0.97 ± 0.186 | 0.877033 |
| Lysine | 1 ± 0.069 | 1.03 ± 0.042 | 0.713689 |
| Lyxose | 1 ± 0.066 | 0.56 ± 0.035 | 0.000161 |
| Malic acid | 1 ± 0.138 | 1.55 ± 0.023 | 0.002871 |
| Maltitol | 1 ± 0.180 | 1.08 ± 0.051 | 0.678478 |
| Maltose | 1 ± 0.191 | 0.73 ± 0.041 | 0.194988 |
| Methionine | 1 ± 0.158 | 1.27 ± 0.108 | 0.190173 |
| Octadecanoic acid | 1 ± 0.034 | 0.93 ± 0.024 | 0.132096 |
| Ornithine | 1 ± 0.151 | 1.01 ± 0.022 | 0.970357 |
| Phosphoric acid | 1 ± 0.030 | 0.92 ± 0.027 | 0.060869 |
| Proline | 1 ± 0.158 | 0.9 ± 0.073 | 0.571072 |
| Putrescine | 1 ± 0.076 | 0.88 ± 0.068 | 0.261981 |
| Pyruvic acid | 1 ± 0.059 | 0.98 ± 0.051 | 0.808753 |
| Raffinose | 1 ± 0.129 | 0.53 ± 0.025 | 0.004891 |
| Serine | 1 ± 0.050 | 0.98 ± 0.016 | 0.772012 |
| Succinic acid | 1 ± 0.154 | 1.64 ± 0.192 | 0.026448 |
| Sucrose | 1 ± 0.031 | 0.91 ± 0.026 | 0.044509 |
| Threonine | 1 ± 0.095 | 1.37 ± 0.065 | 0.010052 |
| Trehalose | 1 ± 0.175 | 0.72 ± 0.051 | 0.151665 |
| Tyrosine | 1 ± 0.082 | 1 ± 0.011 | 0.96423 |
| Valine | 1 ± 0.064 | 1.3 ± 0.082 | 0.016065 |

Mean numbers of three independent replicas ± standard errors

studies. The callus lacks the complex structure of the fruit, and its physiological characteristics. For instance, although both aconitase forms, mitochondrial and cytosolic, are detected in the callus, developmental changes in their activities characterized of the fruit (Sadka et al. 2000a), are absent the callus (Fig. 1c). Nevertheless, because citrate is the major acid in the callus, and factors which affect its content in the fruit, such as arsenic compounds and iron limitation, act similarly in these cells, the callus is likely to provide experimental system to study organic acid metabolism in fruit pulp (Sadka et al. 2000a; Shlizerman et al. 2007).

We recently showed that iron limitation induces citrate levels by inhibiting the cytosolic aconitase (induced during acid decline stage), and its application might also reduce fruit acidity towards fruit harvest (Shlizerman et al. 2007). In the present work, we demonstrated that citramalate and oxalomalate, when added to callus growth medium, reduce the extractable activity of the enzyme (Fig. 1a, b), and induce citrate accumulation (Fig. 2). As already mentioned, inhibition of the reaction catalyzed by citrus aconitase by the effector citramalate has been shown to be reversible (Bogin and Wallace 1966). The inhibitory effect of the compound was also apparent on the isozyme gel; therefore, it can be assumed that the compound remains bound to the enzyme during electrophoresis, despite the fact that this binding is non-covalent. Because the inhibitor also induces aconitase protein synthesis, it is also possible that the newly synthesized protein is inactive due to a missing positive factor, rather than inhibitor binding. Similarly to citramalate, oxalomalate has also been shown to bind reversibly to both animal and plant aconitases (Adinolfi et al. 1971; Eprintsev et al. 1995). In animal cells, oxalomalate has been demonstrated to inhibit both aconitase and NADP-ICDH (Adinolfi et al. 1971; Ingebretsen 1976). Although oxalomalate is below detection level in control or treated fruit, oxalomalate may inhibit both the cytosolic aconitase and the NADP-ICDH. This point demonstrates the perennial complexity of using inhibitors in plant metabolism: the possibility that the inhibitors are non-specific. Moreover, the inhibitors themselves might be metabolized, adding additional complexity. Nevertheless, as discussed below, the overall effects of the inhibitors, especially oxalomalate, on the metabolism of the fruit cell were in agreement with the previous findings, and therefore the general metabolic picture resulting from these studies would appear to be valid.

As in tomato (Roessner-Tunali et al. 2003), citramalate is a naturally abundant compound of citrus fruit. We, therefore, tested the potential for its use as a practical tool to block acid decline in citrus varieties which tend to




◀ **Fig. 5** Citrate and citramalate concentrations in the juice of citrus fruit. The concentrations of citrate and citramalate was determined in the pulp of acid lemon (**a**), acidless lime (**b**), Minneola tangelo (**c**), Shamouti orange (**d**) and grapefruit (**e**) during fruit development

reduce their acidity below commercially desirable levels. Indeed, 48 h after the application of citramalate to mandarin trees early in fruit development (during the increasing acid stage), there was a significant reduction in the activity of the mitochondrial aconitase (Fig. 3). Interestingly, this represents the predominant form of aconitase at this developmental stage (Sadka et al. 2000b). Increases in the citrate level and in total acidity of the fruit were detected a few weeks later. However, unlike the effect of arsenic-like compounds, which can be applied early in fruit development and alter citrate level and fruit acidity throughout all stages of fruit development (Sadka et al. 2000a), the effect of citramalate was transient. Later treatments, during stages of peak and declining acid levels, were ineffective. This was, however, perhaps unsurprising since during the second half of fruit development, the cytosolic aconitase, which is not affected by citramalate, is the predominantly active form (Sadka et al. 2000b).

The effects, generated by citramalate and oxalomalate, on citrate levels and aconitase activity in citrus callus (Figs. 1, 2) were similar to those observed following reductions in iron level (Shlizerman et al. 2007). However, while iron limitation affected only the cytosolic aconitase, with no detectable effect on the mitochondrial enzyme, citramalate exclusively inhibited the mitochondrial form. The expression of three citrus aconitase genes was recently described in a variety of acid and acidless cultivars (Terol et al. 2010). The mRNAs levels of two of the genes, *CCAco1* and *CCAco2*, usually coincided with changes in acid levels, while that of CCAco3 was relatively low. However, no evidence was provided as to the localization of their products. Therefore, whether the two forms of the plant enzyme are encoded by two different genes, as in animals (Gruer et al. 1997), or by one gene, as in *Trypanosoma* (Saas et al. 2000), remains an open question. Analysis of the *Aco1* mutant of the wild tomato *Solanum pennellii* revealed that mutation in this gene affects both cytosolic and mitochondrial aconitase activities (Carrari et al. 2003), and knockouts of the three genes in *Arabidopsis* also affected both its cytosolic and mitochondrial activities (Arnaud et al. 2007). Regardless of the situation in citrus, previous and current results suggest that the two forms of aconitase display distinct properties, at least with respect to their sensitivity to iron limitation and to inhibition by citramalate. Similarly, while the activity of the cytosolic aconitase in *Arabidopsis* was reduced in *atm3*

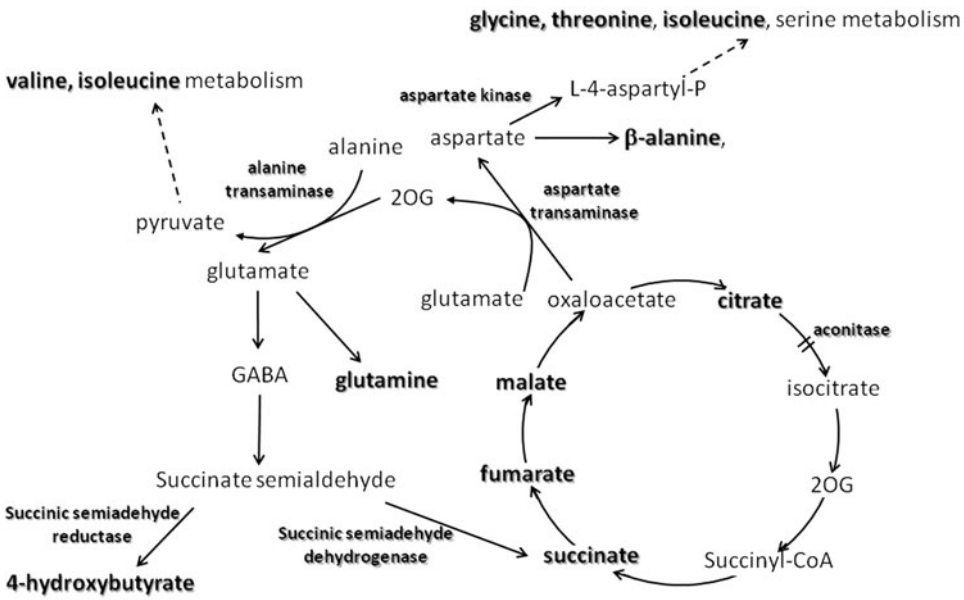



**Fig. 6** Proposed metabolic flow in oxalomalate-treated cells. Metabolites which are induced by the treatment are in *bold*, and enzymes assayed in this work are shown

mutants due to a defect in iron–sulfur cluster assembly, that of the mitochondrial and plastid aconitases was unaffected (Bernard et al. 2009). Some induction in protein content, but not in the mRNA level of aconitase was detected following treatment with both citramalate and oxalomalate. This further supports the notion that the citrus aconitase is regulated mostly at the post-transcriptional and post-translational levels. The induction in protein level might serve as a feedback control mechanism to overcome, at least in part, the inhibitory effects of citramalate and oxalomalate.

Is citramalate the endogenous inhibitor of aconitase?

If the notion that citramalate promotes citrate accumulation via inhibition of mitochondrial aconitase is valid, then a correlation between increasing citramalate and citrate accumulation would be expected. However, the changes in endogenous citramalate concentration in five different fruits, i.e. the drop in its concentration early in fruit development concomitant with an increase in citrate concentration, clearly places this hypothesis into question (Fig. 5). Acid accumulation is a complex process involving several steps, such as citrate synthesis in the mitochondria and transport across the mitochondrial and vacuolar membranes. In addition, citrate accumulation in the vacuole must be coordinated with proton accumulation (Müller et al. 1996). It may well be that high levels of citramalate are required to partially block the mitochondrial aconitase at early stages of fruit development, while during subsequent stages, another mechanism is responsible for maintaining acid accumulation.

Aconitase inhibition results in a metabolic shift toward amino acids and the GABA shunt

In addition to the increase in citrate, there was a significant increase in the levels of three organic acids: succinate, fumarate and malate (Table 1). We cannot exclude the possibility that this increase resulted from a direct effect of aconitase inhibition leading to an increase in the TCA cycle intermediates, as supported by the increase in sucrose, glucose and glucose-6-P, possible source molecules for the synthesis of organic acids. However, our results could also suggest that the induction of succinate, fumarate and malate levels was a secondary effect resulting from induction of the amino acids, as supported by previous reports (see further on). Thus, aconitase inhibition might lead to an increase in citrate as well as oxaloacetate (Fig. 6). Assuming this to be the case, oxaloacetate might then be converted with glutamate by the enzyme aspartate transaminase, which was found to be induced by the treatment (Fig. 4c), into aspartate and 2OG. The activity of alanine transaminase was also induced (Fig. 4b). If this enzyme is operating predominantly in the direction of alanine breakdown, 2OG and alanine will be converted into pyruvate and glutamate. It should be noted that because the direction in which the transaminases operate is governed in vivo by substrate availability, the above metabolic steps are dependent on increases in the relevant metabolic intermediates. The increase in 4-hydroxybutyrate, a side product of the GABA shunt and succinate (Table 1), suggests that this shunt was activated. The GABA shunt is composed of three main reactions catalyzed by glutamate decarboxylase (GAD), GABA transaminase and SSAD, and an additional

side reaction catalyzed by SSAR. Changes in flux through the shunt can be rapid and are predominantly regulated by calcium/calmodulin activation of GAD (Fait et al. 2007). Nevertheless, the extractable specific activity of SSAD showed some induction upon treatment with the inhibitor, whereas that of SSAR showed stronger induction. SSAR, which is present in mitochondria and cytosol, catalyzes the conversion of glyoxylate to glycolate, and it has a considerably higher affinity for glyoxylate than for succinate semialdehyde (SSA) (Hoover et al. 2007; Simpson et al. 2010). SSAD is probably localized only in the mitochondria (Busch and Fromm 1999). Because SSA is synthesized in the mitochondria, it must be transported into the cytosol and plastid (reviewed in Allan et al. 2009). Regardless of this complexity and the lack of data on the effect of the aconitase-inhibiting treatment on each of the SSAR forms, these results support the notion that the GABA shunt is activated. Therefore, through the activities of enzymes of the entire GABA shunt, glutamate is subsequently shuttled into succinate. Succinate, which was also elevated by the treatment, enters the TCA cycle, leading to an increase in the downstream intermediates of this pathway—fumarate and malate. An additional result which might be related to induction of the GABA shunt is that glutamate was below the limits of detection. This result is interesting in light of the finding that glutamate is an abundant amino acid in plants, and in leaves, for instance, it is maintained at relatively constant concentrations, suggesting glutamate homeostasis in plants (Forde and Lea 2007; Stitt and Fernie 2003). The low level of glutamate might result from the high rate of its catabolism, supported by an increase in glutamine, synthesized by glutamine synthetase (Fig. 6). Alternatively, or in addition to high catabolism, a block in the TCA cycle, although most likely partial, might result in a low rate of 2OG synthesis, which in turn might also cause low levels of glutamate; indeed, 2OG was also below the limits of detection. These observations might be related to the induction in the GABA shunt, which is operative in control cells (as supported by the case of fruit, see further on), and is further induced in treated cells, thereby providing a strong sink for glutamate and 2OG. Alternatively, or in addition, if the cytosolic NADP-ICDH is not inhibited by oxalomalate, it could provide a shunt to bypass inhibition of the mitochondrial aconitase, leading to citrate catabolism and amino acid synthesis.

Induction of the GABA shunt is associated with biotic and abiotic stress responses, and possibly with other cellular processes, such as primary metabolism (Shelp et al. 1999; Bouché et al. 2003; Fait et al. 2007; Allan et al. 2009; Simpson et al. 2010). Moreover, several stresses, such as salinity, drought and cold, have been reported to elevate the level of 4-hydroxybutyrate (Allan et al. 2008). Therefore, it might be that the treatment with inhibitor is associated with a stress response, although no arrest in cell growth was detected.

Other amino acids were also induced by the treatment, and the following scenarios are suggested to explain their increase. Aspartate, produced by the activity of aspartate amino transferase, is likely converted into 4-aspartyl-P by aspartate kinase, the activity of which was also induced by the inhibitor (Fig. 4a). This pathway might explain the induction in glycine, threonine and isoleucine, and possibly also $\beta$-alanine (Azevedo et al. 2006; Joshi et al. 2006). It should be noted, however, that plants have several isozymes of aspartate kinase—threonine-sensitive and lysine-sensitive—the latter also sensitive to inhibition by *S*-adenosylmethionine (Azevedo et al. 2006). Although threonine increased in the oxalomalate-treated cells, aspartate kinase activity was similarly induced. This may suggest that either the lysine-sensitive isozyme was induced, or there was a balance between aspartate kinase inhibition by threonine and citrate removal through this pathway. In addition, the levels of two branched amino acids, valine and isoleucine, were induced by the aconitase-inhibiting treatment. Although their synthesis is subject to complex regulation, including partial end-product inhibition, it is possible that pyruvate, potentially generated by the induced activity of alanine transaminase, serves as a primary intermediate for their production (Duggleby and Pang 2000; McCourt and Duggleby 2006).

Based on the results of the current study, we cannot yet identify the molecular causes of the aforementioned changes; doing so would clearly require a massive yet important experimental undertaking, including analysis of each of the various cellular forms of the above enzymes, to understand the flow of metabolites between the various cellular compartments. Nevertheless, together, the above results clearly support previous suggestions that couple citrate catabolism to amino acid biosynthesis and such support stems from the increased activity of cytosolic NADP-ICDH, which converts isocitrate to 2OG and similarly, induction in the mRNA levels of a few amino acid-metabolizing genes, including alanine and aspartate transaminases and glutamine synthetase (Sadka et al. 2000c; Cercos et al. 2006). Moreover, acid catabolism during the acid decline stage has been associated with an increase in the mRNA levels of all GABA shunt enzymes (Cercos et al. 2006). Similarly, in a combined proteomic and metabolomic analysis, we recently identified an increase in amino acids and in the levels of their corresponding biosynthetic proteins during the second half of fruit development (Katz et al. unpublished). That said, the aforementioned linkage between organic and amino acids in citrus fruit might be a special case of the general linkage between C and N metabolism in plants, suggested to be coordinated by ICDH and 2OG dehydrogenase (Hodges 2002; Hodges et al. 2003; Araujo et al. 2008).

In summary, our results demonstrate that by inhibiting the activity of aconitase, a key enzyme in citrate metabolism, it is possible to manipulate fruit acidity, although more research is needed for its practical application. In addition, we show that during aconitase inhibition, the excess citrate is converted to amino acids with induction of the GABA shunt, further supporting previous reports on the fate of citrate during the stage of acid decline.

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