

Chapter 5

General discussion

The aim of this project was to improve the understanding of the role of succinyl CoA ligase on photosynthetic metabolism of plants. In the general introduction of **Chapter 1** are presented the importance of tomato plant as model system, as well as a critical synthesis of the current understanding of the mitochondrial contribution to the metabolism of the illuminated leaf. The focus was mainly on the TCA cycle from the perspective of its role(s) within specific biological processes and the extensive interactions that occur between this pathway and many other pathways of the cell. At the beginning of the project, the functional characterisation of several genes from the TCA cycle was lacking. Therefore, as described in **Chapter 3**, a list of cDNAs coding for enzymes from the TCA cycle were analyzed from a tomato EST collection and screened for full-length. Among the successfully cloned full-length cDNAs, were the two α - and one β -subunits of succinyl CoA ligase. This chapter also detailed a novel assay to measure succinyl CoA ligase activity in the direction of succinate formation in total plant protein extracts. With this assay it was possible to perform preliminary characterisation of the kinetic properties of succinyl CoA ligase and also screen tomato transformants with decreased succinyl CoA ligase activity. These tomato plants expressed the complete SCoAL α - and β subunits separately, in antisense orientation or RNAi technology.

The transformed selected plants exhibiting decreased SCoAL β subunit expression and decreased enzyme activity were characterized at phenotypical, biochemical and physiological levels. The detailed characterisation was described in **Chapter 4**. Generally, little effect on photosynthesis was observed in the transformants when compared to the antisense mMDH lines (Nunes-Nesi *et al.*, 2005a) and the *Aco1* mutant (Carrari *et al.*, 2003). However, significant consequences were obvious on the secondary metabolism of the transformants. Pigments such as chlorophylls, β -carotene, xanthophylls, and lutein are components of the isoprenoid biosynthetic pathway and share a common precursor. All pigments revealed a tendency to be increased in the transformants, and a significant increase was obtained for chlorophyll *a* and β -carotene in two transgenic lines. Similarly, products of the shikimate pathway such as the aromatic acids tyrosine and phenylalanine were significantly increased. Also intermediates of this pathway, such as quinate and its by-product, chlorogenate, were increased in the transformants. These results led to the hypothesis that these alterations were consequence of an increased flux into the shikimate and the isoprenoid pathways.

The increase in aromatic amino acids and isoprenoid levels in the antisense and RNAi SCoAL transformants was shown not to be an indirect effect due to alterations in photosynthesis, or carbohydrate content. Decreased SCoAL activity did also not lead to a general increase in amino acids, rather only to aromatic amino acids. These results highlight two important features of the interaction between primary and secondary metabolism. First, fluxes in secondary metabolism can be favoured by an alteration of primary metabolism that causes an increased precursor supply, also shown in Henkes and collaborators (Henkes *et al.*, 2001). This shows that major changes in secondary metabolism require appropriate reprogramming of primary metabolism. Secondly, in a more general concept, the discrimination between primary and secondary metabolism leads to question about the regulation of what were previously regarded as independent and unrelated biological processes. Indeed, few metabolic pathways work in isolation. Different pathways share the same metabolic intermediates such that pathways become interdependent and connected with one another. Therefore, metabolism constitutes an example of dynamic network, not a static entity but rather one that is constantly rewired and reconfigured to suit the prevailing physiological demands placed upon the cell at a given moment (Sweetlove and Fernie, 2005). The utility of genomic scale profiling technologies such as those described in this thesis is beginning to allow fully understanding of these connections.

This work also highlighted that a decreased activity in SCoAL activity led to an increased content in α -tocopherol in the transformants, which is also product of the isoprenoid pathway with nutritional value. These results are of particular interest given that a negative correlation between succinyl CoA ligase transcript level and tocopherol were already observed in an integrated transcript and metabolite profile in potato tuber systems (Urbanczyk-Wochniak *et al.*, 2003). Interestingly, the same relationship was obtained by a similar approach analysing Arabidopsis leaves (Bruedigam, Studart-Guimarães and Fernie, unpublished, **Figure 5.1**). Therefore, these results suggest that the correlation between a decrease in SCoAL β -subunit transcript level (and total SCoAL activity) and tocopherol metabolite level is a general phenomenon in plants. Beside the important biological aspect of these findings, these results show the power of combining metabolite and transcript in discriminate for candidate genes to manipulate important metabolite levels (Tohge *et al.*, 2005).

In plants several genes have been annotated on the basis either of correlations between transcript and metabolite levels or simply on the basis of altered metabolite profiles. To date these approaches have proven more informative in secondary metabolism with clear annotations possible for genes associated with the isoflavonoid and triterpenoid pathways of *Medicago truncatula* (Suzuki *et al.*, 2005; Achnine *et al.*, 2005), the methylketone pathway of tomato (Fridman *et al.*, 2005), pyridine alkaloid biosynthesis in tobacco (Goossens *et al.*, 2003) and glucosinolate, flavonoid and sterol biosynthesis (Hirai *et al.*, 2004; Tohge *et al.*, 2005; Morikawa *et al.*, 2006). In those examples altered metabolite profiles could be correlated to specific genes and on subsequent heterologous over-expression gene function could be proven.

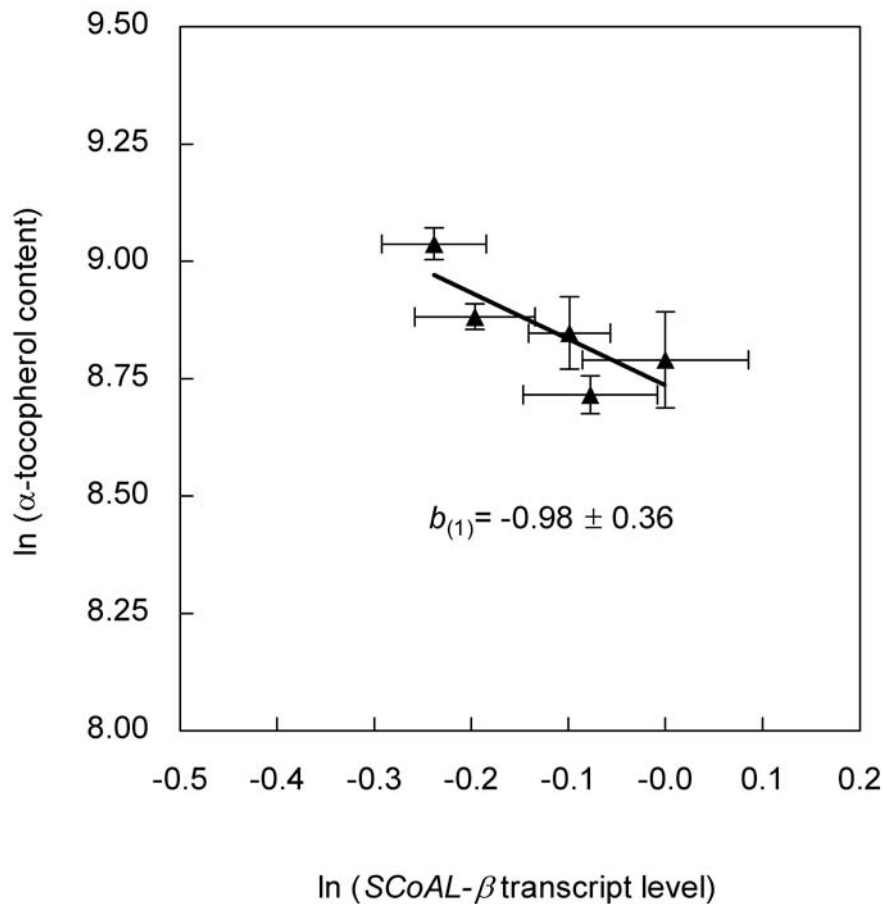


Figure 5.1 Relationship between *SCoAL* β subunit transcript level and α -tocopherol accumulation in *Arabidopsis thaliana* leaves. Data were plotted on a natural logarithmic scale. $b_{(1)}$ gradient of the slope. The correlation coefficient (r) for this line equals -0.81 ($p = 0.09$). Data points represent four transgenic lines and wild type.

Another feature revealed in the transformants with decreased SCoAL activity was a slight decrease in rate of respiration when compared to antisense mMDH lines and antisense fumarase lines (Nunes-Nesi and Fernie, unpublished). The rate of respiration was indirectly deduced from a flux analysis of Glc through glycolysis, OPPP and the TCA cycle after incubation of leaf discs in buffer containing differentially positionally labelled ^{14}C -Glc molecules. Calculations of the respiration rate are based on the ratio C1 to C3:4 after analysis of the evolution of $^{14}\text{CO}_2$. This ratio is proportional to the decrease in the TCA cycle function given that less $^{14}\text{CO}_2$ is being released from ^{14}C [3:4]Glc in the TCA cycle than from other carbohydrate oxidation pathways. Comparative analysis of respiration rate on antisense mMDH lines and fumarase lines are presented in **Table XI**.

Table XI. Comparison of respiration rates on transformed tomato plants. Percentage of decreased respiration rates are represented for tomato plants transformed with antisense mitochondrial malate dehydrogenase (mMDH; Nunes-Nesi *et al.*, 2005a); with antisense fumarase (Nunes-Nesi and Fernie, unpublished) and antisense and RNAi succinyl CoA ligase, and their respective transgenic lines. Data were extracted from the evaluation of the relative rates of carbohydrate oxidation.

Plants	Antisense mMDH		Antisense fumarase		Antisense and RNAi SCoAL		
Lines	AL8	AL21	FL11	FL41	AL18	RL40	RL25
% of respiration rate	40	38	26	49	10	43	20

From **Table XI** it is clear that the respiration rate in the SCoAL transformants is not much affected. When data from dark respiration were considered, a similar conclusion can be taken because the respiration rate is impaired in 30% in the SCoAL transformants even in the strongest line, whilst the antisense mMDH lines is 70% impaired in the strongest line despite having a less dramatic reduction in activity. Metabolite and enzyme activities determinations suggest that the GABA shunt was activated to bypass the SCoAL deficiency and replenished succinate levels in the TCA cycle. On the basis of these data I propose that the GABA shunt maintains the respiration rate with the only difference that instead of succinate and ATP produced by SCoAL activity, succinate and NADH are produced. In spite of the loss of this substrate level phosphorylation the total ATP content is unaffected in the transformants (**Table IX**). Succinic semialdehyde dehydrogenase (SSADH) a key enzyme of the GABA shunt operates in the mitochondria and catalyzes the final reaction producing succinate and NADH from succinic semialdehyde (SSA).

Cloning and kinetic characterisation of Arabidopsis SSADH showed interesting features of its possible regulation (Busch and Fromm, 1999). In this work the influence of different organic acids, such as intermediates of the TCA cycle and the GABA shunt, was investigated on SSADH reaction rate. Results revealed that GABA, Glu, pyruvate and succinate had no effect at concentrations from 1 μ M to 10 mM, but product inhibition was observed for NADH. Interestingly, the initial-rate studies on SSADH kinetics showed also that an inhibition caused by AMP and ADP was dependent on NAD⁺ concentration, whereas the inhibitory effect of ATP was independent of it (Busch and Fromm, 1999).

The initial aim of this project was to elucidate the influence of the TCA cycle metabolism on photosynthesis by altering the activity of one TCA cycle enzyme. Results presented appear to be redundant, however interesting hypothesis arose. First, in **Chapter 3** a preliminary characterisation of SCoAL kinetic properties was performed. Results of the investigation of the effects of different intermediates of the TCA cycle, among other compounds, on SCoAL reaction rate revealed an allosteric regulation of SCoAL activity. Available evidence showed above suggests that the GABA shunt is probably not under allosteric regulation considering that succinate, GABA nor Glu had any effect on SSADH reaction rate. However, a more complete study of allostery is required for the other reactions of the shunt. Second, GENEVESTIGATOR databank shows that genes associated to the GABA shunt are differently regulated than genes associated to the TCA cycle. Whilst this does not constitute a definitive proof, it is consistent with my hypothesis. Further obvious experiments are suggested: (i) Create tomato SCoAL α and β subunits double antisense and to enzymes of the GABA shunt. (ii) Submit plants to stress conditions and analyse GABA shunt activity. GABA is a known stress metabolite and the GABA shunt activity is rapidly increased in plants under biotic and abiotic stresses (Snedden and Fromm, 1999; Shelp *et al.*, 1999). In response to several stress conditions, such as hypoxia, temperature shock, and mechanical manipulation induced rapid GABA accumulation (Wallace *et al.*, 1984; Shelp *et al.*, 1995). Interestingly, many of the same stresses that stimulate GABA synthesis in plants also cause fluxes in cytosolic Ca²⁺ (Knight *et al.*, 1991), which is involved in the physiological responses to a variety of environmental stimuli. The activity of the first enzyme of the GABA shunt, glutamate decarboxylase (GAD), is modulated by Ca²⁺ / CaM (Snedden *et al.*, 1995), and increases also by stresses that cause rapid increase of GABA. SCoAL transformants are under a stress condition, but in this case is a long term condition and constant situation.

These experiments would be to test the hypothesis that the long-term stress condition caused by decreased SCoAL activity in the transformants induced GABA shunt activation.

All the experiments performed in this work are based on the analysis of illuminated leaves. Little is known about the physiological effect of decrease in SCoAL in the tomato fruit. Results from the phenotype analysis performed in the tomato plants revealed a differentiated development of the flowers, smaller fruits in all lines and less total number of fruits in the transformants. From the Northern blot analysis (**Figure 9**) and the SCoAL activity measurements of different tomato organs (**Chapter 3**); it is clear that the transcript level of SCoAL and also the activity of SCoAL in tomato floral tissues are stronger when compared to the other organs. GABA content in fruits is high (Roessner-Tunali *et al.*, 2003), but little is known on its functions. The analysis of transgenic tomato fruits would be important for two reasons: (i) Respiration has a fundamental importance in fruit ripening. Especially in climacteric fruits, such as tomatoes, which are distinguished from non-climacteric fruits by their increased respiration and ethylene biosynthesis rates during ripening (Giovannoni, 2001). A detailed metabolite profiling and complementing studies could be performed in order to have experimental evidences for the function of the GABA shunt in fruits. Interesting starting information that shows the differences in leaf and fruit metabolism was in Purnell *et al.* (1997) and confirmed in the previous chapter, is that no activity of glutamate dehydrogenase is detected in fruits. (ii) Secondly, the great importance of tocopherol in the human diet suggests the fruit would be worthy of study. Tocopherol content is increased in leaves of SCoAL transformants, but humans and animals only consume tomato fruits. Therefore it would be important and interesting to evaluate tocopherol content in fruits.

Transgenic plants expressing SCoAL α 1 subunit in antisense orientation and exhibiting decreased enzyme activity were also characterized in parallel to the analysis of tomato SCoAL β -plants. Studies on bacterial succinyl CoA ligase demonstrated that the α subunit was the catalytic subunit and the β subunit determined the nucleotide specificity. Intriguingly, the transformants expressing tomato SCoAL α 1 subunit in antisense orientation showed no phenotype and metabolite profile revealed any significant differences when compared to wild type. It appears likely could be that the degree of decrease of SCoAL activity achieved with the SCoAL β subunit downregulation was higher than that achieved using the SCoAL α 1 subunit.

That said plants show altered metabolite content when SCoAL activity is reduced a certain threshold. Second, it is possible that in plants SCoAL β subunit plays a regulatory role and not merely a role in determining the nucleotide specificity.

In a parallel attempt to understand the role of SCoAL in plants, Arabidopsis plants were co-transformed with yeast succinyl CoA ligase (LSC) α and β subunits to obtain plants with increased SCoAL activity. Transformants were screened by enzyme activity and selected lines were subjected to phenotypical and physiological characterisation. Interestingly, these transformants revealed impaired photosynthetic rate, including an altered CO₂ assimilation and chloroplast electron transport rates. Together with data from a detailed metabolite profiling on these transformants, complementary information could be obtained to better understand SCoAL regulation in plants. That said, this study represents the first reverse genetic characterisation of SCoAL and in this study I was able to characterize the enzyme at the genetic, biochemical and functional basis. The latter was performed exclusively in the illuminated leaves since previous studies indicated an important role for the TCA cycle in the photosynthetic process. The fact that antisense and RNAi of SCoAL produced remarkably differentiated phenotype than that observed in *Aco1* mutant and antisense mMDH plants suggest that as in yeast the TCA cycle of plants operates in distinct modules.