Short title: Variation in Mature Leaf Respiration at Night

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Article Title: Variation in Leaf Respiration Rates at Night Correlates with Carbohydrate and Amino Acid Supply

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One Sentence Summary:
Mature Arabidopsis leaves display substantial variation in night-time respiration rates that is metabolically linked to daytime carbon and nitrogen assimilation but not night-time protein synthesis.

Author Contributions:
B.M.O, O.K.A and A.H.M. conceived of the research plans. B.M.O., and C.P.L. performed the experiments. B.M.O., R.C. and T.B.B. analysed the data. B.M.O., O.K.A. and A.H.M wrote the article.

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Abstract

Plant respiration can theoretically be fuelled by and dependent upon an array of central metabolism components; however, which ones are responsible for the quantitative variation found in respiratory rates is unknown. Here, large scale screens revealed twofold variation in night-time leaf respiration rate \( (R_N) \) among mature leaves from an Arabidopsis natural accession collection grown under common favourable conditions. \( R_N \) variation was mostly maintained in the absence of genetic variation, which emphasized the low heritability of \( R_N \) and its plasticity towards relatively small environmental differences within the sampling regime. To pursue metabolic explanations for leaf \( R_N \) variation, parallel metabolite level profiling and assays of total protein and starch were performed. Within an accession, \( R_N \) correlated strongly with stored carbon substrates, including starch and dicarboxylic acids, as well as sucrose, major amino acids, shikimate and salicylic acid. Among different accessions metabolite-\( R_N \) correlations were maintained with protein, sucrose and major amino acids but not stored carbon substrates. A complementary screen of the effect of exogenous metabolites and effectors on leaf \( R_N \) revealed that i) \( R_N \) is stimulated by the uncoupler FCCP and high levels of substrates, demonstrating that both adenylate turnover and substrate supply can limit leaf \( R_N \), and ii) inorganic nitrogen did not stimulate \( R_N \), consistent with limited night-time N assimilation. Simultaneous measurements of \( R_N \) and protein synthesis revealed that these processes were largely uncorrelated in mature leaves. These results indicate that differences in preceding daytime metabolic activities are the major source of variation in mature leaf \( R_N \) under favourable controlled conditions.
Introduction:
Few plant metabolic fluxes are readily accessible to routine measurement. The major exceptions to this are fluxes involving gas exchange, such as respiration and photosynthesis. Measurements of respiratory gas exchange (i.e. mitochondrial O$_2$ uptake or CO$_2$ release in the absence of photorespiration) are useful from a metabolic perspective because they can be interpreted in terms of the underlying carbon fluxes and the generation of ATP by oxidative phosphorylation (Sweetlove et al., 2013). The biochemical reactions of respiration that produce CO$_2$ and lead to O$_2$ consumption are well understood (Plaxton and Podestá, 2006; Sweetlove et al., 2010; Millar et al., 2011; Tcherkez et al., 2012). The stoichiometries of the reactions and the connections between them are known, which provides us with a metabolic map of respiration that includes glycolysis, the oxidative pentose phosphate pathway (OPPP), the citric acid cycle (TCA cycle), the mitochondrial electron transport chain (mETC), ATP synthase and several other surrounding reactions. In plants, carbohydrates are the dominant respiratory substrates whereas lipids are rarely respired (Plaxton and Podestá, 2006). Carbohydrate oxidation proceeds via organic acids which serve many purposes in plant cells. Two metabolic fates for organic acids are intimately linked to respiration: i) further oxidation to CO$_2$ via the TCA cycle providing reductant to the mETC and fuelling oxidative phosphorylation; and, ii) the provision of carbon skeletons needed for the assimilation of nitrogen into amino acids. Because the metabolic pathways supporting ATP production and carbon skeleton production for amino acid biosynthesis largely overlap, respiration is considered to be fulfilling both functions simultaneously in plants. How these two processes are regulated to meet changing cellular demands and integrated diurnally with photosynthesis (carbon reduction) lies at heart of understanding plant primary carbon metabolism.

As an easily measurable metabolic flux, determining how variation in rates of leaf respiration are linked to genetics, development, metabolite levels and enzymes activities is also an opportunity to better predict rates of carbon use in plants. Determinants of respiration have been widely studied in plants, motivated by the need to: i) better understand plant growth and performance in variable environments; ii) model plant growth in managed and natural ecosystems; and, iii) predict the impact of future climate change on carbon exchange between vegetation and the atmosphere (Leakey et al., 2009). The fraction of daily fixed carbon respired is substantial (varying from 20-80%, depending on the species), with around half of whole plant respiration taking place in leaves (Atkin et al., 2007). Thus, variations in the rate of leaf respiration are quantitatively important, not only for individual plants, but also for issues such as the speed and scale of future climate warming. Because of this, variations in respiration need to be accounted for in model frameworks, including those designed to model crop and natural ecosystem metabolic fluxes. Currently in such models,
respiration is commonly predicted as a scalar of photosynthesis or nitrogen content (Ryan, 1991; Cannell and Thornley, 2000; Reich et al., 2008; Atkin et al., 2015). However, the causal mechanisms of these relationships are not well described nor are they thought to adequately capture the complex 16-fold variation in respiration rate observed from leaves of differing genetic, environmental and developmental backgrounds (Wright et al., 2004; Wright et al., 2006; Reich et al., 2008; Atkin et al., 2015). Importantly, much of the total variation in leaf respiration persists among co-occurring species within one environmental site (Atkin et al., 2015), suggesting that genetics strongly determines respiratory flux. Understanding the mechanistic basis of genotypic and environmental variability in leaf respiration is needed if the predictive capacity of crop/natural ecosystem models is to be improved.

Conceptualization of variation of respiration in plants typically leads to the proposal that changes in respiratory rate are primarily due to altered supply of substrates or altered demand for respiratory products, namely, ATP and carbon skeletons (Cannell and Thornley, 2000; Noguchi, 2005). Changes in environment can also cause an adjustment (i.e. acclimation) of total respiratory capacity (Leakey et al., 2009). However, respiratory metabolism is not thought to be limited by enzyme capacity at warm temperatures in vivo because respiration of harvested tissue or isolated mitochondria can be stimulated to run faster (Atkin and Tjoelker, 2003). The demands on respiration are often categorized by the terms growth or maintenance respiration (Thornley, 1970; Amthor, 2000; Thornley, 2011) to help conceptualize the different usage of respiratory derived ATP for macromolecule biosynthesis in growing vs full-grown tissues. However, subdivision of ATP usage in any form suffers from a difficulty in making any confirmatory empirical measurements. This is especially troublesome in plants where the ATP yield from respiration can vary dramatically depending on the relative activity of cytochrome vs. alternative pathways in the mETC (Millar et al., 2011). Furthermore, the demands which can be placed upon respiration are not equally distributed throughout the night and day (Cannell and Thornley, 2000). For example, in leaves, the energy costs closely associated with nitrogen assimilation and amino acid synthesis are mostly borne by photosynthesis in the day and protein synthesis is also greater during the day (Matt et al., 2001; Pal et al., 2013). Overall, more rigorous experimentation involving novel lines of empirical evidence are needed to support advancement in modelling plant respiration and quantitatively assessing its determinants.

For this study, we have specifically chosen to study leaf respiration rates at night ($R_N$), rather than dark respiration rates ($R_d$) measured during the day under artificial darkness. Arabidopsis leaves undergo diurnal cycles of carbohydrate, amino acid and organic acid accumulation and leaf primary metabolism is strongly regulated in a diurnal fashion. Therefore, it is likely a source of error to
consider that the metabolic status underlying $R_d$ measurements is representative of true night-time metabolism (Florez-Sarasa et al., 2012). No extensive measurement of leaf night-time metabolic fluxes (e.g. metabolic flux analysis) has been performed; however, Cheung et al. (2014) proposed a model based on flux balance analysis to reconcile observations from day and night leaf metabolism. From the model and much additional research, the following examples of important features of leaf night-time metabolism can be deduced. Firstly, night-time respiration is thought to function largely in an energy generating capacity involving a cyclic flux through the TCA cycle (Sweetlove et al., 2010; Cheung et al., 2014). Secondly, leaves continuously export sucrose and amino acids throughout the diurnal cycle to support the growth of heterotrophic tissues at a substantial cost of ATP (Bouma et al., 1995; Kallarackal et al., 2012). Thirdly, assimilation of nitrogen and thus de novo amino acid synthesis at night is greatly reduced in leaves because nitrate reduction is low to nil and ammonium assimilation is also less than in daytime (Canvin and Atkins, 1974; Matt et al., 2001; Nelson et al., 2014). Lastly, night-time metabolism appears synchronized to the amount of carbohydrate (e.g. starch) stored during the day, such that when demand is sufficient, starch reserves are metabolized evenly through the night and set to be nearly exhausted at dawn (Graf et al., 2010). What is not clear, however, is how the above factors combine to account for the often reported variation in respiratory rates seen in controlled environment and field-based studies (Atkin et al., 2015).

Cellular respiration measurements in plants have traditionally been performed using $O_2$ electrodes to measure $O_2$ consumption or infra-red gas analyzers to measure CO2 evolution. Mass spectrometry based measurements are also performed and have a particular use in analysing $O_2$ isotope discrimination by the cytochrome versus the alternative pathways of the mitochondrial electron transport chain (Cheah et al., 2014). Though providing robust measurements of respiration, these procedures are not high-throughput and this has limited the scope of experiments aimed at better understanding plant respiration. Recently fluorometric $O_2$ sensors have been utilised in multiplexed experiments to provide measurement of changes in $O_2$ concentration in solution (Sew et al., 2013) and in gas phase (Scafaro et al., 2017). Here, our study used high-throughput fluorometric measurements to perform large scale surveys of leaf $R_n$ amongst Arabidopsis accession populations grown under a common favourable environment. We evaluate the contribution of genetic, environmental and developmental differences to the observed variation in respiration rates. By coupling $R_n$ measurements to an extensive metabolomic analysis, we analysed key patterns of metabolite correlation with and stimulation of $R_n$ and discuss the degree to which substrate supply or output demand is driving variation in leaf respiration at night.
Results

Diurnal and developmental standardization of leaf tissue selection

The technical requirements for a robust high-throughput O$_2$ consumption rate measurement are linearity with time, a high signal-to-noise ratio and rapid sample preparation. Oxygen consumption measurements of leaf discs performed in air by the Q2 fluorophore based oxygen sensor satisfy these requirements. Figure 1A shows a representative O$_2$ consumption curve for a vial containing three Arabidopsis leaf discs (1 cm$^2$ total) harvested at night, and an empty control vial. From 0.5 to at least 3 h after sealing the vials, oxygen depletion was essentially linear with time; therefore, this timespan was selected for future gas phase respiration measurements. Experiments relying on exogenous chemical additions utilized vials containing single leaf discs floated on top of respiration buffer. In the absence of added metabolites, these measurements were also linear with time between 0.5 and 3 h (Fig. 1B). The addition of respiratory inhibitors KCN plus SHAM (0.4 and 20 mM, respectively) reduced leaf O$_2$ consumption by 90±1% (n=12; Fig. 1C). The O$_2$ depletion within the measurement vials was therefore assumed to be due almost entirely to leaf respiration and will henceforth be referred to as night respiration (R$_n$).

To minimize diurnal and developmental variation of R$_n$ in our screens, we standardized the leaf tissue to be routinely harvested. As shown previously, Arabidopsis leaf respiration rates were significantly higher if the leaf discs contain the mid-vein but were otherwise similar across the rest of the leaf blade (Supplemental Fig. 1) (Sew et al., 2013). Therefore, all experiments used leaf discs excised from either side of the mid-vein. Arabidopsis leaf R$_n$ also varied throughout development, gradually decreasing with leaf age (Supplemental Fig. 1). Leaf selection was standardized by harvesting from the youngest four leaves that had reached the outside edge of each rosette (e.g. leaves 7-10 in Supplemental Fig. 1). Lastly, a time course experiment was performed to assess variation in R$_n$ throughout the night (Fig. 2). Leaf disc samples harvested from the Arabidopsis accession Landsberg erecta, but not Col-0, showed significant differences in R$_n$ between a maximum at 6 hours and a minimum 10-12 hours into the 16 h night. However, in both cases R$_n$ was relatively stable between 1 and 4 hours into the night. Therefore, leaf tissues were routinely harvested between 2 - 4 hours into the dark period and our R$_n$ measurements were assumed to be independent of sampling time.

Screens of respiration rates and growth rate in Arabidopsis natural accessions
A sequence of three independent respiration screens was performed on Arabidopsis plants grown under common favourable conditions in single growth cabinets to minimize environmental variation. Accession screen 1 and accession screen 2 both involved collections of Arabidopsis accessions while the third Col-0 screen included only the accession Columbia-0 (see Materials and Methods and
Supplemental Table 1 for details of accessions included within each screen). In each screen four sets of leaf discs from four mature leaves from each harvested plant were assayed for \( R_N \) (Table 1). Directly afterwards, the fresh mass of each set of leaf discs was measured. Test measurements performed before and after the approximately 3 h period of \( R_N \) measurement showed that leaf disc fresh mass did not significantly change during this time (data not shown). Subsequently, total soluble protein content for each set of leaf discs was measured. The mean values for each plant were calculated and the correlations between plant mean values within each screen are summarized in Table 1. The observed area-based rates of \( R_N \) approximated a normal distribution (Shapiro-Wilk; \( p>0.05 \)) and exhibited a 2.3-fold, 2.0 fold and 2.0 fold range between the highest and lowest respiring plants in the accessions screens 1 and 2 and the Col-0 screen, respectively. There was a consistent positive correlation between area-based rates of \( R_N \) and protein amount.

Several findings support the view that the variation in \( R_N \) observed in the screens was mostly not due to genetic differences among accessions. In accession screen 2, two ecotypes were highly replicated, Col-0 (n=17) and Ag-0 (n=11). By comparing the distribution of \( R_N \) measurements within and between accessions the heritability (\( H^2 \)) of area-based and mass-based \( R_N \) under the growth conditions of our screen was estimated to be 0.29 and 0.30 respectively; indicating the proportion of variation due to genetic differences. Furthermore, there was low reproducibility of the relative respiratory performance of genotypes across the two accession screens: there was no correlation among area- or mass-based \( R_N \) values or the relative rank of matching accession measurements between accession screens 1 and 2. A genome wide association study to examine the causal genetic loci was performed for both screens. However, they did not identify any significant genetic loci as determinants of \( R_N \) rates, likely due to a lack of power given the small degree of heritability of the trait. Lastly, the Col-0 screen which does not include genetic variation displays a similar overall level of variation in \( R_N \) as screens 1 and 2 (Table 1).
The rate of change in total plant leaf area was also captured by photography during the first 35 days of growth in accession screen 2. Beyond 35 days, leaf overlap within and between pots precluded calculations of total leaf area. Notwithstanding changes occurring between dates of measurement, there were no strong correlations ($r^2>0.05$) between area-based rates of $R_n$ and total leaf area, leaf area expansion rate or relative leaf area expansion rate (Supplemental Table 2).
Metabolite levels correlate strongly with R\textsubscript{N}

To evaluate the relationship between metabolite levels and R\textsubscript{N}, metabolomic analyses were performed on leaf disc samples which matched those used in each respiration screen. A subset of 80 plants in screens 1 and 2 were chosen to reflect the range of R\textsubscript{N} rates observed. Twenty-one plants were selected from the Col-0 screen. From each plant, four samples of leaf discs were subjected to targeted GC-MS metabolite analysis and the relative metabolite abundance data was averaged for each plant and log transformed. Many metabolites levels correlated positively with area-based rates of R\textsubscript{N} and the highest correlations observed from each screen are shown in Table 2. The full dataset of metabolites and correlations is shown in Supplemental Table 3. Most metabolite-R\textsubscript{N} correlations were stronger in the Col-0 screen, which indicated that intra-species Arabidopsis genetics affected some metabolite-R\textsubscript{N} relationships. However, there were obvious similarities across screens, as several metabolites, in particular alanine, pyroglutamate, glutamic acid, glutamine, sucrose and gluconic acid, were consistently among the strongest correlating of compounds from each screen. Note that pyroglutamate is formed from the cyclization of either Glu or Gln and thus represents a combination of these amino acids. No significant negative correlations (r<-0.25) were identified in accession screens 1 or 2 but in the Col-0 screen three unidentified compounds (likely di saccharides or trisaccharides) showed significant negative correlations with R\textsubscript{N} (Supplemental Table 3).

The amino acid alanine displayed the most consistently strong correlation with R\textsubscript{N} across all screens (Table 1, Fig. 3). Alanine levels are often considered to be tightly linked with pyruvate levels via alanine aminotransferase (Miyashita et al., 2007). However, correlations between alanine and respiration were consistently much stronger than the correlations between pyruvate and R\textsubscript{N}, thus indicating a dis-equilibrium or distinct subcellular localization between pyruvate and alanine pools in the leaf cells (Fig. 3 and Supplemental Table 3).

As the major storage carbohydrate in Arabidopsis leaves, it was also of interest to determine the relationship between starch and R\textsubscript{N}. Starch assays were performed on sets of four replicate leaf samples from each of 57 plants from accession screen 1, and 41 plants of the Col-0 screen. Starch levels among Col-0 plants during the early night correlated strongly with R\textsubscript{N} ($r^2$=0.73) (Fig. 3). Whereas, among different accessions, starch levels were only weakly correlated with R\textsubscript{N} ($r^2$=0.07).

Using multiple linear regression analysis, it was calculated that protein and Alanine amounts were consistently the two strongest predictors of R\textsubscript{N} in the inter-accession screens and together with fresh mass they explained 63% and 65% of the variation in screens 1 and 2, respectively ($p$<0.001). In the Col-0 screen, starch was the strongest single metabolic predictor of R\textsubscript{N}, while alanine was the most significant additional predictor increasing the variation explained to 80%. However, in all cases,
rigorous interpretation of the multiple linear regression analysis was not possible because of the high amounts of co-variance among metabolite, starch and protein levels.

Exogenous substrates can rapidly stimulate leaf respiratory rate

The strong correlations of some metabolites with $R_N$ could be related to substrate supply. It was therefore tested whether exogenous addition of metabolites could stimulate $R_N$ in Arabidopsis leaf discs. Respiratory $O_2$ consumption measurements were performed as above but with single leaf discs of the accession Col-0 floating on 10 mM or 100 mM buffered metabolite solutions (Fig. 4). At the high 100 mM exogenous substrate concentration, many but not all metabolites tested had a stimulatory effect on $O_2$ consumption, including several amino acids, carbohydrates and organic acids. At the lower concentration of 10 mM no metabolites displayed a significant stimulation of $R_N$. Incubations with external nitrogen sources consisting of 10 mM KNO$_3$, or 1-10 mM NH$_4$Cl were also performed but had no significant effect on $R_N$ (Fig. 4). For selected metabolites of interest additional concentrations were also assayed (Supplemental Fig. 2). These results clearly demonstrate that some metabolites have a concentration dependent stimulatory effect on night-time leaf $O_2$ consumption. No metabolite solution included in the study displayed $O_2$ consumption in the absence of leaf tissue and all metabolite solutions were sensitive to the respiratory inhibitors cyanide and SHAM, indicating that any stimulation of $O_2$ depletion was mostly attributable to $R_N$. As differences in metabolite uptake rate or endogenous metabolite levels were not accounted for, comparisons between metabolite stimulatory effects were not performed.

The uncoupler FCCP rapidly stimulates night respiration rate

Compounds which dissipate the electrochemical proton gradient across the mitochondrial inner-membrane (known as uncouplers) have frequently been used to assess whether $O_2$ consumption
rate is limited by being coupled to oxidative phosphorylation of ADP to ATP by ATP-synthase. Here, the uncoupler FCCP stimulated Arabidopsis leaf \( R_N \) in a rapid and concentration dependent manner (Fig. 5). This stimulation indicates that limited ADP supply to ATP synthase is contributing to restricting respiration rate (often referred to as adenylate control of respiration). In the presence of
2 µM FCCP, the addition of 100 mM of stimulatory metabolites led to significant further stimulations of respiration in all cases tested except for Ala (Fig. 5). Ser and Thr were not significantly stimulatory on their own or in the presence of FCCP. Compounds which increase respiration in the presence of FCCP are likely acting upstream of the mETC either as respiratory substrates or activators of oxidative metabolism. Conversely, the initial stimulation of respiration by 100 mM Ala in the absence of FCCP appears to be due to effects downstream of the mETC, presumably by increasing ATP turnover.

The relationship between $R_n$ and protein synthesis
We used cycloheximide, an inhibitor of cytosolic ribosomal translation, to evaluate the role of protein synthesis in determining \( R_N \). However, the results were uninterpretable as cycloheximide on its own stimulated \( R_N \) in a time and concentration dependent fashion (Supplemental Fig. 3). This effect of cycloheximide have been documented previously in certain tissues but the mechanism is unknown (Ellis, 1970; McMahon, 1975).

Instead, further investigations into the relationship between night-time protein synthesis and respiration were performed by measuring \( O_2 \) consumption of leaf discs floating on respiration buffer containing 1 µM of \(^{14}\text{C}-\text{Leu} \) (0.1 µCi). The incorporation of exogenous radiolabelled \(^{14}\text{C}-\text{Leu} \) into protein which occurred during the respiration assay was taken as a simultaneous measure of protein synthesis rate. Averaged measurements of four leaf disc samples from 24 mature leaves of different Col-0 plants revealed that there was only a weak positive correlation between respiration and protein synthesis (Fig. 6). Young developing Arabidopsis leaves are known to have higher rates of protein synthesis per area than mature leaves (Ishihara et al., 2015), which is thought to contribute to higher rates of respiration. In a comparison between developing and mature leaves from the accession Col-0, the simultaneous measurements clearly revealed that developing leaf blade tissue has higher rates of both protein synthesis and respiration (Fig. 6).
Discussion:

Substantial variation in leaf respiratory rates is maintained in a common environment. Respiratory flux of leaves can be expected to vary depending on the environment, tissue, developmental stage, time of day and the species being studied (Atkin et al., 2015). We have sought
to limit as many of these factors as possible in order to consider the role of metabolic determinants under controlled, non-stressful conditions. We identified and attempted to eliminate variation in respiration that was associated with time of night, leaf blade location and leaf developmental stage. We did not detect a correlation between the total leaf area expansion rate for a plant and the respiratory rate of its developed leaf blades, suggesting that variations in shoot growth rate do not have a direct effect on respiratory rates of mature leaves. However, unintended differences in the growth rate of individual leaves, which were not captured by our total plant growth measurements, remains a possible source of variation in $R_N$. The final two-fold variation in mature leaf blade $R_N$ observed amongst Arabidopsis accessions was only weakly associated with genetic differences and thus could be mostly reproduced in a genetically uniform population of Col-0 plants. The low heritability of $R_N$ determined here (0.31) is consistent with previous low measures of leaf respiration heritability in _Hordeum spontaneum_ (Poorter et al., 2005). In comparison, a much greater amount of the variation could be linked to metabolic status which provides a distinct perspective on the source of variation in $R_N$.

**Correlations between carbon substrates and $R_N$**

Respiration has often been described as being driven by substrate supply or ATP demand (Cannell and Thornley, 2000). Using simple chemical and substrate treatments of leaf discs we could show that, contrary to some previous observations (Noguchi, 2005; Li et al., 2013) leaf $R_N$ was rapidly stimulated in situ by both high levels of several carbon substrates and the uncoupler FCCP (Fig. 4 and Fig. 5). Thus, it is experimentally possible to demonstrate in situ control of respiration by both substrate supply and adenylate restriction. However, the effect of substrate supply on respiration was concentration dependent, and it is probable that the levels of some exogenous metabolites needed to promptly stimulate respiration are not encountered in vivo. Furthermore, not all compounds that stimulated $R_N$ necessarily acted solely as respiratory substrates. For example, stimulation by Ala was sensitive to the uncoupler FCCP, indicating that Ala stimulates ATP consumption. Nevertheless, as the stimulations of many potential substrates were qualitatively additive to the stimulation caused by FCCP (Fig. 5), the conclusion remains that Arabidopsis leaf $R_N$ can, in practice, be stimulated by substrate supply.

In the early night, following a day of favourable photosynthetic conditions, carbohydrates and organic acids are plentiful and constitute the main stores of carbon fuelling $R_N$ (Plaxton and Podestá, 2006). Robust correlations between carbohydrates and $R_N$ have been observed several times previously, following experiments which subjected plants to varying photosynthetic conditions.
during the preceding light period (Azcon-Bieto et al., 1983; Noguchi, 2005; Florez-Sarasa et al., 2012; Peraudeau et al., 2015). In addition to starch, Arabidopsis plants also accumulate the dicarboxylic acids fumarate and malate during the day (Pracharoenwattana et al., 2010). Here, following relatively uniform photosynthetic conditions, we also observe that stored carbon substrates for respiration like starch, the starch breakdown product maltose, malate and to a lesser extent fumarate are all strongly correlated with $R_w$ and could explain the bulk of variation in $R_w$ among Col-0 plants. The levels of succinate, fumarate and malate displayed strong co-variance with starch ($r^2 = 0.72, 0.63$ and $0.50$ respectively; Supplemental Table 3) which likely results because of their mutual dependence of photosynthetic carbon fixation (Pracharoenwattana et al., 2010). Sucrose also displays a consistent correlation with $R_w$; however, sucrose is unlikely to represent a major respiratory substrate in leaves because it is actively synthesized and exported from leaves at night and respiration of newly synthesized sucrose would invoke a wasteful (futile) cycle of ATP hydrolysis, though it may still occur to some degree. Rather, sucrose synthesis and export represents a substantial ATP cost borne by leaves at night, previously estimated to account for 29% of leaf $R_d$ (Bouma et al., 1995). Therefore, a strong case can be made that $R_w$ variation is largely related to photosynthetically produced substrate availability and this is discussed in the context of diurnal metabolism below.

**Protein synthesis is not a major determinant of mature Arabidopsis leaf $R_w$**

The metabolite group which correlated most strongly and consistently with $R_w$ across all screen were the major (i.e. abundant) amino acids: Glu, Asp, Ala, Gln and Thr. Through our experiments we can evaluate three possible causal reasons for these correlations: substrate supply, allosteric regulation and respiratory costs associated with amino acids.

Firstly, amino acids in leaves are not known to be oxidized for respiration except during carbon starvation or senescence (Hildebrandt et al., 2015). Given that stores of starch and organic acids remain at early points in the night, it is unlikely that amino acids would represent major substrates for the night respiration measurements performed in this study. Secondly, amino acids can also act as potent allosteric regulators of respiratory enzymes. In particular, Glu and Asp are potent allosteric effectors of the key regulatory glycolytic enzymes phosphoenolpyruvate carboxylase and cytosolic pyruvate kinase. However, the pattern of known regulatory effects of Asp and Glu and other amino acids on respiratory enzymes would not explain the consistently positive correlation of amino acids with $R_w$ (O’Leary and Plaxton, 2015). Thirdly, there may be respiratory costs associated with amino acids: specifically rates of i) amino acids synthesis, ii) amino acid export
or, iii) protein metabolism. In leaves the rate of inorganic N assimilation into amino acids and the associated costs of ATP and carbon skeletons are reduced at night compared to the daytime. This is because NO$_3^-$ assimilation is not thought to occur at night and NH$_4^+$ assimilation is sharply reduced, greatly limiting capacity for *de novo* amino acid synthesis (Canvin and Atkins, 1974; Matt et al., 2001; Nelson et al., 2014). In our study, neither exogenous NO$_3^-$ nor NH$_4^+$ stimulated night-time O$_2$ consumption, which is consistent with inorganic N not being appreciably assimilated and amino acid synthesis costs being limited (Fig. 4). As source tissues, mature leaves continually export amino acids to sink tissues, but the export rate and indirect ATP cost of amino acid transport from leaves that occurs at night has not been quantified and experimental methods to address this question are lacking (Kallarackal et al., 2012). Therefore, it remains unclear whether this process is a cost that contributes greatly to the observed variation in R$_N$. Lastly, protein synthesis and degradation continue at night in plant leaves and are thought to represent major cellular demands for respiratory ATP production (Bouma et al., 1994; Cannell and Thornley, 2000; Pal et al., 2013). However, the correlation between R$_N$ and protein synthesis observed here within mature leaves was weak (Fig. 6), indicating that variation protein synthesis is not a major determinant of the variation in R$_N$. This suggests that, in our target mature Arabidopsis leaves, protein synthesis is a comparatively minor sink for ATP consumption at night. This is consistent with recent measurements of proteome wide turnover rates in Arabidopsis leaves, which estimated protein biosynthesis costs to account for 13% of the ATP budget in mature leaves, while up to 38% in actively growing leaves (Li et al., 2016). Altogether, within our current understanding of leaf night-time metabolism, we find no compelling rationale to conclude that the relationship between major amino acid levels and Arabidopsis R$_N$ is causative in mature leaves.
An alternative explanation to the above is that the correlation between major amino acids and \( R_N \) is indirect and not causal. The major amino acids may reflect other metabolic activities that are themselves determinants of \( R_N \). Relative to other amino acids, the major amino acids Glu, Asp, Ala, Gin, Ser and Gly are thought to exist as single metabolically active pools which are closely linked to primary metabolism (Nelson et al., 2014; Ishihara et al., 2015). For example, the Ala pool is the most rapidly and completely labelled amino acid pool following \(^{13}\text{CO}_2\) or \(^{15}\text{NH}_4\) application to photosynthesizing Arabidopsis or barley leaves (Nelson et al., 2014; Ishihara et al., 2015; Szecowka et al., 2015). Major amino acids could therefore display a strong correlation with \( R_N \) as a result of the sensitivity of their pool size to changes in both C and N assimilation. As total free amino acids accumulate during the light period and are depleted during the night period in Arabidopsis and tobacco (Fritz et al., 2006; Gibon et al., 2006; Watanabe et al., 2014), amino acid levels in the early night, not unlike starch, could be an indicator of the previous day’s aggregate metabolic activity (Fig. 7).

\( R_N \) scales with daytime metabolic activity

Respiration has previously been observed to scale with photosynthesis. A link with daytime metabolic productivity may also partly explain the consistent correlation between respiration and N content, which itself is thought to reflect protein content. The protein-respiration correlation has been attributed to: i) protein levels scaling with metabolic activity, in particular photosynthesis; and, ii) increased protein turnover costs to maintain higher amounts of protein (Ryan, 1991; Bouma et al., 1995; Reich et al., 2008). In our study, we found the correlation between \( R_N \) and protein content in mature leaves to be much stronger than that between \( R_N \) and protein synthesis. This suggests that
the scaling of protein amount with metabolic activity underlies the correlation between $R_N$ and protein (or N) in mature leaves (Fig. 7).

The metabolic correlations observed here between $R_N$ and protein, carbon stores and amino acid levels all support a model where increased daytime assimilatory activity is responsible for the variation in $R_N$ in mature leaves. As summarized in Figure 7, increased daytime C and N assimilation is linked mechanistically to $R_N$ most obviously by increased sucrose export costs at night (the costs of amino acid export being unknown). The ATP costs associated with sucrose export have been estimated to account for a wide range of total $R_N$ output, with an estimated average of 29% (Bouma et al., 1995). Here, the levels of sucrose, starch and major amino acids, which relate to assimilation activity, and protein, which relates to assimilation capacity, could explain most of the variation in $R_N$, while protein synthesis predicted very little. Hence we hypothesize that differences in export costs, driven by differences in the availability of exportable nutrients, may be the main source of variation in $R_N$ in mature leaves. Ultimately, the proposed variation in assimilatory activity within leaf blade tissue may be due to small differences in environmental conditions such as light or N supply.

$R_N$ supports biosynthesis

We do not exclude the possibilities that other ATP consuming processes may also influence $R_N$ or that substrate supply directly enhances respiratory O$_2$ consumption to some extent. Indeed the observed correlations between shikimic acid, a precursor of plastidic aromatic metabolism, hexadecanoic acid and octadecanoic acid, products of plastidic fatty acid synthesis and intermediates of glycerolipid synthesis, and gluconic acid/6-phosphogluconate, an intermediate in the NADPH generating pentose phosphate pathway all suggest a relation between $R_N$ and biosynthetic pathways. However, non-photosynthetic aspects of plastid biosynthesis like fatty acid synthesis and the shikimate pathway are also downregulated at night (Entus et al., 2002, Sasaki et al., 1997), and whether any remaining flux through these pathways would represent a substantial nocturnal ATP cost is not known. An alternative explanation is that fatty acids and shikimate co-vary strongly with other products of plastid daytime metabolism like starch whose levels more directly influence $R_N$ (Supplemental Table 3).

Lastly, the correlation between $R_N$ and the plant signalling compound salicylic acid is intriguing but we can only speculate as to a reason for this relationship. Mechanistically, at low levels salicylic acid has been observed to uncouple respiration and activate succinate dehydrogenase in isolated mitochondria, and upregulate alternative oxidase expression (Norman et al. 2004; Belt et al. 2017). Salicylic acid has long been known to be involved in controlling thermogenic respiration in Arum
species (Raskin et al., 1987), and the strong correlation obtained here suggest it could affect the rate of non-thermogenic plant respiration.

Summary

There has long been known to be a diversity of respiration rates throughout the plant kingdom. An important finding of this study was that the extent of intra-species variation in RN of mature leaves remained sizable even under a favourable controlled environment. A combination of high-throughput measurements provided a fresh experimental approach and new empirical data that quantitatively assess underlying metabolic reasons for this variation. The genetic interaction with respiration is undoubtedly complex given the size of the pathway, but under standard test conditions used here it accounts for a rather small amount of natural intraspecies variation of RN. The results suggest that differences in mature leaf RN relate largely to a combination of daytime metabolic activities and associated night-time export costs and less so to night-time protein synthesis rates. As such, variation in leaf RN should not be viewed as a question of supply and demand because both respiratory substrates and export costs depend on assimilated carbohydrate levels. Rather, RN in mature leaves should be viewed as scaling with daytime metabolism (Figure 7). This concept provides a more mechanistic basis for the known scaling of RN with photosynthesis but our results also indicate that daytime N assimilation into amino acids, besides C assimilation into carbohydrates, could be quantitatively important in explaining or predicting RN. More research into night-time use of amino acids in leaves is needed to further mechanistically understand this relationship.
Materials and Methods:

Plant growth

Arabidopsis thaliana seeds were sown into a 3:1:1 mix of potting soil, perlite, and vermiculite, supplemented with slow release fertilizer and covered with a transparent plastic cover until established. Plants were grown in a controlled environment growth chamber maintaining a short day photoperiod of 8 h light to 16 h dark (23:00-7:00 light), a photon flux of 150 µmol m⁻² s⁻¹, a relative humidity of 75% and a day-night temperature cycle of 22°C-17°C. Soil was kept well moistened with regular watering. Unless otherwise indicated leaf tissue was harvested from 5-7 week old plants as described for the respiration screen below.

Arabidopsis respiration screens

Three different night respiration screens were performed. Accession screens 1 and 2 were performed on sets of plants from an Arabidopsis natural accession collection (Li et al., 2010), grown in a single growth chamber. In accession screen 1, 226 plants were sampled, each a single replicate of a separate natural accession. Our decision to use a single plant for each accession was motivated by the need to assess as wide a range of intra-species genotypic variation in Rₙ as possible. Following the completion of the first screen, we decided to perform a second screen using a growth cabinet with in-built photographic capabilities, enabling assessment of plant growth rate; however, this required switching from fluorescent to LED lighting. In screen 2, 190 plants were sampled, representing 162 singly replicated accessions and 17 and 11 replicate plants of the accessions Columbia-0 (Col-0) and Ag-0, respectively, which were used to estimate the heritability of Rₙ. The lists of accessions sampled in screens 1 and 2 are provided in Supplemental Table 1, with 86 accessions being sampled in both screens. Following the completion of screen 2, a third screen was performed to focus on intra-genotype variation in Rₙ. Screen 3 was performed in a different growth cabinet with fluorescent lighting and 41 plants of the accession Col-0 were harvested.

For all screens, seeds were pre-treated with 10 µm gibberellic acid for 7 days at 4°C to encourage uniform germination. Between 37 and 46 days after sowing, four leaves were harvested from plants selected at the time of harvesting on the basis of leaf size (approx. > 6 cm²). No leaf senescence had begun at the time of harvesting in any of the plants. The leaves chosen from each plant were carefully selected to represent the four youngest leaves that had reached the outer edge
of the rosette. Importantly, under the growth conditions used, mature Arabidopsis leaves continue slowly expanding, thus eliminating the possibility of using fully-expanded leaves as the standard.

Leaf tissue was harvested 2-4 hours into the night under dim green light (<1 µmol photons m² s⁻¹). Tissue harvest was completed using a 6.5 mm diameter cork borer to punch six leaf discs from each leaf, equalling 2 cm² of leaf blade. Three discs (1 cm²) were used for the O₂ consumption measurement (see below), followed by fresh mass measurement, then snap-frozen in liquid N₂ and stored for protein measurements. The other three discs were immediately snap-frozen in liquid N₂ and stored at -80°C for metabolite analysis.

Respiration measurements

Respiration measurements were performed on a Q2 oxygen sensor (Astec-Global), in sealed 850 µL capacity tubes containing three leaf discs totalling 1 cm². Oxygen concentration measurements were made at 3 min intervals. The slope of oxygen consumption was calculated between 0.5 and 3 h after the start of the run. Standards containing normal air and 100% N₂ were used to calibrate to 100% and 0% atmospheric oxygen. The O₂ partial pressure was determined to be 20.95% of atmospheric pressure, and the ideal gas law was used to calculate molar O₂ consumption rates (Scafaro et al. 2017).

To test the effect of metabolite additions, single leaf discs harvested at four hours into the night period were floated on top of 600 µL of respiration buffer (50 mM HEPES, 10 mM MES pH 6.6 and 200 µM CaCl₂) with or without additional metabolites within an 850 µL volume tube. To allow time for metabolite uptake, respiration measurements were calculated as the slope between 1 and 3 hours after the start of the run. A minimum of 5 leaves from different plants were assayed for each treatment.

Genome wide association study

Genome wide association study of respiration per leaf area and respiration per fresh mass were performed as previously described (Cheng et al., 2010). The 0.05 genome-wide threshold was determined using the Bonferroni method, which proved to be close to the empirical threshold found using the permutation test (Churchill and Doerge, 1994).
**Leaf area expansion measurements**

Leaf area expansion was measured using the TraitCapture imaging and segmentation pipeline (Brown et al., 2014). Images with a resolution of approximately 25 pixels mm\(^{-2}\) were captured with two Canon EOS700D, DSLR digital cameras mounted in the centre of each side of the chamber about 1.5 m from the plants. Images were corrected for colour and distortion and then segmented using the TraitCapture segmentation code to calculate leaf area for each plant at each time point.

**Metabolite analysis**

Frozen leaf discs were ground in a bead mill and metabolites immediately extracted in 200 µL of 85% v/v methanol, 15% ddH\(_2\)O and 8 µg mL\(^{-1}\) of ribitol as an internal standard. Samples were incubated on a thermomixer for 15 min at 60°C and 1400 rpm, followed by centrifugation for 10 min at 20 000 g. For starch assays the pellet was further washed with ethanol and assayed for starch as described previously (Smith and Zeeman, 2006). For GC-MS metabolite analysis, the supernatant was transferred to a new tube and centrifuged for 5 min at 20 000 g. Exactly 40 µL of supernatant was transferred to a glass vial and dried in a vacuum concentrator without heat.

Samples were derivatized by incubation in 10 µL of 20 mg/mL methoxyamine hydrochloride in pyridine for 90 min at 37°C with agitation at 750 rpm, followed by the addition of 15 µL of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and incubation at 37°C for 30 min with agitation at 750 rpm. Next 5 µL of alkane standards were added to each sample. In screen 2 but not screen 1, sample derivatization was performed online using a Gerstel sample preparation robot.

Metabolites were fractionated and detected using an Agilent 7890A GC, equipped with a Varian CP9013-Factor 4 column (40 m × 0.25 µm inner diameter), coupled to an Agilent 5975 quadrupole MS detector. Helium acted as carrier gas at a constant flow rate of 1 mL min\(^{-1}\). The injection temperature was 250°C; the transfer line and ion source were set at 250°C. The oven temperature was constantly increased at 15°C min\(^{-1}\) from 70°C to 325°C. After a solvent delay of 5 min, mass spectra were recorded at 50 Hz with a scanning range of 40–600 m/z. GC-MS data was analysed using MetabolomeExpress (https://www.metabolome-express.org) (Carroll et al., 2010).

**Protein quantification**

Frozen leaf discs were ground in a bead mill, then mixed with 500 µL of 50 mM HEPES pH 8.0, 0.1% (v/v) triton-x 100 and 1% (w/v) polyvinylpolypyrrolidone and processed again in the bead mill.
Samples were centrifuged for 10 min at 20,000 g and 200 µL of supernatant was transferred to new tube and snap frozen in liquid N₂ and stored at -80°C. Protein quantification was performed using a BCA protein assay kit (Bio Rad) following the manufacturer’s instructions.

Protein synthesis quantification

Relative protein synthesis rates were measured using a modified version of a published method (Van der Werf et al., 1992). Radio-labelled Leu is used as a protein synthesis indicator as the ¹⁴C label from Leu is not rapidly metabolised into other metabolites besides protein (Van der Werf et al., 1992). Leaf discs harvested at two hours into the night period were floated on top of 400 µL of respiration buffer containing 0.1 µCi of uniformly labelled ¹⁴C-Leu (300mCi/mMole; Perkin Elmer) for four hours in sealed Q2 respiration vials. Directly afterwards, leaf discs were rinsed, then frozen in liquid N₂. Leaf discs were ground in a bead mill and protein was extracted with 200 µL of 0.1 M NaOH for 15 min at 65°C and 1400 rpm. Following centrifugation at 20,000 g for 15 min, the supernatant was collected and the pellet re-extracted by the same method. The combined supernatants were precipitated with 5% TCA at 4°C overnight to precipitate protein but not free ¹⁴C-Leu. The samples were centrifuged for 15 min at 20,000 g and the pellet washed with acid ethanol (0.1M HCl:ethanol = 1:11 [v/v]). The pellet was resolubilized in 0.1 M NaOH containing 0.5% SDS and mixed with 5 mL of Ultima Gold (Perkin Elmer) followed by scintillation counting.

Supplemental Data

Supplemental Table 1. List of Arabidopsis accessions used in measurements from each screen.

Supplemental Table 2. Correlations between growth and respiration rate.

Supplemental Table 3. Full list of metabolite correlations with Rₙ.

Supplemental Figure 1. Age and location dependent variation in Arabidopsis leaf Rₙ rates

Supplemental Figure 2. Concentration dependent stimulation of leaf night respiration by select metabolites.

Supplemental Figure 3. The effect of cycloheximide (CHX) on Rₙ rate in leaf discs.

Acknowledgements
We gratefully acknowledge the assistance of Dr. Adam Carroll, Australian National University, for his assistance in analysing the metabolomics data using MetabolomeExpress. We thank Dr. Clarissa Alves Negrini, Dr. Andrew Scafaro, Yuzhen Fan and Matthew Spence, Australian National University, for their assistance with respiration measurements.
**Table 1**: Summary data from the screens of Arabidopsis leaf R_\text{N}. Significant correlations between the plant mean values are indicated in bold (p<0.01).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Col-0</th>
<th>Screen 1</th>
<th>Screen 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plants sampled</td>
<td>41</td>
<td>226</td>
<td>190</td>
</tr>
<tr>
<td>Genotypes sampled</td>
<td>1</td>
<td>226</td>
<td>162</td>
</tr>
<tr>
<td>Mean O_2 consumption rate ± std. dev (µmol O_2 m(^{-2}) s(^{-1}))</td>
<td>0.59±0.09</td>
<td>0.58±0.09</td>
<td>0.76±0.10</td>
</tr>
<tr>
<td>Mean O_2 consumption rate ± std. dev (nmol O_2 g(^{-1}) s(^{-1}))</td>
<td>3.4±0.5</td>
<td>3.5±0.6</td>
<td>3.6±0.5</td>
</tr>
<tr>
<td>Correlation coefficients (r)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O_2 consumption rate (_{\text{per Area}}) vs fresh mass</td>
<td>0.17</td>
<td>0.16</td>
<td><strong>0.35</strong></td>
</tr>
<tr>
<td>O_2 consumption rate (_{\text{per Area}}) vs protein amount</td>
<td><strong>0.60</strong></td>
<td><strong>0.55</strong></td>
<td><strong>0.58</strong></td>
</tr>
<tr>
<td>Fresh mass vs protein amount</td>
<td>0.36</td>
<td>0.10</td>
<td><strong>0.31</strong></td>
</tr>
</tbody>
</table>
Table 2: Identified metabolites detected by GC-MS which correlate most strongly with \( R_N \).

Coefficients of determination \( (r^2) \) between averaged leaf disc metabolite levels and \( R_N \) per area are given. Metabolites with significant correlations and coefficients of determination larger than \( r^2=0.2 \) in any one screen are listed. All correlations are positive. Statistically significant correlations \( (p<0.01) \) are indicated in bold.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Col-0 Screen</th>
<th>Accession Screen 1</th>
<th>Accession Screen 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino Acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Alanine</td>
<td>0.53</td>
<td>0.36</td>
<td>0.42</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>0.34</td>
<td>0.18</td>
<td>0.34</td>
</tr>
<tr>
<td>Pyroglutamic acid</td>
<td>0.62</td>
<td>0.12</td>
<td>0.27</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>0.61</td>
<td>0.20</td>
<td>n.d.</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>0.49</td>
<td>0.16</td>
<td>0.27</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>0.28</td>
<td>0.24</td>
<td>0.20</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.45</td>
<td>0.11</td>
<td>0.07</td>
</tr>
<tr>
<td>L-Valine</td>
<td>0.02</td>
<td>0.15</td>
<td>0.28</td>
</tr>
<tr>
<td>β-Alanine</td>
<td>0.24</td>
<td>0.02</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>Dicarboxylic Acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Malic acid</td>
<td>0.49</td>
<td>0.01</td>
<td>0.22</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>0.62</td>
<td>0.04</td>
<td>0.16</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>0.30</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Carbohydrates and related</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Gluconic acid</td>
<td>0.40</td>
<td>0.18</td>
<td>0.31</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.49</td>
<td>0.28</td>
<td>0.31</td>
</tr>
<tr>
<td>6-Phosphogluconic acid</td>
<td>0.49</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.48</td>
<td>0.04</td>
<td>0.14</td>
</tr>
<tr>
<td>L-Threonic acid</td>
<td>0.34</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>L-Ascorbic acid</td>
<td>0.08</td>
<td>0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>Hexose Phosphate</td>
<td>n.d.</td>
<td>0.12</td>
<td>0.22</td>
</tr>
<tr>
<td><strong>Phenolics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shikimic acid</td>
<td>0.61</td>
<td>0.02</td>
<td>0.32</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>0.64</td>
<td>n.d.</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Fatty acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octadecanoic acid</td>
<td>0.44</td>
<td>0.03</td>
<td>0.14</td>
</tr>
<tr>
<td>Hexadecanoic acid</td>
<td>0.44</td>
<td>0.01</td>
<td>0.11</td>
</tr>
</tbody>
</table>

† n.d.: not detected
Figure legends

Figure 1: Representative measurements of leaf O$_2$ consumption rate. Measurements of O$_2$ depletion from leaf discs in air (A) or on top of respiration buffer solution (B) are shown in black; empty sealed control vials are shown in grey. Leaf O$_2$ consumption between 0.5 and 3 h after sealing the vial is linear and the coefficient of determination is indicated. (C) Measurement of leaf disc O$_2$ consumption before and after opening the vials for the addition of CN and SHAM to the respiration buffer.

Figure 2: Time course of respiration measurements throughout the night period. Five replicate measurements each containing three leaf discs from different leaves of Landsberg erecta (black circles) or Col-0 (grey squares) were taken at the indicated time points throughout the night. Error bars indicate standard error and the brackets indicates significant differences between time points ($p < 0.05$).

Figure 3: Alanine, pyruvate and starch correlations with R$_N$ within and among accessions. A-D) Relative leaf disc levels of Ala and Pyr as measured by GC-MS are plotted against R$_N$ per area. The averaged values per plant from accession screen 2 (A,C) and the Col-0 screen (B,D) are shown. E-F) Starch levels (glucose equivalents) as measured by enzyme assay are plotted against R$_N$ per area. Plant averaged values are taken from accession screen 1 (E) and the Col-0 screen (F).

Figure 4: A screen of the effect of exogenous metabolites on leaf disc R$_N$ was performed. Chemical additions to the respiration buffer were made at 10 mM (grey bars) and 100 mM (black bars) for each compound except for KNO$_3$ and NH$_4$Cl which were made at 10 mM. Values are expressed relative to untreated controls. The compounds are generally sorted as carbohydrates and glycolytic intermediates (A), organic acids (B) amino acids (C) and inorganic nitrogen (D). Asterisks denote significant differences ($p<0.05$) between treatments and non-treated controls.

Figure 5: FCCP stimulates leaf R$_N$. A) The mitochondrial uncoupler FCCP was supplied exogenously at several concentrations to single leaf discs. The average relative rates of O$_2$ consumption are shown from a minimum of six replicates and significant increases compared to control assays ($p<0.05$; ANOVA) are indicated by asterisks. B) Leaf discs in the presence of 2 µM FCCP were assayed for respiration in the presence or absence of 100 mM of select metabolites. The relative increase caused
by the metabolite additions over measurements with FCCP alone are shown and asterisk indicate significant increases ($p<0.05$; paired t-test; $n\geq6$).

**Figure 6:** Simultaneous measurement of protein synthesis and $R_{n}$ in leaf discs. Net $O_2$ depletion and dissociations per minute (DPM) from scintillation counts of $^{14}$C-Leu incorporation into protein were determined following a 4 h incubation of leaf discs. A) The relationship between $R_{n}$ and $^{14}$C-Leu incorporation within mature leaves. Each data point represents the average of 4 leaf disc measurements taken from a single mature leaf. The coefficient of determination of the linear regression is indicated. B) Measurements from individual leaf discs from young developing leaves (grey circles) and mature leaves (black squares) are compared.

**Figure 7:** A model depicting how aspects of day metabolism are involved in the variation of $R_{n}$ rates under a common environment in mature leaves. Dashed boxes represent metabolic processes, while solid boxes represent metabolite pools and protein. Where measured, coefficients of determination between aspects of metabolism and $R_{n}$ are shown. Inputs into day assimilatory metabolism, shown as bold arrows, are assumed to be constant. Major amino acids and carbohydrate stores are known to accumulate during the day and become depleted during the night. Stored carbohydrates are linked to export and respiration, with sucrose levels indicative of carbohydrate export. Night-time major amino acid pools are linked to protein synthesis and export, but the costs associated with amino acid export are unknown. The correlation observed with protein is attributed mostly to a relationship between protein and daytime assimilatory capacity as the correlation between $R_{n}$ and with protein synthesis is low. Organic acid metabolism and other metabolic processes are omitted for simplicity, but may be quantitatively important in determining respiration.
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Thornley JH (2011) Plant growth and respiration re-visited: Maintenance respiration defined - it is an emergent property of, not a separate process within, the system - and why the respiration : Photosynthesis ratio is conservative. Ann Bot 108: 1365-1380


