Chapter 2

Material and Methods

2.1. Chemicals

All 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). Molecular biological reagents and kits were purchased from Amersham Bioscience (Freiburg, Germany) or Invitrogen (Karlsruhe, Germany). Radiolabeled sodium bicarbonate and D-[1-^{14}C]-, D-[3:4-^{14}C]-, and D-[6-^{14}C]Glc were from American Radiolabeled Chemicals (St. Louis); and the L-[1-^{14}C]Glutamic Acid from Amersham (Freiburg, Germany). All enzymes were obtained from Roche Diagnostics (Mannheim, Germany) with the exception of glycerol kinase which was obtained from Sigma-Aldrich (Taufkirchen, Germany).

2.2. Bacterial and yeast strains

Escherichia coli strains

Xl-1 Blue	Stratagene, La Jolla, CA, USA (Bullock *et al.*, 1987)
TOP10	Invitrogen, Karlsruhe, Germany

Agrobacterium tumefaciens strains

GV2260	Deblaere *et al.* (1985)
GV3101	Koncz and Schell (1986)

Saccharomyces cerevisiae strains

BY4741	Euroscarf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index Przybyla-Zawislak *et al.* (1998)

2.3. Expression vectors

pFL61	Drager *et al.* (2004)
pK2WG7	www.plantgenetics.rug.ac.be/gateway/index.html Karimi *et al.* (2002)
pK7GWIWG2(I)	www.plantgenetics.rug.ac.be/gateway/index.html Karimi *et al.* (2002)

2.4. Transformation and cultivation of bacteria

Chemically competent *E.coli* cells were transformed by heat-shock as described by Hanahan (1983). After transformation, cells were grown at 37°C on LB medium supplemented by selective antibiotic as described by Sambrook *et al.* (1989). Competent *A. tumefaciens* cells were prepared according to Höfgen and Willmitzer (1990) and transformed by electroporation according to Miller *et al.* (1988). The cells were grown at 28°C on YEB medium supplemented with selective antibiotic according to Vervliet *et al*. (1975).

2.5. Complementation, transformation and cultivation of yeast

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. The above mentioned yeast strains were maintained on YPD (10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone, 20 $g.L^{-1}$ glucose, 15 $g.L^{-1}$ agar) medium supplemented with 200 $mg.ml^{-1}$ of geneticin. Complementation assays were performed by transforming the mutant yeast strains with a modified version of the pFL61 vector. The yeast mutant ΔLSC1 was transformed with vectors independently containing *Sl*SCoAL α1 and α2, 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 based on 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 media (SS, 0.7 % yeast nitrogen base, 2 % glucose, 0.7 g/L Drop out supplement, 20mg/L histidine, 20 mg/L leucine and 0.05 % yeast extract; 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 (4μL) were plated onto solid SS media, replacing the glucose by 3% glycerol as sole carbon source and adding 2 % agar (Difco) and incubated for 4-6 days at 30˚C. A non fermentable carbon source had to be used because it is known that the synthesis of the TCA cycle enzymes are repressed during fermentation of glucose, and are subsequently induced by growth on non fermentable carbon sources, such as glycerol, acetate, pyruvate and ethanol (Przybyla-Zawislack *et al.*, 1998).

2.6. Yeast mitochondria isolation

To obtain a crude mitochondrial preparation 50 ml of an overnight culture (OD_{600} 1 – 2) was centrifuged for 5 min at 3000 rpm. The pellet was resuspended in 300 µL of extraction buffer (0.6 M sorbitol; 20 mM HEPES-KOH pH 7.4), transferred to a 1.5 mL Eppendorf tube and added V/2 of glass beads (0.25 – 0.5 mm; ROTH, Karlsruhe, Germany) and 2 mM PMSF. The mixture was left on ice for 5 min, and then 3 times repetition of 1 min high speed vortexing and 1 min on ice. The mixture was centrifuged for 5 min at 2500 rpm. The supernatant was transferred into a new Eppendorf tube and again centrifuged for 12 min at 9000 rpm. The supernatant was transferred to a new tube (post-mitochondrial fraction), and the pellet was resuspended in 20 - 50 µL of extraction buffer (mitochondria).

2.7. DNA manipulation

DNA manipulations were performed essentially as described by Sambrook *et al.* (1989). Plasmid DNA was extracted using the NucleoSpin Exctraction kit (Macherey-Nagel GmbH, Germany) and PCR mix was purified using NucleoSpin purification kit (Macherey-Nagel GmbH, Germany). For microarrays, first- and second-strand cDNA synthesis mix were cleaned up by Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) and concentrated using a Microcon YM-30 column (Millipore).

2.8. Plant material

Solanum lycopersicum cv. Moneymaker was obtained from Meyer Beck (Berlin, Germany). Plants were handled as described in the literature (Carrari *et al.*, 2003). *Arabidopsis thaliana* cv Col 0 seeds were surface-sterilized (ethanol 70% 2 min; 6 % Na-hypochlorite + 0.1 % triton X-100 solution, 15 min; wash 3 time in sterile water) and plated onto AMOZ media (0.24 % Murashige and Skoog-Medium; 0.055 % MES; 0.7 % agar) supplemented with 1 % sucrose and 50 µg.ml^{-1} kanamycin (for selection). Plates were kept in growth chamber (250 µmol photons.m^{-2}.s^{-1}, 22°C under a 16-h/8-h day light) for about 7 days or until it was possible to differentiate seedlings that were kanamycin resistant. Seedling were transferred to a vermiculite-soil mixture and kept in the greenhouse under following conditions: minimum of 250 µmol photons.m^{-2}.s^{-1} light intensity, 22°C under a 16-h/8-h day light regime.

2.9. Screening of putative full-length cDNA from the tomato EST collection

Full length cDNAs encoding for selected TCA genes were isolated after screening the tomato EST collection. This EST collection was obtained from Clemson University Genomics Centre (http://www.genome.clemson.edu) and was described by van der Hoeven and collaborators (van der Hoeven *et al.*, 2002). Its database is publicly available and was generated with support of the National Science Foundation Plant Genome Program (http://www.tigr.org/tdb/tgi/lgi; http://www.sgn.cornell.edu). For the initial large scale screening, bacteria containing the EST clones annotated to encode for TCA genes were picked from a 384-well plate and inoculated into 96-well plate containing LB liquid medium supplemented with 100 $mg.L^{-1}$ ampicilin. The resultant cultures were used to inoculate PCR, which contained *Taq* DNA polymerase 10X buffer (Invitrogen, USA), 0.1 mM of each dNTP, 1.5 mM $MgCl_2$, 0.30 μM of each Lac Z specific primers (forward LacZ1 5'GCTTCCGGCTCGTATGTTGTGTG 3' and reverse LacZ2 5' AAAGGGGGATGTGCTGCAAGGCG 3') and Taq DNA polymerase (Invitrogen, USA). The amplified fragments were run on agarose gels and if the size of the amplified product was similar at least to the respective Arabidopsis gene, the DNA was partially sequenced (SeqLab, Germany). For confirmation of the fragment size plasmid DNA from EST clones was extracted using the NucleoSpin kit (Macherey-Nagel GmbH, Germany) and digested using the restriction enzymes *Eco* RI/*Xho* I (Roche Diagnostics, Germany).

The criterion of selection of the putative full length clones was the homology with the orthologous genes mainly from *Arabidopsis thaliana*, because its genome is publicly available. **Table I** shows the list of genes of interest selected, the number of ESTs that were initially analyzed and finally the full-length clones found.

Table I. List of the genes of the TCA cycle and the number of EST that were initially analysed. The last column represents the number of confirmed full-length that were found. Abbreviations: ACO: aconitase, 2-OGC. 2-oxoglutarate complex and the respective subunits; SCoAL: succinyl CoA ligase and the subunits, SDH: succinate dehydrogenase; MDH: malate dehydrogenase (here are the following isoforms included: mitochondrial, cytosolic, chloroplastic and glyoxysomal); PDC: pyruvate dehydrogenase complex and its subunits.

Enzyme	No of ESTs	No. of full length clones
ACO	45	-
2-OGC E1	3	-
2-OGC E2	22	1
2-OGC E3	27	1
SCoAL α1	26	1
SCoAL α2	7	1
SCoAL β1	18	1
SDH iron sulphur	20	1
SDH flavoprotein	8	-
MDH isoforms	23	1 (for each isoform)
PDC E1 α	43	1
PDC E1 β	45	1
PDC E2	11	-

2.10. Subcloning of selected cDNA encoding TCA genes from tomato into pENTR vector

The full-length cDNAs selected were used for further cloning using the GATEWAY system (Invitrogen Life Technology, USA). The cloning into the pENTR vector was performed utilizing the pENTR Directional TOPO cloning kit for the Gateway system. Firstly, the full-length coding regions were amplified by PCR from plasmid DNA using gene specific primers (**Table II**) and 1 unit of *Pfu* DNA polymerase (Stratagene, Amsterdam). The primers were designed omitting the stop codon to permit carboxyl terminal fusions (for example with GFP). The amplification was done under following conditions: initial denaturation for 5 min at 95°C, and 30 cycles, each consisting of

denaturation for 1 min at 95°C, annealing for 1 min at the melting temperature for each pair of primers (**Table II**), and elongation for 2 min at 72°C. The last round of elongation was for 10 min at 72°C. Ten ng of purified amplification product were added to the cloning reaction mix and after 5 min incubation the mix used for heat-shock transformation of TOP10 chemically competent *E. coli*. The bacterial culture (50 and 200 µL) was plated onto selective LB solid medium supplemented with 50 $mg.L^{-1}$ kanamycin. Four to five colonies were inoculated into selective LB liquid medium for over night incubation. Plasmid DNA of the putative Entry clones was extracted and the presence of the coding region was verified by PCR using the gene-specific primers described before. Finally, the sequences obtained were introduced into the Genbank (accession numbers are listed in **Table II**).

The full-length coding regions contained in the pENTR vectors were transferred, by homology recombination, into the expression vectors (**Item 2.3**), in a one-step reaction, following GATEWAY cloning protocol. The reaction mix was then used to transform *E. coli* TOP10 strain. Four to five colonies were confirmed by PCR.

2.11. Subcellular localisation of tomato SCoAL α1, α 2 and β subunits, and mitochondrial-, cytosolic-, chloroplastic- and glyoxysomal MDH isoforms in Arabidopsis

The full-length tomato SCoAL (*SlSCoAL*) *α1*, *α2* and *SlSCoAL β* coding regions were cloned into pK7FWG2 vector (**Item 2.3**) and transformed into *A. thaliana* cv. Col-0. After kanamycin selection, putative stable transformed plants were identified using a stereoscope microscope which allows screening using a GFP specific filter. Positive plants were used to isolate protoplasts to have a more detailed picture of the protein targeting. For this, young leaves were cut in small pieces and immediately transferred to a Petri dish containing 0.5 M mannitol solution for 1 hour. 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 hours. The resultant isolated protoplasts were treated with 1µM 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).

The same strategy was used for MDH isoforms for subcellular localization, but in these plants, whole leaf tissue was analysed under the confocal microscope.

Table II. **Full-length cDNAs of genes cloned into the cloning vector pENTR**. This table includes the name of the EST from were the full-length cDNA was obtained and the specific primers used for amplification. As complement information, the annealing temperature for PCR was added and in the last column are included the accession number of each gene in the Genbank. (See Table I for abbreviations).

Gene	EST	Primers	Temp (°C)	Genbank
2-OGC E3	cLES19N7	F 5' TAATGGCGATTGGGAGCTTAG R 5' CATGTGAATGGGCTTGTCCTAA	49	AAN23154
SCoAL α1	cLES12N15	F 5'CACCATGGCTCGCCAAGCG R 5'TTTCACAAGACCCCTCTGTTTGAAC	51.6	AY167586
SCoAL α2	cLEY17J6	F 5'CACCATGGCTCGCCAAGCC R 5' CGCAAGACCCCTCTGTTTG	52.8	AY650029
SCoAL β	cTOF2K12	F 5'CACCATGCTGCGTAAACTTGCCAATC R 5' AGCTAAGGCCTTGACTGCC	50	AY180975
mMDH	cLEI12D19	F 5' CACCATGAGGACCTCCATGTTG R 5' GTTTTCTTTGGCAAACTTGATTC	51.5	AY725474
chMDH	cTOC4D24	F 5' CACCATGGCGGTGGCAGAGTTT R 5' CATTTCTCCAGGGAGCATTGA	53.8	AY725477
gMDH	cTOE5A3	F 5' CACCATGCAGCCATCAGGTGCA R 5' ATTCCTGATGAAGGAAATTCCTTTC	54.3	AY725476
cMDH	cLEC31P18	F 5' CACCATGAATATAGAAAAGGATCCC R 5'GTTTAGGCATGAATACGCTAAGG	50.7	AY725475
SDH iron sulphur	cLPP11A2	F 5' CACCATGGCGACTAGTTTAATCCGACG R 5' AGGTGCCATCTCCAGCTTCTTG	57	-

2.12. Phylogenetic analysis tomato SCoAL α1, α 2 and β subunits

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 I only selected sequences from eukaryotes and prokaryotes with fully sequenced genomes. I also used the tBLASTn 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.

2.13. Expression analysis tomato SCoAL α1, α 2 and β subunits

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 in 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.

2.14. Analysis of enzyme activities

Enzyme extract were prepared as described previously (Tauberger *et al.*, 2000). Except where stated all activities were determined as described in Nunes-Nesi *et al.* (2005a).

2.14.1. Succinyl CoA ligase enzyme assay

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 and 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.

ADP (ATP free) was prepared mixing in a 10 mL Falcon tube: 4 mL of ADP solution 25 mM; 0.5 mL of 1 M Tricine/ KOH pH 8.0; 0.05 mL of 1 M MgCl2; 0.2 mL of 1 M glycerol; 0.1 mL of 50 $u.ml^{-1}$ glycerokinase; 0.2 mL of 500 $u.ml^{-1}$ G3POX. The mix was incubated in the closed Falcon tube at 37°C overnight under constant shaking. To stop the reaction and destroy the DAP produced (but not the ADP) the mix was incubated at 95°C for 20 min. The final solution was aliquoted in Eppendorf tubes and maintained at -80°C.

SCoAL protein extract was frozen in liquid nitrogen and stored at -80°C to test the enzymes stability. SCoAL activity could still be detected after 5 days at -80°C.

2.14.2. Glutamate Decarboxylase (GAD) enzyme assay

GAD activity was measured basically as described by Turano and Fang (1998). Fresh collected tomato leaf tissue (150-200 mg) was extracted in 400 µL of extraction buffer (50 mM TES, pH 5.8; 5 mM EDTA, 1 mM $MgCl_2$; 0.1 mM PLP ; 1 mM DTT; 4 mM L-cystein; 1 mM PMSF;) in a mortar, adding a spatula tip of PVP-40. Samples were kept on ice for 30 min, transferred to Eppendorf tubes and centrifuged (10 min, 13,000 rpm, 4 °C) to remove debris. Supernatant was added to 7 ml PC bottles (4.5 x 1.5 mm, Sarstedt, Australia) containing reaction buffer (100 mM pyridine-HCL, pH 5.8; 10 mM NaCL; 0.1 mM PLP; 1 mM CaCl2; 0.4 µM CaM and 20 mM glutamate containing 0.02 µCi / µmol L-[1-^{14}C]Glu) in a final volume of 500 µL. Prior to incubate, two glass microfibre filters (21 mm diameter, Whatman, UK) were fixed to a toothpick and imbibed with 180 µL 1 M KOH solution. The glass microfibre filters was hang in the glass vial and closed with laboratory film (Parafilm, American National Can, Greenwich, England). Samples were incubated under constant shaking at RT for 1 h. The reaction was stopped with 100 µL 1 M KOH. The vials were immediately transferred to ice for 1 h. The glass microfibre filters were transferred to small scintillation vials and quantified by liquid scintillation counting. Activity was firstly determined using different amount of extract to test the linearity of the enzyme, and controls omitting extracts or denaturating the extracts (5 min at 95°C) were also performed. GAD enzyme activity was detected adding 20 µL of tomato total protein extract. Background processes producing $^{14}CO_2$ were not active since the label was not detected in the controls. Activity was normalized against protein concentration which was determined according to Bradford (1976) using BSA as standards. Total initial label was measured from 100 µL of the reaction mix and GAD enzyme activity calculated as a percentage of the initial label that was released as $^{14}CO_2$ and trapped in the KOH solution.

2.14.3. Glutamate Dehydrogenase (GDH) enzymatic assay

GDH activity was determined basically as described in Purnell *et al.* (2005) measured in the direction of glutamate formation (aminating direction). Leaf tissue (50 - 80 mg) ground in liquid nitrogen and kept at -80 °C was extracted in 300 µL of extraction buffer (100 mM Tris-HCl, pH 8.0; 2 mM EDTA, pH 8.0; 1 mM DTT; 1 mM reduced glutathione; 0.1 % Triton X-100; a spattle tip of PVPP and PVP-40 were added to the samples). The extracts were mixed and centrifuged at 4°C for 15 min at 13.000 *g*. GDH activity was measured

adding the extract to the reaction mix (100 mM Tris, pH 8.0; 15 mM 2-OG; 250 mM NH_4Cl; 1 mM $CaCl_2$ and 0.25 mM NADH) in a final volume of 300 µL. The mix was incubated for 2 min at RT and read at 340 nm in a microtitre plate reader (Biolise) for 30 min. Different amount of extract (5-10-20-40 µL) were used to check the linearity of the enzyme activity as well as controls omitting either 2-OG or the extract were performed. GDH enzyme activity could be detected adding 30 µL of tomato total protein extract.

2.15. Oxygen consumption measurements in yeast mutant strains complemented with tomato SCoAL α1, α 2 and β subunits

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 (YPD replacing the glucose with 3 % glycerol) and oxygen consumption was recorded under continuous stirring at 25°C.

2.16. Determination of metabolite levels in transgenic and wild type tomato leaves

Metabolites were extracted and measured from leaf samples frozen in liquid nitrogen exactly as described by Nunes-Nesi *et al.* (2005a). Hexose phosphates, 3PGA and acetyl CoA were determined in trichloracetic acid extracts using the cycling assays defined in Gibon *et al.* (2002). The determination of the levels of chlorophylls A and B, β-carotene, lutein, neoxantin, violaxantin and zeaxantin were performed in 80% acetone extracts as described in Thayer and Björkman (1990). Ascorbate and dehydroascorbate were measured using bipyridyl method based on the Kampfenkel *et al.* (1995) and adapted to microtitre plate reader. Tocopherols were measured from 100 mg of frozen leaf tissue in HPLC according to the method described by Thompson and Hatina (1979).

2.17. Measurements of photosynthetic parameters

Fluorescence emission was measured in vivo using a PAM fluorometer (Walz, Effeltrich, Germany) on 6-week-old plants maintained in the dark for 30 min to measure the chlorophyll fluorescence yield at different light intensities, and relative ETR was calculated using the WinControl software package (Walz). Gas-exchange measurements were performed in special custom-designed open system (Lytovchenko *et al.*, 2002). The ^{14}C-labelling pattern of sucrose, starch and other cellular constituents was performed by illuminating leaf discs (10-mm diameter) in a leaf-disc oxygen electrode (Hansatech, Kings Lynn, Norfolk, UK) in saturating $^{14}CO_2$ at a PDF of 600 $\mu mol.m^{-2}.s^{-1}$ of photosynthetically active radiation at 20ºC for 30 min, and subsequent fractionation was performed exactly as described by Lytovchenko *et al.* (2002).

2.18. Measurement of respiratory parameters

Estimations of the TCA cycle flux on the basis of $^{14}CO_2$ evolution were carried out following incubation of leaf discs (6 mm diameter) in 10 mM MES-KOH solution, pH 6.5, 0.3 mM Glc supplemented with 0.0625$\mu Ci.ml^{-1}$ of [1-^{14}C]-, [2-^{14}C]-, [3,4-^{14}C]-, or [6-^{14}C]-Glc, with a specific activity of 7.5 $MBq.mmol^{-1}$. $^{14}CO_2$ liberated was trapped in a 10% KOH solution and quantified by liquid scintillation counting. The results were interpreted following ap Rees and Beevers (1960).

2.19. Microarray

Messenger RNA (mRNA) from wild type and a transgenic line was extracted from 200 mg grinded tomato leaves and cDNA was synthesized exactly as described by Degenkolbe *et al.* (2005). Quality control of the synthesized double-stranded cDNA Real-Time-PCR was performed using the tomato specific primers for GAPDH (Lower 3'SlGAPDH 5' TAAGATCGACAACGGAGACATCAG; Upper 3' SlGAPDH 5' TTCAACATCATCCCTAGCAGCACT). Two µL of a 1/10 dilution of double-stranded cDNA was used for PCR amplification mix in final volume of 25µL. Concentrated double stranded of 0.7 µg were used for co-hybridization on glass slides containing arrayed tomato ESTs (TOM1) obtained directly from The Center of Gene Expression Profiling

(CGEP) at the Boyce Thompson Institute (BTI), Cornell University, The Geneva Agricultural Experiment Station, the USDA Federal Plant and Nutrition Laboratory, following the description of Degenkolbe *et al.* (2005). Signal intensities of four independent hybridization were quantified using GeneSpotter software version 2.3 (MicroDiscovery, Berlin, Germany) and analyzed and normalized using the special statistical software package R (R Development Core Team, 2004). Final treated data were visualized using MAPMAN software (Thimm *et al.*, 2004; Usadel *et al.*, 2005).

2.20. Statistical analysis

The *t* tests were performed using algorithm embedded into Microsoft Excel (Microsoft, Seattle). The term significant is used in the text only when the change in question has been confirmed to be significant ($P < 0.05$) with the *t* test.