Respiration and nitrogen assimilation: targeting mitochondria-associated metabolism as a means to enhance nitrogen use efficiency

Christine H. Foyer1, Graham Noctor2,* and Michael Hodges2

1 Centre for Plant Sciences, Faculty of Biology, University of Leeds, Leeds LS2 9JT, UK
2 Institut of Biologie des Plantes, UMR8618 CNRS/Université de Paris-Sud 11, Bâtiment 630, Université de Paris-Sud 11, F-91405 Orsay cedex, France

* To whom correspondence should be addressed: E-mail: graham.noctor@u-psud.fr

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Abstract

Considerable advances in our understanding of the control of mitochondrial metabolism and its interactions with nitrogen metabolism and associated carbon/nitrogen interactions have occurred in recent years, particularly highlighting important roles in cellular redox homeostasis. The tricarboxylic acid (TCA) cycle is a central metabolic hub for the interacting pathways of respiration, nitrogen assimilation, and photorespiration, with components that show considerable flexibility in relation to adaptations to the different functions of mitochondria in photosynthetic and non-photosynthetic cells. By comparison, the operation of the oxidative pentose phosphate pathway appears to represent a significant limitation to nitrogen assimilation in non-photosynthetic tissues. Valuable new insights have been gained concerning the roles of the different enzymes involved in the production of 2-oxoglutarate (2-OG) for ammonia assimilation, yielding an improved understanding of the crucial role of cellular energy balance as a broker of co-ordinate regulation. Taken together with new information on the mechanisms that co-ordinate the expression of genes involved in organellar functions, including energy metabolism, and the potential for exploiting the existing flexibility for NAD(P)H utilization in the respiratory electron transport chain to drive nitrogen assimilation, the evidence that mitochondrial metabolism and machinery are potential novel targets for the enhancement of nitrogen use efficiency (NUE) is explored.

Key words: Carbon–nitrogen interactions, isocitrate dehydrogenase, mitochondria, nitrate assimilation.

Introduction

Whilst the pathways of primary nitrogen assimilation, respiration, and the associated process of photorespiration are well defined it is only since the application of reverse genetic strategies that the high level of interaction between them has begun to be fully appreciated (Bauwe et al., 2010; Sweetlove et al., 2010). However, in terms of whole cell energy status the exact contribution of respiration is dependent on cell type. Moreover, fundamental questions remain, particularly concerning the degree of inhibition of the tricarboxylic acid (TCA) cycle in the light. While TCA cycle flux is reduced in leaves in the light when compared to that seen in the dark, data obtained from enzyme measurements (Tovar-Mendez et al., 2003) and isotope labelling profiles (Tcherkez et al., 2005) are not easily reconciled with the results from transgenic plants (Fernie et al., 2004). The operation of different flux modes in the light has been suggested as a possible solution to this problem (Sweetlove et al., 2010).

Whilst the co-ordination of the pathways of primary nitrogen assimilation and respiration has been presumed for decades, our understanding of the precise details of how this is achieved remains incomplete. In tissues that lack photosynthesis such as roots, the reliance on mitochondrial oxidative phosphorylation to meet the energy demands of the cell, including nitrogen assimilation, greatly simplifies matters.
However, even such general assumptions may be challenged as it appears that some enzymes of the photorespiratory pathway could be expressed in a root-specific fashion (Nunes-Nesi et al., 2010). Moreover, while certain mitochondrial TCA cycle reactions are required to support cytosolic/vacuolar nitrate accumulation (Nunes-Nesi et al., 2007), a recent study has suggested a major role of night-stored molecules in providing 2-OG for glutamate synthesis in illuminated rape seed (Gauthier et al., 2010). Some of these issues might be resolved by genetic manipulation of individual enzymes of the TCA cycle, as discussed below. Whilst our understanding of related signal transduction cascades and associated retrograde signalling from the mitochondria to the nucleus remains fragmentary, there is increasing sophistication in the approaches that are being applied to gain a better resolution of metabolism and its regulation (Niittylä et al., 2009).

Increasing evidence demonstrates the importance of maintaining the cellular energy balance in the co-ordination of primary nitrogen assimilation, respiration, and photorespiration. For example, an Arabidopsis thaliana mutant deficient in the expression of the uncoupling protein AtUCP1, which is an integral component of the inner mitochondrial membrane, revealed a specific inhibition of photorespiration (Sweetlove et al., 2006). Moreover, the ucp1 mutants displayed dramatically reduced rates of carbon dioxide assimilation linked to a reduced rate of photorespiratory glycine oxidation (Sweetlove et al., 2006). Components of the mitochondrial electron transport chain such as the non-phosphorylating bypass proteins (alternative oxidases and internal NADH dehydrogenases) and the UCP therefore appear to be essential for the proper maintenance of intracellular redox gradients. In this context, uncoupling proteins might be engaged when the demand for oxidation of NADH is high, and so allow increased TCA cycle flux (Smith et al., 2004). Moreover, engagement of the respiratory alternative oxidase and the internal NADH dehydrogenases would provide mechanisms whereby electron transport could proceed without proton translocation and therefore could fulfil similar roles to the UCP. The following consideration of the complex interactions between respiration and nitrogen metabolism will therefore focus on aspects of respiratory metabolism and associated processes that generate biosynthetic precursors and also regulate the cellular energy balance in order to ensure an efficient appropriate allocation of essential resources. The review considers two key related aspects: first, current knowledge of the different enzymes that contribute the key C skeleton, 2-oxoglutarate (2-OG) to ammonia assimilation and, second, recent studies that have analysed how genetic manipulation of the TCA cycle and mitochondrial electron transport components impacts on N metabolism and related processes.

Respiratory production of carbon skeletons for amino acid synthesis

Ammonium can be produced by a number of metabolic pathways and enzymatic reactions, such as the reduction of nitrate to nitrite via the action of nitrate and nitrite reductases (NR, NiR), the action of glycine decarboxylase (GDC) during the photorespiratory cycle, and the degradation of proteins during senescence, to name but three. Irrespective of the ammonium-producing pathway, in plant cells, it is (re)assimilated into organic molecules mainly by the action of the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle, although it could also possibly be assimilated via glutamate dehydrogenase (GDH) (Ireland and Lea, 1999). In the first step of the GS/GOGAT cycle, ammonium is fixed to glutamate by GS and ATP is consumed (Fig. 1). Then, GOGAT takes a molecule of glutamine and makes two molecules of glutamate by a reaction that requires carbon skeletons in the form of the organic acid, 2-OG. This reaction also consumes a reductant, either reduced ferredoxin (Fd) via photosynthetic electron transfer or NADH depending on the form of GOGAT (Fd or NADH-GOGAT). In the case of GDH, ammonium is directly fixed to 2-OG to form glutamate and either NADH or NADPH are oxidized. However, GDH is not a major ammonium-assimilating route in plant cells but a stress/senescence-associated enzyme involved in the production of 2-OG from glutamate. By contrast, net ammonia assimilation requires a source of 2-OG. Current understanding suggests that this 2-OG is generated through the partial respiration of sugars or sugar-phosphates in which the final step is catalysed by isocitrate dehydrogenases (IDH). This section of the review discusses this small family of 2-OG-producing enzymes and their roles in N assimilation and in redox-stress related NADPH production.

IDH produces 2-OG and liberates CO2 by the oxidative decarboxylation of isocitrate (Fig. 1). This requires a pyridine nucleotide co-factor (NAD or NADP) and a divalent cation (e.g. magnesium or manganese). Plants contain two types of IDH that use different co-factors. One is NADP-dependent (NADP-IDH), while the other is NAD-dependent (NAD-IDH). Therefore, IDH activity also generates either NADPH or NADH. The NAD-IDH is located uniquely in the mitochondria of eukaryotic cells where it is part of the TCA cycle. On the other hand NADP-IDH is found in several cell compartments. Work in several species has established that these are the cytosol, plastids, mitochondria, and peroxisomes (Gálvez et al., 1994, 1996, 1998; Corpas et al., 1999). Their exact physiological function(s) are still under debate (see Gálvez et al., 1999; Lancien et al., 2000; Hodges, 2002; Hodges et al., 2003, for reviews).

Current knowledge of Arabidopsis genes encoding IDH is summarized in Fig. 2. NAD-IDH is heteromeric and each subunit (of approximately 35-37 kDa) is encoded by a different gene. In Arabidopsis thaliana, five NAD-IDH genes are expressed and by homology with the well-characterized yeast enzyme, where IDH1 encodes a regulatory subunit and the catalytic subunit is encoded by IDH2 (Keys and McAlister-Henn, 1990). Arabidopsis contains two genes for ‘catalytic’ and three genes for ‘regulatory’ subunits. In Arabidopsis rosettes, four of these genes (At4g35260, At2g17130, At5g03290, and At3g09810) are expressed while the fifth gene (At4g35650) is expressed mainly in pollen.
At least three NAD-IDH genes have been found in *Nicotiana tabacum* (tobacco), with a single gene encoding a 'catalytic' subunit and two genes encoding 'regulatory' subunits (Lancien et al., 1998). To obtain a catalytic activity, the plant NAD-IDH must contain at least one of each subunit type. This has been shown by the complementation of yeast IDH mutants (Lancien et al., 1998; Lemaitre et al., 2007), the production of recombinant tobacco IDH proteins in bacterial cells (Lemaitre, 2005), and the analysis of *Arabidopsis* knock-out mutants (Lemaitre et al., 2007). On the other hand, NADP-IDH is homomeric (composed of a 47 kDa subunit). In *Arabidopsis*, only three genes exist for four isoenzymes with the cytosolic and peroxisomal forms encoded by their own gene (At1g65930 and At1g54340, respectively) while a third gene (At5g14590) encodes both the mitochondrial and plastidial isoforms (McKinnon et al., 2009; Fig. 2). To date, the factors that regulate the proportion of each isoform within a given organelle are unknown.

In the literature two hypotheses have been put forward concerning the involvement of different IDH enzymes in GS/GOGAT functioning. It was proposed that mitochondria are the source of 2-OG, through NAD-IDH (Miflin and Lea, 1980). In this scenario, 2-OG would leave the mitochondria, probably via a dicarboxylate-tricarboxylate carrier named DTC (Picault et al., 2002), to be imported into the plastids, where the leaf GS/GOGAT cycle is located, by a transporter named DiT1 (Weber et al., 1995; Fig. 1). The second hypothesis involves the cytosolic NADP-IDH (Chen and Gadal, 1990). In this case, citrate is exported from the mitochondria (again by DTC) and transformed to isocitrate via an aconitase and then into 2-OG by the cytosolic NADP-IDH (Fig. 1). According to this second scenario, 2-OG of cytosolic origin would again be imported into the plastids via DiT1 in exchange for malate. A second transporter, DiT2, exports glutamate made in the plastid, again in exchange for malate (Renne´ et al., 2003). While it was used to be thought that the provision of carbon skeletons for the GS/GOGAT cycle...
could be dominated by one of these two routes, the data below will show that investigations over many years have failed to produce such evidence and that the principles governing the flux between the two pathways is still unclear.

NAD-IDH and ammonium assimilation

Are mitochondria the source of the 2-OG required for GS/GOGAT functioning? This organelle contains both NAD-IDH and NADP-IDH, but Miflin and Lea (1980) proposed that NAD-IDH is responsible for making 2-OG required for ammonium assimilation. To date, this role for NAD-IDH remains under debate since the TCA cycle enzyme is still poorly characterized.

The best characterized NAD-IDH is the yeast enzyme. Two genes encode the IDH1 and IDH2 subunits required for an active enzyme composed of four IDH1–IDH2 dimers (Panisko and McAlister-Henn, 2001). Its essential role in the TCA cycle was shown by the ‘acetate’ phenotype of yeast NAD-IDH mutants. However, such mutants were not glutamate auxotrophs due to the presence of the cytosolic and mitochondrial NADP-IDH enzymes (Zhao and McAlister-Henn, 1996). Mammalian NAD-IDH is also heteromeric and three subunits exist (α, β, and γ) with the α subunit being equivalent to the yeast IDH2 ‘catalytic’ subunit. Recombinant mammalian NAD-IDH composed of all three subunits showed the highest activity, although a combination of NAD-IDHα with one of the other two subunits was active (albeit 10-fold lower) (Kim et al., 1999). In plants, NAD-IDH shows a low measurable in vitro activity and the complex is unstable, thus hampering our understanding of its function in plant systems. As in the yeast and mammalian systems, plant NAD-IDH activity requires at least a ‘catalytic’ and a ‘regulatory’ subunit for enzymatic activity (Lancien et al., 1999).

Evidence for the role of NAD-IDH in ammonium assimilation is scarce. NAD-IDH transcript levels were found to increase when N-starved tobacco plants were resupplied with nitrate or ammonium. These changes were co-ordinated with the expression of other TCA cycle genes (citrate synthase and aconitase but not fumarase) and N-assimilatory genes (NR and GS). Since the response kinetics were correlated to the nitrate assimilatory capacity of each organ, it was proposed that the nitrate status or metabolites associated with its metabolism could be acting as signals (Lancien et al., 1999). NAD-IDH expression and localization have been investigated in rice roots with respect to N-nutrition and compared with those of NADH-GOGAT and GDH. Only IDHa and IDHc1 transcripts (encoding a catalytic and a regulatory subunit) responded to the different N-regimes while IDHc2 and GDH did not. Under ammonium nutrition, NAD-IDH protein co-localized with NADH-GOGAT in the epidermis and the exodermis, suggesting that it could play a role in N-assimilation (Abiko et al., 2005). Recently, tomato antisense lines with a reduced NAD-IDH activity of around 40% were shown to have a modified capacity to assimilate nitrate, as judged by an increase in leaf nitrate levels and reduced amino acid levels. The NAD-IDH mutants contained lower NADH (and NADPH levels) in the leaves, which could impact on NR activity. These mutants also exhibited a reduced TCA cycle flux, decreased starch levels, less chlorophyll, and lower organic acid amounts that suggest an N-limitation. However, 2-OG levels were not affected and plant growth was not significantly altered. Interestingly, the mutation did affect

Fig. 2. Genes encoding isocitrate dehydrogenase (IDH) in Arabidopsis and subcellular localization of the encoded proteins. For the six NAD-IDH genes, ‘pg’ indicates pseudogene, ‘r’ regulatory subunit, and ‘c’ catalytic subunit. At least one catalytic and one regulatory subunit are required to form a functional NAD-IDH.
tomato fruit yield. Although fruits were smaller and had a lower fresh weight, fruit number was not modified (Sienkiewicz-Porzucek et al., 2010).

A number of observations, however, argue against a mitochondrial NAD-IDH origin for the 2-OG used to fuel the GS/GOGAT cycle. NAD-IDH exhibits a low measurable activity in vitro and relatively high $K_m$ values for isocitrate (280–850 μM) and NAD (150–800 μM) (see Gálvez et al., 1999, for more details) when compared to NADP-IDH (see below). Furthermore, the enzyme is inhibited by NADH ($K_i$=70–420 μM) which could accumulate in leaf mitochondria in the light via the action of the photosrespiratory enzyme, GDC. However, this scenario may not take place because GDC is also inhibited by NADH ($K_i$=15 μM; Oliver, 1994). Of course, the in planta NAD-IDH activity is perhaps higher than that measured in vitro and NADH might not accumulate due to the need to channel reducing power to the cytosol and the peroxisomes via a malate/oxaloacetate shuttle driven by the activity of a dicarboxylate carrier as described by Palmieri et al. (2008). In illuminated leaves, TCA cycle decarboxylations can be reduced by 80% and the decarboxylation reaction of the pyruvate dehydrogenase is decreased by 30% when compared to dark respiration (Tcherkez et al., 2008). It is possible that this activity is not sufficient to make 2-OG for N-assimilation. However, in photosynthetic tissues, the activity of a complete TCA cycle appears to be reduced and a non-cyclic TCA pathway is probably more important in the light (illuminated leaves) due to the transport of organic acids out of the mitochondria (Tcherkez et al., 2009; Sweetlove et al., 2010). Recently, experiments using isotopic double-labelling ($^{13}$C/$^{15}$N) and NMR analyses have indicated that the carbon skeletons used for day N-assimilation originate from stored organic acids (probably malate or citrate) made during the night period (Gauthier et al., 2010).

The study of three Arabidopsis knock-out mutants for different NAD-IDH subunits (encoded by At5g03290, At4g325260, and At2g17130) also suggests that NAD-IDH is not essential in producing 2-OG for N-assimilation. Under standard, non-limiting growth conditions, the mutants, which had only 8%, 40% or 57% of normal NAD-IDH activity in their rosette leaves, did not show any growth or developmental phenotype. However, the mutants did contain reduced amounts of certain amino acids in their leaves but this did not include Glu and Glu. Interestingly, the content of NAD$^+$ and NADPH was reduced by the modified NAD-IDH activity thus indicating a perturbation in cell redox level (Lemaître et al., 2007).

### NADP-IDH and ammonium assimilation

Although an NADP-IDH is found in plastids (Gálvez et al., 1994), the principal site of ammonia assimilation, it was proposed by Chen and Gadal (1990) that the cytosolic isoform is important in producing the 2-OG for amino acid biosynthesis. As yet, no convincing evidence supporting this hypothesis has been reported in the literature although a number of observations have been interpreted in its favour. Cytosolic NADP-IDH is the major isoform in green leaves. For example, it accounts for 95% of the total tobacco leaf NADP-IDH activity (Gálvez et al., 1994). It has been shown to be the predominant NADP-IDH in 15 plant species (Chen, 1998) including tomato fruit (Gallardo et al., 1995), potato (Fieuw et al., 1995) and it is the only detectable activity in pine cotyledons (Palomo et al., 1998). These observations consolidate the idea that the cytosol has the capacity to be the predominant source of 2-OG in the leaf. All NADP-IDH isoforms have similar low $K_m$ values for isocitrate (11–80 μM) and NADP (4–16 μM) that reflect their highly conserved protein sequences (see Gálvez et al., 1999, for more details).

A number of data have linked cytosolic NADP-IDH to amino acid biosynthesis. An analysis of NR-deficient tobacco plants accumulating both nitrate and 2-OG, showed an increase in cytosolic NADP-IDH transcript levels and a number of N-assimilation and organic acid-metabolism genes, while the starch-biosynthesis pathway enzyme, ADP-glucose pyrophosphorylase, was down-regulated. These observations were interpreted as a functional co-ordination of a cytosolic NADP-IDH 2-OG pathway with that of N-assimilation (Scheible et al., 1997). This was in agreement with the preferential export of citrate from malate-fed intact mitochondria extracted from photosynthetically active tissues (Hanning et al., 1999). In pine, a correlation was found between cytosolic NADP-IDH and GS transcripts during seed germination and with GS and Fd-GOGAT transcripts during chloroplast biogenesis. However, such a correlation was not conserved during the advanced stages of cotyledon development, thus leading the authors to suggest that, in pine, cytosolic NADP-IDH had several functions (Palomo et al., 1998). A similar conclusion was reached when cytosolic NADP-IDH expression was examined in potato (Fieuw et al., 1995). The modification of NADP-IDH transcript and activity levels by light and mimicked by nitrate and sucrose (as also observed for N metabolism genes: Stitt, 1999) suggested a role in N-assimilation. Induced leaf senescence also led to an increase in NADP-IDH activity and stable NADP-IDH protein levels while many other protein levels decreased. Stable leaf NADP-IDH transcript levels were also reported in ageing tobacco plants (Masclaux et al., 2000). These observations were interpreted in terms of a role for cytosolic NADP-IDH in the cycling, redistribution, and export of amino acids during leaf senescence. A similar function was attributed to this NADP-IDH isoform during tomato fruit ripening due to a 2–3-fold increase in NADP-IDH activity associated with glutamate accumulation (Gallardo et al., 1995).

Despite these elements, as with NAD-IDH, studies using transgenic plants (either antisense or T-DNA knock-out mutants) with reduced or absent cytosolic NADP-IDH activity were not able to show an essential role for this enzymatic activity in N-assimilation. Potato (Kruse et al., 1998) and tobacco (S Gálvez and M Hodges, unpublished data) retaining less than 10% of their total NADP-IDH activity, exhibited no deleterious growth phenotype. A detailed analysis of the antisensed potato plants showed no noticeable alterations in either C or N metabolism while amino acid content was unchanged. Recently, Arabidopsis
mutants lacking cytosolic NADP-IDH activity have been isolated and characterized (Mhamdi et al., 2010). Three allelic T-DNA knock-out lines grew normally and did not show any significant developmental phenotype, although plants did appear to be slightly smaller than their wild-type counterparts. Metabolic profiling by GC-TOF-MS and HPLC revealed that the loss of cytosolic NADP-IDH activity did not have a large impact on leaf compounds associated to C and N metabolism, although fructose was reduced by 50% and citrate was increased by 50% in air-grown plants. When grown under non-photorespiratory conditions (high CO2), the mutant plants had decreased levels of sucrose and malate as well as three amino acids—mainly serine but also alanine and threonine to a lesser extent. Total amino acid pools did not appear to be affected by the absence of cytosolic NADP-IDH (Mhamdi et al., 2010). A recent study of tomato plants with only partial decreases in cytosolic NADP-IDH activity reported considerable shifts in metabolism characterized by decreases in TCA cycle intermediates, total soluble amino acids, starch, and NAD(P)H but only a small change in plant growth (Sulpice et al., 2010).

When tobacco plants were subjected to N-starvation followed by nitrate or ammonium resupply, cytosolic NADP-IDH transcript levels were not affected (Lancien et al., 1999). Such observations appear to be in contradiction to those made using the tobacco NR mutants (Scheible et al., 1997) but the differences are probably due to the different experimental conditions and plant history, with nitrate assimilation almost absent in the latter case but fully active in the former. It has also been shown that cytosolic NADP-IDH is localized mainly in the vascular tissues of higher plant leaves (Galvez et al., 1996) and roots (Boiffin et al., 1998). This is not consistent with the major ammonium assimilatory site. Interestingly, vascular tissues do contain cytosolic GS (GS1) and NADH-GOGAT and, therefore, the presence of cytosolic NADP-IDH suggests a role in plant metabolic functions associated with N-metabolism. In pine, the spatial expression of NADP-IDH was not found to coincide with that of other key N-assimilatory enzymes, and it was concluded that NADP-IDH was a housekeeping enzyme (Pascual et al., 2008). In conclusion, it is probable that no specific IDH is involved in the production of 2-OG for N-assimilation. A similar situation has been found in yeast cells: indeed, glutamate auxotrophy requires the absence of NAD-IDH and the mitochondrial and cytosolic NADP-IDH isoenzymes (Zhao and McAlister-Henn, 1996).

NADP-IDH in redox homeostasis and related processes

Over the last decade, there are ever-increasing reports, especially in mammalian cells, that NADP-IDH could have a major role in the production of NADPH required for protection against oxidative stress. Mitochondrial NADP-IDH has been shown to be important in the cellular response to heat shock (Shin et al., 2007), cadmium (Kil et al., 2006), and other treatments that lead to an oxidative stress and eventually to apoptosis (cell death) in different types of mammalian cell (Jo et al., 2001). In the case of a Cd stress, even though Cd binds to mitochondrial NADP-IDH and induces a loss of activity, it was shown that mitochondrial NADP-IDH activity was important in protecting glutaredoxin from Cd and thereby reducing cell damage (Kil et al., 2006). Resistance against the adverse effects of selenium has also been shown to be decreased in cells with reduced mitochondrial NADP-IDH levels (Kil et al., 2010). IDP1 (mitochondrial NADP-IDH) was found to be important in producing NADPH in the fungal pathogen Cryptococcus neoformans, thus protecting it against nitrosative stress and mitochondrial damage (Brown et al., 2010). Cytosolic NADP-IDH has also been shown to play an important role in the protection against oxidative stress in mice renal cells (Sh Lee et al., 2010). The analysis of yeast idp2 (cytosolic NADP-IDH) mutants showed that this isoform also has an antioxidant role. The effects were more severe in idp2/huc-6-PDH double mutants, thus suggesting that cytosolic NADP-IDH is not the only enzyme that produces NADPH for such antioxidant functions (Minard and McAlister-Henn, 2001). In plants, it has been found that mitochondrial NADP-IDH is involved in the reductive activation of alternative oxidases. Indeed, the overexpression of this NADP-IDH isoform led to the presence of more reduced, active alternative oxidase subunits (Gray et al., 2004). A role for cytosolic NADP-IDH in plants was suggested from the recent study of Arabidopsis knock-out lines. When crossed with a catalase (cat2) mutant that induces an increased oxidative stress triggered by enhanced H2O2 availability, knockout mutations for cytosolic NADP-IDH caused more marked accumulation of oxidized glutathione and enhanced pathogen-related responses compared with the cat2 parent line (Mhamdi et al., 2010). Pathogenesis-related responses were also observed in single NADP-IDH mutants. These results suggest that cytosolic NADP-IDH could play a role in redox or other signalling linked to pathogen responses (Mhamdi et al., 2010).

In mammalian systems, it appears that both mitochondrial and cytosolic NADP-IDHs are protected against oxidative stress by a process called protein S-glutathionylation. Indeed, NADP-IDH activity is susceptible to inactivation by a number of thiol-modifying reagents. It was shown that mitochondrial NADP-IDH can become glutathionylated on Cys269, thus leading to an inactive enzyme. However, it was reactivated by glutaredoxin2 in the presence of GSH (Kil and Park, 2005). Glutathionylated mitochondrial NADP-IDH was found to be less susceptible to ROS-induced proteolysis during oxidative stress. Similar observations have been made concerning the cytosolic NADP-IDH in mice kidney cells where Cys269 was also shown to become glutathionylated (Shin et al., 2009). In plant systems, there is no reported evidence of such a process occurring, although plant NADP-IDH proteins do contain the equivalent Cys residue.

Regulation of the oxidative pentose phosphate pathway

The metabolic pathway of N assimilation involves the reduction of nitrate to nitrite by the cytosolic enzyme
nitrate reductase (NR) at the expense of NADH produced from malate shuttled from the mitochondrion or chloroplast (Foyer and Noctor, 2002). In leaves, nitrite then enters the chloroplast either as the neutral acid (HNO₂) or via a transporter (Sugiura et al., 2007), where it is reduced by the ferredoxin system to ammonium with the evolution of 1.5 O₂ per nitrite reduced. Thereafter, the ammonium is combined with 2-OG in the GS/GOGAT system to produce glutamate. While many higher plant species predominantly undertake this pathway in leaves, others assimilate nitrate mainly in the root and many species such as white lupin and soybean use both organs with about 45% of the nitrate assimilation taking place in the roots (Cen et al., 2001; Cen and Lazzell, 2003). Estimates of carbon partitioning in roots suggest that about 15% of root carbon catabolism is coupled to ammonium assimilation under ammonium nutrition, whereas under nitrate nutrition about 5% of the root carbon catabolism is coupled to nitrate absorption from the soil, 15% to nitrate assimilation, and 3% to ammonium assimilation (Bloem et al., 1989, 1992, 2002).

The reducing power provided by photosynthesis drives primary nitrogen assimilation in green tissues in the light, but in the dark and in non-green tissues, the necessary reducing power is supplied by the oxidative pentose phosphate pathway (OPPP; Bowsher et al., 1992; Esposito et al., 2003). The interdependency of nitrogen assimilation and respiratory carbon assimilation in terms of reducing power and carbon metabolism has been studied intensively for many years (Emes and Fowler, 1979; Bowsher et al., 1989, 1993; Wright et al., 1997). While the complete OPPP is found in plastids, the non-oxidative reactions also occur in the cytosol. The first committed step of the pathway is catalysed by glucose 6-phosphate dehydrogenase (G6PDH), which is considered to be the major control point of the pathway and 6-phosphogluconate dehydrogenase (6PGDH). Together, these enzymes convert glucose 6-phosphate (Glc6P) to ribulose 5-phosphate with the production of two molecules of NADPH. Of the six G6PDH gene family members in A. thaliana, four are predicted to encode plastidial proteins (Wakao and Benning, 2005). At the protein level, two plastidial isoforms, P1-G6PDH and P2-G6PDH, have been the most studied (Kruger and von Schaewen, 2003).

All eukaryotic G6PDHs studied to date are subject to feedback inhibition by NADPH and are therefore presumed to act as cellular redox sensors. While G6PDH transcripts and activity have been shown to be induced by nitrogen in various plants, the cytosolic G6PDH forms are largely insensitive to nitrogen status and to thiolredoxin (TRX)-mediated redox regulation (Fickenscher and Scheibe, 1986). By contrast, the plastid isoenzymes are subject to both forms of control. P1-G6PDH is controlled by light-regulated reductive inactivation through the stromal TRX system and by changes in the stromal NADPH/NADP ratio (Wenderoth et al., 1997) but it is not greatly affected by nitrogen. The P2-G6PDH form is directly responsive to tissue nitrogen status and is more resistant to changing NADPH levels than P1-G6PDH (Esposito et al., 2001, 2005). Unlike leaf Fd-GOGAT activities, which are enhanced in the presence of nitrogen, root Fd-GOGAT activity and protein are substantially unchanged by nitrogen availability. The induction of the P2-G6PDH form by nitrogen leading to enhanced P2-G6PDH-mediated OPPP activity is considered to be particularly important in driving root nitrogen assimilation linked to NADH-GOGAT (Esposito et al., 2001, 2005).

While the oxidation of Glc6P by the OPPP in the roots is capable of supporting nitrite reductase (NiR; Bowsher et al., 1989) and GOGAT (Bowsher et al., 1992) activities alone, the capacity of the pathway is insufficient to support the simultaneous operation of both NiR and GOGAT (Bowsher et al., 2007). The capacity of the OPPP in root plastids is therefore insufficient to meet the demands of nitrogen assimilation and the flux through the OPPP constrains the nitrogen assimilation ability of root plastids (Bowsher et al., 2007). The oxidation of Glc6P, rather than the activities of nitrogen assimilation enzymes, limits root nitrogen assimilation. If the requirements of primary nitrogen assimilation are combined with the reductive requirements of other pathways in non-photosynthetic plastids, then it is likely that the rate of carbohydrate oxidation by the OPPP activity represents a major limitation. G6PDH is generally considered to be an important source of NADPH in non-photosynthetic tissues other than roots, particularly those that produce fatty acids at high rates, such as oil seeds. However, while the loss of cytosolic G6PDH adversely affected the metabolism of developing seeds it appeared to do so by increasing the availability of carbon substrates for the synthesis of storage compounds rather than by decreasing the NADPH supply for fatty acid synthesis (Wakao et al., 2008).

Plastidial G6PDH belongs to a group of enzymes that respond rapidly to reductive regulation by the chloroplast TRX system. This group of chloroplast enzymes also includes the ATP-synthase and aggregates of phosphoribulokinase and non-regulatory glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that are linked by the redox-regulated CP12 protein. G6PDH is inactivated by TRX in the light, thereby turning off the OPPP and preparing acceptor molecules for carboxylation. The redox potentials of the different enzymes would suggest that this process would occur before the reductive activation of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) activase and the regulatory form of GAPDH that would initiate the carboxylation reactions that begin the reductive pentose phosphate pathway (Calvin Cycle). The reductive inactivation of the G6PDH forms is presumed to prevent unnecessary carbon oxidation when there is sufficient NADPH generation by photosynthesis. It is perhaps not surprising that G6PDH transcript and activity levels increase upon exposure to oxidative stresses such as that induced by methyl viologen, and by inhibitors of the photosynthetic electron transport system.

In addition to the regulation of the activities of G6PDH and thiol-modulated reductive pentose phosphate pathway enzymes, the plastid TRX system also participates in the control of starch turnover through reductive activation of ADP-glucose pyrophosphorylase (AGPase). The AGPase from potato tubers and leaf chloroplasts is activated by TRXs f and m allowing the enzymes of the reductive pentose
phosphate pathway and of starch synthesis to be regulated in a co-ordinate manner (Hendriks et al., 2003; Kolbe et al., 2005). A second TRX system, involving NADP-TRX reductase C (NTRC), whose activity is triggered in response to sucrose, also operates in plastids. The NTRC system facilitates redox regulation of AGPase in roots and in leaves and other green tissues in the dark (Michalska et al., 2009). The combined operation of the NTRC and Fd/TRX systems may provide a mechanistic link between reactions in the chloroplasts and the amyloplasts and other plastids of heterotrophic tissues through the sucrose triggered activation of AGPase. Redox activation of AGPase is induced by sucrose and other sugars in leaves in both the light and dark (Hendriks et al., 2003). In this way, starch synthesis in organs far removed from the leaves is responsive to signals from the chloroplasts that contain information regarding the carbon status of the plant (Kolbe et al., 2005). NTRC is considered to protect leaf enzymes against enhanced cellular oxidation, particularly under low light conditions or when leaves are subjected to prolonged darkness, but there is as yet no information on how this system might interact with the TRX-dependent oxidative activation of G6PDH and the functioning of the OPPP in chloroplasts in the dark or in root plastids to allow the provision of sufficient carbon for respiration.

### Regulation of the respiratory electron transport chain

The plant respiratory electron transport chain consists of four multi-subunit oxidoreductases, Complexes I to IV, and the ATP synthase complex (Complex V) with additional oxidoreductases. The alternative oxidase (AOX) allows electrons to bypass Complexes III to IV of the cytochrome pathway and provides a mechanism for oxidation of the ubiquinone pool. This is considered to prevent the build-up of electrons within the electron transport system and to minimize the risk of electron donation to oxygen and superoxide formation. In addition, there are up to four different ‘rotenone-insensitive’ type II NAD(P)H dehydrogenases, which circumvent Complex I of the electron transport chain. All the complexes apart from succinate dehydrogenase (Complex II) participate in the formation of supercomplexes, which have the potential to add another layer of complexity to the regulation of respiratory electron transport. For example, supercomplexes involving complexes I, III and IV might operate in a single functional unit allowing a direct electron channel or path from NADH to cytochrome c (Eubel et al., 2004). This type of supercomplex has been called a ‘respirasome’ because it can carry out respiratory electron flow in the presence of ubiquinone and cytochrome c in an autonomous manner (Dudkina et al., 2005). Supercomplex formation might also assist in the regulation of alternative respiratory pathways via electron channelling. Like the ‘alternative’ and accessory components, such supercomplexes confer energetic flexibility on the system by allowing adjustments in ADP:O ratios (Rasmusson et al., 2004). Interestingly, comparisons of the root and shoot mitochondrial proteomes have failed to reveal any major differences in the abundance of mitochondrial electron transport chain components. In marked contrast, the abundance of enzymes involved in the TCA cycle and in photorespiration were different in the root and shoot mitochondrial proteomes (CP Lee et al., 2010). Such results suggest that changes in the abundance of proteins involved in the TCA cycle and in photorespiration can be sufficient to allow adaptation of mitochondria to leaf and root metabolism without changes in the content or composition of components of the electron transport system (CP Lee et al., 2010).

The type II NAD(P)H dehydrogenases and AOX are considered to function in higher plants as a defence against metabolic fluctuations. It is perhaps not surprising therefore that the expression of the NAD(P)H dehydrogenases and AOX genes is influenced by the availability of nitrate and ammonium. In Chlamydomonas reinhardtii, for example, the expression of Aox1, the major AOX, is strongly down-regulated by ammonium and stimulated by nitrate in line with changes in the rate of respiration (Baurain et al., 2003). In A. thaliana, the expression of the NAD(P)H dehydrogenase and AOX genes was decreased in the presence of nitrate and increased by ammonium (Escobar et al., 2006). The switch from nitrate to ammonium enhanced respiration rates and increased AOX activity and protein as well as calcium-dependent external NADH oxidation, trends consistent with an increased capacity of respiratory bypass pathways after switching from nitrate to ammonium (Escobar et al., 2006).

The impact of the mitochondrial electron transport chain on photosynthetic and respiratory metabolism has long been studied in leaves and protoplasts using pharmacological methods (Krömer et al., 1988, 1993; Raghavendra and Padmasree, 2003). Recent years have witnessed several analyses of such questions using reverse genetics approaches (Noctor et al., 2007). For example, Aox1a knockout mutants accumulate sugars but have lower organic and amino acids compared with the wild type, consistent with the view that, in the absence of AOX and alternative non-phosphorylating respiration, the respiratory chain cannot alleviate the tight constrictions of adenylate control (Giraud et al., 2008). Knockouts in Complex I and uncoupling proteins have been shown to exert a strong influence on photosynthesis (Dutilleul et al., 2003; Sweetlove et al., 2006), as do decreases in components of the TCA cycle (Carrari et al., 2003; Nunes-Nesi et al., 2005, 2007).

Over the last 10 years, a range of studies on Complex I mutants has unequivocally demonstrated that the path of NADH oxidation, through either Complex I or the alternative NAD(P)H dehydrogenases, has a significant impact on nitrogen metabolism and carbon/nitrogen interactions. Two mutants have proved to be particularly useful tools in revealing this relationship. These are the Nicotiana sylvestris CMSII mutant, which carries a mutation in the mitochondrial nad7 gene (Gutierrez et al., 1997; Pineau et al., 2005), and the A. thaliana ndufs4 that lacks the fragment S subunit 4 18 kDa subunit of Complex I. As a consequence of these mutations, both lines are completely deficient in respiratory Complex I. The Complex I-deficient mutants grow more slowly than their
Mitochondrial redox cycling as a key player determining the rate of nitrate assimilation

In addition to integration with respiratory C flow, N assimilation in the leaves of C3 species is also intimately associated with photorespiratory C flow, an interaction that further complicates the photosynthetic C and N interaction (Stitt et al., 2002; Foyer et al., 2009). Under most conditions, the photorespiratory cycle is much more important and rapid than primary nitrate reduction. Photorespiration can influence N assimilation in several ways (Foyer et al., 2009). Ammonia liberated during the conversion of Gly to Ser in leaves is recycled through Gln and Ghu, and this process probably occurs through the same GS and GOGAT isoforms as those involved in primary ammonia assimilation (Hirel and Lea, 2002). Photorespiration also involves reductant cycling. NADH is produced by Gly oxidation in the mitochondria. While stoichiometrically equal amounts of NADH are required during steady-state operation of the photorespiratory pathways for glyceraldehyde 3-phosphate dehydrogenase (Hanning and Heldt, 1993), the enhanced generation of light-dependent reductant in both the chloroplasts and mitochondria could contribute to increasing cytosolic NADH concentrations. These concentrations are rather low during most conditions, and the cytosolic NADH pool, estimated at about 0.7 mM, has been considered to be more than 99% NAD+ (Heineke et al., 1991). For comparison, the $K_m$ NADH of NR is 1–5 μM (Campbell, 1999).

For the above reasons, the ability of chloroplasts or mitochondria to deliver reducing equivalents to the cytosol could influence the rate of N assimilation (Fig. 3; Kroemer and Heldt, 1991; Hanning and Heldt, 1993). Low photosynthesis rates could limit NR activity through decreased reductant availability, as well as through post-translational inactivation (Kaiser et al., 2000, 2002). Nitrate reduction is significantly stimulated by anoxia, conditions that are expected to favour export of mitochondrial reductant to the cytosol. Such export is likely to be favoured in conditions in which the complex redox cycling of photosynthesis is active and GDC activity is significant (Fig. 3). Indeed, photosynthesis has been reported to be necessary for optimal rates of nitrate assimilation in several crop species (Rachmilevitch et al., 2004; Bloom et al., 2010). This may involve improved availability of reductant in both the cytosol and chloroplast when photosynthesis is favoured (Bloom et al., 2010). Fundamental mechanistic questions remain, however, concerning the links between photorespiration, respiration, and nitrogen assimilation. Delivery of reductant from the chloroplast to the cytosol could also be favoured by the operation of triose-phosphate/3-phosphoglycerate shuttles (Fig. 3). This is predicted to be favoured by high photosynthesis rather than high photorespiration, and an important role for such shuttles is consistent with some literature observations (Kaiser et al., 2000). Assuming that triose-phosphate is re-oxidized through the classical glycolytic NAD-dependent glyceraldehyde-3-phosphate dehydrogenase, the operation of this shuttle would augment the production of cytosolic NADH above that linked to triose phosphate metabolism through to phosphoenolpyruvate (Fig. 1). By contrast, a requirement of high rates of photorespiration for optimal nitrogen assimilation rates (Rachmilevitch et al., 2004; Bloom et al., 2010) could be explained by enhanced redox transfer to the cytosol through the chloroplastic envelope or mitochondrial malate/oxaloacetate shuttles (Fig. 3).

An important point concerning amino acid synthesis is that this process requires simultaneous C oxidation and nitrate reduction. Thus, any perturbation of redox cycling could radically alter C/N partitioning through concerted and opposing effects on the two processes. Organic acid synthesis may also be subject to redox modulation in the C/N interaction because, as discussed in the first part of this review, the production of compounds such as 2-OG requires oxidation through respiratory pathways involving the cytosol and mitochondria (Foyer et al., 2003, 2009; Hodges, 2002; Noctor et al., 2007).

It remains unclear whether part of the Complex I deficiency on nitrogen metabolism is mediated through effects on photorespiration. The mitochondrial electron transport chain oxidizes NADH generated by various soluble mitochondrial enzymes, notably GDC and TCA cycle dehydrogenases (Rasmusson et al., 2004; Noctor et al., 2007). Mitochondria lacking Complex I have a decreased capacity for glycine...
oxidation (Sabar et al., 2000), possibly due to increased redox control over glycine decarboxylase. Despite this, the glycine-serine ratios were not increased in leaves of the Complex I mutants in air (Dutilleul et al., 2003, 2005), although the decreased mesophyll conductance might indicate higher rates of photorespiration (Priault et al., 2006).

Mitochondrial targets for the enhancement of nitrogen use efficiency (NUE)

Recent increases in our understanding of the interactions between respiration and nitrogen assimilation have revealed that mitochondrial metabolism is a potential target for enhancing NUE. For example, recent evidence suggests that the effects of Complex I mutations in different species show a strong similarity to each other in terms of effects on nitrogen metabolism and the accumulation of amino acids. These results demonstrate that low Complex I activities favour N-rich phenotypes. Moreover, limitations at the level of the OPPP capacity rather than nitrogen assimilation itself have been identified in roots. Therefore, manipulation of the different G6PDH isoforms to enhance flux through the OPPP in roots is a potential new target for enhancing NUE.

Of the new potential targets for improved NUE, increasing NAD plus NADH availability by manipulating the relative capacities of Complex I and the type II NAD(P)H dehydrogenases is attractive because the availability of NADH exerts a strong influence on nitrogen assimilation and amino acid accumulation. The relationship between Complex I and the type II NAD(P)H dehydrogenases is a flexible parameter and potentially open to manipulation. The factors that relate perturbation of the respiratory electron transport chain to high amino acid contents remain to be clearly identified. This could partly occur through stimulation of nitrate assimilation but other factors may also be involved. There is a close but not unbreakable correlation between amino acids synthesized through different pathways (Noctor et al., 2002; Fritz et al., 2006). Post-translational control of protein redox state could be one influential mechanism. In this connection, it is intriguing that plants engineered to have high glutathione contents through chloroplastic overexpression of the glutathione synthesis pathway showed enhanced accumulation of several amino acids (Noctor et al., 1998).

Recent studies have highlighted the central role for pseudo-response regulators in controlling metabolic homeostasis in the TCA cycle (Fukushima et al., 2009; Nakamichi et al., 2005). Moreover, the teosinte branched1, cycloidea, PCF (TCP) family of transcription factors and the site II cis-acting regulatory elements to which they bind have been shown to link the regulation of genes encoding mitochondrial proteins with the circadian clock in Arabidopsis thaliana (Giraud et al., 2010). These transcription factors appear to be central regulators in circadian clock input sites, the clock oscillator itself, and of clock output sites, directing and regulating the expression of genes encoding cellular energy metabolism components, particularly in mitochondria (Giraud et al., 2010). The demonstration of the involvement of these transcription factors in growth and developmental processes not only provides a molecular link co-ordinating metabolism, growth, and development but it offers new potential targets with regard to enhancing metabolism. For example, the site II elements in the promoter regions of genes encoding mitochondrial, plastid, and peroxisomal proteins provide a direct mechanism for the co-ordination of the circadian clock with the expression...
of genes involved in a variety of organelar functions, including energy metabolism.

Analyses of the different IDHs in supplying 2-OG to ammonia assimilation continue to support ideas of redundancy between the different forms (Lancien et al., 2000). However, the recent identification of activation of pathogenesis responses in mutants lacking the cytosolic NADP-dependent isoform point to new possible additional functions (Mhamdi et al., 2010). Although the mechanisms linking loss of cytosolic NADP-IDH to pathogen responses remain unclear, this finding is one of several over recent years that underline the importance of primary metabolism in pathogen responses (Bolton, 2009). The effect may be partly linked to redox effects mediated by NADPH or secondary effects on respiration downstream of 2-OG. However, it is worth noting that, as well as its role in ammonia assimilation, 2-OG is a cofactor for dioxygenase enzymes that are important in several phytohormone synthesis pathways. It remains unclear whether 2-OG could limit such enzymes at concentrations that are sufficient for ammonia assimilation, although compartmentation is an obvious potentially influential factor. Finally, it is important to note that 2-OG is also an important signal metabolite that is sensed by a protein named PII. When 2-OG is bound to this protein, PII-target protein interactions and hence PII-regulated activities are modified (Uhrig et al., 2009). To date, in plants, PII is known to modify Arginine (Arg) (an N-rich amino acid) biosynthesis by activating the N-acetyl-glutamate kinase (Ferrario-Méry et al., 2006) and by reducing its retroinhibition by Arg (Chen et al., 2006). More recently, a role for PII in regulating fatty acid synthesis has been indicated by its interactions with the BCCP subunit of a plastidial acetyl-CoA carboxylase, which decreases the activity of this enzyme (Feria Bourrellier et al., 2010). PII mutants show altered Arg levels in rosette leaves as well as reduced starch and increased amino acid contents when plants are grown under certain N-regimes, indicating a role for this protein in C/N interactions (Ferrario-Méry et al., 2005). PII and 2-OG levels could have a significant role in controlling the flow of C to either N or lipid metabolic pathways depending upon environmental conditions.

Conclusions and perspectives

Nitrogen is a major factor limiting plant growth in the field. Crop productivity is largely dependent on high nitrogen fertilization rates, but this practice is costly and has negative environmental impacts. An enhanced understanding of the mechanisms that determine NUE is required so that the current levels of nitrogen fertilization can be decreased, together with the identification of new targets for plant improvement. Factors associated with carbon and nitrogen metabolism have a close relationship to crop yields and they are thus important current targets for improving NUE, particularly using quantitative trait loci. To date, studies concerning the improvement of NUE have focused largely on the components of primary nitrogen assimilation and associated carbon metabolism and few have looked at the wider perspective of manipulation of energy utilization as a further means to achieve this goal. It is suggested that exploitation of the flexibility of respiratory pathways has the potential to enhance NUE.

It has long been recognized that respiration and nitrogen assimilation are intimately linked in plant cells, particularly because of energy and metabolite requirements. However, it is only recently that a more comprehensive understanding of the interactions and limitations on these pathways has been demonstrated largely through the application of -omics technologies and forward and reverse genetic techniques. These approaches have not only yielded valuable new insights into the crucial role of cellular energy balance as a broker of co-ordinate regulation but they have also provided evidence that components of the mitochondria are exciting potential novel targets for the enhancement of NUE. Some of the possibilities for manipulation of the respiratory processes have been highlighted here in order to provide a more efficient driving force for nitrogen in plants. There is significant potential for exerting a positive influence on NUE by either genetic manipulation of components of respiratory electron transport chain and/or OPPP, as well as the identification of new molecular markers and aids to quantitative trait loci (QTL) analysis. Forward and reverse genetic approaches, coupled to classical biochemistry and physiology, will remain essential tools for analysis of the roles of mitochondrial targets in improving NUE.

References


