Use of mitochondrial electron transport mutants to evaluate the effects of redox state on photosynthesis, stress tolerance and the integration of carbon/nitrogen metabolism

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Abstract

Primary leaf metabolism requires the co-ordinated production and use of carbon skeletons and redox equivalents in several subcellular compartments. The role of the mitochondria in leaf metabolism has long been recognized, but it is only recently that molecular tools and mutants have become available to evaluate cause-and-effect relationships. In particular, analysis of the CMSII mutant of Nicotiana sylvestris, which lacks functional complex I, has provided information on the role of mitochondrial electron transport in leaf function. The essential feature of CMSII is the absence of a major NADH sink, i.e. complex I. This necessitates re-adjustment of whole-cell redox homeostasis, gene expression, and also influences metabolic pathways that use pyridine nucleotides. In air, CMSII is not able to use its photosynthetic capacity as well as the wild type. The mutant shows up-regulation of the leaf antioxidant system, lower leaf contents of reactive oxygen species, and enhanced stress resistance. Lastly, the loss of a major mitochondrial dehydrogenase has important repercussions for the integration of primary carbon and nitrogen metabolism, causing distinct changes in leaf organic acid profiles, and also affecting downstream processes such as the biosynthesis of the spectrum of leaf amino acids.

Key words: C/N metabolism, complex I, mitochondrial electron transport mutants, photosynthesis, redox state, stress tolerance.

Introduction

There is growing interest in the role of redox state in the orchestration of plant cell metabolism and in the determination of cell fate. Even in the soluble phase of the cell, ‘redox state’ can mean many things, because, under most conditions, the major soluble redox-active couples (e.g. pyridine nucleotides, thioredoxins, glutathione, ascorbate) are unlikely to be in equilibrium with each other (Noctor et al., 2000). Thus, consideration of the influence of redox state depends on the redox couple under discussion. For dehydrogenase activities, the major immediate influence will be the reduction state of NAD or NADP. For the regulation of protein function by thiol/disulphide exchange, the redox states of thioredoxins and/or glutathione are likely to be most important. Other important redox-active compounds that interact with these components are reactive oxygen species (ROS), and their rates of production and destruction. Growing evidence demonstrates that all the above elements are not only key to the control of metabolism but are also sensed to initiate changes in gene expression. The primary aim of this review is to discuss...
recent evidence derived from studies of the *Nicotiana sylvestris* mutant, CMSII, relating to the role of mitochondrial redox state in leaf metabolism and defence.

Leaf mitochondria play key roles in photosynthesis, e.g. glycine decarboxylation in C₃ plants and malate decarboxylation in certain C₄ plants. Moreover, leaf mitochondrial function will depend on whether the cells are photosynthetic or non-photosynthetic. Despite extensive work on purified leaf mitochondria and mitochondrial components (Douce and Neuburger, 1989; Douce et al., 2001; Möller and Rasmusson, 1998; Möller, 2001), much remains to be elucidated concerning the role of the mitochondrial electron transport chain in the integration of leaf metabolism (for reviews, see Krömer, 1995; Hoefnagel et al., 1998; Foyer and Noctor, 2000). The key questions addressed below are: How important is mitochondrial electron transport in the C₃ photosynthetic process? What is the significance of changes in mitochondrial redox state for leaf redox signalling and stress tolerance? Do changes in mitochondrial redox state impact on the co-ordination of carbon and nitrogen assimilation in leaves? An incisive approach to answering these questions is the analysis of plants in which key components of the mitochondrial electron transport chain have been manipulated genetically. Isolated in Orsay, the *Nicotiana sylvestris* mutant, CMSII, is one of the few such stable mutants characterized to date.

**Phenotypic and developmental characteristics of CMSII**

Numerous studies on the consequences of genetic modification of enzyme expression have emphasized the impressive flexibility of leaf metabolism. It might therefore be predicted that leaves possess considerable redundancy in redox exchange between subcellular compartments, and that the impact of non-lethal mutations would be absorbed by metabolic adjustments, preventing marked perturbations or phenotypic effects. The essential feature of CMSII is a deletion mutation in the mitochondrial gene, *nad7*, leading to the absence of functional complex I (Gutierres et al., 1997). Thus, the mutant has lost the function of a major NADH sink, resulting in partial male sterility, i.e. decreased production of pollen. Although the mutation also causes slower shoot growth during the vegetative phase, the final biomass attained by the mutant is not significantly different from the wild type (WT). It is therefore possible to analyse the two genotypes at comparable stages of shoot development, and thus to obtain information on the importance of mitochondrial electron transport status in leaf physiology and metabolism. A comparable stage of development is achieved by sowing CMSII seeds 3 weeks earlier than the WT. Figure 1 compares the morphology of CMSII with the WT at about 10 d prior to the stage at which sampling is performed.

**Respiratory activities of the CMSII mutant**

Despite the absence of complex I function, CMSII does not show decreased rates of leaf respiration in the dark, suggesting that TCA cycle activity is maintained in the mutant by complex II and alternative dehydrogenases that bypass complex I (Sabar *et al.*, 2000; Dutilleul *et al.*, 2003a). In mitochondria isolated from CMSII leaves, O₂ consumption is insensitive to rotenone and ADP/O values are significantly lower than in WT mitochondria (Sabar *et al.*, 2000). Flux to O₂ in mutant mitochondria is probably facilitated by the increased reduction state of mitochondrial NAD(P) pools, which allows the internal alternative dehydrogenases to be engaged (Möller, 2001). At first sight, therefore, the response of respiration to the loss of complex I seems to point to a further example of enzyme redundancy in plants. However, one of the principal themes of this article is to show that, even if respiration is not decreased when complex I is non-functional, the absence of this NADH sink has important consequences for leaf function, and necessitates numerous adjustments in leaf metabolism and gene expression that uncover the potentially critical influence of mitochondrial redox status.

A key observation in isolated CMSII mitochondria is that respiratory capacity relative to the WT is substrate-dependent (Sabar *et al.*, 2000). Gly metabolism is inhibited about 70% relative to the WT, whereas respiration of malate, in the presence of pyruvate, is unchanged (Sabar *et al.*, 2000). Subsequent work showed that, in the dark,
GDC (Douce et al., 2003a). These observations cannot be explained by a possible absence of catalytically active Gly decarboxylase (GDC). First, the mutant shows a pronounced burst of CO$_2$ evolution during the first minute following a light–dark transition and, second, Gly/Ser ratios in the light are not increased relative to the WT (Dutilleul et al., 2003a). Thus, the internal alternative NADH dehydrogenase (NDin) appears to be able to function with malate dehydrogenase and/or malic enzyme but much less efficiently with GDC, probably reflecting the conflicting kinetic properties of NDin and GDC: the $K_{	ext{m}}$ of NDin is about 5-fold higher than the $K_i$ of GDC (Douce et al., 2001; Bykova and Möller, 2001). Hence, in the absence of complex I and significant extra-mitochondrial reductant sinks (i.e. in isolated mitochondria or in leaves in the dark), the accumulation of NADH to a concentration required to engage NDin substantially is incompatible with optimal function of GDC.

Intercompartmental redox shuttles during photosynthesis

The role of mitochondrial matrix enzymes in C$_3$ photosynthesis is well established (Douce and Neuburger, 1989), and work with Arabidopsis and barley ‘photorespiratory’ mutants has demonstrated that GDC and serine hydroxymethyl transferase are indispensable for photorespiratory carbon and nitrogen cycling (Somerville and Ogren, 1981; Blackwell et al., 1990). The fate of the matrix NADH produced by GDC is less clear (Leegood et al., 1995; Hoefnagel et al., 1998; Foyer and Noctor, 2000; Gardeström et al. 2002). Studies in vitro led to the conclusion that the reducing equivalents are shared between the mitochondrial electron transport chain and exchange with the peroxisome via malate/oxaloacetate shuttles (Hanning and Heldt, 1993). If this is so in vivo, the mitochondrial electron transport chain must deal with a considerable flux of electrons from GDC and a large fraction of the reductant necessary for peroxisomal glyceralate synthesis must be produced elsewhere, presumably the chloroplast, via the malate valve (Hanning and Heldt, 1993; Hoefnagel et al., 1998; Scheibe, 2003). When one considers that the malate valve may also be coupled to the mitochondrial electron transport chain via cytosolic and mitochondrial redox shuttles, the picture that emerges is one of chloroplast and mitochondrial reductant export co-existing with peroxisomal and mitochondrial reductant import. Furthermore, in the cytosol, enzymes such asglyceraldehyde-3-phosphate dehydrogenase may also make a contribution to NAD(P)H production, whereas nitrate reductase (NR) acts as an electron sink. According to this view, the roles of the chloroplast and peroxisome in redox exchange in illuminated leaves are relatively clear. Stated simply, the former is a reductant exporter, the latter a reductant importer. Mitochondrial function in the light is a more complex question. Because the mitochondrial NADH/NAD ratio is maintained about 100-fold higher than the cytosolic ratio, Krömer and Heldt (1991) concluded that malate/oxaloacetate exchange must operate in the direction of net reductant export from the mitochondria, and that mitochondrial oxidation of NAD(P)H produced in the cytosol probably occurs through external dehydrogenases (reviewed by Möller, 2001).

In the leaves of most C$_3$ species, photosynthesis (and, in many conditions, the accompanying process of photorespiration) can be very much faster than dark respiration. The light-dependent photorespiratory pathway complicates the measurement of respiration in the light, though most data indicate that non-photorespiratory CO$_2$ evolution is significantly inhibited in the light relative to the dark, whereas inhibition of mitochondrial O$_2$ consumption is less apparent (Avelange et al., 1991; Pärnik and Keerburg, 1995; reviewed by Krömer, 1995; Hoefnagel et al., 1998). Thus, under many conditions, mitochondrial electron transport activity in the light is probably maintained, in part, by processes other than ‘dark’ respiration. What is the predicted effect of photorespiratory Gly oxidation on total mitochondrial electron transport activity? Given that fluxes of carbon into glycollate approach the rate of net photosynthesis under physiological conditions, it might be considered that when C$_3$ photosynthesis is rapid, significant partitioning of reductant from GDC activity to the mitochondrial electron transport chain would entail much higher respiratory electron transfer rates than those operating in darkened leaves. Measurements of respiratory O$_2$ consumption during active photorespiration are technically difficult, notably because ribulose-1,5-bisphosphate oxygenation and associated glycollate oxidation account for most of the observed gross O$_2$ uptake (Rey and Peltier, 1989). Nevertheless, it can be inferred that even during high rates of photosynthesis in air, a substantial fraction of Gly oxidation could be coupled to the mitochondrial electron transport chain without a huge increase in overall electron transfer rates relative to those that are commonly observed in darkened leaves (Table 1). This is because (1) only half the Gly produced must be oxidized, (2) at least part of the reducing equivalents is exported from the mitochondrion, and (3) tricarboxylic acid (TCA) cycle activity in the light is probably significantly less than in the dark (Pärnik and Keerburg, 1995). Nevertheless, the values given in Table 1 suggest that significant coupling of Gly decarboxylation to the mitochondrial chain would cause some increase in overall electron transport rates. An attractively simple mechanism, involving inhibition of pyruvate dehydrogenase by photorespiration-stimulated protein phosphorylation (Gemel and Randall, 1992), has been identified that could explain how, in the light, mitochondria in photosynthetic cells substitute GDC activity for TCA cycle
Table 1. Predicted effect of photorespiration on mitochondrial electron transport rates in the photosynthetic cells of C₃ leaves

The data compare typical rates of leaf O₂ consumption in the dark with maximum and likely rates of mitochondrial O₂ consumption at moderately fast rates of photosynthesis. It is assumed that the ratio of ribulose-1,5-bisphosphate (RuBP) carboxylation:oxygenation is 2.5, which is a likely value for non-stressed C₃ leaves (Keys, 1999); and that ‘dark’ respiration is inhibited 50% by light (see text for references). Maximum [G] and likely [I] rates of Gly-linked O₂ consumption in the light assume that Gly oxidation is coupled either 100% [G] or 40–60% [I] to the mitochondrial electron transport chain. The basic equation for calculating Gly-linked O₂ consumption by the electron transport chain can be represented by 2 Gly + 0.5 O₂ → Ser + CO₂ + NH₃. For purposes of simplicity, O₂ consumption that could be linked to export of reductant from the chloroplast is not considered. For further discussion, see text.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Derivation</th>
<th>Value (µmol m⁻² s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[A] ‘Dark’ respiration (in dark)</td>
<td>Typical leaf respiration rate</td>
<td>1</td>
</tr>
<tr>
<td>[B] ‘Dark’ respiration (in light)</td>
<td>0.5 [A]</td>
<td>0.5</td>
</tr>
<tr>
<td>[C] RuBP carboxylation</td>
<td>Moderately fast C₃ photosynthesis</td>
<td>25</td>
</tr>
<tr>
<td>[D] RuBP oxygenation</td>
<td>[C] / 2.5</td>
<td>10</td>
</tr>
<tr>
<td>[E] Glycine oxidation</td>
<td>0.5 [D]</td>
<td>5</td>
</tr>
<tr>
<td>[F] Net CO₂ uptake</td>
<td>[C] – ([B] + [E])</td>
<td>19.5</td>
</tr>
<tr>
<td>[G] Maximum Gly-linked O₂ consumption</td>
<td>0.5 [E]</td>
<td>2.5</td>
</tr>
<tr>
<td>[H] Maximum total mitochondrial O₂ consumption in light</td>
<td>[B] + [G]</td>
<td>3</td>
</tr>
<tr>
<td>[I] Likely Gly-linked O₂ consumption in light</td>
<td>0.4 [G] to 0.6 [G]</td>
<td>1 to 1.5</td>
</tr>
<tr>
<td>[J] Likely total O₂ consumption in light</td>
<td>[B] + [I]</td>
<td>1.5 to 2</td>
</tr>
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Complex I is required for optimal CO₂ fixation under physiological conditions

Much of the data available on the role of mitochondrial electron transport in photosynthesis has been generated by inhibitor treatment of leaf pieces or protoplasts (Krömper et al., 1988, 1993; Igamberdiev et al., 1997, 1998; Padmasree and Raghavendra, 1999). This and other work has pointed to several mechanisms by which mitochondrial electron transport and associated oxidative phosphorylation could be important in optimizing photosynthesis. First, mitochondrial ATP production could be crucial to support UDP-glucose formation for sucrose synthesis in the cytosol. Second, as discussed above, mitochondrial electron transport is probably responsible for the regeneration of part of the NAD used by GDC. Third, the mitochondria might act as the ultimate acceptors for reductant generated in the chloroplast and, therefore, prevent over-reduction of this organelle.

In the mutant, CMSII, non-function of complex I does not cause decreased photosynthetic capacity. At saturating CO₂ concentrations, where photorespiration is negligible, CMSII displays photosynthetic rates at least equal to the WT (Dutillleul et al., 2003a). Despite the clear restriction over Gly oxidation in CMSII mitochondria in the dark, in illuminated leaves the Gly/Ser ratio is not increased relative to the WT (Dutillleul et al., 2003a). Thus, though inhibited in the dark, Gly metabolism in CMSII is supported in the light by components other than complex I, presumably extra-mitochondrial reductant sinks. As discussed above, NDin is unlikely to be able to substitute completely for complex I, though a contribution from NDin to photorespiratory Gly metabolism cannot be excluded, given that (1) Gly oxidation is only 70% inhibited in isolated CMSII mitochondria (Sabar et al., 2000) and (2) NDin shows some evidence of light-induction in potato leaves (Svensson and Rasmusson, 2001).

Although light restores Gly metabolism in the mutant, this comes at a price for the overall photosynthetic process, suggesting that, in the WT, complex I plays a significant role in regenerating NAD for GDC activity. Carbon fixation is consistently decreased in the mutant by 20–30% in air (Sabar et al., 2000; Dutillleul et al., 2003a). Analysis of C₃ curves and O₂ sensitivity demonstrates that photosynthesis is less inhibited in CMSII, relative to the WT, under conditions where CO₂ fixation (and, therefore, sucrose synthesis) is enhanced. This suggests that the primary cause of decreased photosynthesis in CMSII in air is unlikely to be insufficient mitochondrial ATP production for sucrose synthesis (Dutillleul et al., 2003a). Even more striking than the decrease in steady-state photosynthesis is the effect on the photosynthetic induction period, which is greatly prolonged relative to the WT (Dutillleul et al., 2003a). From these data the following conclusions can be inferred. First, mitochondrial complex I is largely dispensable for photosynthesis when photorespiration is inactive, probably because, in this condition, C₃ photosynthesis is limited to chloroplastic events, accompanied by sucrose synthesis in the cytosol. Second, in air, active complex I plays an important role in the inter-compartmental redox cycling involved in C₃ photosynthesis (Fig. 2A). Third, when extra-mitochondrial sinks are
available in the light, Gly metabolism can continue in the absence of complex I, but greater demand for other reductant sinks impacts on redox cycling in the rest of the cell, decreasing the efficiency of the photosynthetic process (Fig. 2B). This last conclusion is supported by increased activation state of the chloroplast NADP-malate dehydrogenase in CMSII, suggesting that the reduction state of the stroma is increased relative to the WT (Dutilleul et al., 2003a). One interpretation of the inability of the mutant to deploy its photosynthetic capacity as efficiently as the WT is that the photosynthetic cell has a relatively narrow window of redox flexibility and that the mitochondrial electron transport chain plays an important part in setting the width of this window. Other components can compensate for the absence of complex I in maintaining Gly oxidation, but this lowers the rate of photosynthesis that can be achieved.

Mitochondria and leaf redox homeostasis: influence on diurnal regulation and the determination of stress resistance

The redox changes in CMSII are accompanied by induction or diurnal adjustment of antioxidant systems located within and outside the mitochondria (Dutilleul et al., 2003b). Although modulation of antioxidant expression is usually taken as an indicator of oxidative stress, modified expression in CMSII occurs despite the absence of any evidence of increased oxidative load or overall leaf flux to reactive oxygen species (ROS). In fact, the mutant shows decreased leaf H$_2$O$_2$ contents, both in the light, where mitochondrial H$_2$O$_2$ formation is probably a minor component of total leaf production, and in the dark, where the mitochondria are probably a major source of H$_2$O$_2$ (Dutilleul et al., 2003b). Associated with lower leaf H$_2$O$_2$ is significantly enhanced resistance to both biotic and abiotic stress, linked at least in part to a higher capacity of antioxidative enzymes (Dutilleul et al., 2003b). The increased availability of reductant, as discussed above, might also play a significant role in a more general up-regulation of defence metabolism that underpins observations that CMSII has a higher constitutive resistance than the WT to a broad spectrum of environmental constraints and stresses (authors’ unpublished results).

As for several other C$_3$ species, there are three genes encoding catalase in N. sylvestris, and the transcripts of these genes all vary throughout the day/night cycle. Whereas CAT1 is most strongly expressed in the dark, CAT2 and CAT3 transcripts peak in the light (Dutilleul et al., 2003b). These diurnal changes are enhanced in CMSII, as is the rhythm for cytosolic ascorbate peroxidase (cAPX) transcripts, which are highest towards the end of the photoperiod (Dutilleul et al., 2003b). In contrast to these transcripts, for which WT expression and/or light/dark rhythms are reinforced in CMSII, the diurnal pattern of alternative oxidase (AOX) expression is completely inverted in the mutant. At each point measured throughout the light/dark cycle, AOX transcripts are more abundant in CMSII than in the WT. However, AOX transcripts peak in the light in the WT but are more abundant in the dark in CMSII, so that the relative increase in AOX transcripts (CMSII/WT) is about 2-fold in the light but 10-fold or more in the dark (Dutilleul et al., 2003b). The expression of AOX has been shown to be inversely correlated with mitochondrial ROS availability in tobacco (Maxwell et al., 1999), and AOX capacity can play a role in determining cell fate in oxidative conditions (Robson and Vanlerbergh, 2002; Vanlerbergh et al., 2002). While the factors controlling AOX expression remain fully to be elucidated, it is possible that mitochondrial redox state, manifested either as the relative reduction of NAD(P) or of key electron transport components such as ubiquinone, is one of the principal determinants of AOX transcript abundance. Although the intermediaries that link changes in mitochondrial redox state to nuclear gene expression remain to be identified, the initial sensing could involve components such as mitochondrial thioredoxins (Laloi et al., 2001). Engagement (i.e. in vivo activity) of AOX protein is dependent on reductive activation, linked to the reduction status of mitochondrial pyridine nucleotide pools, probably either through the mitochondrial glutathione system or, more likely perhaps, NADPH-dependent reduction of mitochondrial isoforms of thioredoxin (Møller and Rasmusson, 1998; Vanlerbergh and Ordog, 2002). Effects on nuclear transcripts that result from mitochondrial redox sensing could be multifactorial in nature, with thioredoxin(s) perceiving the NADPH reduction state and other components such as ROS relaying information on the status of the electron transport chain itself.

Mitochondria-linked redox modulation impacts on the co-ordination of carbon and nitrogen assimilation

Co-ordination of carbon and nitrogen metabolism in leaves involves intricate cross-talk at the level of gene expression and enzyme activity (Stitt et al., 2002; Foyer et al., 2003). Complementarity and competition also occurs in the use of metabolites and energy, and the interlocking of C and N assimilation entails communication between several compartments of the leaf cell, including the chloroplast, the cytosol, and the mitochondrion (Noctor and Foyer, 1998). Comparison of foliar metabolite profiles in CMSII and the WT indicates that complex I dysfunction impacts strongly on the leaf C/N interaction (authors’ unpublished results). Thus, even though respiration rates in the dark are not decreased in the mutant, the relative amounts of metabolites involved in the TCA cycle are modified. Most strikingly, 2-OG, the carbon skeleton for the glutamine
Redox interactions in mitochondrial complex I mutants

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Key questions concern how such redox changes are sensed. First, by analogy to the chloroplast plastoquinone pool, the redox state of an electron transfer component such as ubiquinone could be important. Second, redox effects on leaf metabolism and function could be mediated by changes in pyridine nucleotide pools, which would directly influence dehydrogenase activities and feed downstream to influence processes located in other subcellular compartments, such as CO₂ fixation and ammonia assimilation. Third, effects could occur through modified protein function and/or gene expression mediated by protein thiol/disulphide exchange linked to thioredoxins and/or protein glutathionylation. Thiol-modulation of proteins is a well characterized mode of post-translational regulation, particularly in chloroplast metabolism, where thiol/dis-
ulphide exchange is linked to the flux of electrons through the electron transport chain. In the mitochondria, thioredoxin is reduced by NADPH, which is generated by enzymes such as NADP-dependent isocitrate dehydrogenase. Finally, modified mitochondrial ROS production might be one factor responsible for the observed changes in nuclear gene expression that result from the absence of a major NADH sink in complex I-deficient plants.

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