Chapter 1

General introduction

1.1 Tomato plant as a model system

Tomato (*Solanum lycopersicum*) is a member of the Solanaceae, which is a large extremely diverse family containing over 3000 species with origins in both the Old (eggplant – China, India) and the New World (pepper/tomato/potato – Central and South America) (Knapp, 2002). The family has both economic and nutritional value. Humans utilize more then 18 species of this family and those that are consumed in the form of vegetable crops provide important dietary sources of vitamin A and C and antioxidants (e.g. lycopene) (Mayne, 1996; Palozza and Krinski, 1996; Bramley, 2000). Many species of the Solanaceae have the same basic chromosome number (x=12), and the genetic content of tomato, potato, pepper, and eggplant remains remarkably similar despite differences in genomic size (about 950 Mb, 1800 Mb, 3000 Mb, and 1100 Mb, respectively) (Bonierbale and Tankley, 1988; Livingstone *et al.*, 1999; Doganlar *et al.*, 2002).

Tomato plants are very valuable because of their fruit production which not only serves for consumption, but also for study in terms of development, maturation, ripening, and associated quality and yield characteristics. Tomato fruits, as well as fruits of cucurbits, avocado, banana, peaches, plums, and apples are considered climacteric fruits because they exhibit increased rates of respiration and ethylene biosynthesis (Giovannoni, 2001). Parallel to the dependence to ethylene biosynthesis, another important feature related to fruit ripening is the transition of photosynthetic to respiratory metabolism in the tomato fruit. Initially tomato fruits contain chloroplasts that are photosynthetically active, being therefore able to fix carbon, but these chloroplasts then differentiate to chromoplasts, which are non-photosynthetic plastids. Many studies focus therefore on tomato fruit ripening at transcript level (Bartley and Ishida, 2002; Fei *et al.*, 2004) and also the regulation and control of ethylene on gene expression supported by selected metabolite profiling for parallel information (Alba *et al.*, 2005) to try to understand these transition steps and its regulation.

Tomato was one of the first plants to be genetically modified utilising recombinant DNA techniques in 1987 by Fillatti (Fillatti *et al.*, 1987). The full sequencing of the gene rich regions of tomato is under way afforded by genome sequencing centres located in different countries throughout the world. However, huge amount of sequenced cDNA clones of genes expressed in tomato is already available.

Expressed Sequence Tags (ESTs) are created by sequencing the 5´and/or 3´ends of randomly isolated gene transcripts that have been converted into cDNA (Adams *et al.*, 1991). EST collections are a relatively quick and inexpensive route for discovery of new genes, confirming coding regions in genomic sequence can sometimes be utilized directly for transcriptome activity and provide the basis for the development of expression arrays on chips (Alba *et al.*, 2004). EST sequencing projects together with other high-throughput phenotypic technologies, are leading to a creation of huge database systematically organized for visualization of data. The Solanaceas Genomics Network (SNG; http://sng.cornell.edu), for example, is dedicated to the biology of Solanaceas species, including tomato, potato, tobacco, eggplant, pepper, and petunia. With such database and advances in computational biology and biostatistics, it is relatively facile to analyze large EST datasets efficiently as demonstrated by the statistical analysis of rice ESTs (Ewing *et al.*, 1999), the virtual display of gene expression in wheat (Ogihara *et al.*, 2003) and the comparative analysis of potato ESTs libraries (Ronning *et al.*, 2003). The tomato EST collection, obtained from Clemson University Genomics Centre (http://www.genome.clemson.edu), was described by van der Hoeven and collaborators (van der Hoeven *et al.*, 2002) and its public database was generated with support of the National Science Foundation Plant Genome Program (http://www.tigr.org/tdb/tgi/lgi; http://www.sgn.cornell.edu). The first dataset utilizing this platform were recently published (Alba *et al.*, 2004; Fei *et al.*, 2004; Uppalapati *et al.*, 2005; Baxter *et al.*, 2005; Carbone *et al.*, 2005; Urbanczyk-Wochniak *et al.*, 2005).

1.2. Plant mitochondria and metabolic respiration

Mitochondria are ubiquitous and vital organelles that play a pivotal role in energy metabolism in the eukaryotic cells. They were identified over 50 years ago as the site of oxidative energy metabolism (Kennedy and Lehninger, 1949), but only in the past decades researchers, predominantly working with yeast, have elucidated the basic mechanism controlling mitochondrial shape, size and number in this organism (Shaw and Nunnari, 2002). Although most work has been carried out in yeast, this is a poor model system for higher plants. Plant mitochondria have evolved specially functions due to differences in evolution compared with other organisms augmenting the flexibility of respiratory pathways (Mackenzie and McIntosh, 1999).

In keeping with this, considerable little attention has been paid to plant mitochondria via proteomic of morphological and oxidative stress studies recently carried out in mitochondria (Kruft *et al.*, 2001; Sweetlove *et al.*, 2002; Logan *et al.*, 2003).

Plants produce carbon-rich products in their chloroplasts by harnessing light energy via photosynthesis, and then respire these compounds in the cytosol and the mitochondria to generate energy and the carbon intermediates necessary for biosynthesis and growth. It is therefore known, that both processes are interdependent in the way that respiration relies on photosynthesis for substrates whereas photosynthesis depends on respiration for a range of compounds (Hoefnagel *et al.*, 1998; Gardeström *et al.*, 2002; Raghavendra and Padmasree, 2003; Noctor *et al.*, 2004; Yoshida *et al.*, 2006). In this general introduction, the metabolic pathways of mitochondrial respiration will be firstly discussed as independent processes. For this purpose, respiration can be divided into three main pathways: glycolysis, mitochondrial tricarboxylic (TCA) cycle and mitochondrial electron transport. Given the large differences between plant mitochondria and those of other organism, it could be anticipated that there are unique features of plant respiratory metabolism. These include [i] multiple entry points into the respiratory pathway from sucrose and starch; [ii] the duplication of pyrophosphate and ATP-dependent phosphorylation of fructose 6-phophate; [iii] the loss of regulation of glycolysis by kinetic effects of ATP on phosphofructokinase and pyruvate kinase reactions, and [iv] the presence of non-phosphorylating electron transport systems (Fernie *et al.*, 2004a). In the following section, the three stages of respiration will be described with most attention focussing on the tricarboxylic acid cycle since this will constitute the major focus of this study.

1.2.1. Glycolysis

Glycolysis has two main functions: it oxidizes hexoses generating ATP, reductant and pyruvate, and also produces building blocks for anabolism (Plaxton, 1996). In the reverse direction this pathway also functions to generate hexoses from low-molecular weight compounds in the energy-dependent pathway gluconeogenesis. In plants, glycolysis is the predominant pathway that fuels plant respiration, because a significant proportion of the carbon that enters the glycolytic and afterwards the tricarboxylic acid cycle pathways are utilized in the biosynthesis of numerous compounds such as secondary metabolites, isoprenoids, amino acids, nucleic acids, and fatty acids.

The conversion of hexoses to pyruvate in plants can occur independently in the cytosol and in the plastids. The fact that glycolysis in plant can occur in the cytosol and in the plastid is one of the features that differentiate plant from other eukaryotes glycolysis, because glycolysis in both compartments can interact through the action of highly selective transporters. The existence of multiple enzymes that allow bypasses and various isozymes of the plant glycolysis acting in the two compartments provides the plant with immense metabolic flexibility. Recent studies on Arabidopsis mitochondria suggest that the entire cytosolic glycolytic pathway is associated with plant mitochondria by attachment to the cytosolic face of the outer mitochondrial membrane (Giege *et al.*, 2003). The authors proposed that this localization of the glycolytic pathway would facilitate that pyruvate is directly provided to the mitochondria to be used as respiratory substrate.

Glycolysis is intimately linked to the oxidative pentose phosphate pathway (OPPP) and the predominant pathway depends on the varying requirements for hexose phosphates, reducing power and energy of the cells. That said under most conditions the rate of glycolysis is approximately four-fold that of the oxidative pentose phosphate pathway. The OPPP generates the reduced co-factor NADPH and precursors for many biosynthetic pathways including those for shikimate acid and nucleotide biosynthesis (Debnam *et al.*, 2004).

1.2.2. TCA cycle

The tricarboxylic acid (TCA, **Figure 1.1**) cycle, also known as the Krebs cycle and the citric acid cycle, comprises the second stage of the process of cellular respiration. In plants, reactions of the TCA cycle are mainly localized within the mitochondrial matrix, but the organic acid intermediates accumulate to a large extend in the vacuole. The TCA cycle promotes the metabolism of organic acids and provides precursor ultimately utilized in the formation of important cellular constituents such as amino acids, fatty acids, flavonoids, alkaloids and isoprenoids and in tandem to the production of energy, reducing power and ATP equivalents. Since its elucidation in pigeon muscle by Krebs and co-workers (Krebs and Johnson, 1937), evidence has accumulated that the same reactions occur in plant cells (Beevers, 1961). Despite this fact the physiological role of this important pathway in plants is still far from clear (Hill, 1997; Siedow and Day, 2000).

Even fundamental questions, such as whether the TCA cycle operates at all in illuminated photosynthetic tissue and if so, whether it contributes to the energy requirements of photosynthetic sucrose synthesis, remain controversial (Graham, 1980; Krömer, 1995; Padmasree *et al.*, 2002).

In addition to its role in energy production the TCA cycle is thought to have a number of other functions, such as meeting the demand for carbon skeletons imposed by anabolic processes such as amino acid synthesis (Douce and Neuberger, 1989; Mackenzie and McIntosh, 1999) and isoprenoid synthesis (Fatland *et al.*, 2005), regulation of cellular redox (Scheibe *et al.*, 2005) and the control of C/N balance (Noguchi and Terashima, 2006).

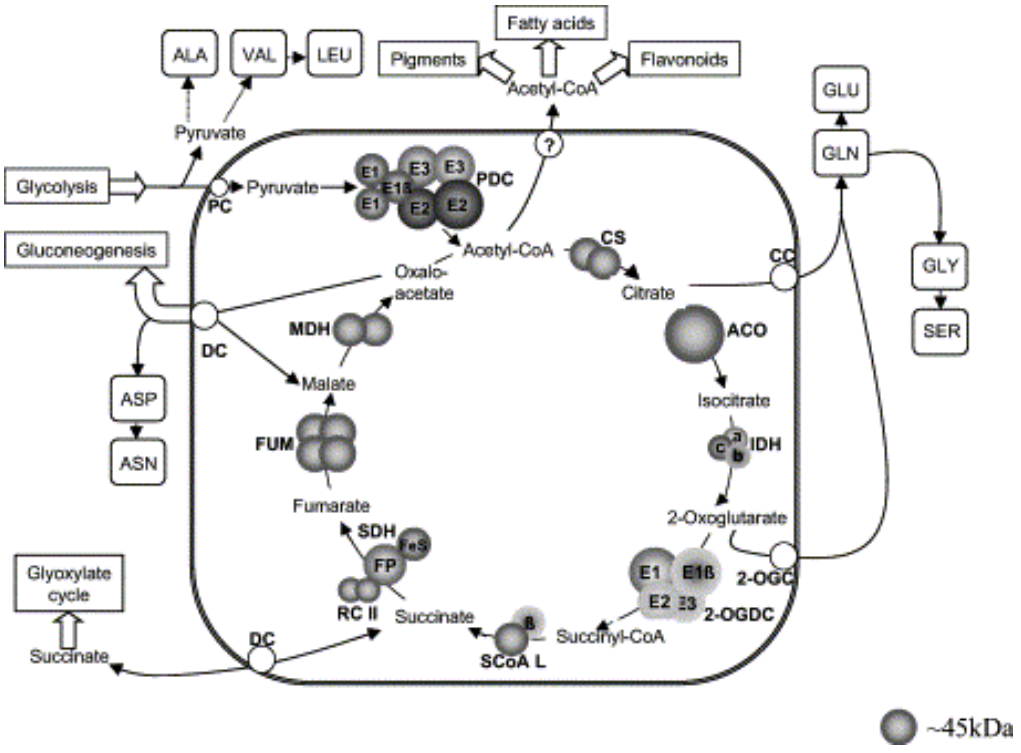

Figure 1.1 Schematic summary of the tricarboxylic acid cycle and its convergent and divergent pathways. Subunits are represented by spheres, where differences represent the heteromeric nature of the enzymes and white spheres represent carriers or antiporters. Abbreviations: CS: citrate synthase; ACO: aconitase; IDH: isocitrate dehydrogenase; 2-OGDC: 2-oxoglutarate dehydrogenase complex; SCoAL: succinyl CoA ligase; SDH: succinate dehydrogenase; FUM: fumarate; MDH: malate dehydrogenase; PDC: pyruvate dehydrogenase complex; PC: pyruvate carrier; DC: dicarboxylate carrier; 2-OGC: 2-oxoglutarate carrier, and CC: citrate carrier.

Moreover, recent studies have revealed that photosynthetic performance can in fact be improved by modifications in either activities of the mitochondrial electron transport chain (Bartoli *et al.*, 2005) or of the TCA cycle (Carrari *et al.*, 2003; Nunes-Nesi *et al.*, 2005a;b) *per se*. The major reasons for limited progress in this area are the extensive subcellular compartmentation of plant cell (ap Rees, 1987) and the difficulties in extracting enzymes and metabolites from plant tissues (ap Rees, 1980). Thus, although almost all genes encoding for the enzymes involved in the TCA cycle have been cloned from different plant species (for review see Hill, 1997; Schnarrenberger and Martin, 2002) and many of the encoded proteins have been biochemically characterized (for example see Millar *et al.*, 1999a; b; Lancien *et al.*, 1998; Thelen *et al.*, 1998), only a relative small number of these proteins have been subjected to molecular physiology studies to date. The first enzyme of the TCA cycle, the mitochondrial pyruvate dehydrogenase, is a multienzyme complex that catalyses the irreversible reaction that converts pyruvate into acetyl-CoA. This enzyme is though to be a key regulatory point for fluxes into the cycle. Acetyl-CoA can then be converted to citrate by citrate synthase enzyme. This reaction step was the target of the initial reverse genetic approach attempted in plants, suggesting an important role for this enzyme in flower development (Landschütze *et al.*, 1995). More recent study that also suggests an import role of the TCA cycle enzyme in flower development was the finding that tobacco plants with reduced levels of the E1 α subunit of pyruvate dehydrogenase complex exhibited male sterility because of failed anther development (Yiu *et al.*, 2003). The enzyme isocitrate dehydrogenase was also the focus of several studies in plants (Lancien *et al.*, 1999; Igamberdiev and Gardeström, 2003), however relatively little research has do yet focused on studying the 2-oxoglutarate dehydrogenase complex, the succinate dehydrogenase, the fumarase or the succinyl CoA ligase reactions.

The schematic representation of the TCA cycle in **Figure 1.1** highlights some of the interconnection with other metabolic pathways giving an idea of the metabolic network of the illuminated leaf. It allows initial understanding of the traffic pattern in the mitochondria, but offers little explanation of why these patterns emerge or how is it possible to control them. A future research goal should therefore be the analysis of the effect of systematic modification of the expression and/or the enzyme activity of each polypeptide of the cycle (and of the mitochondrial carrier proteins) in tomato by utilizing

high-throughput expression constructs based on hairpin gene silencing (Wesley *et al.*, 2001) or characterising mutants in the TCA cycle. The primary characterisation of such genetically diverse plants is based on the visual phenotypic level, looking at among other features growth and fertility of the transformants and mutants. Later, a more detailed characterisation should give some insights if the modulation of one of the TCA cycle enzymes results in a modification in carbon partitioning towards commercially valuable metabolites. However, a more holistic approach incorporating transcriptomic studies, flux measurements and an assessment of protein-protein interactions within the cycle might also be highly informative in such studies.

1.2.3. Mitochondrial Electron Transport

The mitochondrial electron transport or oxidative phosphorylation constitutes the third stage of respiration. It involves the transfer of electrons from reducing equivalents generated from the TCA cycle activity to the final electron acceptor oxygen. In addition to provide NAD and FAD to the TCA cycle and to other pathways, it generates an electrochemical potential which is used to drive ATP synthesis. The mitochondrial electron transport includes four electron carrier complexes with unique composition and each is capable of transferring electrons. Complexes (I) and (II) catalyses electron transfer to ubiquinone from two different electron donors: NADH (Complex I or NADH dehydrogenase complex) and succinate (Complex II or succinate dehydrogenase). Complex III (cytochrome bc_1 complex or ubiquinone-cytochrome c oxidoreductase) carries electrons from ubiquinone to cytochrome c, and Complex IV (cytochrome oxidase) completes the sequence by transferring electrons from cytochrome c to oxygen. The electron transfer through these complexes is tightly coupled to ATP synthase complex which phosphorylates ADP to ATP. Plants possess additional components that serve as alternative non-phosphorylating routes for electron transfer. These includes internal and external NAD(P)H dehydrogenases and, as well as the membrane-potential-dissipating uncoupling proteins. The NADH dehydrogenases constitute non-phosphorylating bypasses in that they don’t contribute to the generation of the inner membrane proton gradient. The alternative oxidase pathway is embedded in the inner mitochondrial membrane and it can oxidise oxygen in the presence of cyanide, an inhibitor of the cytochrome pathway.

Both bypasses allow electron transport to continue even when membrane potential is high, thereby uncoupling electron transport from ATP synthesis.

1.3 Respiratory activity

Mitochondrial respiratory activity in the dark includes the oxidation of carbon compounds in the TCA cycle and of NAD(P)H in the mitochondrial electron transport chain. The TCA cycle releases carbon dioxide and reducing equivalents. During oxidation of these reducing equivalents, oxygen is consumed and a proton gradient created across the inner mitochondrial membrane providing the energy for ATP synthesis in the mitochondria in a process known as oxidative phosphorylation. In the light, the oxidative decarboxylation of glycine to serine occurs in the mitochondrial matrix as a part of the photorespiratory cycle (Ogren, 1984). Plant mitochondria contain a malic enzyme allowing them to operate the TCA cycle independently of glycolysis, the respiratory chain also has several unique features: a cyanide-resistant non-phosphorylating pathway, a rotenone-insensitive oxidation site, and the ability to oxidise external NAD(P)H (Douce and Neuberger, 1989; Møller and Lin, 1986; Rasmusson *et al.*, 1999; Michalecka *et al.*, 2003). However, despite much biochemical and reverse genetic experimentation (Rasmusson and Møller, 1991; Millar *et al.*, 1998), the physiological importance of these features is not entirely clear. The operation of the TCA cycle in the light is affected by a combination of at least two factors: the reversible inactivation of the mitochondrial pyruvate dehydrogenase complex in the light (Budde and Randall, 1990) and the rapid export of TCA cycle intermediates out of the mitochondria (Hanning and Heldt, 1993; Atkin *et al.*, 2000), for utilization in glutamate synthesis (Hodges, 2002). However, the mitochondrial electron transport chain continues to be active, despite the limitations that the above modifications must impose on the TCA cycle, irrespective of illumination (Atkin *et al.*, 2000; Padmasree *et al.*, 2002; Yoshida *et al.*, 2006). Indeed a wide range of evidence has been accumulated that suggests mitochondrial function is not only active in illuminated leaves but is an integral part of photosynthetic metabolism. Much of the early evidence of a link between mitochondrial and chloroplastic function came from studies using specific inhibitors of the mitochondrial cytochrome and alternative respiratory pathways (Krömer and Heldt, 1991; Krömer *et al.*, 1993; Padmasree and Raghavendra, 1999a). However, several further lines of evidence support a considerable mitochondrial component within photosynthetic leaf metabolism.

These include the transgenic studies mentioned above, the observation of light-dependent expression of mitochondrial genes (Svensson and Rasmusson, 2001) and the decreased rate of photosynthesis, under photorespiratory conditions that was observed in the CMSII mutant of *Nicotiana sylvestris* which exhibits a loss of function of mitochondrial complex I (Dutilleul *et al.*, 2003). However, the operation of the TCA cycle in the light remains somewhat contentious with recent studies in broad bean suggesting an almost complete cessation of TCA cycle activity in the light (Tcherkez *et al.*, 2005).

As stated by Krömer (1995) - in her earlier review on the subject - the direct determination of respiratory oxygen uptake of carbon dioxide release is a complicated task due to the multiplicity of oxygen liberating/ consuming and carbon dioxide liberating/ consuming reactions in the leaf. For this reason gas exchange measurements have classically been used in conjuncture with radiolabel studies and mass spectrometry to differentiate rates of evolution and fixation of these gases. Using such approaches the degree of dark respiration in the light has been estimated from carbon dioxide release to be around 25 and 100% of the respiratory rate in darkness (Sharp *et al.*, 1984; Kirschbaum and Farquhar, 1987; Cashin *et al.*, 1988; Rébeillé *et al.*, 1988; Avelange *et al.*, 1991). Whereas some reports based on oxygen consumption suggest an inhibition of respiration in the light (Canvin *et al.*, 1980; Bate *et al.*, 1988), others suggest that the rate of respiration was invariant irrespective of illumination (Gerbaud and Andre, 1980; Peltier and Thibauld, 1985; Weger and Turpin, 1989). Analysis of microarray data sets show a clear trend of reduced expression in the light of genes associated with respiratory processes (Urbanczyk-Wochniak *et al.*, 2005; 2006; Bläsing *et al.*, 2005), however it should be noted that this does not necessarily imply a down-regulation in the flux through the pathway. A recent study by Tcherkez *et al.* (2005) studied the metabolism of ^{13}C enriched glucose or pyruvate by leaves of French bean by determining the $^{13}CO_2$ production in the light. Using differential positional ^{13}C-enrichments these authors estimated that flux through the TCA cycle is reduced by 95%, in the light, whereas the pyruvate dehydrogenase reaction is much less reduced by only 27% or so. Given that the inhibition of the pyruvate dehydrogenase complex is particularly well characterised (Budde and Randall, 1990; Tovar-Mendez *et al.*, 2003), this result is at first sight rather surprising. However, it should be borne in mind that in addition to inhibition of pyruvate dehydrogenase, many of the dehydrogenase enzymes of the TCA cycle would also be expected to be inhibited due to the high mitochondrial NADH level as a consequence of photorespiratory glycine

decarboxylation (Atkin *et al.*, 2000). In a similar vein, experimental evidence has been provided that the mitochondrial isocitrate dehydrogenase is inhibited by the high NADPH/NADP ratios that occur in the light (Igamberdiev and Gardeström, 2003).

Despite these reasons to expect TCA cycle flux to be inhibited in the light, the extent of the reduction reported by Tcherkez and co-workers seems to be somewhat contrary to observations of considerable labelling of organic and amino acids following incubation of illuminated isolated leaf discs of a range of species with $^{14}CO_2$ (Zeeman and ap Rees, 1999; Lytovchenko *et al.*, 2002; Nunes-Nesi *et al.*, 2005a). It is apparent that there is considerable flux to organic acids and amino acids (the biosynthetic precursors of many of which emanate from the TCA cycle), in the light. Moreover, transgenic tomato plants displaying reduced expression of the mitochondrial malate dehydrogenase exhibited a significantly reduced flux to organic and amino acids implicating this enzyme and by extension the TCA cycle, in the synthesis of these compounds in the light. These data and the clear phenotypes produced by the mutation or antisense inhibition of enzymes of the TCA cycle (Carrari *et al.*, 2003; Nunes-Nesi *et al.*, 2005a), as well as studies utilising specific inhibitors of the TCA cycle (Vani *et al.*, 1990), suggest that flux through the TCA cycle is indeed of considerable biological importance during photosynthesis. The exact reason for the lack of consensus between different estimates of TCA cycle flux in the light is unclear. Whilst it is possible that the study of Tcherkez *et al.* (2005) underestimated the flux through the TCA cycle by using a method that did not facilitate the acknowledgement of dilution factors, it is equally possible that the differences in light inhibition of dark respiration are species dependent. However, given the body of evidence suggesting a biologically significant operation of the TCA cycle in the illuminated leaf the exact value of the flux through the pathway is relatively academic. For this reason, in the rest of this **Chapter 1** I will focus on addressing the question – what is (are) the major function(s) of the TCA cycle in the illuminated leaf? Five possibilities are discussed: (i) the provision of ATP to support photosynthetic sucrose synthesis, (ii) the export of carbon skeletons and reducing equivalents required for nitrate reduction in the cytosol, (iii) oxidation of reducing equivalents exported from the chloroplast, (iv) the export of reducing equivalents required for hydroxypyruvate reduction in the peroxisomes and (v) a role in energy metabolism *per se* of the illuminated leaf.

1.4. The provision of mitochondrial ATP to support photosynthetic sucrose synthesis

It has previously been suggested that light may exert an inhibitory effect on the mitochondrial electron transport chain via the cytosolic adenylate pool (Heber and Heldt, 1981). However the cytosolic ATP/ADP ratio is actually lower in the light during steady-state photosynthesis at saturating carbon dioxide concentration than it is in the dark (Gardeström, 1987; Hampp *et al.*, 1982; Stitt *et al.*, 1982), and it is probable that even conditions that increase the cytosolic ATP/ADP ratio are unlikely to affect the respiratory oxygen uptake (Dry and Wiskich, 1982). However, given that the cytosolic ATP pool in illuminated leaves can, in principal, be sustained either by photosynthetic ATP synthesis or by oxidative phosphorylation, it is often reported that a primary function of the TCA cycle operation during the day is the production of ATP to support cytosolic sucrose synthesis. Several lines of evidence offer strong support for the theory that mitochondria supply a large portion of the cytosolic ATP. First, plant mitochondria possess a highly active ATP: ADP translocator that has been characterised to efficiently exports matrix produced ATP to the cytosol (Heldt, 1969) Secondly, specific inhibition of the mitochondrial ATP synthase by oligomycin decreases cytosolic ATP/ ADP ratio in barley leaf protoplasts by approximately 60% (Krömer and Heldt, 1991; Krömer *et al.*, 1993), whereas aminoacetonitrile which inhibits photorespiratory conversion of glycine to serine and thus impairs oxidative phosphorylation caused a decreased cytosolic ATP/ ADP ratio by 25 and 45% at limiting and strictly limiting carbon dioxide concentrations, respectively (Gardeström and Wigge, 1988). That this supply of mitochondrially derived ATP is necessary for sucrose biosynthesis is, however, not so clear. Received wisdom postulates that cytosolic sucrose synthesis is highly dependent on the supply of UTP which could be met by the conversion of mitochondrially produced ATP via the action of a cytosolic nucleoside-5`-diphosphate kinase (NDK; **Figure 1.2**). Based on kinetic arguments it can be hypothesized that a drop in mitochondrial ATP synthesis would have knock-on effects mediated via NDK on UTP levels and consequently on UDPglucose pyrophosphorylase (UGPase) and sucrose phosphate synthase (SPS) activity. Experimental evidence for such a scenario is available: inhibition of oxidative phosphorylation has been demonstrated to result in a decrease of both activity and activation state of SPS (Krömer *et al.*, 1993). Moreover, at least one genetic experiment is in keeping with this theory since when oxidative phosphorylation was inhibited in both the wild type and a starch-less mutant of

Nicotiana sylvestris the mutant, but not the wild type, was impaired in photosynthetic activity. This result indicated that in the wild type the cytosolic ATP level did not limit sucrose synthesis, but it did in the mutant which required an elevated rate of sucrose synthesis to compensate for the block in starch synthesis in order to maintain comparable rates of photosynthate usage (Hanson, 1992).

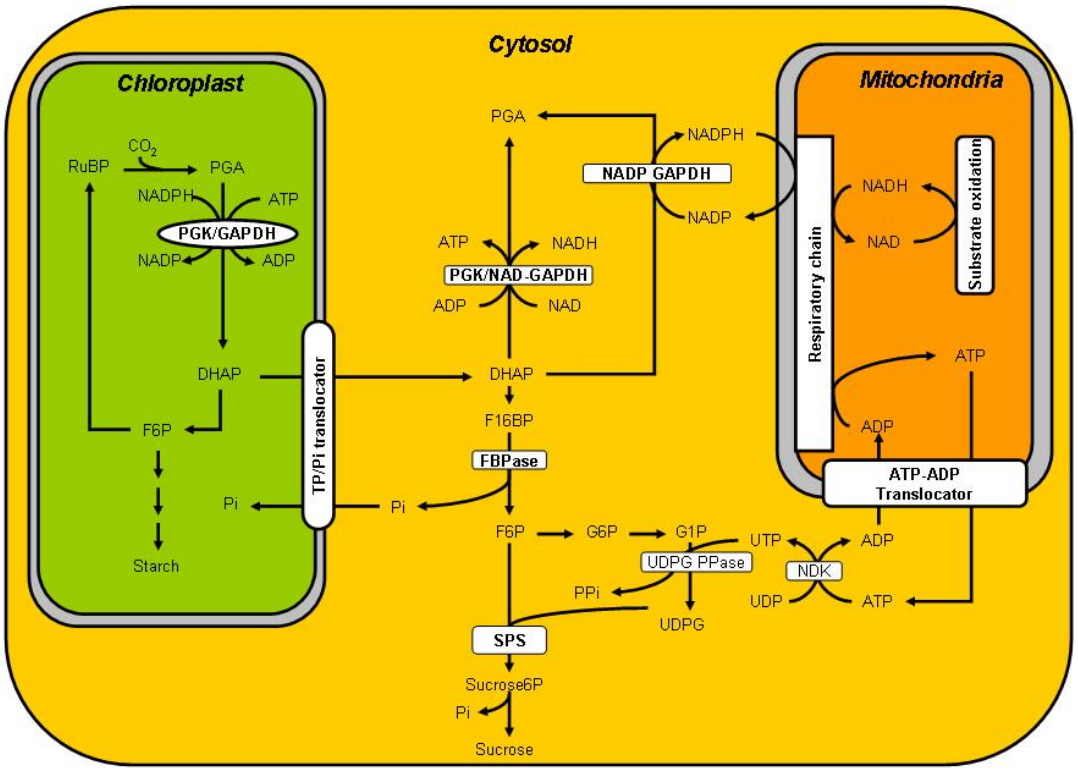

Figure 1.2 Suggested interactions between chloroplasts and mitochondria in the light. NDK, nucleotide-5'-diphosphate kinase; UDPG PPase, UDPglucose pyrophosphorylase; FBPase, fructose 1,6-bisphosphatase; RuBP, ribulose 1,5-bisphosphate; F6P, fructose 6-phosphate; G1P, glucose 1-phosphate; F16BP, fructose 1,6-bisphosphate; F26BP, fructose 2,6-bisphosphatase; UDPG, UDPglucose pyrophosphorylase; PPi, pyrophosphate; TP triose phosphate; Pi, inorganic phosphate. Adapted from Krömer (1995).

However, other genetic interventions seem to contradict this scenario. For example, reduction of the flux through the TCA cycle in the *Aco*1 mutant of *Solanum pennellii* resulted in a considerable decrease in the cellular ATP/ ADP ratio but an enhanced rate of photosynthetic sucrose synthesis (Carrari *et al.*, 2003).

This finding in particular suggests that the production of ATP in the mitochondria is not a prerequisite for the maintenance of high rates of photosynthetic sucrose synthesis and suggests that further experimentation is required to clarify this point. More recently, additional experiments to determine the function of respiration in photosynthetic metabolism have suggested that it is not merely to improve sucrose metabolism but also to modulate the flow of metabolites related to redox status (Dutilleul *et al.*, 2005; Scheibe *et al.*, 2005; Padmasree *et al.*, 2002; Igamberdiev *et al.*, 1998; Padmasree and Raghvendra, 1999b).

1.5. The export of carbon skeletons and reducing equivalents required for nitrate reduction in the cytosol

Nitrate assimilation requires the reduction of nitrate to nitrite in the cytosol, reduction of this nitrite to ammonium in the plastid and the subsequent assimilation of the nitrogen from ammonium into amino acids by the activities of glutamine synthase (GS) and glutamate synthase (GOGAT) in the plastid and/or cytosol (**Figure 1.3**). Since plant mitochondria mainly export citrate (Hanning and Heldt, 1993) and given that there are cytosolic isoforms, or at least localisations, of the enzymes required for the conversion of citrate to 2-oxoglutarate (Chen and Gadal, 1990; Gálvez *et al.*, 1999; Carrari *et al.*, 2003; Abiko *et al.*, 2005) as well as an efficient mechanism of plastidial 2- oxoglutarate uptake (Renné *et al.*, 2003) it is frequently postulated that these reactions are involved in nitrate assimilation (Gálvez *et al.*, 1999; Fernie *et al.*, 2004a). This theory is increasingly supported by an accumulation of experimental evidence ranging from the fact that the regulatory properties of the pyruvate dehydrogenase complex would allow it to operate in support of such a pathway even in conditions where the rest of the TCA cycle were non-operational (Schuller and Randall, 1989; Gemel and Randall, 1992; Krömer, 1995). Several other lines of correlative evidence also support such a role for the TCA cycle (Fieuw *et al.*, 1995; Scheible *et al.*, 1997; Stitt, 1999; Masclaux *et al.*, 2000); however, there are also many experimental observations that appear to contradict a role for the TCA cycle in nitrate assimilation (Gálvez *et al.*, 1996; Kruse *et al.*, 1998; Lancien *et al.*, 1999). Given that this evidence is already critically assessed in an excellent review focussed on the metabolism of 2-oxoglutarate (Hodges, 2002), I will only detail the major points in passing here.

Importantly, the demand for 2-oxoglutarate needed for ammonium assimilation could be met either by cytosolic or mitochondrial isocitrate dehydrogenases or by aspartate amino transferases. Whilst, direct evidence exists for a role of the aspartate transferase in the form of a mutant of the cytoslic isoform of this enzyme exhibiting a retarded growth phenotype and altered aspartate biosynthesis in the light (Schultz *et al.*, 1998), such direct evidence does not exist for any of the isoforms of isocitrate dehydrogenase (Hodges *et al.*, 2003). That said this does not necessarily imply that there is no role for TCA cycle enzymes in nitrogen assimilation since the *Aco*1 mutant of *Solanum pennellii*, described above, exhibited dramatic shifts in amino acid contents that would suggest that the TCA cycle did indeed play such a role.

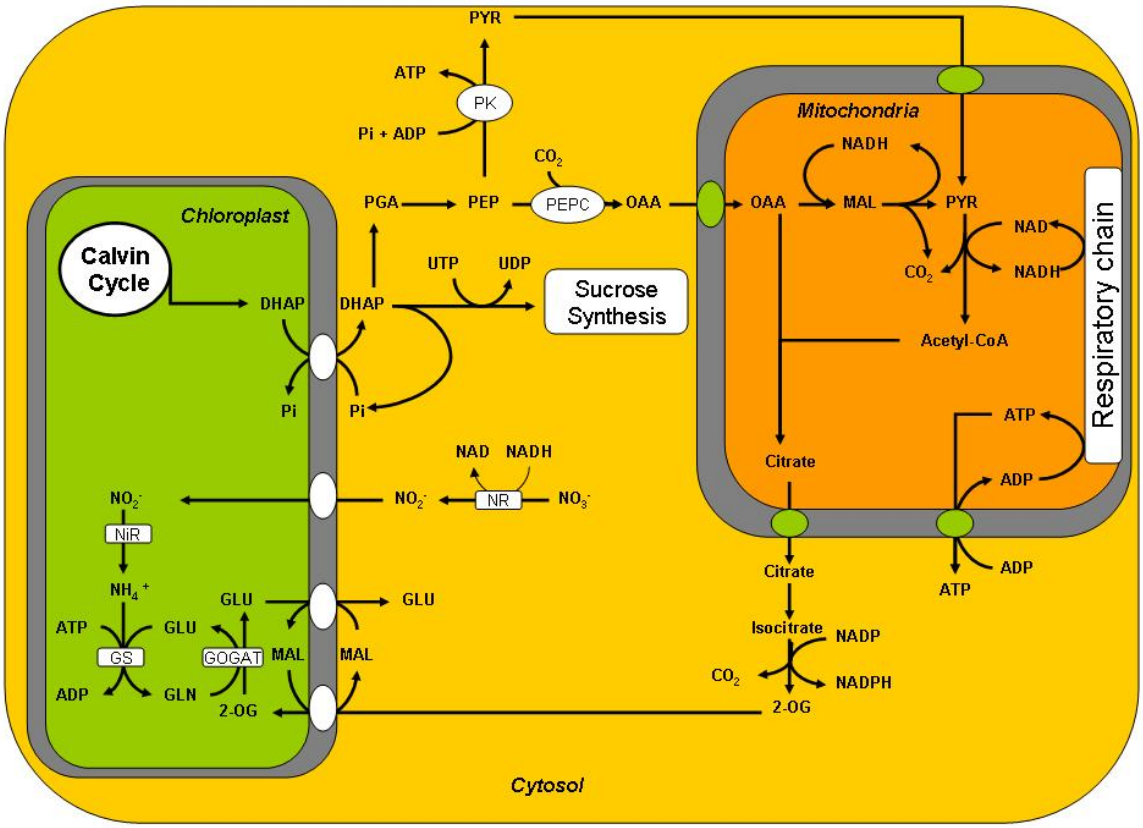

Figure 1.3 Suggested supply of carbon skeletons for photosynthetic nitrate assimilation. NR, nitrate reductase; NiR, nitrite reductase; Pyr, pyruvate; Mal, malate; glu, glutamate; gln, glutamine; GS, glutamine synthase; GOGAT, glutamate synthase; PK, pyruvate kinase; Pi, inorganic phosphate; PEPC, phosphoenolpyruvate carboxylase; DHAP, dihydroxyacetone phosphate; OAA, oxaloacetate; 2-OG, 2-oxoglutarate; GLU, glutamate; GLN, glutamine. Adapted from Krömer (1995).

The identification and preliminary characterisation of the isocitrate dehydrogenase isoforms of the Arabidopsis genome should facilitate reverse genetic analysis of the role of the various isoforms of isocitrate dehydrogenase (Hodges *et al.*, 2003). Such experiments would conceivably also clarify the localisation of the reactions catalysing the conversion of citrate to 2-oxogluterate, since the dual-targeting of aconitase to both cytosol and mitochondria (Millar *et al.*, 2001), renders such conclusions impossible to date.

1.6. Oxidation of reducing equivalents exported from the chloroplast

In addition to the putative role of the TCA cycle in the provision of carbon skeletons for nitrogen assimilation, it is also possible that the TCA cycle is a source of the reductant required for the nitrate- and nitrite-reductase reactions. The conversion of pyruvate to 2-oxoglutarate to provide the carbon skeletons will also generate 2 molecules of NADH. Substantial amounts of NADH are also produced in the mitochondrial matrix as a result of the activity of glycine decarboxylase of the photorespiratory pathway. A recent study of Arabidopsis seedlings that were re-supplied either nitrate or ammonium after a period of nitrogen starvation observed that when nitrate was re-supplied, there was a decrease in the expression of genes encoding specific proteins of the mitochondrial electron transport chain (Escobar *et al.*, 2006). It was suggested that this would restrict entry of NADH into the electron transport chain and promote export of reductant in the form of malate where it can be regenerated in the cytosol by cytosolic malate dehydrogenase.

In summary, the TCA cycle may be important both for the supply of carbon skeletons and reductant to support nitrogen assimilation. However, it is equally conceivable that other routes of carbon skeleton provision (e.g. via aspartate amino transferase) and cytosolic reductant formation operate and further direct genetic experimentation is required to resolve the precise role of the TCA cycle during nitrogen assimilation.

1.7. The export of reducing equivalents required for hydroxypyruvate reduction in the peroxisomes

The oxygenase activity of Rubisco produces phosphoglycolate under atmospheric conditions. In order to prevent wasteful loss of the carbon phosphoglycolate is converted to phosphoglycerate in the photorespiratory pathway.

The reactions of this pathway are spatially spread across three distinct subcellular compartments: the chloroplast, mitochondria and peroxisome (Raghavendra *et al.*, 1998; Padmasree *et al.*, 2002; Bykova *et al.*, 2005; **Figure 1.4**). Within the mitochondria matrix, two molecules of glycine are converted to one molecule of serine with the simultaneous evolution of carbon dioxide and ammonium and the production of NADH. Thus 75% of the carbon in phosphoglycolate molecule is reintroduced into metabolism by the operation of this cycle. Oxidation of NADH produced upon glycine oxidation occurs preferentially over that from other substrates such as malate or succinate (Bergman and Ericson, 1983; Dry *et al.*, 1983). These findings have lead to suggestions that protein complexes located in the vicinity of the complexes of the respiratory chain may give reducing equivalents formed from the oxidation of given substrates facilitated access to the respiratory chain (Krömer, 1995). Recent evidence in support of such a theory has been supplied by the identification using proteomics of supercomplexes consisting of components of the electron transport chain and biosynthetic enzymes (Millar *et al.*, 2005).

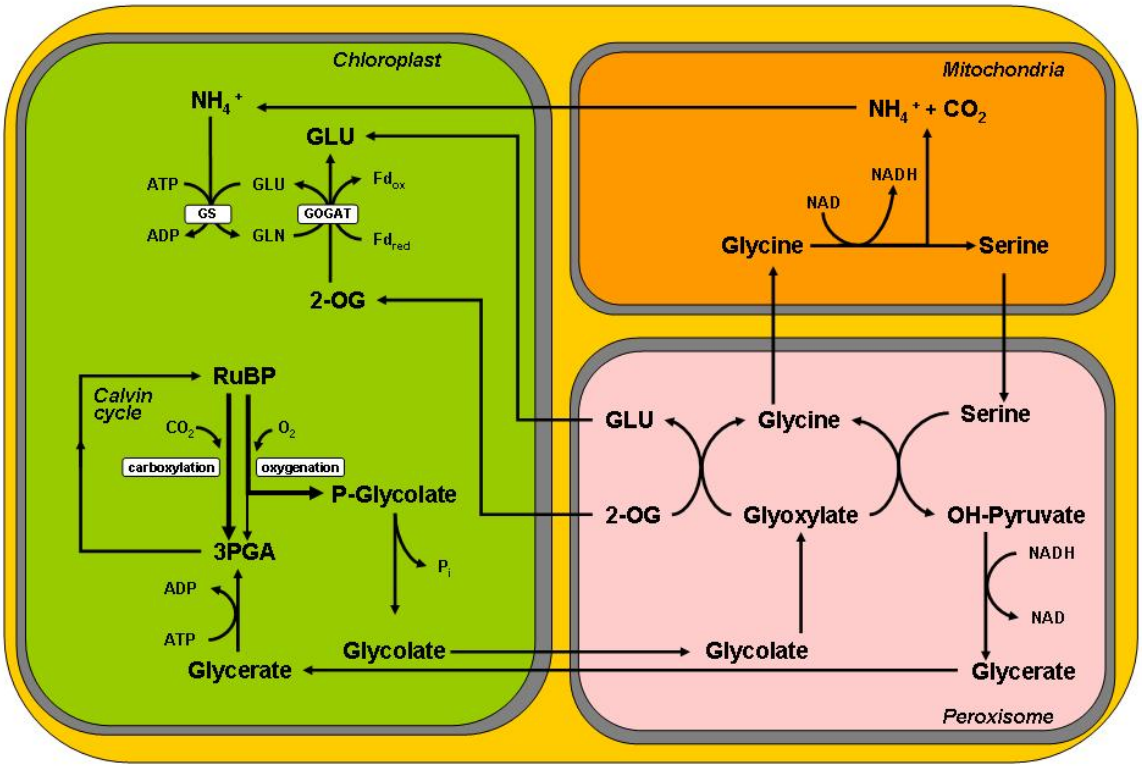

Figure 1.4 The involvement of the mitochondria in the process of photorespiration. Adapted from Hoefnagel *et al.* (1995).

Irrespective of how mitochondria prioritise the oxidation of glycine, the further operation of the photorespiratory cycle requires the delivery of redox equivalents to the peroxisomal matrix for the reduction of hydroxypyruvate. The peroxisomal membrane has porins which allow the diffusion of NADH. However, cytosolic concentrations of NADH (Heineke *et al.*, 1991) would only support 1% of the photorespiratory flux (Reumann *et al.*, 1994). An alternative source of NADH could be the peroxisomal oxidation of malate. Considering the malate is present in concentrations that are ten-fold higher than OAA (Heineke *et al.*, 1991) and that the activity of peroxisomal malate dehydrogenase is sufficient to catalyse observed photorespiratory fluxes (Heldt and Flügge, 1987) suggests that in the illuminated leaf under photorespiratory conditions the demands of the peroxisomal hydroxypyruvate for reductant are met by internal oxidation of malate and not by import of NADH (Reumann *et al.*, 1994). The NADH requirement of peroxisomal hydroxypyruvate reductase is equivalent to the NADH production on glycine oxidation in the mitochondrial matrix leading to the proposal that the NADH produced in the mitochondria is utilised in the peroxisome (Journet *et al.*, 1981). However, given that the export of only 25-50% of the redox equivalents produced in the mitochondrial matrix is insufficient to sustain hydroxypyruvate reduction another source of NADH is required. Experimental evidence suggests that the activity of the malate-OAA shuttle in the chloroplastic envelope is high enough to support this reaction (Hatch *et al.*, 1984) and export of chloroplastic malate is thought to occur to maintain photosynthetic redox balance (Scheibe *et al.*, 2005). It seems likely that both chloroplasts and mitochondria simultaneously allocate reductant to the peroxisomes, although the contribution of the two sources remains to be quantified (Krömer and Scheibe, 1996). Despite the fact that all of the genes encoding the enzymes of the photorespiratory pathway have now been cloned and functionally characterized (Boldt *et al.*, 2005), there remain many gaps in our understanding of the functioning of this crucial pathway. Even fundamental aspects such as the nature of the metabolite shuttles which facilitate it remain unanswered to date (Linka and Weber, 2005; Taira *et al.*, 2004). Once such questions have been answered it will be far easier to assess the importance of mitochondrial function in general, and the mitochondrial TCA cycle in particular, within the photorespiratory process.

1.8. A role in energy metabolism per se *of the illuminated leaf*

Improving plant productivity is now of unprecedented importance given the twin problems of environmental deterioration and the world population explosion (Miyagawa *et al.*, 2001). Whilst many successful manipulations of agronomic yield have involved the modification of sink metabolism (Stark *et al.*, 1992; Regierer *et al.*, 2002; Fridman *et al.*, 2004; Geigenberger *et al.*, 2005; Davuluri *et al.*, 2005), perhaps the major determinant of crop growth and yield is photosynthetic carbon metabolism (Galtier *et al.*, 1993; Sweetlove *et al.*, 1998; Miyagawa *et al.*, 2001; Schauer *et al.*, 2006). However, to date very few molecular approaches aimed at improving the efficiency of these pathways have proven successful. Those strategies that did work generally involved expression of cyanobacterial enzymes in higher plants. The expression of Fru-1,6 sedoheptulose/1,7-bisphosphatase (Miyagawa *et al.*, 2001) in tomato (*S. lycopersicum*); *ict*B, a gene involved in HCO_3^- accumulation in tobacco (*Nicotiana tabacum*); and Arabidopsis (Lieman-Hurwitz *et al.*, 2003) resulted in increased photosynthetic rates and growth under normal and limiting carbon dioxide concentrations, respectively. A recent report showed that overexpression of the gene *ots*A from *Escherichia coli*, encoding trehalose phosphate synthase, resulted in elevated rates of photosynthesis and growth in tobacco leaves (Pellny *et al.*, 2004). Interestingly, however, none of the successful strategies described above has been the result of alterations in the levels of the endogenous plant proteins. As mentioned above, as part of an ongoing project to determine the function of the TCA cycle in the illuminated leaf, previously the tomato wild species (*Solanum pennellii*) mutant *Aco*1, which exhibits a deficiency in expression of one of the two isoforms of aconitase present in the tomato was comprehensively phenotyped (Tanksley *et al.*, 1992; Carrari *et al.*, 2003). Biochemical analyses of the *Aco*1 mutant revealed that it exhibited a decreased flux through the TCA cycle and decreased levels of TCA cycle intermediates but was characterized by elevated adenylate levels and an increased rate of photosynthesis (carbon dioxide assimilation). In addition, although it must be taken into account that *S. pennellii* is a green fruited species bearing very small fruits (Schauer *et al.*, 2005) these plants were characterized by a dramatically increased fruit weight. In order to test the generality of this effect plants exhibiting a reduced expression of the mitochondrial isoform of malate dehydrogenase were generated and characterised (Nunes-Nesi *et al.* 2005a). These studies revealed that transformants exhibiting significantly reduced activities of the malate dehydrogenase also show enhanced photosynthetic activity and aerial growth under atmospheric conditions.

Accumulation of carbohydrates and redox regulated components such as ascorbate were also markedly elevated in the transformants. Interestingly, reanalysis of the GC-MS chromatograms of the *Aco*1 mutant revealed that this was also characterised by elevated ascorbate levels (Urbanczyk-Wochniak *et al.*, 2006). In contrast, metabolic profiling of other transgenic lines with different photosynthetic rates (e.g. lines expressing an acetyl CoA hydrolase which exhibited reduced rates of photosynthesis and a stunted phenotype) suggest that the linkage between ascorbate levels and photosynthetic rate is not universal (Bender-Machado *et al.*, 2004). Nevertheless, the altered photosynthetic rate in TCA cycle mutant and transgenic plants corroborates data from other researchers (Raghavendra and Padmasree, 2003) that suggests that the importance of the respiratory pathways in photosynthetic metabolism is greater than once imagined. That ascorbate levels are increased following a restriction of flux through the TCA cycle is fascinating particularly in light of recent reports demonstrating that the terminal enzyme of ascorbate biosynthesis - L-galactono-1,4-lactone dehydrogenase (GLDH) - is coupled to the cytochrome pathway (Bartoli *et al.*, 2000). Intriguingly, the malate dehydrogenase transformants exhibit an elevated capacity for the synthesis of ascorbate from L-galactono-lactone. Much is known concerning the importance of ascorbate within photosynthesis. Ascorbate acts in the Mehler peroxidase reaction with ascorbate peroxidase to prevent photo-oxidative stress and as a co-factor for violaxanthin deepoxidase, an enzyme involved in xanthophyll cycle involved in photoprotection (Smirnoff and Wheeler, 2000; Danna *et al.*, 2003). The ascorbate-redox state has also recently been shown to control guard cell signalling and stomatal movement (Chen and Gallie, 2004) as well as the expression levels of both nuclear and chloroplastic components of the photosynthetic apparatus (Smirnoff, 2000; Kiddle *et al.*, 2003; Pastori *et al.*, 2003). An additional function of ascorbate is as a co-factor for proxyl hydroxylase, an enzyme that post-translationally hydroxylates Pro residues in the cell wall Hyp-rich glycoproteins required for cell division and expansion (Smirnoff and Wheeler, 2000). The exact mechanism linking ascorbate to the rate of photosynthesis is currently unclear. However, experiments in which exogenous ascorbate was supplied to leaf discs prove that the photosynthetic assimilation is responsive to ascorbate levels and that this response is rapid (within 2h; Nunes-Nesi *et al.*, 2005a). Microarray analyses of the transgenic malate dehydrogenase line and of the *Aco*1 mutant revealed large-scale modification of the transcription of genes associated with both the Calvin cycle and the light reactions of photosynthesis (Urbanczyk-Wochniak *et al.*, 2006).

It is conceivable that the increase of photosynthesis occurs in compensation for the reduced flux through the TCA cycle in order to elevate chloroplastic energy and redox production. The broad *in vivo* effects of altering redox status as assessed by transcriptomics and metabolite profiling have recently been reported in Arabidopsis (Kolbe *et al.*, 2006). These, and recent proteomic studies (Balmer *et al.*, 2004) suggest that the role of redox in controlling metabolic and cellular processes may be even more widespread than previously thought. However, many more experiments will be required to clarify such a hypothesis. Not least of these will be to elucidate the precise mechanism by which ascorbate or any other mitochondrially-derived signal affects the rate of photosynthesis (**Figure 1.5**).

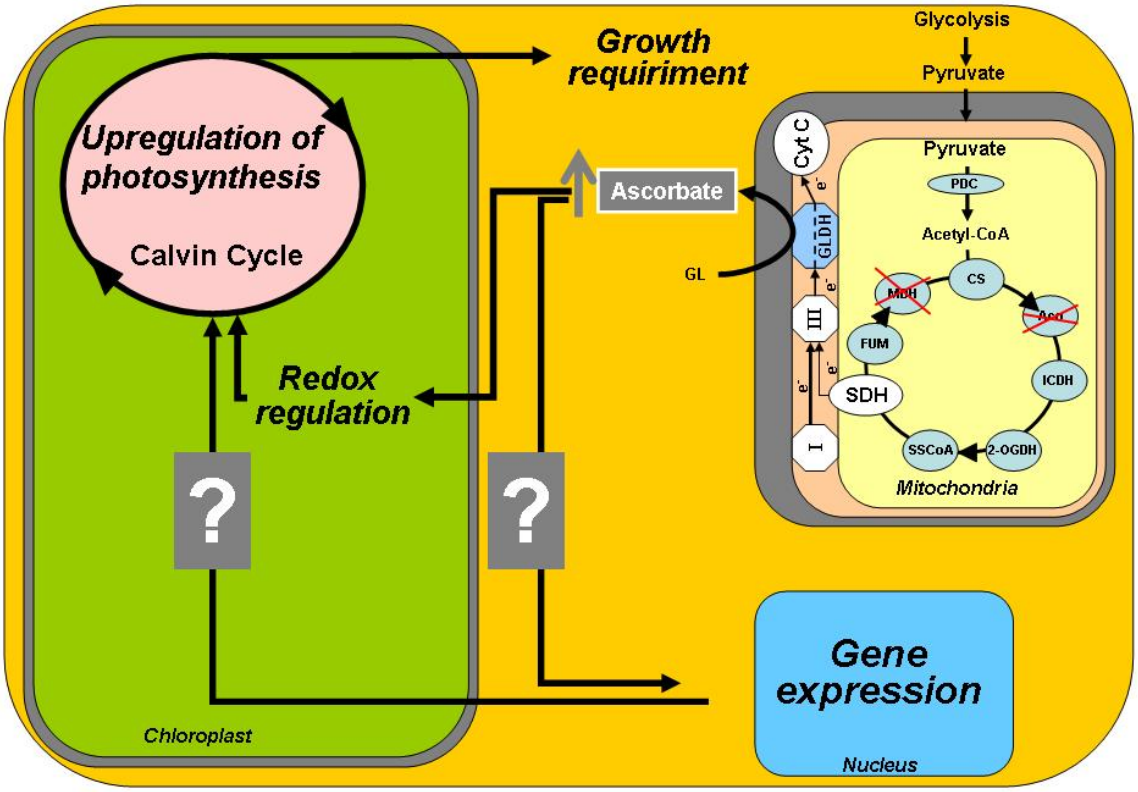

Figure 1.5 Schematic representation of the consequences of deficiency in either aconitase or mitochondrial malate dehydrogenase activity on chloroplastic photosynthesis. We hypothesize that the observed increased in vivo activity of GLDH (L-galactono-1,4-lactone dehydrogenase), which catalyses the conversion of galactono-lactone to ascorbate and is coupled to the mitochondrial electron transport chain leads to an up-regulation of photosynthesis by an as yet unknown mechanism involving either modulation of gene expression, redox regulation or merely efficient removal of photosynthate to support enhanced growth. SDH, succinate dehydrogenase; cyt c, cytochrome c; GL, L-galactono-1,4-lactone.

Positive effects of respiratory processes on photosynthesis are not confined to manipulation of the TCA cycle itself with many examples of the interlinking of the mitochondrial electron transport chain and the efficiency of photosynthesis (Krömer *et al.*, 1988; Padmasree *et al.*, 1999a; b; Bartoli *et al.*, 2005; Ishizaki *et al.*, 2005). Whilst current knowledge suggests several different mechanisms that may link mitochondrial and chloroplastic metabolism the most commonly postulated are redox homeostasis (such as described above for the mitochondrial malate dehydrogenase example) and the malate valve (Scheibe *et al.*, 2005; Scheibe, 2004). Suffice to say that given malate can be easily transported around the plant cell through recently identified transporters (Taniguchi *et al.*, 2002; Renné *et al.*, 2003; Emmerlich *et al.*, 2003) and that the mitochondria contain an effective capacity to use up excess reducing-equivalents produced by the chloroplast. In mediating the dissipation of these molecules from the chloroplast they are able to prevent thylakoid membrane damage (Gilmore, 1997; Niyogi *et al.*, 1998; Niyogi, 1999).

Mechanisms by which mitochondrial function facilitates optimum rates of photosynthesis under stress conditions has become the focus of much recent research in the field. One such example is provided by the recent finding that alternative oxidase expression is highly upregulated under drought conditions (Bartoli *et al.*, 2005). This upregulation provides an effective sink for reducing power, thereby preventing the accumulation of excess reducing equivalents and thereby decreasing the probability of loss of photosynthetic function. Similarly, mitochondria have been reported to oxidise excess photosynthetic reducing equivalents under cold, bright conditions (Raghavendra *et al.*, 1994; Saradadevi and Raghavendra, 1994; Hurry *et al.*, 1995; Atkin *et al.*, 2000). Moreover, the use of inhibitors of both cytochrome and alternative pathways produce a strong decrease in the rate of photosynthesis (Padmasree *et al.*, 1999a; b; Krömer *et al.*, 1988; 1993) as well as highlighting a major role of these pathways in protecting photosynthesis against photoinhibition (Saradadevi and Raghavendra, 1992). A recent study in broad bean using the same inhibitors revealed that inhibition of the alternative oxidase causes decreases in the rate of photosynthetic oxygen evolution even under low irradiances and suggest that this pathway provides chloroplasts with flexible strategies against photoinhibition (Yoshida *et al.*, 2006).

It has become increasingly apparent that the non proton-translocating mitochondrial electron transport proteins are upregulated during such stress conditions and moreover,

that this upregulation is driven by coordinated increases in transcript abundance (Clifton *et al.*, 2005). Meanwhile, strong evidence for the critical role of complex I was provided by the characterisation of cytoplasmic male sterile mutants lacking one of the subunit of complex I and displaying markedly reduced rates of photosynthesis (Sabar *et al.*, 2000; Dutilleul *et al.*, 2005). A final example which has recently come to light is the fact that the mitochondrial electron-transfer flavoprotein complex plays an important role in the degradation of chlorophyll during dark induced senescence (Ishizaki *et al.*, 2005). When taken together the multiplicity of mechanisms linking mitochondrial and plastidial (and cytosolic and peroxisomal) function serve to reinforce the high degree of metabolic coordination inherent in the plant cell. These examples furthermore highlight the essentiality of the mitochondria to plant photosynthetic metabolism. Intriguingly, the fact that modifying the mitochondrial electron transport chain has different effects on photosynthesis than modifying the TCA cycle further suggests that these effects of mitochondrial function are likely independent of one another.

1.9. Aim

The aim of this work was to characterize the enzyme succinyl Coenzyme A ligase and to investigate at different levels tomato plants exhibiting decrease in succinyl CoA ligase activity. For this, different strategies were followed: (I) isolation of tomato full-length cDNA corresponding to succinyl CoA ligase; (II) generation of transgenic tomato plants expressing succinyl CoA ligase α and β subunits in antisense or RNAi orientation and (III) biochemical and physiological analysis of the transgenic plants that showed decreased succinyl CoA ligase enzyme activity. Item (I) is discussed in Chapter 3 and describes also the primarily characterization of tomato succinyl CoA ligase; whilst items (II) and (III) are included in Chapter 4. Each of these chapters includes a short introduction and discussion making them independent entities; however at the end of the thesis, a general discussion summarizes the results in a more general context. These studies directed to provide further information concerning the function and regulation of the TCA cycle in the illuminated leaf of the tomato.