Disruption of the mitochondrial alternative oxidase (AOX) and uncoupling protein (UCP) alters rates of foliar nitrate and carbon assimilation in Arabidopsis thaliana

Anthony Gandin, Mykhaylo Denysyuk and Asaph B. Cousins*

School of Biological Sciences, Molecular Plant Sciences, Washington State University, Pullman, WA 99164-4236, USA

* To whom correspondence should be addressed. E-mail: acousins@wsu.edu

Received 30 January 2014; Revised 10 March 2014; Accepted 13 March 2014

Abstract

Under high light, the rates of photosynthetic CO2 assimilation can be influenced by reductant consumed by both foliar nitrate assimilation and mitochondrial alternative electron transport (mAET). Additionally, nitrate assimilation is dependent on reductant and carbon skeletons generated from both the chloroplast and mitochondria. However, it remains unclear how nitrate assimilation and mAET coordinate and contribute to photosynthesis. Here, hydroponically grown Arabidopsis thaliana T-DNA insertional mutants for alternative oxidase (AOX1A) and uncoupling protein (UCP1) fed either NO3– or NH4+ were used to determine (i) the response of NO3– uptake and assimilation to the disruption of mAET, and (ii) the interaction of N source (NO3– versus NH4+) and mAET on photosynthetic CO2 assimilation and electron transport. The results showed that foliar NO3– assimilation was enhanced in both aox1a and ucp1 compared with the wild-type, suggesting that foliar NO3– assimilation is probably driven by a decreased capacity of mAET and an increase in reductant within the cytosol. Wild-type plants had also higher rates of net CO2 assimilation (A net) and quantum yield of PSII (ϕPSII) under NO3– feeding compared with NH4+ feeding. Additionally, under NO3– feeding, A net and ϕPSII were decreased in aox1a and ucp1 compared with the wild type; however, under NH4+ they were not significantly different between genotypes. This indicates that NO3– assimilation and mAET are both important to maintain optimal rates of photosynthesis, probably in regulating reductant accumulation and over-reduction of the chloroplastic electron transport chain. These results highlight the importance of mAET in partitioning energy between foliar nitrogen and carbon assimilation.

Key words: Alternative oxidase, ammonium, energy balancing, nitrate assimilation, reductant, uncoupling protein.

Introduction

Nitrogen (N) availability is a major determinant of plant growth and productivity (Reich et al., 1997, 2006a). However, N fertilizers also represent a large economical cost and source of ground water pollution in agriculture systems. In higher plants, nitrate (NO3–) and ammonium (NH4+) are the two primary forms of assimilated inorganic N. The latter is typically assimilated directly into amino acids through the glutamine synthetase–glutamate synthase complex, whereas the former is first reduced to nitrite by cytosolic nitrate reductase and then to NH4+ by plastidic nitrite reductase (Epstein and Bloom, 2005). The assimilation of NO3– has a higher energetic requirement compared with NH4+ assimilation (Noctor and Foyer, 1998; Scheurwater et al., 1999; Escobar et al., 2006) and in the leaves the cytosolic reduction of nitrate to nitrite may consume reductant exported from either the chloroplast or the mitochondria (Foyer et al., 2011). On the other hand,
nitrite reduction and NH$_4^+$ assimilation occur in the chloroplast stroma and consume reduced ferredoxin. Therefore, the complexity and compartmentalization of these pathways necessitates balancing photosynthetic energy supply and demand to optimize rates of NO$_3^-$ and CO$_2$ assimilation.

The availability of N is known to affect rates of photo-synthesis and respiration (Reich et al., 2006b; Atkinson et al., 2007), and there is a strong correlation between leaf N content and rates of respiration (Terashima and Evans, 1988; Makino and Osmond, 1991; Byrd et al., 1992; Lusk and Reich, 2000). For example, the tricarboxylic acid (TCA) cycle enzymes fumarase, NAD-dependent isocitrate dehydrogenase (Makino and Osmond, 1991), and NAD-dependent malic enzyme (Noguchi and Terashima, 2006) are up-regulated under low N. Additionally, the expression and activity of several glycolytic and TCA cycle enzymes were differentially influenced following NH$_4^+$ or NO$_3^-$ feeding (Larsen et al., 1981; Scheible et al., 1997; Lancien et al., 1999; Stitt, 1999). These changes in respiration are linked to the supply of carbon skeletons (e.g. 2-oxoglutarate, isocitrate, and citrate) from the TCA cycle to maintain optimum N assimilation and amino acid biosynthesis (Larsen et al., 1981; Scheible et al., 1997; Lancien et al., 1999). Furthermore, the N source (NO$_3^-$ versus NH$_4^+$) has been shown to change gene expression of the mitochondrial electron transport chain, particularly the alternative oxidase (AOX), type II NAD(P)H dehydrogenases, and uncoupling proteins (UCPs) of the mitochondrial alternative electron transport (mAET) (Escobar et al., 2006; Patterson et al., 2010). Additionally, the capacity and protein amount of AOX, a major component of mAET, increase under low NO$_3^-$ conditions (Sieger et al., 2005; Watanabe et al., 2010; Hachiya and Noguchi, 2011). The mAET bypasses one or more of the multiprotein complexes of the ‘classic’ electron transport chain, minimizing proton pumping across the inner membrane (Vanlerberghe and McIntosh, 1997; Rasmusson et al., 2004). Consequently, mAET oxidizes NAD(P)H uncoupled from ATP production and has been proposed to dissipate excess reductant (Raghavendra and Padmasree, 2003). For example, mAET plays an important role in the response to several environmental constraints such as cold (Armstrong et al., 1988; Gandin et al., 2007), as well as other reactive oxygen species-inducing stress conditions (Maxwell et al., 1999). Additionally, it has been suggested that the capacity of mAET responds to changes in NO$_3^-$ assimilation (Dutilleul et al., 2005; Escobar et al., 2006).

Reducant availability within the leaf cytoplasm probably often limits rates of de novo NO$_3^-$ assimilation. This is in part due to the low cytosolic NADH availability (0.3–0.7 μM), which is far below the 7 μM $K_m$ of nitrate reductase for NADH (Kaiser et al., 2000; Heineke et al., 2001). Therefore, conditions such as high light or perhaps high rates of photorespiration that increase cytosolic NADH concentrations have been suggested to increase rates of foliar NO$_3^-$ assimilation (Bloom et al., 2002; Searles and Bloom, 2003; Rachmilevitch et al., 2004; Guo et al., 2007). Alternatively, NO$_3^-$ assimilation decreases under conditions that restrict the export of reductant via the malate shuttle from the chloroplast. It has also been reported in the literature that changes in mitochondrial electron transport, particularly the alternative non-phosphorylating pathways, could influence the de novo assimilation of N (Watanabe et al., 2008). However, it remains unclear how the alternative non-phosphorylating pathways of the mitochondrial inner membrane influence foliar NO$_3^-$ assimilation.

Additionally, it has been shown that both the alternative non-phosphorylating pathways of the mitochondrial inner membrane and N assimilation oxidize excess reductant produced by photosynthesis, photorespiratory glycine oxidation, and the TCA cycle (Sweetlove et al., 2006; Gandin et al., 2012). Therefore, the energy partitioning in the cell is probably balanced in part by both mitochondrial electron transport and NO$_3^-$ assimilation (Hachiya and Noguchi, 2011), avoiding chloroplast over-reduction and maintaining optimal rates of photosynthesis. The role of the mAET and NO$_3^-$ assimilation in energy consumption and dissipation is well accepted; however, their respective contribution and coordination remain unclear. Therefore, the aim of this research is to test the hypothesis that changes in mAET and NO$_3^-$ assimilation influence energy partitioning between N and carbon metabolism. To test this hypothesis, this study (i) investigated the influence of changes in mAET capacity on de novo NO$_3^-$ assimilation and (ii) determined the response of photosynthetic CO$_2$ assimilation and electron transport to changes in N source (NO$_3^-$ versus NH$_4^+$) in plants with disrupted mAET [wild type (WT) versus aox1a and ucp1].

**Materials and methods**

*Plant material and growth conditions*

WT and T-DNA insertion lines for AOX1a (SALK_084897) and UCPI (SAIL_536G01) plants of Arabidopsis thaliana (Sweetlove et al., 2006; Giraud et al., 2008; Gandin et al., 2012) were grown hydroponically in a controlled environment growth chamber (Biochambers GC-16, Winnipeg, Manitoba, Canada) at a photosynthetic photon flux density (PPFD) of 160 μmol quanta m$^{-2}$ s$^{-1}$ at plant height, relative humidity of 50%, and air temperature of 23 °C and 18 °C during the day and night, respectively, with a 10 h day. The SAIL_563G01 line was obtained from the TAIR collection and homozygous lines were screened by PCR of genomic DNA using the GACGAAGATGTAAGTACGACC/ TACGATCTGAAATTTTCAACCACTCGATACAC and GACGGAAGTGAAGTACGACC/TACGATCTGAAATTTTCAACCACTCGATACAC primer pairs. Homozygous lines were selfed and screened again by PCR using the same primer pair. Seeds were germinated on Rockwool cylinders (GroDan Cubes, Rockwool BV, Roemond, The Netherlands) for 7 d. Subsequently, seedlings were transferred to 14 litre containers filled with aerated nutrient solution containing 0.2 mM NH$_4$Cl, 0.2 mM KNO$_3$, 1.25 mM CaSO$_4$, 0.75 mM MgSO$_4$, 0.5 mM K$_2$HPO$_4$, 0.04 g 1$^{-1}$ FeDPTA, and micronutrients (Gibeaut et al., 1997). Nutrient solution was replaced every 2 d.

**Growth parameters, chlorophyll contents, and Rubisco activity**

Total leaf area, leaf number, and rosette size were measured from digital pictures of whole plants using Image J software (NIH, Bethesda, MD, USA). Rosette size was calculated according to
Nitrate uptake, content and assimilation

WT, aox1a and ucp1 A. thaliana plants were grown as described above in a hydroponic solution containing 0.2 mM NO₃⁻ at natural abundance ¹⁵Ν⁻. Subsequently, plants were grown for 24 h on a nutrient solution depleted of N (as above). Half of the plants were shifted to a nutrient solution supplemented with 0.2 mM NO₃⁻ enriched 25% with ¹⁵NO₃⁻ and the other half were fed natural abundance ¹⁵NO₃⁻ as a control. In the first set of experiments, plants were labelled for up to 9 h to look at the time course of NO₃⁻ uptake and assimilation (time effect, Supplementary Fig. S1 available at JXB online). Subsequent experiments were limited to 6 h of feeding (irradiance effect, Fig. 1). After labelling, plants were rinsed in ultra-pure water then separated into shoots and roots, oven-dried, and ground to a fine powder in a mortar and pestle. Total ¹⁵Ν enrichment was measured using an elemental analyser (ECS 4010, Costech Analytical, Valencia, CA, USA) connected directly to a continuous flow isotope ratio mass spectrometer (Delta PlusXP, ThermoFinnigan, Bremen, Germany) (Brenna et al., 1997). Isotopic reference materials are interspersed with samples for isotope ratio calibration and acetylene was used in a multipoint correction for N%.

Free NO₃⁻ was extracted from powder plant material, and ¹⁵Ν enrichment of free NO₃⁻ was estimated by converting NO₃⁻ to N₂O using the denitrifying Pseudomonas aureofaciens (Sigman et al., 2001; Casciotti et al., 2002). The headspace N₂O was sampled with a two-holed needle mounted on an autosampler (GC-PAL, CTC Analytics, Switzerland) and connected to a GasBench II (ThermoFinnigan) interface. Samples were cleaned of water and volatile organic compounds (VOCs) through a liquid nitrogen/ethanol slush trap (~110 °C), and of CO₂ and H₂O through an ascariate/magnesium perchlorate trap. Further removal of VOCs was achieved with a Supelco type F trap following the slush trap. Purified samples were separated through a Poraplot Q GC column (Varian, 25 μm x 0.32 mm ID), run through a final nafion water trap (Permapure LLC, NJ) for trace water removal, and analysed by a continuous flow isotope ratio mass spectrometer (Delta PlusV, ThermoFinnigan) (Brenna et al., 1997). NO₃⁻ assimilation was thus estimated as NO₃⁻ assimilation=total ¹⁵Ν⁻¹⁵NO₂⁻. Finally, total free NO₃⁻ was quantified from powder using high-pressure liquid chromatography (Thayer and Hufnaker, 1980).

Amino acid analysis

Leaf tissues were ground in 0.25 ml of 0.1 M HCl using a micropestle, and 400 μM aminobutyric acid was added as internal control. Samples were centrifuged for 20 min at 5000 g at 4 °C and the supernatant was collected and the pellets re-extracted with 0.25 ml of 0.1 M HCl. Supernatants were combined and filtered through a 45 μm polyvinylidene difluoride (PVDF) filter and stored at −80 °C until analysis. Amino acids were derivatized using 4-fluoro-7-nitro-2,1,3-benzenoxadizole (NBD-F) according to Aoyama et al. (2004) by incubating 5 μl of the extract with 50 mM borate buffer pH 9.5 and 3 mM NBD-F reagent at 60 °C for 10 min. Reactions were terminated by addition of 333 mM tartarate buffer pH 2.0 and derivatized amino acids were separated using an Alliance® HPLC System (2695 Separations Module, Waters, Milford, MA, USA) and fluorometrically detected at 540 nm with excitation at 470 nm (2475 Multi-Wavelength Fluorescence Detector, Waters).

Feeding system for gas exchange and chlorophyll fluorescence

Twenty-four hours before the gas exchange measurements, plants were transferred to nutrient solution depleted in N source (neither NH₄⁺ nor NO₃⁻) for starvation. Subsequently, plants were individually transferred 12 h before measurements to stainless steel cuvettes sealed with Teflon caps. The root cuvettes were fed with a continuous flow of nutrient solution supplemented with either NO₃⁻ or NH₄⁺ using a custom-built multipant feeding system. Nutrient solution was equally distributed between each of the six cuvettes using electronically controlled solenoid valves (ASCO RedHat II 8262, Florham Park, NJ, USA). Each individual stainless steel cuvette was housed within a sealed PVC tube containing temperature-controlled circulating water to maintain the root system at 25 °C.

Gas exchange and chlorophyll fluorescence

Gas exchange and chlorophyll fluorescence measurements were made on fully expanded leaves using a LI6400 (LICOR Biosciences, Lincoln, NE, USA) leaf chamber (LI6400-40). Gas exchange measurements were made at a Pₒ₂ of 18.6 kPa, a leaf temperature of 25 °C, a saturating light intensity of 1000 μmol quanta m⁻² s⁻¹ PAR (photosynthetically active radiation), and a CO₂ partial pressure of 37.2 Pa. Light–response curves were made by decreasing light from 2000 to 1500, 1200, 1000, 800, 500, 200, 100, 40, and 20 μmol quanta m⁻² s⁻¹ PAR. The O₂ response curves were made by modulating Pₒ₂ inside the chamber using two mass flow controllers (Aalborg, Orangeburg, NY, USA) to mix N and O₂ gas proportional to 46.6, 32.6, 18.6, 9.3, and 1.9 kPa Pₒ₂. The order of Pₒ₂ during the measurements was randomized. Simultaneously, chlorophyll fluorescence measurements were made using a LI6400-40 pulse-modulated light emitting diode (LED) illuminator and monitored with a Multi-Wavelength Fluorescence Detector (Waters) using a 470 nm LED excitation coupled with a 685 nm LED for the chlorophyll fluorescence measurements.
fluorometer and multiple flash protocol (Loriaux et al., 2013). The quantum yield of photosystem II ($\phi_{\text{PSII}}$) and photochemical quenching (qP) were determined as $(F_{m}'-F)/F_m$ and $(F_{m}'-F)/(F_{m}'-F_s)$, respectively. The rate of linear electron transport through PSII ($J$) was calculated from chlorophyll fluorescence measurements as $J=\phi_{\text{PSII}}\times 41.8\times 0.48$, where $Abs$ is leaf absorption (=0.85). $I$ is the incident irradiance, and assuming a relative excitation distribution to PSII of 0.48 (Laisk and Loreto, 1996). Furthermore, the rate of electron transport required to sustain the photosynthetic carbon reduction and photorespiratory cycles ($J_e$) was calculated from gas exchange measurements as $J_e=A_{\text{net}}+R_d$ $(4C_4+8\Gamma^*)/(C_4^\ast+\Gamma^\ast)$ where $A_{\text{net}}$ is net CO$_2$ assimilation rate, $R_d$ is dark-type respiratory rate, $C_4$ is the chloroplastic CO$_2$ partial pressure, and $\Gamma^\ast$ is the CO$_2$ compensation point in the absence of dark-type respiration.

**Results**

**Leaf characteristics**

Measurements of growth, leaf chlorophyll content, and Rubisco activity were made to characterize WT, aox1a, and ucp1 Arabidopsis thaliana plants grown in hydroponic conditions. Shoot dry weight was 9 and 10% lower in ucp1 compared with the WT and aox1a, respectively (Table 1). However, leaf number, diameter of the rosette, LMA, and root biomass were similar between genotypes. Additionally, leaf chlorophyll content and Rubisco activity were similar, with an average chlorophyll $a/b$ ratio of 1.8 and Rubisco activity of 48 mmol m$^{-2}$ s$^{-1}$, suggesting similar photosynthetic capacity in all three genotypes (Table 1).

**Nitrate uptake, accumulation, and assimilation**

To estimate the impact of AOX1a and UCP1 function on de novo N uptake, rates of NO$_3^-$ uptake and assimilation were measured by feeding hydroponically grown plants $^{15}$N-enriched NO$_3^-$. The uptake and accumulation of NO$_3^-$ were quantified from measurements of bulk leaf $^{15}$N and free $^{15}$NO$_3^-$, respectively, relative to plants fed natural abundance NO$_3^-$. The assimilation of NO$_3^-$ was estimated from differences between total leaf $^{15}$N minus $^{15}$NO$_3^-$ content. Rates of NO$_3^-$ uptake and assimilation were measured in WT plants at three time points after initiating feeding (3, 6, and 9 h) to determine the optimum time. Rates of shoot NO$_3^-$ uptake, assimilation, and accumulation were similar after 3, 6, and 9 h (Supplementary Fig. S1 at JXB online). However, rates of root NO$_3^-$ uptake and assimilation were higher at 3 h compared with 6 h and 9 h.

Therefore, NO$_3^-$ uptake, assimilation, and content were measured in WT, aox1a, and ucp1 plants after 6 h of $^{15}$NO$_3^-$ feeding. Under 160 μmol quanta m$^{-2}$ s$^{-1}$ (growth irradiance), shoot NO$_3^-$ uptake was higher in ucp1 compared with aox1a and WT plants. However, shoot assimilation of NO$_3^-$ was higher in both aox1a and ucp1 compared with the WT, while shoot NO$_3^-$ content was similar between all three genotypes (Fig. 1). Regardless of the genotype, the uptake, assimilation, and content of NO$_3^-$ in the roots were similar (Supplementary Fig. S2 at JXB online). After 6 h under high light (1000 μmol quanta m$^{-2}$ s$^{-1}$), shoot NO$_3^-$ uptake and assimilation were higher in ucp1 compared with the WT and aox1a; however, NO$_3^-$ content was lower (Fig. 1). Under the high light treatment, the root NO$_3^-$ uptake, assimilation, and content were similar between all three genotypes (Supplementary Fig. S2).

**Amino acid analysis**

Nineteen amino acids were quantified in leaves of A. thaliana WT, aox1a, and ucp1 fed either NO$_3^-$ or NH$_4^+$ as sole N source under both growth (160 μmol quanta m$^{-2}$ s$^{-1}$) and high (1000 μmol quanta m$^{-2}$ s$^{-1}$) irradiance. Asparagine, aspartate, and lysine contents were higher in the aox1a mutant plants compared with the WT, regardless of N source or irradiance (Fig. 2; Supplementary Table S1 at JXB online). However, only asparagine was significantly higher in ucp1 compared with the WT plants. In contrast, under growth irradiance, cysteine, glycine, and serine were lower in ucp1 compared with the WT, regardless of the N source. However, under saturating irradiance, glycine and serine were significantly decreased but cysteine was not in ucp1 compared with WT plants.

**Response of photosynthesis**

Measurements of $A_{\text{net}}$, $\phi_{\text{PSII}}$, and the excitation pressure on the chloroplast electron transport chain (1–qP) were measured in WT, aox1a, and ucp1 plants fed either NO$_3^-$ or NH$_4^+$ to test the impact of two major electron sinks on photosynthesis. In WT plants, $A_{\text{net}}$ and $\phi_{\text{PSII}}$ increased while 1–qP decreased under NO$_3^-$ compared with NH$_4^+$ feeding (Fig. 3). However, in the aox1a and ucp1 plants the change in N source had no effect on $A_{\text{net}}$, $\phi_{\text{PSII}}$, or 1–qP. Under NO$_3^-$ and saturating irradiance, $A_{\text{net}}$ was lower in ucp1 compared with WT and aox1a plants; however, under non-saturating irradiances, $A_{\text{net}}$ was similar between genotypes. Furthermore, under NO$_3^-$, the measured $\phi_{\text{PSII}}$ was lower and 1–qP higher in the ucp1

| Table 1. Growth characteristics, chlorophyll ratio, and Rubisco activity in wild-type, aox1a, and ucp1 Arabidopsis thaliana |
|---------------------------------------------------------------|
| **Root biomass (mg)** | **Shoot biomass (mg)** | **No. of leaves** | **Rosette diameter (cm)** | **LMA (g m$^{-2}$)** | **Chl a/b** | **Rubisco (μmol m$^{-2}$ s$^{-1}$)** |
| WT | 88.6 ± 6.9 | 412.3 ± 21.5 | 21 ± 1 | 12.3 ± 1.2 | 68.7 ± 5.4 | 1.7 ± 0.3 | 49.0 ± 3.7 |
| aox1a | 89.8 ± 5.7 | 415.1 ± 18.1 | 21 ± 1 | 12.6 ± 0.8 | 67.1 ± 3.2 | 1.9 ± 0.4 | 44.4 ± 6.4 |
| ucp1 | 76.5 ± 5.0 | 375.1 ± 16.0 | 20 ± 1 | 11.3 ± 0.3 | 67.5 ± 5.9 | 1.8 ± 0.3 | 51.0 ± 4.2 |

Values represent the means ± SE of 10 biological replicates for growth characteristics and five biological replicates for chlorophyll and Rubisco measurements. ANOVA results are indicated; different letters indicate significant differences between genotypes at $P<0.05$. 


Mitochondrial alternative electron transport influences N and C assimilation

Mitochondrial alternative electron transport influences N and C assimilation and aox1a plants compared with the WT plants, regardless of irradiance. Under NH₄⁺ feeding, Aₜₙₑᵦ, Φₚₛₛᵦ, and 1–qP were not significantly different between ucp1, aox1a, and WT plants across all irradiances. There was a significant oxygen response of Aₜₙₑᵦ but not Φₚₛₛᵦ and 1–qP for all three genotypes regardless of N form (Fig. 4). In WT plants Aₜₙₑᵦ and Φₚₛₛᵦ were higher while 1–qP was lower under NO₃⁻ compared with NH₄⁺ feeding across all pO₂; however, there was no difference in these parameters between N form in the ucp1 and aox1a plants (Fig. 4). Additionally, there was a significant difference in Aₜₙₑᵦ, Φₚₛₛᵦ, and 1–qP between WT and both mutant lines (ucp1 and aox1a) at all pO₂ under NO₃⁻ but not NH₄⁺.

The rate of linear electron transport estimated from chlorophyll fluorescence (Jₑ) was compared with the electron transport demand required to sustain rates of CO₂ assimilation and photorespiration (J₉). The relationship between Jₑ and J₉ is linear in response to irradiance and did not differ between NO₃⁻ and NH₄⁺ feeding for all three genotypes (Fig. 5A). Additionally, the relationship between Jₑ and J₉ under NO₃⁻ feeding was similar among all three genotypes in response to pO₂; however, under NH₄⁺ feeding, there was a significantly higher slope in the ucp1 plants (1.18) compared with aox1a (0.95) and WT (0.97) plants (Fig. 5B).

**Discussion**

The disruption of mAET had significant impacts on both foliar N and carbon metabolism in A. thaliana. For example, the loss of the uncoupling protein UCP1 or the alternative oxidase AOX1a increased rates of foliar NO₃⁻ assimilation compared with WT plants. Additionally, Aₜₙₑᵦ was lower in ucp1 plants compared with the WT and aox1a lines in NO₃⁻-fed plants. However, under NH₄⁺, rates of Aₜₙₑᵦ were not significantly different between genotypes. As discussed below, these data demonstrate that UCP1 and AOX1a are important for balancing the energy partitioning between N and carbon metabolism.

**Disruption of alternative mitochondrial electron transport enhances foliar nitrate assimilation**

The rates of foliar NO₃⁻ assimilation were higher in the aox1a and ucp1 plants compared with the WT under the non-saturating growth light (160 μmol quanta m⁻² s⁻¹) conditions (Fig. 1). Low light conditions would probably decrease the amount of reductant exported from the chloroplast, and the competition for reductant between NO₃⁻ assimilation and mitochondrial electron transport would be high. Under these conditions, the availability of NADH for the cytosolic conversion of NO₃⁻ to NO₂⁻ and the chloroplastic reduction of NO₂⁻ to NH₄⁺ by ferredoxin would be limiting. Therefore, the increase in NO₃⁻ assimilation in the ucp1 and aox1a plants under low light is probably attributed to an increased availability of cytosolic reductant because of decreased consumption by the mitochondria. The increased NO₃⁻ assimilation in the ucp1 and aox1a plants also corresponded to a higher amino acid content (Fig. 2, Supplementary Table S1 at JXB online) and lower total leaf carbon to N ratio as previously observed in tobacco plants deficient in the mitochondrial complex I (CMS mutant) (Dutilleul et al., 2005) and in AOX1a (Watanabe et al., 2008). Alternatively, at saturating light (1000 μmol quanta m⁻² s⁻¹), the export of excess reductant from the chloroplast into the cytosol will be high and will not limit rates for NO₃⁻ assimilation. In fact under high light, there was only a small increase in NO₃⁻ assimilation with the
loss of UCP1 and there was no change in NO$_3^-$ assimilation with the absence of AOX1a.

Generally, the effect of lack of UCP1 on N metabolism was more marked than that of the lack of AOX1a. The expression of UCP1 has been shown to be up-regulated in the aox1a mutant (Watanabe et al., 2008, 2010). Given the potentially overlapping function of these two respiratory components, UCP1 may partly compensate for the loss of AOX1a, which would therefore diminish the impact of the genetic manipulation. However, AOX content has been shown to decrease in the ucp1 mutant (Sweetlove et al., 2006). The higher rates of foliar NO$_3^-$ assimilation in the ucp1 and aox1a plants also require an increase in carbon skeletons for the de novo synthesis of amino acids. The present results show a significant increase in aspartate and asparagine levels in mutants compared with the WT. The aspartate pathway drives the synthesis of several amino acids that may contribute to generate additional energy under stress conditions (Galili, 2011). This pathway has been suggested to operate in combination with the TCA cycle in inducing the catapleurotic fluxes with energy deprivation conditions. However, anapleurotic fluxes can also supply amino acid synthesis with carbon skeletons. In aox1a and ucp1 mutants, it is likely that asparagine family synthesis was driven by reductant accumulation and carbon skeleton availability leaking out of the TCA cycle. It has been demonstrated that the carbon needed for the synthesis of amino acids comes primarily from the partial operation of the TCA cycle (Chen and Gadot, 1990; Tcherkez et al., 2009; Sweetlove et al., 2010). Additionally, it has been reported that there is an increase in the anaplerotic production of carbon skeletons through an incomplete TCA cycle with increased NO$_3^-$ assimilation (Scheible et al., 1997; Stitt, 1999). Therefore, the increase in NO$_3^-$ assimilation and overall amino acid content observed in the ucp1 and aox1a lines probably increased the TCA production of carbon skeletons. This would further increase NADH production available via the malate shuttle for de novo NO$_3^-$ assimilation.

Root NO$_3^-$ assimilation was not altered by the loss of UCP1 and AOX1a, suggesting a more important role for AOX1a and UCP1 in photosynthetic tissue. This is supported by 6-fold higher AOX1a transcripts in the shoot compared with the root in A. thaliana; however, the UCP1 expression level is similar between root and shoot tissues (Watanabe et al., 2010). Furthermore, the AOX1d transcript level is enhanced in the root of aox1a mutants, but not in the shoot, suggesting a potential compensatory effect.
for the loss of AOX1a in the roots (Watanabe et al., 2010). Taken together, AOX1a and potentially UCP1 appears to have a more predominant role in shoot than root tissues and plays an important role in regulating de novo shoot assimilation of NO3⁻, particularly when the reductant availability is limiting.

*Alternative mitochondrial electron transport and nitrate assimilation synergistically optimize photosynthesis*

The present results demonstrate that both NO3⁻ assimilation and mAET optimize rates of photosynthetic CO2 assimilation; however, independently neither is sufficient to influence A_{net}. For example, A_{net} was higher under NO3⁻ compared with NH4⁺ feeding in WT plants but not in the aox1a and ucp1 mutants, despite increased NO3⁻ assimilation in these plants. Additionally, under NH4⁺ feeding, there was not a significant difference in A_{net} between the WT and the two mutant lines (ucp1 and aox1a). This suggests that the increase in A_{net} under NO3⁻ is dependent on functional AOX1A and UCP1, and that NO3⁻ assimilation alone is insufficient to alter A_{net}. Furthermore, the loss of AOX1A and UCP1 had no effect on A_{net} or leaf photochemistry under NH4⁺.

In WT plants, A_{net} and \( \phi_{PSII} \) were 12–17\% higher under NO3⁻- compared with NH4⁺-fed plants, respectively. An increase in A_{net} and \( \phi_{PSII} \) under NO3⁻ versus NH4⁺ feeding has been previously described in barley (Bloom et al., 1989), wheat (Bloom et al., 2002), tomato (Searles and Bloom, 2003), and maize (Cousins and Bloom, 2003). The foliar reduction of NO3⁻ to NH4⁺ requires cytosolic NADH, typically generated from reductant exported from the chloroplast, and ferredoxin within the chloroplast. Therefore, NO3⁻ assimilation may compete for reductant with Rubisco-mediated assimilation of CO2 (Bloom et al., 2002). However, under the conditions used here and as previously reported (Bloom et al., 1989, 2002; Cousins and Bloom, 2003; Searles and Bloom, 2003), the rates of A_{net} were higher in NO3⁻- versus NH4⁺-fed plants. This suggests that the consumption of reductant via NO3⁻ assimilation stimulates rates of net CO2 assimilation and \( \phi_{PSII} \), probably through optimizing the ATP/NADPH production within chloroplasts. Additionally, the present data indicate that NO3⁻ assimilation contributes to protect the chloroplast electron transport chain from over-reduction and therefore ensures optimal rates of CO₂ assimilation.

The measured A_{net} was lower in NO3⁻-fed ucp1 mutants compared with WT plants; however, there was no difference...
in $A_{\text{net}}$ between ucp1 and WT plants under NH$_4^+$ feeding (Fig. 3). The decrease in $A_{\text{net}}$ seen under NO$_3^-$ feeding is consistent with these plants grown in soil as reported by Sweetlove et al. (2006). These authors attributed the decrease in $A_{\text{net}}$ in the ucp1 compared with the WT to a restricted flux through the photorespiratory pathway and an associated limited regeneration of ribulose-1,5-bisphosphate. A decrease in the glycine to serine conversion could affect methionine synthesis through C1 metabolism. Both serine and methionine are precursors of cysteine synthesis, which is at a low level in ucp1 compared with the WT. These data are similar to those of Sweetlove et al. (2006), who also showed a lower amount of glycine and serine in the ucp1 mutant compared with the WT (Fig. 2). However, it was found that the difference in $A_{\text{net}}$ between ucp1 and the WT was constant in response to O$_2$ availability (from 1.9 kPa to 46.6 kPa O$_2$). This suggests that UCP1 optimizes $A_{\text{net}}$ regardless of rates of photorespiration and probably plays an important role in balancing the energy supply with demand between N and carbon metabolism within the leaf. The N feeding experiments demonstrated that NO$_3^-$ assimilation could compensate for the lack of excess reductant consumption by the mitochondria in the absence of UCP1. However, $A_{\text{net}}$ was not enhanced in aox1a and ucp1 mutants fed with NO$_3^-$ as seen in WT plants, suggesting that the stimulation of CO$_2$ fixation by NO$_3^-$ assimilation is dependent on a fully functional mAET.

In the ucp1 mutants fed with NH$_4^+$, the electron production by the chloroplastic electron transport chain increased significantly with decreasing oxygen compared with the electron demand for $A_{\text{net}}$ and photorespiration (Fig. 5B). This shift between electron production and demand indicates extra electron transport to alternative chloroplastic sinks such as the Mehler reaction. In addition to the Mehler reaction, other alternative electron sinks such as the cyclic electron flux and chlororespiration have been reported to optimize ATP synthesis, balance the production of the chloroplastic ratio of ATP/NADPH, and avoid over-reduction of the photosynthetic electron transport chain (Johnson, 2005). In ucp1 mutants fed NH$_4^+$ under non-photorespiratory conditions, the change in $J_f$/$J_g$ suggests that the Mehler reaction has a greater influence on linear electron flow compared with other genotypes and treatments. This would avoid excess NADPH accumulation within the stroma due to the loss of two other major electron sinks (mitochondria alternative pathways and NO$_3^-$ assimilation) while still maintaining rates of linear electron transport.

### Conclusion

In summary the importance of mAET in foliar de novo NO$_3^-$ assimilation in A. thaliana, particularly under conditions that limit reductant availability (low light), is demonstrated.
in the present work. Additionally, the data show that both mAET and NO\textsubscript{3} assimilation influence rates of photosynthetic CO\textsubscript{2} assimilation and electron transport. mAET and NO\textsubscript{3} assimilation appear to function synergistically to avoid excess reduc tant accumulation and over-reduction of the chloroplast. Finally, the data demonstrate that mitochondrial respiration significantly contributes to the energy balancing between N and carbon metabolism.

**Supplementary data**

Supplementary data are available at JXB online.

**Figure S1.** Rates of NO\textsubscript{3} uptake and assimilation, and free NO\textsubscript{3} content in wild-type shoots and roots of A. thaliana fed with \textsuperscript{15}NO\textsubscript{3} for 3, 6, or 9 h.

**Table S1.** Amino acid levels (nmol mg\textsuperscript{-1} DW) in shoots of wild-type, aox1a, and ucp1 Arabidopsis thaliana fed either NO\textsubscript{3} or NH\textsubscript{4} as sole N source.

**Figure S2.** Rates of NO\textsubscript{3} uptake and assimilation, and free NO\textsubscript{3} content in wild-type, aox1a, and ucp1 roots of A. thaliana fed with \textsuperscript{15}NO\textsubscript{3} for 6 h.

**Acknowledgements**

The authors thank Charles A. Cody for his technical assistance with growth chambers, Professor James Whelan (University of Western Australia) for supplying seeds of the aox1a line, and Professor Sanja Roje for her help with the amino acid analysis.

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