Invited Expert Review

On the role of the tricarboxylic acid cycle in plant productivity

Running head: The role of the TCA in the plant productivity

Youjun Zhang¹,² and Alisdair R. Fernie¹,²*

¹ Max-Planck-Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Potsdam-Golm, Germany
² Center of Plant System Biology and Biotechnology, 4000 Plovdiv, Bulgaria

*Correspondence: fernie@mpimp-golm.mpg.de

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Abstract

The tricarboxylic acid (TCA) cycle is one of the canonical energy pathways of living systems, as well as being an example of a pathway in which dynamic enzyme assemblies, or metabolons, are well characterized. The role of the enzymes have been the subject of saturated transgenesis approaches, whereby the expression of the constituent enzymes were reduced or knocked out in order to ascertain their \textit{in vivo} function. Some of the resultant plants exhibited improved photosynthesis and plant growth, under controlled greenhouse conditions. In addition, overexpression of the endogenous genes, or heterologous forms of a number of the enzymes, has been carried out in tomato fruit and the roots of a range of species, and in some instances improvement in fruit yield and postharvest properties and plant performance, under nutrient limitation have been reported, respectively. Given a number of variants, in nature, we discuss possible synthetic approaches involving introducing these variants, or at least a subset of them, into plants. We additionally discuss the likely consequences of introducing synthetic metabolons, wherein certain pairs of reactions are artificially permanently assembled, into plants, and speculate as to future strategies to further improve plant productivity by manipulation of the core metabolic pathway.
INTRODUCTION

Considerable evidence has accumulated that mitochondrial respiratory function is associated with proper maintenance of cellular metabolism, as a whole (Araujo et al. 2012a). That this holds in non-photosynthetic organisms is perhaps unsurprising given that they depend on the mitochondria for the vast majority of their energy requirements. Reverse genetic approaches have, however, additionally established the physiological and metabolic basis for mitochondrial function in photosynthesizing plant cells (Nunes-Nesi et al. 2013). Indeed, such studies demonstrated that mitochondrial metabolism is of fundamental importance in processes such as photosynthesis, photorespiration, nitrogen metabolism, redox regulation and signalling (Heazlewood et al. 2004; Foyer et al. 2011; Bauwe et al. 2012) (See also Figure 1). That being said, quite remarkably, since it was demonstrated almost 60 years ago that exactly the same reactions occur in plant cells that were first described by Hans Krebs in pigeon muscle (Beevers 1961), relatively little is known concerning the regulation and control of the plant pathway (Szal and Podgorska 2012; Tcherkez et al. 2012).

The plant tricarboxylic acid (TCA) cycle is composed of a set of eight enzymes primarily linking the oxidation of pyruvate and malate (generated in the cytosol) to CO$_2$ with the generation of NADH for the oxidation by the mitochondrial respiratory chain (Fernie et al. 2004). The genomic organization and the subcellular localization of the enzymes involved in the Arabidopsis TCA cycle have been expertly reviewed elsewhere (Millar et al. 2011). However, here, we intend to focus solely on the mitochondrial reactions, as this is the only plant organelle in which a full cycle can, at least theoretically, operate (Sweetlove et al. 2010) and, moreover, it is the most comprehensively characterized.

The presence of organic acids, particularly TCA cycle intermediates, in all plants is known to support numerous and diverse functions within and beyond cellular metabolism. However, the level of accumulation of the various organic acids is extremely variable between species, developmental stages and tissue types (Fernie and Martinoia 2009), suggesting that the enzymes involved in the interconversion of these metabolic intermediates are subject to tight regulatory control. The sequestration of organic acids into the vacuole and their secretion into the rhizosphere additionally represent important mechanisms of regulating the level of abundance of these intermediates (Meyer et al. 2010).
Insight into the direct regulation of the TCA cycle was provided by a recent metabolic control analysis which showed that much of the control through this pathway is resident in fumarase, malate dehydrogenase (MDH) and 2-oxoglutarate dehydrogenase (Araujo et al. 2014), suggesting that these would be likely targets for flux regulation. However, a dearth of subcellular information concerning the levels of intermediates of the cycle (Sweetlove and Fernie 2013), effects us from being able to assess the potential of the constituent enzymes to play regulatory roles. That being said, there is a growing body of information concerning allosteric and post-translational regulation of specific enzymes from which mechanisms of regulation can be inferred. Historically, these can be divided into traditional studies, investigating the role of enzyme effectors, and broad-based studies in which changes in transcripts, protein abundance, post-translational modification of proteins or metabolite levels were assessed.

Based on these combined studies, a wide range of regulatory mechanisms has been proposed for the TCA cycle, including phosphorylation, redox regulation, light-dependent regulation, phytochrome and nitric oxide regulation, sensitivity to oxidative stress and acetylation (Nunes-Nesi et al. 2013). Three of these regulatory mechanisms are particularly prominent. First, the phosphorylation of the pyruvate dehydrogenase complex has long been described to be one of the mechanisms by which flux through the TCA cycle is downregulated; considerable in vitro evidence is available to support of this notion (Rapp et al. 1984). Secondly, more recently, both in vitro and reverse genetic studies have revealed an important role for the mitochondrial (and cytosolic) thioredoxin system in the regulation of the TCA cycle, through deactivating both succinate dehydrogenase and fumarase, as well as upregulating the cytosolic enzyme ATP-citrate lyase (Schmidtmann et al. 2014; Daloso et al. 2015; Geigenberger et al. 2017). Thirdly, a range of contemporary molecular and cell biology approaches have revealed that the plant TCA cycle (Zhang et al. 2017; Zhang et al. 2018), like that of microbial (Meyer et al. 2011) and mammalian (Robinson and Srere 1985) systems, form dynamic enzyme assemblies or metabolons. Indeed, the metabolon concept was established on studying the TCA cycle in rat liver, with the impetus to look for such an enzyme assembly being the fact that the levels of organic acids, within this tissue, were not consistent with the properties of the enzymes when evaluated in isolation (Srere 1985).
A wide range of theories to the function of such assemblies have been postulated, including increasing local concentrations of intermediates, decreasing the concentrations of enzymes required to maintain a given flux, and sequestration of toxic and highly reactive intermediates (Jorgensen et al. 2005). However, a less often explored possibility is that they are an important mechanism for channelling flux at important branch-points of metabolism (Sweetlove and Fernie 2018). In this vein, studies in plants revealed the presence of substrate-channeling, occurring following the dynamic assembly of an aconitase-citrate-synthase-malate dehydrogenase metabolon, amongst a total of 132 interactions between subunits of enzymes of the TCA cycle (Zhang et al. 2017) and 125 extra pathway interactions (Zhang et al. 2018) (Figure 2). We will return to discussing this phenomenon in the synthetic biology section below. However, before we do so, we will detail approaches to improve plant productivity that are based on transgenic manipulation of the native plant pathways, beginning with those studies related to enhancement of photosynthesis, but also including the improvement of root function and the manipulation of fruit and seed traits.

THE ROLE OF THE TCA CYCLE IN PHOTOSYNTHETIC TISSUES

Photosynthesis and respiration have long been known to be highly entwined, given that they share carbon and oxygen, as substrate and product, or product and substrate, respectively (Nunes-Nesi et al. 2011). Despite this and the fact that the core reaction schemes of photosynthesis, respiration and photorespiration are well defined, it is only via wide reverse genetic experimentation that the extreme level of interaction between these pathways has begun to be realized (Bauwe et al. 2010; Sweetlove et al. 2010; Obata et al. 2016; Timm et al. 2016). However, the exact contribution of each pathway to the cellular energy status is dependent upon both the cell type (Nunes-Nesi et al. 2010) and prevailing environmental conditions (Florian et al. 2014). Surprisingly, despite intensive research efforts, even fundamental questions, such as the degree of inhibition of the TCA cycle in the light, remain somewhat controversial (Tcherkez et al. 2009; Nunes-Nesi et al. 2011). In this section, we will review our current understanding of the influence of the TCA cycle on photosynthetic metabolism, focusing almost exclusively on the illuminated leaf of C3 plants, given that the majority of work to date has been carried out using transgenics and mutants of tomato and Arabidopsis.
It has long been assumed that the TCA cycle in the illuminated leaf is almost completely inhibited at the reaction catalyzed by the pyruvate dehydrogenase complex (Tovar-Mendez et al. 2003). However, there is at first sight some conflict between both *in vitro* measurements (Rapp et al. 1984) and flux profiles (Tcherkez et al. 2005) with the results described below from the transgenic plants. These findings can be reconciled if it is borne in mind that the TCA cycle actually rarely operates in a cyclic flux mode (Sweetlove et al. 2010), and especially not during the light phase (Gauthier et al. 2010).

One of the earliest proposed roles for mitochondria, during photosynthesis, was that they supply a large proportion of the ATP required to sustain high rates of sucrose synthesis (Kromer et al. 1993). Only partial support for this notion has been provided, with conflicting results being reported for the cytoplasmic male sterile (CMSI) mutant of tobacco and the Aco1 mutant of the wild species tomato *S. pennellii* (Nunes-Nesi et al. 2011). More recently, evidence has accumulated that certain mitochondrial reactions are crucial to support cytosolic nitrate accumulation (Nunes-Nesi et al. 2007). However, results of an elegant study in *Brassica napus* question the absolute requirement of a direct requirement for concurrent nitrate assimilation facilitating photosynthesis (Gauthier et al. 2010).

Given the lack of consensus regarding the above two postulates, several other roles for mitochondria, during photosynthesis, have been proposed, including the balance of cellular redox status (Raghavendra and Padmasree 2003; Scheibe 2004), buffering of metabolism by photorespiration, and/or the alternative oxidase (Rasmusson et al. 2009; Bauwe et al. 2010), or retrograde signaling (Zarkovic et al. 2005; Alhagdow et al. 2007). That being said, as we detail below, a renaissance of work related to guard cell metabolism (Daloso et al. 2017) has revealed that, counter to the perceived wisdom at the turn of the century (Outlaw 2003), malate does play a considerable role in guard cell function (Lee et al. 2008; Araujo et al. 2011).

As stated above, the most complete series of experiments has been carried out in a range of transgenics of tomato individually inhibited in the expression of one of the mitochondrial isoforms of the eight enzymes of the TCA cycle proper. These are detailed in Figure 1, namely aconitase, malate dehydrogenase, fumarase, succinylcoA ligase, citrate synthase, isocitrate dehydrogenase, succinate dehydrogenase and 2-oxoglutarate dehydrogenase (Carrari et al. 2003; Nunes-Nesi
et al. 2005a; Nunes-Nesi et al. 2007; Studart-Guimaraes et al. 2007; Sienkiewicz-Porzucek et al. 2008; Sienkiewicz-Porzucek et al. 2010; Araujo et al. 2011; Araujo et al. 2012b). Of these transgenics, only four were characterized by dramatic changes in their rates of photosynthesis and growth, namely those plants exhibiting decreases in the expression of aconitase (unpublished data which mirrors that of the Aco1 mutant), the mitochondrial isoforms of malate dehydrogenase (Nunes-Nesi et al. 2005b), fumarase (Nunes-Nesi et al. 2007), and the iron-sulphur subunit of succinate dehydrogenase (Araujo et al. 2011), which exhibited increased, increased, decreased and increased rates of assimilation, respectively.

Evaluation of Arabidopsis knockout mutants of the mitochondrial isoforms of malate dehydrogenase (Tomaz et al. 2010), and the flavoprotein subunit of succinate dehydrogenase (Fuentes et al. 2011), revealed that the latter were also characterized by improved growth performance, via the same mechanism (defined below), but that the malate dehydrogenase mutants (knockout) conversely exhibited reduced photosynthesis and growth. As yet no detailed characterization of the mitochondrial fumarase of Arabidopsis has been reported.

Whilst the reasons for increased plant performance in the aconitase lines are unclear, we consider that two different mechanisms underlie the changes observed in the other tomato lines, which we summarize in Figures 2A and B. First, the mitochondrial malate dehydrogenase lines were characterized by elevated synthesis of ascorbate and increased rates of assimilation (up to 11%), and shoot, leaf and fruit biomass (up to 19%), but a reduced root biomass (Nunes-Nesi et al. 2005b). Detailed studies demonstrated that this was likely due to the mitochondrial electron transport chain utilizing L-galactono-lactone, the terminal precursor of ascorbate biosynthesis, as an alternate electron donor given the reduced availability of electrons from the TCA cycle (Figure 3A). This led Nunes-Nesi and co-workers to propose that ascorbate acts as a signal that allows the coordination of plant energy metabolism between the mitochondria and the chloroplast.

Consistent with this hypothesis is the fact that both nuclear and plastid gene expression of the photosynthetic machinery is upregulated following feeding with ascorbate (Smirnoff 2000; Smirnoff and Wheeler 2000; Nunes-Nesi et al. 2005b), and more importantly, that the rate of C assimilation is also upregulated under these conditions (Nunes-Nesi et al. 2005b). Moreover, the phenotype is somewhat
dependent on the length of the photoperiod (Alhagdow et al. 2007); however, this is likely due to a multitude of reasons, including the general importance of the TCA cycle under conditions wherein less photosynthesis is possible.

The recent identification of a plastid ascorbate transporter (Miyaji et al. 2015) has provided clues as to how this signal may be relayed, although the exact nature of its transduction requires considerable further research (Fernie and Toth 2015). These results were observed in the greenhouse, but may well be transferable to field conditions wherein the plants were exposed to a greater pressure from (a)biotic stresses, but such studies have not yet been carried out.

The second mechanism was first suggested by studies in transgenic tomatoes, in which fumarase was constitutively downregulated (Araujo et al. 2011). These studies revealed that plant performance was retarded by a deficiency in fumarase expression, with a 50% reduction in carbon assimilation corresponding to around 20% reduction in stem, leaf and fruit biomass. Furthermore these plants were characterized by a decreased stomatal conductance, leading the authors to suggest that the lines were compromised in this parameter, due to an increase in malate (and fumarate) levels.

Two subsequent papers provided considerable support for this hypothesis. First, the cloning and characterization of the AtABCB14 transporter and demonstration that plants deficient in its expression had less efficient photosynthesis (Lee et al. 2008), highlights the importance of malate efflux from the guard cell. Secondly, studies of plants deficient in succinate dehydrogenase expression were characterized by decreased levels of malate and fumarate and an upregulated stomatal conductance and carbon assimilation (Araujo et al. 2011). However, only when the deficiency of succinate dehydrogenase activity occurred in the mesophyll cells, no effect on these parameters was noted in plants in which the activity was altered in a guard-cell-specific manner (Figure 3B). Similarly, Arabidopsis plants deficient in the expression of the flavoprotein subunit of succinate dehydrogenase were characterized by similar effects on photosynthesis, as well as better growth under nitrogen-limiting conditions (Araujo et al. 2011).

Interestingly, the downregulation of AtQUAC1, an R-type anion channel responsible for the release of malate from guard cells, also resulted in a similar
photosynthetic phenotype (Medeiros et al. 2016), whereas the downregulation of the vacuolar malate transporter had little effect on either stomatal conductance or photosynthesis, with seemingly prioritizing malate for maintaining photosynthesis at the cost of photosynthesis (Medeiros et al. 2017). Although we have previously expressed caution as to the applicability of enhancing photosynthesis by altering malate levels to field grown crops, two recent studies suggest it may also be relevant for such plants.

A meta-analysis of metabolite and photosynthetic profiles of 14 species revealed the level of malate to be a highly pertinent link (Gago et al. 2016). Similarly, comparison of metabolite profiles with ground and remote sensing measurements of photosynthesis, in a Mediterranean grapevineyard, additionally revealed that malate is an important determinant of stomatal conductance, even under water-limited conditions (Gago et al. 2017). By contrast to the above examples, altering the expression of citrate synthase (Sienkiewicz-Porzucek et al. 2008), isocitrate dehydrogenase (Sienkiewicz-Porzucek et al. 2010), succinyl CoA ligase (Studart-Guimaraes et al. 2007), and oxoglutarate dehydrogenase (Araujo et al. 2012b) had no effect on the rate of assimilation, whereas the closely associated enzyme, pyruvate dehydrogenase, has not been subject to detailed reverse genetic studies.

Another enzyme intimately related to the TCA cycle is the mitochondrial glycine decarboxylase complex. As one of the most abundant mitochondrial proteins, this enzyme has, for many decades, been the subject of detailed study (Douce et al. 2001). Arabidopsis knockouts of the L-protein, a mitochondrial dihydrolipoyl dehydrogenase that mainly participates in the glycine decarboxylase complex revealed that they were characterized by reduced levels of TCA cycle and photorespiratory intermediates, yet they had enhanced rates of photosynthesis and growth (Timm et al. 2015). Collectively, these examples underscore the importance of mitochondrial reactions in proper chloroplast, and hence cellular function, and highlight that the manipulation of certain steps of the pathway can result in enhanced aerial growth, and indeed, crop yield.

ROLE OF THE TCA CYCLE IN ROOT DEVELOPMENT
It has traditionally been assumed that understanding the role of respiration, in plants, is considerably easier in roots than leaf tissues, given that they rely exclusively on mitochondrial oxidative phosphorylation to meet the energy demands of the cell. However, recent demonstrations that photosynthesis occurs in germinating seeds (Borisjuk and Rolletschek 2009; Galili et al. 2014), along with the fact that some enzymes of photorespiration are expressed in a root-specific fashion (Nunes-Nesi et al. 2014b), combine to suggest that such generalizations should be made with caution.

Evidence for at least some species suggests that the activity of the TCA cycle is very important for generating the organic acids that are exuded into the rhizosphere (De la Fuente et al. 1997). The first observation of this phenomenon was reported in experiments in tobacco by the group of Luis Herrera-Estrella in 1987 (De la Fuente et al. 1997). A detailed characterization of the roots of the transgenic tomato lines, described above, revealed that while they were uniformly compromised in terms of biomass this could not be attributed to alterations in organic acid exudation. Indeed, the reduction in biomass of both malate dehydrogenase and fumarase plants were concluded to be due to a reduction in the rate of cell wall synthesis (Van der Merwe et al. 2009). However, a follow-up study, comparing plants that were independently inhibited in all eight enzymes, revealed that the differential root branching exhibited by some of these lines was attributable to altered phytohormone relations, and that neither root biomass nor structure was influenced by the root exudates of any of the lines (Van der Merwe et al. 2010).

Irrespective of these findings, as mentioned above, other studies have demonstrated that the overexpression of citrate synthase in tobacco roots was able to enhance both the tolerance of these transgenic lines, upon exposure to aluminium ions (De la Fuente et al. 1997), and phosphorus uptake, with both phenomena attributed to the elevate exudation of citrate into the rhizosphere.

The mechanisms underlying aluminium tolerance have, at least to some extent, been clarified. The fact that the majority of soils in tropical and sub-tropical climes are acidic renders soil a major determinant of crop productivity. Aluminium cations (particularly Al^{3+}), are largely responsible for disruption of water and nutrient uptake, either by exclusion or tolerance mechanisms (Nunes-Nesi et al. 2014a)(See also Figure 4). The tolerance mechanism is, arguably, the best characterized and involves
secretion of organic acids, including citrate, malate and oxalate, which is activated by the presence of aluminium ions in the root apex, with the organic acids likely chelating aluminium ions and, thus, preventing its uptake (De la Fuente et al. 1997).

Increasing organic acid exudation has been tested as a possible means to enhance Al tolerance; for example, through increasing the activities of the following enzymes: (i) citrate synthase (Barone et al. 2008; Deng et al. 2009; Han et al. 2009), (ii) malate dehydrogenase (Tesfaye et al. 2001; Wang et al. 2010), (iii) malic enzyme (Sun et al. 2014), (iv) pyruvate phosphate dikinase (Trejo-Tellez et al. 2010) and (v) the combination of phosphoenolpyruvate carboxylase and citrate synthase (Wang et al. 2012). It should be mentioned that oxalate has also been characterized within the exudates of many species (Ma 2000; Kochian et al. 2004).

Despite the number of such examples, this approach is not without controversy, particularly given that aluminium-sensitive plants have also been characterized to excrete large amounts of organic acids (Ishikawa et al. 2000; Pineros et al. 2005). An aluminium-induced secretion of organic acids cannot account for genotypic differences between aluminium tolerance in buckwheat, soybean or maize cultivars (Pineros et al. 2005; Zheng et al. 2005). However, via broad screens, a wide number of proteins and transcription factors have been identified to be associated with aluminium tolerance, including the Al-activated malate transporter in wheat (Sasaki et al. 2004), Arabidopsis (Hoekenga et al. 2006) and rape (Ligaba et al. 2006). In addition, genes involved in Al-activated secretion of citrate and malate have been identified in barley (Furukawa et al. 2007), sorghum (Magalhaes et al. 2007), maize (Maron et al. 2013) and Arabidopsis (Liu et al. 2009; Carvalho et al. 2016). Thus, engineering organic acid exudation appears to represent a reasonable strategy for securing crop yields in acidic soils.

Numerous studies have also focused on the role of organic acid secretion in phosphate acquisition, with a seminal study again coming from the laboratory of Luis Herrera-Estella (Lopez-Bucio et al. 2000). This study revealed that tobacco plants overexpressing citrate synthase were better able to cope with phosphate poor soil. In support of this finding, correlations between phosphate uptake and organic acid exudation have been reported for a range of species, including maize (Strom et al. 2002), castor bean (Jeschke et al. 1997) and Arabidopsis (Narang et al. 2000). Furthermore, a range of other transgenic plants overexpressing either citrate
synthase or malate dehydrogenase in tobacco (Lu et al. 2012), pigeonpea (Hussain et al. 2016) and cotton (Wang et al. 2015) have similarly support the notion that enhancing organic acid secretion can improve biomass under phosphate-limited conditions.

It is important to note, however, that this support should be viewed as conditional, given with the finding from a detailed study of wheat cultivars which failed to find a correlation between exudation and biomass productivity (Ryan et al. 2014). This study lead several researchers to conclude that the mechanisms underlying this relationship is likely to be far more complex than initially assumed, and thus, considerable further work is required in order to better understand this phenomenon.

**TTCA CYCLE ROLE IN OTHER HETEROTROPHIC TISSUES**

The effect of manipulating the TCA cycle on other tissues is relatively poorly studied. There are a few reports that: (i) downregulation of the mitochondrial citrate synthase leads to cytoplasmic male sterility (Landschutze et al. 1995; Zou et al. 1999); (ii) link activity of the TCA cycle to aspects of seed metabolism and development (Fait et al. 2006; Angelovici et al. 2011; Fait et al. 2011); (iii) downregulating the TCA cycle activity has consequences on heterotrophic tissues other than roots, flowers and seeds. Given that the final item on this list is the best understood, we will focus this section on these studies, largely assessing the effects observed in tomato fruits and potato tubers.

It has been long known that tomato is classified as a climacteric fruit; i.e., their ripening is characterized by an ethylene-mediated boost in respiration. Data from kinetic profiling of wild type tomato (Carrari et al. 2006; Wang et al. 2009) and known ripening mutants (Osorio et al. 2011) revealed that, during ripening, changes in organic acid metabolism are highly correlated with transcriptomics, metabolomics and developmental shifts. However, a comparative analysis of the climacteric fruit, tomato, versus the non-climacteric fruit, pepper, revealed that whereas there were species-specific patterns of network regulatory behavior, a coordinate regulation of transcripts was also observed by malate and citrate in potato (Szecowka et al. 2012).

Having demonstrated the above-described correlation in tomato, our group set about characterizing whether the alterations in organic acid metabolism was a cause
or consequence of the altered rates of ripening. In order to do so, fumarase or malate dehydrogenase were independently silenced, in a fruit-specific manner (Centeno et al. 2011)(see also Figure 5). While these genetic perturbations had relatively little effect on total fruit yield, they had dramatic consequences for fruit metabolism, as well as unanticipated changes in postharvest shelf life and susceptibility to bacterial infection. Detailed characterization suggested that the rate of ripening was essentially unaltered, but those lines containing higher malate were characterized by lower levels of transitory starch and a lower soluble sugars content, at harvest, whereas those with lower malate contained higher levels of these carbohydrates.

Analysis of the activation state of ADP-glucose pyrophosphorylase revealed that it correlated with the accumulation of transitory starch. Taken together with the altered activation state of the plastidial malate dehydrogenase and the modified pigment biosynthesis of the transgenic lines, these results suggest that the phenotypes are due to an altered cellular redox status. The combined data reveal the importance of malate metabolism in tomato fruit metabolism and development, and confirm the importance of transitory starch in the determination of agronomic yield in this species. Interestingly, in support of the above conclusions, alteration of the interconversion of pyruvate and malate, in the plastid or cytosol of ripening tomato fruit, invoked diverse consequences on sugar, but similar effects on cellular organic acid metabolism and transitory starch accumulation(Osorio et al. 2013).

Surprisingly, a similar effect was not seen following the downregulation of fumarase expression in potato (Szecowka et al. 2012), suggesting that the physiological regulation of AGPase-mediated starch synthesis is context dependent. That being said, few other reverse genetic manipulations of the TCA cycle have been carried out in potato, using tuber-specific promoters, so whether this is a general phenomenon, or only linked to this specific reaction, remains to be established. Worthy of note here is the downregulation of the NAD$^+$ dependent malic enzyme (Jenner et al. 2001), which resulted in no change in TCA cycle activity, but significant increase starch yield, whereas it seems likely that this result is associated with a redox regulation of AGPase, experimental evidence to confirm this is lacking. Thus, the main effect of altered organic acid metabolism in fruit and tubers relates to the ripening and post-harvest effects of manipulations in organic acid metabolism and the knockon consequences on sugar metabolism.
PERSPECTIVES FOR SYNTHETIC BIOLOGY APPROACHES AT MODIFYING THE TCA CYCLE

We now turn our attention to the advent and widespread adoption of synthetic biology approaches, aided by multigene transformation, which has shifted the focus of next-generation metabolic engineering strategies towards multi-gene interventions (Kopka and Fernie 2018; Sonnewald and Fernie 2018). In order to increase productivity and yield of a valuable molecule, the metabolic engineering in living cells needs to be balanced, a condition which is hard to achieve as it requires controlling gene expression levels, translation, scaffolding, compartmentation and metabolic flux (Sweetlove and Fernie 2013; Sweetlove et al. 2017).

With nature’s strategies to increase metabolic efficiency in mind, metabolic engineers are, thus, increasingly trying to engineer artificial multienzyme complexes, where the enzymes performing consecutive reactions are spatially organized to the sequential complex. Due to the enforced proximity of the enzyme active sites, and the formation of enzyme microdomains built as a consequence of co-clustering of multiple enzymes into higher aggregates, catalytic efficiency and metabolic pathway performance can arguably be improved (Pröschel et al. 2015). The metabolic engineering of the bacterial TCA cycle has frequently been used for decades to produce chemicals such as citrate, L-glutamate, ethylene gas and succinate (Vuoristo et al. 2016). Moreover, several synthetic biology approaches have been taken in which the TCA cycle has been re-routed, to great effect, in bacterial systems (for a recent review see [Aslan et al. 2017]).

Returning to plants, an interesting perspective of the role of organic acids has recently been presented (Igamberdiev and Eprintsev 2016). These authors maintain that organic acids are “a result of the incomplete oxidation of photosynthetic products and represent the stored pools of fixed carbon accumulated due to different transient times of conversion of carbon compounds in metabolic pathways”. Furthermore, they state that whereas the citrate- and malate-valves support nitrogen assimilation and redox balancing, respectively, other secondary reactions result in accumulation of other organic acids derived from intermediates of the cycle such as trans-aconitate, hydroxycitrate and 4-hydroxy-2-oxyglutarate, oxalate, malonate (Igamberdiev and Eprintsev 2016), as well as a compound they do not mention - maleate (Sweetlove et al. 2007).
In addition to these auxiliary reactions, a range of other reactions, which occur in non-plant systems, are worthy of discussion. For instance, in eukaryotes, the conversion of isocitrate to 2-oxoglutarate is catalyzed by the NAD\(^+\)-dependent isocitrate dehydrogenase, whereas prokaryotes employ an NADP\(^+\)-dependent isocitrate dehydrogenase. In addition, conversion of malate to oxaloacetate is catalyzed in eukaryotes by the NAD\(^+\)-dependent malate dehydrogenase, yet most prokaryotes utilize the quinone-dependent version of the enzyme (Van der Rest et al. 2000). On a similar note, most organisms utilize the ubiquitous NAD\(^+\)-dependent 2-oxoglutarate dehydrogenase, but some bacteria utilize a ferredoxin-dependent 2-oxoglutarate synthase (Baughn et al. 2009).

Other organisms, including obligately autotrophic and methanotrophic bacteria and archaea, bypass succinyl-CoA entirely, and convert 2-oxoglutarate to succinate via succinate semialdehyde, using 2-oxoglutarate decarboxylase and succinate-semialdehyde dehydrogenase. Similarly, the conversion of succinyl CoA to succinate is subject to considerable variation, in that whereas most organisms use an ADP forming succinate CoA ligase, in mammals a GDP forming succinate CoA ligase also operates (Lambeth et al. 2004). Moreover, some acetate-producing bacteria utilize a completely different enzyme - succinyl-CoA:acetate CoA-transferase which links the TCA cycle to acetate metabolism (Mullins et al. 2008). In other bacteria, including *Helicobacter pylori*, employ yet another enzyme - succinyl-CoA:acetoacetate CoA-transferase - for this conversion (Corthesy-Theulaz et al. 1997; Steinhauser et al. 2012).

In addition to the variation within the individual reactions, *per se*, there are other variations of note, not least of which being the Anon-Buchanan cycle, in which the TCA cycle runs in the reverse direction in a wide range of bacteria (Buchanan et al. 2017). There are also a range of shunts and bypasses which occur in non-plant systems, including the citramalate shunt (Filatova et al. 2005), the bypass of the 2-oxoglutarate dehydrogenase reaction uncovered recently in cyanobacteria (Zhang and Bryant 2011; Steinhauser et al. 2012), and the recently described acetate shunt (Kwong et al. 2017), as well as variants of the glycoxylate shunt (Vuoristo et al. 2016) which is confined to specialized peroxisomes, known as glycoxysomes in plants and absent in mammals. A maleate shunt has also been described in several bacteria and the compound is detectable in plants and correlates with levels of malate and
fumarate, yet the genes/enzymes responsible have not yet been characterized in plants (Sweetlove et al. 2007; Sweetlove et al. 2017).

A promising strategy in plants would, thus, likely be to do the same as described above for bacteria; as mentioned earlier, a wide range of variant TCA cycles are known in nature, and the major variants are summarized in Figure 6. Introduction of some of these may prove a more effective future strategy at improving plant performance, as these may well be less subject to feedback control mechanisms. Another possibility, as mentioned above, is the generation of artificial metabolons. Recently, several studies have demonstrated the physical assembly of TCA cycle enzymes to form metabolite channels or metabolons (Wu and Minteer 2015; Zhang et al. 2017). The sequential enzymes of the metabolic pathway can be associated into a temporary structural-functional complex by both by non-covalent interactions, anchorage to membranes, and by encapsulation of enzymes in protein-coated microcompartments (Figure 2).

It has been proposed that metabolons may improve the efficiency of metabolic pathways, by increasing the concentration of intermediates, decreasing the enzyme concentration, directing the products of a pathway to a specific subcellular location, or minimizing the escape of reactive intermediates (Jorgensen et al. 2005). Several metabolons have been proposed to mediate substrate channelling in diverse organisms; for instance, branched chain amino-acid metabolism in human mitochondria (Islam et al. 2007), the glycolytic pathways of mammals, yeast and plants (Al-Habori 1995; Brandina et al. 2006; Graham et al. 2007), and a wide variety of specialized metabolic pathways, including polyamine (Panicot et al. 2002), isoprenoid (Leivrad et al. 2005), alkaloid (Winzer et al. 2015), phenylpropanoid (Achnine et al. 2004) and cyanogenic glucoside (Bassard et al. 2017; Fujino et al. 2018) biosynthesis in plants. That being said, experimental evidence for these is often rather fragmentary, and the experimental manipulation of the metabolon remains relatively scarce. However, the technologies exist which enable multienzyme complexes to be organized by scaffold proteins, which contain multiple modular interaction domains; for example, protein-protein interaction domains, or motifs (Good et al. 2011).
Synthetic scaffold proteins provide the possibility for highly flexible rational assembly of enzymes, in a defined and controllable manner. So the main advantage of the scaffold-based multienzyme complex formation strategy is the modularity. A major advantage of this approach is that the scaffold architecture, and therefore, the enzyme stoichiometric ratios can be easily controlled in a modular fashion (Pröschel et al. 2015). In a recent example, artificial, peptide scaffolded, synthetic metabolons of hexokinase (HXK) and glucose-6-phosphate dehydrogenase (G6PDH), were proven to be more efficient than the free enzymes for metabolic engineering (Liu et al. 2017). Such studies suggest that synthetically varying the proportion of TCA cycle enzymes that are complexed, as opposed to unbound, likely represents an interesting strategy for future studies.

CONCLUSIONS AND FUTURE PROSPECTS AT MODIFYING PLANT PRODUCTIVITY VIA MODIFYING THE ACTIVITY OF THE TCA CYCLE

In this review, we have summarized the state-of-the-art in manipulating plant productivity through engineering of the TCA cycle. To date, approaches have been confined to single gene manipulations; however, several of the genes whose manipulation proved successful have been taken up into next-generation engineering strategies (Sonnewald and Fernie 2018). The first results based on these approaches will soon become available. The first generation strategies demonstrated that manipulation of the TCA cycle can either enhance assimilation and shoot growth, resulting in increased yields in greenhouse crops, improve root growth under nutrient limited or acidic soils, and improve the post-harvest physiology of spoilable crops.

Currently, the majority of these examples have only been demonstrated in one or a handful of species, and furthermore, very few of the conditions under which organic acid levels are dramatically altered have been evaluated in detail in order to ascertain the physiological function of the individual metabolites of the cycle. However, the widespread adoption of genome wide association mapping and quantitative trait loci approaches that utilize both metabolomics and yield-associated phenotyping has begun to identify (Riedelsheimer et al. 2012; Wen et al. 2015; Tohge et al. 2016; Peng et al. 2017), and will certainly continue to find, further associations of enzymes
involved in the interconversion of organic acids and a range of other traits associated with plant productivity.

In parallel, as we argue above, once we have gained a more complete understanding of the exact function of the metabolites themselves, in a range of tissues and cellular circumstances, adoption of synthetic biology approaches may prove a more effective way of rationally engineering their levels. While the synthetic biology approach may well circumvent problems associated with feedback regulation of the plants native enzymes, another aspect that will need a great deal of attention is the fact that several of the intermediates of, or intimately related to, the TCA cycle have recently been demonstrated to have signaling functions (Finkemeier et al. 2013; Timm et al. 2013; Gilliham and Tyerman 2016). Bearing this in mind, the idea of expressing synthetic metabolons may well ultimately represent the best practice for engineering plant metabolism with as great a precision as possible.

AUTHOR CONTRIBUTIONS

Y.Z. and A.R.F. wrote the manuscript.

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**Figure 1. Schematic overview of the TCA cycle and the complex metabolic network into which it is embedded**

The large text and the arrows represent the intermediates and reactions, respectively. The enzyme names are shown in blue rectangles. Subunits of the enzymes are represented by spheres with the size roughly proportional to the molecular mass of the protein. Names of subunit proteins are described as text next to the spheres. Abbreviations: PDC, pyruvate dehydrogenase complex; ME, malic enzyme; CSY, citrate synthase; ACO, aconitase; IDH, isocitrate dehydrogenase; ODC, oxoglutarate dehydrogenase complex; SCoAL, succinyl-CoA ligase; SDH, succinate dehydrogenase; FUM, fumarase; MDH, malate dehydrogenase; AcCoA, acetyl-CoA; 2OG, 2-oxoglutarate; SucCoA, succinyl-CoA; MPC, mitochondrial pyruvate carrier; SFC, succinate/fumarate carrier; and DC, dicarboxylate carrier.
Figure 2. The plant TCA cycle metabolon

The enzymes are depicted as circles and the interactions of any subunits of sequential enzymes are shown as green arrows. The interactions of catalytic subunits that potentially mediate metabolite channelling are described next to the arrows. Metabolites drawn as red, grey and black text are channelled, not channelled and not tested, respectively (Zhang et al. 2017).

Figure 3. Influence of the TCA cycle in the illuminated leaves

(A) The ETC utilizes the L-galactono-lactone, the terminal precursor of ascorbate biosynthesis, as an alternate electron donor. The L-galactono-lactone could later be catalyzed by GLDH to the vitamin C (ascorbate) in plants. Ascorbate could upregulate the photosynthetic machinery after importing into chloroplast. (B) The malate (fumarate) produced by the TCA cycle is transported to the vacuole, where it is stored. By an unclear mechanism, the level of organic acid is altered in the subsidiary cells, leading to an increased (decreased) concentration in the guard cells.
that culminates with the closing (opening) of stomata. Abbreviations: ETC, mitochondrial electron transport chain; GLDH, L-galactono-1,4-lactone dehydrogenase.

Figure 4. The influence of organic acid exudation on ion exchange in the root

The mitochondrial oxidative phosphorylation not only provides energy for root growth and development, but also provides the organic acid (such as citrate and malate) for ion exchange (such as aluminium ions (Al$^{3+}$) and phosphate (PO$_4^{3-}$)) into the root for plant growth and development. Studies established that several Al-induced tolerance genes (such as Al-activated malate transporter 1 (AtALMT1) and multidrug and toxic compound extrusion 1 (AtMATE1)) could improve Al$^{3+}$ exclusion by associating with the exudation of organic acid (i.e., citrate, malate, or oxalate). In addition, ALS1 is implicated in Al$^{3+}$ sequestration in the vacuole after chelation of Al$^{3+}$ with OA in the cytosol. Abbreviations: ALMT, aluminum-activated malate transporter; ALS3, aluminum-sensitive 3; DTC, dicarboxylate/tricarboxylate carrier; OA, organic acid; ALS1, acetolactate synthase 1.

Figure 5. Model of the influence of mitochondrially-derived malate on tomato fruit starch, soluble sugar content, postharvest shelf life and bacterial infection
Mitochondrial MDH was inhibited in tomato lines (A) (increased malate), or fumarase (B) (decreased malate). (1) Alterations in mitochondrial redox status are transmitted, either within the same cell type or from adjacent tissues, to the plastid via the malate valve, as described by Scheibe (2004). (2) Altered plastidial redox status results in a reduced (MDH) or enhanced (fumarase) activation state of the reaction catalyzed by AGPase (as well as similar changes in the activation state of the plastidial MDH); whether this is mediated by thioredoxin or the NTR-C pathway is currently unknown. (3) This leads to redox-mediated alterations in pigment biosynthesis during ripening. (4) Starch is rapidly broken down, leading to a reduced soluble solid content in red-ripe fruit in the MDH lines and an increased soluble solid content in the fumarase lines. (5) Potentially, as a result of differences in cellular osmolarity, the transgenic sets oppositely display an increased water loss and wrinkling (MDH) or a decreased water loss and wrinkling (fumarase) that appears to be cell wall-independent. (6) These changes in water loss and wrinkling correlate positively to the rate of opportunistic pathogen infection in the transgenic sets, whereas the MDH lines are increasingly susceptible to B. cinerea infection (Modified from Centeno et al 2011).

Figure 6. Natural TCA cycle variants

The classical TCA cycle is represented by the unbroken black line. (1) the citramalate shunt, yellow broken arrows; (2) the glyoxylate shunt, green broken arrows; (3) The TCA cycle variant newly discovered in cyanobacteria, blue broken arrows; (4) the GABA shunt, red broken arrows; (5) the closing reaction (without glyoxylate shunt) of the previously thought to be incomplete cyanobacterial TCA cycle, grey broken arrows; and (6) the acetate shunt, broken violet arrows. For clarity, cofactors have been omitted. Abbreviations: AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; GABA-T, GABA aminotransferase; GAD, glutamate decarboxylase; ICL, isocitratelyase; MS, malate synthase; OGDC, 2-oxoglutarate decarboxylase; SSADH, succinic semialdehydedehydrogenase; CMS, citramalate synthase; and ASCT, acetate:succinate CoA-transferase.