Chapter 4

Physiological characterisation of plants with reduced succinyl CoA ligase activity

4.1. Introduction

Determining the interaction between the mitochondrial TCA cycle and photosynthetic processes in illuminated tissues is part of an ongoing project. Previously, the phenotyping of the tomato wild species (*Solanum penellii*) mutant *Aco*1, which exhibits a deficiency in expression of one of the two isoforms of aconitase present in tomato, was published (Tanksley *et al.*, 1992; Carrari *et al.*, 2003). Molecular and biochemical analysis of the *Aco*1 mutant revealed that it exhibits a decreased flux through the TCA cycle and decreased levels of TCA intermediates but was characterized by elevated adenylate levels and an increased rate of carbon dioxide assimilation. More recently, another publication reported the molecular physiological study of transgenic tomato (*Solanum lycopersicum*) plants with alteration in the expression of the mitochondrial malate dehydrogenase (mMDH) gene (Nunes-Nesi *et al.*, 2005a). In this study, transgenic tomato plants expressing a fragment of *mMDH* gene in the antisense orientation and exhibiting reduced activity of this isoform of MDH showed enhanced photosynthetic activity and aerial growth under atmospheric conditions (360 ppm CO_2). In comparison to wild-type plants, carbon dioxide assimilation rates and total plant dry matter were up to 11% and 19% enhanced in the transgenics, respectively, when assessed on a whole-plant basis. Accumulation of carbohydrates and redox-related compounds such as ascorbate was also markedly elevated in the transgenics. Also increased in the transgenic plants was the capacity to use L-galactono-lactone, the terminal precursor of ascorbate biosynthesis, as respiratory substrate. Experiments in which ascorbate was fed to isolated leaf discs also resulted in increased rates of photosynthesis providing strong indication for an ascorbate-mediated link between the energy-generating processes of respiration and photosynthesis. This report thus showed that the repression of this mitochondrially localized enzyme improved both carbon assimilation and the aerial growth in a crop species. Recent microarray experiments also revealed a general increase in the level of expression of genes associated with photosynthesis in the *Aco*1 mutant and in the mMDH transgenic lines (Urbanczyk-Wochniak *et al.*, 2006). These results suggested an important role of the TCA-cycle function in the regulation of photosynthesis. Other studies have also recently demonstrated that photosynthesis is strongly dependent on mitochondrial metabolism (Gardeström *et al*., 2002; Raghavendra and Padmasree, 2003; Noctor *et al*., 2004; Yoshida *et al*., 2006).

Other enzymes then mMDH and aconitase have been the subject of molecular genetic approaches for example citrate synthase (Landschütze *et al.*, 1995; Koyama *et al.*, 2000) and isocitrate dehydrogenase (Kruse *et al.*, 1998), however these other studies were not focused on photosynthetic metabolism. To date, no such functional analysis approach has been reported for succinyl CoA ligase.

In this chapter I turn my attention to the analysis of the role of succinyl CoA ligase (SCoAL) in photosynthetic metabolism. In **Chapter 3** I reported the characterisation of the α and β subunits of succinyl CoA ligase of tomato. Here I describe the generation and characterisation of transgenic tomato plants exhibiting decreased expression in SCoAL β subunit gene and reduced SCoAL activity. The results obtained are discussed in the context of the regulation of energy metabolism in illuminated leaves and in the activity of alternative pathways that produce succinate and overcome the deficiency of SCoAL.

4.2. Results

4.2.1 Screening of SCoAL β tomato lines and morphological characterisation

The activity of SCoAL was determined in the direction of succinate formation as described in the previous chapter in 60 6-week-old plants obtained after *Agrobacterium tumefaciens*-mediated transformation. Screening of the lines for a reduction in SCoAL activity yielded 15 lines that exhibited a reduction in activity, but I selected RL25 and RL40 with RNAi construct and AL18 with antisense construct for further characterisation because they also showed a decreased expression level of SCoAL β subunit gene (**Figure 4.1 B**). The SCoAL activity of the selected lines and the wild-type tomato plant are shown in **Figure 4.1 C**.

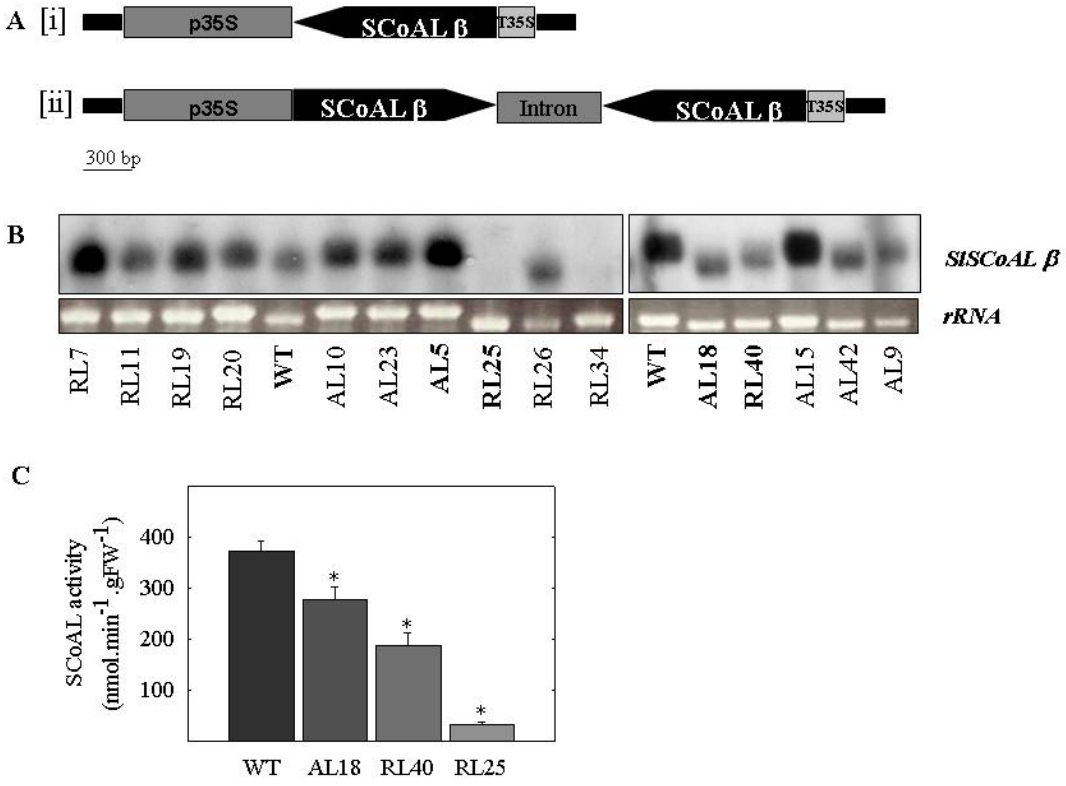


Figure 4.1. Characterisation and expression of tomato SCoAL β subunit. A, Construction of a chimeric gene for the expression of tomato SCoAL β subunit antisense RNA (subsection i) or RNA interference (RNAi, subsection ii) consisting of fragment encoding the CaMV 35S promoter and a 1017-bp (antisense) or two 1017-bp tandem fragments separated by a stem loop (RNAi; T35S terminator). B, Northern –blot analysis of leaves of transgenic plants preliminary screened by SCoAL activity with altered expression of SCoAL β subunit. C, SCoAL activity determined in 6-week-old fully expanded source leaves taken from selected transgenic plants with altered expression of SCoAL β subunit as compared with wild type (WT) plants. Values are presented as mean ± SE of determination on six individual plants per line; an asterisk indicates values that were determined by the *t* test to be significantly different ($P< 0.05$) from the wild type. AL: antisense lines; RL: RNAi lines.

For phenotypic characterisation, the plants were propagated *in vitro* to obtain at least 6 plants for each line and transferred to the greenhouse where they grew side by side with the wild type control plants. The lines were indistinguishable from wild type at early developmental stages, but 8 weeks after transferring to the greenhouse, the transgenic lines

were taller (**Figure 4.2 A**) and RL25 showed significantly longer internodes. Close examination of the transgenic plants revealed that they had a lower production of leaves and fruits which was reflected in dry weight determination (**Figure 4.2 B**). Transgenic plants appeared to have smaller fruits and also the flowers exhibit an altered morphology (**Figure 4.2 C**).

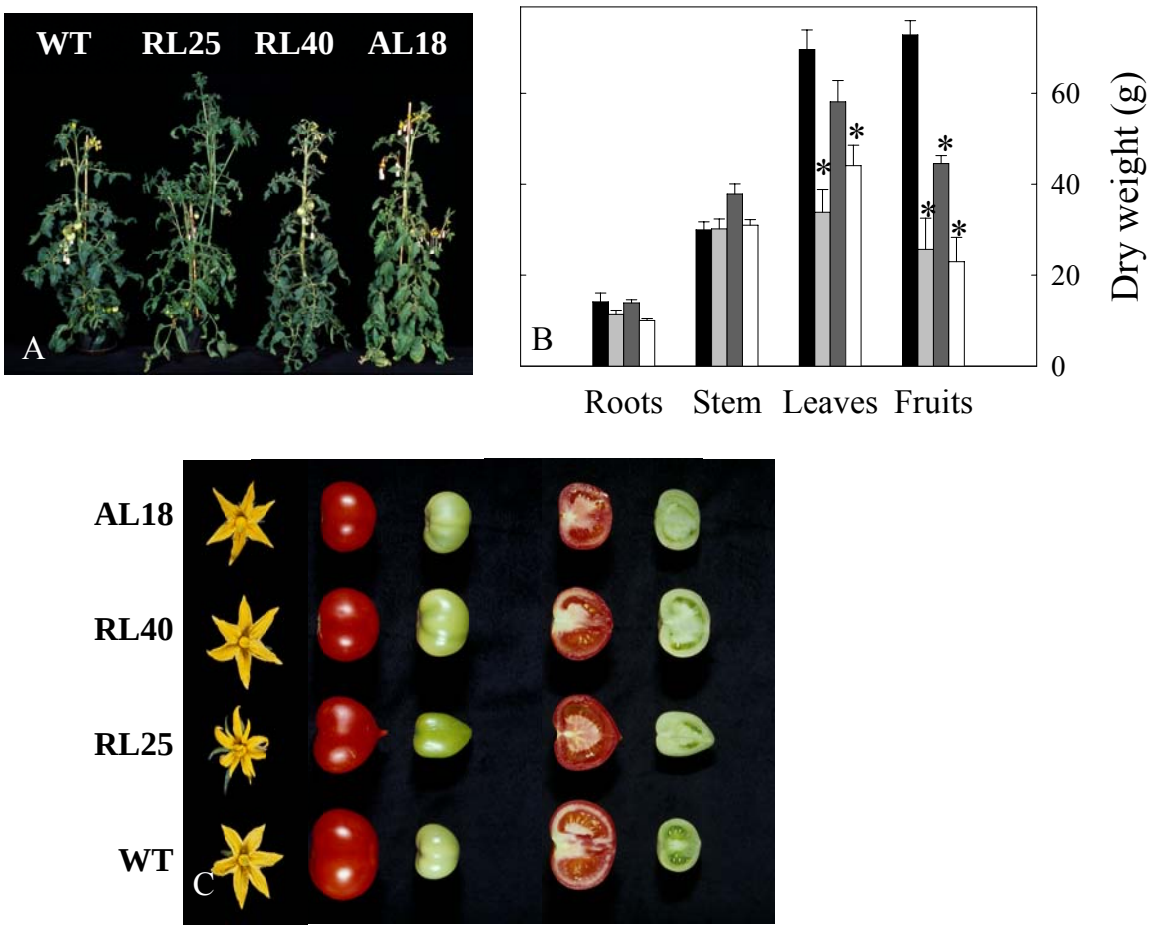


Figure 4.2. Growth and fruit phenotype of antisense and RNAi SlSCoAL tomato plants. Transgenic plants showed an increased stem elongation, but a decreased aerial biomass and fruit production with respect to the wild type. A, Photograph showing representative plants after 8 weeks growth. B, Biomass (in grams dry weight) of various plant organs on six plants per line after maturity. Values are presented as mean ± SE of determination on six individual plants per line; an asterisk indicates values that were determined by the *t* test to be significantly different ($P < 0.05$) from the wild type. Wild type, black bar; AL18 light grey bar; RL40, dark grey bar; RL25, white bar. C, Photograph of representative flowers and fruits of tomato plants.

Whilst the total biomass present in leaves and fruits decreased in the transgenic plants, analysis of root and stem biomass revealed no apparent difference when compared with wild type (**Figure 4.2 B**). When taken together these findings imply that decreased photosynthetic tissue production could lead to a decreased fruit production in the transformants.

4.2.2. Reduction of SCoAL activity results in little effect on carbon assimilation and chloroplastic electron transport rate

Given a decrease in the aerial yield in the transformants, I next analyzed if they exhibited altered photosynthetic rates. *In* vivo fluorescence emission was measured using a pulse amplitude modulation (PAM) fluorometer in order to calculate relative electron transport rates (RETRs). When exposed to different irradiance (photon flux density [PFD]) the SCoAL β subunit plants exhibited unimpaired RETRs (**Figure 4.3 A**). Other parameters such as photochemical and non-photochemical quenching were also invariant in the transformants. Interestingly, however, the transformants exhibited assimilation rates that were slightly lower than the wild type under conditions of high irradiances (800 and 1000 $\mu mol.m^{-2}.s^{-1}$, significantly so in the case of RL25, **Figure 4.3 B**). The internal carbon dioxide concentrations were also estimated to be slightly increased under light-saturated conditions in the transgenic.

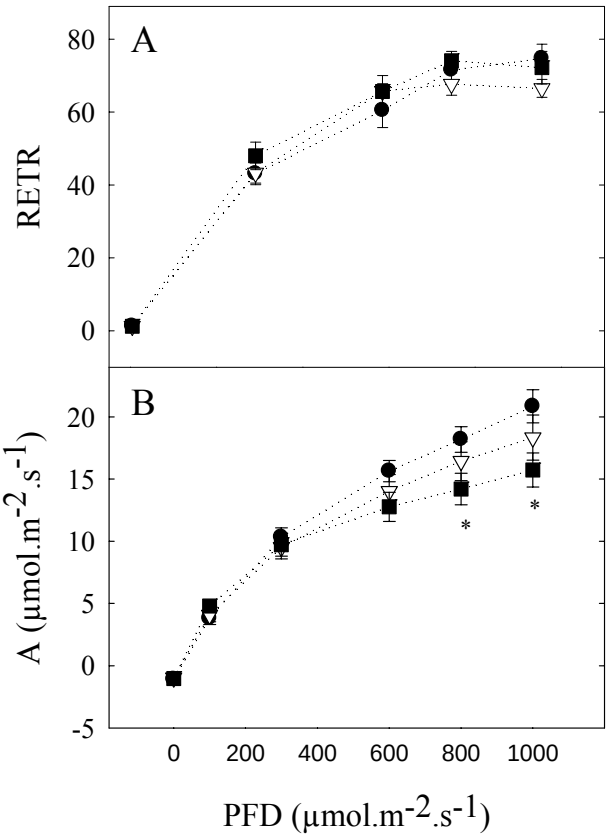


Figure 4.3 Effect of decrease in SCoAL activity on photosynthesis. A, In vivo fluorescence emission was measured as an indicator of the electron transport rate (ETR) by use of a PAM fluorometer at different PFDs in a light curve. B, Assimilation rate. Plants used: wild type, black circles; RL40, white triangles; RL25, black squares. Values are presented as mean ± SE of determination on five individual plants per line; an asterisk indicates values that were determined by the *t* test to be significantly different ($P< 0.05$) from the wild type.

Although assimilation rate was largely invariant it is possible that the carbon partitioning was altered in the transformants. In order to test this possibility I studied the metabolism of $^{14}CO_2$ in illuminated (at PFD 600 µmol.m^{-2}.s^{-1}) leaf discs excised from wild type and transformants. The total assimilation was invariant in the transformants (**Figure 4.4**) and a decrease in SCoAL activity did also not lead to a different partitioning into sugars, starch, amino acids, and organic acids and phosphorylated intermediates. These results implied no effect on the assimilation rate and carbon partitioning in the transformants under light intensities lower then 600 µmol.m^{-2}.s^{-1}.

From these results it is possible to deduce that transformants with decreased activity in SCoAL showed unimpaired assimilation and chloroplastic electron transport rates.

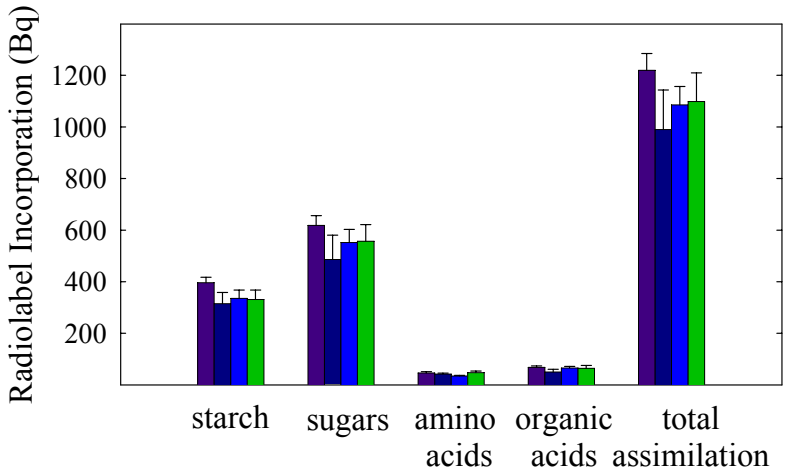


Figure 4.4. Effect of decrease in SCoAL activity photosynthetic assimilation and carbon partitioning at the onset of illumination. Leaf discs were cut from five untransformed plants and five plants of each transgenic line after 30 min in the dark, and illuminated at 600 µmol photosynthetically active radiation $m^{-2}.s^{-1}$ in an oxygen electrode chamber containing air saturated with $^{14}CO_2$ after 30 min the leaf discs were frozen in liquid nitrogen for further extraction and fractionation. Plants used: wild type, violet bars; AL18, dark blue bars; RL40, bright blue bars; RL25, green bars. Values are represented as mean ± SE of determinations on five individual plants per line.

4.2.3. Measurements of key enzymes activities of carbohydrate metabolism

Analysis of maximal catalytic activities of a broad range of important enzymes of photosynthetic carbohydrate metabolism revealed no changes in important enzymes of photosynthesis (Rubisco and Calvin cycle enzymes) (**Table VIII**). No significant differences were observed in the maximal catalytic activities of AGPase, pyruvate kinase, transketolase and phosphoglycerate kinase. However, an increase in the activities of both pyrophosphate-dependent and ATP-dependent PFK and of phosphoribulokinase was observed and a strong decrease in the activities of UGPase and $NADP^+$ -dependent glyceraldehyde phosphate dehydrogenase (NADP-GAPDH) was observed in two lines.

Table VIII. Enzymes activities in SCoAL lines. Enzyme activities were determined in source leaves samples from 6-week-old plant. The data presented are the mean ± SE of determination on six individual plants per line. Values in bold type indicate those that were determined by *t* test to be significantly different ($P < 0.05$) from the wild type. AGPase: ADPglc pyrophosphorilase; UGPase: UDPglc pyrophosphorilase; PFK: phosphofructokinase; NADP-GAPDH: $NADP^+$ -dependent glyceraldehyde phosphate dehydrogenase

	Activities ($nmol.min^{-1}.gFW^{-1}$)			
Enzymes	WT	AL18	RL40	RL25
AGPase	507 ± 68	591 ± 64	792 ± 177	602 ± 61
UGPase	8.48 ± 1.2	8.64 ± 0.9	**4.81 ± 1.2**	**3.97 ± 1.0**
PFK ATP-dependent	69.9 ± 6.1	**94.9 ± 7.7**	78.7 ± 7.9	65.5 ± 2.0
PFK PPi-dependent	0.21 ± 0.05	0.19 ± 0.04	0.28 ± 0.03	0.16 ± 0.03
NADP-GAPDH	0.83 ± 0.07	**0.54 ± 0.03**	1.02 ± 0.15	**0.30 ± 0.12**
pyruvate kinase	1.55 ± 0.15	1.45 ± 0.1	1.67 ± 0.18	1.46 ± 0.12
Rubisco initial activity	3.67 ± 0.55	2.31 ± 0.48	2.79 ± 0.33	2.94 ± 0.80
Rubisco total activity	5.33 ± 0.59	3.51 ± 0.60	5.17 ± 0.59	4.66 ± 0.90
Rubisco activation state (%)	67.0 ± 4.6	64.6 ± 3.4	59.5 ± 11.4	58.7 ± 6.0
transketolase	4.14 ± 0.45	3.40 ± 0.49	3.42 ± 0.62	3.43 ± 0.81
phosphoribulokinase	1.62 ± 0.18	**2.29 ± 0.22**	**2.79 ± 0.23**	2.02 ± 0.26
phosphoglyceratekinase	0.87 ± 0.18	0.99 ± 0.12	1.29 ± 0.11	0.60 ± 0.11

4.2.4. Inhibition of SCoAL activity results in slightly reduced rates of respiration

Close examination of the gas exchange in the dark, considering only dark respiration, revealed an approximately 30% of inhibition of dark respiration in the strongest line RL25. For a direct evidence of an alteration in the TCA cycle, the relative rates of carbohydrate oxidation were directly evaluated in the transformants. For this purpose, the evolution of $^{14}CO_2$ following incubation of leaf discs in positionally labelled ^{14}C-Glc molecules was recorded. Leaf discs taken from plants in the light were incubated and supplied with [1-^{14}C]-, [2-^{14}C]-, [3:4-^{14}C]-, or [6-^{14}C]-Glc over a period of 6 hours. During this time, the $^{14}CO_2$ evolved was collected at hourly intervals. Carbon dioxide can be released from the C1 position by the action of enzymes that are not associated with the

mitochondrial respiration, but carbon dioxide evolution from the C3,4 positions of Glc cannot (ap Rees and Beevers, 1960; **Figure 4.5**).

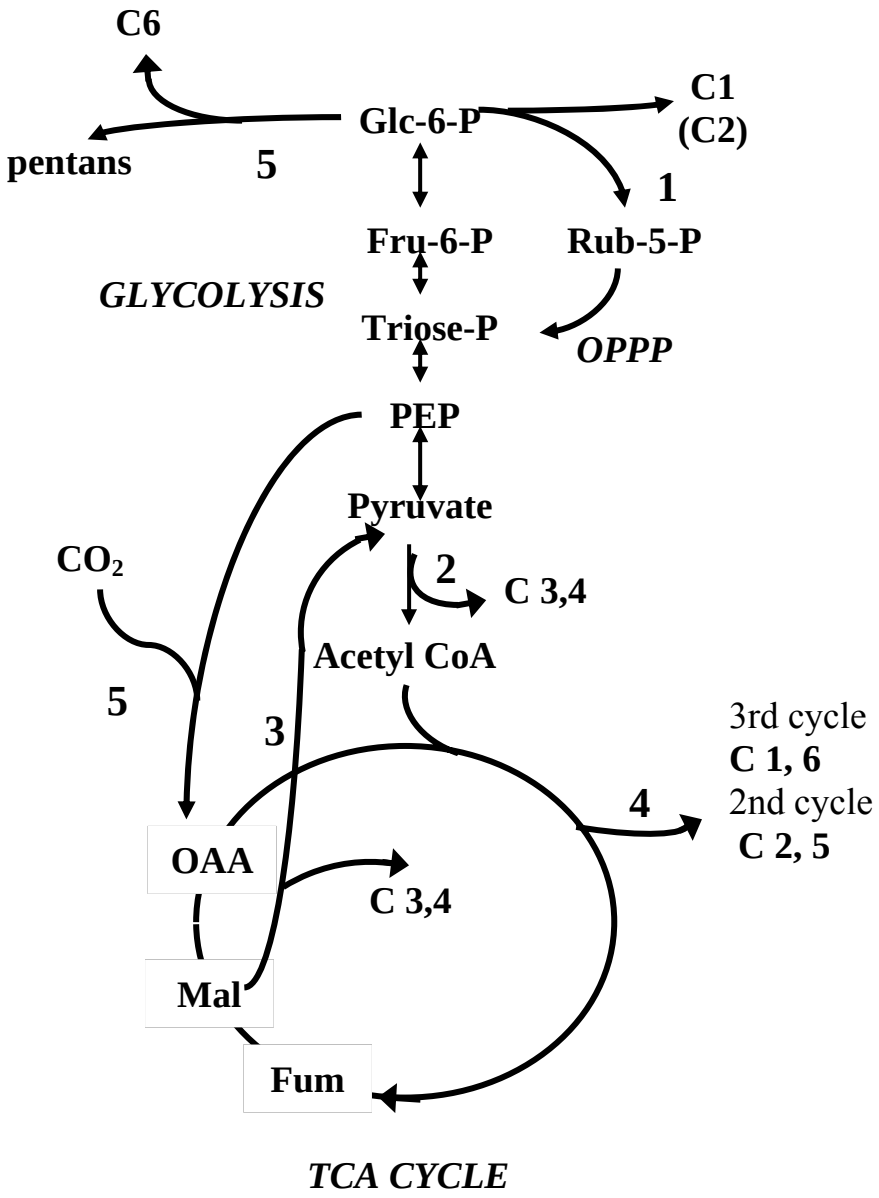


Figure 4.5. A simplified metabolic map of the dominant pathways of carbohydrate oxidation showing the sites of CO_2 release from specific atoms of glucose. The carbon of glucose that is released by decarboxylation reactions are represented by C1-6. Not all reactions between metabolic intermediates are shown. Enzymes of the pathway section are: 1: 6-phosphogluconate dehydrogenase; 2: pyruvate dehydrogenase complex; 3: NAD^+-malic enzyme; 4: isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase complex; 5: carboxyl lyase.

Thus, the ratio of carbon dioxide evolution from C1 and C3,4 positions of Glc provides an indication of the relative rate of other processes of carbohydrate oxidation with respect to the rate of the TCA cycle. The rate of $^{14}CO_2$ evolution from leaves incubated in [1-^{14}C] Glc was always highest; however, the absolute rate of carbon dioxide evolution from the C1 position of the transgenic lines was in excess when compared to the wild type (**Figure 4.6**). The release from C3,4 positions were apparently not much lower in the transgenic plants than in the wild type in absolute terms, but in relative terms, the C1/C3,4 ratios in the transgenic plants were higher. Therefore, these results indicate a lower proportion of carbohydrate oxidation is carried out by the TCA cycle in the transgenic lines. Carbon dioxide evolution from C2 and C6 positions was relatively similar across the transgenic lines and the wild type.

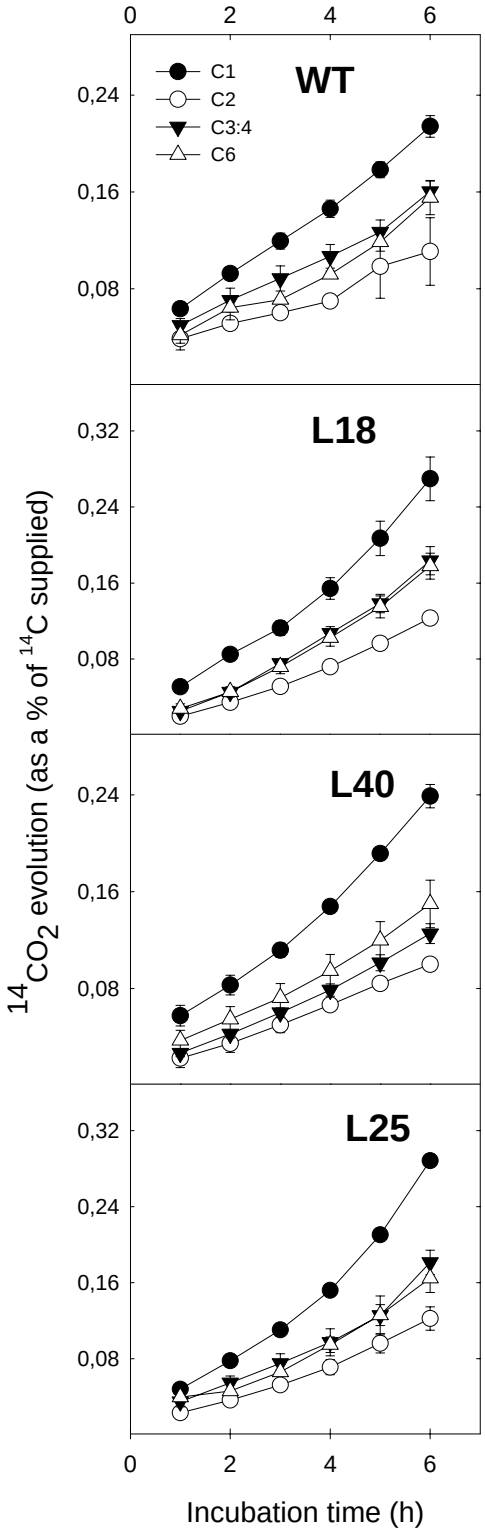


Figure 4.6 $^{14}CO_2$ evolutions from isolated leaf discs in the light as respiratory parameter of the SCoAL lines. Leaf discs were taken from 10-week-old plants and were incubated in 10 mM MES-KOH solution, pH 6.5, 0.3 mM Glc supplemented with [1-^{14}C]-, [2-^{14}C]-, [3,4-^{14}C]-, or [6-^{14}C]-Glc (at a specific activity of 7.5 MBq.mmol^{-1}). The $^{14}CO_2$ liberated was captured (in hourly intervals) in a 10% KOH trap and the amount of radiolabel released was subsequently quantified by liquid scintillation counting. Values represent as mean ± SE of determinations on six individual plants per line.

4.2.5. Photosynthetic carbon metabolism and metabolite levels in the SCoAL transformants

Analysis of the **c**arbohydrate content of leaves from 7-week-old plants during a diurnal cycle revealed no significant change in Suc and starch (**Figure 4.7**) as well as for Glc and Fru in the transformants. Despite the fact the there was little change in Suc and starch and on carbon assimilation I next decided to carry out a broader metabolite profile in order to search for metabolic perturbation consequent of the genetic manipulation. First, the levels of adenylates and uridinylates were determined by HPLC in trichloracetic acid extracts from leaves samples from the transformants (**Table IX**). There were no significant differences in the transformants. In addition to the nucleotide measurements, I determined the levels of key photosynthetic metabolites such as Glc 6-phosphate, Glc 1-phosphate, Fru 6-phosphate, 3-phosphoglycerate using a sensitive cycling assay that had been previously established (Gibon *et al.*, 2002). These assays revealed significantly higher levels of Glc 6-phosphate, but no significant differences in Glc 1-phosphate, Fru 6-phosphate and inorganic phosphate in the transformants. However, the mechanism by which this occurred could not be solved in the present study. Levels of acetyl-CoA and reduced CoA were also measured in the transformants to have an insight about the total CoA content in the plant, considering that SCoAL reaction involves the transfer of CoA, but no differences were obtained neither in the levels of acetyl-CoA nor in reduced CoA.

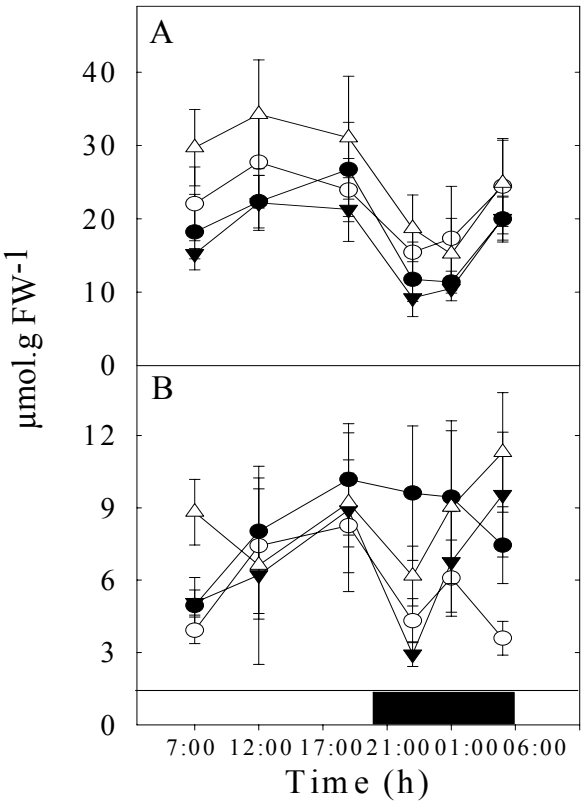
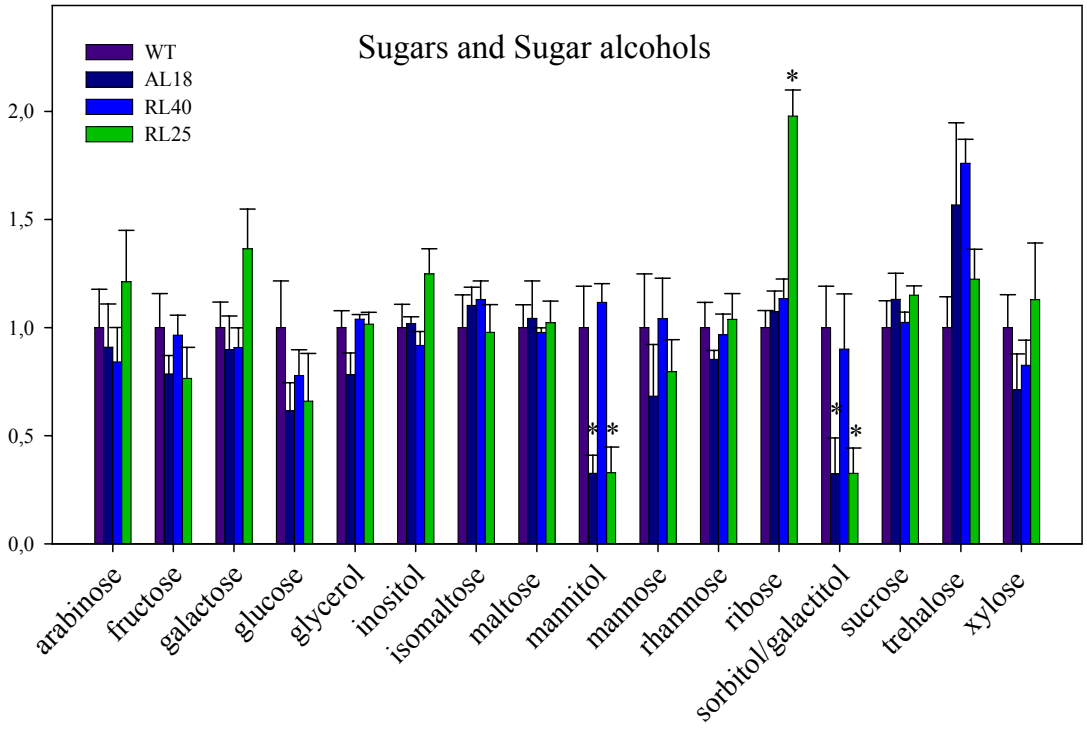


Figure 4.7. Diurnal changes in Suc (A) and starch (B) content in leaves of 7-week-old plants. At each time point, samples were take from fully expanded source leaves, and the data represent the mean ± SE of measurements from six individual plants per line. The lines used were: wild type, black circles; AL18, white circles; RL40, black triangles; RL25, white triangles. Black bar represents the dark period; white bars indicate the light period. Values represent as mean ± SE of determinations on six individual plants per line.

Table IX. **Nucleotides, phosphorylated intermediates and acetyl-CoA levels in SCoAL lines.** Metabolites were measured from illuminated fully expanded source leaves of 6-week-old plants. Sample harvested for all metabolite measurements were exactly the same used for enzyme determination represented in **Table VIII**. Data represented are mean ± SE of determination on six individual plants per line. Values in bold type indicate those that were determined by *t* test to be significantly different ($P < 0.05$) from the wild type.

	Quantification in wild type and the SCoAL lines (nmol.gFW^{-1})			
Metabolite	WT	AL18	RL40	RL25
Glc6P	76.2 ± 3.6	**99.6 ± 4.6**	67.7 ± 10.4	**94.9 ± 5.8**
Glc1P	20.6 ± 2.1	16.4 ± 1.7	13.4 ± 2.5	22.5 ± 2.6
Fru6P	17.9 ± 2.0	23.6 ± 2.1	17.8 ± 2.9	17.2 ± 2.8
3-PGA	506 ± 105	534 ± 50	465 ± 34	591 ± 29
Pi	3.1 ± 0.7	4.1 ± 0.5	2.5 ± 0.4	4.2 ± 0.6
∑ hexose -P	115.8 ± 7.3	**144.1 ± 3.1**	98.7 ± 14	132.7 ± 8.8
UDP-Gluc	26 ± 1.6	26 ± 0.5	26 ± 1.0	27 ± 1.1
UDP	44 ± 4.5	50 ± 4.5	43 ± 3.3	33 ± 2.3
UTP	11 ± 0.6	10 ± 0.6	11 ± 0.5	13 ± 0.3
∑ Uridinylates	83 ± 6.3	75 ± 11.2	80 ± 4.5	72 ± 2.8
UTP / UDP	0.3 ± 0.03	0.2 ± 0.03	0.3 ± 0.02	0.4 ± 0.03
ADP	6 ± 0.5	4 ± 0.4	6 ± 0.4	5 ± 0.6
ATP	18 ± 1.6	17 ± 0.9	16 ± 0.8	19 ± 0.9
∑ Adenylates	24 ± 1.8	21 ± 0.9	21 ± 1.1	24 ± 1.3
ATP / ADP	3.1 ± 0.4	4.0 ± 0.4	2.9 ± 0.2	4.0 ± 0.3
CDP	55 ± 4.3	53 ± 2.6	54 ± 1.9	49 ± 4.1
CTP	2.6 ± 0.4	2.5 ± 0.3	2.5 ± 0.4	2.6 ± 0.3
GDP	5.8 ± 0.9	**3.2 ± 0.3**	4.6 ± 1.2	4.5 ± 1.2
GTP	3.2 ± 0.3	3.5 ± 0.5	2.6 ± 0.5	3.6 ± 0.2
Acetyl -CoA	7.7 ± 1.0	6.9 ± 0.5	7.3 ± 1.0	8.4 ± 0.7
CoASH	2.0 ± 0.3	1.8 ± 0.3	1.8 ± 0.6	2.0 ± 0.5
Total CoA	9.7 ± 1.3	8.7 ± 1.1	9.1 ± 0.8	10.4 ± 0.4

As a second procedure to examine the consequence of altering the activity of SCoAL on the TCA cycle, I evaluated the levels of many intermediates of the TCA cycle and other pathways utilizing an established gas-chromatography-mass spectrometry (GC-MS) protocol for metabolic profiling (Fernie *et al.*, 2004b). These studies revealed an increase in TCA cycle intermediates upstream from succinate (isocitrate, citrate and 2-oxoglutarate, **Figure 4.8**). No differences were observable in the levels of other intermediates of the cycle not even in succinate in spite of it being one of the reaction products of SCoAL. The significant increase in Glc 6-phosphate observed in the previous experiment was also observed using this protocol. When amino acids were analyzed, glutamate and GABA showed a tendency to increase (significantly for RL25), potentially suggesting an activation of the GABA shunt in the transformants. Interestingly, a significant decrease in proline content was additionally observed in the lines AL18 and RL25. This could potentially be explained by a higher degradation of proline to produce glutamate in the transformants.



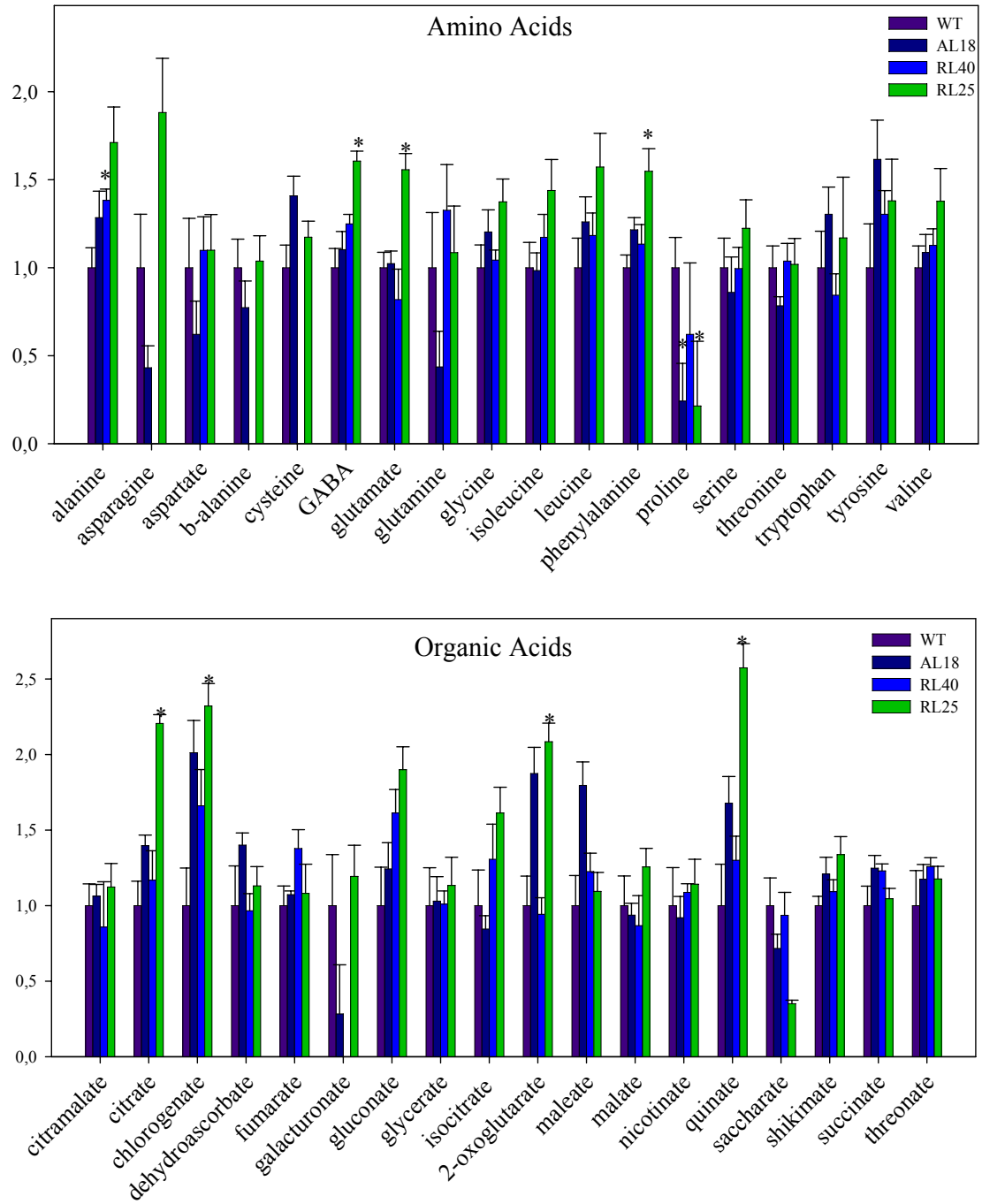


Figure 4.8. Relative metabolite content of fully expanded leaves from 6-week-old plants of SCoAL lines. Metabolites were determined as described in "Materials and Methods". Data are normalized with respect to the mean response calculated for the wild type (to allow statistical assessment individual samples of this set of plants were normalized in the same way). Values are represented as the mean ± SE of determinations of six individual plants per line; asterisk indicates values that were determined by the *t* test to be significantly different ($P < 0.05$) from the wild type.

4.2.6. Measurement of key enzyme activities of the GABA shunt

Results obtained from the analysis of metabolite levels in the transformants suggested an activation of the GABA shunt. To confirm this, I measured the maximal catalytic activities of the first enzyme of the GABA shunt, glutamate decarboxylase (GAD), which catalyzes the conversion of glutamate to GABA and glutamate dehydrogenase (GDH). The latter catalyzes the reductive amination of 2-oxoglutarate producing glutamate. For GDH results on the two lines with the strongest decreased SCoAL activity revealed an increase in the activity in GDH, significant so for RL25 (**Figure 4.9 A**). Similar results were obtained for GAD maximal catalytic activity determination which revealed also an increase in GAD activity, significantly so for RL25 (**Figure 4.9 B**).

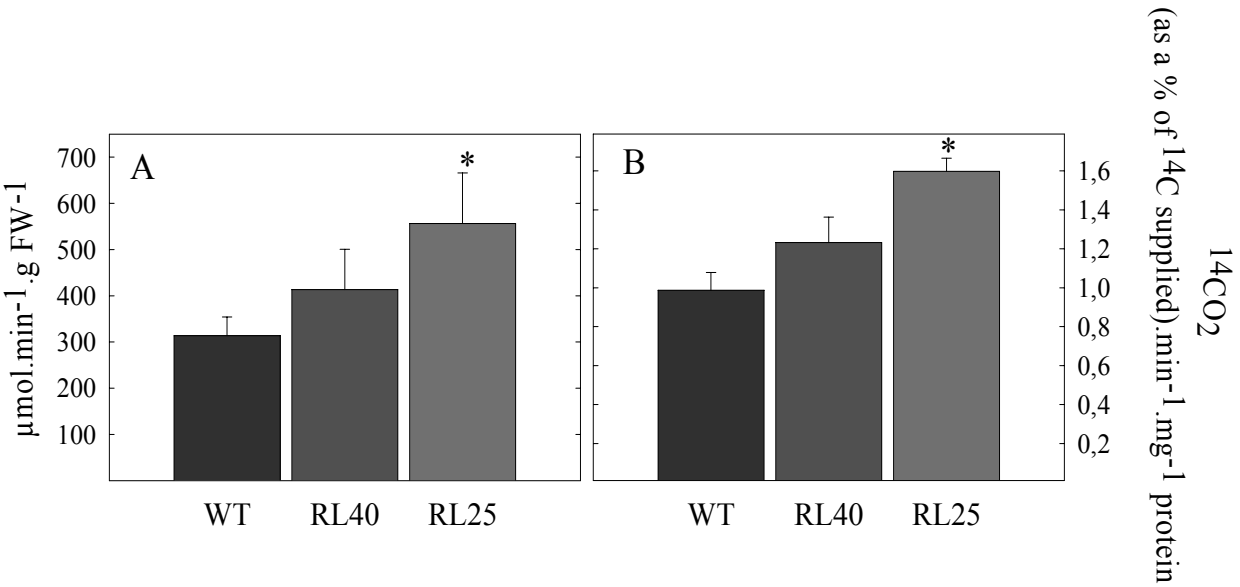


Figure 4.9. Measurement of GDH and the first enzyme of the GABA shunt in SCoAL lines. (A) Glutamate dehydrogenase (GDH) and (B) glutamate decarboxylase (GAD) enzyme activities. GDH samples were taken from 6-week-old frozen leaf material, and GAD samples were taken from fresh 7-week-old mature source leaves. Values are represented as the mean ± SE of determinations of five individual plants per line; asterisks indicate values that were determined by the *t* test to be significantly different ($P< 0.05$) from the wild type.

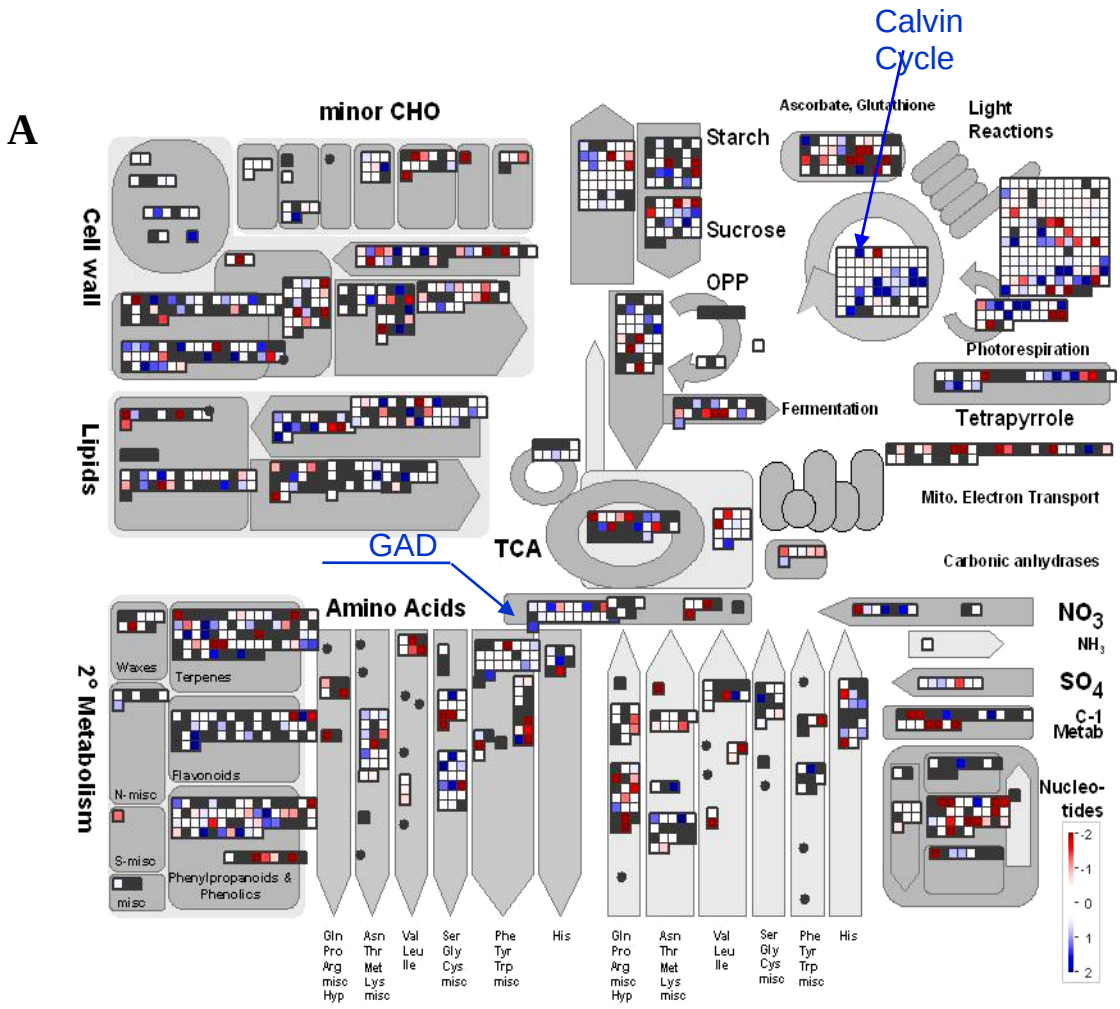
4.2.7. Evaluation of changes in transcript level between wild type and RL25

To identify genes that were differentially expressed in the tomato wild type and in the transformed line with strongest decrease in SCoAL activity, I used DNA microarray technology co-hybridizing labelled cDNAs against the commercially available TOM1 chip.

Given the huge number of transcripts measured in a single experiment, to evaluate and visualize the data I used the recently modified MapMan visualization software (Thimm *et al.*, 2004; Usadel *et al.*, 2005; Urbanczyk-Wochniak *et al.*, 2006) which offers the possibility to paint out microarray or metabolite profiling experiments onto functional pathway or process maps. Firstly, an analysis of transcripts related to metabolism (**Figure 4.10 A**) was performed. The SCoAL transgenic RL25, when analyzed on the BIN level using the Wilcox test, was characterized by a large number of changes in transcripts for genes associated with nucleotide metabolism, followed by the Calvin cycle, the amino acids metabolism, the secondary metabolism, the mitochondrial electron transport and redox, ascorbate and glutathione. The *Aco*1 mutant (Carrari *et al.*, 2003) and the well characterised malate dehydrogenase antisense line AL9 (Nunes-Nesi *et al.*, 2005a) revealed a similar pattern of change in transcription (Urbanczyk-Wochniak *et al.*, 2006) to that observed in the SCoAL line RL25. However, the SCoAL line displayed a markedly reduced proportion of changes in photosynthetic light reactions and in the TCA cycle. At the level of individual genes, the majority of the genes involved in the Calvin cycle had increased transcript levels as well as most genes encoding for proteins of the photosynthetic light reactions, photorespiration, and flavonoid metabolism. A trend for decrease in transcription levels of genes involved in amino acid synthesis is also observable, but this also holds true for genes involved in their degradation. In contrast, almost all genes involved in the mitochondrial electron transport chain have a decrease in transcription level, and there is a clear trend for decreased transcript levels of genes involved in nucleotide metabolism and in the TCA cycle. Interestingly, most of the genes annotated for GAD, which is directly involved in the GABA shunt, showed increased transcription in the transgenic line showing that regulation of GAD activity occurs at transcript level. This is in close agreement with the data that suggested a correlation between *in vitro* GAD activity and the levels of the GAD transcript in developing leaves and germinating seeds of petunia (Chen *et al.*, 1994).

Having a close examination to other BINs, such as the tetrapyrrole synthesis, showed that transcript levels of genes involved in the synthesis of chlorophylls are increased. Transcripts that encode for the enzymes glutamyl tRNA synthase, hydroxymethylbilane synthase and S-adenosyl-L-methionine:Mg-protoporphyrin IX methyltransferase showed an increased level in line RL25. Similarly, transcripts for key enzymes for the common pathway of carotenoid and tocopherols biosynthesis isopentenyl

diphosphate isomerase and geranylgeranyl diphosphate synthase are increased in line RL25. Furthermore, the levels of transcript for the enzyme phytoene synthase (the initial enzyme specific of the carotenoid biosynthesis pathway) were also increased in the transgenic line. Moreover, similar to the *Aco* 1 mutant but not to the malate dehydrogenase antisense line, the SCoAL transgenic line revealed a general decrease in transcript level of genes associated with the phenylpropanoids and cell wall metabolism (although it should be noted that the transcript level of some of the genes of these BINs also increased). For completeness the genes associated with regulatory processes are provided in **Figure 4.10 B**. The huge number of genes exhibiting altered transcript levels was somewhat surprising considering that the transformants did not exhibit a strong phenotype. Comparison of the transcription changes following modulation in the expression of three TCA cycle enzymes revealed some commonality, but also many differences – as would be expected since at a gross phenotypic level the SCoAL plants were not as strongly affected as the *Aco*1 mutant and the malate dehydrogenase antisense line.



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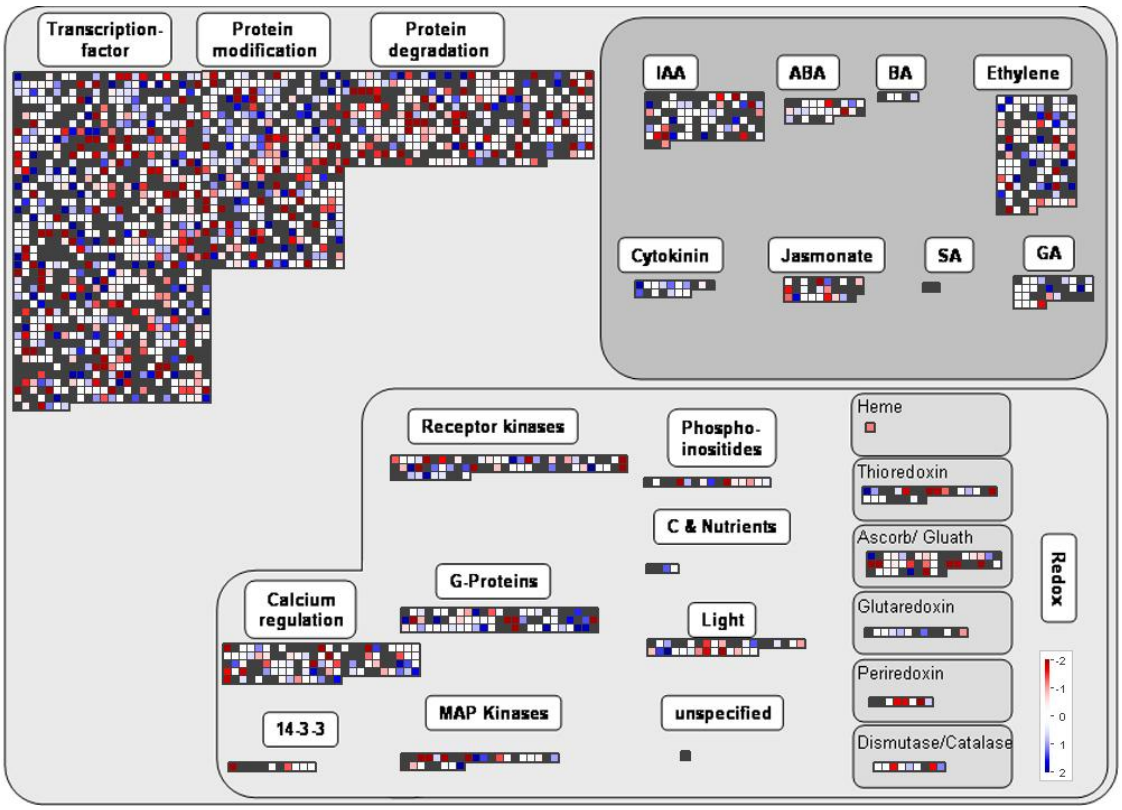

Figure 4.10 Transcript profiling in leaves. Differences in transcript levels between wild type and RL25 for genes associated with (A) metabolism and (B) regulation was determined. Both sets of material were harvested in the middle of the day from plants grown in the glasshouse. Blue and red represent an increase or a decrease of expression respectively, in the wild type relative to RL25. The colour scale which was used is reproduced in the figure.

4.2.8. Shikimate and isoprenoid pathways

The shikimate pathway links primary carbohydrate metabolism to the biosynthesis of aromatic compounds (Hermann and Weaver, 1999). As described above the relative metabolic content levels of aromatic amino acids (mainly of phenylpropanoid and tyrosine) are increased. Interestingly, also the levels of secondary products of this pathway such as quinate and chlorogenate were increased. Another common product of the isoprenoid pathway, phytyl pyrophosphate, and chlorophyllide, a compound originated from glutamate, are essential intermediates in chlorophyll biosynthesis. Given these changes and the altered transcript levels described above in the SCoAL plants lead me to investigate what happened to pigment content. These determinations revealed higher levels of β-carotene and chlorophyll *a* in leaves from the transformants (significantly for RL25 and

RL40, **Figure 4.11**). A similar effect was previously reported for *Aco 1* mutant but not for the mMDH transformants (Carrari *et al.*, 2003; Nunes-Nesi *et al.*, 2005a).

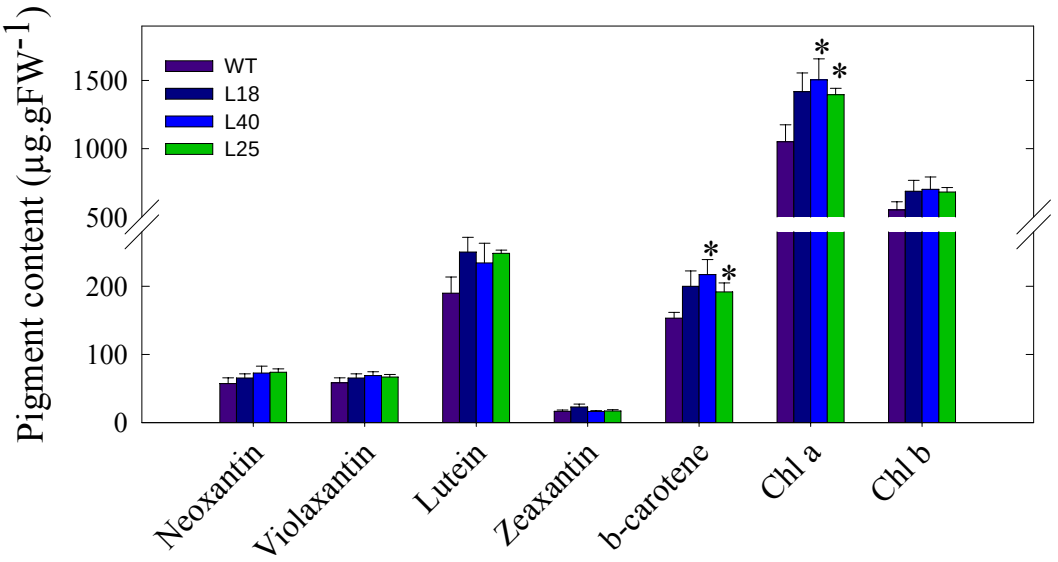

Figure 4.11 Pigment content in leaves. Chl a and Chl b: Chlorophyll *a* and *b*, respectively, and the other pigments were measured in leaves taken from 6-week-old plants. Values are represented as the mean ± SE of determinations of five individual plants per line; asterisks indicate values that were determined by the *t* test to be significantly different ($P< 0.05$) from the wild type.

Tocopherols are synthesized from precursors of two pathways: the isoprenoid pathway, which provides the hydrophobic tail, and that of homogentisic acid formation, starting from tyrosine, which provides the head group (Hirschberg, 1999). Previous integrated studies of potato transcript and metabolite profiling showed a negative correlation between tocopherols and succinyl CoA ligase expression (Urbanczyk-Wochniak *et al.*, 2003). For this reason, I next determined the levels of tocopherols in leaves of the transformants. Tocopherols were measured by HPLC analysis of a diethylether extraction. An increased level of α-tocopherol was observed in all lines, significantly so for lines AL18 and RL40 (**Table X**). The less abundant isomer γ-tocopherol was also detected in the HPLC runs, and a significant increase of this isomer was observed in line AL18.

Table X. **Tocopherol measurements in SCoAL plants.** Tocopherols were measured from illuminated fully expanded source leaves of 6-week-old plants. Samples harvested for tocopherol measurements were exactly the same used for enzyme determination and metabolite measurements represented in **Table VIII** and **IX**. Data represented are mean ± SE of determination on six individual plants per line. Values in bold type indicate those that were determined by *t* test to be significantly different ($P < 0.05$) from the wild type.

	$\mu g.gFW^{-1}$			
	WT	AL18	RL40	RL25
α-tocopherol	34.8 ± 1.5	**43.3 ± 1.9**	**41.7 ± 1.6**	39.1 ± 2.8
γ-tocopherol	1.3 ± 0.3	**3.7 ± 0.5**	1.6 ± 0.3	1.3 ± 0.2

4.3. Discussion

In this chapter I described the first physiological characterisation of tomato plants with decreased activity of succinyl CoA ligase (SCoAL). This exclusively mitochondrial enzyme catalyses the conversion of succinyl CoA to succinate with a substrate level phosphorylation of ADP producing ATP. Tomato plants with decrease activity of SCoAL were obtained by transforming plants with tomato SCoAL β subunit making use of RNAi and antisense approaches. Previous related work characterising the molecular physiology of plants with decreased activity in other enzymes of the TCA cycle, as was the case of the mitochondrial malate dehydrogenase (mMDH) (Nunes-Nesi *et al.*, 2005a) and the aconitase (Carrari *et al.*, 2003), revealed the importance of the respiratory pathways in photosynthetic metabolism. These early results are in close agreement with data of other researchers (Gardeström *et al.*, 2002; Raghavendra and Padmasree, 2003; Noctor *et al.*, 2004; Yoshida *et al.*, 2006).

SCoAL transformants showed a decrease in leaf biomass and in fruits production, but an increase in high. However, the reduction of SCoAL activity showed a relatively mild phenotype when compared to the mMDH plants and the *Aco1* mutants. Analysis of photosynthetic metabolism of the SCoAL transformants revealed that carbon assimilation rate was largely invariant and chlorophyll fluorescence measurements indicated that the photochemical efficiency of the photosystem II was unimpaired. Soluble sugars and starch were measured enzymatically in a diurnal cycle, but no differences were observed. These results were confirmed by GCMS metabolic profiling. The malate dehydrogenase antisense

lines were characterised by an increase level of ascorbate and dehydroascorbate (Nunes-Nesi *et al.*, 2005a). Based on these results, ascorbate and dehydroascorbate were measured spectrophotometrically in the SCoAL transformants and in the wild type, but they showed no significant change when compared with the wild type. Analysing data from respiration parameters and comparing them with the one obtained of respiration rate in plants with decrease activity of mMDH (Nunes-Nesi *et al.*, 2005a) revealed that SCoAL lines had less compromised activity of the TCA cycle. In keeping with this, decreased SCoAL activity in plants intriguingly revealed little effect in the TCA cycle function. The data on the SCoAL plants did not contradict the statement about the importance of the respiratory pathways in photosynthetic metabolism, but on the contrary, they reinforce the importance in keeping the respiratory pathways well working supported by complementary pathways or reactions that replenish the intermediates of the cycle and allow maintenance of cellular levels. An important feature of primary metabolism in plants is that characterised by a huge redundancy and flexibility with there often being multiple routes to the same end (ap Rees and Hill, 1994; Rontain *et al.*, 2002). This metabolic flexibility is perhaps best exemplified by genetic engineering experiments in which post-transcriptional gene silencing technologies have been utilized to partially or fully eliminate an enzyme traditionally considered to be essential, and yet the resulting transgenic plants that were able to grow and develop more or less normally (Plaxton, 1996; Plaxton and Podestà, 2006).

Surveying the levels of a wide range of primary metabolites revealed increased levels of 2-oxoglutarate, glutamate and GABA, suggesting that the SCoAL transgenic plants were profiting of their metabolic flexibility as discussed above. A short pathway that bypasses two of the steps of the TCA cycle, namely the conversion of 2-oxoglutarate (2-OG) to succinyl CoA, catalyzed by the 2-oxoglutarate dehydrogenase complex, and the reaction of conversion of succinyl CoA to succinate catalyzed by SCoAL is known as the GABA shunt (**Figure 4.12**). The GABA shunt produces γ-aminobutyric acid (GABA), and metabolizes it to produce succinate (Satyanarayan and Nair, 1990; Bouché *et al.*, 2003a). It is composed of three enzymes: the cytosolic glutamate decarboxylase (GAD) and the mitochondrial enzymes GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH). Glutamate dehydrogenase and glutamate decarboxylase were measured and their increased catalytic activity in the transformants was, together with the increase in glutamate and GABA levels, complementing evidence that the GABA shunt was activated in the transformants. Studies show that GABA shunt activity is enhanced in

response to biotic and abiotic stresses (Shelp *et al.*, 1999; Snedden and Fromm, 1999). This was confirmed in Arabidopsis mutants with T-DNA insertion in the SSADH, which showed accumulation in reactive oxygen intermediates and cell death in response to light and heat stresses (Bouché *et al.*, 2003b). Other report shows the importance of a gradient in GABA concentration for normal pollen tubes establishment and development (Palanivelu *et al.*, 2003).

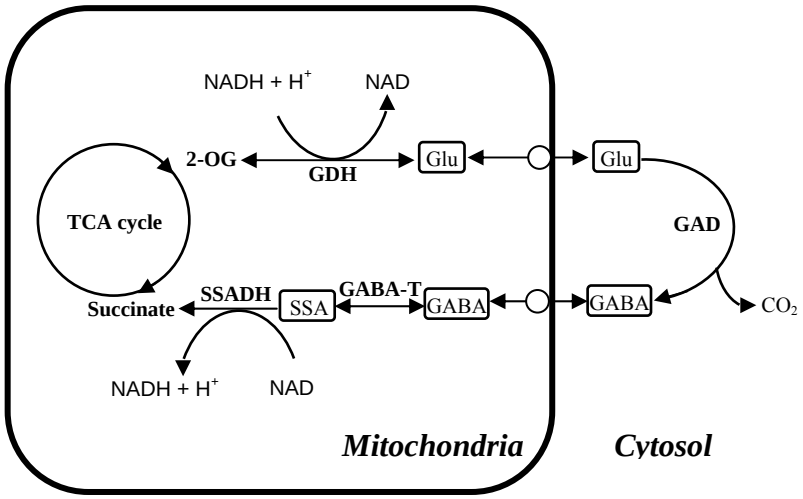

Figure 4.12 Metabolic reactions of the GABA shunt. The Glu produced in the mitochondria is transported to the cytosol where it is decarboxylated to GABA by GAD. GABA is transported to the mitochondria and metabolized to SSA and finally to succinate which re-enters the TCA cycle. 2-OG: 2-oxoglutarate; GDH: glutamate dehydrogenase; Glu: glutamate; GAD: glutamate decarboxylase; GABA: γ-aminobutyric acid; GABA-T: GABA transaminase; SSA: succinic semialdehyde; SSADH: succinic semialdehyde dehydrogenase.

Glutamate dehydrogenase (GDH, E.C. 1.4.1.2) catalyses the reversible amination of 2-OG to Glu, and subcellular localization experiments have demonstrated that GDH is preferentially located in the mitochondria (Dubois *et al.*, 2003). Before the discovery of the glutamine synthase/glutamate synthase (GOGAT) cycles, GDH was believed to be the main portal for ammonium assimilation. Nevertheless, there is evidence that if GDH operated in the anabolic direction, it may reassimilate a small amount of photorespiratory ammonium and/or help assimilate excessive exogenous ammonium. Conversely, if GDH operates in the catabolic direction, it may help fuel the TCA cycle under conditions of

carbon deficit and/or help balance cellular carbon: nitrogen ratios (Purnell *et al.*, 2005). Glutamate decarboxylase (GAD, E.C. 4.1.1.15) catalyses the decarboxylation of L-Glu forming CO_2 and GABA. In plants, GABA is produced almost exclusively by GAD and its activity is modulated by Ca^{2+}/calmodulin (Snedden *et al.*, 1995). Previous work revealed that increases in GAD activity were observed shortly after the imposition of stresses known to cause an increase in GABA content (Wallace *et al.*, 1984; Satyanarayan and Nair, 1986). More recent work of a parallel analysis of transcript and metabolic profiles of potato tuber systems revealed several correlations between the level of gene expression and the level of nutritionally important metabolites, such as tocopherols (Urbanczyk-Wochniak *et al.*, 2003). Correlating my data from enzyme activity and metabolite profiling, I performed a preliminary correlation study, which in some cases showed reproducibility when compared to data published by Urbanczyk-Wochniak *et al.* (2003).

Other alternative routes for the conversion of succinyl CoA to succinate must be considered in order to understand the mild phenotype of SCoAL plants studied here. One of the routes is the glyoxylate cycle, with the enzyme isocitrate lyase which catalyzes the conversion of isocitrate to glyoxylate and succinate in the peroxisome and in plants it is considered to serve an anaplerotic function in plants (Eastmond and Graham, 2001). The succinate produced is transported into the mitochondrion through the succinate-fumarate translocator (Catoni *et al.*, 2003) where it re-enters the TCA cycle to be further oxidized by the enzyme succinate dehydrogenase. In plants, the glyoxylate cycle operates mainly during mobilization of seed triglycerides for seedling growth, where it has a key role in conversion of acetyl CoA from fatty acid β-oxidation into oxaloacetate, and subsequently into sugar and therefore represents an important source of succinate in the illuminated tomato leaf.

Another possible bypass of SCoAL deficiency may occur via a group of enzymes termed CoA transferases, which catalyze the transfer of CoA moieties from one compound to another. One CoA transferase which is present in plant mitochondria, acetyl CoA hydrolase (Zeiher and Randall, 1990), has a broad substrate specificity for different thioesters, including propionyl CoA, butyryl CoA, acetyl CoA, palmytoyl CoA. Less, but nevertheless still activity was detected with oleolyl CoA, succinyl CoA and crotonyl CoA (Zeiher and Randall, 1990). Acetyl CoA hydrolase are though to play a role in hydrolyzing excess of acetyl CoA that may accumulate during oxidative metabolism (Söling and Rescher, 1985). This enzyme may play a role in the hydrolysis of excess succinyl CoA that

might accumulate in SCoAL plants with deficient SCoAL activity. A second possible but more speculative role of acetyl CoA hydrolase is that it might be able to function as a CoA transferase; instead of hydrolyzing succinyl CoA to form succinate, it could transfer the CoA to acetate to form acetyl CoA. The relationship between hydrolases and transferases was demonstrated for thioesterase II, where a single amino acid change was sufficient to give to this hydrolase an acyltransferase activity (Witkowski *et al.*, 1994). However, the lack of change in the levels of acetyl CoA and CoA in the transformants is not in keeping with this hypothesis. That said, further experimental evidence would be required to formally exclude this.

The activation of the shikimate and the isoprenoid pathway in plants with decreased SCoAL activity was at first sight surprising since these pathways are quite removed from that of the TCA cycle. The shikimate pathway (**Figure 4.13**) is defined as the seven metabolic steps beginning with the condensation of phosphoenolpyruvate and erythrose 4-phosphate and ending with the synthesis of chorismate (Hrazdina and Jensen, 1992; Hermann and Weaver, 1999). Chorismate is further metabolized as the precursor of the three aromatic amino acids phenylalanine, tryptophan and tyrosine, and several other compounds of primary metabolism. The shikimate pathway constitutes a part of metabolism that is found only in microorganisms and in plants, but not in animals. One of the branches of the shikimate pathway main trunk is the conversion of dehydroquinate to quinate catalyzed by quinate dehydrogenase. Quinate can accumulate in plants as an alicyclic carbon reservoir for aromatic compound biosynthesis and is also a precursor of the plant secondary product chlorogenate. Chlorogenate is made by combining quinate with caffeate, a late intermediate of phenylpropanoid metabolism. Chlorogenate is believed to protect against fungal attack (Maher *et al.*, 1994). The relationship between the decrease in SCoAL activity and the increase in quinate and chlorogenate in the plants studied here is not clear, but analyzing the correlation between the SCoAL activity and the levels of these metabolites revealed a strong negative correlation for quinate and chlorogenate (R^2 values of 0.941 and 0.964, respectively). Another interesting negative correlation found out was between SCoAL activity and tyrosine content which suggested that the level of this aromatic amino acid increased proportionally with level of decrease in SCoAL activity in the transgenic lines.

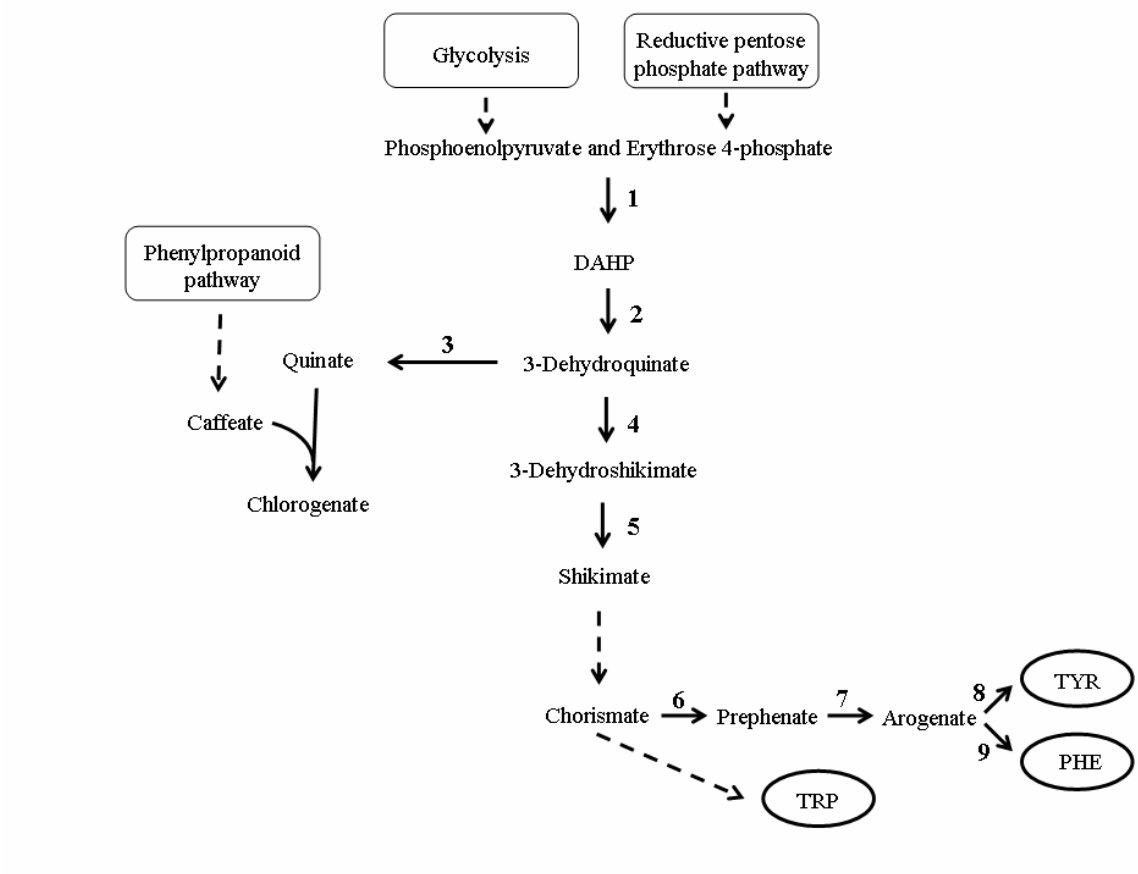

Figure 4.13 The shikimate pathway in plants. The shikimate pathway main trunk is represented until the endpoint of chorismate production, which represents the starting point of aromatic amino acids production. A branch point of the main trunk is also represented with the production of quinate and chlorogenate. Metabolite abbreviations: DAHP: 3-Deoxy-D-*Arabino*-Heptulosonate 7-Phosphate; TYR: Tyrosine; PHE: Phenylalanine; TRP: Tryptophan. Enzymes of the pathway section are: 1: 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; 2: 3-dehydroquinate synthase; 3: quinate dehydrogenase; 4: 3-dehydroquinate dehydratase; 5: shikimate dehydrogenase; 6: chorismate mutase; 7: prephenate aminotransferase; 8: NADP dependent arogenate dehydrogenase; 9: arogenate dehydratase.

Similarly, plastidial synthesized isoprenes are altered in the transformants. The isoprenoid biosynthesis pathway is the main pathway of secondary metabolism that occurs in the plastids and serves to the synthesis of many important compounds such as carotenoid, monoterpenes, diterpenes, isoprene, gibberellins, sterols and the prenyl side chains of chlorophyll and plastoquinone (Dixon, 1999; Hirschberg, 1999; Botella-Pavía and Rodríguez-Concepción, 2006). All isoprenoids are built from the 5-carbon precursor isopentenyl diphosphate, which is itself made from pyruvate and glyceraldehyde-3-

phosphate. **Figure 4.14** represents the main trunk of the isoprenoid pathway indicating that the production of geranylgeranyl diphosphate represents the branch point in the production carotenoid, chlorophylls and tocopherols. Carotenes, and their oxygenated derivatives xanthophylls, are synthesized from geranylgeranyl diphosphate which is metabolized by phytoene synthase to phytoene. Phytoene is substrate of lycopene production and with lycopene the pathway is branched between δ-carotene, α-carotene and lutein, and β-carotene and the xanthophylls zeaxanthin, antheraxanthin and violaxanthin. At the biological function level, chlorophylls are also pigments essentials for photosynthesis, but structurally they are tetrapyrroles (Eckhardt *et al.*, 2004). Chlorophylls and carotenoids share a common precursor from the isoprenoid pathway, which is geranylgeranyl diphosphate (**Figure 4.14**).

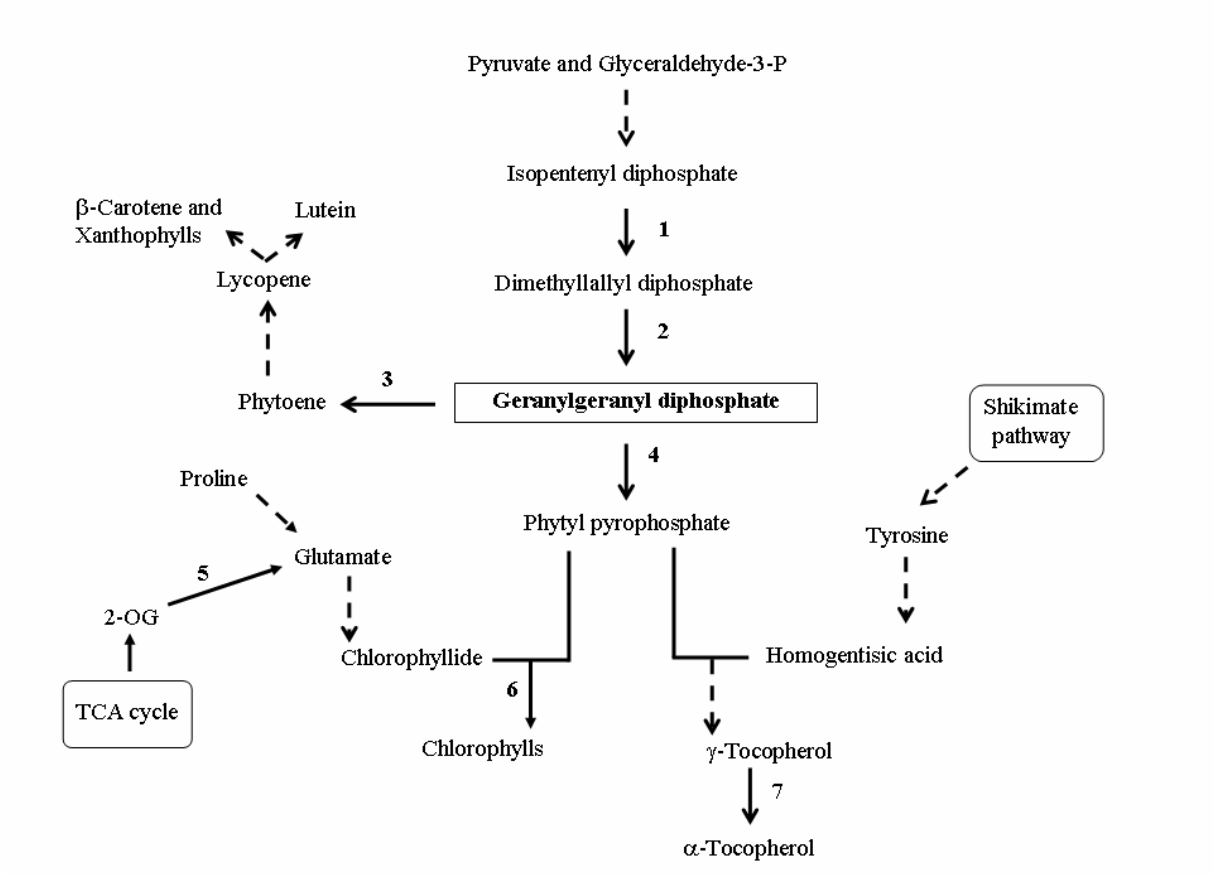

Figure 4.14 Pathways of isoprenoid biosynthesis. The main trunk of the isoprenoid pathway and the branches to the production of carotenoid and other pigments, chlorophylls and tocopherols (showing also the interactions with intermediates of other pathways) is represented. Enzymes of the pathway section are: 1: isopentenyl diphosphate isomerise; 2: geranylgeranyl diphosphate synthase; 3: phytoene synthase; 4: geranylgeranyl reductase; 5: glutamate dehydrogenase; 6: chlorophyll synthase; 7: γ-tocopherol methyl transferase.

Tocopherols, known as vitamin E, are a class of lipid-soluble antioxidants, which are essential in human nutrition (Traber and Sies, 1996) and are synthesized only by photosynthetic organisms. The biosynthesis of this amphiphilic lipid requires precursors derived of two pathways, the shikimate pathway as well as the isoprenoid pathway (Hirschberg, 1999; Bergmüller *et al.*, 2003; Collakova and DellaPenna, 2003; DellaPenna and Last, 2006; DellaPenna and Pogson, 2006; **Figure 4.14)**. In the biosynthetic pathway, geranylgeranyl diphosphate is converted to phytyl diphosphate by geranylgeranyl diphosphate reductase, and this phytyl sidechain is added to homogentisic acid. In the last step α-tocopherol synthesis, γ-tocopherol is methylated by γ-tocopherol methyltransferase, yielding α-tocopherol. Some of the genes that encode for enzymes of the tocopherol biosynthesis pathway were cloned and used in genetic manipulation of plants aiming increase in the total tocopherol level (Garcia *et al.*, 1997; Norris *et al.*, 1998; Keller *et al.*, 1998). On the other hand, one successful manipulation with increase in the α- to γ-tocopherol ratio and an increase in total vitamin E in seeds was obtained in *Arabidopsis* with the overexpression of γ-tocopherol methyltransferase (Shintani and DellaPenna, 1998). More recently, interesting results were obtained from the analysis of a tocopherol-deficient Arabidopsis mutant lacking tocopherol cyclase activity (Porfirova *et al.*, 2002), that suggested that the absence of tocopherol had no largely impact on photosynthesis or plant viability. Similar results were obtained from the characterization of Arabidopsis mutants deficient in γ-tocopherol methyltransferase revealing that a shift in tocopherol composition or the absence of tocopherol did not affect the photosynthetic apparatus at high light and had no major impact on the amount of specific fatty acids or on lipid hydrolysis (Bergmüller *et al.*, 2003). In another study, transgenic tobacco plants with antisense repression of the geranylgeranyl reductase gene, *ChlP*, have decreased contents of total and phytylated chlorophyll as well as of tocopherol (Tanaka *et al.*, 1999). In the present work, although the genetic manipulation was directed to the TCA cycle, an alteration in the tocopherol pathways was observed. A negative correlation between succinyl CoA β subunit transcript and the tocopherol level was determined in previous work (Urbanczyk-Wochniak *et al.*, 2003). Searching in the Comprehensive Systems-Biology Database (http://csbdb.mpimp-golm.mpg.de/csbdb/dbcor/ath/html) for correlations of the *Arabidopsis* gene At2g20420, which encodes for SCoAL β subunit, it was found that it correlates negatively with the gene At1g74470, which encodes for geranylgeranyl reductase. These are evidences for the relationship between SCoAL and

tocopherol and chlorophylls. In agreement with this result, a close examination of the transcript profiling at the terpenes BIN, RL25 revealed a decrease in the transcript level of γ-tocopherol methyltransferase (**Figure 4.10 A**). A potential hypothesis for this could be a feedback inhibition of γ-tocopherol methyl transferase transcription by the end product. Irrespective of the mechanism this result is highly exciting since it validates a previous proof of concept study utilizing a combination of transcript and metabolic profiling to the identification of candidate genes for biotechnology. Interestingly plant hormone gibberellin is also a constituent of this upregulated branch of secondary metabolism potentially explaining the increased internodes length of the transformants.

As stated above transformants revealed increased levels of 2-oxoglutarate, GABA, carotene and some xanthophylls, of chlorophyll *a* and of tocopherols, but since it is dangerous to try to estimate fluxes from changes in the steady-state levels of metabolites, I would such as to be cautious about generate hypotheses concerning plant physiological regulation on the GABA shunt an other metabolic pathways caused by decrease in SCoAL activity. For these reason, the next step would be the measurement of flux, following the flow of atoms through metabolite systems using radioactive isotopes or stable isotopes of non-natural mass. The metabolic flux analysis in tomato wild type and in SCoAL transgenic lines RL40 and RL25 has already been started. For this purpose, tomato leaflets were cut and incubated in buffer containing ^{13}C-labelled glutamate and acetate, at different concentrations for a period of 1 and 6 h. After incubation, leaf discs were isolated from the leaflets and immediately frozen in liquid nitrogen to afterwards been processed for GCMS. After analysis of GCMS chromatograms and in addition to ^{14}C glutamate and ^{14}C acetate feeding experiments, I expect to have more information concerning the GABA shunt, and to confirm the physiological link between succinyl CoA ligase and nutritionally important metabolites such as tocopherols and β-carotene.

Despite the fact that some questions were not fully answered in this work, the data presented allow several important conclusions to be made:

[i] Plants with decreased SCoAL activity consequent of a decreased expression of *SCoAL β* gene exhibited a lack of photosynthetic and growth phenotype. Photosynthetic parameters were measured directly through *in vivo* gas-exchange measurement in leaf and indirectly through evaluation of the CO_2 assimilation rate of leaf discs. Growth phenotype was recorded through a detailed measurement of plant organs, which were also dissected

and individually analyzed. The lack of phenotype led to search for putative alternative routes that complemented the deficiency of SCoAL activity.

[ii] The GABA shunt, which provides succinate to the mitochondria, was suggested to be the alternative route that complemented the SCoAL deficiency in the transformants. Metabolite profiling of intermediates of the GABA shunt, such as 2-OG, Glu and GABA, as well as the measurements of enzyme activities that catalyze the GABA shunt confirmed the activation of this route in the transformants and consequently the replenishment of succinate for further oxidation through the TCA cycle.

[iii] Further analysis of metabolite profiling suggested the activation of other pathways such as shikimate and the isoprenoid pathways in the transformants. The content of one nutritionally important end product of the isoprenoid pathway, α-tocopherol, was increased in the transformants, so the case also for other end products of this pathway such as carotenoids and chlorophylls. This showed that the deficiency in SCoAL activity caused an increase in α-tocopherol, carotenoids and chlorophylls.

The relevance of this *in vivo* properties of SCoAL determined in the previous chapter in light of these findings will form the focus of the final chapter.