

Mild reductions in cytosolic NADP-dependent isocitrate dehydrogenase activity result in lower amino acid contents and pigmentation without impacting growth

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Abstract Transgenic tomato (*Solanum lycopersicum*) plants were generated targeting the cytosolic NADP-dependent isocitrate dehydrogenase gene (*SIICDH1*) via the RNA interference approach. The resultant transformants displayed a relatively mild reduction in the expression and activity of the target enzyme in the leaves. However, biochemical analyses revealed that the transgenic lines displayed a considerable shift in metabolism, being characterized by decreases in the levels of the TCA cycle intermediates, total amino acids, photosynthetic pigments, starch and NAD(P)H. The plants showed little change in photosynthesis with the exception of a minor decrease in maximum photosynthetic efficiency (F_v/F_m), and a small decrease in growth compared to the wild type. These results reveal that even small changes in cytosolic NADP-dependent isocitrate dehydrogenase activity lead to noticeable alterations in the activities of enzymes involved in primary nitrate assimilation and in the synthesis of 2-oxoglutarate derived amino acids. These data are discussed within the context of current models for the role of the various isoforms of isocitrate dehydrogenase within plant amino acid metabolism.

Keywords Cytosolic isocitrate dehydrogenase · *Solanum lycopersicum* · Mitochondria · TCA cycle · Nitrogen metabolism · Amino acid biosynthesis

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Introduction

An important role for 2-oxoglutarate (2OG) has been proposed within ammonium assimilation on the basis of studies of plants exposed to various environmental or genetic perturbations (Hodges 2002; Hodges et al. 2003; Dutilleul et al. 2005; Fritz et al. 2006; Schneidereit et al. 2006). 2OG is required as the primary carbon acceptor for ammonium in the GOGAT pathway. It has been proposed that this 2OG is either synthesized by NAD-dependent isocitrate dehydrogenase (IDH), which catalyses a tricarboxylic acid (TCA) cycle reaction wherein isocitrate is oxidized and decarboxylated to yield 2OG, NADH and CO₂ (Siedow and Day 2000) or by NADP-dependent isocitrate dehydrogenase (ICDH; Chen 1998). Whilst the NAD-dependent IDH is located exclusively in the mitochondrial matrix, there are several isoforms of the NADP-dependent isoforms, located in different organelles, such as the chloroplasts (Gálvez et al. 1994), mitochondria (Gálvez et al. 1998), peroxisomes (Corpas et al. 1999) and in the cytosol (Gálvez et al. 1996).

In a previous study, we evaluated the role of the mitochondrial NAD-dependent IDH by generating transgenic plants displaying mild deficiency in the expression of this gene (Sienkiewicz-Porzucek et al. 2010). These plants exhibited slight decreases in the levels of amino acids, intermediates of the TCA cycle, photosynthetic pigments, starch, and NAD(P)H, increased levels of nitrate and increased total protein hinting at compensatory mechanisms to maintain 2OG synthesis. The vast majority of the NADP-requiring ICDH activity is due to a cytosolic isoform. This accounts for 95% of total NADP-ICDH activity in green tobacco leaf tissue, and is the predominant isoform of at least 15 species (Gallardo et al. 1995; Gálvez et al. 1996; Palomo et al. 1998; Chen, 1998). Similarly, although

the potato cytosolic NADP-ICDH gene was active in all plant organs, its expression was highest in green tissues, flowers and roots (Fieuw et al. 1995). Chen and Gadal (1990) proposed that NADP-ICDH function in GS/GOGAT-dependent nitrogen assimilation, as a part of a cytosol-localized pathway that produces carbon skeletons for ammonium assimilation in circumstances in which TCA cycle activity is decreased. Due to the preferential expression of this isoform in mature leaf veins, NADP-ICDH has also been postulated to be involved in cycling, redistribution and export of amino acids, in addition to its primary function in GS/GOGAT-dependent nitrogen assimilation. It was, therefore, surprising to find that both transgenic potato (Kruse et al. 1998) and tobacco (Gálvez et al. 1999) antisense plants displaying only 6–10% residual ICDH activity showed neither growth nor developmental phenotypes. Since then, considerable research effort has been expended in an attempt to elucidate the metabolic and physiological functions of the various isoforms of isocitrate dehydrogenase (Hodges et al. 2003; Lemaitre et al. 2007). In light of the strategic positional importance of the cytosolic ICDH, studies investigating its metabolic importance in plants remain surprisingly scarce, and generally correlative in nature (Scheible et al. 2000; Bläsing et al. 2005; Urbanczyk-Wochniak et al. 2005).

As part of our ongoing project to determine the function of the TCA cycle in illuminated leaves, we have characterized the *Aco1 Solanum pennellii* mutant deficient in aconitase expression (Carrari et al. 2003), as well as transgenics of cultivated tomato deficient in the expression of the mitochondrial isoforms of malate dehydrogenase (Nunes-Nesi et al. 2005), fumarase (Nunes-Nesi et al. 2007), succinyl-CoA ligase (Studart-Guimarães et al. 2007) citrate synthase (Sienkiewicz-Porzucek et al. 2008) and NAD-isocitrate dehydrogenase (Sienkiewicz-Porzucek et al. 2010). These studies along with those in potato tuber material in which the activity of the 2-oxoglutarate dehydrogenase complex (2OGDH) was chemically inhibited (Araújo et al. 2008), have allowed us to establish important roles for constituent enzymes of the TCA cycle in the maintenance of photosynthetic efficiency, and in the supply of carbon for amino acid biosynthesis. In this study, we turn our attention to the evaluation of the importance of the cytosolic NADP-dependent isocitrate dehydrogenase for metabolism in the illuminated tomato leaf. We describe here the generation of tomato transgenic plants exhibiting mild decreases in the expression of this gene. The transgenic lines generated were characterized at transcriptional, biochemical and physiological levels. The present results are discussed in the context of current models concerning the importance of the TCA cycle and of the coordination of plant CN metabolism in the illuminated leaf.

Results

Transgenic NADP-dependent ICDH plants showed no change in time of flowering or growth rate

A 527-bp fragment of the tomato gene encoding *SIICDH1* was cloned using the RNA interference (RNAi) design (pK7GWIWG2; Karimi et al. 2002). The RNAi construct was inserted between the cauliflower mosaic virus (CaMV) 35S promoter and the octopine synthase (*ocs*) terminator. Following *Agrobacterium tumefaciens*-mediated transformation, we transferred 25 ICDH transformed tomato plants to the greenhouse. A first screening of the lines was performed by measuring total cellular ICDH activity (data not shown). These preliminary studies allowed the identification of two lines that showed a statistically significant and specific reduction in ICDH1 expression and total ICDH activity in leaves. These two transgenic lines were clonally propagated and transferred to the greenhouse alongside wild type controls. Following a period of 5 weeks growth, leaves were harvested in the middle of the light period, and total ICDH and NAD-dependent IDH activities were measured in order to confirm the reduced activity of NADP-dependent ICDH (Fig. 1a) and its effects on total NAD-dependent IDH activity (Fig. 1b). To verify the specificity of the constructs as well as to ensure that no compensatory effect involving increased expression of the others isoforms occurred, a secondary screen was performed at the mRNA level, using an established quantitative RT-PCR protocol (Czechowski et al. 2004). This revealed that only *SIICDH1* expression was significantly reduced in the transgenic lines. Moreover, the expression of non-targeted isoforms was unaltered in the transformants (Fig. 1c). When taken together, these results demonstrate that the two lines (ICDH10 and ICDH20), are suitable for assessing the transcriptional, biochemical and physiological effects of a mild reduction of the cytosolic ICDH isoform activity.

For phenotypic characterization, we grew the transgenic plants in the greenhouse side by side with wild type controls. After 5 weeks growth no clear difference could be observed in the growth of aerial parts of the plant (data not shown). Close inspection of the transformants revealed only a slight decrease in root dry mass (Fig. 1d), stem (Fig. 1e) and leaf (Fig. 1f) biomass. From these results it follows that the transformants displayed an unaltered total biomass accumulation (Fig. 1g). This decrease was non-significant for line ICDH20 and only weakly significant for line ICDH10. Given that antisense inhibition of citrate synthase led to inhibition of flower formation in potato plants (Landschütze et al. 1995) and changes in fruit yield were observed in several tomato genotypes deficient in TCA cycle enzymes (Carrari et al. 2003; Nunes-Nesi et al.

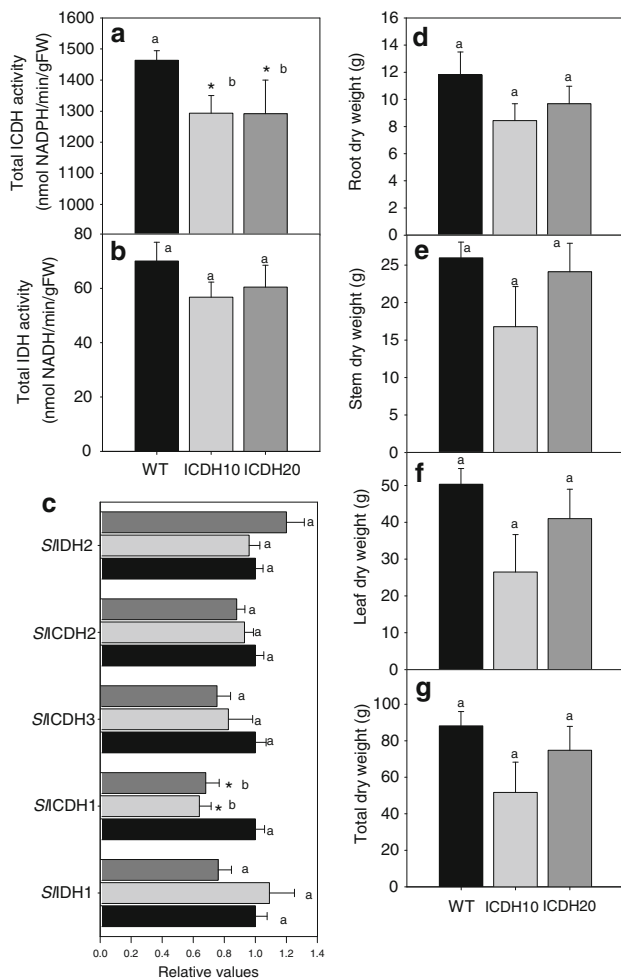


Fig. 1 Characterization and expression of tomato cytosolic isocitrate dehydrogenases. Total NADP-dependent (a) and NAD-dependent (b) isocitrate dehydrogenase activities were determined in 5-week-old leaves taken from fully expanded source leaves of transgenic plants with altered expression of *S/ICDH1* as compared to wild type. c The relative transcript abundance of the various cellular isoforms of isocitrate dehydrogenase. The abundance of isocitrate dehydrogenase mRNAs was measured by quantitative RT-PCR. Growth phenotype of ICDH transgenic tomato plants: d total root dry weight, e total stem dry weight, f total leaf dry weight and g total plant dry weight of 6-weeks old plants. Values are presented as mean \pm SE ($n = 5$). An asterisk indicates significantly different values obtained for each line in comparison to wild type as determined by the *t* test. Values marked with a different letter were determined by an ANOVA-Tukey test to be significantly different ($P < 0.05$) from each other. *S/IDH1* (TC193092), *S/ICDH1* (TC202045); *S/ICDH3* mitochondrial ICDH like protein (TC196623); *S/ICDH2* (TC164449); *S/IDH2* (TC198615). Black bar wild type, light gray bar line ICDH10, gray bar line ICDH20

2005, 2007; Studart-Guimarães et al. 2007) we next evaluated flower and fruit production in the transgenic lines. For this purpose, we grew the transgenics until full maturity counting daily the number of flowers produced. This study showed that the flowering time was not affected by the reduction in cytosolic ICDH. The total number of flowers produced by the transgenic and wild type plants

were similar (data not shown), resulting in an unaltered number of fruits produced (data not shown).

Photosynthetic carbon metabolism in the cytosolic ICDH transformants

Analysis of the carbohydrate content of leaves from 5-week-old plants during a diurnal cycle revealed that the transformants were characterized by unchanged levels of sucrose (Fig. 2a), glucose and fructose (data not shown), but a 30% reduction in the level of starch (Fig. 2b).

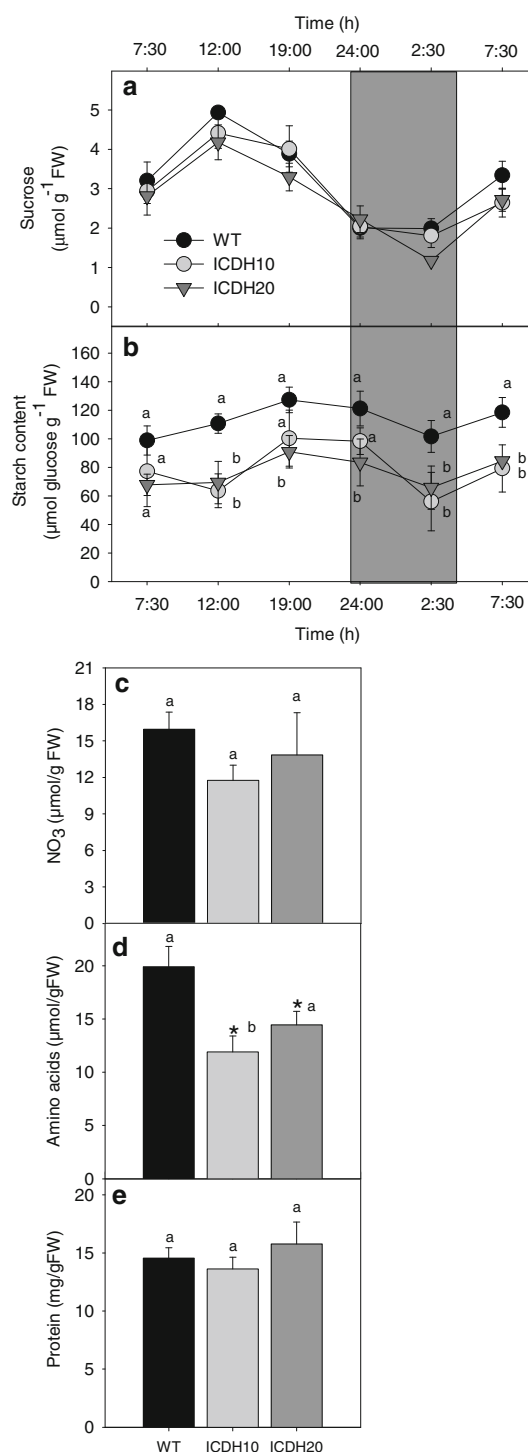
In order to verify whether the reduction in the activity of ICDH results in changes in other major pathways of primary metabolism we used an established GC-MS protocol for metabolite profiling which allows us to quantify the relative levels of around 70 metabolites (Fernie et al. 2004). This study revealed considerable differences between the two transgenic lines and the wild type (Table 1). There were several notable changes in the contents of a range of amino acids. Namely, alanine (both lines), glutamine (both lines), arginine, asparagine, aspartate, glutamate, glycine and proline (both lines) were all significantly reduced, whilst tyramine (line ICDH10) and isoleucine (line ICDH20) were significantly increased. It is noteworthy that glutamine decreased by more than 50%, even though 2OG is required to allow further metabolism of glutamine to glutamate. It is also intriguing that glutamate (which is the product of the reaction in which 2OG reacts with glutamine) decreased by only 20–25% whereas other amino acids showed larger decreases.

Levels of 2OG and isocitrate were unchanged in the transformants. This may be because interconversion of 2OG and isocitrate can be catalyzed by multiple isoforms of ICDH, and IDH. Despite this, other TCA cycle intermediates such as fumarate (both lines) and succinate (line ICDH10) were significantly reduced in the transformants. Several other interesting changes were also observed such as the significant reduction in threonate (line ICDH10), maleate (both lines), glycerate (line ICDH10), citramalate (line ICDH10) and an increase in pyruvate (both lines).

Reduction of cytosolic ICDH activity results in slight decreases in nitrate levels and reduced total amino acids

Given the considerable changes in amino acid metabolism we next decided to evaluate nitrate assimilation and metabolism in leaves of the transformants. For this purpose, we analyzed by spectrophotometry the content of nitrate, total amino acids and total insoluble protein in leaves. These studies revealed slight decrease in the levels of nitrate (Fig. 2c). The total amino acid levels were significantly reduced in both lines (Fig. 2d), whilst protein levels were unaltered in both lines (Fig. 2e).

Fig. 2 Diurnal changes in sucrose (a) and starch (b) content in leaves of 6-week-old ICDH transgenic tomato plants. At each time point, samples were taken from mature source leaves, and the data presented are mean \pm SE ($n = 6$). Nitrate (c), total amino acids (d) and protein (e) contents in leaves of ICDH transgenic tomato plants. The leaf material was harvested in the middle of the light period from 5-week-old plants. The data presented are mean \pm SE ($n = 6$). An asterisk indicates significantly different values obtained for each line in comparison to wild type as determined by the *t* test. Values marked with a different letter were determined by an ANOVA-Tukey test to be significantly different ($P < 0.05$) from each other. The lines used were: wild type, black circles; ICDH10, gray circles; ICDH20, gray triangles. Shaded background indicates the dark period



Pigment contents in the transgenic lines

Considering the above results, we determined the levels of the photosynthetic pigments because these compounds have often been reported as important indicators of nitrogen deficiency (Gaude et al. 2007; Sienkiewicz-Porzucek et al. 2008). In accordance with our previous studies with mitochondrial citrate synthase antisense plants (Sienkiewicz-Porzucek et al. 2008), high-performance liquid chromatography (HPLC) analysis revealed a general decrease in pigment content in the transformants. Chlorophylls *a* (both lines) and *b* (line ICDH20) were both significantly reduced in the transformants as was lutein (line ICDH20), violaxanthin (both lines) and neoxanthin (line ICDH20), while the levels of β -carotene, zeaxanthin and antheraxanthin were unaltered (Fig. 3a).

Reduction of cytosolic ICDH activity has no effect on the relative electron transport or assimilation rates and a minor reduction in the maximum efficiency of PSII

Since we used the constitutive CaMV 35S promoter to drive transgene expression in this study, we concentrated the current investigation on leaf metabolism. In addition to that, we have previously reported alterations in photosynthetic performance in tomato genotypes displaying reduced activity of aconitase (Carrari et al. 2003), mitochondrial malate dehydrogenase (Nunes-Nesi et al. 2005) and fumarase (Nunes-Nesi et al. 2007), as well as altered dark respiration in genotypes deficient in citrate synthase (Sienkiewicz-Porzucek et al. 2008). Given this, we next analyzed whether the ICDH transformants exhibited alterations in these parameters. For this purpose, we performed direct measurement of gas exchange under photon flux densities (PFD) that ranged from 0 to $1,200 \mu\text{mol m}^{-2} \text{s}^{-1}$. In contrast to our previous studies with lines with decreased activities of other enzymes involved in organic acid metabolism, we observed no major differences in either assimilation rate or dark respiration in the ICDH antisense lines (data not shown).

Moreover, when we measured the rate of chloroplastic electron transfer, this parameter was found to be unaltered in the transformants (data not shown). However, when we closely evaluated chlorophyll fluorescence parameters a minor but significant reduction in the PSII maximum efficiency after dark adaptation (F_v/F_m) was observed in both lines (WT, 0.81 ± 0.001 ; ICDH10, 0.79 ± 0.002 ;

Table 1 Relative metabolite content of fully expanded illuminated leaves of 5-week-old plants of the ICDH transgenic tomato plants

	WT	ICDH10	ICDH20
Amino acids			
β -Alanine	1.000 \pm 0.027a	0.831 \pm 0.081a	1.002 \pm 0.050a
Alanine ^a	1.000 \pm 0.170a	0.505 \pm 0.127a	0.475 \pm 0.081a
Arginine	1.000 \pm 0.096a	0.589 \pm 0.135b	1.062 \pm 0.079a
Asparagine	1.000 \pm 0.047a	0.367 \pm 0.053b	1.293 \pm 0.384a
Aspartic acid	1.000 \pm 0.048a	0.361 \pm 0.055b	0.931 \pm 0.107a
GABA	1.000 \pm 0.036a	1.063 \pm 0.060a	0.944 \pm 0.074a
Glutamic acid	1.000 \pm 0.024a	0.798 \pm 0.059b	1.052 \pm 0.047a
Glutamine	1.000 \pm 0.152a	0.421 \pm 0.139b	0.482 \pm 0.118b
Glycine	1.000 \pm 0.063a	0.496 \pm 0.083b	0.915 \pm 0.055a
Isoleucine ^a	1.000 \pm 0.069a	1.287 \pm 0.145a	1.284 \pm 0.023a
Phenylalanine	1.000 \pm 0.051a	1.119 \pm 0.081a	0.846 \pm 0.064a
Proline	1.000 \pm 0.065a	0.230 \pm 0.032c	0.533 \pm 0.070b
Serine	1.000 \pm 0.045a	0.794 \pm 0.093a	1.017 \pm 0.057a
Tryptophan	1.000 \pm 0.087a	1.297 \pm 0.233a	0.998 \pm 0.114a
Tyramine	1.000 \pm 0.083a	1.574 \pm 0.194b	1.015 \pm 0.070a
Tyrosine	1.000 \pm 0.058a	1.349 \pm 0.189a	0.961 \pm 0.081a
Valine	1.000 \pm 0.054a	1.338 \pm 0.158a	1.076 \pm 0.045a
Organic acids			
Citric acid	1.000 \pm 0.059a	1.053 \pm 0.048a	0.928 \pm 0.068a
Fumaric acid ^a	1.000 \pm 0.065a	0.618 \pm 0.120b	0.740 \pm 0.090a
2OG	1.000 \pm 0.098a	0.982 \pm 0.055a	1.250 \pm 0.225a
Glyceric acid	1.000 \pm 0.063a	0.712 \pm 0.040b	1.117 \pm 0.083a
Glycolic acid	1.000 \pm 0.044a	0.850 \pm 0.069a	1.030 \pm 0.081a
Isocitric acid	1.000 \pm 0.051a	0.868 \pm 0.054a	0.847 \pm 0.154a
Lactic acid	1.000 \pm 0.060a	0.919 \pm 0.055a	0.967 \pm 0.087a
Maleic acid	1.000 \pm 0.074a	0.625 \pm 0.038b	0.675 \pm 0.066b
Citramalic acid	1.000 \pm 0.046a	0.739 \pm 0.048b	0.848 \pm 0.060a
Malic acid	1.000 \pm 0.022a	0.935 \pm 0.037a	1.007 \pm 0.057a
Pyruvic acid ^a	1.000 \pm 0.067a	1.485 \pm 0.104b	1.247 \pm 0.055a
Saccharic acid	1.000 \pm 0.091a	0.957 \pm 0.092a	1.500 \pm 0.264a
Succinic acid ^a	1.000 \pm 0.080a	0.746 \pm 0.075a	0.835 \pm 0.047a
Threonic acid	1.000 \pm 0.087a	0.372 \pm 0.047b	0.924 \pm 0.024a
Sugars			
Fructose	1.000 \pm 0.036a	1.057 \pm 0.039a	1.060 \pm 0.063a
Glucose	1.000 \pm 0.029a	0.938 \pm 0.054a	1.052 \pm 0.040a
Maltose ^a	1.000 \pm 0.047a	0.848 \pm 0.036a	1.180 \pm 0.101a
Psicose	1.000 \pm 0.031a	0.946 \pm 0.085a	0.717 \pm 0.067b
Sucrose	1.000 \pm 0.035a	1.044 \pm 0.032a	1.059 \pm 0.062a
Trehalose	1.000 \pm 0.045a	0.858 \pm 0.087a	0.830 \pm 0.092a
Xylose ^a	1.000 \pm 0.076a	0.780 \pm 0.053a	0.967 \pm 0.103a

Values set in bold type were determined by the *t* test to be significantly different from the wild type and values marked with a different letter were determined by the ANOVA-Tukey test to be significantly different (*P* < 0.05) from each other

^a Metabolite values where the significance by ANOVA-Tukey and *t* test differed

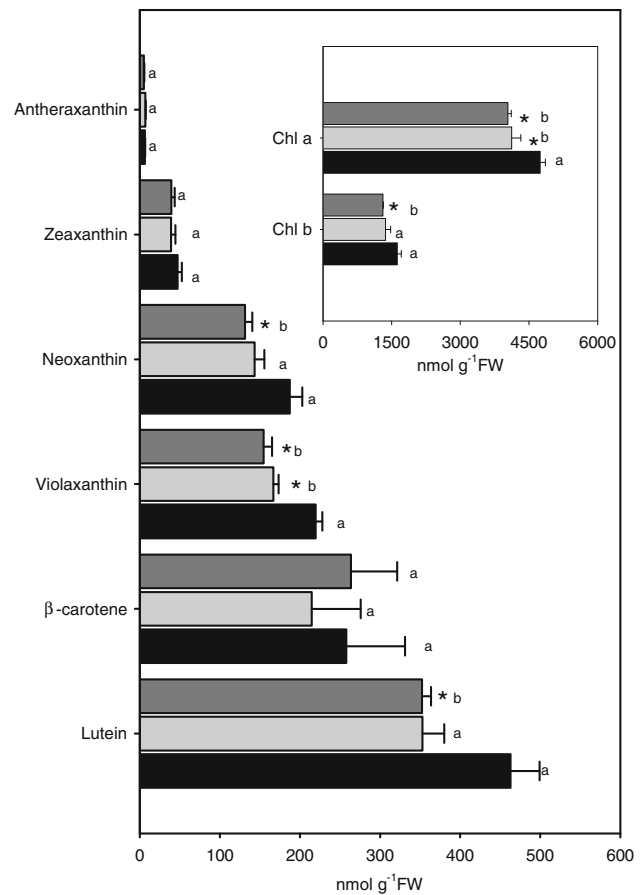


Fig. 3 Pigment content in leaves of ICDH transgenic tomato plants determined in 6-week-old fully expanded source leaves harvested in the middle of the day. Values presented are mean \pm SE of six individual plants per line. An asterisk indicates significantly different values obtained for each line in comparison to wild type as determined by the *t* test. Values marked with a different letter were determined by an ANOVA-Tukey test to be significantly different (*P* < 0.05) from each other. The lines used were: wild type, black bars; ICDH10, light gray bars; ICDH20, gray bars

ICDH20, **0.79 \pm 0.004**; numbers set in bold type are significantly different from the wild type as determined by the *t* test (*P* < 0.05).

Pyridine nucleotide content in ICDH transgenic plants

Since the oxidative decarboxylation of isocitrate to 2OG catalyzed by both IDH and ICDH also results in the production of reduced coenzyme NAD(P)H, it might be expected that reduction in the activity of these enzymes affects the redox balance in the transformants. We therefore decided to assay the levels of pyridine dinucleotides in the leaves of wild type and transformant plants. Interestingly, there was a significant 19–33% decrease of NADH (WT, 53.8 \pm 2.4; ICDH10, **36.1 \pm 2.6**; ICDH20,

Table 2 Enzyme activities in ICDH transgenic lines (nmol min⁻¹ g⁻¹ FW)

	WT	ICDH10	ICDH20
Citrate synthase	138.41 ± 15.18a	133.38 ± 16.17a	148.92 ± 19.78a
Aconitase	351.34 ± 40.94a	334.80a ± 58.79a	464.59 ± 71.03a
GDH (NAD)	226.60 ± 25.16a	289.35 ± 18.06a	327.94 ± 13.82b
Nitrate reductase	321.05 ± 32.76a	351.73 ± 48.42a	368.18 ± 33.05a
Pyruvate kinase	1,108.64 ± 94.20a	897.31 ± 132.58a	1,211.20 ± 83.26a
PEP Carboxylase	1,432.68 ± 96.30a	1,086.12 ± 160.94a	1,390.21 ± 111.76a
Fumarase	5,121.71 ± 853.18a	2,646.24 ± 483.53b	3,400.07 ± 350.61a
Glycerate kinase	1,963.57 ± 135.33a	2,175.45 ± 180.81a	2,226.96 ± 200.21a
MDH (NAD)	75,304.24 ± 4,558.54a	81,992.53 ± 5,414.97a	73,068.12 ± 4,914.24a
MDH total (NADP)	5,464.99 ± 184.15a	4,483.07 ± 186.77b	5,167.04 ± 245.29a
MDH initial (NADP)	795.72 ± 26.40a	657.85 ± 19.51b	703.25 ± 17.74b
MDH activation ^a	0.146 ± 0.003a	0.148 ± 0.007a	0.137 ± 0.007a

Activities were determined in 5-week-old fully expanded source leaves harvested 6 h into the photoperiod. Data presented are mean ± SE of measurements from six independent plants per genotype. Values set in bold type were determined by the *t* test to be significantly different from the wild type and values marked with a different letter were determined by the ANOVA-Tukey test to be significantly different ($P < 0.05$) from each other

FW Fresh weight, PEP phosphoenolpyruvate, MDH malate dehydrogenase, GDH glutamate dehydrogenase

^a Ratio of initial NADP-MDH activity to total NADP-MDH activity

43.6 ± 3.5 nmol NADH/gFW; numbers set in bold type are significantly different from the wild type as determined by the *t* test ($P < 0.05$) in the transgenic lines. There was also a significant, but much smaller, decrease of NADPH (WT, $26.6 ± 0.9$; ICDH10, **$23.5 ± 0.9$** ; ICDH20, **$22.4 ± 1.4$** nmol NADPH/gFW; numbers set in bold type are significantly different from the wild type as determined by the *t* test ($P < 0.05$), NAD and NADP levels were essentially unaltered in all genotypes (data not shown). As a result, a decrease in the NADH/NAD (WT, $1.18 ± 0.11$; ICDH10, **$0.64 ± 0.05$** ; ICDH20, **$0.77 ± 0.06$** ; numbers set in bold type are significantly different from the wild type as determined by the *t* test ($P < 0.05$) and unaltered NADPH/NADP ratios was observed (WT, $0.50 ± 0.04$; ICDH10, $0.51 ± 0.04$; ICDH20, $0.51 ± 0.08$).

Effect of reduction of cytosolic ICDH on the activities of other enzymes of primary metabolism

To better understand the above described changes in metabolites, we next analyzed the maximal activities of a wide range of key enzymes of carbon and nitrogen metabolism (Table 2). Our previous results on the mitochondrial isocitrate dehydrogenase suggested a reduced capacity for nitrate assimilation in the IDH lines (Sienkiewicz-Porzućek et al. 2010). As seen previously for IDH, NAD-dependent glutamate dehydrogenase (GDH) activity was significantly increased (line ICDH20), suggesting that glutamate might be oxidized in the mitochondria in order to sustain the supply of 2OG to the TCA cycle, concomitant to the release of NH₄⁺ (Robinson et al. 1991; Aubert

et al. 2001). We also assayed glycerate kinase, which is a key enzyme of the photorespiratory cycle (Boldt et al. 2005). This enzyme was only slightly increased in both transgenic lines. Considering TCA cycle enzymes, there were no significant differences in the total activities of citrate synthase, aconitase and NAD-dependent malate dehydrogenase. However, the activity of fumarase was reduced (significantly in line ICDH10). As described before, both transgenics lines showed lower NAD(P)H levels, reduced pigment levels and minor reduction in the efficiency of PSII. For that reason we expected changes in the chloroplastic redox state (Scheibe 2004). To further test this hypothesis, we assayed both initial and maximal activities of NADP-dependent malate dehydrogenase and from these data calculated the activation state of the enzyme. The results revealed that both total and initial NADP-MDH activities were reduced in the both ICDH lines, however, no change in the activation state of NADP-MDH was observed.

Discussion

Much research effort has been recently invested in the study of plant photosynthetic energy metabolism in order to better understand the functional role of respiratory metabolism in the illuminated leaf. Studies in which components of the mitochondrial electron transport chain were manipulated indicate that its correct functioning is required to sustain optimal rates of photosynthesis (Igamberdiev et al. 2001; Dutilleul et al. 2003; 2005; Bartoli

et al. 2005; Sweetlove et al. 2006). A similar conclusion has been reached in pharmacological approaches (Raghavendra and Padmasree 2003). Mitochondria ATP synthesis contributes to maintenance of the ATP/ADP ratio in the cytosol in the light, at least under photorespiratory conditions (Wigge et al. 1993; Igamberdiev and Gardeström 2003). In the current article, we focussed our effort on understanding the influence of NADP-dependent isocitrate dehydrogenase on photosynthetic metabolism. Inhibition of the cytosolic NADP-dependent isocitrate dehydrogenase resulted in unaltered rate of assimilation and chloroplastic electron transport, but the maximal efficiency of photosystem II (F_v/F_m) was slightly reduced in the transformants. Consistent with this alteration are the changes observed in pigment content such as the reduction in chlorophyll *a* and *b*, violaxanthin, zeaxanthin and lutein (Fig. 3). There are two possible explanations for the reduced levels of pigments. They could reflect the reduced rate of nitrate assimilation and thus reduced supply of precursors, such a hypothesis is supported by the metabolic profiling of these plants which shows large reductions in organic acids and the amino acid pools which they support (Table 1). Alternatively, they could be caused by the altered redox balance of the transgenics, given that their biosynthesis is subject to redox-regulated control (Isaacson et al. 2004).

Despite these rather minor changes in photosynthesis, considerable changes were observed in metabolism. One of the most striking effects was a general decrease in starch accumulation (Fig. 2). It is already well known that diurnal changes in starch as well as organic acid biosynthesis are synchronized to nitrate assimilation (Scheible et al. 1997; Chia et al. 2000; Scheible et al. 2000). Downregulation of the cytosolic ICDH also led to a decrease in total amino acid levels, decrease in fumarate and succinate, increased pyruvate, but did not affect nitrate levels or protein levels (Fig. 2). Many of these changes are similar to those observed following inhibition of the mitochondrial citrate synthase (Sienkiewicz-Porzucek et al. 2008). Interestingly, there are similarities and differences to the response in tomato plants with decreased expression of the mitochondrial NAD-dependent IDH (Sienkiewicz-Porzucek et al. 2010), which in effect catalyses a redundant reaction with respect to 2OG formation, except that the reaction occurs in a different compartment and with a different redox couple. In both cases, a small decrease in activity led to lower starch content, decreased NADH and NADPH, a decreased NADH/NAD ratio, a marked decrease in fumarase activity, and a trend to higher GDH activity. However, whereas a decrease in IDH activity had little effect on the total amino acid content and the levels of most of the individual amino acids, and resulted in an increased nitrate and protein content, a decrease in ICDH activity led to a

50% decrease of the total amino acid content, decreased levels of many of the individual amino acids, a lower nitrate content and an unaltered protein content. Moreover, unlike the situation observed in the mitochondrial NAD-dependent isocitrate dehydrogenase antisense lines there was no effect on the activity of nitrate reductase.

Despite the importance of 2OG in respiration, photorespiration and nitrogen metabolism, it is not yet clear where the major site of production of 2OG resides. This organic acid can in principle either be produced from sugar respiration or by amino acid transamination, following the concerted action of isocitrate dehydrogenases, aminotransaminases and GDH (Lancien et al. 2000). When the current results are taken alongside those of our previous study (Sienkiewicz-Porzucek et al. 2010), it can clearly be stated that both the mitochondrial NAD-dependent isocitrate dehydrogenase and the cytosolic NADP-dependent isocitrate dehydrogenase appear to have an important role in 2OG production in the light. The lines described in the present study displayed a small yet specific significant reduction of the cytosolic NADP-dependent isocitrate dehydrogenase activity, without any effect on the mitochondrial NAD-dependent isocitrate dehydrogenase activity. This did not result in reduction of absolute 2OG levels. However, there were clear reductions in the levels of the amino acids alanine, glutamine and proline, a smaller decrease in glutamate, and decreases of many other amino acids. A small reduction in IDH activity also had no marked effect on 2OG levels, but led to a decrease of proline, glycerate and smaller changes of other organic acids (Sienkiewicz-Porzucek et al. 2010). The minimal change in the level of 2OG in these genetic perturbations is in agreement with previous studies, which have shown that 2OG levels are remarkably constant across a wide range of treatments (Fritz et al. 2006 and refs therein). These results indicate that the 2OG level is tightly controlled, and that decreases in synthesis via one pathway may be compensated by increased synthesis via another pathway.

Two features in the data of the present study and Sienkiewicz-Porzucek et al. (2010) are worthy of note with respect to mechanisms that may compensate for decreased expression of cytosolic ICDH or mitochondrial IDH. Firstly, it is interesting that the RNAi cytosolic NADP-dependent isocitrate dehydrogenase lines and the antisense mitochondrial NAD-dependent isocitrate dehydrogenase lines display elevated levels of GDH. Under the perception of nitrogen-limiting conditions, this enzyme might provide glutamate and maintain nitrogen metabolism (Tercé-Laforgue et al. 2004). Evidence for such a compensatory mechanism has been provided by antisense inhibition of ferredoxin-dependent glutamine-2-oxoglutarate aminotransferase (Fd-GOGAT) tobacco plants (Ferrario-Mery et al. 2002). Secondly, the RNAi cytosolic ICDH lines and

the antisense mitochondrial IDH lines show decreased levels of NADH and NADPH, and a decreased NADH/NAD ratio. It has been proposed that a high ATP or, more probably, a high redox state may restrict the synthesis of 2OG, based on studies of a *N. sylvestris cms* mutant in which mitochondrial complex I is impaired (Dutilleul et al. 2003, 2005), and a meta-analysis of 2OG and glutamate levels in low and high light in wild type tobacco and *rbcS* antisense lines (Fritz et al. 2006). In this case, the decrease in the NADH/NAD and NADPH/NADP ratios in the antisense IDH and RNAi ICDH lines might be part of a compensatory response to maintain 2OG synthesis. Thus, the cytosolic ICDH is involved in the regulation of pyridine dinucleotide, isocitrate and 2OG (Igamberdiev and Gardeström 2003). The deficiency of the cytosolic NADP-dependent isocitrate dehydrogenase clearly results in an alteration of not only the total cellular redox balance but also, as indicated by the activities of the chloroplastic malate dehydrogenase, of the subcellular redox poise. However, whilst these alterations are likely, at least partially, responsible for the large metabolic shifts observed in these plants they have little effect on plant performance or fertility.

Recently, Pracharoenwattana et al. (2010) recently showed that cytosolic fumarase (*FUM2*) accounts for most of the total fumarase activity in Arabidopsis, and that *fum2* knock-out lines have decreased levels of fumarate, lower levels of amino acids in the light, and impaired growth on high nitrogen. Intriguingly, the RNAi cytosolic ICDH lines and the antisense mitochondrial IDH lines showed a 33–50 and >50% decrease in fumarase activity, respectively, and the ICDH lines had significantly lower levels of fumarate. These results add to the evidence for a close link between fumarate accumulation and nitrogen assimilation.

To summarize, in this article, we have demonstrated that minor changes in activity of cytosolic NADP-isocitrate dehydrogenase in tomato leaves has dramatic metabolic consequences but these have little effect on growth or plant performance. Despite this fact, this study allows several important conclusions to be made. It demonstrates that both the mitochondrial NAD-dependent isocitrate dehydrogenase and the cytosolic NADP-dependent isocitrate dehydrogenase have important and partly non-redundant roles in nitrate assimilation. Furthermore, it suggests the operation of compensatory mechanisms to maintain 2OG production which underlines the importance of this metabolite for maintenance of normal cellular function. It is clear that a mild reduction in the cytosolic NADP-dependent isocitrate dehydrogenase led to a slow-down of the TCA cycle, and consequently a likely reduction in the mitochondrial levels of NADH and NADPH. When assessed in the context of studies of the TCA cycle in general the fact that antisense lines of different enzymes of the cycle have

dramatically different phenotypes is highly interesting. When considered together alongside recent genome scale models of Arabidopsis metabolism (Poolman et al. 2009; de Oliveira Dal'Molin et al. 2010) and the lack of congruence between the expression levels (Urbanczyk-Wochniak et al. 2005) and maximum catalytic activities of the constituent enzymes (Gibon et al. 2004), gives strong support for the contention that, as in bacteria, the TCA cycle of plants operates in a modular manner (Sweetlove et al. 2010; Gauthier et al. 2010; Tcherkez et al. 2009). Within such a context it is clear from our work that citrate synthase, the mitochondrial NAD-dependent isocitrate dehydrogenase, the cytosolic NADP-dependent isocitrate dehydrogenase and perhaps also 2OG dehydrogenase would form such a functional module within the illuminated leaf with responsibility for the provision of the precursors for amino acid biosynthesis during nitrate assimilation.

Methods

Materials

Tomato (*Solanum lycopersicum*) 'Moneymaker' was obtained from Meyer Beck. Plants were handled as described in the literature (Carrari et al. 2003). All chemicals and enzymes used in this study were obtained from Sigma and Roche Diagnostics.

cDNA cloning and expression

A 527-bp fragment of the cytosolic *SIIICDH1* was cloned using the RNAi design (pK7GWIWG2; Karimi et al. 2002) between the CaMV 35S promoter and the *ocs* terminator into the vector pBINAR (Liu et al. 1999). These constructs were introduced into plants by an *Agrobacterium*-mediated transformation protocol, and plants were selected and maintained as described in the literature (Tauberger et al. 2000). Initial screening of 25 lines was carried out on the basis of total enzyme activity determinations. These screens allowed the selection of two lines, which were taken to the next generation. Total IDH and ICDH activity and expression of the various isoforms of IDH and ICDH were confirmed in the second harvest of these lines.

RNA extraction and quantification and qRT-PCR analysis

The RNA and qRT-PCR were determined as described in Zanor et al. (2009). Relative quantification of the target expression of IDH and ICDH genes in WT and transgenic lines was performed using the comparative Ct method. For

analysis of *SIIIDH1* transcript levels (GenBank accession no. TC163092), the following primers, forward 5'-CAGTGAAGCGTGTTATTGCCG-3', reverse 5'-GACAGCATCCGTGATTTCTTGG-3', and *SIIIDH1* (GenBank accession no. TC202045), forward 5'-TCCTGATGGCAAGACCATTGA-3', reverse 5'-TGTGCTGGTTTCACCTCCTTTC-3', for *SIIIDH3* (GenBank accession no. TC196623), forward 5'-ATGCACGAACATTCCCAGGA-3', reverse 5'-TGGCACGATACTGATCACCAAA-3', for *SIIIDH2* (GenBank accession no. TC164449), forward 5'-GAGCAGCCCATGGAACTGTT-3', reverse 5'-TCCACGAGTCCAAGCAAAAATC-3', and for *SIIIDH2* (GenBank accession no. TC198615) forward 5'-CTTTGAGCGATACGAAGTGCG-3', reverse 5'-GCCCTCCTTTCAGACAAACCTT-3'. To normalize *genes* expression for differences in the efficiency of cDNA synthesis, transcript levels of the constitutively expressed elongation factor 1- α of *S. lycopersicum* (GenBank accession no. X14449) and ubiquitin3 (GenBank accession no. X58253) were measured using the following primers, forward, 5'-ACCACGAAAGCTCTCCAGGAG-3', reverse, 5'-CATTGACCAAATGTCAC-3', Schaarschmidt et al. (2006) for elongation factor 1- α and forward, 5'-AGGTTGATGACACTGGAAAGGTT-3', reverse, 5'-ATCGCCTCCAGCCTTGTTGTA-3' for ubiquitin3 (Wang et al. 2008).

Analysis of enzyme activities

Enzyme extracts were prepared as described previously (Gibon et al. 2004), except that Triton-X 100 was used at a concentration of 1% and glycerol at 20%. Citrate synthase, isocitrate dehydrogenase (NAD), phosphofruktokinase (ATP) fumarase and pyruvate kinase were assayed as described in Nunes-Nesi et al. (2007). Malate dehydrogenase (NADP) was assayed as described in Scheibe and Stitt (1988). NADP-isocitrate dehydrogenase, nitrate reductase, PEPC and GDH (NAD) were assayed as described in Gibon et al. (2004). Glycerate kinase was assayed as described in Huege et al. (2007).

Determination of metabolite levels

Leaf samples were taken at the time point indicated, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis. Extraction was performed by rapid grinding of tissue in liquid nitrogen and immediate addition of the appropriate extraction buffer. The levels of starch, sucrose, fructose, and glucose in the leaf tissue were determined exactly as described previously (Ferne et al. 2001). The levels of all other metabolites were quantified by GC-MS exactly following the protocol described by Roessner et al. (2001) with the exception that peak identification was optimized to tomato tissues (Roessner-Tunali

et al. 2003) and extended to include newly identified peaks (Kopka et al. 2005; Schauer et al. 2005). Nitrate was determined as detailed in Fritz et al. (2006). The procedure of extraction and assay of NAD, NADH, NADP and NAPH was performed according to the method described by Gibon and Larher (1997). Photosynthetic pigments were determined exactly as described in Bender-Machado et al. (2004).

Measurements of photosynthetic parameters

Four to 5-week-old plants maintained at fixed irradiance of 250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ were used for gas exchange using a Licor-6400 gas exchange system (Li-Cor; <http://www.licor.com/>) under different light intensities (as described in the text), 400 ppm CO_2 , and a leaf temperature of 25°C . Fluorescence emission measurements to estimate the actual flux of photons driving photosystem II (ETR) was performed using a leaf chamber fluorometer (Model 6400-40, Li-Cor).

Statistical analysis

The *t* tests were performed using the algorithm embedded into Microsoft Excel (Microsoft) and analysis of variance and ANOVA-Tukey test were performed using the R software (R Development Core Team 2006). The term significant is used in the text only when the change in question has been confirmed to be significant by the *t* test ($P < 0.05$) in order to aid comparison to previous studies of TCA cycle transgenics. However, results from the ANOVA-Tukey test are superimposed on the data.

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