Diel and temperature driven variation of leaf dark respiration rates and metabolite levels in rice

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Summary

- Leaf respiration in the dark (R_{dark}) is often measured at a single time during the day, with hot-acclimation lowering R_{dark} at a common measuring temperature. However, it is unclear whether the diel cycle influences the extent of thermal acclimation of R_{dark}, or how temperature and time of day interact to influence respiratory metabolites.
- To examine these issues, we grew rice at 25/20°C (day/night), 30/25°C and 40/35°C, measuring R_{dark} and changes in metabolites at five time-points spanning a single 24-hour period.
- R_{dark} differed among the treatments and with time of day. However, there was no significant interaction between time and growth temperature, indicating that the diel cycle does not alter thermal acclimation of R_{dark}. Amino acids were highly responsive to the diel cycle and growth temperature, and many were negatively correlated with carbohydrates and with organic acids of the tricarboxylic acid (TCA) cycle. Organic TCA intermediates were significantly altered by the diel cycle irrespective of growth temperature, which we attribute to light-dependent regulatory control of TCA enzyme activities.
- Collectively, our study shows that environmental disruption of the balance between respiratory substrate supply and demand is corrected for by shifts in TCA-dependent metabolites.

Introduction

Leaf respiration in the dark (R_{dark}) provides plants with ATP, reducing equivalents, and carbon skeletons needed to support growth and cellular maintenance (Atkin & Macherel, 2009, Gonzalez-Meler *et al.*, 2004, Lambers & Ribas-Carbo, 2005, Noguchi & Yoshida, 2008). Rates of R_{dark} increase exponentially with rising temperature in the short-term (i.e. time scales of seconds to minutes) (Amthor, 2000, Atkin *et al.*, 2005b, Reich *et al.*, 2016, Smith & Dukes, 2013). With sustained exposure to different growth temperatures (e.g. over periods of days to weeks), R_{dark} often acclimates, resulting in lower rates when measured at a common measuring temperature in warm/hot-grown plants compared to their cooler-grown counterparts (Atkin *et al.*, 2005a, Reich *et al.*, 2016, Slot & Kitajima, 2015, Smith & Dukes, 2017). Acclimation of

R_{dark} is common (Slot & Kitajima, 2015), and likely contributes to global patterns in R_{dark} (Atkin*et al.*, 2015, Vanderwel *et al.*, 2015), with acclimation having the potential to dampen the effects of future climate warming on plant respiratory carbon release (Huntingford *et al.*, 2017, Reich *et al.*, 2016, Smith & Dukes, 2013). Importantly, studies assessing acclimation invariably rely on measurements made at a single time-point in a day, implicitly assuming that acclimation capacity is constant through a 24-hour diel cycle. This assumption remains untested, even though R_{dark} supports leaf metabolism which varies greatly during the day. For example, photosynthesis varies with diel fluctuations in light intensity, temperature, and evaporative demand, likely affecting carbon fluxes in cellular metabolism (Steer, 1973). Given the strong coupling between respiratory and photosynthetic metabolism (Kromer, 1995, Shameer *et al.*, 2019, Tcherkez *et al.*, 2017), diel and growth-temperature dependent variations in photosynthetic activity might be expected to influence rates of R_{dark}. Time of day is also likely to influence concentrations of metabolites involved in glycolysis, the tricarboxylic acid (TCA) cycle, the shikimate pathway and several other related metabolic networks (Gibon *et al.*, 2009, Hurry *et al.*, 2005, Urbanczyk-Wochniak *et al.*, 2005); however, whether diel variations in these metabolites is affected by growth temperature remains unclear. This interaction between the diel cycle and respiratory responses to growth temperature is important in understanding the effects of climate warming on plant carbon fluxes, both in natural and managed ecosystems. This is especially important when considering the disproportionate increase in night temperatures over the past half century (Davy *et al.* 2017). The impact of growth temperature and time of day on leaf respiratory metabolism is of particular interest in globally important crops such as rice, where rising growth temperatures (particularly during the night) are leading to reduced yields (Peng *et al.*, 2004, Welch *et al.*, 2010).

Photosynthesis generates sugar substrates for respiration, but the evidence for substrate-dependent changes in leaf R_{dark} is inconclusive. In some studies, glucose and sucrose concentrations can positively correlate with rates of R_{dark} (Ogren, 2000). For example, higher sugar content was linked to faster R_{dark} in leaves of *Quercus rubra* (Griffin *et al.*, 2002, Whitehead *et al.*, 2004), as well as in *Spinacia oleracea* and *Alocasia macrorrhiza* (Noguchi *et al.*, 1996). However, sugars were not correlated to R_{dark} in two alpine perennials, *Bisorta bistortoides* and *Campanula rotundifolia* (McCutchan & Monson, 2001). Moreover, thermal acclimation often alters temperature-normalized rates of R_{dark}, but such changes can occur without concomitant changes in concentrations of soluble sugars (Zaragoza-Castells *et al.*, 2007). Indeed, sugar concentrations are often relatively constant across a range of environmental treatments (Ayub *et al.*, 2011, Martínez-Vilalta *et al.*, 2016), likely as a

consequence of the synthesis and degradation of starch (Hüve *et al.*, 2012). Starch turnover is regulated by a circadian clock (Graf *et al.*, 2010, Graf & Smith, 2011) and increases during the day as photosynthetic assimilates are typically stored as starch in leaves if not immediately used or exported (Pilkington *et al.*, 2015, Smith & Stitt, 2007). Starch is broken down at a constant rate during the night to provide a relatively constant supply of sugars for R_{dark} and growth in the absence of photosynthesis, with approximately 90-95% of the starch consumed by dawn (Graf *et al.*, 2010, Pilkington *et al.*, 2015, Smith & Stitt, 2007). If this circadian regulation of starch stabilizes sugar concentrations over a 24-hour cycle, leaf sugars are unlikely to limit R_{dark} and contribute to diel variation. Nevertheless, R_{dark} may be affected by diel changes in the availability of other substrates of respiration, specifically amino acids, proteins, organic acids and/or lipids (O'Leary *et al.*, 2011, O'Leary *et al.*, 2017). As described in O'Leary *et al.* (2017), such metabolites may drive respiration by supplying intermediates to the TCA cycle, reductant for ATP synthesis via oxidative phosphorylation, and carbon skeletons required for biosynthesis or nitrogen (N) assimilation into amino acids. Thus, substrate-product relationships across respiratory network must be considered when assessing the interactive effect of growth temperature and time of day on leaf R_{dark} and to identify key metabolites that correlate strongly with R_{dark}.

Metabolomics may provide insights into factors underpinning variability in fluxes through the respiratory pathways, and thus how R_{dark} varies with temperature and diel cycle. One interesting observation from metabolomic studies published to-date is the amount to which metabolite concentrations vary with temperature. Sicher (2015) showed that 28 of 43 total metabolites in soybean leaflets were affected by an increase in growth temperature. With the exception of raffinose and γ-aminobutyric acid (GABA), most of the organic and amino acid metabolites decreased in response to elevated growth temperature. In wheat leaves under warmer night temperatures, the abundance of fumarate and alanine increased, while glutamine, glutamate and GABA remained constant (Impa *et al.*, 2019). Glaubitz*et al.* (2015) found that warmer day (30°C) and night (28°C) temperatures increased the abundance of amino and organic acids in temperature-sensitive rice cultivars (e.g. DR2 and M202) but not in intermediate (e.g. IR64 and IRRI123) and temperature-tolerant cultivars (e.g. IR72 and Taipei309). In addition to being affected by growth temperature, amino acids change in response to the diel cycle. For example, Urbanczyk-Wochniak *et al.* (2005) found amino acid levels increased in the light and decreased in the dark in potato (*Solanum tuberosum*) leaves. The metabolites mentioned above are regulated by the TCA cycle which is central to respiration,

suggesting that the strong response of many metabolites to temperature and light are likely to be associated with alterations in respiratory rates.

TCA cycle intermediates can also respond to changes in growth temperature and the time of day. There is evidence that malate and fumarate accumulate during the day, while citrate, aconitate and succinate accumulate at night (Gibon *et al.*, 2006, Watanabe *et al.*, 2014). Further, light suppresses the pyruvate dehydrogenase complex (PDC) (Tcherkez *et al.*, 2009), potentially reducing PDC activity and its decarboxylation of pyruvate, reducing entry of carbon into the TCA cycle (Tovar-Mendez *et al.*, 2003) with consequences for the concentration of TCA cycle intermediates. Moreover, in some cases the TCA cycle can be supplemented through the action of light-induced phosphoenolpyruvate carboxylate (PEPC) that produces oxaloacetate and malate (Araújo *et al.*, 2012, Marsh *et al.*, 2003, Sullivan *et al.*, 2004). In this pathway, PEPC generally replenishes the TCA cycle intermediates that are depleted when α-ketoglutarate is used for amino acid synthesis. In addition to these day/night effects, measuring temperature can also alter the abundance of TCA cycle intermediates. For example, the concentrations of α-ketoglutarate, fumarate, malate, and citrate increased when Arabidopsis leaves were cooled from 20°C to 4°C (Cook *et al.*, 2004). In Arabidopsis grown at 15°C for 24 hours relative to 20°C controls, succinate, fumarate, and malate accumulated at the beginning of the day but otherwise fluctuated similarly to the 20°C controls during the light period (Florian *et al.* (2014). Further, metabolites that typically vary diurnally – such as alanine, phenylalanine and glutamine – maintain their diurnal oscillations even when leaves are chilled at 4°C (Espinoza *et al.*, 2010). The aforementioned studies imply that there are distinct respiratory metabolite adjustments to temperature and to light/dark cycles. However, more in-depth experimentation of the interaction between temperature and the diel cycle is lacking and should be explored

In the current study, we investigated whether thermal acclimation of respiratory metabolism interacts with the day/night cycle in rice (*Oryza sativa,* cultivar IR64). Rice is one of the most important food crops yet yields are declining, likely from rising growth temperatures (Wassmann *et al.*, 2009). In a recent study, we assessed the effect of growth temperature on acclimation of photosynthesis and respiration in the IR64 cultivar (Rashid *et al.*, 2020); in that study, growth temperature-dependent changes in leaf carbon exchange were assessed at a single time point during the day. Our current study used metabolite profiling to assess how growth temperature and the diel cycle might influence the concentrations of metabolites in the respiratory network of rice. We explore whether adjustments in the abundance of individual metabolites provide insights into how growth temperature affects

regulatory control of respiratory metabolism. We specifically examined: (1) if rates of leaf R_{dark} (measured as O_2 uptake in darkness) vary over a day, (2) whether the extent of the diel variation is influenced by growth temperature, (3) if diel variations in R_{dark} are linked to concomitant changes in substrate availability, and (4) the extent to which growth temperature and day/night cycles influence the metabolome profile.

Material and Methods

Plant material and temperature treatments

Rice (*Oryza sativa* L.) cultivar IR64 was grown hydroponically in a glasshouse facility at the Australian National University in Canberra, Australia. Growth conditions were the same as those reported in Rashid *et al*. (2020). The hydroponic solution consisted of NH_4NO_3 (1.4 mM), $NaH_2PO_4.2H_2O$ (0.6 mM), K_2SO_4 (0.5 mM), $CaCl_2.2H_2O$ (0.2 mM), $MgSO_4.7H_2O$ (0.8 mM) and micronutrients: Fe-EDTA (0.07 mM), H_3BO_3 (0.037 mM), $MnCl_2.4H_2O$ (0.009 mM), $ZnSO_4.7H_2O$ (0.00075 mM), $CuSO_4.5H_2O$ (0.0003 mM), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.0001 mM), NH_4VO_3 (0.000138 mM) and Na_2SiO_3 (0.0012963 mM). Twelve 20 l hydroponic tanks holding a maximum 20 plants were placed in a temperature-controlled glasshouse at 30°C during the day and 25°C (30/25°C) at night under a natural photoperiod. The sunset and sunrise times were 19:30 and 06:30 ± 15 min, respectively. Photosynthetically active radiation (PAR) measured between 10:00 and 15:00 hours ranged from 400 and 1200 µmol quanta m^{-2} s^{-1}. After two weeks at 30/25°C, eight tanks were randomly chosen and shifted to adjacent glasshouse rooms set to 25/20˚C or 40/35˚C temperature regimes (four tanks in each room). Another four tanks were maintained in the original 30/25°C room as controls. Each plant within a separate tank was considered as a biological replicate (Supporting Information Figure S1). Sampling for all experiments unless stated otherwise occurred over a single 24-hour period at 04:30, 08:30, 13:00, 17:30 and 21:30 hours. To minimise developmental differences, all leaf samples for respiratory and metabolite profiling were taken from young fully expanded leaves on days seven and eight after temperature transfer from plants previously unsampled which developed under the prevailing temperature regime.

Dark respiration measurements

Rates of dark respiration (R_{dark}) were measured using a high-throughput fluorophore O_2-sensor system (Astec Global, Maarssen, The Netherlands) as previously described (Coast *et al.*, 2019, O'Leary *et al.*, 2017, Scafaro *et al.*, 2017). Four independent plants, each from a separate tank,

from each growth temperature treatment were placed in a container with a small volume of hydroponic solution and brought back to the laboratory to be processed. After 30 min in darkness two separate 2 cm^2 sections from the middle part of the lamina were cut and placed in separate 2 ml capacity O_2-sensor sampling tubes. One sample tube was measured at a common temperature of 30°C, and the other at the leaf at the prevailing growth temperature. R_{dark} was calculated from an O_2 consumption slope between 30 min to two hours after initiation of the fluorophore O_2-sensor system. At the cessation of measurements, leaf sections were collected, stored in paper bags, oven-dried at 70°C and weighed to obtain dry mass. The dried leaf materials were subsequently used in the determination of starch and soluble sugar concentrations as described below. For expediency of sample collection and processing we did not measure leaf area. For metabolite profiling over the diurnal cycle, leaves from the same plants chosen for respiration were harvested in the glasshouse, immediately frozen in liquid N_2 and stored at minus 80°C.

Determination of soluble sugars, starch and nitrogen concentrations

To determine the concentrations of soluble sugars and starch, dried rice leaf sections were ground to a fine powder and 5-10 mg placed in a 2 ml microfuge tube. 0.5 ml of 80% (v/v) ethanol was added and vigorously vortexed for 20 sec. The tissues were incubated at 80°C while being orbitally shaken at 500 rpm for 20 min on a Thermomixer (Eppendorf). The samples were then centrifugated for 5 min at 12,000 rpm, and the resulting supernatant and pellet were separated. This procedure was repeated twice again on the same pellet, and supernatant pooled. The supernatant and pellet were used for determination of the concentrations of soluble sugars and starch using a Fructose Assay Kit (Sigma-Aldrich) and a Total Starch Assay Kit (Megazyme), respectively, following manufacturer's instructions. A standard curve for soluble sugars was generated using a series of known concentrations of sucrose, glucose and fructose (Sigma-Aldrich). Measurements were collected using a microtitre plate reader (Infinite M1000Pro; Tecan).

To determine the concentration of nitrogen (N), approximately 1-2 mg dried rice leaf sections were ground and placed in a tin-made capsule. The analysis was conducted using combustion, combining an elemental analyser 15 (Heraeus CHN-O Rapid), a Finnigan MAT Trapping box HT and a Finnigan MAT mass spectrometer (delta D) with a dual inlet at a precision of 0.1 ‰. N concentrations on leaf dry mass basis were calculated based on Gebauer and Schulze (1991).

Gas Chromatography-Mass Spectrometry (GC-MS) metabolite analysis

To quantify metabolites involved in carbon metabolism a mass spectrometry approach using GC-MS metabolite profiling was adopted. The extraction was carried out according to the procedure previously described in Che-Othman *et al.* (2020) with some modifications. Frozen leaf tissue was ground to powder and approximately 25 mg transferred into frozen 2 ml microfuge tubes. 0.5 ml of cold extraction buffer was added, and incubated in a Thermomixer for 20 min at 75°C and 1400 rpm mixing. All tubes were then centrifuged at 20,000 ´ *g* for 10 min at room temperature. 60 µl of supernatant was dried down in 200 µl glass insert for derivatization prior to metabolite profiling using Gas Chromatography-Mass Spectrometry (GC-MS). The derivatisation was performed using the MPS2 XL-Twister autosampler, Gerstel GmbH & Co. KG). 1 µl of derivatised sample was immediately vaporised in the inlet at 250°C and injected onto the chromatography column in split-less mode. Helium was used as carrier gas. Compounds were eluted by the following temperature gradient: hold for 1 min at 70°C then ramp with 7°C/min to 325°C and hold for 3.5 min. The ion transfer line was heated to 280°C and the ion source and quadrupole were at 150°C and 230°C respectively. The resulting peaks were analysed using MassHunter Workstation Software Quantitative Analysis Version B.07.1 / Build 7.1.524.0 for GCMS (Agilent Technologies). The integrated area of the quantifier ion for each peak was compared between samples after normalisation. Metabolites were normalised against the weighted and averaged signal of 3 internal standards followed by weighting against the average measured signal across all samples for each compound before statistical analysis was performed. Metabolite data is presented in Supporting Information dataset S1.

Statistical Analyses

Data were analysed using one-way analyses of variance (ANOVA), two-way ANOVA, principal component analysis (PCA), weighted correlation network analysis, permutational multivariate analysis of variance and multiple comparison tests using the SigmaPlot v11.0 software (Systat Software Inc) and/or R v3.4.4 software (R Core Team, 2018) with RStudio. In R v3.4.4 software, data preparation and plotting were done with packages tidyverse (Wickham, 2017). The PCA was carried out with R packages 'FactoMineR' (Le *et al.*, 2008), factoextra (Kassambara & Mundt, 2017) and ade4 (Dray & Dufour, 2007). Metabolite levels were centred and scaled with standard score. Significance testing was done with R package 'vegan' (Oksanen *et al.*, 2018). Weighted correlation network analysis was conducted using R package 'WGCNA' (Langfelder & Horvath, 2008). Permutational multivariate analysis of

variance was carried out using Euclidean distance matrices of metabolites compared pairwise (Anderson, 2001, McArdle & Anderson, 2001). The analysis produces test statistic analogous to Fisher's F-ratio, and calculates P-value through permutations.

Results

R_{dark} varies independently with time of day and temperature

When measured at the prevailing growth temperature of each treatment, R_{dark} varied with time of day (Fig. **1a**), with rates typically being at their lowest in the early evening (noting that night temperatures were 5°C lower than those during the day). R_{dark} was faster in the hottest treatment (40/35°C) and slower at the two lower growth temperatures (warm, 30/25°C; cool, 25/20°C), and this effect was consistent through the day with no significant growth temperature time interaction (Fig. **1a**, Supporting Information Table S1).

Despite prevailing rates of R_{dark} rising with growth temperature (Fig. **1a**), there was evidence that acclimation did occur, as the hot-grown plants exhibited lower temperature-normalized rates of R_{dark} (i.e. lower rates measured at 30°C) than their warm- and cool-grown counterparts (Fig. **1b**, Supporting Information Table S1). This finding supported our previous findings of respiratory thermal acclimation in rice, quantified via measurements of respiratory CO_2 release in darkness during the daylight period of 10:00-14:00 hours (Rashid *et al.*, 2020). The acclimation response (determined as the difference among the three growth temperatures in temperature-normalized rates of leaf R_{dark}) appeared greater at 17:30 hours (2 h before sunset) and lesser during the night (Fig. **1b**). However, the interaction between growth temperature and time of day was not statistically significant (P=0.065), suggesting that the effect of growth temperature on R_{dark} is relatively similar through the diel cycle.

Diel variations in R_{dark} are not linked to concomitant changes in sugar substrate availability

Changes in starch and soluble sugar content (Fig. **2**) did not mirror the acclimation of – or the diel variation in – rates of R_{dark} measured at 30°C (Fig. **1b**). The concentration of individual and total leaf soluble sugars remained constant throughout the day (Figs **2a-d**), with no particular sugar exhibiting a statistically significant response to time of day. Total soluble sugar concentration was affected by growth temperature, being lower in the cold (25/20°C), mainly due to lower glucose and fructose levels in leaves grown in colder temperatures (Figs **2a-d**). Unlike soluble sugars, starch concentration varied diurnally irrespective of growth temperature, decreasing through the night and increasing through the day, with a minimum value near

sunrise and maximum value near sunset (Fig. **2e**). Low growth temperature resulted in significantly greater leaf N concentrations compared to warm- and hot-grown plants (Fig. **2f**); while there was a significant main effect of time on leaf N, there was no clear diel pattern.

Metabolite profiles: relationships among metabolites

Two general findings were observed from metabolite profiling. Firstly, principal component analysis (PCA) of correlations among metabolites (Fig. **3a**) revealed a negative correlation between amino acids and carbohydrates/organic acids. Secondly, changes in metabolites in response to a day/night cycle or growth temperature were mostly independent of one another (Figs **3b**,**c**, Table 1). Independence was evident from metabolite responses to day and night being separated mostly along the first principal component. In contrast, metabolite responses to growth temperature were grouped mostly along the second principal component (Fig. **3b,c**). Apart from the shikimate pathway derived tryptophan and tyrosine, a two-way ANOVA corroborated that there was little significant interaction among metabolites responding to changes in day/night cycle and growth temperature (Table 1).

Metabolite profiles: The influence of growth temperature and day/night cycles

Since there was minimal interaction between the diurnal cycle and growth temperature in terms of metabolite adjustments, we performed separate one-way ANOVA, one for the diel cycle, and the other for growth temperature changes in metabolites. We displayed these changes in relation to the respiratory pathway (Figs **4** and **5**). Generally, the diel changes in metabolites and metabolite responses to growth temperature did not align with specific metabolic pathways (i.e. glycolysis, TCA cycle, shikimate pathway, amino acid synthesis). For example, a change in the TCA cycle intermediate, aconitate, was not preceded by a change in the antecedent metabolite citrate, nor were changes in aconitate followed by the changes in subsequent metabolites succinate, fumarate, and malate. The most striking general diel and temperature driven change in metabolites was that many branched chain and aromatic amino acids increased in abundance during the night and in response to warmer temperatures (Figs **4** and **5**). By contrast, short chained amino acids such as alanine, glycine and serine, as well as the N side chain amino acids glutamine and asparagine, were in greater abundance during the day and at cooler growth temperatures.

Independent of the growth temperature, most of the TCA cycle intermediates changed with diel cycle (Fig. **4**). The intermediate substrates at the beginning of the glycolysis pathway (glucose-6-phosphate and fructose-6-phosphate) varied significantly through the 24-hour cycle,

with abundances highest at mid-day and lowest at night (Fig. **4**). Citrate accumulated at night, α-ketoglutarate accumulated during the day, and malate depleted during the day. The amino acid alanine was more abundant during the day and depleted at night, but aspartic acid was reduced in the day and more abundant at night. Tryptophan, phenylalanine and tyrosine, aromatic amino acid products of the shikimate pathway, were significantly reduced at night (Fig. **4**).

In contrast to diel cycle, growth temperature alone affected fewer metabolites (Fig. **5**). This may be a consequence of the diel cycle having both an irradiance and temperature component (with nights being 5°C cooler than that during the day). The metabolite analyses revealed that growth temperature treatment subtly changed relative abundance of soluble sugars, confirming the enzymatic assay of total soluble sugars (Fig. **2**). Cold temperature reduced abundance of glucose and fructose, albeit not to a significant level. By contrast, increasing growth temperature greatly reduced levels of glucose-6-phosphate and fructose-6-phosphate. As with diel cycle, phenylalanine and tyrosine were significantly affected by temperature with lower abundance in the colder grown rice. Intermediates of the TCA cycle did not significantly vary with growth temperature apart from aconitate which increased with increasing growth temperature. Many amino acid pools generated from TCA intermediates significantly changed in response to growth temperature, with lysine and GABA increasing at the hottest temperature of 40/35°C, proline and isoleucine decreasing at the lowest temperature of 25/20°C, and glutamine increasing at 25/20°C (Fig. **5**).

Metabolite profiles: relationships with R_{dark}

Weighted correlation network analysis identified seven metabolites that correlated with changes in R_{dark} measured at a common 30°C (Fig. **6**, Supporting Information Table S3). Correlations between R_{dark} and metabolites were observed with changes in growth temperature, the diel period, or both. Weighted correlation network analysis describes correlation patterns in data (Zhang & Horvath, 2005) and has been successfully used to analyse biological data such as gene co-expression (Stuart *et al.*, 2003, Weston *et al.*, 2008) and protein-protein interactions (Jeong *et al.*, 2001). Applied here, it described the interactions among metabolites by grouping similarity responding metabolites into clusters and identifying seven key metabolites with responses representative of the clusters. This allowed for a test of relationship between the key metabolites and R_{dark} at 30°C (Fig. **6**), without correcting for multiple comparisons necessary if all metabolites were tested. Metabolites xylitol, aconitate, and erythritol increased with declining R_{dark} at 30°C, primarily in association with growth

temperature (Fig. **6**). Serine, putrescine, and fructose-6-phosphate increased with rising R_{dark}, associated with both temperature and diel responses. Succinate increased with rising R_{dark} primarily associated with diel responses alone (Fig. **6**).

Discussion

Our study has pointed to a surprising role of TCA-dependent metabolites – particularly amino acids – in the acclimation response of respiration. Glycolysis and the TCA cycle derived xylitol, erythritol, serine, and putrescine were strongly correlated (although not necessarily due to a causal relationship) with adjustments in R_{dark} to growth temperature and the day/night cycle (Fig. 6). More expectedly, some of the intermediates of glycolysis (fructose-6-phosphate) and the TCA cycle (aconitate and succinate) correlated with adjustments in R_{dark}, although others (malate and citrate) did not (Fig. **6**). Many of the metabolites that did change in abundance to growth temperature or the diel cycle did so for one and not the other (Table 1), supporting separate influences of growth temperature and diel perturbations on respiration. The limited interaction between metabolite responses to growth temperature and a day/night cycle was evident in respiratory flux measurements, with the acclimation phenotype largely been held across the diurnal cycle (Fig. **1b**). However, of interest, the shikimate derived aromatic amino acids were the only metabolites that significantly adjusted in relative abundance to both growth temperature and the diel period. These observations provide insights into the likely metabolic determinants of respiratory acclimation and its response to the day/night cycle.

Does time of day influence our conclusion of whether acclimation has occurred?

While temperature-normalized rates of R_{dark} of rice leaves differed among the growth temperature treatments (indicating that thermal acclimation occurred) and varied through the diurnal cycle, the effect of growth temperature did not vary significantly with time (Fig. **1b**, Supporting Information Table S1). Thus, it appears that – at least for rice used in our study – respiratory thermal acclimation can be assessed using measurements made at a single time point during the day. Expressing R_{dark} on a leaf area basis is unlikely to alter the result as leaf mass per area did not change in rice exposed to the same experimental conditions (Rashid *et al.* 2020). As noted in the Introduction, temperature-normalized rates of R_{dark} – measured at a single time point during the day – are commonly used to quantify thermal acclimation and biogeographical patterns in leaf respiration (Atkin *et al.*, 2005a, Huntingford *et al.*, 2017, Reich *et al.*, 2016, Slot & Kitajima, 2015, Smith & Dukes, 2013). Such studies point to decreases in

temperature-normalized R_{dark} with sustained exposure to higher growth temperature, and lower rates of temperature-normalized R_{dark} in the plants growing in the tropics than the arctic (Atkin et al. 2015). Our results suggest that those conclusions are unlikely to be affected by the time of day when rates of leaf temperature-normalized R_{dark} are measured.

Specific metabolites that correlate with R_{dark}

Changes due to growth temperature in the sugar alcohols xylitol and erythritol, and the TCA organic acid aconitate, had the strongest correlation with R_{dark} at 30°C (Fig. **4**). In a recent study, warmer night temperatures led to an increase in the sugar alcohol myo-inositol in spikes of a relatively heat tolerant wheat cultivar (Impa *et al.*, 2019). The authors attributed this to the role of myo-inositol to osmolyte protection. The increase in xylitol and erythritol that we observed is likely to be a similar osmolyte protection response to heat. Increases in these sugar alcohols and reduced R_{dark} at 30°C – while inversely correlated - may be independent responses to increased *T*. Alternatively, the accumulation of osmolytes for heat protection may in some way supress respiratory requirements of the leaf. Stabilisation of membranes as a result of osmolyte protection during heat acclimation is postulated (Gauthier *et al.*, 2014, Sung *et al.*, 2003). As such, a greater maintenance of proton motive force, through reduced membrane leakiness, is a conceivable way in which respiratory costs could be reduced with increases in xylitol and erythritol.

While growth temperature had no significant effect on succinate levels (Fig. **5**), time of day did significantly alter the abundance of this TCA intermediate, with succinate concentrations declining through the night (Fig. **4**). When succinate abundance was plotted against R_{dark} at 30°C, (Fig. **6**), a significant positive correlation was observed, suggesting that succinate may be a key control point in coordinating the TCA cycle with respiratory adjustments over a day/night cycle. This is likely associated with the light dependent regulation of succinate dehydrogenase activity and its influence over succinate content (Daloso *et al.*, 2015, Eprintsev *et al.*, 2013).

The amino acid serine, the amino acid breakdown product putrescine, and the phosphorylated sugar fructose-6-phosphate were the metabolites with strongest correlation between R_{dark} at 30°C associated with both growth temperature and the diel cycle (Fig. **6**). It is interesting that an amino acid and its associated amino acid breakdown product correlate with R_{dark} as strongly as a sugar substrate of glycolysis. The correlation between fructose-6-phosphate and R_{dark} at 30°C, both over the diel cycle, and growth temperature, is understandable as this initial substrate of glycolysis is a regulatory point of respiration (Plaxton

& Podestá, 2006, Stitt, 1990). Serine and putrescine, on the other hand, are not intermediates of the TCA cycle. As discussed below, there seems to be a tight coordination between amino acid synthesis and respiration which may account for this close association between serine, putrescine, and the respiratory flux at 30°C. Additionally, serine is a product of photorespiration, which may explain its variability across growth temperatures and the day and night periods (Fig. 6). Photorespiration would not occur in the dark and would be stimulated by heating (Walker *et al.* 2016). Levels of serine were lower at night in agreement with reduced photorespiration. However, serine levels were often lower at 40°C than 25°C in the day (Fig. 6), at odds with photorespiratory expectations. This highlights the difficulty in characterising the relationships between metabolite pools and environmental perturbations. Many metabolites will have interconnected pathways that are influenced separately by temperature and light, such as the involvement of serine in photorespiration and protein synthesis.

Assimilate utilisation

Starch accumulated during the day in leaves, and was depleted during the night, reaching a minimum in the early morning (Fig. **2e**) - a pattern that is well documented (Graf *et al.*, 2010, Graf & Smith, 2011). Interestingly, starch concentrations exhibited a similar pattern irrespective of growth temperature, with no significant interaction between temperature and the starch diurnal cycle. Increased catabolism of sugars did not supplement R_{dark} substrate supply as soluble sugar content was not depleted at 40/35°C (Fig. **2a-d**). Thus, starch was depleted from a similar starting point and at a similar rate between 30/25°C and 40/35°C grown plants despite the latter having much faster R_{dark}. $^{14}CO_2$ pulse-chase experiments demonstrate that faster rates of R_{dark} are associated with faster depletion of starch (Hüve *et al.*, 2012). However, transport of starch out of leaves, and utilisation of starch for growth, also have a major impact on leaf starch content (Stitt & Zeeman, 2012). Rice that we grew under the same experimental conditions do have faster leaf elongation rates at 40/35°C compared to the cooler temperatures, across the entire diel cycle (Rashid *et al.* 2020).The similar pattern of starch drawdown despite differences in R_{dark} is therefore likely due to temperature mediated shifts in starch transport (as sucrose) out of leaves to other organs, and different rates of utilisation due to temperature-dependent differences in leaf biomass accumulation. In other words, the levels of starch and soluble sugars were relatively constant across growth temperatures, but the fluxes of these respiratory substrates likely increased with heat to match stimulated growth.

Amino acids respond strongly to the diurnal cycle and growth temperature

Although metabolite levels are not equivalent to fluxes, increased abundance of amino acids in the dark seems counterintuitive, as N incorporation into amino acids predominantly occurs in the light (Canvin & Atkins, 1974, Fritz *et al.*, 2006, Nelson *et al.*, 2014). Toxic ammonia is initially incorporated into glutamine and glutamate by glutamine synthetase and glutamate 2-oxoglutarate aminotransferase, an energy expensive process likely supplemented by photosynthetic energy generation (Plaxton & Podestá, 2006, Tcherkez *et al.*, 2017). However, we saw a greater accumulation of certain amino acids at night. In particular, the abundance of longer chained and aromatic amino acids, such as the shikimate pathway derived aromatics phenylalanine, tyrosine and tryptophan; the oxaloacetate derived aspartic acid and lysine; and the α-ketoglutarate derived proline and GABA, all significantly increased at night or with warmer growth temperature (Fig. **4, 5**). Of further interest, this increase in long chained and aromatic amino acids had a strong negative relationship with carbohydrate/organic acid pools (Fig. **3a**) This suggests that N was likely fixed into amino acids during the light, but catabolised at night (more so when nights were warmer) through the respiratory pathways to synthesise a wider array of amino acids, at the expense of carbohydrate and organic acid levels. Alanine is the prime candidate for night-time N recycling, as it is more abundant than other amino acids in the day and significantly depleted at night. It also has the shortest anabolic/catabolic pathway, being converted between alanine and pyruvate by alanine aminotransferases (Hildebrandt *et al.*, 2015). Furthermore, the *de novo* synthesis of alanine in the light is faster than other amino acids (Ishihara *et al.*, 2015, Nelson *et al.*, 2014, Szecowka *et al.*, 2013), indicating its role in initial N storage and carbon skeleton synthesis. Glycine and serine are highly abundant, short-chained amino acids that accumulate during the day and are depleted at night (Fritz *et al.*, 2006), likely playing a similar role to alanine in night-time N and carbon recycling. Recycling of organic carbon skeletons through the TCA cycle may coordinate substrate supply with environmental dependent shifts in energy requirements, maintaining balanced redox poise (Igamberdiev & Eprintsev, 2016).

TCA cycle organic acid intermediates

Neither irradiance nor growth temperature led to a uniform shift in TCA cycle intermediates (Fig. **4**, **5**, Table 1). This is likely attributable to light-dependent regulation of TCA enzymes. Succinate dehydrogenase and fumarase are downregulated in the light via phytochrome A (Eprintsev *et al.*, 2013, Eprintsev *et al.*, 2016) as well as deactivated by a thioredoxin enzyme (Daloso *et al.*, 2015) leading to malate, fumarate and succinate increasing during high-light,

highly-reduced conditions. This tightly controlled light-dependent regulation of succinate dehydrogenase is the likely reason that diel variations in succinate exhibited the strongest correlation with R_{dark} at 30°C (Fig. **4**). In addition, the anaplerotic pathway involving the phosphorylation by PEPC (O'Leary *et al.*, 2011, Tcherkez *et al.*, 2009, Werner *et al.*, 2011) could also contribute to the accumulation of malate and fumarate. Aconitate isomerase activity is greater in the day than at night which may explain a decrease in citrate levels during the day (Igamberdiev & Eprintsev, 2016). We can therefore attribute the significant shifts in TCA intermediates like malate, citrate, fumarate and succinate between day and night (Fig. **4**) to shifts in enzyme velocities regulated by the day night cycle. Interestingly, the limited effect of growth temperature on succinate, malate or citrate levels (Fig. **5**) indicates that these day/night enzyme activity dependent fluctuations are not disrupted by longer-term acclimation to growth temperature.

The accumulation of malate during the day and its depletion at night is linked to enzymatic regulation, with evidence that NAD-malic enzyme is inactive in the light but not dark, coordinating malate storage and utilization with the day/night cycle (Hill, 1992). Additionally, a link between photosynthesis, malate concentrations and leaf respiration is evident in Arabidopsis where reduced plastid NAD-malate dehydrogenase activity results in higher night-time malate and starch content and reduced R_{dark} (Beeler *et al.*, 2014). Similarly, malate depletion due to overexpression of NADP-malate dehydrogenase in Arabidopsis chloroplasts leads to a suppression of R_{dark} and ultimately plant starvation (Zell *et al.*, 2010). Our observation of malate declining at night can thus be attributed to malate synthesis during the day as a storage of photosynthesis reducing potential, followed by oxidation at night to facilitate respiration. The use of stored malate as a TCA substrate at night would likely be another contributor to the disparity between growth temperature dependent R_{dark} and starch drawdown we observed.

Conclusions

From this work we have shown that R_{dark}, glycolysis, and the TCA cycle are highly responsive to both the day/night cycle and temperature acclimation. However, there is little interaction, with minimal influence of the diel cycle on temperature acclimation. Shikimate pathway derived aromatic amino acids were the only metabolites to interact in response to both growth temperature and the day/night cycle. The glycolysis substrate fructose-6-phosphate and the TCA cycle intermediates aconitate and succinate correlate strongly with environmental driven acclimation in R_{dark}. Thus, there is evidence of the tight coordination of glycolysis and the TCA

cycle to respiratory acclimation. However, the relationship between R_{dark} acclimation and the metabolites xylitol, erythritol, putrescine, and serine suggests that by-products of the respiratory pathways are similarly associated with R_{dark} acclimation to temperature and photoperiod. Our results point to a coordinated and complex regulatory system, whereby environmental shifts in redox potential and ATP demand are balanced through adjustments in anaplerotic reactions of the TCA cycle and consequential changes in R_{dark}. How these metabolite adjustments ultimately influence plant growth, development and reproductive fitness may have significant implications on plant adaptation to a warmer climate and crop yields.

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Author Contribution

FAAR, APS, RCD, JM, NLT, and OKA conceptualised experiments. FAAR, APS, SA, and RF performed experimentation and/or data analysis. FAAR, APS, SA, NLT and OKA drafted the manuscript with contributions from all authors.

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Supporting Information

Dataset S1. Metabolite profiles quantified by Gas Chromatography-Mass Spectrometry (GC-MS) for leaf samples collected for each growth treatment and over a diel cycle.

Fig. S1 Photograph of rice plants in their hydroponic containers just prior to sampling.

Table S1. Two-way ANOVA of growth temperature and time effects on dark respiration measured at the prevailing day-time growth temperature and at a common temperature of 30°C.

Table S2. Two-way ANOVA of temperature and diurnal effects on the content of glucose, fructose, sucrose, total soluble sugar, starch and nitrogen in rice leaves.

Table S3. Results of linear regression on dark respiration rates (R_{dark}) measured at 30°C and key metabolites identified in weighted correlation network analysis as presented in Figure 4.

Figure legends

Figure 1. Dark respiration (R_{dark}) of rice leaves on a dry mass basis measured over the diurnal cycle at; (**a**) the respective day-time growth temperature of each treatment; and (**b**), a common temperature of 30°C. Measurements were conducted on rice leaves grown at 25/20°C (blue squares), 30/25°C (control; green circles) and 40/35°C (red triangles). Area shaded in grey represents the night-time. The *P*-values from a two-way ANOVA for the temperature (Temp.), time of sampling (Time) and the interaction between the two variables (Temp. ´ Time) are presented on the graphs. The *F*-values of the two-way ANOVA are presented in Supporting Information Table S1. Data represents means of four biological replicates ± SE.

Figure 2. Rice leaf content of glucose (**a**), fructose (**b**), sucrose (**c**), total soluble sugar (**d**), starch (**e**), and nitrogen (**f**). Leaves were grown at different temperatures of 25/20°C (blue squares), 30/25°C (control; green circles) and 40/35°C (red triangles). Area shaded in grey represents the night-time. The *P*-values from a two-way ANOVA for the temperature (Temp.), time of sampling (Time) and the interaction between the two variables (Temp. ´ Time) are presented on the graphs. The *F*-values of the two-way ANOVA are presented in Supporting Information Table S2. Data represents mean of three biological replicates ± SE.

Figure 3. Principal component analyses (PCA) portraying relationships among metabolites in relation to the diurnal cycle and growth temperature. (**a**) Each thin arrow represents a metabolite, and for each arrow, the direction and length from the centre represent a correlation with principle components (x- and y-axis) and the quality of representation (loading) on the principle component plane. For example, Xylitol (labelled as 29) is well represented by both first and second principle components and is more correlated with the first than the second, while putrescine (labelled as 30) is not well represented by the first two components but is more correlated with the second than the first. Different colours of metabolites and their associated arrows denote different metabolite classes; amino acids in red, carbohydrates in green, organic acids in blue, and others in purple. (**b**) Metabolite responses in the first and

second dims of rice leaves to sampling time (day/night), with day indicated in red and night in blue. (**c**) Metabolite responses in the first and second dims of rice leaves to growth temperature with 25/20°C in blue, 30/25°C in green and 40/35°C in red.

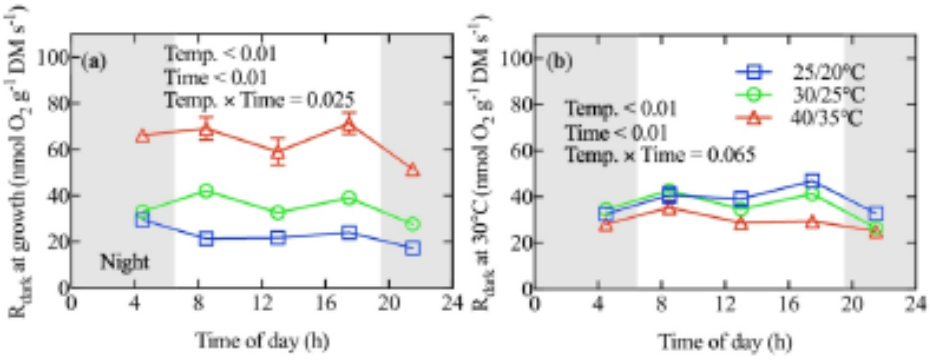
Figure 4. Effect of the diurnal cycle on the relative concentrations of metabolites in rice leaves associated with carbohydrates, glycolysis, amino acids and the tricarboxylic acid (TCA) cycle. Rice leaves were sampled at five different time points throughout light/dark cycle: 13:00 (red), 17:30 (yellow), 21:30 (green), 04:30 (blue) and 08:30 (purple) hours. Data represents mean of four biological replicates. One-way ANOVA and post-hoc test were conducted and different letters show significant differences according to Tukey test (P=<0.05).

Figure 5. Effect of growth temperature treatments on the relative abundance levels of metabolites in rice leaves associated with carbohydrate metabolism, glycolysis, amino acid metabolism and tricarboxylic acid (TCA) cycle. Measurements were conducted on rice leaves grown in 25/20°C (blue), 30/25°C (green) and 40/35°C (red). Data represents mean of four biological replicates. One-way ANOVA and post hoc test were conducted and different letters show significant differences according to Tukey test (P=<0.05).

Figure 6. Metabolites that significantly correlated with R_{dark} at 30°C identified through weighted correlation network analysis. For aconitate, xylitol and erythritol, correlations were mostly attributable to growth temperature. For serine, putrescine and fructose-6-phosphate, correlations were attributable to both growth temperature and the diel cycle. Succinate correlation was attributable to the day/night cycle. Regression analysis P and r^2 values for each metabolite are presented in Supporting Information Table S3.

Table 1. Two-way ANOVA of temperature and diurnal effects on the metabolite level of rice leaves grown at different temperatures. Asterisks (*) denote statistically significant difference (P=<0.05) among the levels of temperature treatments and time points.

Metabolite	*P*-value		
	Temperature	Diurnal	Temperature x Diurnal
Alanine	0.1130	5.25×10^{-7}*	0.2665
Valine	0.2452	0.0242*	0.4354
Isoleucine	0.0281*	0.2110	0.3534
Proline	0.0116*	0.7936	0.2217
Succinate	0.0108*	7.28×10^{-9}*	0.6848
Fumarate	0.0363*	0.0007*	0.9844
Threonine	0.9219	0.0747	0.1243
Malate	0.1522	2.22×10^{-13}*	0.2691
Aspartic acid	0.0829	1.07×10^{-6}*	0.2295
4-Aminobutyric acid	0.0028*	0.9579	0.5610
α-ketoglutarate	3.32×10^{-5}*	$<2.2 \times 10^{-16}$*	0.1675
Phenylalanine	3.54×10^{-6}*	0.0003*	0.0741
Asparagine	0.9157	0.2746	0.2260
Putrescine	2.37×10^{-5}*	0.1720	0.4763
Aconitate	3.75×10^{-9}*	0.0016*	0.6774
Glutamine	1.04×10^{-5}*	0.0279*	0.3447
Shikimic acid	0.5227	0.1504	0.4783
Citrate	0.0919	8.19×10^{-5}*	0.7641
Fructose	0.1620	0.1418	0.8123
Glucose	0.4284	0.0189*	0.9153
Lysine	1.36×10^{-6}*	2.04×10^{-5}*	0.4090
Tyrosine	2.01×10^{-8}*	5.83×10^{-12}*	0.0092*
Tryptophan	0.2963	1.35×10^{-5}*	0.0166*
Fructose-6-phosphate	4.74×10^{-14}	1.59×10^{-7}*	0.5368
Glucose-6-phosphate	5.62×10^{-12}	5.18×10^{-6}*	0.9891
Sucrose	2.66×10^{-5}*	0.0287*	0.7049



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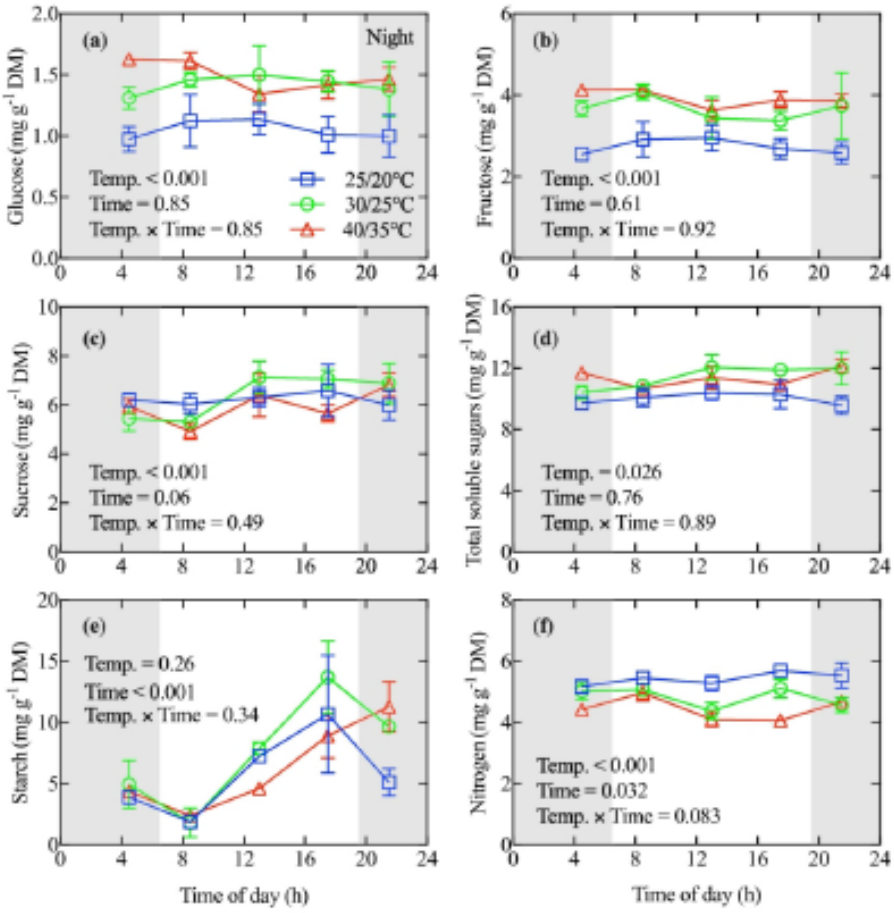

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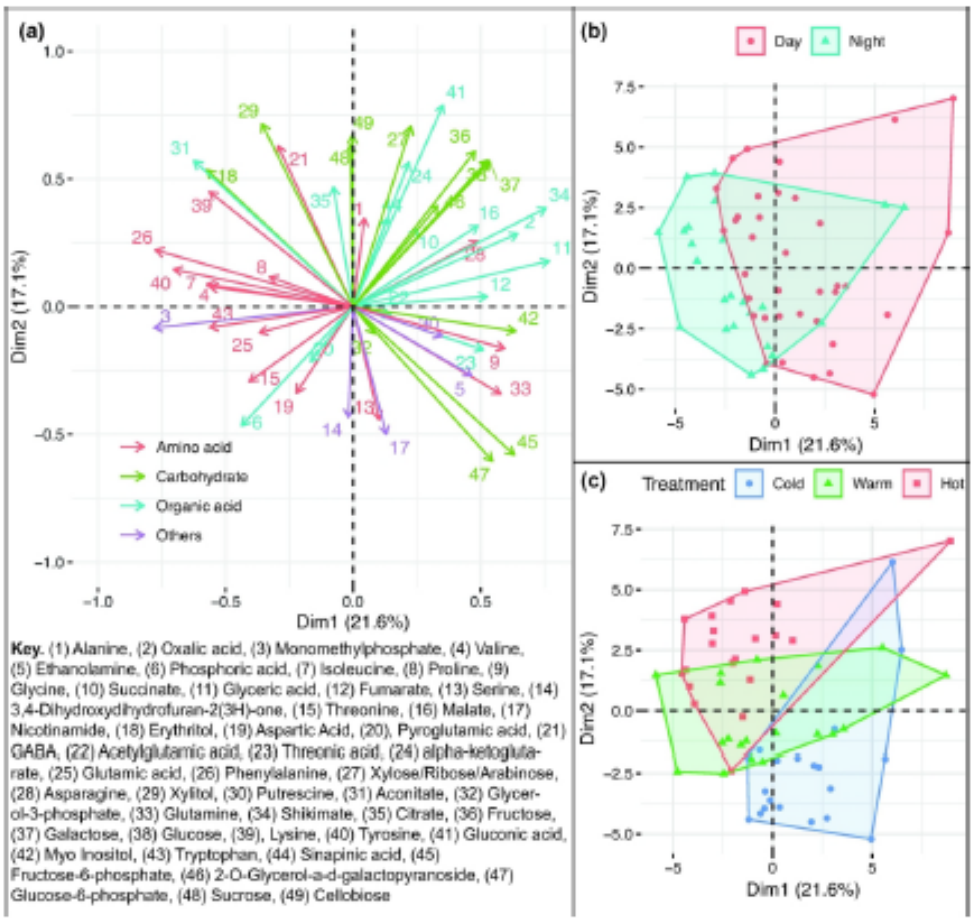

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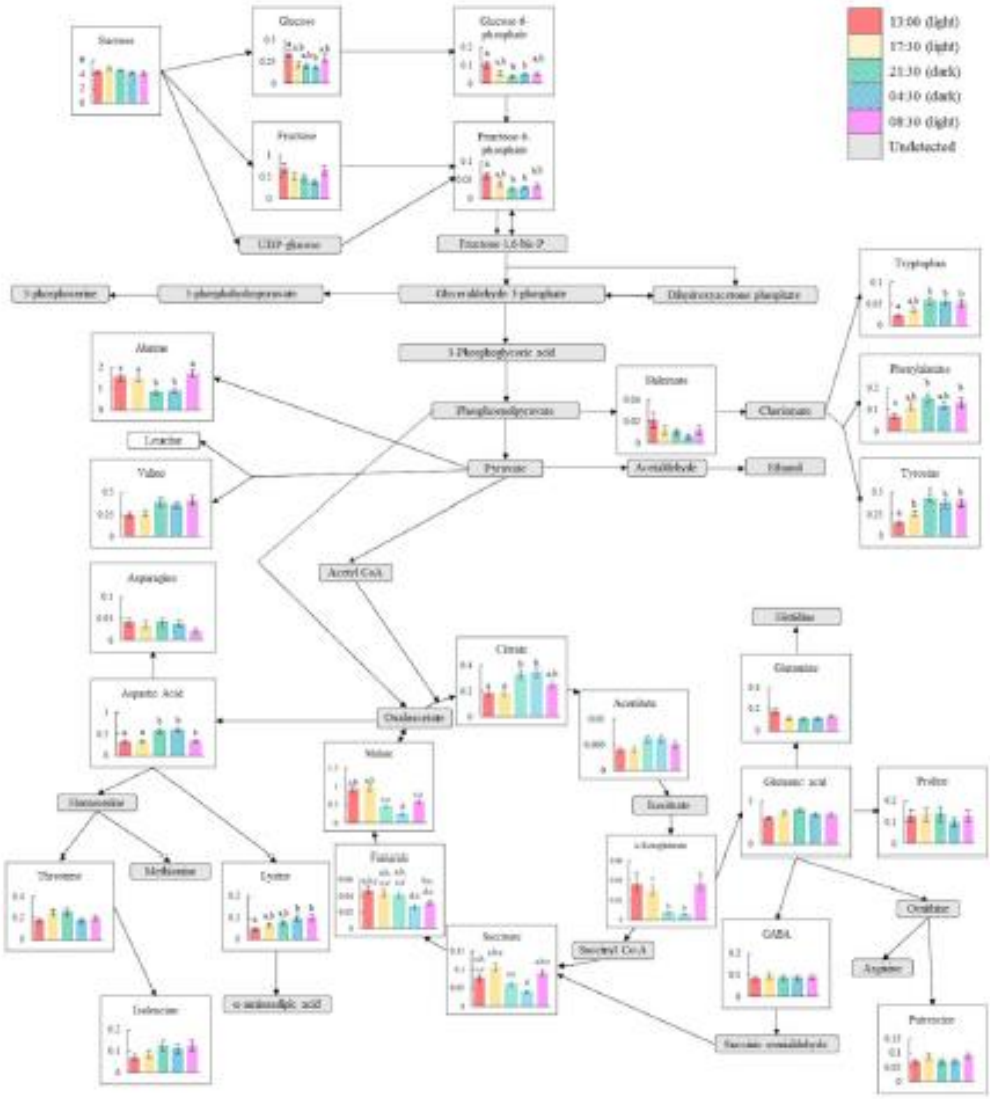

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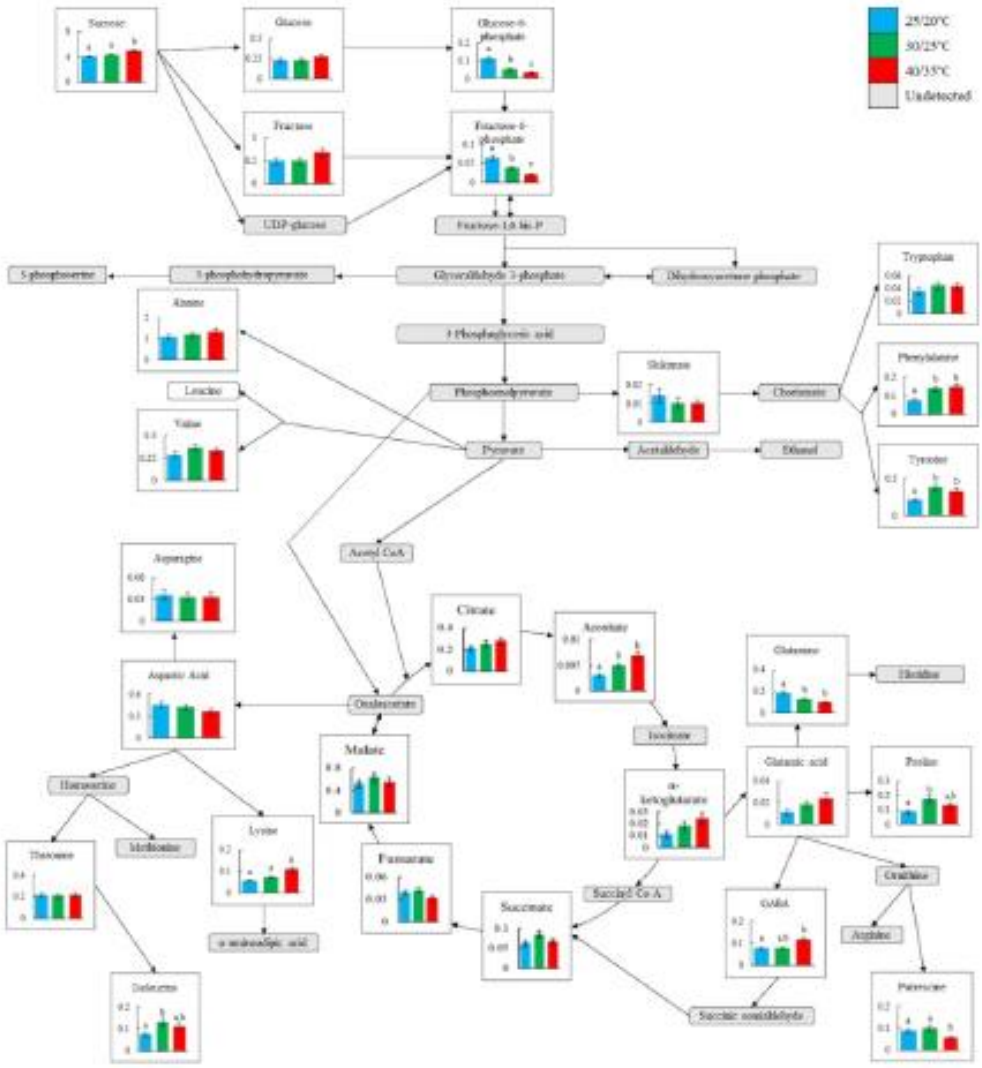

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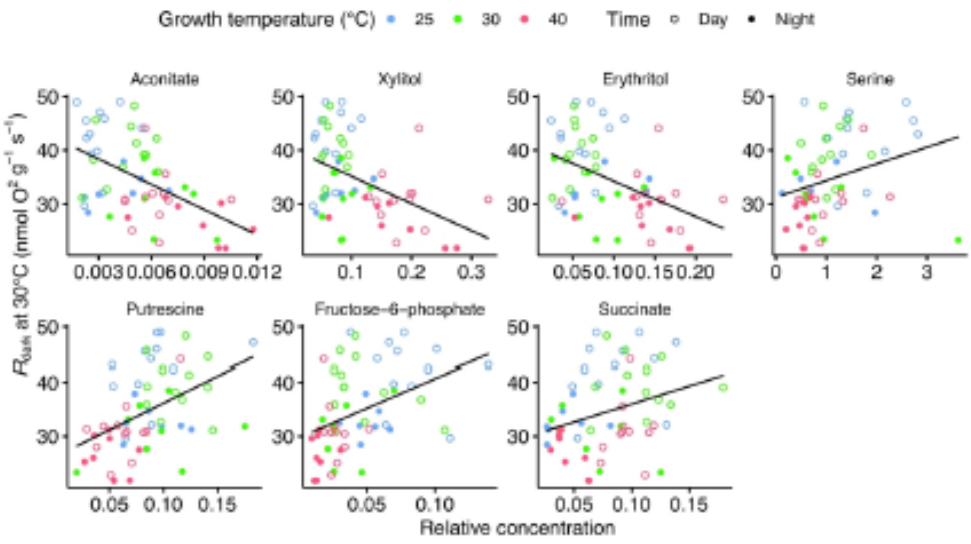

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