Plant, Cell & Environment

Plant, Cell and Environment (2012) **35**, 1–21 doi: 10.1111/j.1365-3040.2011.02332.x

Metabolic control and regulation of the tricarboxylic acid cycle in photosynthetic and heterotrophic plant tissues

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ABSTRACT

The tricarboxylic acid (TCA) cycle is a crucial component of respiratory metabolism in both photosynthetic and heterotrophic plant organs. All of the major genes of the tomato TCA cycle have been cloned recently, allowing the generation of a suite of transgenic plants in which the majority of the enzymes in the pathway are progressively decreased. Investigations of these plants have provided an almost complete view of the distribution of control in this important pathway. Our studies suggest that citrate synthase, aconitase, isocitrate dehydrogenase, succinyl CoA ligase, succinate dehydrogenase, fumarase and malate dehydrogenase have control coefficients flux for respiration of –0.4, 0.964, –0.123, 0.0008, 0.289, 0.601 and 1.76, respectively; while 2-oxoglutarate dehydrogenase is estimated to have a control coefficient of 0.786 in potato tubers. These results thus indicate that the control of this pathway is distributed among malate dehydrogenase, aconitase, fumarase, succinate dehydrogenase and 2-oxoglutarate dehydrogenase. The unusual distribution of control estimated here is consistent with specific non-cyclic flux mode and cytosolic bypasses that operate in illuminated leaves. These observations are discussed in the context of known regulatory properties of the enzymes and some illustrative examples of how the pathway responds to environmental change are given.

Key-words: metabolic control analysis; metabolic regulation; respiration; *Solanum lycopersicum* (tomato); TCA cycle.

INTRODUCTION

The main role of the mitochondrion in the plant cell, like in all eukaryotic cells, is production of ATP, reducing equivalents and metabolic intermediates for use in biosynthesis elsewhere in the cell (Saraste 1999; Fernie, Carrari & Sweetlove 2004; Nunes-Nesi *et al*. 2008). In addition to representing the primary site of energy transduction and ATP generation in plant cells, the mitochondrion participates in many other important cellular processes such as meeting the demand for carbon skeletons imposed by anabolic processes such as amino acid and isoprenoid syntheses (Fatland, Nikolau & Wurtele 2005), carbon-nitrogen interactions and balance (Noguchi & Terashima 2006; Sienkiewicz-Porzucek *et al*. 2010), biotic stress response (Amirsadeghi, Robson & Vanlerberghe 2007), photosynthetic optimization (Nunes-Nesi *et al*. 2008) and plant cell redox homeostasis and signalling (Scheibe *et al*. 2005; Noctor, De Paepe & Foyer 2007). Moreover, the mitochondria are intimately involved in the production of reactive oxygen species and the processes of programmed cell death (Vianello *et al*. 2007; Logan 2008), flower development (Carlsson *et al*. 2008), seed germination (Macherel *et al*. 2007) and fruit ripening (Sweetman *et al*. 2009; Centeno *et al*. 2011). In addition to ATP, the mitochondrion produces various biosynthetic precursors, ascorbate, vitamin co-factors and several other metabolites which are used in many fundamental metabolic processes during growth and maintenance of the cell (Bartoli, Pastori & Foyer 2000; Plaxton & Podesta 2006; Sweetlove *et al*. 2007). These include fatty acid synthesis (Gueguen *et al*. 2000), synthesis of vitamins and their co-factors such as ascorbate (Bartoli *et al*. 2000) and folate (Mouillon *et al*. 2002), synthesis and export of iron-sulphur clusters (Kushnir *et al*. 2001), and degradation of branched chain amino acids, phytol and lipids (Ishizaki *et al*. 2005; Baker *et al*. 2006; Araújo *et al*. 2010). Notwithstanding these key roles, mitochondrial metabolism supports several light-associated processes including photosynthesis, photorespiration, nitrogen metabolism, reductant transport and the maintenance of photosynthetic redox balance (Rasmusson & Escobar 2007; Nunes-Nesi *et al*. 2008). All these factors considered, it is clear that this organelle is of key importance to cellular function.

Since the elucidation of the tricarboxylic acid (TCA) cycle in pigeon muscle by Krebs & Johnson (1937), evidence has accumulated suggesting that the near-exact same reactions occur in plant cells (Beevers 1961). However, the precise physiological role of the constituent enzymes of this cycle in

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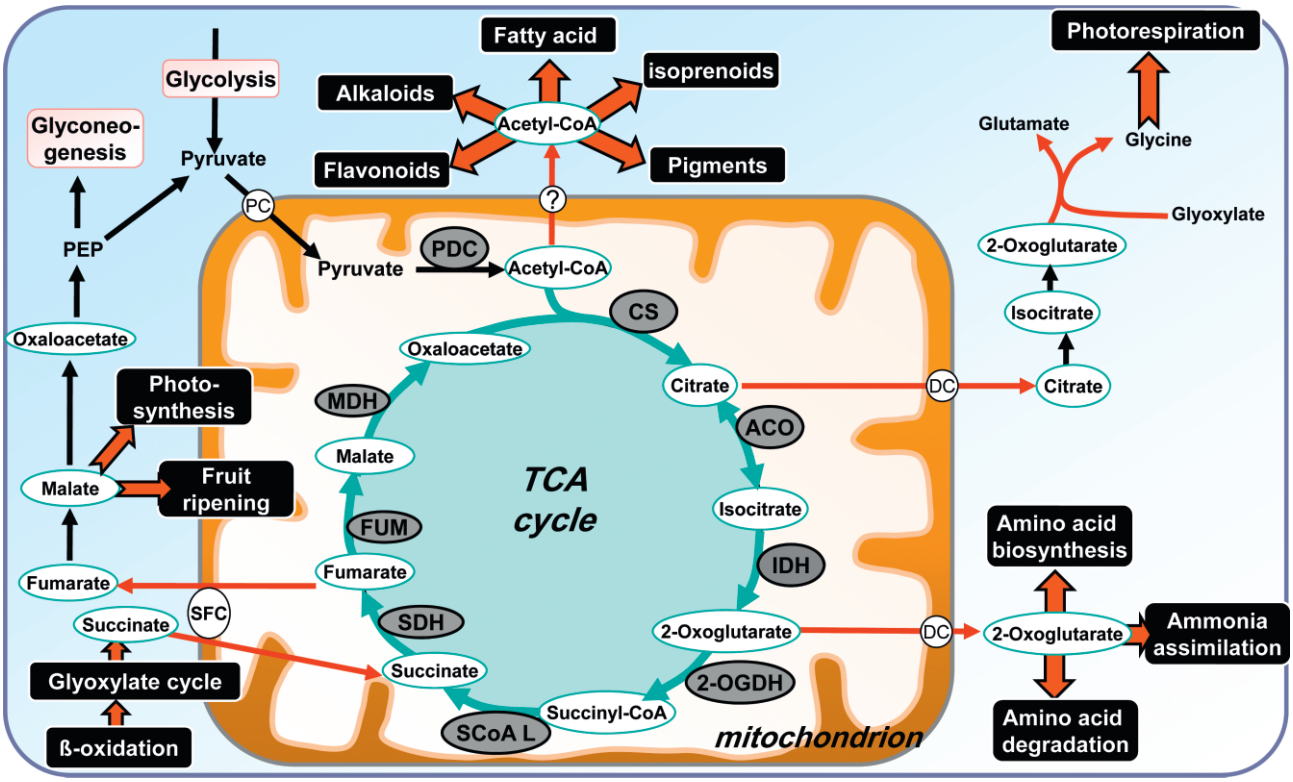

Figure 1. Schematic summary of the TCA cycle and its convergent and divergent pathways showing that the TCA cycle is embedded in a complex metabolic network. White spheres represent carriers or antiporters. For clarity, cofactors have been omitted – the PDC and 2-OGDH reactions both require CoA; the CS and SCoA L reactions produce CoA; NAD+ is converted to NADH by thePDC, IDH, 2-OGDH and MDH reactions; NADP+ is converted to NADPH by a specific isoform of IDH; ADP is converted to ATP by the SCoA L reaction. CS,citrate synthase; ACO, aconitase; IDH, isocitrate dehydrogenase; 2-OGDH, 2-oxoglutarate dehydrogenase complex; SCoA L, succinyl CoA ligase; SDH, succinate dehydrogenase; FUM, fumarase; MDH, malate dehydrogenase; PDC, pyruvate dehydrogenase complex; PC, pyruvate carrier; DC, dicarboxylate carrier; SFC, succinate/fumarate carrier; PEP, phosphoenolpyruvate; TCA cycle, tricarboxylic acid cycle.

plants has, until recently, remained far from clear (Hill 1997; Siedow & Day 2000; Carrari *et al.* 2003b; Nunes-Nesi & Fernie 2007; Nunes-Nesi *et al.* 2008). As a consequence, until recently, relatively little was known concerning the regulation and control of the pathway (Fernie *et al.* 2004). That said, recent years have witnessed great efforts in establishing the importance of the mitochondrial electron transport chain (Møller & Rasmusson 1998; Rasmusson, Geisler & Møller 2008; Rasmusson & Wallström 2010) and a wide range of work has additionally been focused on the role of the TCA cycle enzymes in respiration (Fernie *et al.* 2004; Plaxton & Podesta 2006; Noctor, De Paepe & Foyer 2007). In addition, understanding of the complex pathways through which organic acids are metabolized as well as how these are regulated *in vivo* remains incomplete (Fernie *et al.* 2004; Sweetlove *et al.* 2007, 2010; Fait *et al.* 2008). Bearing this in mind, it is perhaps unsurprising that intensive efforts are currently devoted to elucidating the metabolic regulation of the TCA cycle (Fahnenstich *et al.* 2008; Fukushima *et al.* 2009; Araújo *et al.* 2010; Sweetlove *et al.* 2010; Tomaz *et al.* 2010; Zell *et al.* 2010) as well as its interactions with photosynthesis (Nunes-Nesi, Sweetlove & Fernie 2007b; Nunes-Nesi, Araujo & Fernie 2011), photorespiration (Bauwe, Hagemann & Fernie 2010) and nitrate assimilation (Foyer, Noctor & Hodges 2011). The TCA cycle is a sequence of catabolic reactions that support ATP synthesis, but at the same time it is clearly embedded in a wider metabolic network that allows TCA cycle activity to contribute to other aspects of metabolism (Fig. 1). While the presence of organic acids in all plants is known to support numerous and diverse functions within and beyond cellular metabolism, the level of accumulation of the various organic acids are extremely variable between species, developmental stages and tissue types (Sweetman *et al.* 2009), providing further support that the enzymes involved in the interconversion of these metabolic intermediates are subject to tight regulatory control.

Despite the presence of the numerous metabolic activities which occur or originate in the mitochondrion, there is no doubt that respiration is the dominant metabolic activity of this organelle in heterotrophic tissues. However, in this article, we will not cover the entire respiratory pathway in detail as this has more than adequately been performed elsewhere (Plaxton & Podesta 2006; Sweetlove *et al.* 2007; Rasmusson *et al.* 2008). Rather, it is our intention to review in detail the key aspects of the control and regulation of the TCA cycle as well as to place these events within the

broader context of various cellular environments. For the purpose of this review, we used Fell's definitions whereby *control* indicates the influence of the rate of a metabolite or transport reaction on the flux (e.g. respiration), whereas *regulation* refers to the modulation of an enzyme activity pathway in response to the change in the level of a metabolite (Fell 1997).

STRUCTURE AND OPERATION OF THE TCA CYCLE

The TCA cycle is comprised of a set of eight enzymes in the mitochondrial matrix that couple the product of oxidation of pyruvate and malate (generated in the cytosol) to CO_2 with the generation of NADH for the oxidation by the respiratory chain (Fernie *et al*. 2004). The genomic organization and the subcellular localization of the enzymes involved in the TCA cycle are summarized in the Table 1; however, we will not describe this in detail as it has been recently reviewed elsewhere (Millar *et al*. 2011). In the next section, we will briefly describe each enzyme in detail; moreover, for the purpose of the discussion, we have also included the pyruvate dehydrogenase reaction which is not strictly part of the TCA cycle but is, nevertheless, intimately associated with it.

In plants, pyruvate enters the mitochondrial matrix via an as yet unidentified pyruvate carrier, which is anticipated to operate within the mitochondrial inner membrane. Once within the matrix, pyruvate is oxidatively decarboxylated and converted into acetyl CoA via the action of the mitochondrial pyruvate dehydrogenase complex concomitantly releasing CO_2 and reducing NAD^+ to NADH (Budde & Randall 1990). This enzyme is thought to be a key regulatory point for fluxes into the TCA cycle and to display a general importance with respect to flower development – a fact which was confirmed by the finding that tobacco plants with reduced levels of the E1a subunit of pyruvate dehydrogenase exhibited male sterility (Yui *et al*. 2003).

Acetyl CoA is subsequently converted to citrate by citrate synthase, which is often regarded as the first committed step of the TCA cycle (Fernie *et al*. 2004). Functional studies have implicated the mitochondrial citrate synthase to have important roles in floral development (Landschutze, Willmitzer & Müller-Röber 1995), in the process of organic acid excretion to facilitate nutrient uptake (de la Fuente *et al*. 1997; Delhaize *et al*. 2003) and as a source of carbon skeletons for nitrogen assimilation (Sienkiewicz-Porzucek *et al*. 2008).

Aconitase next converts citrate to isocitrate via the bound intermediate *cis*-aconitate (Moeder *et al*. 2007). Aconitase (Verniquet *et al*. 1991; Lehmann *et al*. 2009) as well as pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase (Taylor *et al*. 2004; Winger *et al*. 2007) are very sensitive to oxidative stress. Analysis of the *Aco*1 *Solanum pennellii* mutants has suggested that aconitase plays a crucial role in regulating carbon metabolism (Carrari *et al*. 2003a). This fact is in agreement with early studies suggesting a role for the TCA cycle in the illuminated leaf (Krömer, Malmberg & Gardestrom 1993; Gardestrom & Lernmark 1995; Hurry *et al*. 1995; Krömer 1995).

Isocitrate can subsequently be oxidatively decarboxylated to 2-oxoglutarate by either NAD^+- or $NADP^+$-dependent isocitrate dehydrogenases, generating CO_2 and NADH or NADPH, respectively (Lemaitre *et al*. 2007; Sienkiewicz-Porzucek *et al*. 2010; Sulpice *et al*. 2010; Foyer *et al*. 2011). This enzyme is believed to be a key regulatory step of the TCA cycle as well as to play an important role in the maintenance of the 2-oxoglutarate level and therefore in the regulation of nitrogen assimilation (Lancien *et al*. 1999; Stitt & Fernie 2003).

Another oxidative decarboxylation reaction – that catalyzed the 2-oxoglutarate dehydrogenase complex – converts 2-oxoglutarate to succinyl CoA, producing more CO_2 and NADH (Bunik & Fernie 2009). Surprisingly, given the fact that a multitude of studies in *Arabidopsis*, tomato and tobacco have been reported for other enzymes of the TCA cycle [for an overview, see Fernie *et al*. (2004) and Sweetlove *et al*. (2007)], there are, as yet, no reports of studies employing mutagenic or transgenic approaches to genetically manipulate the activity of this multienzyme complex.

Table 1. Genomic organization of the enzymes of the TCA cycle

Enzymes	Number of genes[a]	Subcellular localization
Citrate synthase	2	Mitochondria and peroxyssome
Aconitase	2	Mitochondria and cytosol
Isocitrate dehydrogenase	5	Mitochondria, cytosol and peroxyssome
2-oxoglutarate dehydrogenase	6	Mitochondria
Succinyl-CoA ligase	3	Mitochondria and cytosol
Succinate dehydrogenase	11	Mitochondria
Fumarase	2[b]	Mitochondria and cytosol
Malate dehydrogenase	4	Mitochondria, chloroplasts, peroxisomes, and the cytosol

[a]The number of genes that encode polypeptide components in *Arabidopsis thaliana* is shown.
[b]In tomato, rice and poplar, only a single gene encoding fumarase, predicted to be mitochondrial, has been identified to date.
TCA, tricarboxylic acid.

Thus, the metabolic impact of 2-oxoglutarate dehydrogenase in plants remains relatively poorly characterized. Nevertheless, it has been recently demonstrated that the inhibition of 2-oxoglutarate dehydrogenase complex, via specific chemical inhibitors, limits respiration in potato tubers and that the enzyme plays an important role in nitrogen assimilation (Araújo *et al.* 2008). Additionally it has been observed that in plants the metabolic perturbation following its inhibition is generally similar to that observed in species of other kingdoms (Bunik & Fernie 2009).

Plant succinyl-CoA ligase subsequently couples the synthesis of ATP from ADP and Pi, with the concomitant conversion of succinyl-CoA to succinate (Johnson *et al.* 1998; Studart-Guimarães *et al.* 2005). It is, in this respect, different to its mammalian and microbial counterparts which prefer GDP as substrate (Fraser *et al.* 1999, 2006; Lambeth *et al.* 2004). Recent evidence using transgenic tomato plants in which the expression of succinyl-CoA ligase was impaired imply that an up-regulation of the γ-aminobutyric acid (GABA) shunt can adequately complement this deficiency and uncovered that the GABA shunt effectively operates as an alternative source of mitochondrial succinate (Studart-Guimarães *et al.* 2007).

Succinate dehydrogenase (SDH) catalyzes the oxidation of succinate to fumarate and the simultaneous reduction of ubiquinone to ubiquinol (Hagerhall 1997; Figueroa *et al.* 2001). Therefore SDH, often also referred to as complex II, has a dual function being important within both the TCA cycle and the respiratory chain. To date, several forward or reverse genetic strategies have been employed to study the function of complex II in plants (Leon, Holuigue & Jordana 2007; Roschzttardtz *et al.* 2009; Araújo *et al.* 2011). These studies have revealed that the disruption of the expression of the *SDH1-1* gene results in alterations in gametophyte development, pollen abortion and reduced seed set (Leon *et al.* 2007), whereas the absence of SDH2-3 in mutant *Arabidopsis* seeds appears to inhibit their germination (Roschzttardtz *et al.* 2009). In addition, the antisense inhibition of the iron–sulphur subunit of *SDH* in tomato plants increases photosynthesis and biomass via an organic acid-mediated effect on stomatal aperture (Araújo *et al.* 2011).

The reaction catalyzed by fumarase (fumarate hydratase) next converts fumarate to malate (Nunes-Nesi *et al.* 2007a), which is then oxidized to oxoloacetate by NAD^+-dependent malate dehydrogenase (Nunes-Nesi *et al.* 2005). Fumarase activity has been documented to be extremely high in guard cells of *Vicia faba* and *Pisum sativum* (Hampp, Outlaw & Tarczynski 1982; Outlaw 2003). Furthermore, antisense tomato (*Solanum lycopersicum*) plants with a reduced expression and activity of fumarase have been characterized to exhibit reduced growth phenotype observed on a whole plant basis. Further experimentation has suggested that the observed phenotype could be linked to impaired stomatal functioning (Nunes-Nesi *et al.* 2007a), rather than a direct metabolic effect, resulting in CO_2 limitation of photosynthesis in contrast to the situation observed in SDH antisense tomato plants described previously (Araújo *et al.* 2011). Furthermore, measurements of apoplastic and protoplastic organic acid concentrations in SDH transformants (Araújo *et al.* 2011), and also in transformants with deficient stomatal functioning (Nunes-Nesi *et al.* 2007a), have revealed a negative correlation between the concentrations of fumarate and gas exchange through the stomata and provide strong evidence to support that modulation of guard cell malate and fumarate concentration can greatly influence stomatal opening.

The TCA cycle is then completed by the action of malate dehydrogenase which catalyzes the reversible oxidation of malate to produce oxaloacetate (OAA) (Nunes-Nesi *et al.* 2005). The mitochondrial malate dehydrogenase seems to be important not only for NADH oxidation but also as a component of the malate-aspartate and malate-OAA shuttles for the exchange of substrate, and reducing equivalents across the mitochondrial membrane (Scheibe 2004; Nunes-Nesi & Fernie 2007). It is clear, however, that the exchange through the membranes is strictly controlled, as large redox differences in NAD(H) pools exist between compartments (Igamberdiev & Gardestrom 2003). Tomato plants with reduced expression of malate dehydrogenase displayed an enhanced biomass and photosynthetic performance (Nunes-Nesi *et al.* 2005). A range of metabolites, such as ascorbate, also accumulated in the tomato antisense plants and this increase could be linked to an enhancement of photosynthesis because ascorbate feeding to leaves can also increase photosynthetic performance (Nunes-Nesi *et al.* 2005). These results furthermore functionally confirmed the previously hypothesized link between ascorbate biosynthesis and the mitochondrial electron transport chain (*see* Bartoli *et al.* 2000). It was additionally observed that these transgenic plants had altered partitioning of resources between shoot and root (van der Merwe *et al.* 2009), leading to a lower root dry weight and lower root respiratory rate.

The comprehensive analysis of the TCA cycle function in tomato revealed a surprising spectrum of transcriptional, metabolic and phenotypic alterations following perturbation of gene expression and activity of almost all of the constituting enzymes. However, these observations are consistent with the assumed importance of this pathway. A summary of observed metabolic and morphological phenotypes for the different transgenic lines of the different enzymes of the TCA cycle is presented in Table 2. The wide diversity that has been observed when comparing the various genotypes exhibiting deficiency in expression of enzymes of the TCA cycle, however, provides strong support for the contention that the TCA cycle often operates in a modular fashion in plants – i.e. not all reactions in the pathway carry the same flux (Sweetlove *et al.* 2010) – because if this was not the case, suppression of any given enzyme would be expected to reduce the cyclic flux to the same extent as any other and thus lead to similar metabolic and phenotypic consequences. In some circumstances, non-cyclic flux modes may quantitatively be more important than cyclic ones (Sweetlove *et al.* 2010). Similar conclusions of the operation of an incomplete cycle have previously been made in microbial and mammalian systems (McCammon *et al.* 2003; Tian *et al.* 2005; Singh *et al.* 2009; Lemire *et al.* 2010),

Table 2. Summary of observed phenotypes for the different transgenic lines of the enzymes of the TCA cycle

	Phenotype	
Enzyme	Morphological	Metabolic
Citrate synthase	No visible alteration in growth	Few changes in photosynthetic parameters; decreased flux through the TCA cycle; increased rate of respiration; inhibition of nitrate assimilation
Aconitase	Stunted phenotype at early stages of development	Decreased flux through the TCA cycle; reduced levels of TCA cycle intermediates; elevated adenylate levels; enhanced rate of CO_2 assimilation; increased fruit yield
Isocitrate dehydrogenase	No visible alteration in growth	Few changes in photosynthetic parameters; decreased flux through the TCA cycle; decreases in the levels of amino acids, intermediates of the TCA cycle, photosynthetic pigments, starch and NAD(P)H; increased levels of nitrate and protein
2-oxoglutarate dehydrogenase	n.d.	Dramatic reduction of the rate of respiration; alterations in levels of the TCA cycle intermediates and amino acids crucial to nitrate assimilation
Succinyl-CoA ligase	No visible alteration in growth	Few changes in photosynthetic and respiratory parameters; up-regulation of an alternative pathway for succinate production, the GABA shunt
Succinate dehydrogenase	Increased growth on a whole plant basis	Enhanced assimilation rate; decreased flux through the TCA cycle; alterations in the levels of metabolites associated with the TCA cycle
Fumarase	Reduced biomass on a whole plant basis	Reduced rate of CO_2 assimilation; decreased flux through the TCA cycle; reduced levels of TCA cycle intermediates
Malate dehydrogenase	Increased growth on a whole plant basis	Enhanced assimilation rate; accumulation of carbohydrates and redox-related compounds such as ascorbate

Data source is given in parenthesis: citrate synthase (Sienkiewicz-Porzucek *et al*. 2008); aconitase (Carrari *et al*. 2003a); isocitrate dehydrogenase (Sienkiewicz-Porzucek *et al*. 2010); 2-oxoglutarate dehydrogenase (Araújo *et al*. 2008); succynil-CoA ligase (Studart-Guimarães *et al*. 2007); succinate dehydrogenase (Araújo *et al*. 2011); fumarase (Nunes-Nesi *et al*. 2007a); malate dehydrogenase (Nunes-Nesi *et al*. 2005). n.d. not determined; TCA, tricarboxylic acid; GABA, γ-aminobutyric acid.

revealing that the cycle displays a modular structure, in which different parts of the pathway can have diverse metabolic functions. The conclusive implication is that different steps in the TCA cycle have functions other than merely maintaining cyclic flux, and that the fine metabolic balancing of such functions is likely to depend on the physiological context in which the pathway is operating (Sweetlove *et al*. 2010).

The organization of the TCA cycle is well established at the protein level (Carrari *et al*. 2003b), and all genes which encode the constituent enzymes have been identified and cloned in plants. That said, many open questions concerning the control of pathway flux and the operation of different flux modes remain (Sweetlove *et al*. 2010; van Dongen *et al*. 2011). Nevertheless, the generation of a battery of transgenic plants with altered expression of almost all of the enzymes of the TCA cycle has enabled the elucidation of the contribution of each constituent enzyme to the control of pathway flux. As yet, these results have not been summarized into an overview of flux control at the level of the entire pathway, such as that already carried for photosynthetic starch metabolism (Stitt & Sonnewald 1995) or the sucrose to starch transition (Geigenberger, Stitt & Fernie 2004). For this reason, here we aim to provide such a synthetic review of the distribution of flux control in the reactions of the TCA cycle in addition to discussing what is known concerning its physiological regulation. Particularly, attention is given to the adaptation of this pathway to environmental conditions including trophic status, carbon and nitrogen limitations, and low oxygen stress.

METABOLIC CONTROL ANALYSIS

The concept of biochemical pathway regulation encompasses the mechanisms by which the distribution of fluxes within the pathway are altered in response to some external signal or internal perturbation (Liguzinski & Korzeniewski 2006). On the other hand, flux control often refers to the strength with which a given enzyme activity affects the flux through the entire pathway (Korzeniewski 2001). Metabolic

Table 3. Flux control coefficients for the mitochondrial isoforms of the enzymes of the TCA cycle

Enzyme	Flux control coefficient	
	Quadratic hyperbola	Deviation index
Citrate synthase	–0.4	–4.61 ± 2.93
Aconitase	0.964	0.951 ± NA
Isocitrate dehydrogenase	–0.123	–0.204 ± 0.842
2-oxoglutarate dehydrogenase	0.786	0.541 ± 0.254
Succinyl-CoA ligase	0.0008	–0.0609 ± 0.146
Succinate dehydrogenase	0.289	–1.594 ± 1.38
Fumarase	0.601	0.30 ± 0.19
Malate dehydrogenase	1.76	–1.0629 ± 3.7557

The flux control coefficients (FCCs) were estimated by two approaches: (1) the second column uses the parameters obtained from the best fit to the quadratic hyperbola at the first available decreased enzyme activity (together with the corresponding fluxes); (2) the third column gives the deviation index at the first available decreased enzyme activity; for all enzymes, dark respiration is used as a proxy for the flux. The estimates for the FCCs at different steady states are given in the Supporting Information Table S1. The source of data used for the calculations are presented in the legend of Fig. 2.
TCA, tricarboxylic acid.

control analysis (MCA) is a widely used theoretical framework describing the extent to which a given enzyme controls the metabolite flux through a pathway (Kacser & Burns 1973; Heinrich & Rapoport 1974; Fell 1992, 1997; Geigenberger *et al*. 2004). The framework of MCA has already been coupled with experimental measurements of fluxes and enzyme activities to determine the importance of well-characterized enzymes in plant metabolism (Neuhaus & Stitt 1990; Stitt 1990; ap Rees & Hill 1994; Sweetlove *et al*. 1998; Sweetlove & Hill 2000; Geigenberger *et al*. 2004).

In the framework of MCA, the contribution of particular enzymes to the control over the flux is quantified by a parameter called the flux control coefficient (FCC). This is defined as the ratio of the relative change in flux and the relative change in enzyme amount (Moreno-Sanchez *et al*. 2010). FCCs can be experimentally determined, for instance, by decreasing the amount of a given enzyme, either by genetic manipulation or by using inhibitors and then measuring the impact on the steady-state flux through the pathway (Neuhaus & Stitt 1990). Regardless of the type of experimental setup, the following conditions must hold in order for the MCA findings to be applicable: (1) the system is sufficiently close to a steady state; (2) the enzyme of interest acts only in one reaction; and (3) in mutants and transgenic organisms, it is crucial that only the enzyme in question be changed, excluding any side effects of the transformation. These conditions are the assumptions of the numerical approach described next. In the calculation of the FCC, we employed the mean of the dark respiration as a proxy to the overall flux through the system.

To provide an estimate of the FCC at a steady state determined by the combination of enzyme activity and flux through the system, we used two analytical approaches. Following Small & Kacser (1993b), in the first approach, we employed non-linear regression to determine the best fit for the quadratic hyperbola (see Fig. 1):

$$J = \frac{A \cdot E}{B + E}.$$

The quadratic hyperbola for the flux J, given in the previous equation, is fully determined by the parameters A and B. By differentiating this equation, and substituting the estimates for the parameters A and B, one obtains a value for the FCC. In the column titled Quadratic hyperbola, Table 3, dark respiration is used as a proxy for the flux. To further establish the robustness of the estimates, the parameters A and B are determined by two independent R implementations of non-linear regression for quadratic hyperbola, *mm.2*, from the drc package, and *micmen*, from the VGAM package. We note that accurate estimates of the FCC according to this approach require that the enzyme activity in the mutant is very close to that of the wild type (WT), and that the relationship between fluxes and enzyme activities can be described well with the help of quadratic hyperbola.

In the second approach, we relied on the deviation index. For a linear pathway, for which fluxes are measured at widely separated enzyme levels (activities), E_1, corresponding to WT, and E_2, corresponding to a mutant, the *deviation index*, as previously proposed (Small & Kacser 1993a,b), allow the approximation of the FCC at enzyme level E_1. The deviation index handles the effect of large changes and can serve as a proxy for the FCC. Note that the scaling in the deviation index is with respect to E_2/J_2, i.e. the mean value of the deviation index does not rely on the small-change estimates of the FCC:

$$C_J = \frac{E_2(J_2 - J_1)}{J_2(E_2 - E_1)}.$$

Moreover, the deviation of this expression relies on the assumption that the fluxes of all reactions in the system are effectively linear functions of the involved metabolites (Small & Kacser 1993a). The mean values of the deviation index in Table 1 are obtained by using the flux and enzyme activity values from the WT (J_1 and E_1, respectively) and those from the mutant with enzyme activity closest to that of the WT (i.e. J_2 and E_2). The standard deviations are obtained according to the formula (A11), appearing in the appendix of Small & Kacser (1993a).

Theoretically, in an unbranched pathway, the FCCs are always positive and smaller than or equal to 1. Moreover, following the summation theorem, the FCCs must sum up to 1, implying that changes in the concentration of one enzyme induces changes on the entire system through redistribution of control (Kacser & Burns 1973). Therefore, the pattern of control in a given system, determined by the distribution of metabolic control among different enzymes, varies across different steady states. According to MCA theory, it is, at least theoretically, possible for a single enzyme to have an FCC of 1 and all others in the pathway to have zero. However, based on experimental measures of FCC no enzyme determines the flux uniquely and, thus, there are no so-called 'rate-limiting' steps (whose FCC equals 1). Despite this theoretical claim, in many situations some steps can be 'practically rate-limiting', with the value of the FCC approximating 1 (Groen *et al*. 1982; Westerhoff & Arents 1984). Taken together, these points warrant caution in interpreting the results of MCA derived from experimental data.

It is however important to point out that MCA of non-plant respiration suggest that the contribution of the several enzymes to the overall flux control is highly dependent upon the organism, tissue and physiological condition (Fothergill-Gilmore & Michels 1993; Cornish-Bowden & Cornish-Bowden 1995; Thomas & Fell 1998). Therefore, it is clear that small changes in cellular circumstance can cause a pathway-wide redistribution of the control that each enzyme exerts on respiratory carbon flux. It is highly likely that this finding will also be applicable to plant respiration. Nevertheless, it is important to stress that previous respiratory flux based on glycolitic enzymes of potato tubers, using both experimental and control analysis, suggests that strong co-operative feedback inhibition of enzymes serves to move flux control downstream of the inhibiting metabolite, phosphoenolpyruvate (Thomas *et al*. 1997a,b), and that the majority of the flux controls do appear to reside somewhere in the reactions downstream of glycolysis. Moreover, the application of MCA to plant respiration is greatly complicated by the inherent flexibility of plant respiratory metabolism and mitochondrial electron transport chain (Plaxton & Podesta 2006). Nevertheless, as discussed next, MCA has provided some important contributions to our understanding of the control of plant metabolism and it thus remains likely that application of this framework will prove informative in the case of plant respiration.

FLUX CONTROL ANALYSIS OF RESPIRATION IN THE TCA CYCLE

As outlined in the section 'STRUCTURE AND OPERATION OF THE TCA CYCLE', genes encoding each of the enzymes involved in the TCA cycle have been cloned, and a large number of antisense or RNAi transformants created in which the expression of the encoded enzyme is reduced. Biochemical and physiological analyses of these lines have pointed out the specific contribution of the enzyme constituents of the pathway. We used the results of these previously published data (see figure legend for citations) to generate standard plots (Fig. 2). Each plot shows the relation between the amount of a particular enzyme (estimated via measurement of activity in optimal standard conditions) and the respiration rate. Interestingly, reduction of up to 50% of the activity of citrate synthase promoted a decrease in the relative flux through the TCA cycle and a concomitant increased rate of respiration (Fig. 2) (Sienkiewicz-Porzucek *et al*. 2008). By contrast, studies of the wild species tomato (*S. pennellii*) mutant *Aco*1, deficient in the expression of aconitase, demonstrated a 60% decrease in the activity of aconitase with similar effect on respiration rates (Carrari *et al*. 2003a). Additionally, large decreases of the activity of the mitochondrial NAD-dependent isocitrate dehydrogenase and succinyl-CoA ligase have little, if any, impact on respiration rate (Fig. 2). The antisense inhibition of succinate dehydrogenase reduced the respiration rate in approximately 15% despite large reductions in the activity (~60%). By contrast, a large reduction in the respiration rate was observed in both the antisense inhibition of either fumarase or malate dehydrogenase (Nunes-Nesi *et al*. 2005, 2007a) and the chemical inhibition of the 2-oxoglutarate dehydrogenase complex (Araújo *et al*. 2008). However, it is also important to note that several recent studies have demonstrated that there are several functionally active, alternative donors to the plant mitochondrial electron transport chain (Ishizaki *et al*. 2005; Araújo *et al*. 2010; van Dongen *et al*. 2011) and some control will, at least under certain circumstance, reside in these steps.

Control coefficients were estimated using the approaches described previously (section 'METABOLIC CONTROL ANALYSIS'), and are summarized in Table 3. Theoretically, under the simplifying assumptions regarding the enzyme kinetics, described previously, the deviation index is equivalent to the FCC estimated from the parameters of the fitted quadratic hyperbola. However, as pointed out in Supporting Information Table S1, it may happen that no robust fits to a quadratic hyperbola can be obtained (indicated by the lack of convergence of at least one of the employed fitting procedures). In this case, the parametric estimates of the FCC do not fall in the range of values assumed by the deviation index. While the values for the FCCs presented in Table 3 rely on the mutant which is closest to the WT with respect to enzyme activity, one can alternatively calculate the FCC by using the available data for the remaining mutants

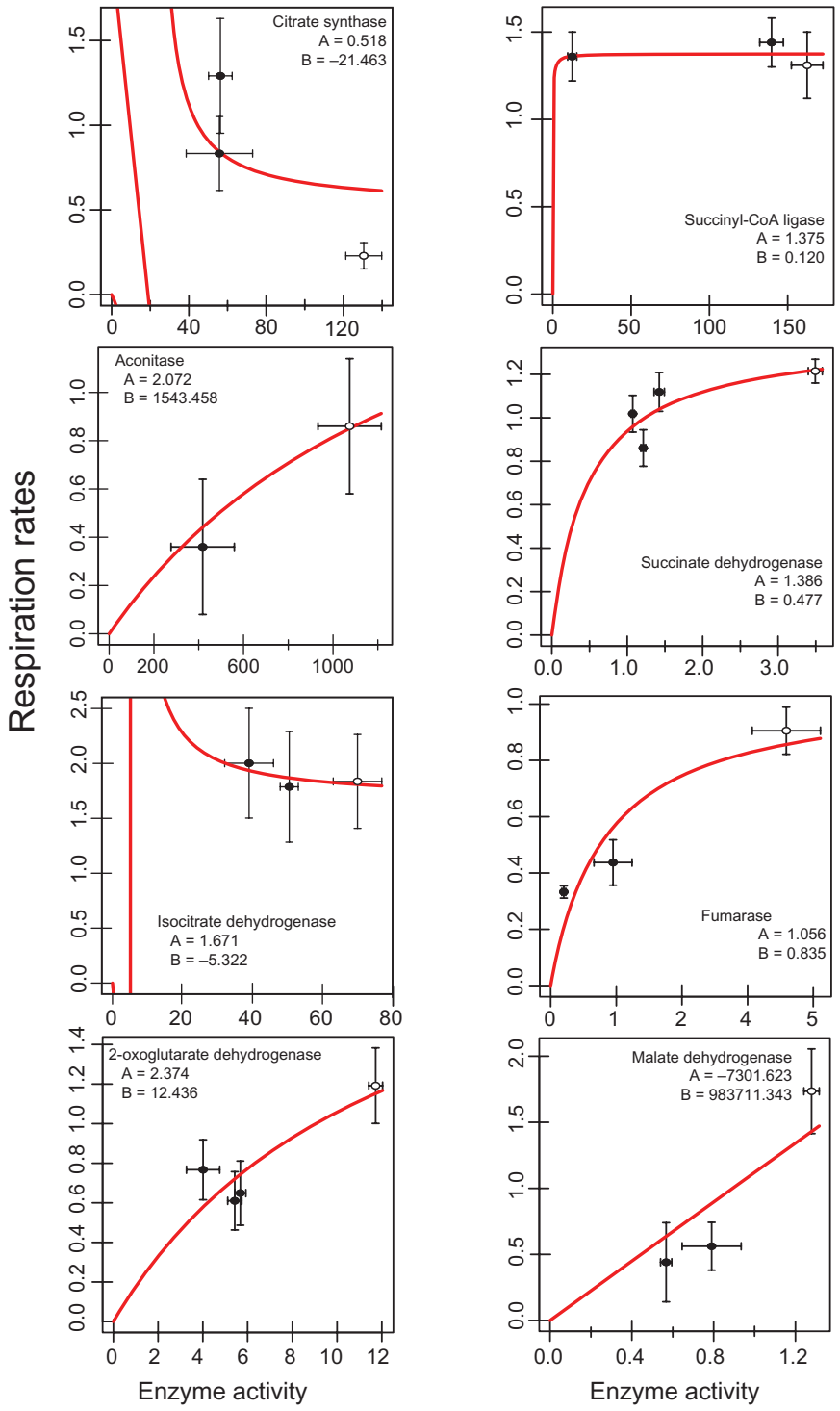

Figure 2. Flux control coefficients (FCCs) of respiration. The FCCs were estimated as described in Table 2. Closed circles represent values obtained for transgenic plants while open circles are from the respective wild-type plants. The relation between enzyme activity and respiration for the following tricarboxylic acid cycle enzymes (data source is given in parenthesis): citrate synthase (Sienkiewicz-Porzucek *et al*. 2008); aconitase (Carrari *et al*. 2003a); isocitrate dehydrogenase (Sienkiewicz-Porzucek *et al*. 2010); 2-oxoglutarate dehydrogenase (Araújo *et al*. 2008); succynil-CoA ligase (Studart-Guimarães *et al*. 2007); succinate dehydrogenase (Araújo *et al*. 2011); fumarase (Nunes-Nesi *et al*. 2007a); malate dehydrogenase (Nunes-Nesi *et al*. 2005).

(i.e. different steady states). The estimates of the FCCs at different steady states are also given in the Supporting Information Table S1.

Large reductions in the activity of succinyl-CoA ligase have no impact on the FCC results with values of 0.0008 (Table 3). Additionally, reductions of nearly 50% in the activity of the mitochondrial NAD-dependent isocitrate dehydrogenase without significant alterations in respiration rates generated an FCC of –0.123. In sharp contrast, citrate synthase had clearly increased respiration rates and therefore a more negative value (–0.4) for FCC was observed. On the other hand, the enzymes aconitase, 2-oxoglutarate dehydrogenase, succinate dehydrogenase, fumarase and malate dehydrogenase show a pronounced effect, with FCC values of 0.964, 0.786, 0.286, 0.601 and 1.76, respectively. The said comparison was conducted based on the FCC estimated from the parameters of the fitted quadratic hyperbola. Note that similar conclusions can be elicited when the deviation index is employed to rank the enzymes according to the control they exert on the flux distribution. However, some of the results based on the deviation index warrant caution, especially when the fitting procedure failed to converge. Typical example is the behaviour of citrate synthase, for which the FCC value presented in the first column of Table 2 does not fall in the corresponding range of values for the deviation index. By inspecting the plot of citrate synthase in Fig. 1, one can clearly observe that the relationship between the flux and enzyme activity can hardly be modelled by a quadratic hyperbola, resulting in unreliable estimates of the FCC with any of the analytical approaches employed in our empirical analysis.

The control coefficients sum to more than 1 which may be expected, for instance, in a branched pathway [similar results were also observed for the MCA of the conversion of sucrose to starch (Geigenberger *et al*. 2004)]. It is therefore clear that the difficulty in determining the FCCs from experimental data resides in several factors. Firstly, the data come from plants with a relatively large reduction in expression and enzyme activity, which could lead to altered distributions of control in the transgenic plants compared with WT. Secondly, the data are derived from plants growing during most of their life with reduced activity of an enzyme involved in the TCA cycle, a key metabolic hub, a disruption which could promote pleiotropic effects that remain undetected. Thirdly, it should be taken into account that the TCA cycle is involved in a complex network (Fig. 1) containing different branch points and substrates (Rocha *et al*. 2010; Sweetlove *et al*. 2010), and thus FCCs may have negative as well as positive values, making the interpretation of the estimated coefficients less straightforward. Finally, it should be noted that using dark respiration as a measure of mitochondrial respiration is the most tractable approach currently available to us. However, a caveat is that while cellular oxygen consumption is massively dominated by mitochondrial respiration, other more minor processes such as the α and β oxidation of fatty acids occurring in the peroxisomes (Footitt *et al*. 2007) and reactions such as that catalyzed by catalase (Yang & Poovaiah 2002) also consume or generate oxygen which produces considerably amounts of oxygen. An additional potential problem is that some of the transgenic manipulations result in alterations in stomatal aperture (explaining their affect on photosynthesis). If respiration is similarly limited by gas exchange, then this would effectively substantially change the metabolic system (altered substrate supply) and would invalidate flux control analysis. However, it appears that this is not a problem in this case because the changes in respiration do not correlate with stomatal aperture. Both fumarase and succinate dehydrogenase have altered stomatal aperture (Nunes-Nesi *et al*. 2007a; Araújo *et al*. 2011) but are associated with decreased respiratory flux.

Despite these important caveats, a closer examination of the FCCs that were obtained from the transgenic plants remains a worthwhile exercise. As one might expect for a central pathway of primary metabolism, control is shared. Among the enzymes considered here, malate dehydrogenase, aconitase, 2-oxoglutarate dehydrogenase and fumarase have most of the control (45, 25, 20 and 15% of the summed FCCs, respectively). Surprisingly, some enzymes have near zero FCCs (isocitrate dehydrogenase and succinyl CoA ligase) and succinate dehydrogenase accounts for only 7% of the summed FCCs.

These FCCs are measured with respect to CO_2 evolution from leaves after 30 min of darkness. In interpreting the observed distribution of control, it is important to understand the metabolic state in this slightly unusual scenario. It is generally assumed that respiration in this condition represents normal dark respiration (Padmasree, Padmavathi & Raghavendra 2002). However, it is known that changes in conditions during the previous light period affect respiration in this early dark period (Atkin, Evans & Siebke 1998), and recently it has been shown that respiration and general metabolic state in this period resemble that in the light (Florez-Sarasa *et al*. unpublished results). The TCA cycle has completely different flux modes between light and dark respirations (Sweetlove *et al*. 2010), and is therefore likely to have a very different distribution of control. In the dark, carboxylic acid metabolism operates in the classical cyclic mode of the TCA cycle. However, in the light, pyruvate dehydrogenase is partially inhibited and there is a very low flux through succinyl CoA ligase and succinate dehydrogenase (Tcherkez *et al*. 2009). Effectively, the pathway is non-cyclic and consists of two branches, one operating from stored citrate to 2-oxoglutarate and one operating in the reverse direction from OAA (generated in the cytosol from phosphoenolpyruvate carboxylase) to fumarate.

The distribution of flux control presented here is consistent with the operation of the TCA cycle in the light; the very low FCCs of succinyl CoA ligase and succinate dehydrogenase to be expected if these enzymes are carrying a negligible flux. Moreover, low FCCs of enzymes in the branch between citrate and 2-oxoglutarate may be explained by the operation of cytosolic bypasses of the TCA cycle (Sweetlove *et al*. 2010), and in particular, the presence of cytosolic isoforms of aconitase and isocitrate dehydrogenase that are thought to be important in the provision of

2-oxoglutarate for nitrogen assimilation (Hodges 2002; Foyer *et al*. 2011). While the exact balance between the use of cytosolic versus mitochondrial routes for synthesis of 2-oxoglutarate remains unknown, the recent discovery that the carbon for nitrogen assimilation in leaves comes from carbon stored from the previous night (most likely organic acids such as citrate stored in the vacuole and released to the cytosol) (Gauthier *et al*. 2010) raises the possibility that 2-oxoglutarate for nitrogen assimilation is generated predominantly in the cytosol and the mitochondrial enzymes make a quantitatively minor contribution (Sweetlove *et al*. 2010). This would explain the negative near-zero FCCs for isocitrate dehydrogenase and the even more negative FCC for citrate synthase. The relatively high FCC for aconitase can be explained by the fact that mitochondrial and cytosolic isoforms of aconitase in tomato leaves are believed to be encoded by a single gene (Carrari *et al*. 2003a). Hence, both isoforms are reduced in the *Aco*-1 mutant and therefore used to estimate the FCC (Carrari *et al*. 2003a). Thus, one would expect a non-zero FCC estimate for aconitase, even if the majority of the flux is through the cytosolic isoform. In addition, the high FCC for aconitase must be viewed with caution because of peculiarities of *S. pennellii* as this specie displays dramatically elevated activities of aconitase in comparison with the cultivated tomato (Steinhauser *et al*. 2010). The very high FCC for malate dehydrogenase, on the other hand, may be related to a redox perturbation as its main role in the light is thought to be to facilitate the synthesis of malate which is then exported in exchange for OAA in a metabolite shuttle that leads to net export of reducing equivalents (Hanning & Heldt 1993).

Overall, the distribution of control of respiration within the TCA cycle estimated here is consistent with the very specific non-cyclic flux mode and cytosolic bypasses that operate in illuminated leaves. It will now be important to extend this analysis to leaves throughout the dark period or to heterotrophic tissues such as roots or fruits. One might expect in such tissues, where a conventional cyclic flux mode of the TCA cycle is known to operate, that control would be more evenly shared. It would also be interesting to know how the distribution of control would be altered under stress conditions. Again, one could make predictions. It would seem likely that enzymes such as aconitase, pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase, which are known to be oxidatively inhibited under stress conditions, would have high FCCs.

THE REGULATION OF THE TCA CYCLE ACTIVITY

While FCCs provide a quantitative breakdown of the control of pathway flux at the level of enzyme amount (and so is relevant to developmental or stress-induced changes), they do not necessarily indicate steps which are physiologically regulated (which often occurs at the post-translational level). One way to approach the physiological regulation is to identify reactions that are effectively irreversible *in vivo* because these reactions are often the site of regulation. For instance, during the conversion of sucrose to starch, the reactions catalyzed by plastidial phosphoglucomutase and AGPase, as well as the amyloplastidic ATP/ADP transporter share the majority of the control (Geigenberger *et al*. 2004). The best approach to address the potential for *in vivo* regulation is, however, the assessment of disequilibrium constants as previously performed for the sucrose to starch transition (Geigenberger *et al*. 2004). Following this approach, the mass action ratio (the ratio between the *in vivo* concentration of substrates and products) is divided by the equilibrium constant (K_{eq}, the ratio between the products and substrates when the reaction is at thermodynamic equilibrium and net flux is zero). This calculation reveals how far each reaction is removed from equilibrium. Ratios highly removed from unity thus represent irreversible reactions. A prerequisite to use this approach in a pathway which is spread across multiple compartments, such as is the case for the TCA cycle, is the ability to determine subcellular metabolite concentrations. In the specific case of the TCA cycle this is only partially available, i.e. the exchange of pools of organic acids between the mitochondria and cytosol, via the mitochondrial carrier family proteins, suggests that these metabolites are likely to be in equilibrium (Palmieri *et al*. 2011) and thus these values could be used as a proxy. Therefore, mitochondrial levels of NAD, ATP and malate are available for leaves (Stitt, Wirtz & Heldt 1980; Stitt *et al*. 1989; Benkeblia, Shinano & Osaki 2007) whilst NAD, ATP, citrate, malate and succinate are available for potato tubers (Farre *et al*. 2001; Tiessen *et al*. 2002; Farre, Fernie & Willmitzer 2008). Unfortunately, this information is insufficient to carry out a systematic analysis. Indeed, we actually do not currently have enough data to even assess a single enzyme of the TCA cycle. Despite the clear need for the experimental data which would facilitate such an assessment on the basis of data accrued, it can safely be assumed that the reactions catalyzed by the enzymes citrate synthase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase are essentially irreversible under normal conditions. This is not, however, always the case as it has been shown that in developing embryos of *Brassica napus*, the isocitrate dehydrogenase reaction was unambiguously identified as reversible after labelling with [U-$^{13}C_5$] glutamate (Schwender, Shachar-Hill & Ohlrogge 2006). Nevertheless, the *in vivo* reversibility of isocitrate dehydrogenase in *B. napus* may in part be attributed to the very high tissue concentration of CO_2 in developing seeds (Goffman *et al*. 2004), which in turn may shift the reaction equilibrium towards carboxylation. Although there is insufficient data to formally establish regulatory enzymes in the TCA cycle based on disequilibrium ratios, there is a wealth of information about allosteric and post-translational regulation of specific information from which the sites and mechanisms of regulation can be inferred. In the following paragraphs, we will discuss the known regulatory features of each enzyme in turn.

The reactions of the pyruvate dehydrogenase complex interconnect the metabolic pathways of glycolysis, gluconeogenesis and fatty acid synthesis to the TCA cycle

Table 4. Known characteristics of regulation of enzymes of the TCA cycle

Enzyme of the pathway		Effectors	Inhibitors	References
Citrate synthase	EC 2.3.3.1	ADP	ATP, NADH and succinyl-CoA	Siedow & Day 2000
Aconitase	EC 4.2.1.3	n.d	NADH, H_2O_2	Verniquet *et al*. 1991
Isocitrate dehydrogenase	EC 1.1.1.41	ADP, NAD^+ and Ca^{2+}	ATP and NADH	Behal, Neal & Oliver 1996; Falk *et al*. 1998; Igamberdiev & Gardestrom 2003
2-oxoglutarate dehydrogenase	EC 1.2.4.2	Thiamine pyrophosphate, AMP, ADP; NAD^+ and Ca^{2+}	NADH	Bunik & Fernie 2009
Succinyl-CoA ligase	EC 6.2.1.5	n.d	NADH	Studart-Guimarães *et al*. 2005
Succinate dehydrogenase	EC 1.3.5.1	ATP, ADP, NADH, FAD,QH2,	OAA; NADH	Oestreicher, Hogue & Singer 1973; Singer *et al*. 1973; Affourtit *et al*. 2001
Fumarase	EC 4.2.1.2	n.d	NADH, pyruvate and 2-oxoglutarate	Behal & Oliver 1997
Malate dehydrogenase	EC 1.1.1.37	n.d	NADH	Siedow & Day 2000

While glycolysis and glycogen metabolism are under complex systems of allosteric and hormonal control, the TCA cycle in contrast is regulated by three simple mechanisms: (1) substrate availability; (2) product inhibition; and (3) competitive feedback inhibition.
n.d., not determined; TCA, tricarboxylic acid.

(Plaxton & Podesta 2006). As a consequence, the activity of the pyruvate dehydrogenase complex is highly regulated by a range of allosteric effectors and by covalent modification (Plaxton & Podesta 2006). Mitochondrial pyruvate dehydrogenase and TCA cycle dehydrogenases, such as isocitrate dehydrogenase, demonstrate product inhibition by NADH *in vitro* (Table 4) (Igamberdiev & Gardestrom 2003; Tovar-Mendez, Miernyk & Randall 2003; McDonald & Vanlerberghe 2006). Because three reactions of the TCA cycle as well as the intimately associated pyruvate dehydrogenase complex utilize NAD^+ as a co-factor, it is not difficult to understand why the cellular ratio of NADH/NAD^+ or more correctly stated that the mitochondrial NADH/NAD^+ ratio has a major impact on the flux of carbon through the TCA cycle. Their *in vivo* activities are almost responsive to the NADH/NAD^+ ratio, which provide a sensitive mechanism by which to balance the rate of pyruvate oxidation by the pyruvate dehydrogenase and the TCA cycle with the rate of oxidative phosphorylation (Plaxton & Podesta 2006).

Citrate synthase is confined to the mitochondrial matrix, except in tissues converting fatty acids into sugars in which a glyoxysomal isoform is also operative (Pracharoenwattana, Cornah & Smith 2005). Interestingly, this glyoxysomal isoform was found to be up-regulated following the antisense repression of the mitochondrial isoform in tomato, and this isoform was furthermore able to functionally compensate for the mitochondrial one (Sienkiewicz-Porzucek *et al*. 2008). Structural and expression characteristics of mitochondrial isoforms from diverse species have been subject to detailed studies (Landschutze *et al*. 1995), and the identification of potential inter-domain disulfides in higher plant mitochondrial citrate synthase suggest paradoxical differences in redox-sensitivity relative to its animal counterpart (Stevens *et al*. 1997). This enzyme is also regulated by the cellular NADH/NAD^+ ratio, the ATP/ADP ratio and succinyl-CoA levels (Table 4).

Aconitase catalyze the reversible hydration of *cis*-aconitate to either citrate or isocitrate. In plants, aconitase has been characterized in several tissues (Pickworth Glusker 1971; Sadka *et al*. 2000; Carrari *et al*. 2003a) and it has been demonstrated that at least two aconitase isoenzymes are present in intact plant cells: one is readily released after stripping the cell membrane and is present in the cytosol (Table 1), the other being confined within mitochondria. (Brouquisse, Gaillard & Douce 1986; Brouquisse *et al*. 1987). That said, following the identification of the genes encoding aconitase, it has become apparent that while the number of genes encoding aconitase varies between plant species – with some harbouring two and others three genes – the gene products are often dual-targeted to both the mitochondria and the cytosol (Gangloff, Marguet & Lauquin 1990; Carrari *et al*. 2003a; Arnaud *et al*. 2007). The aconitase enzyme is highly sensitive to oxidative stress (Verniquet *et al*. 1991; Lehmann *et al*. 2009) and thus far there is no evidence of any allosteric effectors (Table 4).

The metabolic branch point of 2-oxoglutarate, which is either irreversibly degraded by the 2-oxoglutarate dehydrogenase complex or provides carbon skeletons for nitrogen assimilation, is clearly of general importance in nitrogen-autotrophic organisms such as plants. This is further supported by the rich regulation of the 2-oxoglutarate dehydrogenase complex including allosteric responses to second messengers and metabolic indicators, such as Ca^{2+}, ATP/ADP, NADH/NAD^+ and thiamine pyrophosphate (Table 4). This multi-enzyme complex is very similar to the pyruvate dehydrogenase complex in the context of its

protein organization, cofactors, and its mechanism of action (Bunik & Fernie 2009). Similarly to the situation observed for the pyruvate dehydrogenase complex, the reactions of the 2-oxoglutarate dehydrogenase complex proceed with a large negative standard free energy change. Although the 2-oxoglutarate dehydrogenase complex is not subject to covalent modification, its metabolic regulation is quite complex, with activity being regulated by energy charge, the NAD^+/NADH ratio, and effector activity of substrates and products (Bunik & Fernie 2009). In addition to this, 2-oxoglutarate has itself been postulated to play a role as a signal metabolite in plants (Lancien, Gadal & Hodges 2000; Ferrario-Méry *et al*. 2001; Feria Bourrellier *et al*. 2009). This role is, however, largely based on analogy to the important role it plays in conjuncture with the plastidial PII protein in plants (Uhrig, Ng & Moorhead 2009). Reports to date suggest that while PII may regulate a small number of enzyme systems in plants including *N*-acetyl-glutamate kinase (Ferrario-Mery *et al*. 2006; Feria Bourrellier *et al*. 2009) and plastidial acetyl-CoA carboxylase (Feria Bourrellier *et al*. 2010) its role is unlikely to be as pivotal in plants as in non-plant systems. That said, it is clear that the production of 2-oxoglutarate in the mitochondria or cytosol is an important determinant of some plastidial activities.

The reverse reaction of the enzyme succinyl-CoA ligase has been demonstrated as has the fact that succinyl-CoA ligase is feedback inhibited by intermediates of the pathway of porphyrin biosynthesis which it supplies (Wider & Tigier 1971), as well as being competitively inhibited by malonate (Palmer & Wedding 1966). Characterization of the regulatory properties of this enzyme suggests that allosteric control may regulate the flux through the TCA cycle in a manner that would allow a high cyclic flux in times when carbon is in rich supply but a reduced flux in times of carbon deficiency (Studart-Guimarães *et al*. 2007).

The regulation of succinate dehydrogenase has been investigated using coupled mitochondria by simultaneously measuring the oxygen uptake rate and the ubiquinone reduction level (Affourtit *et al*. 2001). This study has revealed that the activation state level of the enzyme is unambiguously reflected in the kinetic dependence of the succinate oxidation rate upon the ubiquinone redox poise. Kinetic results indicated that succinate dehydrogenase is activated by both ATP and ADP (Affourtit *et al*. 2001). The results of this work further demonstrated that the activity of succinate dehydrogenase *in vitro* is modulated by the proton motive force, leading the authors to speculate that the widely recognized activation of the enzyme by adenine nucleotides in plants is mediated in this manner (Affourtit *et al*. 2001).

The allosteric properties of purified pea fumarase, which revealed inhibition by physiological concentrations of pyruvate, 2-oxoglutarate and the adenine nucleotides ATP, ADP and AMP, are consistent with the step being an important control point of the TCA cycle (Behal & Oliver 1997). Furthermore, the importance of the reaction catalyzed by fumarase has been demonstrated in correlation studies showing that this enzyme activity is a reliable diagnostic for the degree of seed dormancy in several tree species (Shen & Oden 2002). Although, Pracharoenwattana and co-workers have recently described that the *Arabidopsis* genome additionally encodes a cytosolic fumarase (Pracharoenwattana *et al*. 2010), as yet enzymatic characterization of this isoform has not yet been reported.

The action of malate dehydrogenase which catalyzes the reversible oxidation of malate to produce OAA (Nunes-Nesi *et al*. 2005) completes the TCA cycle. While the equilibrium position favours malate and NAD^+ production, the *in vivo* removal of OAA by citrate synthase, coupled with the removal of NADH by the respiratory chain, causes the reaction to function in the direction of malate oxidation in most tissues (Nunes-Nesi & Fernie 2007). Therefore, it is clear that NADH would lead to an inhibition of the mitochondrial malate dehydrogenase.

Nevertheless, similarly to the situation observed in studies in which glycolytic enzymes were affected, reduction in the activity of enzymes of the TCA cycle, such as aconitase, isocitrate dehydrogenase or succinyl CoA ligase, displays relatively small alterations in the rate of respiration within photosynthetic tissues (Carrari *et al*. 2003a; Studart-Guimarães *et al*. 2007; Sienkiewicz-Porzucek *et al*. 2008). However, the chemical inhibition of the 2-oxoglutarate dehydrogenase complex in potato tubers had a clear impact on respiration (Araújo *et al*. 2008), and more pronounced effects were also observed following the inhibition of these enzymes in tomato roots (van der Merwe *et al*. 2010). This comparison is, however, not made on an equal basis, and given that the difference between photosynthetic and heterotrophic metabolism is so great, it is important to note that a fairer comparison will likely be possible in the future once transgenic plants deficient in the expression of 2-oxoglutarate dehydrogenase are available.

IN VIVO REGULATION OF THE TCA CYCLE ACTIVITY

The regulation of TCA cycle activity in living tissues is very complex as different steps of the pathway have different roles beyond the maintenance of the cycle activity, and it seems that the balance of these roles strongly depends on the physiological context within which the cycle is operating (Sweetlove *et al*. 2010). One of the physiological contexts for which the pathway has been extensively studied is illuminated leaves. Nevertheless, despite years of efforts, the function of TCA cycle in the illuminated leaves is still not fully understood and its operation in the light remains contentious (Nunes-Nesi *et al*. 2007b). While some studies indicate an almost complete inhibition of the TCA cycle in illuminated leaves (Tcherkez *et al*. 2005, 2008), others suggest significant operation of the TCA cycle in the light (Nunes-Nesi *et al*. 2007b). However, recently the same group has estimated an inhibition of nearly 30% for the decarboxylation reaction catalyzed by the pyruvate dehydrogenase in the light compared with darkness (Tcherkez *et al*. 2009). Indeed, the operation of the pathway in the light is thought to be affected by reversible inactivation of the

mitochondrial pyruvate dehydrogenase complex (Tovar-Mendez *et al*. 2003) as well as by the rapid export of TCA cycle intermediates out of the mitochondria (Hanning & Heldt 1993), particularly for utilization in nitrogen assimilation (Hodges 2002). In addition, many dehydrogenases of the pathway are expected to be inhibited because of high mitochondrial NADH level as a result of photorespiratory glycine decarboxylation (Atkin *et al*. 2000b). Furthermore, mitochondrial isocitrate dehydrogenase is supposed to be inhibited in the light by the high mitochondrial NADPH/NADP ratios (Igamberdiev & Gardestrom 2003). Thus, the rates of respiration in the light are lower than in the dark, despite that daytime respiration plays an important role in carbon and nitrogen metabolism and arguably provides the ATP necessary for sucrose synthesis (Raghavendra & Padmasree 2003; Carrari *et al*. 2003a) as well as at least some of the 2-oxoglutarate that is required for nitrogen assimilation (Nunes-Nesi, Fernie & Stitt 2010). Nitrogen assimilation, through the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway, generates the primary amino donors, glutamate and glutamine, needed for the biosynthesis of nitrogenous compounds in plants. Therefore, nitrogen assimilation must closely interact with respiration, as GS requires ATP and GOGAT requires carbon skeletons and reductant in the form of 2-oxoglutarate and reduced ferredoxin or NADH, respectively (Stitt *et al*. 2002). Moreover, studies that have analysed how genetic manipulations of the TCA cycle and mitochondrial electron transport components can affect N metabolism and related processes have been recently revisited (Foyer *et al*. 2011). Nevertheless, a growing body of evidence indicates that alternative routes can provide carbon skeletons for glutamate production (Nunes-Nesi *et al*. 2010). Experiments in which the aconitase expression levels were altered revealed changes in the level of isocitrate, but not in glutamate and glutamine (Nunes-Nesi *et al*. 2007b), suggesting that aconitase was not limiting for 2-oxoglutarate production. Further evidence emanating from experiments in which illuminated leaves of *B. napus* were incubated with $^{13}CO_2$ and ^{15}N-ammonium nitrate, and nuclear magnetic resonance (NMR) was used to determine the metabolic fate of the ^{13}C and ^{15}N label, suggested that while there is considerable *de novo* incorporation of nitrogen into glutamate and glutamine, the majority of the carbon in these metabolites is not derived from current CO_2 assimilation (Gauthier *et al*. 2010). Taken together, these results indicate that the remobilization of metabolites accumulated during the previous light or dark period are likely to provide much of the 2-oxoglutarate that is used to support glutamate synthesis in the light.

Recently, the impact of ambient CO_2 and O_2 concentrations on the activity of the TCA cycle was investigated. Using isotope-tracing techniques, Tcherkez and co-workers verified that glycolysis and the TCA cycle activities are inversely related to the ambient CO_2/O_2 ratio (Tcherkez *et al*. 2008). In this study, respiratory metabolism during the light period was up-regulated under low CO_2 conditions wherein photorespiration was favoured. Further analysis, whereby dihydroxyacetone phosphate and Glc-6-P were quantified, suggested that the products of photosynthesis exert control on day respiration. In other words, these results indicate that high dihydroxyacetone phosphate-to-Glc phosphate ratios can lead to reduction of TCA cycle activity and that under low CO_2 environment, the commitment of TCA cycle activity and glutamate production is increased (Tcherkez *et al*. 2008).

Another interesting aspect of the regulation of TCA cycle was recently demonstrated in transgenic tomato plants, with reduced activity of the β-subunit of succinyl-coenzyme A ligase (Studart-Guimarães *et al*. 2007). This study demonstrated that despite the strong reduction in the activity of the target enzyme, only a mild alteration in the respiration rate was observed (Fig. 2). Further analysis of the transgenic plants revealed that, whereas considerable activity of this enzyme appears to be dispensable, the reason for such a mild phenotype in highly inhibited lines was a compensatory up-regulation of an alternative pathway for succinate production, which was offered by the GABA shunt. Similar effects were also observed in heterotrophic tissues, where the role of the 2-oxoglutarate dehydrogenase complex in the TCA cycle was studied by specific chemical inhibition of the enzyme (Araújo *et al*. 2008). The GABA shunt bypasses two steps of the TCA cycle: the conversion of 2-oxoglutarate to succinyl-CoA and the subsequent formation of succinate. While the role of GABA in plants is not yet fully understood, it is clearly an important pathway under stress conditions (Bouche & Fromm 2004; Fait *et al*. 2008), being associated with numerous physiological responses, including the regulation of cytosolic pH, carbon fluxes into the TCA cycle, nitrogen metabolism, osmoregulation, plant pathogen interaction and signalling (Bouche & Fromm 2004). Moreover, bioinformatic analyses have revealed that the TCA cycle and GABA shunt are differentially regulated at the level of gene expression (Fait *et al*. 2008). It thus seems reasonable to assume that the increased GABA levels are occurring in response to changing environmental conditions (Fait *et al*. 2008) and represent another adaptive mechanism in the attempt to maintain the rate of respiration under certain detrimental situations.

ENVIRONMENTAL EFFECTS ON RESPIRATORY PROCESS

A large number of measurements have been made concerning gas exchange (i.e. rates of photosynthesis, respiration and transpirations) of different plants growing under contrasting conditions (Lambers, Chapin & Pons 2008). These measurements have yielded a mass of experimental results, some of which have been previously discussed. The enormous variety of alternative respiratory substrates and metabolic pathways makes plant respiration remarkably flexible especially in response to changing environmental circumstance. For instance, it has been shown that oxygen isotope discrimination during plant respiration seems to be independent of temperature over the range of temperature normally encountered during growth (Macfarlane *et al*. 2009). These authors also observed that there is a relatively large

temperature dependence of the respiration rate, suggesting that there was little substrate limitation to respiratory rate in the leaves of healthy plants (Macfarlane *et al.* 2009). Thus, it seems reasonable to assume that enzyme capacity is the main limitation of respiratory rate, and the reduction state of the ubiquinone pool varies little or none with measurement of temperature.

It has been suggested that higher temperatures reduce net carbon gain by increasing plant respiration more than photosynthesis. In fact, the light-saturated photosynthesis rate of C_3 crops such as wheat and rice is at a maximum for temperatures from about 20–32 °C, whereas total crop respiration shows a steep non-linear increase for temperatures from 15 to 40 °C, followed by a rapid and nearly linear decline (Porter & Semenov 2005). Although the stimulation of C_3 photosynthesis by growth at elevated atmospheric [CO_2] can be somewhat predicted with confidence, the nature of changes in respiration remains uncertain (Leakey *et al.* 2009b). The primary reason for uncertainty is that the mechanisms of plant respiratory responses to elevated [CO_2] are not fully understood (Gifford 2003; Leakey *et al.* 2009b). In fact, the results observed in the literature are somehow contradictories and have shown that plant respiration may increase as much as 37%, decrease as much as 18%, or even not change at all with increased [CO_2] (e.g. (Drake *et al.* 1999; Gifford 2003; Leakey *et al.* 2009a). In a recent free air carbon dioxide enrichment (FACE) study where soybean was grown at elevated [CO_2] (550 ppm), the stimulated (37%) rate of night-time respiration was associated with the additional carbohydrate available from enhanced photosynthesis at elevated CO_2 (Leakey *et al.* 2009a). Although at the leaf and plant scales, stimulated respiration at elevated [CO_2] may reduce net carbon balance, it is possible, nevertheless, that such stimulation could facilitate increased yield by providing greater energy for export of photoassimilate from source organs to sink tissues. However, the precise role of plant respiration in augmenting the sink capacity remains fragmented (Gonzalez-Meler, Taneva & Trueman 2004).

Considerable research effort has additionally been directed towards the adaptive responses of respiratory metabolism to low oxygen concentrations. An important environmental stress condition that rapidly leads to the depletion of molecular oxygen within plant organs is flooding or water logging of the soil (Bailey-Serres & Voesenek 2008). The most immediate effect on soil flooding is a decline in the oxygen concentration and a consequent decrease in aerobic root respiration leading to a restriction in ATP production. Furthermore, low availability of oxygen to plant cells can also occur under optimal growth conditions, because of the relatively high resistance to diffusion of oxygen through plant tissues (van Dongen *et al.* 2011). Steep oxygen gradients have been observed in various plant tissues such as roots, stems, seeds or tubers (Armstrong *et al.* 1994; Geigenberger *et al.* 2000; van Dongen *et al.* 2003; Borisjuk & Rolletschek 2009; Zabalza *et al.* 2009). Moreover, during development, local oxygen concentrations can vary, depending on the metabolic activity of the tissue (van Dongen *et al.* 2003; Benamar *et al.* 2008). Therefore, the metabolic responses to low oxygen are directly involved in optimizing the plant's energy status while consuming as little oxygen as possible (van Dongen *et al.* 2011).

It is well known that both metabolic and anatomical adjustments are important strategies in order to allow plants to cope with spatial and temporal variations of the oxygen availability. The major structural change is an increased formation of aerenchyma to lower the resistance to oxygen diffusion into the respiring tissue (Drew, He & Morgan 2000; Jiang *et al.* 2010). From a metabolic perspective, the hypoxic responses includes the down-regulation of a suite of energy-, and therefore, oxygen-consuming, metabolic pathways (Geigenberger 2003). Examples of such metabolic adaptations to hypoxia include the down-regulation of storage metabolism (Geigenberger *et al.* 2000), the switch from invertase to sucrose synthase routes during sucrose hydrolysis (Bologa *et al.* 2003; Huang, Colmer & Millar 2008) and the inhibition of mitochondrial respiration (Gupta, Zabalza & van Dongen 2009; Zabalza *et al.* 2009). It seems reasonable to assume that these responses are already initiated before oxygen becomes limiting as a substrate for respiration. Therefore, it has been suggested that these metabolic changes are important components of the survival strategy as they considerably extend the period of hypoxia that a plant can withstand (van Dongen *et al.* 2011).

Limited water availability, on the other hand, impairs plant growth and is one of the main issues of future climate changes (Ciais *et al.* 2005; Loreto & Centritto 2008). Several studies on the effect of severe drought stress on respiratory pathways have revealed contrasting results, as respiration remained unaltered in soybean (Ribas-Carbo *et al.* 2005), increased in wheat (Bartoli *et al.* 2005), and decreased in bean and pepper (Gonzalez-Meler, Matamala & Penuelas 1997). Nevertheless, the effects of mild to moderate water stress were relatively small on the mitochondrial activity of several key TCA cycle enzymes in two CAM species (Herppich & Peckmann 2000). However, changes in the *in vivo* activities of the cytochrome oxidase (COX) and alternative oxidase (AOX) pathways, measured with the oxygen isotope fractionation technique that has been demonstrated to be the most reliable technique for the studies of electron partitioning between the two main respiratory pathways (Ribas-Carbo *et al.* 1995; Day *et al.* 1996), have been reported by Ribas-Carbo and colleagues (Ribas-Carbo *et al.* 2005; Flexas *et al.* 2006). In their study on soybean (Ribas-Carbo *et al.* 2005), a decrease in COX activity was detected in leaves during severe drought stress, while AOX activity increased. Accordingly, despite complex I dysfunction and hence altered redox balance, the CMSII mutant seems to be able to adjust its photosynthetic machinery during and after drought stress to reduce photo-oxidation and to maintain the cell redox state and the ATP level (Galle *et al.* 2010). Notwithstanding, identifying whether, and to what extent, plant species-specific factors and/or experimental conditions affect *in vivo* respiratory pathways, particularly the TCA cycle, under drought stress, awaits further studies.

It is clear, however, that although there have been a range of studies analysing changes in respiratory rates in response

to light, temperature and CO_2 (Day *et al*. 1985; Atkin *et al*. 1997; Scheurwater *et al*. 2000; Kruse, Rennenberg & Adams 2011), our knowledge of the environmental impact on plant respiration and the TCA cycle remains fragmented. Although the global response of respiration is well characterized, the specific response of the TCA cycle enzymes and intermediates has only been described in a limited number of conditions. Nevertheless, the molecular, enzymatic and metabolic responses were observed in the case of biotic stress such as to moderately low nitrogen (Tschoep *et al*. 2009), low carbon (Gibon *et al*. 2006, 2009; Osuna *et al*. 2007; Usadel *et al*. 2008b), low potassium (Armengaud *et al*. 2009), small decreases in temperature (Usadel *et al*. 2008a) and water deficit (Hummel *et al*. 2010), and this was interpreted as an adaptive response to maintain carbon flux through the TCA cycle. Moreover, a robust link between circadian-clock function and metabolic homeostasis in the TCA cycle was recently suggested (Fukushima *et al*. 2009). Further studies are clearly needed to explore the interactions of mitochondrial non-phosphorylating pathways with photosynthetic processes and cell homeostasis under stressful conditions.

In summary, it is evident that there are several modes of regulation of the TCA cycle activity. For instance, proteomic studies have indicated that the TCA cycle enzymes (aconitase, succinyl-CoA ligase isocitrate, malate, pyruvate and succinate dehydrogenases) are potential targets for redox regulation (Balmer *et al*. 2004). These results, associated with the allosteric properties of succinyl CoA ligase (Studart-Guimarães *et al*. 2005) and with the ability to assess free, as opposed to bound, NADH levels (Kasimova *et al*. 2006) when coupled with observations that glycolitic enzymes are functionally associated to the outer mitochondrial membrane (Giege *et al*. 2003), suggest that many aspects of the regulation of TCA cycle remain to be elucidated. Additionally, there is a wealth of evidence suggesting that the TCA cycle is inhibited in the light as well as being transcriptionally down-regulated; however, it is also equally clear that respiration remains active at considerable levels in illuminated leaves (Atkin *et al*. 2000a; Nunes-Nesi *et al*. 2008). Therefore, it seems likely that the physiological purpose for regulation is not the control of respiration *per se* but of other metabolic processes mediated by respiratory metabolism.

ACKNOWLEDGMENTS

Fundings from the Max Planck Society (to WLA and ARF), the Deutsche Forschungsgemeinschaft (grant no. DFG–SFB429 to ARF) and the Federal Ministry of Education and Research (GoFORSYS project no. 0313924 to ZN) are gratefully acknowledged.

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Received 11 February 2011; received in revised form 18 March 2011; accepted for publication 21 March 2011

SUPPORTING INFORMATION

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

Table S1. Data used for FCC calculations and summary of FCC.

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