Chapter 3

Molecular cloning of tomato TCA cycle full-length cDNAs and characterisation α and β subunits of succinyl CoA ligase

3.1. Introduction

The operation and the localization of the complete TCA cycle was demonstrated in plant cells decades ago (Beevers, 1961) and almost all genes encoding enzymes involved in this cycle have been cloned, but their functional role in the control of primary metabolism in plants is so far from clear (Hill, 1997; Siedow and Day, 2000). Specifically the regulatory actions of the steps catalysed by the enzymes of the pyruvate dehydrogenase complex (PDC), enzymes of the 2-oxoglutarate complex (2-OGC), succinyl CoA ligase (SCoAL) and succinate dehydrogenase (SDH) have not been studied *in planta* until now. This study was focused in the succinyl CoA ligase because it catalyzes the substrate level phosphorylation of ADP, and as such is hypothesized to play an important role in the tricarboxylic acid (TCA) cycle in the mitochondria. It has been shown before, that altering the expression of genes from enzymes involved in the TCA cycle or altering their activity affects the photosynthetic rate of the plant (Carrari *et al.*, 2003; Nunes-Nesi *et al.*, 2005a). Therefore, succinyl CoA ligase could also be closely involved in the plant photosynthetic metabolism. At the beginning of this work, malate dehydrogenase (MDH), particularly the mitochondrial isoform which participates also in the TCA cycle catalyzing the conversion of malate to oxaloacetate, was also an aim of study in our group. Therefore, I contributed for that project cloning the tomato full-length cDNAs of the mitochondrial, cytosolic, chloroplastic and glyoxysomal MDH isoform, obtaining the complete sequences (Genbank accession numbers, see **Table II**), and performing a preliminary study on subcellular localization in Arabidopsis.

Succinyl CoA ligase (SCoAL; E.C. 6.2.1.5), also known as succinyl-CoA synthetase, succinate thiokinase and succinate-CoA ligase, catalyses the interconversion of succinyl CoA, inorganic phosphate and dinucleotide to succinate, trinucleotide and CoA (Johnson *et al.*, 1998), as in the following reaction:

Succinyl-CoA +Pi +NDP ↔ Succinate + NTP + CoA,

where NDT and NTP denotes ADP or GDP and ATP or GTP, respectively. SCoAL requires the metal-ion cofactors Mg^{+2}, Mn^{+2} or Co^{+2} for activity, which was firstly verified by Palmer and Wedding (Palmer and Wedding, 1966).

The forward direction occurs in the TCA cycle to produce succinate and regenerate ATP/GTP through substrate level phosphorylation of ADP/GDP. The reverse direction produces succinyl-CoA for ketone body activation and heme synthesis in animal cells. Little is known of the plant enzyme; however, all SCoALs studied consist of two types of subuniț α; with a molecular mass of 29-34 kDa and a single β subunit with a molecular mass of 41-45 kDa. There is growing evidence that in mammalian cells there are two ligases; one specific for ADP, the other for GDP and that the latter catalyses the synthesis of succinyl CoA during ketone body formation (Ryan *et al.*, 1997). Recent evidence suggests that the β-subunit is important for conferring nucleotide specificity to the mammalian SCoAL, with relative transcript and polypeptide levels of the GDP and ADP specific ligases differing greatly with tissue type in the rat (Lambeth *et al.*, 2004). In contrast *Saccharomyces cerevisiae* and *Escherichia coli* contain only single β-genes but are able to utilize both GDP and ADP (Przybyla-Zawislak *et al.*, 1998; Fraser *et al.*, 1999). In contrast to the microbial and mammalian enzymes which can also, by varying means, use guanine nucleotides as substrate, the plant ligase is specific for adenine nucleotides (Palmer and Wedding, 1966). The plant enzyme has been purified from spinach (Kaufmann and Alivisatos, 1955), artichoke (Palmer and Wedding, 1966) and soybean (Wider and Tigier, 1971), however, at the beginning of this work functional identification of the genes encoding the enzymes was lacking for plant species. Recent studies on proteomic identification of plant mitochondria suggest that SCoAL α is the subunit responsible for metal binding (Herald *et al.*, 2003).

The aim of the work described in this chapter was the characterisation of tomato SCoAL including a functional identification by yeast complementation, of the three open reading frames encoding its constituent subunits. The strategy used to obtain the full-length cDNA from a set of the tomato EST collection is described, as is the cloning of the tomato mitochondrial, glyoxysomal, cytosolic and chloroplastic isoforms of malate dehydrogenase.

3.2. Results and Discussion

3.2.1. Cloning of tomato full-length coding regions for 2-OGC E2 and E3 subunits, SCoAL α and β subunits, SDH iron sulphur subunit, PDC E1α and β subunits, and all MDH isoforms

Searching tomato EST collections (Van der Hoeven *et al.*, 2002), on the basis of nucleotide structure and fragment length facilitated the isolation of 12 putative full-length cDNAs, including the 1164-bp *Sl*SCoAL α1, the 1322-bp *Sl*SCoAL α2 and the 1427-bp *Sl*SCoAl β, as described in **Chapter 2**. In **Table I** are listed all 16 TCA cycle cDNAs that were initially analyzed, considering all isoforms of MDH, and those putative full-length that were selected. Only four coding regions of the initially selected genes are missing (**Table I**): The E2 subunit of the PDC, the SDH flavoprotein subunit, the E1 subunit of the 2-OGC and the ACO. However, as described in Alba *et al.* (2004) EST collections also have limitations. Problems could derive because EST collections do not exactly reflect the mRNA population and low abundance transcripts are merely represented. This could be the case of SDH flavoprotein subunit, PDC E2 subunit and 2-OGC E1 subunit. *In digital* expression analysis performed using the annotations from the TIGR tomato EST collection (**Table III**) showed that few ESTs homologous to these genes are present in this collection and this suggests that their transcript could be lowly represented. In the case ACO and 2-OGC E1 subunit, another problem could rely on the size of the genes (2.8-3.0 kb) therefore the probability to obtain a full-length EST very low. Other sources of errors could also arise during storage, handling, replication, and management of the EST collection reflected in the fact that for example the bacteria failed to grow, and in other cases it was not possible to make a plasmid extraction. Full-length coding regions were further cloned into pENTR vector as described in **Chapter 2** and subcloned into plant- or yeast-specific expression vectors.

Table III. *In digital* expression analysis. This analysis is based on the ESTs annotation from the TIGR Tomato Gene Index (http://www.tigr.org/tdb/tgi/lgi/) Fr: fruit; Fl : flower; R: root; L: leaf. Numbers denote the numbers of ESTs homologous to each gene by the in digital screening of about 278016 ESTs. SCoAL: succinyl CoA ligase and its subunits; PDC: pyruvate dehydrogenase complex and it subunits; 2-OGC: 2-oxoglutarate complex and its subunits; SDH: succinate dehydrogenase and its subunits; ACO: aconitase

Tomato tissues	**Genes**										
	SCoAL β	**SCoAL α**	**PDC E1α**	**PDC E1β**	**PDC E2**	**2-OGC E3**	**2-OGC E2**	**2-OGC E1**	**SDH iron**	**SDH flavo**	**ACO**
Crown gall	1		1	3	1	1	2		1	1	3
Callus and suspension cult.	2	3	7	3		7	2	1	4		5
Fl 0-3mm buds	3	1		3			2		1		
Fl 3-8mm buds	1	1	1	3		1	1		1	1	
Fl buds anthesis			4	2		1					
Shoot/meristem	1	1	5	2	1					1	1
Fr mature green	1		2	1	4				1		3
Fr red ripe		3	1	2			1		2		
Fr breaker	1	2	2	3			2		3	1	
Fr developing/immat			3	3	1						4
Ovary		1	2	2	1	2		1	2	1	5
R nutrient deficient	1	2	4	2		4	1				
R etiolated radicle	1				1		2			1	2
R plants at pre-anthesis		1	2	2	2		2				1
R plant at fruit set	1	1	1	1			2				2
Wild pollen							1		6		
Germinating seed	1			1		3	1		2		7
L *Pseudomonas* resistant	4	2	3	2		1				1	3
L *Pseudomonas* susceptible		2	4	2		4	1	2			2
L mixed elicitor	3	3	5	4		3					3

3.2.2. Subcellular localization of SCoAL α and β subunits and MDH isoforms

To validate the cloning strategy and to investigate the functionality of the coding regions cloned, I used GFP fusion vectors in order to follow the protein targeting and subcellular localization.

A. Subcellular localization of tomato SCoAL in Arabidopsis

For tomato SCoAL (*SlSCoAL*) *α1* and *α2*, and *SlSCoAL β* genes, I first analyzed the peptide sequences using publicly accessible prediction softwares like TargetP (Emmanuelson *et al.*, 2000), Predotar (Small *et al.*, 2004), and SignalP (Bendtsen *et al.*, 2004). Both *SlSCoAL α* and *SlSCoAL β* were predicted to be targeted to the mitochondria (**Table IV**). In this table are included the corresponding Arabidopsis genes to compare the result of this bioinformatic approach, because the Arabidopsis proteins were already detected in isolated mitochondria (Millar *et al.*, 2001). Similar results were obtained with this analysis for the tomato and the Arabidopsis genes.

Table IV. Prediction of subcellular localization. Amino acid sequences of SlSCoAL α1, SlSCoAL α2, SlSCoAL β and the Arabidopsis accessions corresponding to the same subunits of tomato were introduced into TargetP software for prediction of subcellular localization. Abbreviations: cTP: cytosolic and mTP: mitochondrial transit peptide; SP: secretion peptide; M: mitochondria; C: cytoplasma; Loc: location; RC, reliability coefficient.

Name	Length	cTP	mTP	SP	Loc	RC
SlSCoAL α1	332	0.386	**0.512**	0.064	**M**	5
SlSCoAL α2	337	0.428	**0.802**	0.015	**M**	4
SlSCoAL β	417	0.014	**0.948**	0.016	**M**	1
At5g23250 (SCoAL α1)	341	0.450	**0.885**	0.005	**M**	3
At5g08300 (SCoAL α2)	347	0.710	0.697	0.010	C	5
At2g20420 (SCoAL β)	421	0.020	**0.876**	0.014	**M**	2

To confirm these results in Arabidopsis the complete coding regions were fused at the carboxyl terminal end, to an enhanced green fluorescent protein (EGFP, Karimi *et al.*, 2002; **Figure 3.1**) and stably expressed in Arabidopsis plants. GFP fluorescence was detected in protoplasts prepared from leaves of these plants in coincidence with the MITOTrack™ dye (**Figure 3.1 C**). These data are in keeping with the documentation of both succinyl CoA isoforms in the mitochondrial proteome (Millar *et al.*, 2001; Sweetlove *et al.*, 2002), and in fact they even suggest an exclusive mitochondrial localisation of these proteins.

B. Subcellular localization of *MDH* isoforms in Arabidopsis

In the case of the tomato malate dehydrogenase (*SlMDH*) the complete coding regions for the mitochondrial (m), chloroplastic (ch), cytosolic (c) and glyoxysomal (g) isoforms were cloned into the same vector used for SCoAL described above and stably expressed in Arabidopsis plants (**Figure 3.2**). GFP protein was analyzed in whole leaf tissue and detected exclusively in the compartments expected according to the annotation of each isoform. These results confirm that the tomato genes were correctly cloned, they were correctly transcribed and their resulting proteins were correctly targeted.

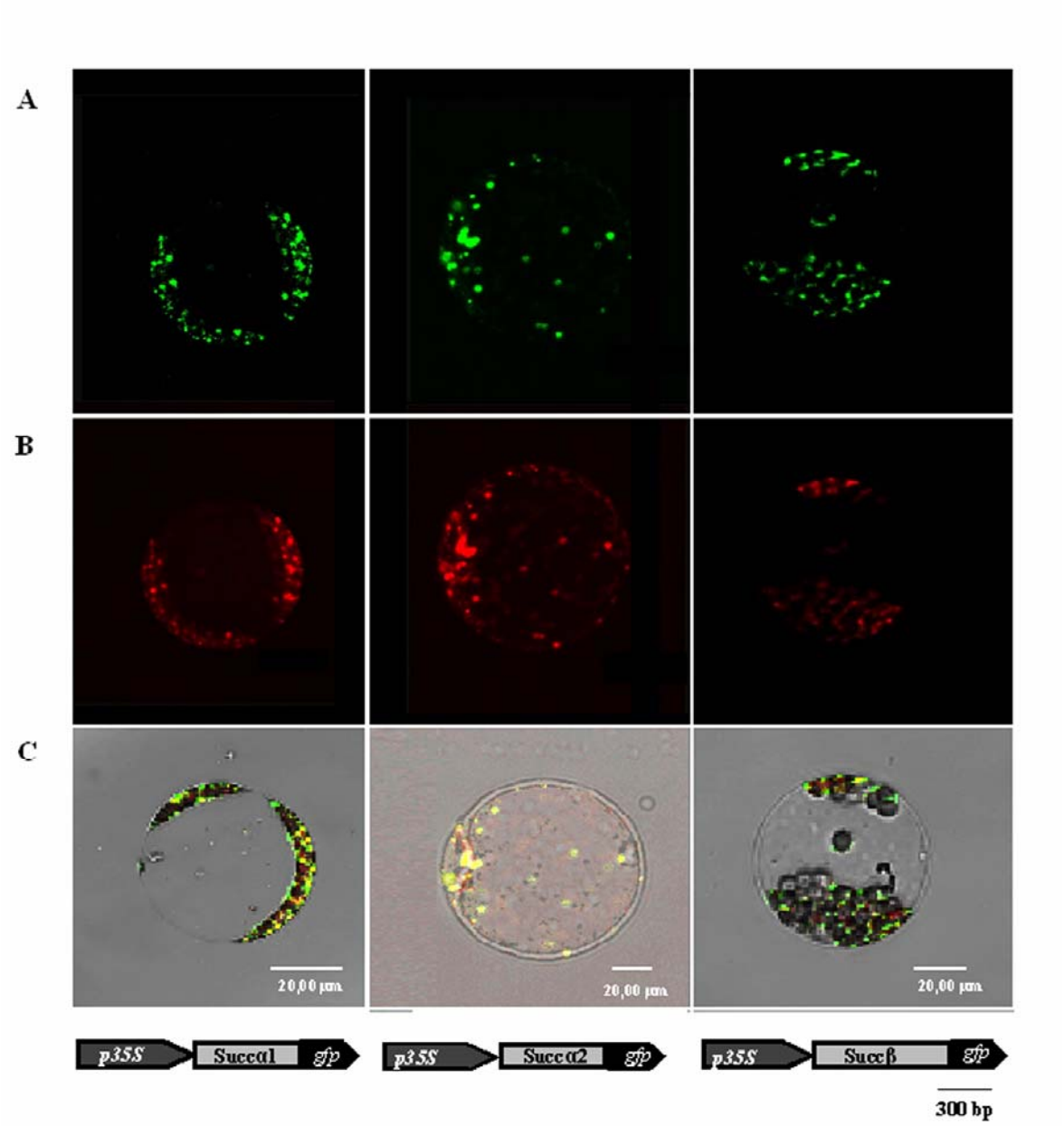


Figure 3.1 Expression of *SlSCoALα1* and *2* and *SlSCoAL β* - GFP fusion in *A. thaliana* protoplasts. SlSCoAL α1-GFP, SlSCoAL α2-GFP and SlSCoAL β-GFP transformed protoplasts were used for confocal microscopy. (A) GFP fluorescence; (B) MitoTracker visualization (fluorescence excitation at 554 nm and emission at 576 nm) and (C) Merged image of A and B. Below each picture schemes of the constructs used to transform *A. thaliana* are shown.

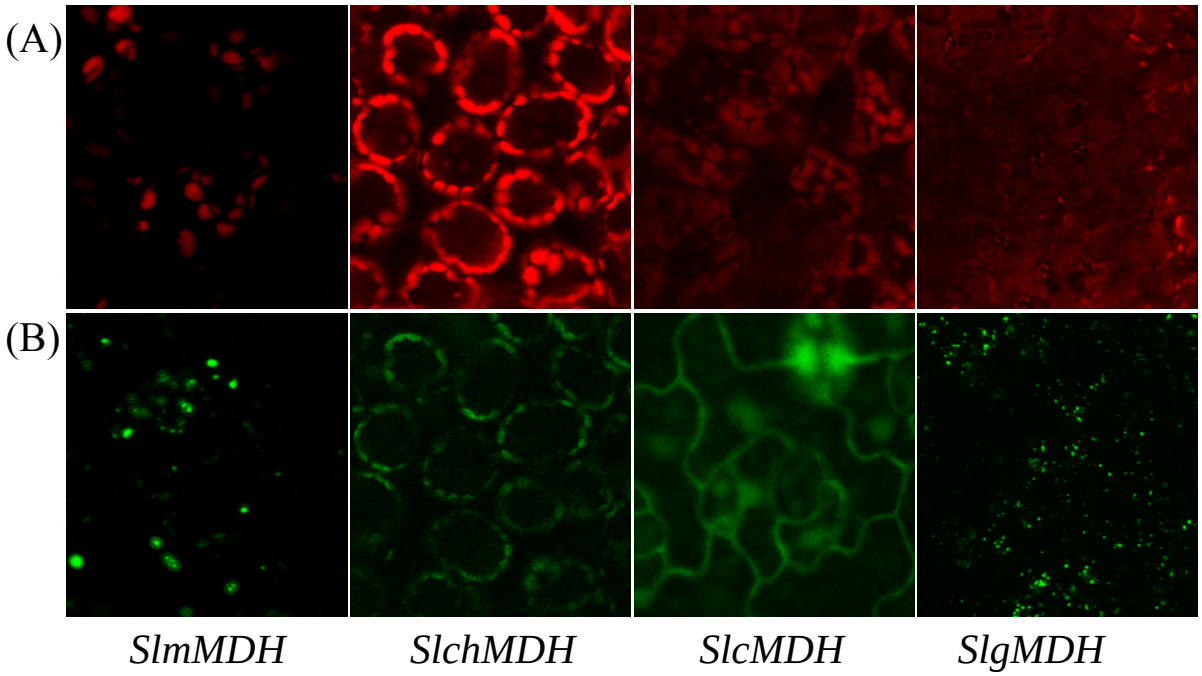


Figure 3.2 Expression of *SlmMDH, SlchMDH, SlcMDH* and *SlgMDH* – GFP fusion. GFP was detected in leaves from stable transformed Arabidopsis plants. (A) Chloroplasts autofluorescence (B) GFP fluorescence.

3.2.3 Identification of plant succinyl CoA ligase subunits by functional complementation of subunit-deficient yeast strains

The *Saccharomyces cerevisiae* strains ΔLSC1 and ΔLSC2 which carry mutations in the α and β subunits of succinyl CoA ligase, respectively (but are otherwise identical to strain BY4741), require high concentrations of glycerol for efficient growth (Przybyla-Zawislak *et al.*, 1998). Expression of *Sl*SCoAL α1 or *Sl*SCoAL α2 complemented growth of yeast ΔLSC1 on limiting media and *Sl*SCoAl β complemented growth of ΔLSC2 (**Figure 3.3**). Dilution drop growth assay indicates that *Sl*SCoAL α1 complements ΔLSC1 slightly better than *Sl*SCoAL α2. Mitochondria were isolated from WT, from complemented yeast mutants and from the mutants to test how the deletion of one of the genes affected the SCoAL activity. The grade of purity of the mitochondrial preparation was tested measuring a mitochondrial specific enzyme, cytochrome c oxidase, in the mitochondria suspension and the post-mitochondrial supernatant. Cytochrome c oxidase activity was detected as expected only in mitochondria enriched preparation.

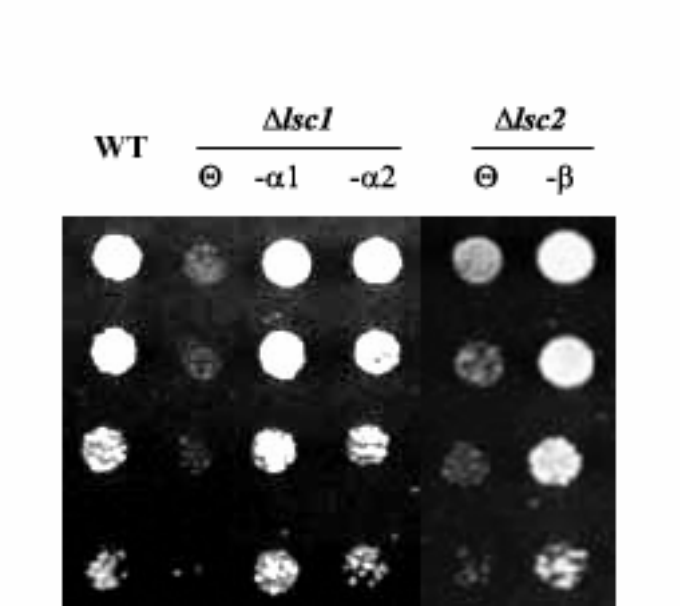


Figure 3.3 Functional complementation of succinyl CoA ligase subunit deficient yeast strains by the *Sl*SCoAL open reading frames. Transformants were grown overnight on SSM with glucose as sole carbon source before being thoroughly washed. The culture was subjected to tenfold serial dilutions and 4 μL of each dilution was spotted onto SSM with glycerol as sole carbon source. Plates were photographed after incubation at 30°C for 4 days. WT: BY4741 Genotype (strain) transformed with the empty vector; Δ*lsc1* (yeast mutant for the α subunit); Δ*lsc1*-θ (mutant transformed with the empty vector); Δ*lsc1*-α1 (mutant complemented with the tomato open reading frame *Sl*SCoALα1); Δ*lsc1*-*α*2 (mutant complemented with the tomato open reading frame *Sl*SCoALα2). *Δlsc2* (yeast mutant for the β subunit); Δ*lsc2*-θ (mutant transformed with the empty vector); Δ*lsc2*-β (mutant complemented with the tomato open reading frame *Sl*SCoALβ).

SCoAL activity was then measured in the mitochondrial suspension and in the post-mitochondrial supernatant. In the post-mitochondrial supernatant, 15 - 20% of total activity in the mitochondrial preparation was observed, suggesting reliability of the yeast mitochondrial preparation protocol. In keeping with the results obtained in the drop test, when the enzyme activity of the yeast strains generated in this study was assayed alongside their respective control strains the total SCoAL activity, assayed in the direction of succinate production, was intermediate between the subunit deficient yeasts and the parental strain (**Table V**). Furthermore, analysis of the respiration rate of the strains here analysed revealed that the mutants were severely compromised whilst complemented mutants regained wild type rates of respiration (**Table VI**).

Table V. Measurement of SCoAL activities in yeast. Yeast mutants strains *Δlsc 1* (*2*), deficient in *SCoAL α* (*β*), were transformed with the empty vector (pFL61) or complemented with the respective tomato SCoAL (*SlSCoAL*) coding regions. Mitochondrial preparations were used for SCoAL activity measurement.

Genotype	Plasmid introduced	% of WT SCoAL activity
Δlsc1	pFL61	5.8
Δlsc1	pFL61-SlSCoALα1	18.6
Δlsc1	pFL61-SlSCoALα2	12.1
Δlsc2	pFL61	37.6
Δlsc2	pFL61-SlSCoALβ	49.9

Table VI. Measurement of yeast respiration rate. Yeast wild type (WT) and mutants stains (*Δlsc*) were transformed with the empty pFL61 vector or with the pFL61 containing the respective *SlSCoAL* coding regions.

Genotype	Plasmid introduced	nmol O_2/mg prot/min ± SE
WT	pFL61	66.87 ± 2.37
Δlsc1	pFL61	35.39 ± 2.27
Δlsc1	pFL61-SCoALα1	49.73 ± 2.46
Δlsc2	pFL61	27.08 ± 5.87
Δlsc2	pFL61-SCoALβ	63.88 ± 7.48

3.2.4. Sequence analysis of plant succinyl CoA ligase subunits

Having identified that the clones encoded functional SCoAL subunits I next carried out DNA sequence comparison of the subunits with each other and with functionally characterised SCoAL subunits from other species. The amino acid sequence from *Sl*SCoAL α1 and α2 is conserved in the known domains of SCoAL alpha from other species (**Figure 3.4**). *SlSCoAL* α1 and *SlSCoAL* α2 only shared 87% sequence similarity with one another suggesting that they are the product of different genes. In keeping with this mapping studies of the cDNAs using a series of tomato introgression lines (Eshed and Zamir, 1994), revealed two independent loci on the Northern arm of chromosome 2 for the *Sl*SCoAL α subunits. In contrast, a single locus, on the Southern arm of chromosome 6 was revealed for the *Sl*SCoAL β subunit. *Sl*SCoAL α1 encodes a protein with an open reading frame of 332 amino acids, whereas *Sl*SCoAL α2 encodes a protein of 337 amino acids and *Sl*SCoAL β a protein of 417 amino acids. At the protein level, *SlSCoAL α1* and *SlSCoAL α2* exhibit 90% identity with the majority of differences being located in the first 43 amino acids, although the *SlSCoAL α2* also contains additional residues at positions 27 and 41 of the *SlSCoAL α1* gene. The *Sl*SCoAL β protein is, in contrast, very distinct from both *SlSCoAL α1* and *SlSCoAL α2* exhibiting no significant similarity. *SlSCoAL α1* and *SlSCoAL α2* (in parenthesis) exhibit 75 (70), 72 (67), 71 (66) and 70 (66), % identity to the functionally characterized succinyl CoA ligases α from *Dictyostelium discoideum*, *Rattus norvegicus*, *Mus musculus* and *Sus scrofa*, respectively, whereas, *SlSCoAL β* showed 56, 53, 52 and 51% identity to the functionally characterized succinyl CoA ligase β from *D. discoideum*, *Gallus gallus*, *M. musculus* and *R. norvegicus*, respectively.

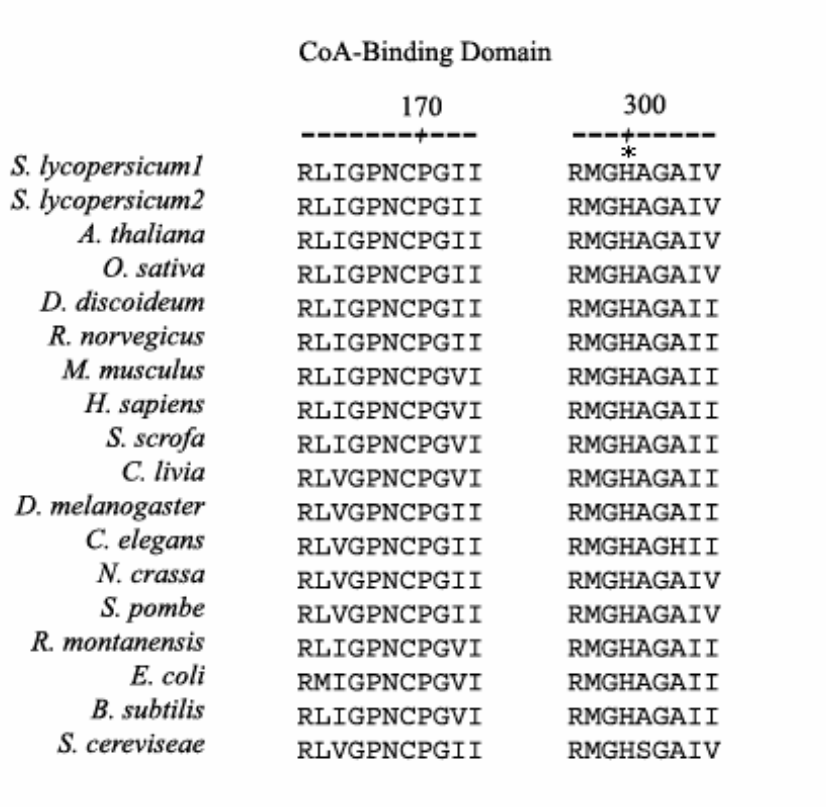


Figure 3.4 Alignment of SCoAL α amino acid sequences. The His residue located in the active site is indicated by an *asterisk* (*). The identification of the CoA-Binding domain and the active His residue is based on Johnson *et al.* (1998). Alignments were produced using MULTALIN software.

Alignment of SlSCoAL α1 and α2 with other functionally characterized SCoALs revealed regions of high sequence conservation corresponding to the CoA binding domain and to the regulatory phosphohistidine residue previously identified via crystallography studies of the *E. coli* enzyme (Wolodko *et al.*, 1994). Furthermore, *in silico* modeling of both possible αβ hetero-dimers predicted from the sequences suggested very similar domain structures and folding patterns to those of the *E.coli* enzyme (Fraser *et al.*, 1999). Analysis of all sequences homologous to the α-subunit of *SlSCoAL* in Genbank revealed that *A. thaliana, Drosophila melanogaster* and *Caenorhabditis elegans* also contained two homologs of this gene. Phylogenetic analysis (**Figure 3.5 A**), however, demonstrates that the four duplications arose independently in the Arabidopsis, tomato, *D. melanogaster* and *C. elegans* lineages. Analysis of sequences homologous to the β-subunit of *SlSCoAL* (**Figure 3.5 B**) suggests that a unique duplication occurred prior to the separation of Drosophila from vertebrates. Therefore, *D. melanogaster* and the vertebrates have two copies of β-subunit, while the rest of the analyzed genomes, including plants, bear only a

single copy of the gene. When this fact is taken into consideration alongside the diverse enzymatic properties reported below suggests considerable differences in the metabolic regulation of this enzyme across species.

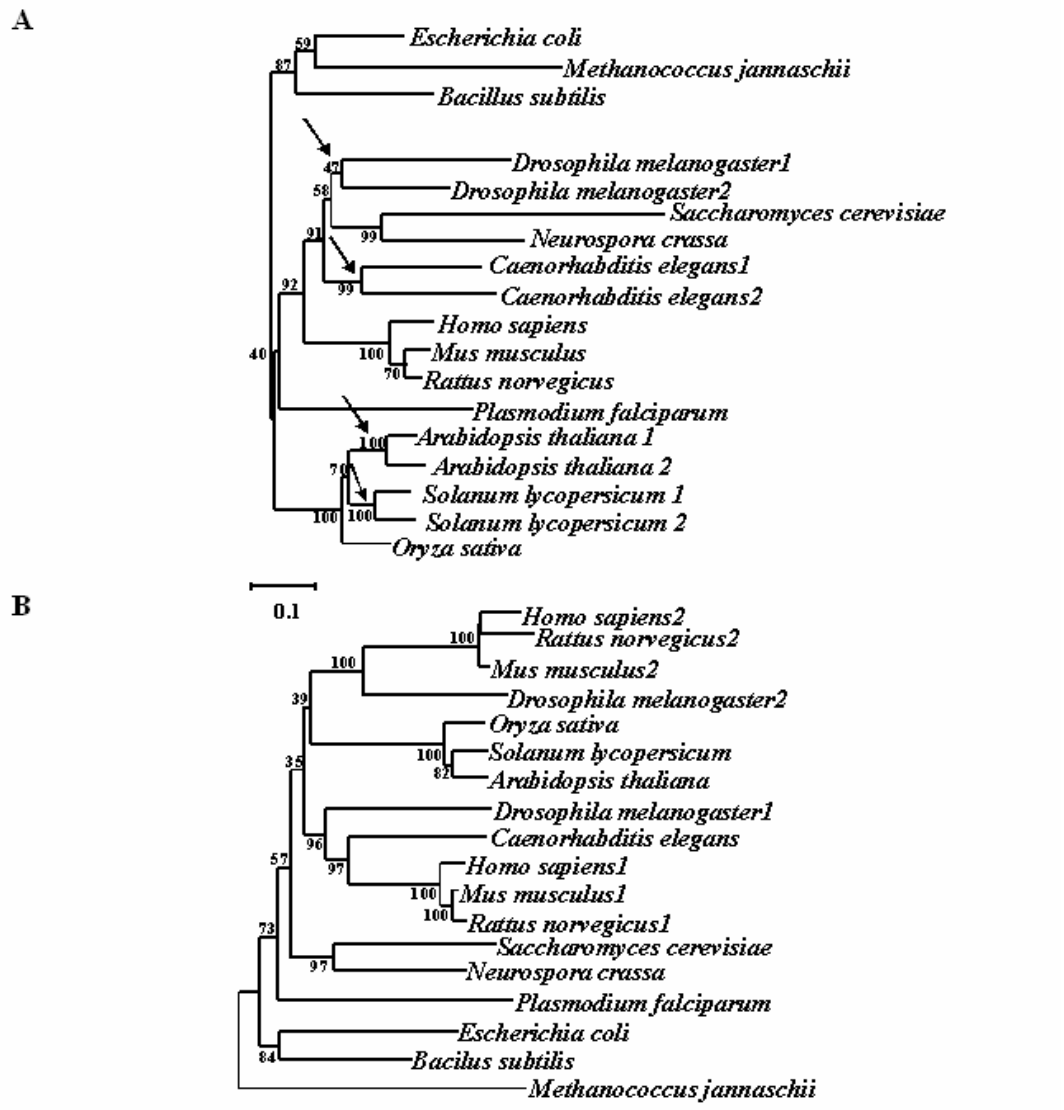


Figure 3.5. DNA sequence analysis of succinyl CoA ligase genes from *S. lycopersicum*. Phylogenetic tree of the α (A) and β (B) subunits of succinyl-CoA ligase. The annotated numbers represent bootstrap values for each node (500 replicates). Independent duplications of the α gene are indicated with arrows.

3.2.5. Establishment of an assay for succinyl CoA ligase in the direction of succinate formation

The basis for the new assay is the enzymic cycle between G3POX and G3PDH (**Figure 3.6**). G3POX catalyses an O_2-mediated conversion of G3P into DAP, and G3PDH converts DAP back into G3P and simultaneously oxidises NADH and NAD^+ (**Figure 3.6 B**).

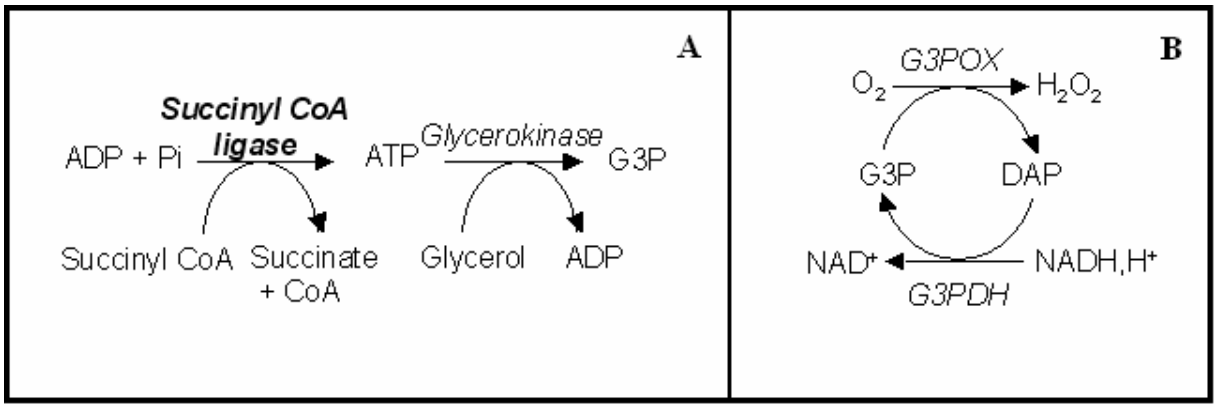


Figure 3.6. Principle of the succinyl CoA ligase assay.

This cycling system has been thoroughly validated and optimised in previous studies (Gibon *et al.*, 2002; Gibon *et al.*, 2004). Here the cycling system was coupled to the operation of SCoAL in the direction of succinate formation by the action of glycerokinase (**Figure 3.6 A**) which stoichiometrically converts the ATP produced by succinyl CoA ligase. The amount of plant material to be included in the assay was optimised to 50 µg per well (namely 5 µL of a 1:2000 dilution; **Figure 3.7 A**), and a recovery of ATP was performed with floral extracts and found to be higher than 90% (**Figure 3.7 D and E**). Linearity with time was also checked. The sensitivity of the assay was found to be at least 50 pmol of ATP in the conditions described in **Chapter 2**, which corresponds to 3 - 4 µg FW. The activity was found to range from 50 to 350 pmol per aliquot, which is far below the linearity range provided by the cycling assay used (3 orders of magnitude).

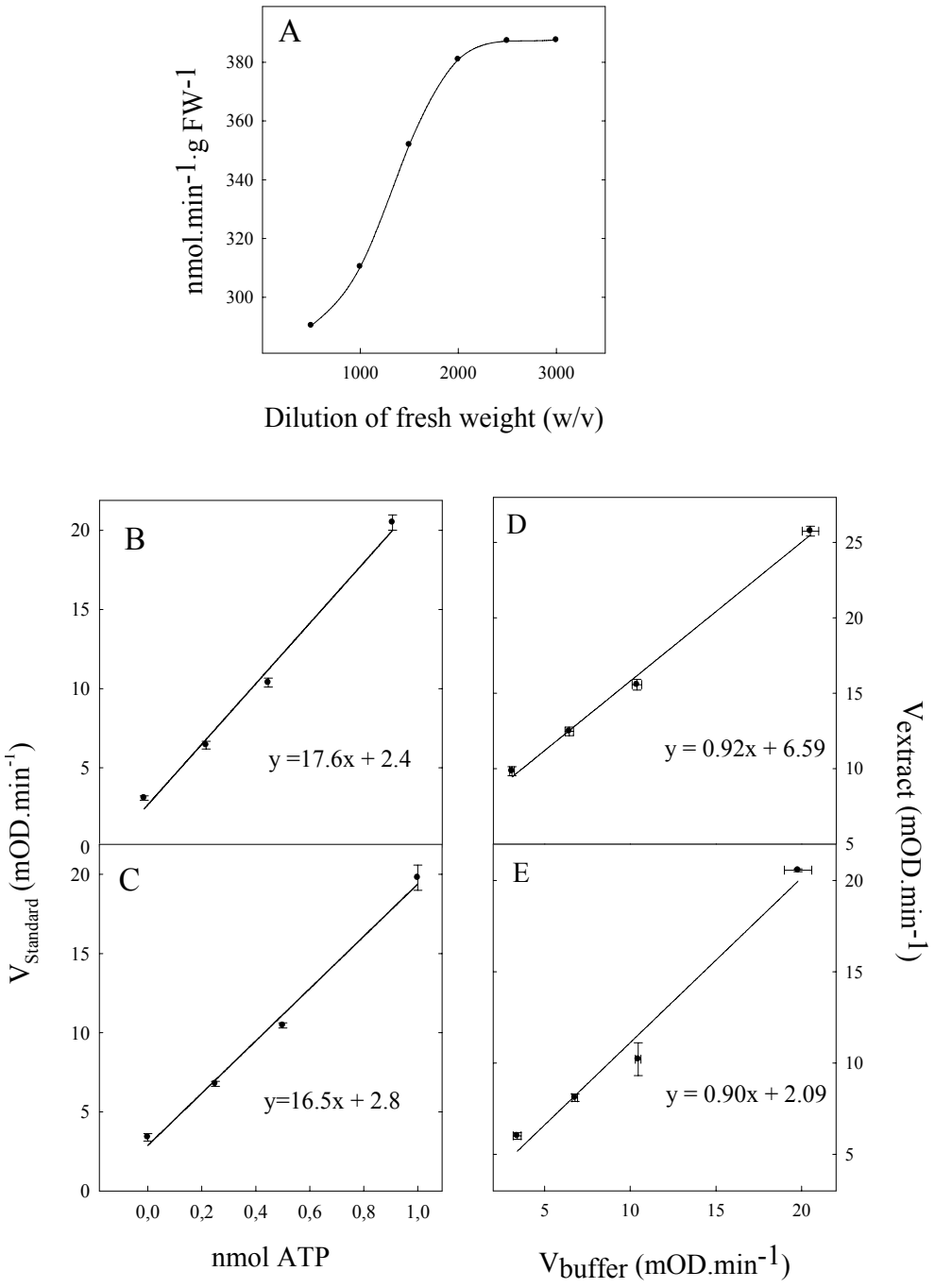


Figure 3.7 Validation of SCoAL enzymatic assay. (A) Relation between the dilution of the extract and the activity determined. Dilutions were made with the extraction buffer before the assay. (B) and (C) ATP standard curves in the 0.1-1 nmol range, with (B) and without (C) succinyl CoA. (D) and (E) Recovery of ATP standards (0, 0.25; 0.5 and 1 nmol) from tomato flower extract set to a 2000-fold dilution of FW (w/v), with (D) and without (E) succinyl CoA. The slope of the lines gives the fraction of ATP recovered. Data are given in mOD. min^{-1} ± SE (n = 3).

After determining that the complemented yeast mutants had elevated activity of succinyl CoA ligase (described above), the activity of succinyl CoA ligase in a range of tomato tissues was evaluated. The highest activity of this enzyme was observed in floral tissues (383 ± 16 nmol.min^{-1}.g FW^{-1}), however the activity was still relatively high in leaves (141 ± 4 nmol.min^{-1}.g FW^{-1}), whilst green (62 ± 3 nmol.min^{-1}.g FW^{-1}) and red fruits (28 ± 4 nmol.min^{-1}.g FW^{-1}) had relatively low activities. The activity in all tissues is very low particularly when it is considered that the activities of aconitase and mitochondrial malate dehydrogenase in the same tissues are typically an order of magnitude higher than this (Carrari *et al.*, 2003; Nunes-Nesi *et al.*, 2005a).

3.2.6. Expression analysis of plant succinyl CoA ligase subunits

The mRNA levels of the *SlSCoAL α1*, *α2* and *β* genes were determined by Northern blots as shown in **Figure 3.8 A**. Single bands were observed when hybridising with *Sl*SCoAL α and *Sl*SCoAL β. Both genes are highly and equally expressed in root, stem and flowers, but are also expressed in leaves and during the whole fruit developmental process, peaking at 55 DAF coincident with the onset of fruit ripening. Given that the high degree of sequence similarity between *Sl*SCoAL α1 and α2 does not allow us to distinguishing differential expression between the two genes I next used an RT-PCR approach to determine the relative steady-state mRNA levels of the *Sl*SCoAL α1 and 2. However, as illustrated in **Figure 3.8 B**, differences in the expression of *Sl*SCoAL α1 and 2 are within the confidence interval (95%), indicating that the expression of the isoforms is similar across all tissues sampled. This is in agreement with what was observed when the analysis of the expression pattern of the genes encoding for the succinyl CoA ligase alpha and beta subunits was performed in Arabidopsis using publicly accessible microarray data (Steinhauser *et al.*, 2004, Zimmermann *et al.*, 2004). The tissue specificity as well as the patterns of induction/repression of these genes under abiotic conditions (especially UV-B, salt and heat stress) follow very similar patterns suggesting that the three genes are also apparently coexpressed in Arabidopsis.

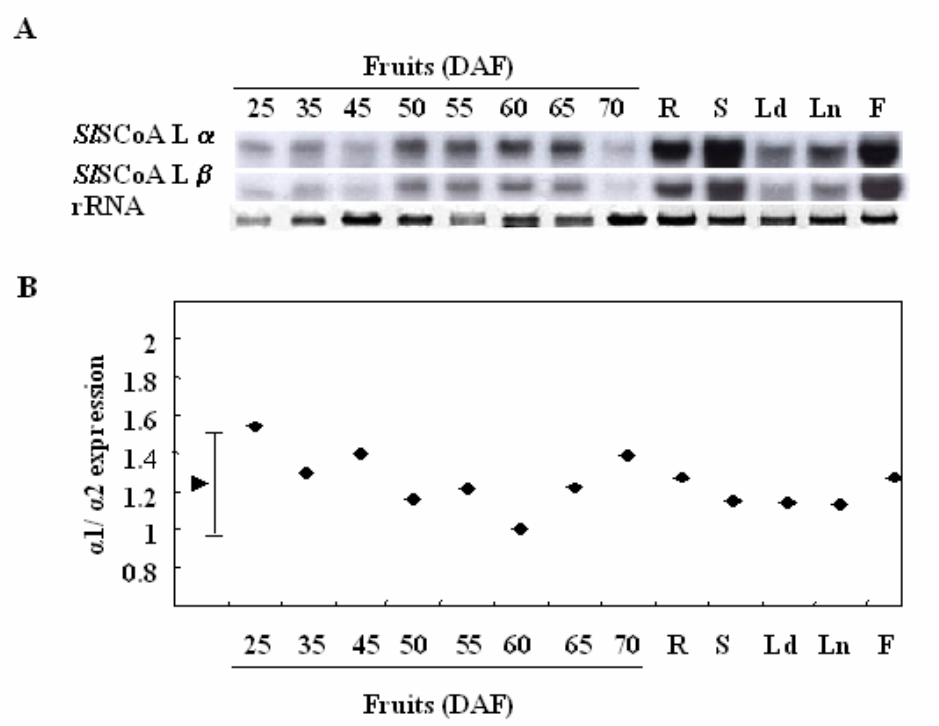


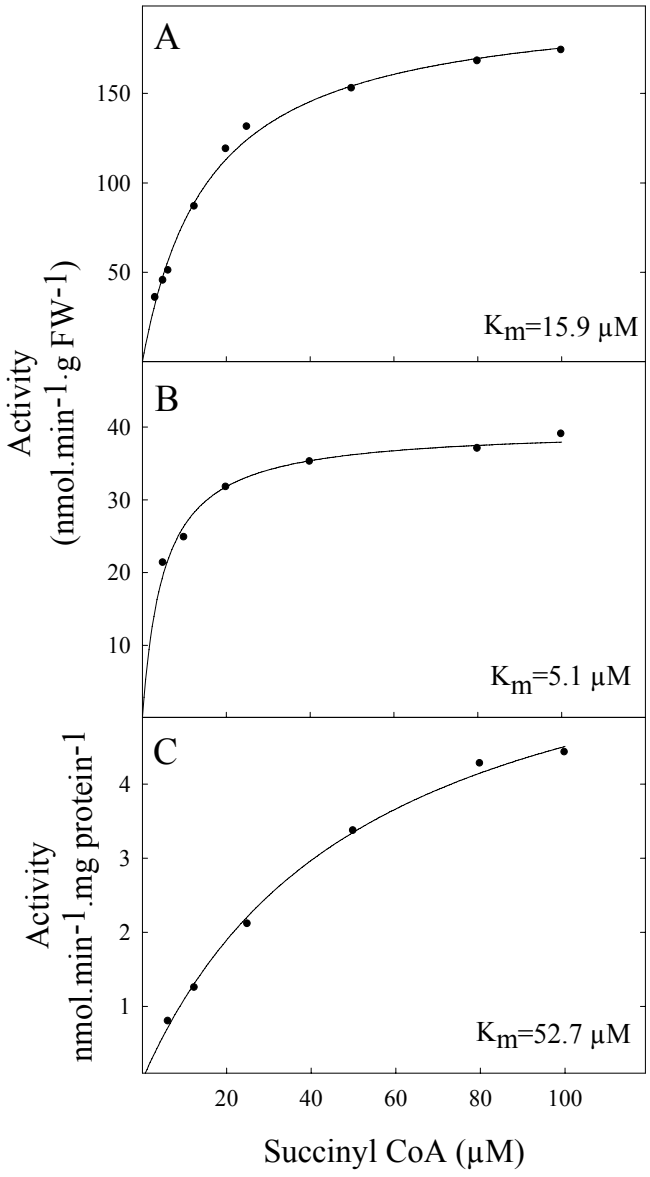
Figure 3.8 Expression analysis of *Sl*SCoAL α and β subunits. (A) Total RNA of tomato fruit at different developmental stages (DAF) and tomato plant organs were hybridised with tomato full-length clones of SCoAL α and β subunits. R: Root; S: Stem; Ld: Leaf collected at day period; Ln: Leaf collected in the night period; F: Flower. Densitometry analysis was performed by using the Scion Image software package (Maryland, USA). (B) Semi-quantitative RT-PCR analysis of the two *SlSCoALα* genes. Relative abundance of α 1 and 2 subunits was measured in tomato plant organs. Values represent the ratios between mean quantifications of PCR band intensities from single tube amplifications using a common forward primer for both isoforms and two specific reverse ones. The vertical bar on the left represents the 95% confidence interval of the mean

3.2.7. Preliminary characterisation of enzymatic properties of plant succinyl CoA ligase

Given that succinyl CoA ligase seems to be present at relatively low activities in plant organs, and that the levels of its transcripts and protein have been observed to be highly variable through development (Urbanczyk-Wochniak *et al.*, 2003), and in response to biological processes such as oxidative stress (Sweetlove *et al.*, 2002), suggests that it may be an important control point of the TCA cycle.

In order to gain preliminary insight into the *in vivo* regulation of the succinyl CoA ligase I next determined K_m for succinyl CoA and ADP for crude extracts from yeast and tomato flower (**Figure 3.9**). These studies revealed that the K_m for both succinyl CoA and

ADP were relatively similar in yeast and tomato, moreover they were also remarkably similar to those determined for other plant species (e.g. potato, **Figure 3.9 B**). The fact that these data of tomato are in such close agreement with the data obtained by Palmer and Wedding (1966) on purified plant enzyme strongly suggests that my results are a good reflection of the actual K_m.



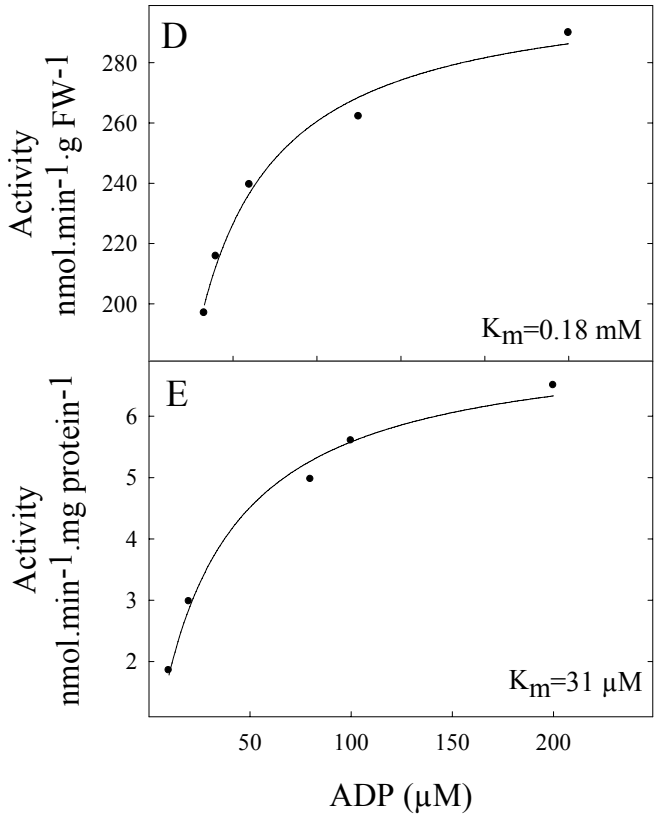


Figure 3.9 Kinetic analysis of the SCoAL in the direction of succinate formation. (A,B,C) Dependence of SCoAL activity from crude extract of tomato flowers (A), potato (B) and yeast mitochondrial preparation (C) on succinyl CoA concentrations. (D,E) Dependence of SCoAL activity from crude extract of tomato flowers (D) and of yeast mitochondrial preparation (E) on ADP concentrations. The K_m where determined by fitting a rectangular hyperbola curve.

Having determined the K_m of succinyl CoA and ADP I next attempted to determine the effects of the presence of varying the concentration of other organic acids, within the assay mixture, on the reaction rate of the enzyme operating in the forward direction. Whilst several early metabolites of the TCA cycle had no effect such as pyruvate and acetyl CoA (**Table VII**), the majority of the TCA cycle intermediates affected the activity of SCoAL. In contrast the cytotoxic product of lipid peroxidation, 4-hydroxy-2-nonenal (HNE), which has previously been demonstrated to inhibit several enzymes of the mitochondrial TCA cycle (Millar and Leaver, 2000), had no effect. The only metabolite that was capable of activating SCoAL was 2-oxoglutarate, whereas citrate and isocitrate and all metabolites downstream of SCoAL apparently inhibit its activity.

However, caution must be taken in interpreting these data since in the majority of cases inhibition was only observed at concentrations far in excess of those reported in the literature (generally two orders of magnitude higher, Schauer *et al.* [2005], Bender-Machado *et al* [2004]). The exceptions to this statement were the activation by 2-oxoglutarate which occurred even at low physiological concentration, succinate and fumarate which inhibit at relatively high physiological concentrations and citrate which inhibit at very high concentrations relative to those reported to be physiological. When taken together these properties of SCoAL suggest that it is inhibited by downstream intermediates of the TCA cycle.

Table VII. I_{50} values for various metabolites in tomato flower protein extract. Assays were carried out in saturating conditions 100 µM Succinyl CoA and 2.5 mM ADP. Nd: not detected. [a] 2-Oxoglutarate activated SCoAL in the range of 0.3 – 10 mM.

Compound	I_{50}
Pyruvate	Nd
Acetyl CoA	Nd
Citrate	33.8 mM
Isocitrate	41.3 mM
2-Oxoglutarate	[a]
Coenzyme A	307.1 µM
Succinate	7.1 mM
Fumarate	11.2 mM
Malate	151.3 mM
Oxaloacetate	1.8 mM
HNE	Nd

3.3. Conclusion

In this first part of the project I searched in the tomato EST collection for the annotation for genes involved in the TCA cycle as the pyruvate dehydrogenase complex E1 α and β, and E2 subunits, aconitase, the subunits of the 2-oxoglutarate complex E1, E2 and E3, succinyl CoA ligase α and β subunits, succinate dehydrogenase iron sulphur and flavoprotein subunits, and mitochondrial, glyoxysomal, cytosolic and chloroplastic isoforms of malate dehydrogenase. Based on the fragment size from many EST for the same annotation and comparing them to the fully sequenced Arabidopsis genes, I was able to obtain the full-length coding regions for 2-oxoglutarate complex E3, succinyl CoA ligase α and β subunits, succinate dehydrogenase iron sulphur subunit, and for all isoforms of malate dehydrogenase. I also performed the first functional characterisation of three plant open reading frames encoding the subunits of SCoAL. Although a more detailed characterisation on purified plant enzymes will be required to confirm and extend these preliminary observations they suggest that the *in vivo* flux through SCoAL may be subject to allosteric regulation in a manner that would allow a high cyclic flux in times when carbon is in rich supply and a reduced flux in times of carbon deficiency. Moreover, the low activity of the enzyme, when considered alongside the observations that the transcription and protein stability of this enzyme vary both developmentally and in response to physiological processes such as oxidative stress (Sweetlove *et al.*, 2002; Urbanczyk-Wochniak *et al.*, 2003), suggest that this may be an important control point of the TCA cycle. Whilst I identified that there were two different genes encoding the α-subunit in plants, there appears to be little functional distinction between them since they are expressed in the same relative level in all plant organs tested and as expected are both targeted to the mitochondria. The yeast complementation indicates that α- or β- subunits can operate independently but the presence of both subunits in all plant organs assayed may indicate a more complex enzyme structure *in planta*. Since this work constitutes the first functional identification of the genes encoding SCoAL in plants it also opens up the possibility to utilize the reverse genetic approach in order to characterise the role of this enzyme in plant metabolism and development.