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Identification and characterisation of the α and β subunits of succinyl CoA ligase of tomato

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Abstract

Despite the central importance of the TCA cycle in plant metabolism not all of the genes encoding its constituent enzymes have been functionally identified. In yeast, the heterodimeric protein succinyl CoA ligase is encoded for by two single-copy genes. Here we report the isolation of two tomato cDNAs coding for α- and one coding for the β-subunit of succinyl CoA ligase. These three cDNAs were used to complement the respective *Saccharomyces cerevisiae* mutants deficient in the α- and β-subunit, demonstrating that they encode functionally active polypeptides. The genes encoding for the subunits were expressed in all tissues, but most strongly in floral and leaf tissues, with equivalent expression of the two α-subunit genes being expressed to equivalent levels in all tissues. In all instances GFP fusion expression studies confirmed an expected mitochondrial location of the proteins encoded. Following the development of a novel assay to measure succinyl CoA ligase activity, in the direction of succinate formation, the evaluation of the maximal catalytic activities of the enzyme in a range of tissues revealed that these paralleled those of mRNA levels. We also utilized this assay to perform a preliminary characterisation of the regulatory properties of the enzyme suggesting allosteric control of this enzyme which may regulate flux through the TCA cycle in a manner consistent with its position therein.

Abbreviations: DAF, days after flowering; DAP, dihydroxyacetonephosphate; EST, expressed sequence tag; G3P, Glycerol-3-Phosphate; G3POX, Glycerol-3-Phophate Oxidase; G3PDH, Glycerol-3-Phosphate Dehydrogenase; ScoAL, Succinyl CoA ligase; TCA, tricarboxylic acid

Introduction

Succinyl CoA ligase (SCoAL; E.C. 6.2.1.5) catalyses the interconversion of succinyl CoA, inorganic phosphate and dinucleotide to succinate, trinucleotide and CoA (Johnson *et al.*, 1998). Little is known of the plant enzyme; however, all SCoALs studied consist of two types of subunit α; with a molecular mass of 29–34 kDa and β with a molecular mass of 41–45 kDa. Catalysis of all reactions in which succinyl CoA participates, proceeds via the intermediate transfer of a phosphoryl group to and from a conserved histidine residue within the α-subunit (Ryan *et al.*, 1997).

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 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, to date functional identification of the genes encoding the enzymes is lacking for plant species (Fernie *et al*., 2004).

Early purification studies on the plant enzyme revealed that it was not only highly specific with regard to nucleotide usage but also with respect to the other substrates of the reverse reaction succinate and CoA (Kaufmann and Alivisatos, 1955). In the reverse direction it has been suggested that SCoAL is feedback inhibited by intermediates of the porphyrin pathway which it supplies (Wider and Tigier, 1971) as well as being competitively inhibited by malonate (Palmer and Wedding, 1966). Here we describe the first functional identification, by yeast complementation, of three plant open reading frames encoding subunits of succinyl CoA ligase. Sequence comparisons, expression analysis and mapping hybridisations confirmed the presence of two α- and one β-subunit of succinyl CoA in tomato. Both bioinformatic analysis and the expression of GFP fusion constructs suggested that the products of all three open reading frames are targeted to the mitochondria in *Arabidopsis thaliana*. Finally following development of a novel assay to measure succinyl CoA ligase activity in the forward direction (that of succinate formation), we performed a preliminary characterisation of regulatory properties of the enzyme.

Material and methods

Material

Solanum lycopersicum cv. Moneymaker was obtained from Meyer Beck (Berlin, Germany). All enzymes were obtained from Roche Diagnostics (Mannheim, Germany) with the exception of glycerol kinase which was obtained from Sigma-Aldrich (Taufkirchen, Germany). All other chemicals used in biochemical assays were obtained from Sigma-Aldrich (Taufkirchen, Germany), with the exception of 5′,5′-diadenosinpentaphosphate obtained from Fluka Chemie Gmbh (Buchs, Switzerland). Putative succinyl CoA ligase ESTs were obtained from the *S. lycopersicum* EST collection of The Clemson University Genomics Centre (http://www.genome.clemson.edu). Molecular biological reagents and kits were purchased from Amersham Bioscience (Freiburg, Germany) or Invitrogen (Karlsruhe, Germany). Yeast mutant strains deficient in either the α- or β-subunit (ΔLSC1, ΔLSC2) of succinyl CoA ligase and the respective wild type strain (BY4741) were ordered from the EUROSCARF collection (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index; Przybyla-Zawislak *et al*., 1998).

Cloning of the full length tomato cDNAs α1, α2, and β succinyl CoA ligase

ESTs with high homology to the functionally characterised succinyl CoA ligase genes of yeast were selected. Full-length coding regions of the *SCoAL* α1, α2 and β genes (corresponding to ESTs cLES12N15, cLEY17J6 and cTOF2K12) were identified and subsequently cloned into the pENTR Directional TOPO vector (Invitrogen, Karlsruhe, Germany) using the following primers: α1F 5′CACCATGGCTCGCCAAGCG; α1R 5′TTTCACAAGACCCCTCTGTTTGAAC; α2F 5′CACCATGGCTCGCCAAGCC; α2R 5′CGCAAGACCCCTCTGTTTG; βF 5′CACCATGCTGCGTAAACTTGCCAATC; βR 5′AGCTAAGGCCTTGACTGCC.

Yeast growth, transformation and selection

The above-mentioned yeast strains were maintained on YPD agar medium supplemented with 200 mg/ml of geneticin. Complementation assays

were performed by transforming the mutant yeast strains with a modified version of the pFL61 vector (Drager *et al*., 2004). The yeast mutant ΔLSC1 was transformed with vectors independently containing *Sl*SCoAL α1 and 2, and the mutant ΔLSC2 was transformed with a vector containing *Sl*SCoAL *β*. Plasmids were transferred into yeast using the lithium acetate heat shock transformation protocol (Schiestl and Gietz, 1989). The transformed colonies were subsequently selected by their ability to grow on uracil deficient medium. For the complementation test, transformed colonies were grown in liquid semi-synthetic (SS) media (Przybyla-Zawislak *et al*., 1998) to an A_{600} of 0.9. Yeast suspensions were centrifuged and washed with water to remove residual glucose. Drops of yeast suspensions were then plated onto solid SS media, containing 3% glycerol as the sole carbon source and incubated for 4–6 days at 30 °C.

Phylogenetic analysis

Protein sequences were retrieved from the GenBank through the BLASTp algorithm using *Sl*SCoAL α1, α2 and *β* subunits as query. With the aim of establishing copy number, we only selected sequences from eukaryotes and prokaryotes with fully sequenced genomes. We also used the tBALSTn algorithm to search for non-annotated proteins. Sequences were aligned using the ClustalW software package (www.ebi.ac.uk/clustalw) using default parameters. Neighbor Joining trees (Saitou and Nei, 1987) were constructed with MEGA2 software (Kumar *et al*., 2001). Distances were calculated using pair-wise deletion and Poisson correction for multiple hits, bootstrap values were obtained with 500 pseudo replicates.

Expression analysis

Total RNA from different tomato organs (root, stem, leaf and flower) and fruits at different developmental stages (25, 35, 45, 50, 55, 60, 65 and 70 days after flowering [DAF]) were extracted using the protocol described by Obiadalla-Ali *et al*. (2004). Northern blot analysis was performed under standard conditions (Sambrook *et al*., 1989). The membrane was probed with the specific PCR fragment of each coding region (0.9 kb for *Sl*SCoAL α and 1.0 kb for *Sl*SCoAL *β*), labelled with α[-^{32}P] dCTP by random priming, using the Random Prime Labelling System (Amersham Bioscience, Freiburg, Germany). For the differential expression analysis of *Sl*SCoAL α1 and α2 a semi-quantitative RT-PCR approach was followed: 3 μg of RNA from the same material were used for cDNA synthesis using SuperScript II reverse transcriptase (Invitrogen, Karlsruhe, Germany). Forward primers were designed on the variable segment of the *Sl*SCoAL α1 (5′ GACCAAACTGATCGCAAATCTGTC 3′) and the *Sl*SCoAL α2 (5′ GGCATCAGTATCGCTACTTTGGATC 3′). The reaction was performed using a common reverse primer (5′ CTTGGGTGTCACTCCACCAACC 3′) at an annealing temperature of 54 °C. The reaction was calibrated by using serial dilutions of the cDNA samples assuming equal efficiency of primer annealing.

Subcellular localisation experiments

The full length *SlSCoAL α1*, *α2* and *SlSCoAL β* coding regions were cloned into pK7FWG2 vector (Karimi *et al*., 2002) and transformed into *A. thaliana* cv. Col-0. Young leaves were cut in small pieces and immediately transferred to a Petri dish containing 0.5 M mannitol solution for 1 h. The solution was then replaced by digestion solution (0.4 M mannitol, 0.33 % Cellulase ''Onozuka'' R −10, 0.17 % Macerozyme, 3 mM MES, pH 5.7 and 7 mM $CaCl_2$) and kept in darkness at 37 °C for 5 h. The resultant isolated protoplasts were treated with 1 μM of the mitochondrial specific dye MitoTracker Orange CMTMRos (Invitrogen, Karlsruhe, Germany) for 1 h in the dark and on ice. Fluorescent signals were analyzed using a Leica TCS SPII confocal laser scan microscope (Leica Microsystems AG, Wetzlar, Germany) as detailed in Carrari *et al*. (2005).

Succinyl CoA ligase enzyme assay

Yeast isolated mitochondria were yeast/isolated in 0.6 M sorbitol, 2 mM PMSF and 20 mM Hepes–KOH (pH 7.4), whilst ground plant tissues were extracted as described in Gibon *et al*. (2004). Succinyl CoA ligase was assayed in the forward direction by measuring the succinyl CoA dependent production of ATP from ADP. Extracts, as well as ATP standards, freshly prepared in the extraction buffer, and ranging from 0 to 1 nmol,

were incubated in a microplate at 25 °C in a medium containing: 100 mM Tricine/KOH pH 8, 10 mM $MgCl_2$, 100 μM EDTA, 1 u ml^{-1} glycerokinase, 10 mM phosphate, 2.5 mM ADP (ATP free), 100 μM 5′,5′-diadenosinpentaphosphate, 120 mM glycerol, 0 (blank) or 100 μM (maximal activity) succinyl CoA. The reaction volume was set to 50 μl. Preliminary tests established that 5′, 5′-diadenosinpentaphosphate exerted no effect on the activity of succinyl CoA ligase. The reaction was stopped with 20 μl of 0.5 M HCl. After neutralisation with 20 μl NaOH, G3P (Glycerol-3-Phosphate) was measured as described in Gibon *et al*. (2002), in the presence of 1.8 u ml^{-1} G3POX (Glycerol-3-Phophate Oxidase), 0.7 u ml^{-1} G3PDH (Glycerol-3-Phosphate Dehydrogenase), 1 mM NADH, 1.5 mM $MgCl_2$ and 100 mM Tricine/KOH pH 8. The absorbance was read at 340 nm and at 30 °C in a Synergy microplate reader (Bio-Tek) until the rates were stabilised. The rates of reactions were calculated as the decrease of absorbance in mOD min^{-1} using KC4 software (Bio-Tek). Inhibitors were added as described in Results and discussion.

Oxygen consumption measurements in yeast strains

An aliquot of 500 μl of a yeast culture in exponential growth face was transferred to the measuring chamber of the liquid phase oxygen electrode (Hansatech, Bachofer, Reutlingen, Germany) containing 1 ml of YPG and oxygen consumption was recorded under continuous stirring at 25 °C.

Results and discussion

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). Searching tomato EST collections (Van der Hoeven *et al*., 2003), on the basis of nucleotide structure and fragment length facilitated the isolation of three putative full length cDNAs: the 1164-bp *Sl*SCoAL α1 (clone cLES12N15), the 1322-bp *Sl*SCoAL α (clone cLEY17J6) and the 1427-bp *Sl*SCoAl β (clone cTOF212). 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 1). Dilution drop growth assay indicates that *Sl*SCoAL α1 complements ΔLSC1 slightly better than *Sl*SCoAL α2. In keeping with this 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 (see below for details), was intermediate between the subunit deficient yeasts and the parental strain (data not shown). Furthermore, analysis of the rate of respiration of the strains analysed here revealed that the mutants were severely compromised whilst complemented mutants regained wild type rates of respiration (data not shown).

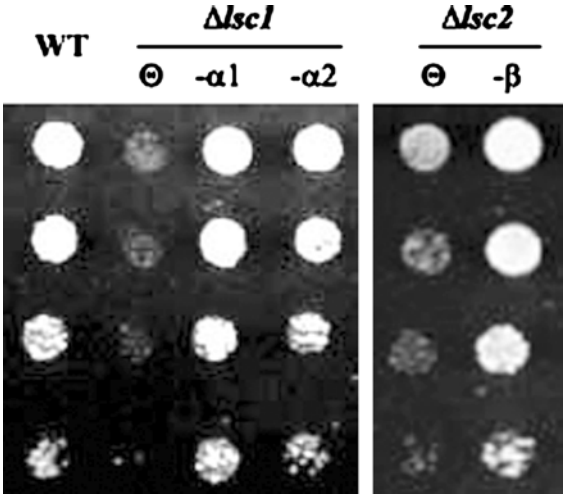

Figure 1. Functional complementation of succinyl CoA ligase subunit deficient yeast strains by the *Sl*SCoAL open reading frames. Transformants were grown overnight on SSM with glucose as sole carbon source before being thoroughly washed. The culture was subjected to ten-fold serial dilutions and 4 μl 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*−α2 (mutant complemented with the tomato open reading frame *Sl*SCoALα2). Δ*lsc2* (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).

Sequence analysis of plant succinyl CoA ligase subunits

Having identified that the clones encoded functional SCoAL subunits we next carried out DNA sequence comparison of the subunits with one another and with functionally characterised SCoAL subunits from other species. The amino acid sequence from *Sl*SCoAL α1 and 2 is conserved in the known domains of SCoAL alpha from other species (Figure 2). *SlSCoAL* α1 (deposited in GenBank as AY167586) and *SlSCoAL* α2 (deposited in GenBank as AY650029) 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 (deposited with GenBank as AY180975). *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.

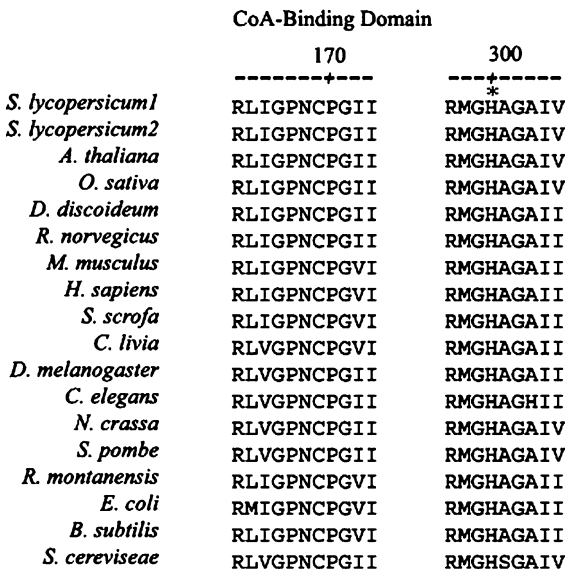

Figure 2. Alignment of SCoAL α amino acid sequences. The His residue located in the active site is indicated by an *asterisk* (*). The identification of the CoA-Binding domain and the active His residue is based on Johnson *et al.* (1998). Alignments were produced using MULTALIN software.

Alignment of SlSCoAL α1 and 2 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 *Sl*SCoAL in GenBank revealed that *A. thaliana, Drosophila melanogaster* and *Caenorhabditis elegans* also contained two homologs of this gene. Phylogenetic analysis (Figure 3A), however, demonstrates that the four duplications arose independently in the Arabidopsis, tomato, Drosophila and *C. elegans* lineages. Analysis of sequences homologous to the β-subunit of *Sl*SCoAL (Figure 3B) suggests that a unique duplication occurred prior to the separation of Drosophila from vertebrates. Therefore, Drosophila 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.

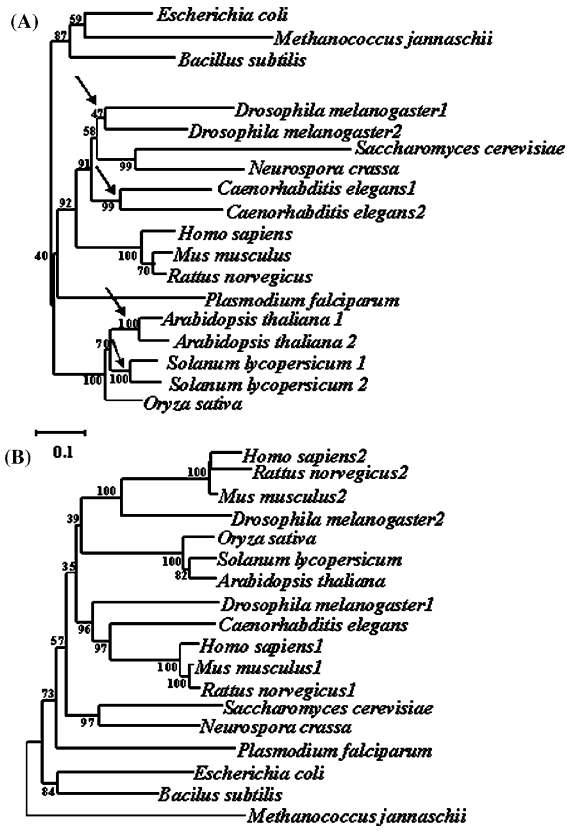

Figure 3. DNA sequence analysis of succinyl-CoA ligase genes from *S. lycopersicum*. Phylogenetic tree of the α (A) and β (B) subunits of succinyl-CoA ligase. The annotated numbers represent bootstrap values for each node (500 replicates). Independent duplications of the α gene are indicated with arrows.

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 4). 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 4B). This cycling system has been thoroughly validated and optimised in previous studies (Gibon *et al*., 2002, 2004), here we coupled the cycling system to the operation of succinyl CoA ligase in the direction of succinate formation by the action of glycerokinase (Figure 4A) 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 (not shown), and a recovery of ATP was

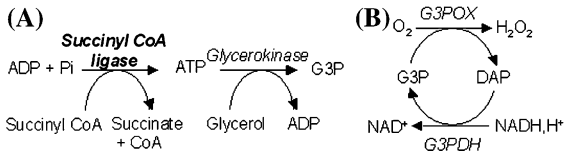

Figure 4. Principles of the succinyl CoA ligase assay.

performed with floral extracts and found to be higher than 90%. 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 Materials and Methods, which corresponds to 3–4 μg FW. The activity was found to range from 50 to 350 pmol per well, which is far below the linearity range provided by the cycling assay used (3 orders of magnitude). After determining that the complemented yeast mutants had elevated activity of succinyl CoA ligase (described above), we next evaluated the activity of succinyl CoA ligase in a range of tomato organs. 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 organs is very low particularly when it is considered that the activities of aconitase and mitochondrial malate dehydrogenase in the same organs are typically an order of magnitude faster than this (Carrari *et al*., 2003; Nunes-Nesi *et al*., 2005).

Expression analysis of plant succinyl CoA ligase subunits

The mRNA levels of the *Sl*SCoAL α1, α2 and β genes were determined by Northern blots as shown in Figure 5A. 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 2 does not allow us to distinguish differential expression between the two genes we next used an RT-PCR approach to determine the relative steady-state mRNA levels of the *Sl*SCoAL α1 and

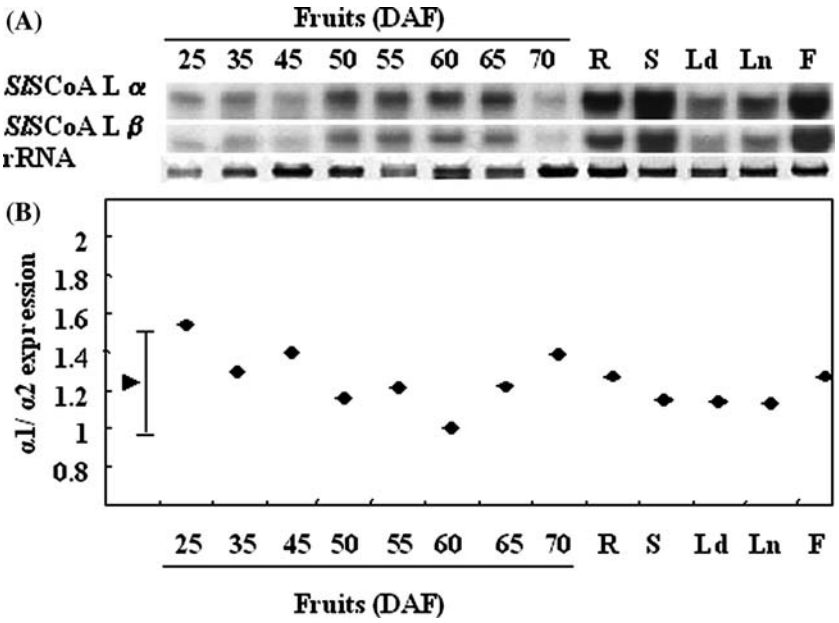

Figure 5. Expression analysis of *Sl*SCoAL α and β subunits. (A) Total RNA of tomato fruit at different developmental stages (DAF) and tomato plant organs were hybridised with tomato full-length clones of SCoAL α and β subunits. R: Root; S: Stem; Ld: Leaf collected at day period; Ln: Leaf collected in 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.

2. However, as illustrated in Figure 5B, 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 organs sampled. This is in agreement with what we observed when we performed the analysis of the expression pattern of the genes encoding for the succinyl CoA ligase alpha and beta subunits in *Arabidopsis thaliana* using publically 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 (specially UV-B, salt and heat stress) follow very similar patterns meaning that the three genes are also apparently coexpressed in Arabidopsis.

Subcellular localisation of succinyl CoA ligase in plants

In order to investigate the cellular localization of the proteins encoded by the *SlSCoAL* α1 and 2 and *SlSCoAL* β genes we first analysed the peptide sequences by bioinformatic prediction (TargetP, Emanuelsson *et al*., 2000; Predotar, Small *et al*., 2004; SignalP, Bendtsen *et al*., 2004). Both *SlSCoAL* α- and the β-subunits were predicted to be targeted to the mitochondria. To confirm these results the complete coding regions were fused at the carboxyl terminal end, to an enhanced green fluorescent protein (EGFP, Karimi *et al*., 2003; Figure 6) 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 6C). 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.

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

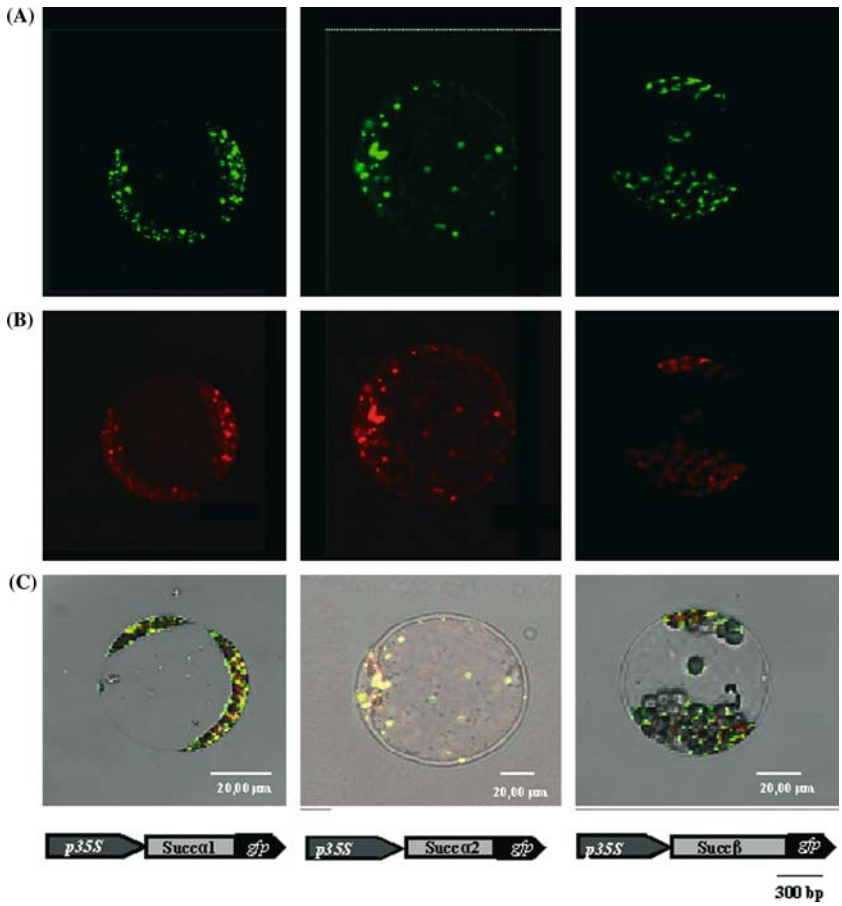

Figure 6. Expression of *SlSCoALα1* and *2* and *SlSCoAL β* - GFP fusion in *A. thaliana* protoplasts. SlSCoAL α1-GFP, SlSCoAL α2-GFP and SlSCoAL β-GFP transformed protoplasts were used for confocal microscopy. (A) GFP fluorescence; (B) MitoTracker visualization (fluorescence excitation at 554 nm and 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.

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 we next determined K_m for succinyl CoA and ADP for crude extracts from yeast and tomato flower (Figure 7 and data not shown). 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, data not shown). The fact that these data are in such close agreement with the data obtained by Palmer and Wedding (1966) on purified plant enzyme strongly suggests that our result are a good reflection of the actual K_m. Having determined the K_m of succinyl CoA and ADP we 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 1), the majority of the TCA cycle intermediates effected 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,

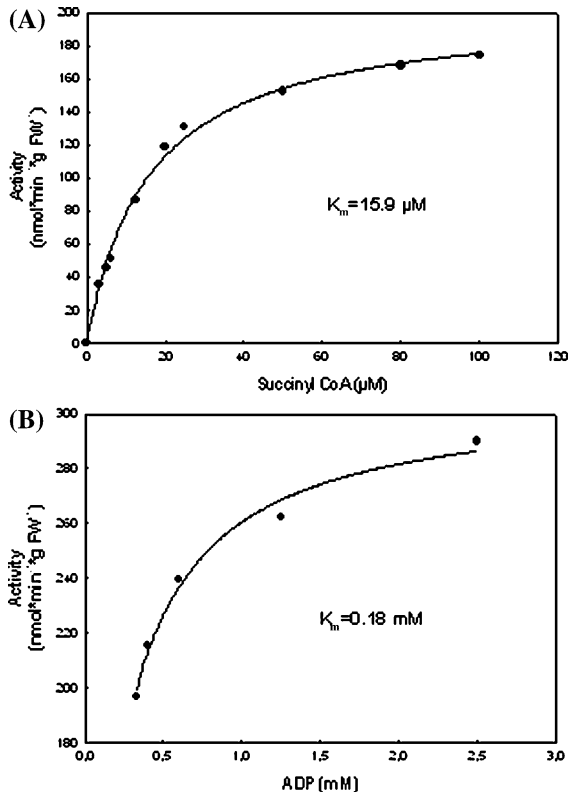

Figure 7. Kinetic analysis of the SCoAL in the direction of succinate formation. (A) Dependence of SCoAL activity from crude extract of tomato flowers on succinyl CoA concentrations. (B) Dependence of SCoAL activity from crude extract of tomato flowers on ADP concentrations. The K_m where determined by fitting a rectangular hyperbola curve.

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 in 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 1. I_{50} values for various metabolites in tomato flower protein extract.

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

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.

In summary, 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 we 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. Our 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.

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