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Publication Date

2023-08-01

DOI

10.1016/j.pbi.2023.102382

Peer reviewed

HHS Public Access

Author manuscript

Curr Opin Plant Biol. Author manuscript; available in PMC 2024 August 01.

Published in final edited form as:

Curr Opin Plant Biol. 2023 August ; 74: 102382. doi:10.1016/j.pbi.2023.102382.

Non-canonical and developmental roles of the TCA cycle in plants

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Abstract

Over recent years, our understanding of the tricarboxylic acid cycle (TCAC) in living organisms has expanded beyond its canonical role in cellular energy production. In plants, TCAC metabolites and related enzymes have important roles in physiology, including vacuolar function, chelation of metals and nutrients, photorespiration, and redox regulation. Research in other organisms, including animals, has demonstrated unexpected functions of the TCAC metabolites in a number of biological processes, including signaling, epigenetic regulation, and cell differentiation. Here, we review the recent progress in discovery of non-canonical roles of the TCAC. We then discuss research on these metabolites in the context of plant development, with a focus on research related to tissue-specific functions of the TCAC. Additionally, we review research describing connections between TCAC metabolites and phytohormone signaling pathways. Overall, we discuss the opportunities and challenges in discovering new functions of TCAC metabolites in plants.

Keywords

Primary metabolism; Plant development; Phytohormones; Tricarboxylic acid cycle

Introduction

The tricarboxylic acid cycle (TCAC) metabolites have long been recognized as vital biomolecules needed to produce cellular energy and synthesize macromolecules such as proteins, lipids, and nucleotides. The TCAC constantly oxidizes carbon to form NADH and $FADH_2$, connecting glycolysis to the electron transport chain and cellular respiration. The TCAC is the central metabolic hub, with many anabolic and catabolic pathways stemming from individual metabolites. In response to cellular demands, TCAC metabolites can be

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alexandra J. Dickinson reports financial support was provided by National Institutes of Health. Alexandra J. Dickinson reports financial support was provided by National Science Foundation. Alexandra J. Dickinson reports financial support was provided by Hellman Foundation.

exported and imported by specific transporters and compartmentalized in organelles instead of being constrained to the mitochondria. The flux through the TCAC pathway is also flexible and is capable of undergoing noncyclic flow [1]. TCAC intermediates can undergo cataplerotic reactions, in which they are consumed for the biosynthesis of macromolecules. The conversion of citric acid to fatty acids is a classic example of cataplerosis. In contrast, anaplerotic reactions replenish the consumed metabolites by converting various precursors, such as pyruvate and amino acids, into TCAC metabolites. Phosphoenolpyruvate (PEP) carboxylase (PEPC) catalyzes the β-carboxylation of PEP to form oxaloacetate [2–5]. In higher plants, PEPC plays a crucial anaplerotic role in replenishing oxaloacetate and malate, which are consumed for nitrogen assimilation and amino acid biosynthesis. The combination of anaplerosis and cataplerosis are important for regulating TCAC intermediate levels. Interestingly, anaplerotic and cataplerotic fluxes vary between different developmental stages of leaves, reflecting differential needs for energy and protein synthesis [6]. By modulating TCAC intermediates, these processes can play an important role in cellular redox state and signaling, as well as aiding recycling pathways and nitrogen trafficking during catabolism [7].

There are a number of excellent reviews that describe the extensive work that has gone into characterizing the regulation and functions of the TCAC in plants [8,9]. Here, we review functions of the TCAC beyond the typically discussed topics of energy production and biosynthesis in non-photosynthetic organisms. First, we summarize research that has elucidated non-canonical roles for the TCAC in plant and animal systems. We then review examples of important functions for the TCAC in plant development, focusing on instances where TCAC metabolites and related genes have been shown to contribute to tissue-specific regulatory processes. Finally, we discuss the intersection of the TCAC with phytohormone signaling pathways, which are critical for development.

Evidence for non-canonical roles of the TCAC in diverse organisms

Emerging work reveals that TCAC metabolites can function as regulatory molecules in diverse biological processes. In mammals, individual TCAC metabolites have been linked to inflammation, tumorigenesis, and development [10–12]. In a critical paper for elucidating novel functions of TCAC metabolites, it was discovered that succinate and AKG are ligands for G-protein coupled receptors, which led to characterization of their roles in cellular signaling [13]. Succinate has also been shown to stabilize Hypoxia Inducible Factor 1 alpha (HIF-a), a DNA-binding complex that is a master regulator of hypoxia [14]. Additionally, changes in TCAC flux have been shown to impact cellular behavior and lead to disease states in animals [15]. In mammalian cancer cell lines, a non-canonical TCAC that relies on using cytosolic enzymes was identified. This rewiring of the TCAC was also found to be required for stem cell differentiation [12]. In addition, alternative forms of the TCAC are generated during different stages of the cell cycle in mammalian cells, resulting in effects on cell proliferation [16].

In plants, TCAC metabolites also have diverse physiological functions. TCAC metabolites and related proteins have been reported to be involved in vacuole physiology [17–19], plastid function [20,21], stomatal behavior [22–25], metal chelation [26–30], nutrient

uptake [31], allosteric effects [32–34], and stress response [35–39]. Citrate and malate are particularly important for vacuolar physiology, their vacuolar levels are regulated by the dicarboxylate transporter, tDT, in Arabidopsis leaves [19]. It was discovered that the affinity of tDT for malate and citrate is dependent on external pH. Malate is critical for regulating stomatal aperture through the vacuolar chloride channel AtALMT9, which is required for normal stomatal opening [40]. Furthermore, starch degradation in guard cells has been shown to serve as a critical malate precursor for modulating stomatal opening [41]. Malate and citrate can also act as metal chelators in the presence of aluminum and hard dissolved inorganic phosphate [26,31]. Moreover, the malate and citrate valves are essential for maintaining the redox equilibrium in cells, which regulate the activity of the TCAC enzymes [42]. TCAC metabolites are also allosteric regulators of proteins, in both plants and animals. For example, the plant NAD-dependent malic enzyme has an allosteric site that can bind either fumarate or malate. The activity of this enzyme is inhibited by malate but promoted by fumarate, which have contrasting effects on its substrate affinity [33]. The TCAC metabolites also contribute to balancing reactive oxygen species (ROS) in subcellular compartments, generating ion gradients, and regulating extracellular pH [42]. Malate, citrate, and oxalate, in particular, are found to be involved in numerous processes in the rhizosphere, such as nutrient acquisition, metal detoxification, alleviation of anaerobic stress in roots, mineral weathering, and pathogen attraction [31]. In addition, genes that modulate biosynthesis and transport of TCAC enzymes are regulated by transcription factors [43], metabolons [44], calcium [45], thioredoxins (TRX) [46], and extra-pathway protein interactions [47] in response to developmental and environmental cues, indicating a wealth of connections between the TCAC and signaling pathways. Researchers have also taken inspiration from animal research to reveal non-canonical roles for the TCAC in plants. For instance, Chen et al. followed up on research in yeast that demonstrated that cleavage of citrate to acetyl-CoA affects histone acetylation in multiple residues of H3 and H4. The authors found that cytosolic acetyl-CoA production also induces histone acetylation in Arabidopsis, primarily at lysine 27 of H3 [48]. In the future, it will be interesting to investigate whether more non-canonical mechanisms of TCAC metabolites are conserved across kingdoms of life.

TCAC metabolites can have specific effects in plant development

The TCAC is an essential process for all eukaryotic cells and the complete knockdown of any of the biosynthetic steps in the cycle is often lethal, as the TCAC connects glycolysis to cellular respiration (Figure 1). Therefore, assessing the more subtle functions of any individual TCAC metabolite is a major challenge. For example, mitochondrial pyruvate dehydrogenase (PDH) plays a key role in linking glycolysis to the production of citrate and acetyl-CoA. Mutants in mitochondrial PDH complex subunits have a number of severe defects, including aberrant development of embryos, pollen, cotyledons, and roots [49–51]. While it would be reasonable to ascribe these deleterious phenotypes solely to reduced ATP production, it was found that pyruvate acts as a substrate for Trp aminotransferases during a key step in the synthesis of the major form of auxin, indole-3-acetic acid (IAA) [52]. The link between TCAC metabolites and phytohormones will be discussed in more detail in subsequent sections, but pyruvate’s role in auxin biosynthesis highlights both the

complications and the potential for understanding the multifaceted developmental effects of primary metabolites.

Each step of the TCAC has been linked to defects in development, beginning with the catalytic formation of citrate from acetyl-CoA and oxaloacetate by citrate synthase (CS). Double mutants for peroxisomal citrate synthase (CS), *csy2csy3*, fail to perform fatty acid respiration, leading to stalled germination [53]. Single mutants were much more robust and were only phenotypically distinguishable by their reduced growth in sucrose-free media [53]. In another study, inhibition of mitochondrial citrate synthase (CS) in potatoes was found to decrease citrate synthase activity and reduce flower formation, while having no impact on vegetative growth [54]. This may suggest tissue-specific roles for citrate – however, changes in enzyme activity or levels do not necessarily change the levels of TCAC metabolites. A challenge of studying the TCAC is that inhibition of biosynthesis can have minimal effects on the cycle, due to replenishing of the pathway by anaplerosis. Furthermore, TCAC carboxylates like malate and citrate can be dynamically sequestered in vacuoles and exported intracellularly to match cellular demand or exudated for defense and nutrient acquisition [19,55–58]. Despite the flexibility of the TCAC pathway, there are a number of examples demonstrating that alterations in elements of the TCAC pathway have tissue-specific effects in plants. Figure 2 summarizes the lethal and tissue-specific effects of alterations in the TCAC that have been characterized in different plant organs across different species. Next, we highlight several examples of this research, focusing on two important steps in the TCAC: isocitrate dehydrogenase and succinate dehydrogenase reactions.

Recently, Isocitrate Dehydrogenases (IDH) was shown to be regulated by redox state, which is critical in developing tissues [59,60]. Mitochondrial IDH is inactivated under oxidation and reactivated by thioredoxindependent reduction in Arabidopsis [61], while cytosolic IDH is regulated by glutathionylation [62]. Overexpression of IDH in poplar trees resulted in taller plants with longer internodes, a phenotype consistent with the increased expression of cataplerotic glutamine synthase seen in these transgenic trees. Additionally, the stem diameter and vascular system width in overexpression (OE) lines were increased compared to wild type [63]. In contrast to poplar, overexpressing maize IDH in Arabidopsis resulted in shorter primary roots and increased sensitivity to salt stress [37]. Other studies have also shown that alterations of TCAC biosynthesis genes can have different effects depending on the species, which raises challenges in studying and engineering the pathway for crop improvement.

Succinate dehydrogenase (SDH), or Complex II of the electron transport chain, is a key enzyme that directly connects the TCAC to cellular respiration. SDH oxidizes succinate to fumarate in the mitochondria and transfers electrons to ubiquinone, the critical substrate for Complex III of the Electron Transport Chain (ETC). SDH can be allosterically regulated by both ATP and ADP [64]. SDH plays complex roles in various physiological processes in plants, including photosynthesis, stomatal function, ROS regulation, root growth, and fungal defense [23,36]. Knock-out of *SDH1-2* in Arabidopsis does not show any obvious phenotypes, but knock-out of *SDH1-1* in Arabidopsis results in failed gametophyte development [65]. Similarly, antisense inhibition of *SDH1-1* leads to pollen abortion and

reduced seed set, showing that SDH1-1 has an important role in reproductive development [65]. Interestingly, while heterozygous *SDH1-1/sdh1-1* shows improved photosynthesis and resiliency in nitrogen-limited conditions due to enhanced stomatal conductance and nitrogen assimilation, homozygous *sdh1* is lethal [66]. In rice, LPS1, which encodes the iron-sulfur subunit *SDH2-1* of succinate dehydrogenase, has been shown to affect both leaf senescence and grain yield [67]. Knockdown of *SDHAF2*, an assembly factor for *SDH1* in Arabidopsis, exhibits an inhibition of primary root growth but has normal leaves with unaffected stomatal conductance and photosynthetic rate. However, knockout of *SDHAF2* leads to seed abortion [68]. High-resolution measurements of TCAC metabolites in developing roots using mass spectrometry imaging revealed that succinate is enriched in the root meristem. In contrast, aconite, malate, and fumarate were all found to be enriched in differentiating tissue. Exogenous succinate treatment leads to increases in meristematic cell divisions and root hair growth [69]. The effects on root hairs correspond with increases in H_2O_2, a predominant form of ROS shown to accumulate in bulging root hairs and necessary to activate Ca^{2+} influx channels for elongation [70,71]. It has also been demonstrated that SDH is a direct source of mitochondrial ROS production capable of affecting broad aspects of plant development including branching, cell signaling, cell cycle and stress response [36]. In mammals, the relationship between SDH and ROS has mainly been studied in the context of elevated ROS in cancer cells [72]. However, a mammalian study demonstrated that succinate can regulate stem cell differentiation through chromatin modifications [73]. Future work may help clarify the contribution of the ETC, ROS, epigenetics, and other mechanisms to the developmental phenotypes induced by alterations in succinate levels and SDH activity.

Crosstalk between the TCAC and phytohormone pathways is critical for development and stress response

A key set of mechanisms that link the TCAC to development are the connections between the cycle and phytohormone signaling. Phytohormones, including auxin, gibberellin (GA), jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA), are critical for the regulation of plant development and stress response. The TCAC metabolites affect the production of hormones and other signaling molecules in plants, while hormones also regulate TCAC metabolism [74]. The following work described represents some of the important research describing this intertwined relationship.

As mentioned previously, there are regulatory links between pyruvate and auxin biosynthesis. Additional research has identified IAR4, a subunit of mitochondrial pyruvate dehydrogenase (PDH), by its resistance to IAA-Alanine treatment in Arabidopsis [75]. It was found that *iar4* plants make fewer root hairs, show reduced lateral root number, and have disruptions in auxin homeostasis. Despite IAA-amino conjugates levels being significantly altered in *iar4* plants, the phenotype can be rescued by overexpressing the YUCCA1 enzyme, which increases auxin biosynthesis [76]. Another phenotype of the *iar4* mutant was increased sensitivity to salt stress, as measured by its inhibited primary root growth and reduced survival. Moreover, salt stress inhibited levels of the auxin transporter (PIN1, PIN2 and PIN3) and DR5-GFP in *iar4* [77]. Treatment with glutathione, a ROS scavenger, and exogenous IAA can both rescue the *iar4* phenotype during salt stress,

indicating that IAR4 may function as a mediator between ROS and auxin to regulate the root growth [77]. Auxin and pyruvate have also been linked through *MAB1*, which encodes a mitochondrial PDH Complex (PDC) E1β subunit with decreases PDC enzymatic activity. The *mab1* mutant has increased pyruvate levels and defects in auxin efflux [51]. Recent enzymatic activity assays confirm that pyruvate participates in the TAA-YUC mediated IAA synthesis pathway. Cytoplasmic localization of TAA1 converts tryptophan to alanine and IPyA, an IAA precursor [52]. Overall, this work demonstrates a direct relationship between auxin and pyruvate.

The knockdown of Succinate Dehydrogenase Assembly Factor 2 (SDHAF2), which is required for activity of the succinate dehydrogenase complex in Arabidopsis, provides another example of TCAC interactions with auxin signaling. Knocking down *SDHAF2* alters pH-dependent root elongation; *sdhaf2* plants have decreased DR5-GUS signal, but increased IAA in the root tip. Increased IAA in *sdhaf2* is thought to be caused by the mutant's effect on ROS levels, which regulates auxin [78]. In addition to auxin, SDH also interconnects with ABA and SA. SDH2-3 expression during seed maturation is regulated by ABA [79] and SDH is shown to be required for SA-dependent H_2O_2 production during stress [80]. In cotton, SDH1-1 has been shown to contribute to SA-mediated systemic resistance to *Verticillium dahlia*, a fungal pathogen [81]. However, many of the molecular mechanisms that enable SDH interactions with phytohormones are still unclear, and it would be informative to investigate the relationships between succinate, SDH, ROS, and hormones in plants.

TCAC metabolites have also been found to interact with other phytohormones pathways that regulate growth, development, abiotic stress response, and defense. Inhibition of mitochondrial malate dehydrogenase (MDH) in tomato reduces GA and ABA levels [82]. In addition, malate is reduced under ABA treatment in *Brassica napus* [83]. Recently, it was demonstrated that brassinosteroid (BR) signaling regulates phosphate-starvation induced malate secretion in Arabidopsis [84]. 2-OG is a precursor of the gibberellin biosynthesis pathway [85] and antisense of 2-OGDH transgenic plants displayed changes in both gibberellin and ABA responses, alterations in the GABA shunt, and perturbed levels of TCAC metabolites [86]. Interestingly, 2-OG can be a substrate for a range of oxidative reactions, including plant hormone biosynthesis. For example, the rice 2-OG-dependent (Fe II) dioxygenase degrades IAA to OxIAA, which is important for regulating IAA homeostasis in plants [87]. In another example, citrate and fumarate prime against early bacterial infections by inducing SA and camalexin accumulation [58]. Finally, citrate binds to trigger a confirmational switch in D3/MAX2, an F-box E3 ubiquitin ligase critical for strigolactone perception and signaling [88]. This switch has a major regulatory effect on strigolactone-mediated protein degradation and is a prime example demonstrating the ability of TCAC metabolites to bind to regulatory proteins and directly affect developmental processes.

Importantly, phytohormone homeostasis also regulates TCAC metabolism. For example, upregulating the auxin signaling gene *SlIAA9* in tomato increases the rate of flux of the TCAC, whereas downregulating auxin signaling diminishes it [89]. In barley, auxin is required for the influx of hexoses in glycolysis and causes increases in pyruvate, citrate,

and succinate levels [90]. As previously described, ABA regulates malate biosynthesis. ABA signaling can also modulate the TCAC metabolism via the SnRK2 pathway [91]. Jasmonate-depletion in Arabidopsis shifts production of the TCAC metabolites. While citrate, aconite, isocitrate, malate and fumarate increase, 2-OG and oxaloacetate are reduced [92]. Finally, SA can bind to the E2 subunit of AKG dehydrogenase, leading to a subsequent decrease in 2-OGDH activity. This reduction in activity then affects both the mitochondrial electron transport chain and basal pathogen resistance [93,94]. Further exploration of the mechanisms that regulate crosstalk between hormones and TCAC metabolism may lead to important regulatory controls and checkpoints in development and stress response.

Conclusion

The TCAC has many non-canonical functions in plants, and elements of the TCAC are important in every known facet of plant development. Evidence suggests that is not only due to their essential as energy sources, but that the metabolites can also bind signaling proteins, affect redox states, and serve as precursors for phytohormone pathways. In turn, numerous hormones have been shown to regulate the TCAC pathway. Optimizing TCAC metabolism in plants to increase growth has shown promise, but still faces many unsolved challenges. For example, while upregulating biosynthetic enzymes in the TCAC pathway can improve growth in certain plants and tissues, there are variable responses to pathway perturbation across tissues and species. Therefore, understanding the mechanisms underlying the effects of TCAC metabolites in specific crops is critical for future agricultural applications. In particular, it will be important to understand how to precisely control spatial distribution of TCAC metabolites in specific tissues and cellular compartments. Furthermore, temporal control of TCAC metabolites may also be essential for promoting desirable effects at specific developmental stages, without compromising overall metabolism during the lifetime of the plant. Finally, elucidating key regulatory mechanisms, such as protein-metabolite interactions, will be vital for robust engineering of desirable traits using the pathway. In animals and plants, TCAC metabolites have been shown to bind to signaling proteins, regulate epigenetic states, modify proteins to alter their function, and contribute to signaling pathways. Due to the challenges of investigating non-canonical functions of TCAC metabolites, there may be many more examples of similar regulatory processes that depend on TCAC metabolites. Elucidation of such mechanisms will be valuable for deepening our understanding of these essential compounds.

Acknowledgments

This work was supported by the National Science Foundation (Collaborative EAGER 2028649 to AJD), the National Institute of Health (NIGMS 1R35GM147216 to AJD, the Hellman Scholars Foundation (to AJD), the Revelle Provost Summer Research Scholarship (to JTP), and the Genentech Scholars Program (to JTP).

Data availability

No data was used for the research described in the article.

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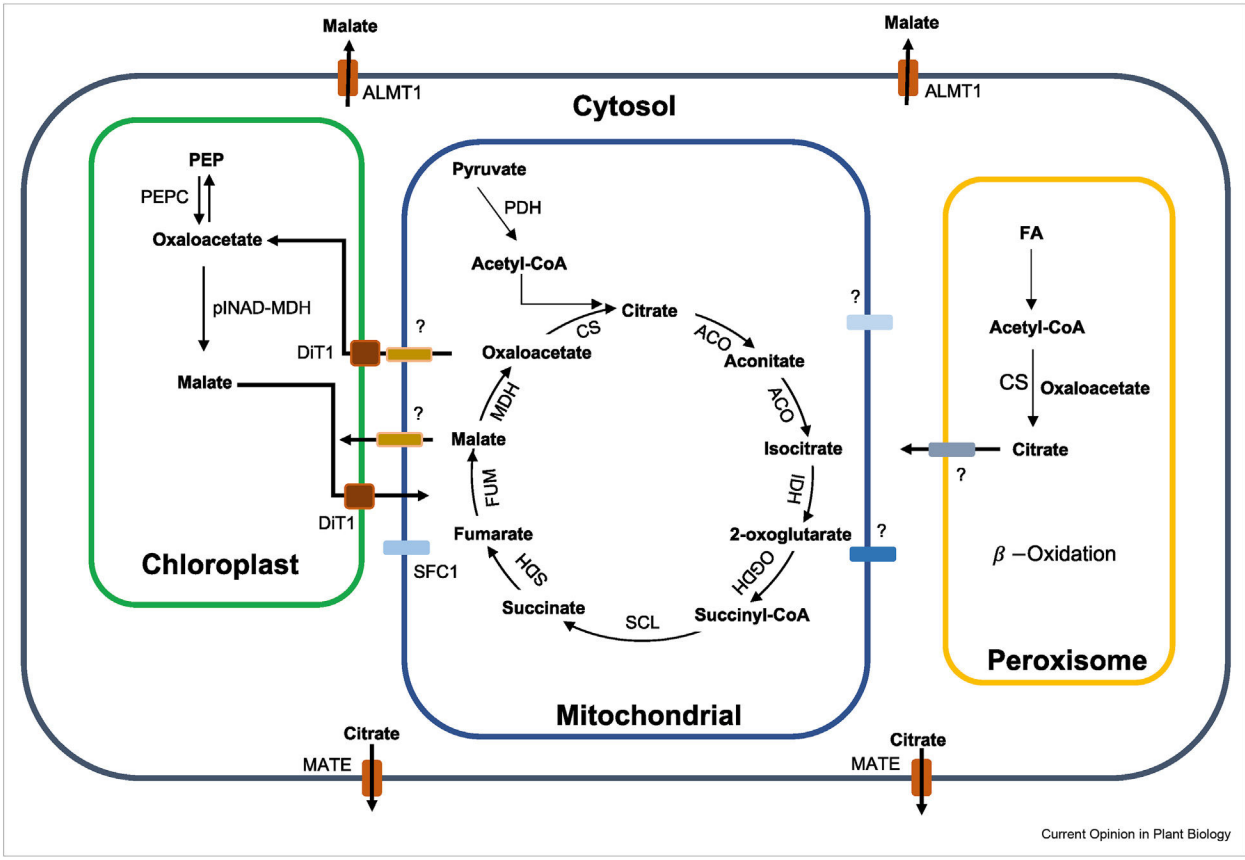

Figure 1.

A model depicting TCAC metabolites biosynthesis and distribution in the plant cell. Abbreviations: PDH, pyruvate dehydrogenase; CS, citrate synthase; ACO, aconitase; IDH, isocitrate dehydrogenase; 2-OGDH, 2-oxoglutarate dehydrogenase; SCL, succinyl-CoA ligase; SDH, succinate dehydrogenase; FUM, fumarase; MDH, malate dehydrogenase; FA, fatty acids. The cytosol, mitochondria, chloroplast, and peroxisome are respectively labeled. The different color boxes shown in the cellular compartments indicate TCAC metabolites transporters or carriers – DiT1, Dicarboxylate transporter 1, located in chloroplast membrane [95]; ALMT1, malate transporter, located in cell membrane; MATE, citrate transporter, located in cell membrane; SFC1, succinate/fumarate carrier 1, AtSFC1 transports citrate, isocitrate and aconite and, to a lesser extent, succinate and fumarate [96]; ?, unidentified TCAC transporters or carriers.

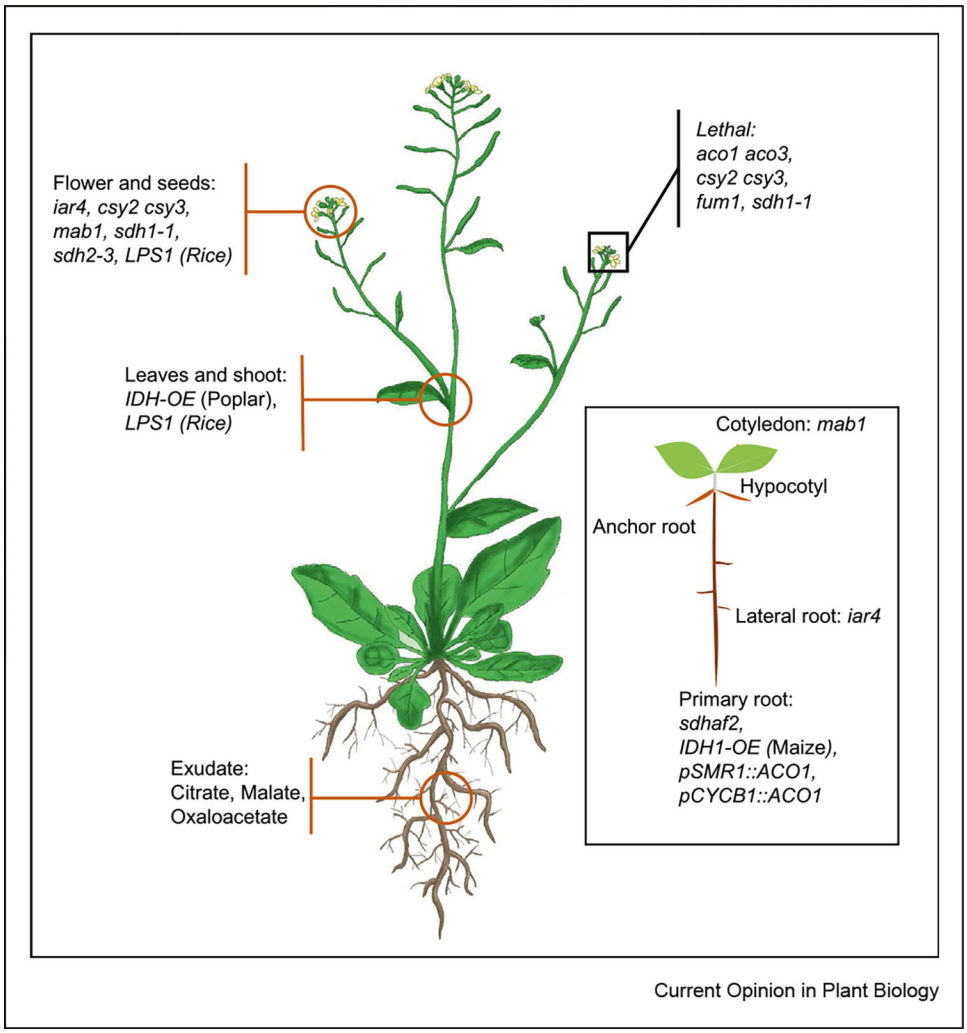

Figure 2.

Schematic showing genetic elements of the TCAC that have specific effects on the growth and development of distinct plant tissues. Plant organs in seedlings and mature plants are labeled and TCAC related genes with effects on those tissues are listed. Unless otherwise labeled, all genes are from Arabidopsis. *IDH1-OE* (Maize) specifically means *IDH1* from maize transformed into Arabidopsis. The other genes from non-Arabidopsis plants were studied in their native context, as described in the text. Citrate, malate, and oxaloacetate are labeled as known TCA metabolites that are exudated in Arabidopsis. Abbreviations: OE, overexpression. All other abbreviations can be found in Figure 1 *iar4* [76], *csy2 csy3* [53], *mab1* [51] *LPS1* (Rice) [67], *fum1* [97], *aco1 aco3* [98], *sdh1-1* [65], *sdh2-3* [79], *IDH-OE* (Poplar) [63], *IDH1-OE* (Maize) [37], *pSMR1::ACO* and *pCYCB1::A-CO1* [69].