

**Functional analysis of the role of
succinyl CoA ligase in the photosynthetic
metabolism of tomato**

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Claudia Rodriguez Studart-Guimarães

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1st Reviewer: Prof. Dr. Thomas Schmüling

2nd Reviewer: Prof. Dr. Bernd Müller-Röber

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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 this work I report the isolation of tomato cDNAs coding for $\alpha 1$ - and $\alpha 2$ - and one coding for the β -subunit of succinyl CoA ligase, for E2- and E3-subunits of 2-oxoglutarate dehydrogenase complex, for iron sulphur-subunit of succinate dehydrogenase, for E1 α - and β -subunits of pyruvate dehydrogenase complex, and for chloroplastic-, cytosolic-, mitochondrial- and glyoxysomal-subunits of malate dehydrogenase. Emphasis was given to the cDNAs coding for $\alpha 1$ - and $\alpha 2$ - and 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 plant organs, but most strongly in flowers and leaves, with the two α -subunit genes being expressed to equivalent levels in all plant organs. 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 plant organs revealed that these paralleled those of mRNA levels. I also utilized this assay to perform a preliminary characterisation of the regulatory properties of the enzyme suggesting allosteric control of this enzyme may regulate flux through the TCA cycle in a manner consistent with its position therein. Transgenic tomato (*Solanum lycopersicum*) plants expressing the complete coding region of $\alpha 1$ - and β -subunit of succinyl CoA ligase separately in antisense orientation and using the RNAi approach were produced. Transformants were screened for reduced succinyl CoA ligase activity and selected lines were used for molecular, biochemical and physiological characterisation. Transgenic tomato plants harbouring the β -subunit of succinyl CoA ligase showed an increase in plant height, and a decrease in fruit production and leaf dry matter, but no change in photosynthetic parameters and about 30% decrease in the respiration rate. Accumulation of glutamate and γ -aminobutyric acid amino acids in leaves, and a higher activity of glutamate dehydrogenase and glutamate decarboxylase in the transformants suggest an alternative route, the GABA shunt which bypasses the succinyl CoA ligase deficiency and supplies the mitochondria with succinate to support the respiratory processes. Further

analysis of other steady-state metabolite levels suggests a link between succinyl CoA ligase activity with other metabolic pathways such as the shikimate and isoprenoid pathways. It was observed that end products of the shikimate pathway, such as aromatic amino acids, were increased, as well as tocopherols, chlorophylls and carotenoids, which are end products of the isoprenoid pathways. Analysis of transgenic tomato plants displaying reduced expression of α 1-subunit of succinyl CoA ligase performed in parallel with the tomato plants described above surprisingly did not show similar phenotype as the first plants, most likely because the selected lines showed not strongly enough decrease in activity.

ZUSAMMENFASSUNG

Trotz der bedeutenden Rolle des Citratzyklus im Stoffwechsel der Pflanze, wurden bisher nur wenige Enzyme innerhalb dieses Stoffwechselweges näher charakterisiert. Das Ziel der vorliegenden Arbeit war es daher eben diese Lücke zu schliessen und die Aktivitäten einiger der relevanten Citratzyklus-Enzyme genauer zu analysieren. Zu diesem Zweck wurden zunächst die cDNAs, die für folgende Enzyme kodieren, aus Tomate isoliert: die α 1-, α 2- und β -Untereinheiten des Enzyms Succinyl Coenzym A-Ligase, die E2- und E3-Untereinheiten des 2-Oxoglutarat Dehydrogenase Komplexes, die Eisen-Schwefel-Untereinheit der Succinat Dehydrogenase, die E1 α - und β -Untereinheiten des Pyruvat Dehydrogenase Komplexes, und die chloroplastidäre-, cytosolische-, mitochondriale- and glyoxysomale-Untereinheiten der Malat Dehydrogenase. Der Schwerpunkt der Arbeit lag dann auf der Untersuchung der Funktionen der α 1-, α 2- und β -Untereinheiten der Succinyl Coenzym A-Ligase. Diese drei cDNAs wurden zunächst zur Komplementation von Hefe-Mutanten (*Saccharomyces cerevisiae*) mit einem entsprechenden Defekt in der jeweiligen Untereinheit verwendet. Es konnte somit gezeigt werden, dass die isolierten cDNAs funktionsfähige Polypeptide kodieren. Hybridisierungsexperimente haben im Weiteren ergeben, dass die Untereinheiten der Succinyl Coenzym A-Ligase in allen pflanzlichen Organen exprimiert werden. Besonders starke Expression fand man für die β -Untereinheit in Blüten und Blättern, wohingegen die beiden α -Untereinheiten in allen Organen zu gleichen Teilen exprimiert wurden. Durch die Analyse der Expression der GFP-fusionierten Untereinheiten konnte weiterhin die erwartete mitochondriale Lokalisierung der Proteine bestätigt werden.

Zur Aktivitätbestimmung des Enzyms Succinyl Coenzym A-Ligase wurde ein neues Protokoll in Richtung der Bildung von Succinat entwickelt. Die Auswertung der gemessenen Enzymeaktivitäten in verschiedenen pflanzlichen Organe ergab, dass diese mit den Transkriptgehalten der Gene korrelierten. Eine umfassende Charakterisierung der regulatorischen Eigenschaften des Enzyms konnte ebenfalls mithilfe des neu entwickelten Enzymassays durchgeführt werden. Dabei ergaben sich Hinweise auf eine allosterische Kontrolle des Enzyms das den Fluss durch den Zyklus übereinstimmend mit seiner Position kontrolliert.

Anschliessend wurden verschiedene, genetisch modifizierte Tomatenpflanzen (*Solanum lycopersicum*) hergestellt, die die vollständige Kodierungsregion der α 1- und β -

Untereinheiten von Succinyl Coenzym A–Ligase einzeln in antisense Orientierung und als RNAi exprimieren. Einzelne Transformanten dieser Pflanzen wurden nach ihrer verminderten Enzymaktivität ausgewählt und dann für molekulare, biochemische und physiologische Analysen verwendet. Hierbei zeigten die für das Gen der β -Untereinheit der Succinyl Coenzym A–Ligase reprimierten Tomatenpflanzen ein gesteigertes Pflanzenwachstum, eine verminderte Fruchtproduktion, ein verringertes Trockengewicht der Blätter und eine ungefähr 30%ige Verminderung der zellulären Atmungsrate. Sie zeigten jedoch keine Änderung der photosynthetischen Parameter (zum Beispiel CO_2 Assimilationsrate). Die Anreicherung an Glutamat und γ -Aminobuttersäure (GABA) in Blättern, und die Erhöhung der Enzymaktivitäten der Glutamat Dehydrogenase und der Glutamat Decarboxylase in diesen Pflanzen, weisen somit darauf hin, dass der „GABA-shunt“, als ein alternativer Weg, den Mangel an Succinyl Coenzym A–Ligase überbrückt und die Mitochondrien mit Succinat beliefert, um somit die Atmungsprozesse zu sichern. Weiterhin durchgeführte Metaboliten-Analysen in Blättern, weisen auf eine Verbindung zwischen der Succinyl Coenzym A–Ligase Enzymaktivität und anderen Stoffwechselwege, wie zum Beispiel dem Shikimat- und dem Isoprenoidweg, hin. So konnte beobachtet werden, dass Endprodukte des Shikimatwegs, wie aromatische Aminosäure, als auch Tocopherol-, Chlorophyll- und Karotenoidmengen des Isoprenoidstoffwechselweges, erhöht waren. Die Analysen der Tomatenpflanzen mit verminderter Expression der Succinyl Coenzym A–Ligase α 1-Untereinheit, die parallel durchgeführt wurden, wiesen überraschenderweise, nicht dieselben Eigenschaften wie die zuvor beschriebenen Transformanten der β -Untereinheit auf. Es besteht hierbei die Möglichkeit, dass die Verminderung der Enzymaktivität der ausgewählten Pflanzen nicht stark genug ausgeprägt war.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ADPglc	ADP glucose
AGPase	ADPglc pyrophosphorylase
ATP	adenosine triphosphate
ATP-PFK	ATP dependent phosphofructokinase
BSA	bovine serum albumin
CaM	calmodulin
CaMV	cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
CoA	Coenzyme A
cv	cultivar
DAP	dihydroxyacetonephosphate
dNTP	deoxynucleotide triphosphate
DTT	1,4-dithiothreitol
DAF	days after flowering
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
Fru	fructose
FW	fresh weight
GABA	γ -aminobutyric acid
G3P	glycerol-3-phosphate
G3PDH	glycerol-3-phosphate dehydrogenase
G3POX	glycerol-3-phosphate oxidase
GC-MS	gas chromatography – mass spectrometry
Glc	glucose
hexose-P	hexose phosphate
mM	milli molar
mRNA	messenger ribonucleic acid
OD	optical density
PAM	pulse amplitude modulation
PCR	polymerase chain reaction
3-PGA	3-phosphoglyceric acid

Pi	inorganic phosphate
PLP	pyridoxal 5-phosphate
PMSF	phenylmethylsulfonylfluoride
PPi-PFK	pyrophosphate dependent PFK
PVP	polyvinyl pyrrolidone
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RT	room temperature
RT-PCR	Reverse transcript PCR
Rubisco	ribulose-1,5-biphosphate carboxylase/oxygenase
SCoAL	succinyl CoA ligase
SE	standard error
Suc	sucrose
TCA	tricarboxylic acid
UDP	uridine diphosphate
UDPglc	UDP glucose
UGPase	UDPGlc pyrophosphorylase
UTP	uridine triphosphate
WT	Wild type