Molecular and physiological responses during thermal acclimation of leaf photosynthesis and respiration in rice

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Abstract:	To further our understanding of how sustained changes in temperature affect the carbon economy of rice (*Oryza sativa*), hydroponically-grown plants of the IR64 cultivar were developed at 30/25°C (day/night) before being shifted to 25/20°C or 40/35°C. Leaf mRNA and protein abundance, sugar and starch concentration, gas-exchange and elongation rates were measured on pre-existing leaves (PE) already developed at 30/25°C, or leaves newly-developed (ND) subsequent to temperature transfer. Following a shift in growth temperature, there was a transient adjustment in metabolic gene transcript abundance of PE leaves before homeostasis was reached within 24 h, aligning with R_{dark} (leaf dark respiratory CO_2 release) and A_n (net CO_2 assimilation) changes. With longer exposure, the central respiratory protein CYTOCHROME C OXIDASE (COX) declined in abundance at 40/35°C. In contrast to R_{dark},

A_n was maintained across the three growth temperatures in ND leaves. Soluble sugars did not differ significantly with growth temperature, and growth was fastest with extended exposure at 40/35°C. The results highlight that acclimation of photosynthesis and respiration is asynchronous in rice, with heat-acclimated plants exhibiting a striking ability to maintain net carbon gain and growth when exposed to heat-wave temperatures, even while reducing investment in energy-conserving respiratory pathways.

Molecular and physiological responses during thermal acclimation of leaf photosynthesis and respiration in rice

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Abstract

To further our understanding of how sustained changes in temperature affect the carbon economy of rice (*Oryza sativa*), hydroponically-grown plants of the IR64 cultivar were developed at 30/25°C (day/night) before being shifted to 25/20°C or 40/35°C. Leaf mRNA and protein abundance, sugar and starch concentrations, gas-exchange and elongation rates were measured on pre-existing leaves (PE) already developed at 30/25°C, or leaves newly-developed (ND) subsequent to temperature transfer. Following a shift in growth temperature, there was a transient adjustment in metabolic gene transcript abundance of PE leaves before homeostasis was reached within 24 h, aligning with R_{dark} (leaf dark

34   respiratory CO_2 release) and A_n (net CO_2 assimilation) changes. With longer exposure, the central

35   respiratory protein CYTOCHROME C OXIDASE (COX) declined in abundance at 40/35°C. In

36   contrast to R_{dark}, A_n was maintained across the three growth temperatures in ND leaves. Soluble sugars

37   did not differ significantly with growth temperature, and growth was fastest with extended exposure

38   at 40/35°C. The results highlight that acclimation of photosynthesis and respiration is asynchronous

39   in rice, with heat-acclimated plants exhibiting a striking ability to maintain net carbon gain and growth

40   when exposed to heat-wave temperatures, even while reducing investment in energy-conserving

41   respiratory pathways.

42

43   **Keywords:** rice, thermal stress, acclimation, respiration, photosynthesis, heat, cold, CYTOCHROME

44   C OXIDASE (COX)

45

46   **Introduction**

47

48   The response of net CO_2 assimilation (A_n) and leaf respiration in the dark (R_{dark}) to changes in

49   temperature (T) is often dynamic. Acclimation – i.e. physiological changes that enable adjustments in

50   the rate of A_n and R_{dark} at a given measuring T in response to sustained changes in growth T – occurs

51   in many species, in natural and controlled environments (Atkin, Bruhn, Hurry, & Tjoelker, 2005; Berry

52   & Bjorkman, 1980; Campbell et al., 2007; Reich et al., 2016; Smith & Dukes, 2017; Tjoelker, Oleksyn,

53   & Reich, 2001). Acclimation can also lead to metabolic homeostasis, where similar rates of A_n and

54   R_{dark} are exhibited by hot- and cold-acclimated plants, when compared at their respective growth Ts.

55   Determining the extent to which R_{dark} and A_n acclimate to sustained changes in T is of growing interest,

56   as global warming is resulting in plants of natural and managed ecosystems experiencing higher

57   average growth Ts, often in conjunction with more frequent and severe heat waves (CSIRO & BOM,

58   2018; Hartmann et al., 2013). The impact of heat on A_n and R_{dark} of cereal crops, including rice (*Oryza*

59   *sativa*), is of particular interest given the need to increase food production to meet the requirements of

60   a growing and more affluent world population (Godfray et al., 2010). Rice contributes substantially to

61   global food demand, particularly in Asia where it makes up more than 30% of all dietary energy intake

62   (Seck, Diagne, Mohanty, & Wopereis, 2012). However, in recent years rice yields have declined in

63   regions such as South-East Asia, with the declines being more strongly correlated with nightly

64   minimum than daytime maximum Ts (Peng et al., 2004; Welch et al., 2010). Reduced yields and grain

65   quality were also observed for rice in North America when exposed to warmer night T (Lanning,

66   Siebenmorgen, Counce, Ambardekar, & Mauromoustakos, 2011). Given this, and the likely

67   importance of A_n and R_{dark} for biomass and grain production (Posch et al., 2019; Yoshida, 1972), it is

68   crucial that we develop a better understanding of how changes in T affect these key metabolic
69   processes in rice.

70   In rice and a range of other crops, RNA sequencing analysis has shown large scale
71   perturbations to the transcript profile of plants exposed to colder or warmer T, with the changes
72   occurring over a period of hours to days and across multiple functional categories, but especially in
73   genes involved in primary metabolism (Bhardwaj et al., 2015; Hu, Sun, Zhang, Nevo, & Fu, 2014;
74   Shen et al., 2014). The vast gene expression response to T-perturbations is likely mediated through
75   heat shock transcription factors, which regulate changes in transcriptional networks. These are induced
76   by heat stress and other abiotic stimuli, changing the protein complement of a cell (Morimoto, 1998;
77   Ohama, Sato, Shinozaki, & Yamaguchi-Shinozaki, 2017). However, changes in protein abundance
78   may not necessarily match changes in transcript abundance due to transcription and RNA turnover
79   rates being influenced by T (Sidaway-Lee, Costa, Rand, Finkenstadt, & Penfield, 2014), and post-
80   transcriptional regulation. In cases where heat stress results in changes in protein abundance, the
81   greatest changes are seen in proteins associated with primary metabolism, with about 50% of all leaf
82   protein abundance changes seeming to be in A_n and R_{dark} metabolic pathways (Scafaro & Atkin, 2016).
83   In rice, cold-stress similarly perturbs a large proportion of energy metabolism pathways (Neilson,
84   Mariani, & Haynes, 2011), emphasising the importance of changes in protein abundance in
85   chloroplasts and mitochondria as part of the thermal acclimation process. There is growing evidence
86   that plants can acclimate to T variations by stimulating energy metabolism at colder T and suppressing
87   energy metabolism at warmer T, either through regulation of enzymatic velocities or changes in
88   enzyme abundance (Armstrong et al., 2008; Badger, Björkman, & Armond, 1982; Campbell et al.,
89   2007; Hikosaka, Ishikawa, Borjigidai, Muller, & Onoda, 2006; Scafaro et al., 2017; Strand et al., 1999;
90   Yamori, Noguchi, & Terashima, 2005). Whether the same is true for rice remains unclear, both for
91   pre-existing (PE) leaves that experience a sustained change in growth T, and in newly-developed (ND)
92   leaves that form under a new thermal regime. In species other than rice, the extent of changes
93   underpinning thermal acclimation (including changes in leaf structure, nitrogen partitioning and
94   organelle abundance), is typically greater in ND than PE leaves (Armstrong et al., 2008; Gorsuch,
95   Pandey, & Atkin, 2010; O'Leary, Asao, Millar, & Atkin, 2018; Tjoelker, Reich, & Oleksyn, 1999;
96   Yamori et al., 2005).

97   Past studies on rice conducted during the vegetative (Glaubitz et al., 2014; Kurimoto, Day,
98   Lambers, & Noguchi, 2004) and reproductive (Bahuguna, Solis, Shi, & Jagadish, 2017; Mohammed,
99   Cothren, & Tarpley, 2013) phases of development have reported limited and variable levels of
100   acclimation of R_{dark}. By contrast, A_n of rice shows strong thermal acclimation, with rates of net CO_2
101   uptake measured at the prevailing growth T being homeostatic or increasing as growth T is increased

3

102   from 15 to 37°C (Nagai & Makino, 2009; Yamori, Noguchi, Hikosaka, & Terashima, 2010). Such

103   studies point to a possible asynchrony in rice acclimation, with a more dynamic A_n than R_{dark} response,

104   although further work simultaneously comparing A_n and R_{dark} in rice is needed. Interestingly, a range

105   of other crops and non-crop species show the opposite – greater R_{dark} than A_n acclimation capacity

106   (Campbell et al., 2007; Drake et al., 2016; Way & Oren, 2010). Although such studies detail the

107   physiological acclimation of energy metabolism in plants, including rice, less is known about the

108   molecular and biochemical responses that underpin this phenotypic T acclimation.

109   It is with the above issues in mind that we conducted a study using the IR64 cultivar of *Oryza*

110   *sativa* to address the following aims: (1) characterise the extent of thermal acclimation of R_{dark} and A_n;

111   and, (2) link physiological acclimation to the underlying processes through analysis of leaf transcript,

112   protein, sugar, and starch abundance, following changes in growth T. We hypothesized that: (1) initial

113   exposure to very high growth T increases and decreases the rates of respiratory CO_2 release and net

114   photosynthetic CO_2 uptake, respectively; (2) subsequent acclimation is associated with recovery of A_n,

115   and reduced rates of R_{dark}; and (3) thermal acclimation of R_{dark} and A_n is associated with dynamic

116   changes in gene expression and protein abundance in key pathways associated with energy capture

117   and use.

118

119   **Materials and methods**

120

121   *Plant material and temperature treatments*

122   Rice (*Oryza sativa*) cultivar IR64 plants were grown hydroponically in a glasshouse facility at the

123   Research School of Biology, Australian National University in Canberra between October and

124   December 2015. Seeds were initially incubated at 40-42°C for two days before soaking in water for

125   eight hours, placed on wet Whatman filter papers in petri dishes and kept in the dark at 30°C for five

126   days. The germinated seedlings were then transferred to trays of vermiculite and placed in temperature-

127   controlled glasshouses (30°C day and 25°C night) under natural sunlight and photoperiod, with

128   photosynthetically active radiation (PAR) between 400 and 1200 µmol m^{-2} s^{-1}. When the third leaf had

129   emerged, seedlings were transplanted from vermiculite to a hydroponic system. Individual plants were

130   placed within PVC tubes with a 3.7 cm diameter and 13 cm height. Tubes were then suspended at the

131   top of 20 L capacity hydroponic tanks (12 tanks in total), with each tank holding a maximum of 20

132   plants. Each tank was filled with hydroponic solution (Table S1) (Hubbart, Peng, Horton, Chen, &

133   Murchie, 2007). The nutrient solution was replaced weekly. Sulphuric acid or sodium hydroxide were

134   used to adjust the pH to 5-6, with pH monitored using a portable pH meter (Rowe Scientific Pty. Ltd.,

135   NSW, Australia). The hydroponic solution was aerated continuously using Infinity AP-950 air pumps

136   (Kong's Pty. Ltd., Ingleburn, Australia).

137   After two weeks of hydroponic growth at 30/25°C ('warm' treatment), the most recently fully-

138   expanded leaves were labelled as pre-existing (PE) leaves. Following labelling, four tanks were

139   randomly chosen and shifted to an adjacent glasshouse room set to 25°C day and 20°C night (25/20°C –

140   'cold' treatment), and four other tanks were moved to a room set at 40°C day and 35°C night (40/35°C

141   – 'hot' treatment); four tanks were retained at 30/25°C as controls. Relative humidity was not

142   controlled. Newly-developed (ND) leaves that emerged under each thermal regime were labelled, with

143   all measurements reported on ND leaves made 21 days after T-transfer.

144

145   *Determination of transcript abundance*

146   Plants were transferred to new thermal regimes three hours after sunrise. To quantify transcript

147   abundance, the labelled pre-existing (PE) leaves were harvested during the photoperiod: two, six and

148   24 h after T-transfer. For each time-point and temperature treatment, approximately 8 cm long

149   segments (less than 100 mg of fresh mass) were sampled half-way along the leaf blade, and

150   immediately frozen in liquid N_2 and stored at -80°C until RNA extraction. Total RNA was extracted

151   using the RNeasy plant mini protocol (Qiagen, Doncaster, VIC, AU) and treated with DNase I (Qiagen,

152   Doncaster, VIC, AU) to remove any contaminating DNA.

153   For qPCR analysis, 1 μg of total RNA in a 10 μL volume was reverse- transcribed into cDNA

154   using SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA), according to

155   the manufacturer's instructions. The reverse-transcribed cDNA samples were diluted 10-fold.

156   Transcript levels of six selected genes and one reference gene (refer to Table S2 for gene accession

157   numbers and primer sequences) were analysed using a Light-Cycler® 480 System (Roche Holding

158   AG, Basel, Switzerland) with SYBR Green I Dye (QIAGEN, Doncaster, Victoria, AU). cDNA

159   samples from each biological replicate were assayed in two technical duplicates. The reaction-mix in

160   each qPCR contained 0.4 μM of each pair of primers, 5 μL of SYBR Master Mix, and 4.6 μL of the

161   diluted cDNA sample in a 10 μL total reaction volume. The raw fluorescence data were analysed using

162   LinRegPCR (Ramakers, Ruijter, Deprez, & Moorman, 2003; Ruijter et al., 2009). Data were

163   normalized to the reference gene, eukaryotic initiation factor 5c (*EIF5C*; LOC_Os11g21990.1), and

164   were expressed as fold-changes against control conditions.

165   RNAseq libraries were prepared using the Illumina Stranded Total RNAseq kit with RiboZero

166   rRNA depletion as per the manufacturer's guidelines (Illumina). Libraries were pooled and sequenced

167   on a HiSeq1500 for 61 cycles in single end mode at the Centre for AgriBioscience, University of

168   LaTrobe. Analysis pipelines for pre-processing and mapping of sequence data are available online on

169   GitHub (https://github.com/pedrocrisp/NGS-pipelines). Quality control was performed with *FastQC*

170   v.0.11.2. Adapters were removed using *scythe* v.0.991 with flags -p 0. and reads were quality trimmed

171   with *sickle* v.1.33 with flags -q 20 (quality threshold), and -l 20 (minimum read length after trimming).

172   The trimmed and quality-filtered reads were aligned to the rice reference genome Os-Nipponbare-

173   Reference-IRGSP-1.0 from the MSU Rice Genome Annotation Project Database v7

174   (http://rice.plantbiology.msu.edu/) using the *subjunc* aligner v1.5.0-p1 with -u and -H flags to report

175   only reads with a single unambiguous best mapping location, -P 3 for phred+33 encoding (Liao, Smyth,

176   & Shi, 2013b). Reads were then sorted, indexed and compressed using *samtools* v1.1-26-g29b0367

177   (Li et al., 2009) and strand-specific bigwig files were generated using *bedtools genomecov* v2.16.1

178   (Quinlan & Hall, 2010) and the UCSC utility *bedGraphToBigWig* for viewing in IGV (Robinson et

179   al., 2011). Summary statistics for each sample are provided in Supplementary Dataset S1: Summary

180   of transcriptomic datasets.

181   For standard differential gene expression testing, the number of reads mapping per IRGSP-

182   1.0 gene loci was summarised using *featureCounts* v1.5.0-p1(Liao, Smyth, & Shi, 2013a) with flags

183   -P and -c to discard read pairs mapping to different chromosomes and the -s flag set to 2 for strand

184   specificity for a strand specific library, multimapping reads and multioverlapping reads were not

185   counted. Reads were summarised to parent IRGSP-1.0 gene loci rather than individual splice variants

186   by summarising to the genomic coordinates defined by the feature "gene" in the IRGSP-1.0.gff

187   reference (last modified 7/2/2012

188   ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/all.dir/all.gff3). Only loci

189   with an abundance of at least 1 CPM (approximately 5 reads) in at least 4 samples were retained.

190   Statistical testing for relative gene expression was performed in R following the "*edgeR-limma-*

191   *voom*" approach (https://www.bioconductor.org/help/workflows/RNAseq123/); using, *edgeR* v.3.4.2

192   (McCarthy, Chen, & Smyth, 2012; Robinson & Oshlack, 2010; Robinson & Smyth, 2007a, 2007b),

193   and *voom* in the *limma* package 3.20.1 (Law, Chen, Shi, & Smyth, 2014; Smyth, Michaud, & Scott,

194   2005).

195

196   *Determination of protein abundance*

197   Samples of frozen PE leaf material six and 24 h after *T* transfer, as well as frozen ND leaf material,

198   were ground to a fine powder using a chilled mortar and pestle, and protein was extracted in extraction

199   buffer containing 100 mM tricine pH 8.0, 1 mM EDTA, 1 mM PMSF, 1x protease inhibitor cocktail,

200   2% (w/v) PVPP, 10 mM (w/v) ascorbate, 5 mM DTT, and distilled water. The sample was then

201   solubilized in a NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA, USA) with 10% (v/v) DTT,

202   then heated for 10 minutes at 95°C, and centrifuged for 30 sec at 12,000 RPM. Thereafter, supernatant

203   was collected and 8 uL were loaded and separated on 4-12% NuPAGE Bis-Tris gel (Invitrogen,

204   Carlsbad, CA, USA) using the MOPS-based buffer system. To blot, proteins from the gel were

205   transferred to Immobilon-P Polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Kilsyth,

206   VIC, AU) using an XCell II Blot module (Invitrogen, Carlsbad, CA, USA). Membranes were then

207   blocked for 2 h with 5% (w/v) skim-milk powder in Tris-buffered saline containing 0.1% (v/v) Tween-

208   20 (TBST). To probe for cytochrome *c* oxidase (*COX*) subunit II, alternative oxidase (*AOX*),

209   uncoupling protein (*UCP*) and voltage-dependent anion-selective channel protein (*VDAC1*-porin), the

210   membranes were incubated for 2 h in primary antibody solution (5% w/v skim milk powder in TBST)

211   containing commercially available polyclonal antibodies (Agrisera, Vännäs, Västerbotten, Sweden).

212   All antibodies were diluted 1:5000. An antibody for ribulose-1,5-bisphosphate carboxylase/oxygenase

213   (Rubisco) was received from Assoc. Prof. Spencer Whitney (Research School of Biology, Australian

214   National University, Canberra, ACT, AU) and used at a dilution of 1:10 000. After washing with TBST,

215   the membranes were incubated for 1 h in goat anti-rabbit antibody solution (5% w/v skim milk powder

216   in TBST) at a dilution of 1:8000. Proteins were then visualized using the AttoPhos AP fluorescent

217   substrate system (Promega, Madison, WI, USA), imaged using a Versa-Doc (Bio-Rad, Hercules, CA,

218   USA) imaging system and quantified using Image Lab software (Bio-Rad, Hercules, CA, USA).

219   Protein concentrations were determined by the Bradford method using Bovine Serum Albumin (BSA)

220   as a standard.

221

222   *Determination of soluble sugar and starch concentrations*

223   Starch and soluble sugars of PE leaves transferred from 30/25°C to 25/20°C or 40/35°C for one and

224   seven days, and ND leaves at the prevailing *T* were collected from separate, previously unsampled

225   plants. Samples were collected at 9:30 to 10:00 am, corresponding to 3 h into the light period, frozen

226   and stored at -80°C before freeze-drying at -105°C for two days (Virtis Benchtop™ "K", SP, Scientific,

227   Gardiner, NY, USA), then ground to a fine powder from which a 5-10 mg aliquot was taken. Five-

228   hundred μL of 80% (v/v) ethanol was added and vortexed for 20 sec. Thereafter, leaf tissues were

229   incubated at 80°C while being centrifuged at 500 RPM for 20 min. Following further centrifugation at

230   12,000 RPM for 5 min, the resulting pellet and supernatant were separated. This procedure was

231   repeated a two more times, and the three pellets and three supernatants were pooled. The pooled

232   supernatants and pellets were used for determination of soluble sugars and starch concentrations, using

233   a Fructose Assay Kit (Sigma-Aldrich, St Louis, MO, USA) and a Total Starch Assay Kit (Megazyme,

234   Chicago, IL, USA), respectively. Following manufacturer's instructions, measurements were made in

235   triplicate, at a wavelength of 340 nm, using a microtitre plate reader (Infinite® M1000 Pro; Tecan US,

236   Morrisville, NC) and standard curves were generated for soluble sugars using sucrose, glucose and

237   fructose (Sigma-Aldrich, St Louis, MO, USA) at known concentrations.

238

239   *Gas exchange measurements using Licor 6400XT 6 cm² cuvettes*

240   Gas-exchange was measured on fully-expanded pre-existing (PE) leaves just prior to- and one, two,

241   three, five and seven days after T transfer, as well as on fully-expanded newly-developed (ND) leaves

242   21 days after transfer, using two matched LI-6400 instruments equipped with 6 cm² cuvettes and a

243   6400-02B red-blue light source (Li-Cor, Lincoln, NE, USA). At each time point, light-saturated net

244   CO_2 assimilation rates (A_n) and then dark respiration rates (R_{dark}) were measured during the light period

245   (between 10 am and 2 pm) in the glasshouses, at the prevailing day-time T of each treatment as well

246   as at a common temperature of 30°C for ND leaves. In call cases, A_n was measured first, with the

247   following settings: 1000 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD), relative humidity

248   of 60–70%, 400 ppm reference CO_2, and a flow rate of 500 μmol s⁻¹. Photosynthesis was measured

249   when CO_2 concentrations in the sample IRGA had stabilized, typically within 10 minutes of exposure

250   to 1000 μmol m⁻² s⁻¹ PPFD. Thereafter, R_{dark} was measured as above but with the flow rate slowed to

251   300 μmol s⁻¹ and turning off the light source for at least 30 minutes of darkness before taking

252   measurements.

253

254   *Gas exchange measurements using* using Walz chambers

255   High resolution temperature response curves of R_{dark} and light-saturated A_n were made on intact ND

256   leaves using two matched LI-6400XT portable gas exchange systems (Li-Cor, Lincoln, NE, USA)

257   each connected to a 14 x 10 cm well-mixed, temperature-controlled Walz Gas-Exchange Chamber

258   3010-GWK1 (Heinz Walz GmbH, Effeltrich, Germany). For each temperature-response curve, leaf T

259   was measured with a small-gauge wire copper constantan thermocouple pressed against the lower

260   surface of the leaf and attached to a LI-6400 external thermocouple adaptor (LI6400-13, Li-Cor Inc.,

261   Lincoln, NE, USA) that enabled leaf temperature to be recorded by the LI-6400XT. As leaves were

262   heated, net CO_2 exchange was recorded at 30 s intervals using the LI-6400XT portable gas exchange

263   systems fitted with an empty and closed 6 cm² chamber that was plumbed into the airstream exiting

264   the Walz leaf chamber (Fig. S1). A_n was measured as described for the 6 cm² cuvette but using a Walz

265   LED-Panel RGBW-L084 light source (Heinz Walz GmbH, Effeltrich, Germany). A_n was monitored

266   as leaves were heated at 1°C min⁻¹ from 20 to 45°C. A water trap was used to remove water vapour,

267   as transpiration from whole intact leaves was incompatible with Licor instrumentation. Therefore,

268   stomatal conductance (g_s) and associated water parameters were not recorded. For R_{dark}, on separate

269   leaves to those used for measuring A_n, the flow rate was reduced to 300 μmol s⁻¹, the light source was

270 turned off, and the chamber was covered with a black cloth, before increasing the leaf temperature in

271 steps of 1°C min^{-1}, from 20 to 60°C. In parallel to quantifying the temperature-response of R_{dark}, we

272 measured minimal chlorophyll *a* fluorescence (F_o) in the presence of a low-intensity far-red light pulse

273 (necessary to maintain PSII in the oxidized state) every 30 sec using a Mini-PAM portable chlorophyll

274 fluorometer (Heinz Walz, Effeltrich, Germany) fitted above the glass surface of the leaf chamber. The

275 temperature at which F_o increased was used as an indication of heat-induced damage to photosystem

276 II, which we hereafter refer to as T_{crit}, calculated using the template of O'Sullivan *et al.* (2013). At the

277 cessation of measurements, leaves were photographed and analysed for leaf area using ImageJ

278 software (Abramoff, Magelhaes, & Ram, 2004). Leaves were stored in paper bags, oven-dried at 70°C

279 for two days and weighed to obtain the dry mass. Quadratic equations were fit to A_n temperature curves

280 and the x- and y-axis values corresponding to the vertex taken as the T-optimum (T_{opt}) and A_n-optimum

281 (A_{opt}) of net assimilation, respectively. For the R_{dark} temperature curves, the x- and y-axis values

282 corresponding to the maximum recorded R_{dark} were taken as the T at which R_{dark} reached a maximum

283 (T_{max}) and the maximum R_{dark} value recorded (R_{max}), respectively.

284

285 *Leaf elongation rates*

286 The leaf elongation rates (LER) of four leaves from separate plants from each temperature regime were

287 measured at five separate time-points over a 24 h period. Measurements were made using a ruler,

288 starting from the ligule of the second youngest leaf to its tip.

289

290 *Statistical analysis*

291 For all T-treatments and collection times, four separate leaves from four separate previously unsampled

292 plants, one plant from each of the four hydroponic tanks (pot replicates) were sampled. One-way

293 Analysis of Variance (ANOVA) was performed on R_{dark} and A_n gas-exchange experiments comparing

294 temperature treatments. Two-way ANOVA was performed on LMA and protein, starch, and sugar

295 concentrations comparing time of sampling and temperature treatments. Gas-exchange and leaf

296 biochemical statistical analysis was performed using GraphPad Prism (v 7) software. Statistical

297 analysis of transcript abundance was performed using R statistical software (v 3.6.1) and packages as

298 mentioned above.

299

300 *Data availability*

301 RNA-seq data is available under the GEO identifier GSE136045.

302

303 **Results**

304

Molecular and biochemical responses of leaves to T

Quantitative PCR was performed on specific genes of interest to elucidate the genetic response of pre-existing (PE) leaves exposed to a change in *T* (Fig. 1). Apart from a sharp rise 6 h into the 40/35°C *T*-transfer, there was a general reduction in transcript abundance of *cytochrome c oxidase* (*cox*), a gene encoding the central respiratory electron transport chain. This reduction occurred in leaves transferred to 25/20°C and 40/35°C, over the seven days post-transfer period, . Two genes encoding respiratory proteins that potentially reduce the production of ATP – *alternative oxidase* (*aox*) and *uncoupling protein* (*ucp*) – both showed an initial increase in expression within the first 24 h of transfer to the hotter 40/35°C, followed by a decline to 30/25°C levels by 48 h. The photosynthetic electron transport gene *ferredoxin NADP reductase* (*fnr*), and the Calvin/Benson cycle gene *phosphoribulokinase* (*prk*) generally showed an increase in expression in the first 48 h at 40/35°C before being supressed for up to 5 days post-transfer. *Sucrose phosphate synthase* (*sps*), involved in the synthesis of sucrose from its precursors, also initially spiked in the first 24 h following transfer to 40/35°C, before being transiently suppressed. Both *sps* and the respiratory and photosynthetic genes – apart from *aox* – followed a similar expression profile when heat-treated, suggesting that assimilate production/consumption and sucrose synthesis were coordinated in response to heat perturbations. In general, the greatest perturbation to gene expression occurred within the first 24 h after transfer.

Based on the qPCR results, we conducted RNA-seq at two, six and 24 h after transfer of PE leaves to new *T*. Following data quality control and filtering, transcript abundance of 19,308 rice genes were retained for differential expression testing. Around 20 M reads were obtained per sample, which were aligned to the Os-Nipponbare-Reference-IRGSP-1.0 rice reference genome (Data Set S1). Principal component analysis showed a substantial treatment effect on gene expression for the heat-exposed leaves (40/35°C) during the first six hours after *T*-transfer compared to the other two 30/25°C and 25/20°C growth regimes (Fig. 2). Globally, there was little gene expression variation between the cold (25/20°C) and the warm (30/25°C) control conditions. After 24 h of growth at new *T*-regimes, limited variation in gene expression was observed between all of the three *Ts* (Table S3).

To assess changes in the expression of individual genes to the hot or cold treatments, differentially expressed genes were identified at each time point by comparison to the warm control expression levels (Data Set S2). There were very few genes differentially expressed under the cold conditions, only six genes in total (Table S3). By contrast, under hot conditions, there were 1,818 and 1,465 genes differentially expressed after two and six hours, respectively. After 24 hours, there were no differentially expressed genes under the hot conditions compared to the control plants. There was

337 a significant overlap between the genes differentially expressed after two and six hours of heat

338 treatment, (Fig. 3a, b). In total, 30% of the genes upregulated after six hours were already upregulated

339 by two hours, and 38% of genes downregulated after six hours were already downregulated by two

340 hours. Many of the remaining genes that were significantly different in transcript abundance only after

341 six hours were already trending in the same direction at the two-hour time point, but the difference

342 compared to the controls did not pass the significance threshold (Fig. 3 c). Overall, these results show

343 that there are significant short-term changes in transcript abundance in rice plants exposed to heat

344 stress. The expression profiles of samples exposed for two and six hours show consistent changes;

345 however, some of the changes peak at two hours and others peak at six hours and most changes

346 dissipate within 24 hours.

347   In total, the heat treatment led to the up- and down-regulation of 1,337 and 1,446 genes,

348 respectively. To investigate the extent to which these genes have a photosynthetic or respiratory

349 function, we first examined expression of genes involved in photosynthesis, glycolysis, TCA and

350 mitochondrial electron transport using MapMan pathway annotations (Fig. S2-4) (Thimm et al., 2004).

351 This qualitative analysis revealed that the expression of only a small number of

352 photosynthetic/respiratory genes were affected. To extract a list of high-confidence differentially

353 expressed respiration-related genes, we manually curated a list of rice loci with homology to

354 Arabidopsis respiration genes (Data Set S3). Using this list, we found that eight genes were

355 differentially expressed at high temperature, with two genes downregulated more than 2-fold: *aox* and

356 ATP-dependent *phosphofructokinase* (Table 1). The seemingly conflicting result of an initial increase

357 in *aox* from the qPCR results, but a decline in *aox* during the same period from the RNA-seq results,

358 can be explained by our qPCR primers targeting the *aox1a* isoform while the RNA-seq identified a

359 decline in the *aox1c* isoform (Data Set S3).

360   It is interesting that in addition to the increase in *aox* and *ucp* gene expression, the expression

361 of an external NAD(P)H dehydrogenase also increased, while that of Complex II decreased

362 significantly (Table 1). Together these changes suggest that an increase in non-phosphorylating

363 electron transport occurred in response to exposure to higher *T*, at the expense of electron transport

364 coupled to ATP synthesis, at least in the short term. The increase in external NAD(P)H dehydrogenase

365 gene expression may also indicate an increased need for mitochondrial oxidation of excess reductant

366 produced in the chloroplast at higher *T*.

367   Given the relatively small effect of the heat treatment on the expression of respiration- or

368 photosynthesis-related genes, we next performed Gene Ontology enrichment analysis. This revealed a

369 notable enrichment for genes involved in primary metabolism (eg GO:0044238) and response to

370 abiotic stimuli (eg GO:0050896), as well as in many biosynthetic pathways (Fig 4).

371   Protein abundance (expressed on a leaf area basis) of key mitochondrial electron transport

372   components – CYTOCHROME C OXIDASE (COX) subunit II, ALTERNATIVE OXIDASE (AOX)

373   and UNCOUPLING PROTEIN (UCP) – were determined by Western blots in PE leaves 6 h and one-

374   day after T-transfer, and in newly developed (ND) leaves that formed under each prevailing growth T

375   (Fig. 5, Fig. S4). The cold (20/25°C) and heat (40/35°C) treatments did not affect the total protein

376   concentration of leaves, at any time after T-transfer (Table S4). As was the case for gene expression,

377   there was a significant decline in COX subunit II protein abundance after 24 h. COX subunit II also

378   declined in abundance in ND leaves when grown at 40/35°C compared to 25/20°C (Fig. 5a). We

379   assume the changes observed in COX subunit II reflect changes in abundance of the entire complex.

380   The abundance of AOX and UCP protein did not vary in response to growth T or duration of exposure

381   to heat for either PE or ND leaves, despite the initial spike in aox and ucp gene expression after transfer

382   to 40/35°C (Fig. 1). Interestingly two bands of AOX that varied relative to one another with

383   temperature treatment were evident in the Western blot (Fig. S5). This is consistent with the

384   fluctuations in expression of different aox genes noted above. Patterns of protein abundance were

385   similar when the analysis was standardised to dry mass or porin abundance (Fig. S6). Porin is a voltage-

386   dependent channel protein located at the outer membrane of mitochondria and is widely used as a

387   proxy for mitochondrial surface area due to its stability under a wide range of environmental conditions

388   (Noguchi, Taylor, Millar, Lambers, & Day, 2005; Shane et al., 2004). Thus, the matching results using

389   leaf area, dry mass or porin abundance indicate that the decline in COX abundance with increased T

390   was not a result of changes in Leaf Mass to Area ratio (LMA) or reduced mitochondria per unit area

391   of leaf. There was a trend for the abundance of Rubisco to decline with the amount of time a leaf

392   developed under 40/35°C (Fig. 5d), although there were no statistically significant T or developmental

393   stage effect.

394   LMA and starch, glucose, fructose and sucrose concentrations were measured in PE leaves one

395   and seven days after T-transfer, and in ND leaves at the prevailing temperature (Table 2). LMA did

396   not change significantly in response to T, for either transferred PE leaves or ND leaves, similar to

397   previous observations in rice over a similar T range (Nagai & Makino, 2009). However, ND leaves

398   did exhibit significantly greater LMA than PE transferred leaves, suggesting an effect of leaf rank on

399   LMA. Transferred PE and subsequently formed ND leaves exhibited consistently lower starch

400   concentrations with increasing T and significantly lower starch with extended duration of development

401   at the prevailing T. Unlike the dynamic responses of leaf starch concentrations to T change,

402   concentrations of soluble sugars were remarkably stable across both PE and ND leaves, in terms of

403   both T-regime and exposure time. Negative correlations between R_{dark} and soluble sugars were

404 observed among leaves within each individual T treatment but not among the three T treatments (Table
405 S5).

406

407 *CO$_2$ flux in responses to T*

408 How molecular changes altered the physiological performance of rice carbon metabolism at differing
409 growth T was investigated through gas-exchange measurements. Rates of A_n and R_{dark} are here
410 presented on a dry mass (DM) basis, noting that the patterns are similar when expressed on a leaf area
411 basis (Fig S7), reflecting the fact that growth T had no significant effect on LMA (Table 2).   A
412 significant change in both A_n and R_{dark} (using mid-sections of leaves placed in Licor 6400 3 x 2 cm
413 chambers) occurred within the first 24 h of transfer to a 40/35°C T-regime for PE leaves, with A_n
414 falling and R_{dark} increasing when measured at the prevailing growth T (Fig. 6). This was followed by
415 stabilisation at the new rate over the subsequent six days. By contrast, A_n and R_{dark} remained relatively
416 constant at both 30/25°C and 25/20°C over a seven-day period monitoring period. Interestingly, rates
417 of R_{dark} for the 30/25°C treated plants decreased from day 3 to 7, compared to the first three days,
418 resulting in slightly lower rates of R_{dark} than for the 25/20°C treated plants by day 7. This possibly
419 reflects temperature-dependent differences in leaf senescence rates. As it could not be controlled,
420 relative humidity in the 40/35°C glasshouse room (Fig. S8) was substantially lower than the other two
421 rooms, leading to reduced humidity during gas-exchange measurements (Fig. 6c). As a consequence,
422 the vapour pressure deficit between the leaf and surrounding air (VPD$_{Leaf}$) increased over the first
423 seven days in PE leaves transferred to 40/35°C, resulting in a dramatic difference by day seven (Fig.
424 6d). The higher VPD$_{Leaf}$ coincided with lower g_s in 40/35°C treated leaves at days three, five and seven,
425 and lower intercellular to ambient CO_2 concentration ratios (C_i/C_a) at days five and seven (Fig. 6e, f).
426 However, for the first two days post transfer both VPD$_{Leaf}$ and g_s were similar between the three growth
427 Ts, Therefore, the decline in A_n and changes in transcript abundance within one day of transfer to
428 40/35°C were not attributable to water relations. Over the longer-term, water relations may have
429 contributed to a slight reduction in C_i/C_a, but not enough to influence A_n, with A_n being stable from
430 one to seven days after transfer irrespective of changes in g_s and C_i/C_a (Fig. 6b). The changes in g_s
431 were not substantive enough to change leaf T, which was stable over the seven days, with both air T
432 and leaf T deviating by less than 2°C from the set room T (Fig. S8).

433 Short-term temperature response curves of entire ND leaves that formed at each prevailing
434 growth T regime were quantified over a 20 to 60°C range using the Walz large leaf chamber (Fig. 7;
435 refer to Figure S9 for area-based rates and Table S6 for quadratic equations fit to curves). Over most
436 of the range of measuring Ts, leaves developed at 25/20°C exhibited higher rates of R_{dark} than those

437   developed under the other two T-regimes. Rates were lowest in leaves developed at 40/35°C (Fig. 7a).

438   When normalised to rates at 30°C, differences in R_{dark} were less pronounced (Fig. 7b), indicating that

439   while R_{dark} at a given measuring T was affected by growth T, the general shape of the R_{dark}-T curves

440   remained largely similar across the three treatments. These observations are consistent with a Type II

441   (changes in baseline) rather than Type I (changes in Q_{10}, the increase in R_{dark} with a 10°C increase in

442   T) respiratory acclimation response (Atkin & Tjoelker, 2003). Importantly, while respiratory thermal

443   acclimation occurred, it was not sufficient to result in R_{dark} being homeostatic across the three growth

444   T treatments. As a result, R_{dark} measured at the growth T was significantly greater in the leaves

445   developed under hot conditions than under the other two treatments (Table 3). Growth T also had a

446   significant effect on the measuring T at which R_{dark} and A_n reached their maximum rates, with leaves

447   developed under high T exhibiting higher T-maxima than control 30/25°C leaves (Table 3). When

448   measured at the prevailing growth T of each treatment, mass-based rates of light-saturated A_n were

449   stable (i.e. homeostatic), further supporting the occurrence of strong thermal acclimation of A_n in ND

450   leaves (Fig. 7), contrary to PE leaves (Fig. 6). The temperature at which PSII lost functionality (T_{crit})

451   tended to increase with growth T (being 3.8°C higher in the hot-grown plants compared to those grown

452   at 25/20°C), although the differences were not statistically significant at $p<0.05$ (Table 3). The high

453   degree of thermal acclimation exhibited by photosynthesis resulted in the ratio of R_{dark} to A_n being

454   lowest in the hot-grown plants, particularly at high measuring T (Fig. 8a); at a measuring T of 40°C,

455   hot-acclimated plants exhibited R_{dark}/A_n ratios that were 50% lower than those measured for their cold-

456   grown counterparts. Further evidence that rice acclimated to heat is seen in the fact that leaf elongation

457   rates – taken over the day and night period – were faster for the 40/35°C grown plants at all times (Fig.

458   8b). Interestingly, A_n of PE leaves ranged from 1.2 to 1.8 μmol g^{-1} DM s^{-1} (Fig. 6), substantially faster

459   than the 0.6 μmol g^{-1} DM s^{-1} in ND leaves (Fig. 7). The former were obtained from measurements on

460   mid-leaf sections placed in a 6 cm^2 chamber, while the latter were obtained from whole leaves placed

461   in a 14 x 10 cm Walz chamber. The lower A_n rates in the latter might reflect a lower proportion of

462   mesophyll cells per unit of area or DM across whole blades compared to the mid-blade section.

463

464   **Discussion**

465

466   Our study investigated the response of photosynthetic and respiratory metabolism to short- and long-

467   term changes in growth T – the highest of which is indicative of heat-wave Ts – to explore: (1) the

468   extent of thermal acclimation of photosynthesis and respiration; and, (2) what underlying changes in

469   gene expression and protein abundance occur during the acclimation process. The results demonstrate

470 that the process of acclimation begins with abrupt changes in gene expression in PE leaves within the

471 first 24 h of heat exposure, followed by a return to homeostatic gene expression (Fig. 1). Importantly,

472 the abundance of the key energy-conserving respiratory protein, COX, declines in abundance when

473 pre-existing leaves are heat-treated for 24 hours, with this phenotype being maintained in newly-

474 developed leaves formed at 40/35°C (Fig. 5). This decline in COX was linked to a slight decline in

475 overall rates of R_{dark} (Fig. 7). The results support the hypothesis that acclimation of photosynthesis

476 and dark respiration are asynchronous in rice, but contrary to observations in non-crop species

477 (Campbell et al., 2007), light-saturated A_n acclimated to a greater extent than R_{dark} (Fig. 7; Table 3).

478 This ability to maintain photosynthetic carbon gain at 40°C is likely to be of crucial importance in

479 helping rice maintain growth during heat-wave conditions.

480

481 *Acclimation to changes in T are rapid and involve a multitude of genes*

482 There was a substantial change in the gene expression profile of rice leaves shifted from 30°C to 40°C

483 within the first 24 h of transfer (Figs. 1, 2, 3, and 4). As might be expected, the largest number of gene

484 expression perturbations were in primary and cellular metabolic processes (Fig. 4). This extensive

485 metabolic response aligns with the instability in R_{dark} and A_n fluxes over the initial 24 h post T-transfer

486 (Fig. 6), which would have contributed to a metabolic imbalance through changes in assimilate supply

487 and demand. Interestingly, the most responsive genes to the initial exposure to heat among upregulated

488 genes were genes involved in biosynthetic processes (Fig. 4) suggesting a stimulation of growth. This

489 is supported by the longer-term increase in leaf elongation rates observed in the 40/35°C grown plants.

490 When analysed in more detail, we observed that heat induced genes linked to energy dissipation

491 (*aox* and *ucp*) over the first 24 h of 40°C heat exposure (Fig. 1, Table 1). AOX and UCP are involved

492 in the diversion of electrons for formation of proton gradients and subsequent ATP synthesis (Krauss,

493 Zhang, & Lowell, 2005; Vanlerberghe, 2013). Past work has shown that overexpressing *aox* in young

494 rice seedlings imparts a benefit on growth under a T of 37°C for eight days, which was attributed to a

495 reduction of excessive proton motive force and reactive oxygen species (Murakami & Toriyama, 2008).

496 Given that AOX and UCP both divert electrons away from ATP synthesis under conditions of high

497 reductant supply, the rapid upregulation of these genes following the initial changes in growth T – with

498 rapid stimulation of R_{dark} and presumably greater reduction of ubiquinone pools (UQ) – indicates that

499 there may have been a temporary imbalance between NAD(P)H supply and demand for ATP. The

500 initial increases in *aox* and *ucp* gene expression (Fig. 1) did not translate into increased total AOX and

501 UCP protein abundance (Fig. 5). However, qPCR results indicate upregulation of the *aox1a* isoform,

502 responsive to abiotic stress in Arabidopsis mitochondria (Clifton, Millar, & Whelan, 2006; Shapiguzov

15

503   et al., 2019), while over the same period RNA-seq analysis indicated a significant decline in a separate

504   *aox1c* isoform. It is possible that the AOX1C isoform is less tolerant of high temperatures and therefore

505   is partially replaced by the AOX1a isoform. In this context it is interesting that in Arabidopsis AOX1a

506   is the major stress-inducible isoform. Since AOX operates as a non-covalently linked dimer (Siedow

507   & Umbach, 2000), the change in the relative expressions of *aox1a* and *1c* isoforms may also indicate

508   a change in the conformation of the AOX dimer, with a different mix of homo- and hetero-dimers in

509   response to heat. This suggests that AOX may have shifted to a more heat-tolerant conformation at

510   40/35°C, at least when the initial shock was imposed. This is an illustration that enzyme isoforms can

511   be an important part of abiotic stress responses that can be easily overlooked when only considering

512   total protein abundance.

513   The limited gene induction when leaves were transferred from 30 to 25°C (Fig. 3c) suggests

514   that a shift to this colder growth T did not significantly perturb metabolic processes in rice leaves,

515   consistent with the limited PE leaf response of R_{dark} or A_n when exposed to the cold (Fig. 6). However,

516   cold-responsive transcriptional regulators and associated changes in metabolism expected from cold

517   exposure (Zhu, Dong, & Zhu, 2007) must have been triggered by the colder Ts. Regulatory adjustments

518   did indeed occur in ND rather than PE leaves, with R_{dark} at a given T being higher in the cold-grown

519   ND leaves (Fig. 7), and homeostasis of A_n being reached in ND leaves when measured at the prevailing

520   growth T (Table 3, Fig. 7).

521

522   *The most evident longer-term acclimation response is reduced COX abundance*

523   The clearest biochemical response to increasing growth T, both in PE and ND leaves, was a decline in

524   the abundance of COX (Fig. 5). A decline in COX has been reported for rice roots when grown at

525   25°C relative to 15°C (Kurimoto, Millar, Lambers, Day, & Noguchi, 2004). Conversely, COX content

526   increased in *Arabidopsis thaliana* leaves grown at 5°C relative to 21°C (Armstrong et al., 2008). In

527   all these cases, COX protein content and rates of respiration at a common measuring T (including in

528   our study; Fig. 5 and Fig. 7) decreased when plants grew at hotter T, suggesting that thermal

529   acclimation results in changes not only in overall rates of respiration but also in the capacity to produce

530   ATP. The acclimation response was rapid as COX declined in abundance by 24 h after 40°C T transfer

531   in PE leaves (Fig. 5).

532   The decline in COX abundance with hotter growth T is intriguing. If COX activity became

533   rate-limiting, it is likely that more ROS would be produced as the UQ pool would quickly become

534   over-reduced. However, other reports suggest that the UQ redox state is relatively stable, including

535   during changes in T, despite higher R_{dark} (Covey-Crump et al., 2007; Wagner & Wagner, 1995). If we

536   assume that UQ redox poise was also stable during the greater R_{dark} at the hottest growth T in our

537   experiments, there are two possible explanations. (1) The absolute flux of electrons through COX

538   actually increased despite the decrease in protein abundance. This could be due to COX capacity being

539   far greater than the capacity of the overall mETC. But since increasing Ts stimulates the relative

540   activity of enzymes (Copeland, 2000), it is possible that the smaller amount of COX protein had higher

541   activity. In other words, the plants could make do with less COX at hotter T. (2) Alternatively,

542   activation of AOX at the higher T may have occurred to supplement COX activity thereby preventing

543   overload of the UQ pool. Measuring T-dependent in $vivo$ [18]O fluxes through COX and AOX, as well

544   as leaf ATP content is required to determine terminal oxidase activity and ATP synthesis.

545   Understanding what, if any, biological benefit arises from synthesising less COX at warmer growth T

546   is another important consideration. Alternatively, a reduction in COX might be a consequence of heat

547   directly interfering with its synthesis. In support of this, a recent report shows that COX abundance

548   and capacity in Arabidopsis is significantly reduced by knocking out a HSP70 isoform, suggesting that

549   heat in some way interacts with COX formation (Wei et al., 2019).

550

551   *Acclimation of R_{dark} and A_n is asynchronous in rice*

552   The R_{dark}/A_n ratio increased with short-term increases in measuring T (Fig. 8), reflecting the fact that

553   R_{dark} is more temperature dependent than is A_n. R_{dark}/A_n ratios were similar in 25 and 30°C grown leaves,

554   when measured at the prevailing growth T of each treatment (i.e. R_{dark}/A_n was homeostatic). Thus, the

555   acclimation process led to the balance between carbon gain and release being maintained across this

556   moderate range of growth Ts (Fig. 8). Acclimation was not, however, sufficient to maintain

557   homeostasis of R_{dark}/A_n in 40°C grown plants (Fig. 8). Similar results of R_{dark}/A_n ratios in leaves and

558   whole plants remaining relatively stable over moderate but not extremely high T have been reported

559   (Atkin, Scheurwater, & Pons, 2006, 2007; Campbell et al., 2007; Drake et al., 2016; Loveys et al.,

560   2003). Different to past studies, our findings in rice show that homeostasis of R_{dark}/A_n is largely the

561   result of maintenance of A_n more than through a marked reduction in rates of R_{dark}. Our results

562   categorically show A_n acclimates to a greater extent than R_{dark} in rice, supporting previous studies of

563   rice that collectively point to greater A_n than R_{dark} acclimation capacity (Bahuguna et al., 2017;

564   Glaubitz et al., 2014; Kurimoto, Millar, et al., 2004; Mohammed et al., 2013; Nagai & Makino, 2009;

565   Yamori et al., 2010), and field studies that infer limited rice R_{dark} acclimation capacity (Peng et al.,

566   2004; Welch et al., 2010). However, for many plant functional types, including temperate grasses, the

567   opposite occurs; R_{dark} acclimates to a greater extent than A_n (Campbell et al., 2007; Ow, Griffin,

568   Whitehead, Walcroft, & Turnbull, 2008; Way & Oren, 2010; Way & Sage, 2008; Yamori et al., 2005).

569   In this context, it should be noted that the previous studies are of species from temperate rather than

570   tropical habitats, raising the question of whether, beyond rice, tropical grasses generally have

571   asynchronous acclimation favouring A_n. The homeostasis of A_n and superior LER of hot-grown ND

572   rice leaves was more remarkable when viewed alongside evidence that prolonged exposure to drier air

573   was closing stomata and presenting slight reductions in CO_2 availability, at least in PE leaves (Fig. 6).

574   There is evidence that stomata close following a T-dependent increase in VPD_{Leaf}, with the mechanism

575   yet uncharacterised but likely involving guard cell sensing of water potential below the epidermis

576   (Peak & Mott, 2010; Shope, Peak, & Mott, 2008). It seems that declining VPD_{Leaf} triggers stomatal

577   closure in rice, even with unlimited root water supply.

578   As noted earlier, in recent years, rice yields have declined in response to increased daily mean

579   Ts, with the decline being more strongly correlated with increasing night rather than day Ts (Peng et

580   al., 2004; Welch et al., 2010). Our finding that A_n is homeostatic across growth T, whereas R_n is not

581   (Table 3) – underpinned by greater acclimation of photosynthesis than respiration – suggests that one

582   reason why yields are declining with increasing night temperatures is because high temperatures

583   stimulate respiratory CO_2 release. This would have a negative effect on daily net carbon gain, and thus

584   the ability to accumulate biomass in the lead up to anthesis.

585

586   *Potential implications of rice leaf acclimation and starch concentration on crop yield*

587   We found that soluble sugar concentrations of rice leaves were remarkably stable, irrespective of

588   growth T or developmental time at each growth T (Table 2). Maintaining soluble sugar homeostasis is

589   an important physiological requirement for many plant species, achieved through balancing CO_2

590   uptake and release in source leaves with sugar export to sink tissues (Rolland, Moore, & Sheen, 2002).

591   Homeostasis of sucrose concentrations in rice leaves has been observed even when carbon demand by

592   sink tissues is limited [e.g. reduced partitioning of sugars to grain (Wang et al., 2008)]. In our study,

593   homeostasis of soluble sugar concentrations occurred even at 40°C, where rates of R_{dark} where

594   significantly higher than in plants at the cooler growth Ts. Associated with the maintenance of sugar

595   concentrations was a T-dependent decline in starch concentration, both in PE and ND leaves (Table

596   2). For PE leaves exposed to 40°C, assimilate supply declined, particularly for 40°C transferred leaves,

597   due to a marked increase in R_{dark} and a decline in A_n (Fig. 6). Starch content also significantly declined

598   with developmental duration under high T, contrary to soluble sugar concentrations (Table 2). It seems

599   likely, therefore, that the reason soluble sugars did not significantly decline at warmer T for PE leaves

600   – even though assimilate supply fell – was a greater draw-down in the starch pool to maintain soluble

601   sugar concentrations (i.e. a reliance on stored assimilate). Other studies [e.g. on the temperate tree

602   *Populus tremula* (Hüve et al., 2012)] have highlighted the importance of starch degradation in

603   maintaining soluble sugar concentrations, particularly under conditions that stimulate CO_2 release by

604   respiration. Interestingly, in our study, ND leaves exhibited reduced starch concentrations while also

605   maintaining assimilate supply; one explanation for this might be that the decline in starch and

606   maintenance of sugars of ND leaves was linked to the increased leaf elongation rates we observed for

607   40°C ND leaves (Fig. 8b), with increased growth (i.e. sink demand) necessitating a greater supply of

608   sugars mediated by the starch pool (Stitt & Zeeman, 2012).

609   The decline in starch concentrations for PE and ND leaves at 40°C (Table 2) has interesting

610   implications for rice development and yield. Starch is stored in the stems in the late vegetative stage

611   of rice, and accounts for a large proportion of the carbon accumulated in seeds, a process that is

612   detrimentally affected by heat stress (Blum, Sinmena, Mayer, Golan, & Shpiler, 1994; Impa et al.,

613   2018; Morita & Nakano, 2011; Yang & Zhang, 2005). Other studies using the IR64 cultivar exposed

614   to hot night temperatures have shown an increase in R_{dark} and associated cost to vegetative growth and

615   starch content of panicles, ultimately reducing yield (Bahuguna et al., 2017; Glaubitz et al., 2014). The

616   reduced storage of starch in leaves with increasing T that we observed at the vegetative stage –

617   assuming it did not reflect diversion of starch to stems – would suggest reduced potential for the storage

618   of starch in stems and a penalty to yield of rice growing in warmer environments. This would be

619   particularly true for rice plants exposed to transient extreme T – such as during heat waves – as we

620   postulate the reduction in starch for PE leaves was due to a reduction in assimilate acquisition due to

621   stimulated R_{dark} and supressed A_n. However, ND leaves did show reduced starch concentration, not as

622   a result of reduced assimilate acquisition, but most likely associated with an increase in growth rates

623   (Table 2; Fig. 8). Thus, it is likely that rice will experience different limitations on yield depending on

624   the duration of thermal changes, with shorter-term exposure to rising T – over a period in which tissue

625   cannot develop anew – likely leading to a greater suppression of yield than leaves developed under the

626   prevailing growth T. Rice may even experience increased yield with sustained mild warming of both

627   night and particularly day T. However, yield potential is dependent on whether heat-dependent changes

628   in growth at the vegetative stage of rice positively contributes to yield, which may be true (Glaubitz et

629   al., 2014; Scafaro et al., 2018), and not simply accelerate development and shorten the time to

630   flowering.

631

632   *Conclusions*

633   Overall, the results we present here demonstrate that both leaf respiration and photosynthesis can

634   acclimate in rice but the extent of acclimation is asynchronous and dependent on the timeframe of T

635   exposure. Warmer growth T of 40°C relative to 25°C will have a greater impact on rice CO_2 flux,

metabolic pathways, starch concentration and ultimately growth. Consequently, rice growing in a

warmer climate with more extreme heating events will likely experience T-dependent alterations in

growth and yield. The duration and intensity of T changes, together with complex interactions between

assimilate acquisition, storage and utilisation will determine if this warmer environment will be

beneficial or detrimental to rice productivity over the coming decades. We suggest that enhancing the

acclimation capacity of R_{dark} for rice at warmer growth T – potentially through COX, AOX and UCP

regulation – could be a key target for improving rice productivity in a warmer world.

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Table 1. Differential expression of respiration genes in leaves after exposure to *T* of 40°C relative to 30°C. Differential expression defined as FDR < 0.05, marked as '*'. Electron transport chain (ETC), Pentose Phosphate Pathway (PPP). No TCA cycle genes were differentially expressed.

Pathway	Gene_name	locus	Log2 fold-change	
			2 hours	6 hours
ETC	Complex II (Succinate dehydrogenase)	LOC_Os08g02640	-0.55*	-0.58*
ETC	External NAD(P)H dehydrogenase	LOC_Os06g47000	0.72*	0.44
ETC	Uncoupling protein	LOC_Os11g48040	0.81*	0.46
ETC	Alternative oxidase	LOC_Os02g47200	-0.99*	-1.91*
glycolysis	ATP-dependent phosphofructokinase	LOC_Os01g53680	-0.11	-1.13*
glycolysis	Phosphoglycerate kinase	LOC_Os02g07260	0.78*	0.39
glycolysis	Enolase	LOC_Os10g08550	0.61*	0.34
PPP	Ribulose 5-phosphate 3-epimerase	LOC_Os09g32810	0.53*	0.87*

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Table 2. Leaf mass per unit area (LMA), starch and soluble sugars of pre-existing (PE) leaves transferred from 30/25°C to 25/20°C or 40/35°C for one and seven days, and leaves newly-developed (ND) at the prevailing T. Data represents mean of three or four separate leaves from separate previously unsampled plants ± SE. The F-values and P-values of a two-way ANOVA comparing T, developmental stage (D) and any interaction ($T \times D$) are reported with asterisks indicating significance at $P<0.05$.

		LMA (g m^{-2})	Starch (mg g^{-1} DM)	Soluble sugar (mg g^{-1} DM)
25/20°C	Day 1	20 ± 3	11.3 ± 1.9	13.5 ± 0.2
	Day 7	19 ± 2	5.5 ± 0.3	11.2 ± 0.1
	ND	30 ± 2	14.4 ± 3.0	11.1 ± 0.2
30/25°C	Day 1	19 ± 2	14.9 ± 2.1	13.0 ± 0.5
	Day 7	21 ± 2	4.9 ± 1.0	10.5 ± 0.2
	ND	28 ± 0.4	9.6 ± 1.6	11.0 ± 0.6
40/35°C	Day 1	18 ± 2	8.5 ± 0.4	11.9 ± 0.2
	Day 7	23 ± 2	3.5 ± 0.3	10.9 ± 0.3
	ND	29 ± 1	6.7 ± 0.5	11.6 ± 0.3
$T \times D$		F=0.7, P=0.6	F=1.7, P=0.14	F=0.01, P=0.99
D		F=28, P<0.001*	F=13, P<0.001*	F=0.14, P=0.94
T		F=0.1, P=0.9	F=4.2, P=0.03*	F=0.01, P=0.99

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Table 3. Summary of key photosynthetic and respiratory parameters generated from temperature-response curves. Parameters are: leaf mass per area; the temperature at which R_{dark} and A_n exhibited maximum rates (T_{max} and T_{opt}, respectively); the maximum rates of R_{dark} and A_n reached (R_{max} and A_{opt}, respectively); rates of R_{dark} and A_n at the prevailing growth temperature; and, the temperature at which PSII lost functionality as determined by an increase in basal fluorescence (T_{crit}). Data represents means of three or four separate leaves from separate plants $\pm$ SE and statistical data (F-value and P-value) based on one-way ANOVA of temperature treatment effect. Superscript letters show significant differences between the T treatments according to a Tukey test.

	25/20°C	30/25°C	40/30°C	F-value	P-value
LMA (g m^{-2})	33±2	30±2	35±3	1.4	0.31
T_{max} (°C)	51± 1[a]	54 ± 1[a,b]	55± 1[b]	4.7	0.04*
T_{opt} (°C)	29± 1[a]	31± 1[a,b]	33 ± 0.3[b]	6.1	0.04*
R_{max} (µmol g^{-1} DM s^{-1} ×10^{-3})	120 ± 5	117 ± 6	121 ± 2	0.16	0.86
A_{opt} (µmol g^{-1} DM s^{-1})	0.65 ± 0.05	0.67± 0.02	0.69 ± 0.04	0.28	0.76
R_{dark} (µmol g^{-1} DM s^{-1} ×10^{-3})	24±3 [a]	27±3 [a]	57±2 [b]	35	<0.001*
A_n (µmol m^{-2} s^{-1})	0.62 ± 0.04	0.67 ± 0.02	0.65 ± 0.04	0.52	0.62
T_{crit} (°C)	46.0 ± 0.6	46.9 ± 0.9	49.8 ± 1.5	3.726	0.089

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Figure Legends

Figure 1. Quantitative PCR analysis of gene expression over the first 168 hours (7 days) after transfer of leaves from 30/25°C to 25/20°C or 40/35°C. Genes analysed were: the respiratory cytochrome *c* complex (*cox*) subunit II, alternative oxidase complex (*aox*) and uncoupling protein (*ucp*); the photosynthetic genes ferredoxin NADH reductase (*fnr*) and phosphoribulose kinase (*prk*); and the sugar metabolism gene sucrose phosphatase synthase (*sps*). Gene expression was revitalised at each time-point to the non-transferred 30/25°C control.

Figure 2. Principal component analysis of normalised RNA-seq expression values for each sample following temperature treatment for (a) 2 hours and (b) 6 hours. Samples are coloured by treatment, day/night temperatures of 30/25°C (control), 40/35°C (hot), and 25/20°C (cold). The y-axis is principle component 1 (PC1) and the x axis is principle component 2 (PC2); the percent of variation explained by each axis is indicated. RNA-seq libraries were normalised using *edgeR* ("TMM" method) and *voom* transformation, scaled by unit variance and clustered using singular value decomposition.

Figure 3. Identification of genes differentially expressed during temperature treatments. (a, b) Common and time point specific differentially expressed genes under heat treatment (40/35°C). The overlap between genes differentially expressed at 2 and 6 h under heat treatment for (a) upregulated genes and (b) downregulated genes. '*' indicates significant overlap p << 0.001, fisher's one-tailed exact test (hypergeometric). (c) Hierarchal clustering of differentially expressed genes. For each time point (2, 6 and 24 h) differentially expressed genes were determined for both the hot (40/35°C) and cold (25/20°C) temperature treatments relative to the 30/25°C control conditions (FDR < 0.05). For each differentially expressed gene, the relative fold-change under each condition over the time series is then displayed on a log2 scale: red = upregulated, blue = downregulated.

Figure 4. Gene ontology (GO) term enrichment among genes differentially Upregulated (a) or downregulated (b) genes after 2 h at 40°C. Ontological annotations downloaded from MSU and ontology enrichment tests performed with topGO in R using the Fisher standard test (on tailed fisher's exact test/ hypergeometric test) with post hoc p value correction for multiple testing using the Benjamini & Hochberg method.

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Figure 5. Abundance of mitochondrial electron transport chain proteins and Rubisco determined by Western blot analysis for rice leaves sampled at different developmental stages of; PE leaves six and 24 h after T transfer to 25/20°C or 40/35°C, and leaves newly developed (ND) post T-transfer. (a) Abundance of CYTOCHROME C OXIDASE (COX) subunit II, (b) ALTERNATIVE OXIDASE (AOX), (c) UNCOUPLING PROTEIN (UCP) and (d) Rubisco large subunit on a leaf area basis with data normalised by adjusting the largest value in each dataset to 100. Data represent mean ± SE of four independent western blots, with each blot representing leaf tissue from a separate plant. The P-values of a two-way ANOVA comparing temperature (T), developmental stage (D) and the interaction between the two ($T{\times}D$) are reported on each graph. Representative blots are presented in Figure S5.

Figure 6. Rates of dry mass (DM) based dark respiration (R_{dark}; a), net assimilation (A_n; b), Relative humidity (RH; c), vapour pressure deficit between the leaf and surrounding air (VPD$_{Leaf}$; d), stomatal conductance (g_s; e), and ratio of intercellular to ambient CO_2 concentrations (Ci/Ca; f) measured at the respective day-time growth temperature of each treatment just prior to (day 0), and 1, 2, 3, 5 and 7-days after transfer of control 30/25°C day/night grown leaves to either 25/20°C, 40/35°C or maintained at 30/25°C. Values are means of four biological replicates ± SE.

Figure 7. Temperature-response curves (a, b) of dark respiration (R_{dark}) and (c, d) net assimilation (A_n), on a dry mass (DM) basis. Values are absolute (a, c) or normalised to values at 30°C (b, d). Measurements were made on whole newly-developed (ND) leaves growing for 21 d at day/night temperatures of 25/20°C, 30/25°C or 40/35°C. Curves fitted to R_{dark} and A_n are quadratic functions. Calculated acclimation parameters from the curves are presented in Table 3. Rates were recorded every 30 sec as leaves were heated at 1°C per minute. Filled area represent standard error of three to four biological replicates.

Figure 8. The percentage of dark respiration (R_{dark}) relative to light-saturated net assimilation (A_n) (a), and leaf elongation rates (LER) over a 24 h day/night cycle (b), for ND leaves growing for 21 d at day/night temperatures of 25/20°C, 30/25°C or 40/35°C. For the R_{dark}/A_n ratio values are calculated from the absolute means presented in Figure 7. For LER the dark (night) period of the 24 h cycle is shaded in grey and values are the means ± SE of four plant replicates.

Summary statement

Leaf respiration and photosynthesis in rice (*Oryza sativa* L.) shows asynchronous acclimation capacity in favour of photosynthesis. Heat acclimation reduced the protein abundance of the respiratory protein cytochrome *c* oxidase (COX), despite respiration and growth being stimulated.
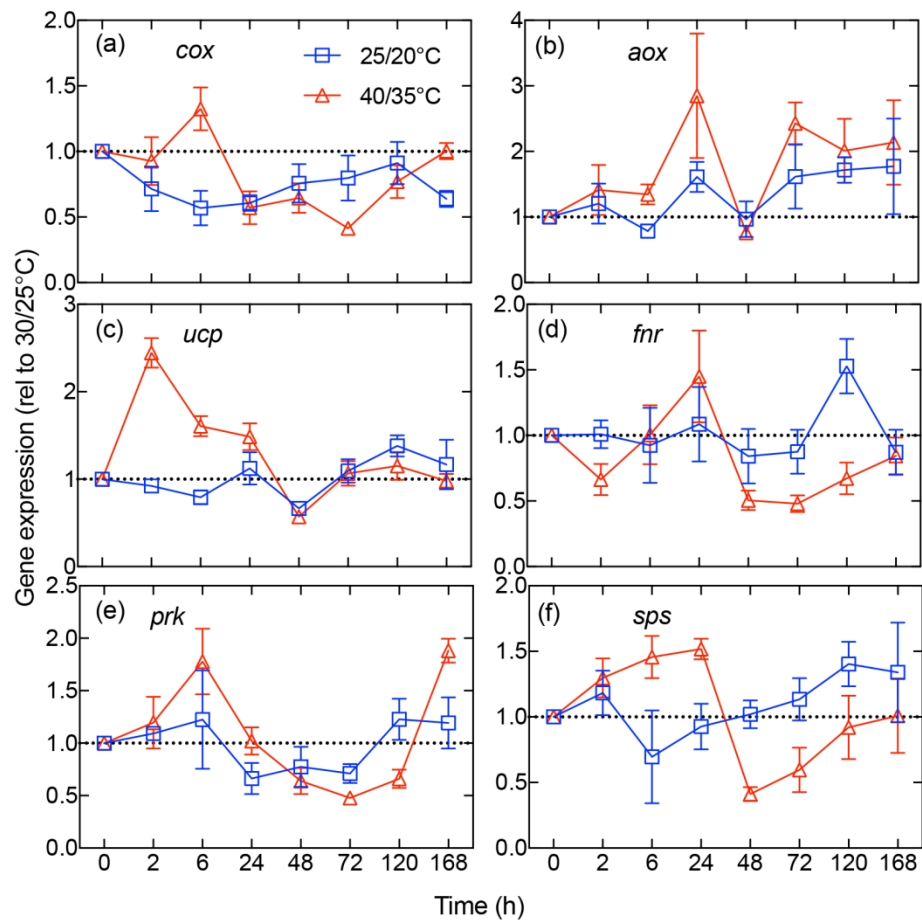
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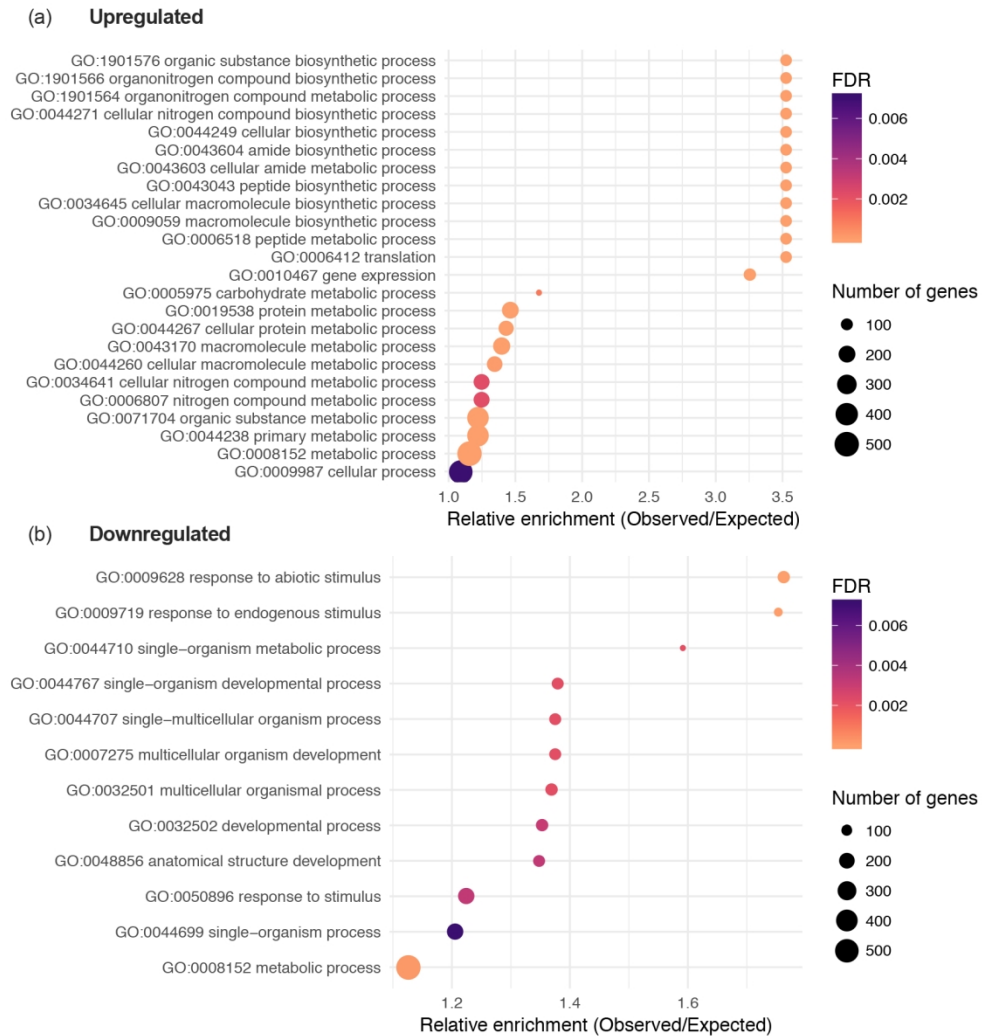
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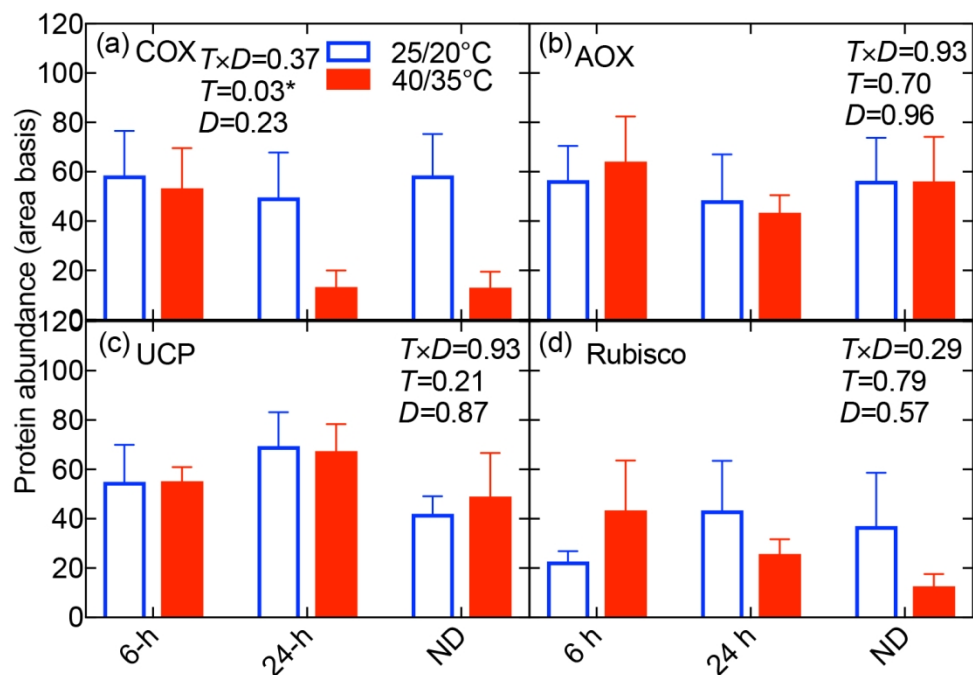
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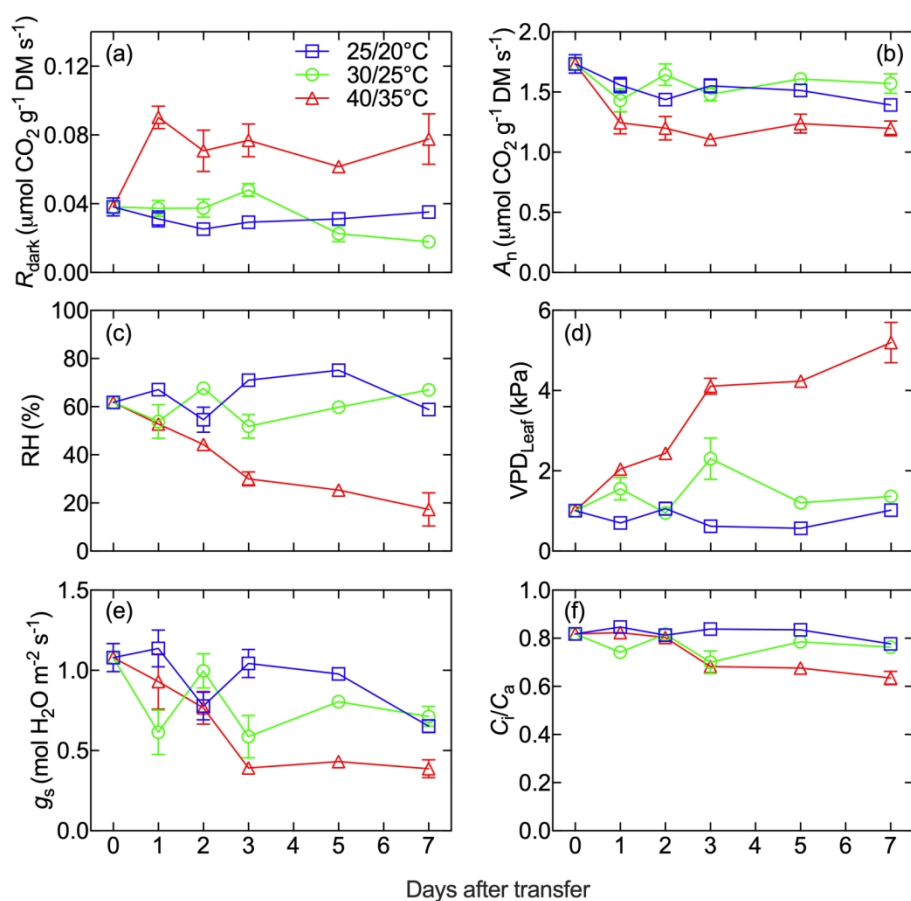
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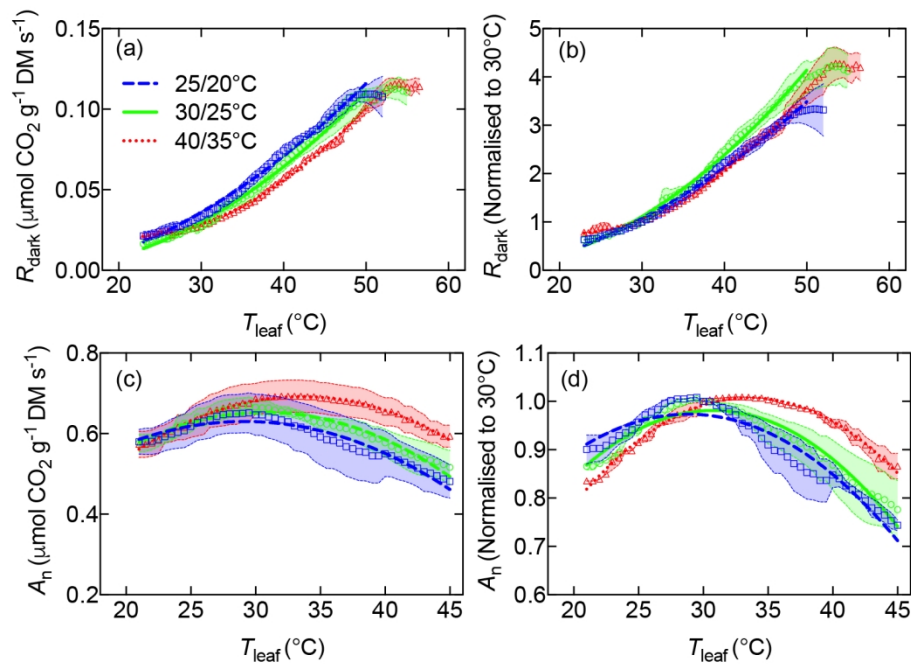
152x141mm (300 x 300 DPI)

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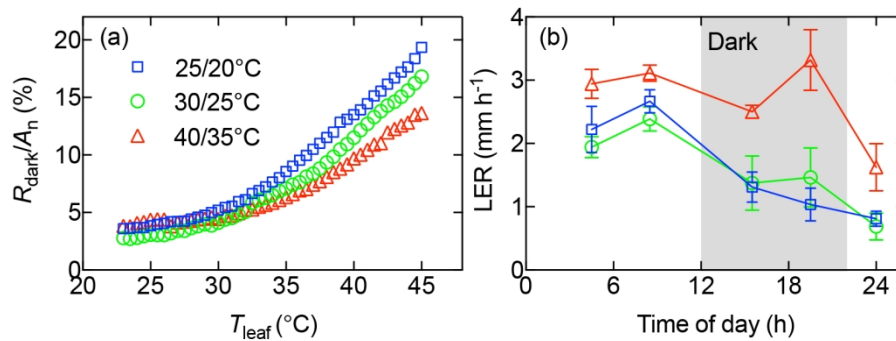
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Supporting Information

Molecular and physiological responses during thermal acclimation of leaf photosynthesis and respiration in rice

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Table S1. Concentrations of essential macro- and micro-nutrients in hydroponic solution for rice plants.

Element	Chemical formula	Concentration (mM)
Macronutrients		
Nitrogen (N)	NH_4NO_3	1.4
Phosphorus (P)	$NaH_2PO_4.2H_2O$	0.6
Potassium (P), Sulfur (S)	K_2SO_4	0.5
Calcium (Ca)	$CaCl_2.2H_2O$	0.2
Magnesium (Mg)	$MgSO_4.7H_2O$	0.8
Micronutrients		
Iron (Fe)	Fe-EDTA	0.07
Boron (B)	H_3BO_3	0.037
Manganese (Mn)	$MnCl_2.4H_2O$	0.009
Zinc (Zn)	$ZnSO_4.7H_2O$	0.00075
Copper (Cu)	$CuSO_4.5H_2O$	0.0003
Molybdenum (Mo)	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0001
Vanadium (V)	NH_4VO_3	0.000138
Silicon (Si)	Na_2SiO_3	0.0012963

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Table S2. Primers used for qPCR gene expression analysis

Protein	Sequence (5'->3')	Length	Start	Stop	Tm	GC %	Size	location	mRNA
Ferredoxin NADP reductase	TCAGGCTCTACTCCATCGCC	20	487	506	58.9	60	152	exon 2	NM_001063105.1
	ACCAGGCTTCAAGTCACAGAGG	22	638	617	60	54.6		exon 3 or 4	
Phosphoribulokinase	TTTGATGCCTTCATTGATCCCC	22	865	886	56.8	45.5	80	exon 3 or 4	NM_001054360.1
	TCATCAGGAATAAGCTGGGTTGG	23	944	922	58.1	47.8		exon 4	
Uncoupling protein	CCTCTACGAGCCCGTGAAATCC	22	348	369	60.6	59.1	134	exon 2 or 3	NM_001075091.1
	TGACAAGGTCAGTGGGGTTGG	21	481	461	60	57.1		exon 4	
Cytochrome oxidase (COX)	GCCATTGCAGGAGTGATGGG	20	73	92	59.2	60	85		LOC_Os12g33946.1
	TCCCACCAAGAATTTGATCGCC	22	157	136	59	50			
Alternative oxidase (AOX)	TGCTTTAGGCCATGGGAGACC	21	416	436	59.9	57.1	84	exon 1 or 2	NM_001060293.1
	CTTGTCGAGCAGCGTCTTGG	20	499	480	59.5	60		exon 1	
Sucrose-phosphate synthase (SPS)	ATGCGAAGCCTGAAACCCCC	20	1317	1336	60.9	60	124	exon 8 or 9	NM_001052401.1
	CACACGGAGGTGCAGAAAAGG	21	1440	1420	59.6	57.1		exon 9	
eIF4-gamma/eIF5/eIF2-epsilon domain containing protein (Eukaryotic initiation facctor 5C)	CTGATGGAGGCTGTTCAGTGG	21	1035	1055	58.5	57.1	81	exon 6 or 7	NM_001074292.1
	AGCCCATGCTTTCACCTGCC	20	1115	1096	61.2	60		exon 7	

Table S3 Differentially expressed genes. At each time point, differential expressed genes were assessed for the hot and cold treated plants relative to the warm grown control plants sampled at the same time ($n = 4$). Genes were considered differentially expressed an FDR adjusted p (padj) < 0.05.

Treatment	Time point	Upregulated	Downregulated
Cold	2 hr	4	0
Hot	2 hr	858	960
Cold	6 hr	2	0
Hot	6 hr	685	780
Cold	24 hr	0	0
Hot	24 hr	0	0

Table S4. Protein concentrations of 25/20°C and 40/35°C day/night grown leaves. Data represents mean of three to four biological replicates ± SE.

	Protein concentration (mg ml^{-1})	
	25/20°C	40/35°C
6 hr [Pre-existing (PE) leaves]	7.7 ± 0.8	7.0 ± 1.0
Day 1 [Pre-existing (PE) leaves]	8.8 ± 1.9	6.9 ± 1.3
Newly-developed (ND) leaves	8.5 ± 0.7	7.8 ± 0.8

Table S5. Pearson's correlation coefficients between rates of dark respiration and concentration of soluble sugars for plants grown at different growth temperature regimes.

	Dark respiration x soluble sugars		
	25/20°C	30/25°C	40/35°C
Day 1			
Correlation coefficient	-0.999	-0.999	0.049
P-value	0.024*	0.032*	0.969
Day 7			
Correlation coefficient	-0.805	-0.990	-0.461
P-value	0.405	0.090	0.695
Newly developed (ND)			
Correlation coefficient	-0.420	-0.560	0.879
P-value	0.724	0.621	0.317

Table S6. Quadratic equations for the short-term temperature (T)-response curves of dark respiration (R_{dark}) and net assimilation (A_n) as presented in Figure 7. The equations were fitted to the mean values of each temperature treatment and their Absolute Sum of Squares (ASS) and goodness-of-fit (R^2) are provided. Equations for R_{dark} were only fitted in the range of 22 to 50°C. R_{dark} and A_n units are in µmol CO_2 g^{-1} DM s^{-1} $\times 10^{-3}$ and T is in °C.

Day/night temperatures	Equation	ASS	R^2
25/20°C	$R_{dark} = -7.2 - 0.094 \times T + 0.051 \times T^2$	2.5×10^{-5}	0.99
	$A_n = 60 + 39 \times T - 0.67 \times T^2$	9.0×10^{-5}	0.92
30/25°C	$R_{dark} = 2.4 - 0.096 \times T + 0.063 \times T^2$	1.7×10^{-5}	0.99
	$A_n = 89 + 48 \times T - 0.72 \times T^2$	6.0×10^{-5}	0.94
40/35°C	$R_{dark} = 5.8 - 3.7 \times T + 0.092 \times T^2$	1.1×10^{-5}	0.99
	$A_n = -240 + 56 \times T - 0.83 \times T^2$	7.3×10^{-5}	0.99

Figure S1 A photo of a LI-6400XT portable gas exchange system (top) and Walz 3010-GWK1 chamber (bottom) used in quantification of short-term temperature-response curves of R_{dark} and A_n in new-developed (ND) leaves. Airline tubes were used to connect the Walz 3010-GWK1 chamber to the LICOR Infrared gas analyser head with a water trap in between to remove excess humidity. Photos are courtesy of www.licor.com and www.walz.com.
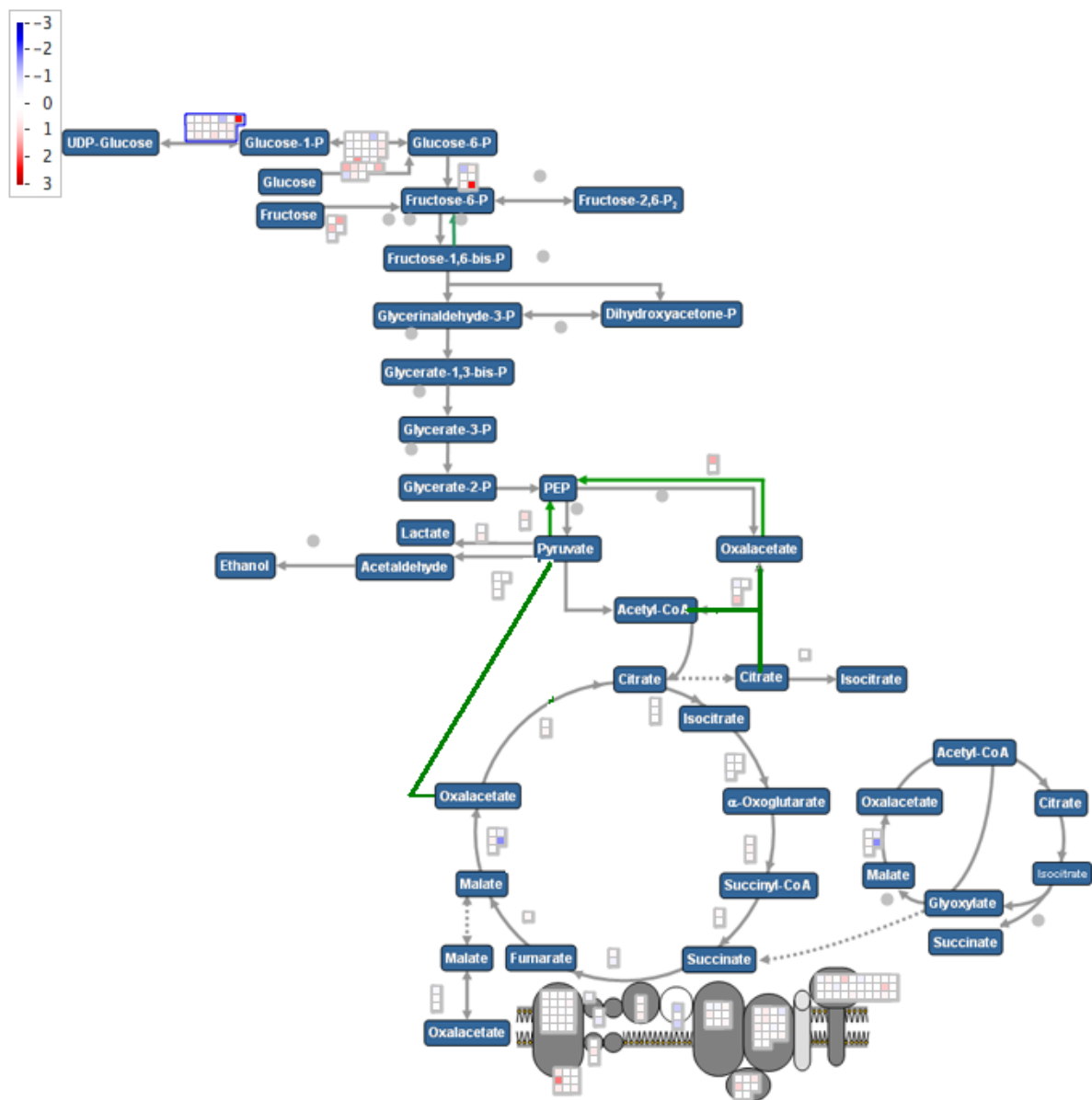
Figure S2 Differential gene expression in the Glycolysis and TCA under hot treatment Gene expression is visualised in a MapMap pathway view of Glycolysis and the tricarboxylic acid (TCA) cycle. Each box represents a rice gene with homology to an Arabidpsis gene annotated in the MapMan pathway. Data show for the comparison of 2 hours hot treatment compared to 2 hours control treatment (warm); red shading represents up-regulation and blue shading down-regulation on a log2 fold-change scale.
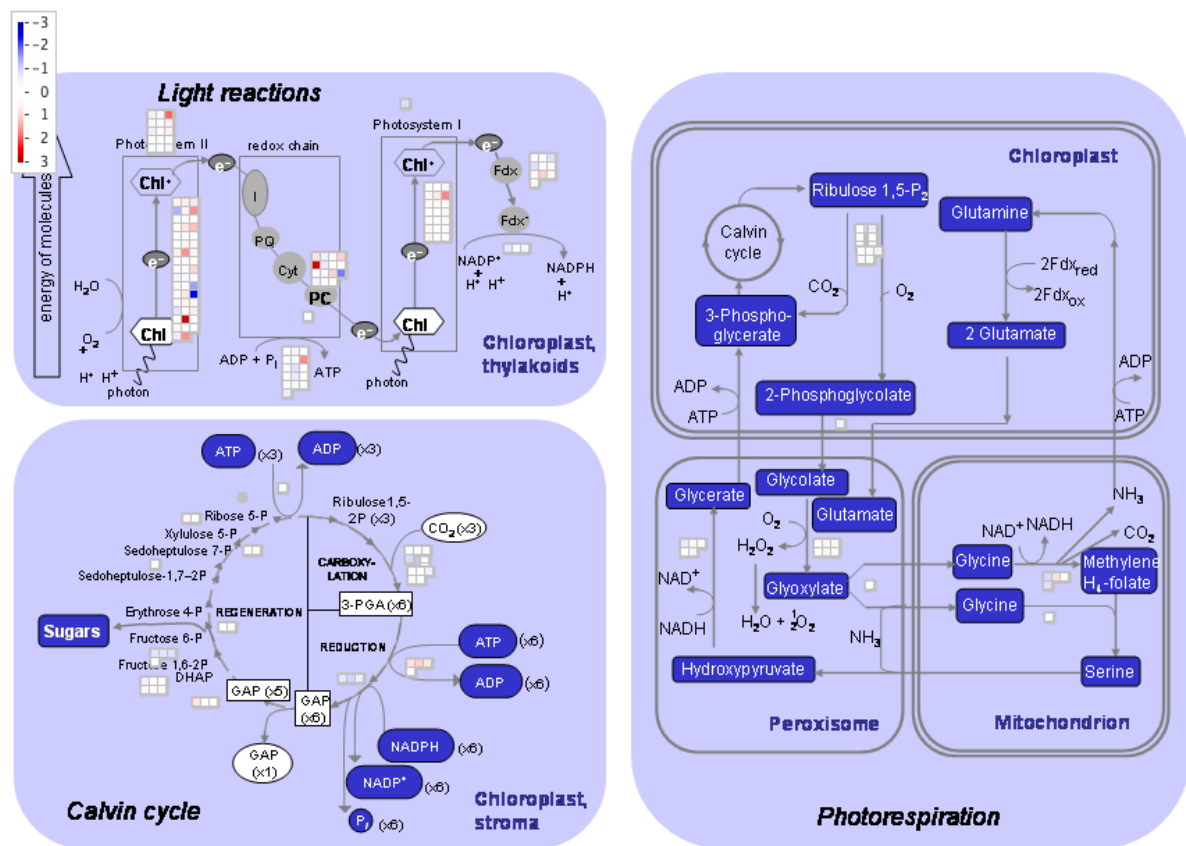
Figure S3. Differential gene expression of photosynthetic genes under hot (40/35°C) treatment. Gene expression is visualised in a MapMap pathway view of photosynthesis. Each box represents a rice gene with homology to an Arabidpsis gene annotated in the MapMan pathway. Data show for the comparison of two hours hot treatment compared to two hours control (30/25°C) treatment; red shading represents up-regulation and blue shading down-regulation on a log2 fold-change scale.
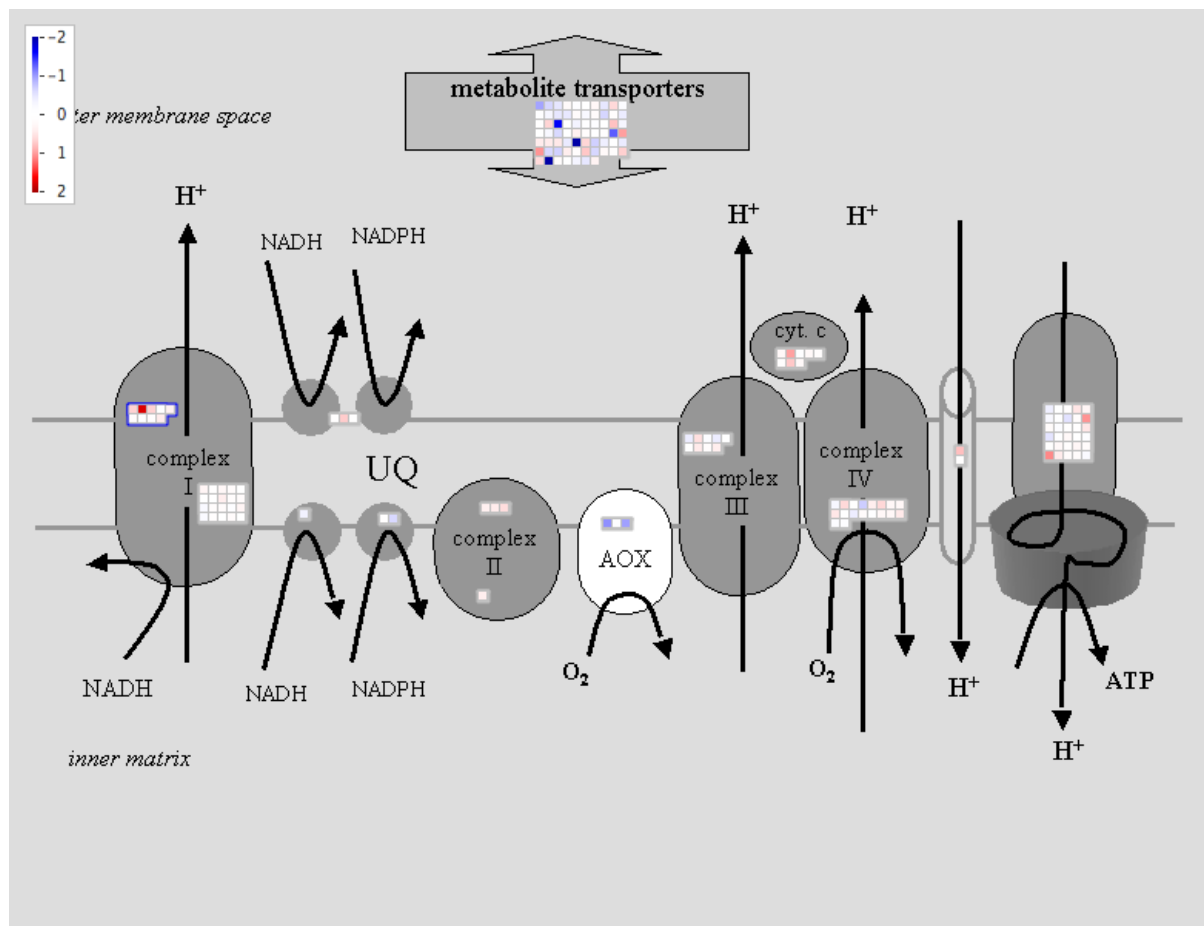
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Figure S4. Differential gene expression in the mitochondrial electron transport chain under hot treatment. Gene expression is visualised in a MapMap pathway view of the mitochondrial electron transport. Each box represents a rice gene with homology to an Arabidopsis gene annotated in the MapMan pathway. Data show for the comparison of two hours hot (40/35°C) treatment compared to two hours control treatment (30/25°C); red shading represents up-regulation and blue shading down-regulation on a log2 fold-change scale.
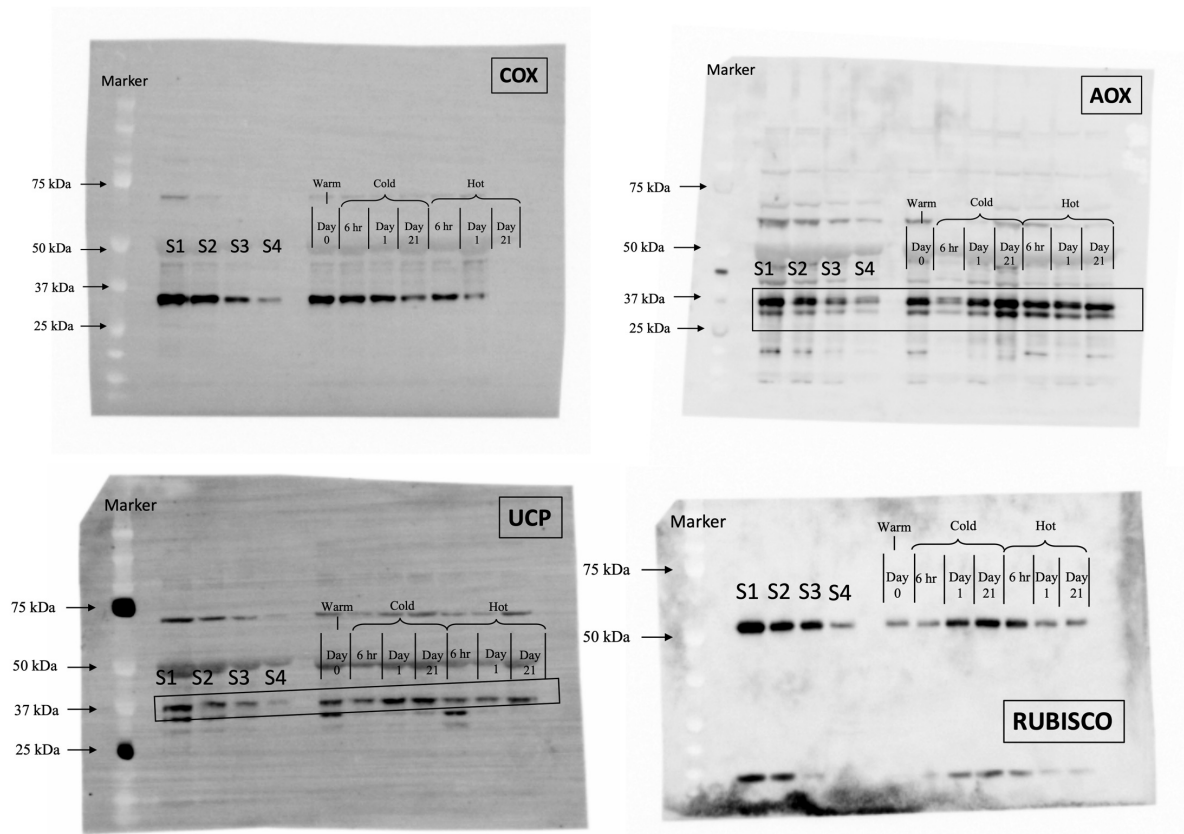
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Figure S5. Representative western blots of cytochrome c oxidase (COX) subunit II, alternative oxidase (AOX), uncoupling protein (UCP), and Rubisco, used for determination of protein content of leaves. S1 to S4 is a dilution series of the warm/control (30/25°C) samples. This is followed by the initial day 0 warm leaves prior to temperature transfer, the 6-h and 1-day times after transfer of PE leaves, and ND leaves after 21 days of temperature transfer, for the cold (25/20°C) and hot (40/35°C) treatments as indicated on the images. For AOX and UCP, the bands within the box are what was classified and analysed as the proteins of interest based on the molecular weight marker markers.
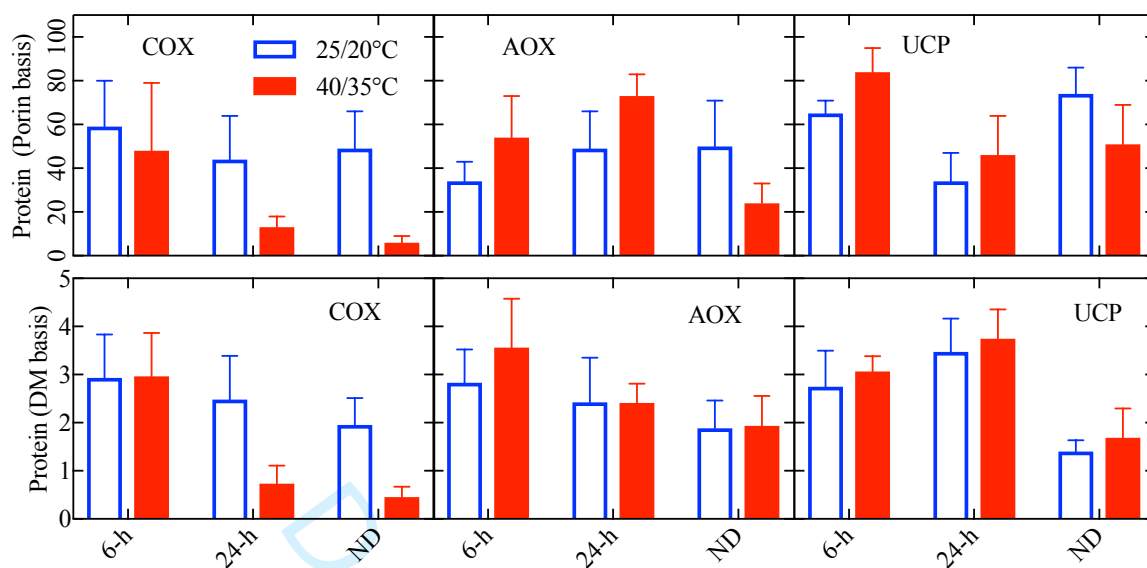
Figure S6. Abundance of mitochondrial electron transport chain proteins determined by Western blot analysis for pre-existing rice leaves sampled at; six and 24 h after *T* transfer to 25/20°C or 40/35°C, and leaves newly developed (ND) post *T*-transfer. The top three panels are protein abundance of cytochrome *c* oxidase (COX), alternative oxidase (AOX), and uncoupling protein (UCP) normalised to porin abundance and adjusting the largest value in each dataset to 100. The bottom three panels are leaf area values normalised to a dry mass (DM) basis by factoring in the leaf mass per area of pre-existing and newly developed leaves as presented in Table 2 of the text. Data represent mean ± SE of four independent western blots, with each blot representing leaf tissue from a separate plant.

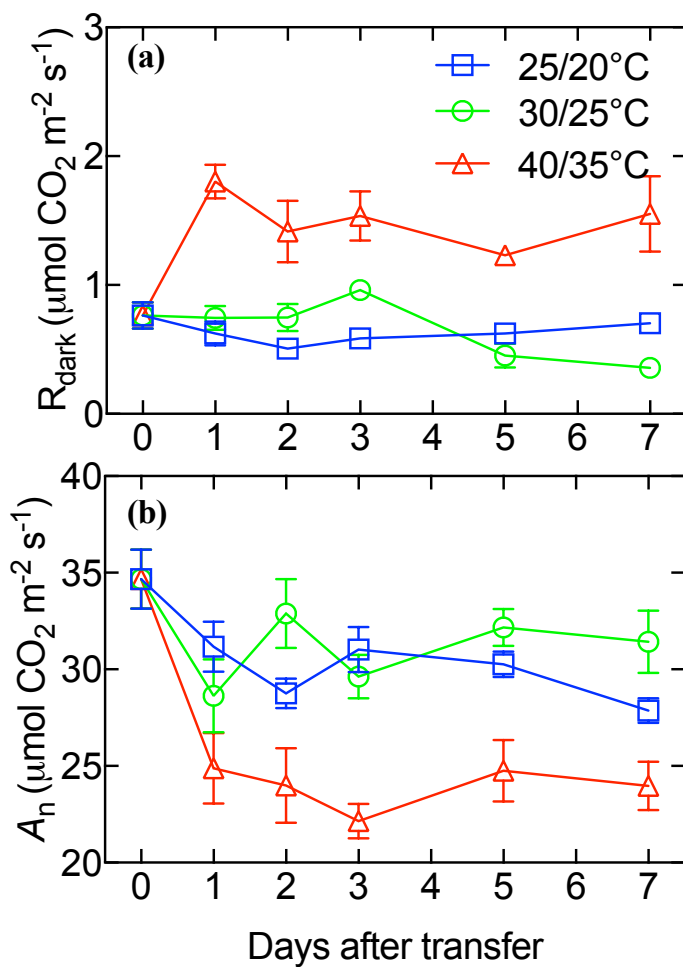**Figure S7. Rates of leaf area based dark respiration (R_{dark}) and net photosynthesis (A_n).** Rates were measured at the respective day-time growth temperature of each treatment just prior to (day 0), and 1, 2, 3, 5 and 7-days after transfer of control 30/25°C day/night grown leaves to either 25/20°C, 40/35°C or maintained at 30/25°C. Values are means of four biological replicates ± SE.
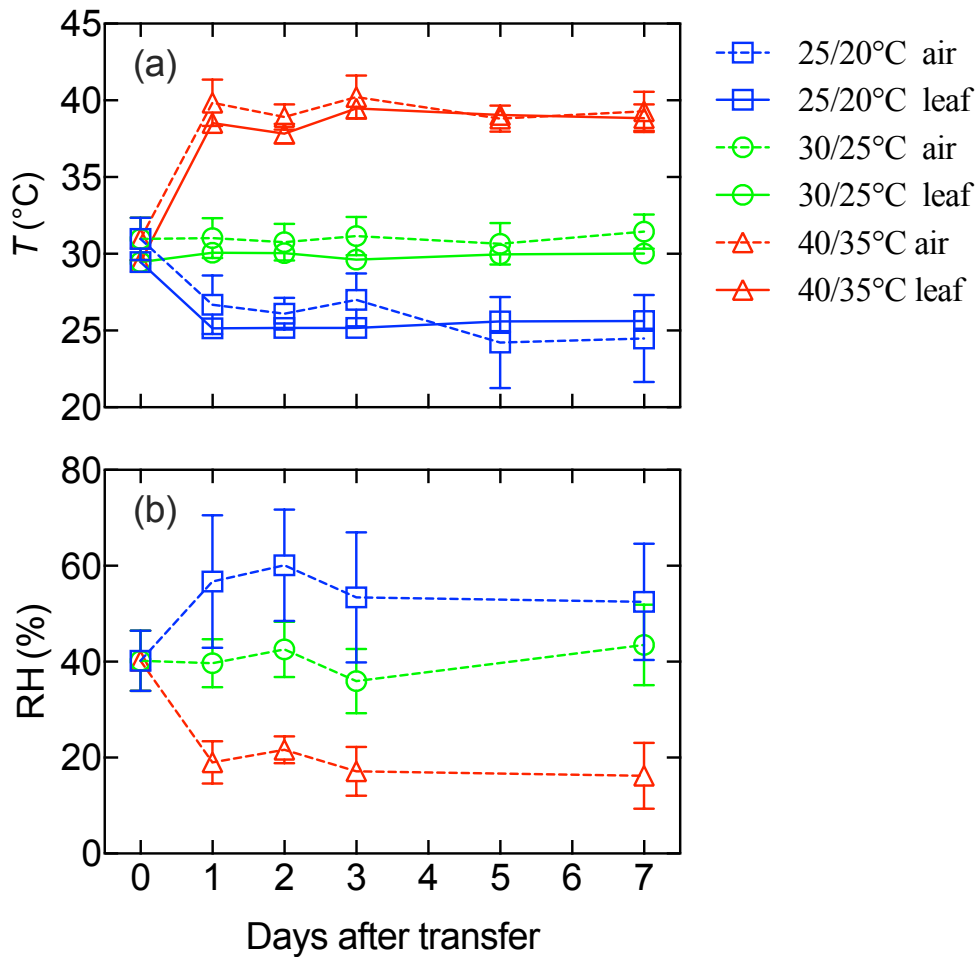
Figure S8. Room (dashed lines) and leaf (solid lines) temperatures and relative humidity (RH) over the steady-state gas exchange experimental period. Temperatures (T) were measured in the room by a thermometer and at the leaf surface by a thermocouple and relative humidity (RH) was measured in the room by a hygrometer. Values are means ± SD.
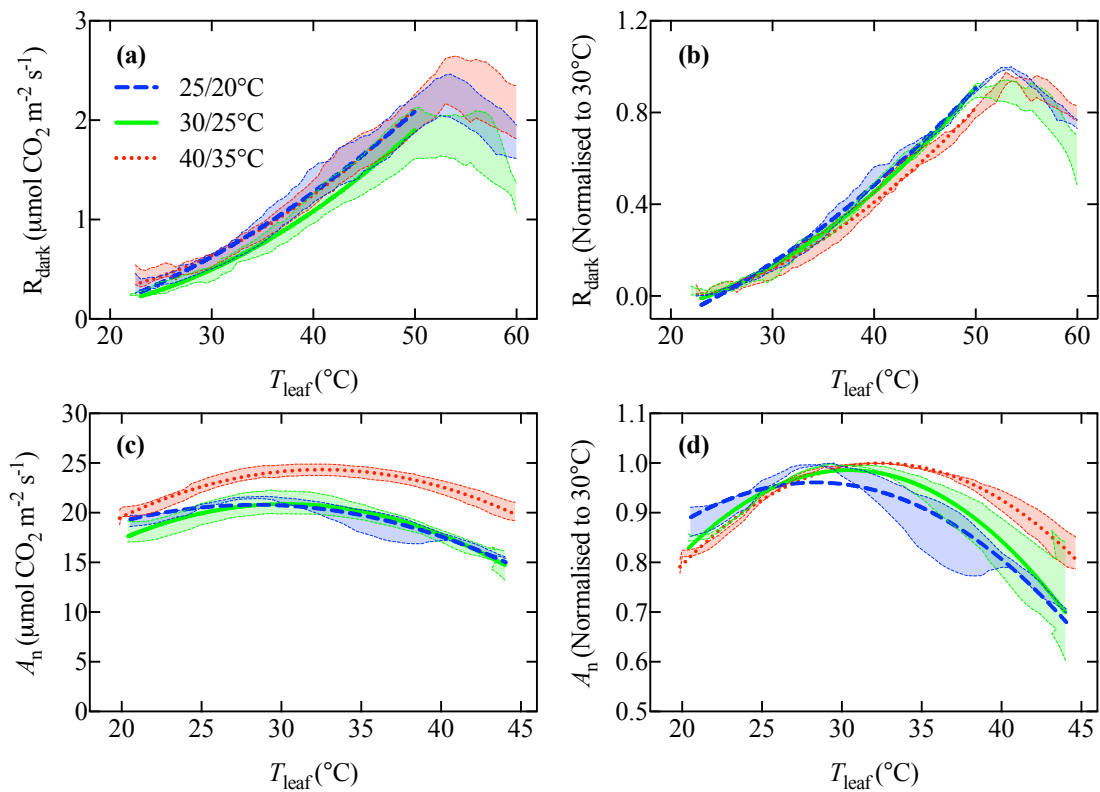
Figure S9. Temperature-response curves (a, b) of dark respiration (R_{dark}) and (c, d) net photosynthesis (A_n), on an area basis. Values are absolute (a, c) or normalised to values at 30°C (b, d). Measurements were made on whole newly-developed (ND) leaves growing for 21 d at day/night temperatures of 25/20°C, 30/25°C or 40/35°C. Curves fitted to R_{dark} and A_n are quadratic functions. Rates were recorded every 30 sec as leaves were heated at 1°C per minute. Filled area represent standard error of three to four biological replicates.

1   **Molecular and physiological responses during thermal acclimation of leaf**

2   **photosynthesis and respiration in rice**

3

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20

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24

25   **Abstract**

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27   To further our understanding of how sustained changes in temperature affect the carbon economy of

28   rice (*Oryza sativa*), hydroponically-grown plants of the IR64 cultivar were developed at 30/25°C

29   (day/night) before being shifted to 25/20°C or 40/35°C. Leaf mRNA and protein abundance, sugar and

30   starch ~~content~~concentrations, gas-exchange and elongation rates were measured on pre-existing leaves

31   (PE) already developed at 30/25°C, or leaves newly-developed (ND) subsequent to temperature

32   transfer. Following a shift in growth temperature, there was a transient adjustment in metabolic gene

33   transcript abundance of PE leaves before homeostasis was reached within 24 h, aligning with R_{dark}

34   (leaf dark respiratory CO_2 release) and A_n (net CO_2 assimilation) changes. With longer exposure, the

35   central respiratory protein CYTOCHROME C OXIDASE (COX) declined in abundance at 40/35°C.

36   In contrast to R_{dark}, A_n was maintained across the three growth temperatures in ND leaves. Soluble

37   sugars did not differ significantly with growth temperature, and growth was fastest with extended

38   exposure at 40/35°C. The results highlight that acclimation of photosynthesis and respiration is

39   asynchronous in rice, with heat-acclimated plants exhibiting a striking ability to maintain net carbon

40   gain and growth when exposed to heat-wave temperatures, even while reducing investment in energy-

41   conserving respiratory pathways.

42

43   **Keywords:** rice, thermal stress, acclimation, respiration, photosynthesis, heat, cold, CYTOCHROME

44   C OXIDASE (COX)

45

46   **Introduction**

47

48   The response of net CO_2 assimilation (A_n) and leaf respiration in the dark (R_{dark}) to changes in

49   temperature (T) is often dynamic. Acclimation – i.e. physiological changes that enable adjustments in

50   the rate of A_n and R_{dark} at a given measuring T in response to sustained changes in growth T – occurs

51   in many species, in natural and controlled environments (Atkin, Bruhn, Hurry, & Tjoelker, 2005; Berry

52   & Bjorkman, 1980; Campbell et al., 2007; Reich et al., 2016; Smith & Dukes, 2017; Tjoelker, Oleksyn,

53   & Reich, 2001). Acclimation can also lead to metabolic homeostasis, where similar rates of A_n and

54   R_{dark} are exhibited by hot- and cold-acclimated plants, when compared at their respective growth Ts.

55   Determining the extent to which R_{dark} and A_n acclimate to sustained changes in T is of growing interest,

56   as global warming is resulting in plants of natural and managed ecosystems experiencing higher

57   average growth Ts, often in conjunction with more frequent and severe heat waves (CSIRO & BOM,

58   2018; Hartmann et al., 2013). The impact of heat on A_n and R_{dark} of cereal crops, including rice (*Oryza*

59   *sativa*), is of particular interest given the need to increase food production to meet the requirements of

60   a growing and more affluent world population (Godfray et al., 2010). Rice contributes substantially to

61   global food demand, particularly in Asia where it makes up more than 30% of all dietary energy intake

62   (Seck, Diagne, Mohanty, & Wopereis, 2012). However, in recent years rice yields have declined in

63   regions such as South-East Asia, with the declines being more strongly correlated with nightly

64   minimum than daytime maximum Ts (Peng et al., 2004; Welch et al., 2010). Reduced yields and grain

65   quality were also observed for rice in North America when exposed to warmer night T (Lanning,

66   Siebenmorgen, Counce, Ambardekar, & Mauromoustakos, 2011). Given this, and the likely

67   importance of A_n and R_{dark} for biomass and grain production (Posch et al., 2019; Yoshida, 1972), it is

68   crucial that we develop a better understanding of how changes in T affect these key metabolic

69   processes in rice.

70   In rice and a range of other crops, RNA sequencing analysis has shown large scale

71   perturbations to the transcript profile of plants exposed to colder or warmer T, with the changes

72   occurring over a period of hours to days and across multiple functional categories, but especially in

73   genes involved in primary metabolism (Bhardwaj et al., 2015; Hu, Sun, Zhang, Nevo, & Fu, 2014;

74   Shen et al., 2014). The vast gene expression response to T-perturbations is likely mediated through

75   heat shock transcription factors, which regulate changes in transcriptional networks. These are induced

76   by heat stress and other abiotic stimuli, changing the protein compleiment of a cell (Morimoto, 1998;

77   Ohama, Sato, Shinozaki, & Yamaguchi-Shinozaki, 2017). However, changes in protein abundance

78   may not necessarily match changes in transcript abundance due to transcription and RNA turnover

79   rates being influenced by T (Sidaway-Lee, Costa, Rand, Finkenstadt, & Penfield, 2014), and post-

80   transcriptional regulation. In cases where heat stress results in changes in protein abundance, the

81   greatest changes are seen in proteins associated with primary metabolism, with about 50% of all leaf

82   protein abundance changes seeming to be in A_n and R_{dark} metabolic pathways (Scafaro & Atkin, 2016).

83   In rice, cold-stress similarly perturbs a large proportion of energy metabolism pathways (Neilson,

84   Mariani, & Haynes, 2011), emphasising the importance of changes in protein abundance in

85   chloroplasts and mitochondria as part of the thermal acclimation process. There is growing evidence

86   that plants can acclimate to T variations by stimulating energy metabolism at colder T and suppressing

87   energy metabolism at warmer T, either through regulation of enzymatic velocities or changes in

88   enzyme abundance (Armstrong et al., 2008; Badger, Björkman, & Armond, 1982; Campbell et al.,

89   2007; Hikosaka, Ishikawa, Borjigidai, Muller, & Onoda, 2006; Scafaro et al., 2017; Strand et al., 1999;

90   Yamori, Noguchi, & Terashima, 2005). Whether the same is true for rice remains unclear, both for

91   pre-existing (PE) leaves that experience a sustained change in growth T, and in newly-developed (ND)

92   leaves that form under a new thermal regime. In species other than rice, the extent of changes

93   underpinning thermal acclimation (including changes in leaf structure, nitrogen partitioning and

94   organelle abundance), is typically greater in ND than PE leaves (Armstrong et al., 2008; Gorsuch,

95   Pandey, & Atkin, 2010; O'Leary, Asao, Millar, & Atkin, 2018; Tjoelker, Reich, & Oleksyn, 1999;

96   Yamori et al., 2005).

97   Past studies on rice conducted during the vegetative (Glaubitz et al., 2014; Kurimoto, Day,

98   Lambers, & Noguchi, 2004) and reproductive (Bahuguna, Solis, Shi, & Jagadish, 2017; Mohammed,

99   Cothren, & Tarpley, 2013) phases of development have reported limited and variable levels of

100   acclimation of R_{dark}. By contrast, A_n of rice shows strong thermal acclimation, with rates of net CO_2

101   uptake measured at the prevailing growth T being homeostatic or increasing as growth T is increased

3

from 15 to 37°C (Nagai & Makino, 2009; Yamori, Noguchi, Hikosaka, & Terashima, 2010). Such studies point to a possible asynchrony in rice acclimation, with a more dynamic A_n than R_{dark} response, although further work simultaneously comparing A_n and R_{dark} in rice is needed. Interestingly, a range of other crops and non-crop species show the opposite – greater R_{dark} than A_n acclimation capacity (Campbell et al., 2007; Drake et al., 2016; Way & Oren, 2010). Although such studies detail the physiological acclimation of energy metabolism in plants, including rice, less is known about the molecular and biochemical responses that underpin this phenotypic T acclimation.

It is with the above issues in mind that we conducted a study using the IR64 cultivar of *Oryza sativa* to address the following aims: (1) characterise the extent of thermal acclimation of R_{dark} and A_n; and, (2) link physiological acclimation to the underlying processes through analysis of leaf transcript, protein, sugar, and starch abundance, following changes in growth T. We hypothesized that: (1) initial exposure to very high growth T increases and decreases the rates of respiratory CO_2 release and net photosynthetic CO_2 uptake, respectively; (2) subsequent acclimation is associated with recovery of A_n, and reduced rates of R_{dark}; and (3) thermal acclimation of R_{dark} and A_n is associated with dynamic changes in gene expression and protein abundance in key pathways associated with energy capture and use.

Materials and methods

Plant material and temperature treatments

Rice (*Oryza sativa*) cultivar IR64 plants were grown hydroponically in a glasshouse facility at the Research School of Biology, Australian National University in Canberra between October and December 2015. Seeds were initially incubated at 40-42°C for two days before soaking in water for eight hours, placed on wet Whatman filter papers in petri dishes and kept in the dark at 30°C for five days. The germinated seedlings were then transferred to trays of vermiculite and placed in temperature-controlled glasshouses (30°C day and 25°C night) under natural sunlight and photoperiod, with photosynthetically active radiation (PAR) between 400 and 1200 µmol m^{-2} s^{-1}. When the third leaf had emerged, seedlings were transplanted from vermiculite to a hydroponic system. Individual plants were placed within PVC tubes with a 3.7 cm diameter and 13 cm height. Tubes were then suspended at the top of 20 L capacity hydroponic tanks (12 tanks in total), with each tank holding a maximum of 20 plants. Each tank was filled with hydroponic solution (Table S1) (Hubbart, Peng, Horton, Chen, & Murchie, 2007). The nutrient solution was replaced weekly. Sulphuric acid or sodium hydroxide were used to adjust the pH to 5-6, with pH monitored using a portable pH meter (Rowe Scientific Pty. Ltd.,

135   NSW, Australia). The hydroponic solution was aerated continuously using Infinity AP-950 air pumps

136   (Kong's Pty. Ltd., Ingleburn, Australia).

137   After two weeks of hydroponic growth at 30/25°C ('warm' treatment), the most recently fully-

138   expanded leaves were labelled as pre-existing (PE) leaves. Following labelling, four tanks were

139   randomly chosen and shifted to an adjacent glasshouse room set to 25°C day and 20°C night (25/20°C –

140   'cold' treatment), and four other tanks were moved to a room set at 40°C day and 35°C night (40/35°C

141   – 'hot' treatment); four tanks were retained at 30/25°C as controls. Relative humidity was not

142   controlled. Newly-developed (ND) leaves that emerged under each thermal regime were labelled, with

143   all measurements reported on ND leaves made 21 days after T-transfer.

144

145   *Determination of transcript abundance*

Plants were transferred to new thermal regimes three hours after sunrise. To quantify transcript

146   abundance, the labelled pre-existing (PE) leaves were harvested during the photoperiod: two, six and

147

148   24 h after T-transfer. For each time-point and temperature treatment, approximately 8 cm long

149   segments (less than 100 mg of fresh mass) were sampled half-way along the leaf blade, and

150   immediately frozen in liquid N_2 and stored at -80°C until RNA extraction. Total RNA was extracted

151   using the RNeasy plant mini protocol (Qiagen, Doncaster, VIC, AU) and treated with DNase I (Qiagen,

152   Doncaster, VIC, AU) to remove any contaminating DNA.

153   For qPCR analysis, 1 µg of total RNA in a 10 µL volume was reverse- transcribed into cDNA

154   using SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA), according to

155   the manufacturer's instructions. The reverse-transcribed cDNA samples were diluted 10-fold.

156   Transcript levels of six selected genes and one reference gene (refer to Table S2 for gene accession

157   numbers and primer sequences) were analysed using a Light-Cycler® 480 System (Roche Holding

158   AG, Basel, Switzerland) with SYBR Green I Dye (QIAGEN, Doncaster, Victoria, AU). cDNA

159   samples from each biological replicate were assayed in two technical duplicates. The reaction-mix in

160   each qPCR contained 0.4 µM of each pair of primers, 5 µL of SYBR Master Mix, and 4.6 µL of the

161   diluted cDNA sample in a 10 µL total reaction volume. The raw fluorescence data were analysed using

162   LinRegPCR (Ramakers, Ruijter, Deprez, & Moorman, 2003; Ruijter et al., 2009). Data were

163   normalized to the reference gene, eukaryotic initiation factor 5c (*EIF5C*; LOC_Os11g21990.1), and

164   were expressed as fold-changes against control conditions.

165   RNAseq libraries were prepared using the Illumina Stranded Total RNAseq kit with RiboZero

166   rRNA depletion as per the manufacturer's guidelines (Illumina). Libraries were pooled and sequenced

167   on a HiSeq1500 for 61 cycles in single end mode at the Centre for AgriBioscience, University of

168   LaTrobe. Analysis pipelines for pre-processing and mapping of sequence data are available online on

169   GitHub (https://github.com/pedrocrisp/NGS-pipelines). Quality control was performed with *FastQC*

170   v.0.11.2. Adapters were removed using *scythe* v.0.991 with flags -p 0. and reads were quality trimmed

171   with *sickle* v.1.33 with flags -q 20 (quality threshold), and -l 20 (minimum read length after trimming).

172   The trimmed and quality-filtered reads were aligned to the rice reference genome Os-Nipponbare-

173   Reference-IRGSP-1.0 from the MSU Rice Genome Annotation Project Database v7

174   (http://rice.plantbiology.msu.edu/) using the *subjunc* aligner v1.5.0-p1 with -u and -H flags to report

175   only reads with a single unambiguous best mapping location, -P 3 for phred+33 encoding (Liao, Smyth,

176   & Shi, 2013b). Reads were then sorted, indexed and compressed using *samtools* v1.1-26-g29b0367

177   (Li et al., 2009) and strand-specific bigwig files were generated using *bedtools genomecov* v2.16.1

178   (Quinlan & Hall, 2010) and the UCSC utility *bedGraphToBigWig* for viewing in IGV (Robinson et

179   al., 2011). Summary statistics for each sample are provided in Supplementary Dataset S1: Summary

180   of transcriptomic datasets.

181   For standard differential gene expression testing, the number of reads mapping per IRGSP-

182   1.0 gene loci was summarised using *featureCounts* v1.5.0-p1(Liao, Smyth, & Shi, 2013a) with flags

183   -P and -c to discard read pairs mapping to different chromosomes and the -s flag set to 2 for strand

184   specificity for a strand specific library, multimapping reads and multioverlapping reads were not

185   counted. Reads were summarised to parent IRGSP-1.0 gene loci rather than individual splice variants

186   by summarising to the genomic coordinates defined by the feature "gene" in the IRGSP-1.0.gff

187   reference (last modified 7/2/2012

188   ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/all.dir/all.gff3). Only loci

189   with an abundance of at least 1 CPM (approximately 5 reads) in at least 4 samples were retained.

190   Statistical testing for relative gene expression was performed in R following the "*edgeR-limma-

191   voom*" approach (https://www.bioconductor.org/help/workflows/RNAseq123/); using, *edgeR* v.3.4.2

192   (McCarthy, Chen, & Smyth, 2012; Robinson & Oshlack, 2010; Robinson & Smyth, 2007a, 2007b),

193   and *voom* in the *limma* package 3.20.1 (Law, Chen, Shi, & Smyth, 2014; Smyth, Michaud, & Scott,

194   2005).

195

196   *Determination of protein abundance*

197   Samples of frozen PE leaf material six and 24 h after *T* transfer, as well as frozen ND leaf material,

198   were ground to a fine powder using a chilled mortar and pestle, and protein was extracted in extraction

199   buffer containing 100 mM tricine pH 8.0, 1 mM EDTA, 1 mM PMSF, 1x protease inhibitor cocktail,

200   2% (w/v) PVPP, 10 mM (w/v) ascorbate, 5 mM DTT, and distilled water. The sample was then

201   solubilized in a NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA, USA) with 10% (v/v) DTT,

202   then heated for 10 minutes at 95°C, and centrifuged for 30 sec at 12,000 RPM. Thereafter, supernatant

203   was collected and 8 uL were loaded and separated on 4-12% NuPAGE Bis-Tris gel (Invitrogen,

204   Carlsbad, CA, USA) using the MOPS-based buffer system. To blot, proteins from the gel were

205   transferred to Immobilon-P Polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Kilsyth,

206   VIC, AU) using an XCell II Blot module (Invitrogen, Carlsbad, CA, USA). Membranes were then

207   blocked for 2 h with 5% (w/v) skim-milk powder in Tris-buffered saline containing 0.1% (v/v) Tween-

208   20 (TBST). To probe for cytochrome *c* oxidase (*COX*) subunit II, alternative oxidase (*AOX*),

209   uncoupling protein (*UCP*) and voltage-dependent anion-selective channel protein (*VDAC1*-porin), the

210   membranes were incubated for 2 h in primary antibody solution (5% w/v skim milk powder in TBST)

211   containing commercially available polyclonal antibodies (Agrisera, Vännäs, Västerbotten, Sweden).

212   All antibodies were diluted 1:5000. An antibody for ribulose-1,5-bisphosphate carboxylase/oxygenase

213   (Rubisco) was received from Assoc. Prof. Spencer Whitney (Research School of Biology, Australian

214   National University, Canberra, ACT, AU) and used at a dilution of 1:10 000. After washing with TBST,

215   the membranes were incubated for 1 h in goat anti-rabbit antibody solution (5% w/v skim milk powder

216   in TBST) at a dilution of 1:8000. Proteins were then visualized using the AttoPhos AP fluorescent

217   substrate system (Promega, Madison, WI, USA), imaged using a Versa-Doc (Bio-Rad, Hercules, CA,

218   USA) imaging system and quantified using Image Lab software (Bio-Rad, Hercules, CA, USA).

219   Protein concentrations were determined by the Bradford method using Bovine Serum Albumin (BSA)

220   as a standard.

221

222   *Determination of soluble sugar and starch* ~~content~~*concentrations*

223   Starch and soluble sugars of PE leaves transferred from 30/25°C to 25/20°C or 40/35°C for one and

224   seven days, and ND leaves at the prevailing *T* were collected from separate, previously unsampled

225   plants. Samples were collected at 9:30 to 10:00 am, corresponding to 3 h into the light period, frozen

226   and stored at -80°C before freeze-drying at -105°C for two days (Virtis Benchtop™ "K", SP, Scientific,

227   Gardiner, NY, USA), then ground to a fine powder from which a 5-10 mg aliquot was taken. Five-

228   hundred μL of 80% (v/v) ethanol was added and vortexed for 20 sec. Thereafter, leaf tissues were

229   incubated at 80°C while being centrifuged at 500 RPM for 20 min. Following further centrifugation at

230   12,000 RPM for 5 min, the resulting pellet and supernatant were separated. This procedure was

231   repeated a two more times, and the three pellets and three supernatants were pooled. The pooled

232   supernatants and pellets were used for determination of soluble sugars and starch

233   ~~contents~~concentrations, using a Fructose Assay Kit (Sigma-Aldrich, St Louis, MO, USA) and a Total

234   Starch Assay Kit (Megazyme, Chicago, IL, USA), respectively. Following manufacturer's instructions,

235   measurements were made in triplicate, at a wavelength of 340 nm, using a microtitre plate reader

236 (Infinite® M1000 Pro; Tecan US, Morrisville, NC) and standard curves were generated for soluble

237 sugars using sucrose, glucose and fructose (Sigma-Aldrich, St Louis, MO, USA) at known

238 concentrations.

239

240 *Gas exchange measurements using Licor 6400XT 6 cm² cuvettes*

241 Gas-exchange was measured on fully-expanded pre-existing (PE) leaves just prior to- and one, two,

242 three, five and seven days after T transfer, as well as on fully-expanded newly-developed (ND) leaves

243 21 days after transfer, using two matched LI-6400 instruments equipped with 6 cm² cuvettes and a

244 6400-02B red-blue light source (Li-Cor, Lincoln, NE, USA). At each time point, light-saturated net

245 CO_2 assimilation rates (A_n) and then dark respiration rates (R_{dark}) were measured during the light period

246 (between 10 am and 2 pm) in the glasshouses, at the prevailing day-time T of each treatment as well

247 as at a common temperature of 30°C for ND leaves. In call cases, A_n was measured first, with the

248 following settings: 1000 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), relative humidity

249 of 60–70%, 400 ppm reference CO_2, and a flow rate of 500 μmol s^{-1}. Photosynthesis was measured

250 when CO_2 concentrations in the sample IRGA had stabilized, typically within 10 minutes of exposure

251 to 1000 μmol m^{-2} s^{-1} PPFD. Thereafter, R_{dark} was measured as above but with the flow rate slowed to

252 300 μmol s^{-1} and turning off the light source for at least 30 minutes of darkness before taking

253 measurements.

254

255 *Gas exchange measurements using* using Walz chambers

256 High resolution temperature response curves of R_{dark} and light-saturated A_n were made on intact ND

257 leaves using two matched LI-6400XT portable gas exchange systems (Li-Cor, Lincoln, NE, USA)

258 each connected to a 14 x 10 cm well-mixed, temperature-controlled Walz Gas-Exchange Chamber

259 3010-GWK1 (Heinz Walz GmbH, Effeltrich, Germany). For each temperature-response curve, leaf T

260 was measured with a small-gauge wire copper constantan thermocouple pressed against the lower

261 surface of the leaf and attached to a LI-6400 external thermocouple adaptor (LI6400-13, Li-Cor Inc.,

262 Lincoln, NE, USA) that enabled leaf temperature to be recorded by the LI-6400XT. As leaves were

263 heated, net CO_2 exchange was recorded at 30 s intervals using the LI-6400XT portable gas exchange

264 systems fitted with an empty and closed 6 cm² chamber that was plumbed into the airstream exiting

265 the Walz leaf chamber (Fig. S1). A_n was measured as described for the 6 cm² cuvette but using a Walz

266 LED-Panel RGBW-L084 light source (Heinz Walz GmbH, Effeltrich, Germany). A_n was monitored

267 as leaves were heated at 1°C min^{-1} from 20 to 45°C. A water trap was used to remove water vapour,

268 as transpiration from whole intact leaves was incompatible with Licor instrumentation. Therefore,

269 stomatal conductance (g_s) and associated water parameters were not recorded. For R_{dark}, on separate

8

270 leaves to those used for measuring A_n, the flow rate was reduced to 300 μmol s^{-1}, the light source was

271 turned off, and the chamber was covered with a black cloth, before increasing the leaf temperature in

272 steps of 1°C min^{-1}, from 20 to 60°C. In parallel to quantifying the temperature-response of R_{dark}, we

273 measured minimal chlorophyll *a* fluorescence (F_o) in the presence of a low-intensity far-red light pulse

274 (necessary to maintain PSII in the oxidized state) every 30 sec using a Mini-PAM portable chlorophyll

275 fluorometer (Heinz Walz, Effeltrich, Germany) fitted above the glass surface of the leaf chamber. The

276 temperature at which F_o increased was used as an indication of heat-induced damage to photosystem

277 II, which we hereafter refer to as T_{crit}, calculated using the template of O'Sullivan *et al.* (2013). At the

278 cessation of measurements, leaves were photographed and analysed for leaf area using ImageJ

279 software (Abramoff, Magelhaes, & Ram, 2004). Leaves were stored in paper bags, oven-dried at 70°C

280 for two days and weighed to obtain the dry mass. Quadratic equations were fit to A_n temperature curves

281 and the x- and y-axis values corresponding to the vertex taken as the T-optimum (T_{opt}) and A_n-optimum

282 (A_{opt}) of net assimilation, respectively. For the R_{dark} temperature curves, the x- and y-axis values

283 corresponding to the maximum recorded R_{dark} were taken as the T at which R_{dark} reached a maximum

284 (T_{max}) and the maximum R_{dark} value recorded (R_{max}), respectively.

285

286 *Leaf elongation rates*

287 The leaf elongation rates (LER) of four leaves from separate plants from each temperature regime were

288 measured at five separate time-points over a 24 h period. Measurements were made using a ruler,

289 starting from the ligule of the second youngest leaf to its tip.

290

291 *Statistical analysis*

292 For all T-treatments and collection times, four separate leaves from four separate previously unsampled

293 plants, one plant from each of the four hydroponic tanks (pot replicates) were sampled. One-way

294 Analysis of Variance (ANOVA) was performed on R_{dark} and A_n gas-exchange experiments comparing

295 temperature treatments. Two-way ANOVA was performed on LMA and protein, starch, and sugar

296 contents concentrations comparing time of sampling and temperature treatments. Gas-exchange and

297 leaf biochemical statistical analysis was performed using GraphPad Prism (v 7) software. Statistical

298 analysis of transcript abundance was performed using R statistical software (v 3.6.1) and packages as

299 mentioned above.

300

301 *Data availability*

302 RNA-seq data is available under the GEO identifier GSE136045.

303

Results

Molecular and biochemical responses of leaves to T

Quantitative PCR was performed on specific genes of interest to elucidate the genetic response of pre-existing (PE) leaves exposed to a change in *T* (Fig. 1). Apart from a sharp rise 6 h into the 40/35°C *T*-transfer, there was a general reduction in transcript abundance of *cytochrome c* ~~complex~~ *oxidase* (*cox*), a gene encoding the central respiratory electron transport chain. This reduction occurred in leaves transferred to 25/20°C and 40/35°C, over the seven days post-transfer period, . Two genes encoding respiratory proteins that potentially reduce the production of ATP – *alternative oxidase* (*aox*) and *uncoupling protein* (*ucp*) – both showed an initial increase in expression within the first 24 h of transfer to the hotter 40/35°C, followed by a decline to 30/25°C levels by 48 h. The photosynthetic electron transport gene *ferredoxin NADP reductase* (*fnr*), and the Calvin/Benson cycle gene *phosphoribulokinase* (*prk*) generally showed an increase in expression in the first 48 h at 40/35°C before being supressed for up to 5 days post-transfer. *Sucrose phosphate synthase* (*sps*), involved in the synthesis of sucrose from its precursors, also initially spiked in the first 24 h following transfer to 40/35°C, before being transiently suppressed. Both *sps* and the respiratory and photosynthetic genes – apart from *aox* – followed a similar expression profile when heat-treated, suggesting that assimilate production/consumption and sucrose synthesis were coordinated in response to heat perturbations. In general, the greatest perturbation to gene expression occurred within the first 24 h after transfer.

Based on the qPCR results, we conducted RNA-seq at two, six and 24 h after transfer of PE leaves to new *T*. Following data quality control and filtering, transcript abundance of 19,308 rice genes were retained for differential expression testing. Around 20 M reads were obtained per sample, which were aligned to the Os-Nipponbare-Reference-IRGSP-1.0 rice reference genome (Data Set S1). Principal component analysis showed a substantial treatment effect on gene expression for the heat-exposed leaves (40/35°C) during the first six hours after *T*-transfer compared to the other two 30/25°C and 25/20°C growth regimes (Fig. 2). Globally, there was little gene expression variation between the cold (25/20°C) and the warm (30/25°C) control conditions. After 24 h of growth at new *T*-regimes, limited variation in gene expression was observed between all of the three *Ts* (Table S3).

To assess changes in the expression of individual genes to the hot or cold treatments, differentially expressed genes were identified at each time point by comparison to the warm control expression levels (Data Set S2). There were very few genes differentially expressed under the cold conditions, only six genes in total (Table S3). By contrast, under hot conditions, there were 1,818 and 1,465 genes differentially expressed after two and six hours, respectively. After 24 hours, there were

10

337 no differentially expressed genes under the hot conditions compared to the control plants. There was

338 a significant overlap between the genes differentially expressed after two and six hours of heat

339 treatment, (Fig. 3a, b). In total, 30% of the genes upregulated after six hours were already upregulated

340 by two hours, and 38% of genes downregulated after six hours were already downregulated by two

341 hours. Many of the remaining genes that were significantly different in transcript abundance only after

342 six hours were already trending in the same direction at the two-hour time point, but the difference

343 compared to the controls did not pass the significance threshold (Fig. 3 c). Overall, these results show

344 that there are significant short-term changes in transcript abundance in rice plants exposed to heat

345 stress. The expression profiles of samples exposed for two and six hours show consistent changes;

346 however, some of the changes peak at two hours and others peak at six hours and most changes

347 dissipate within 24 hours.

348   In total, the heat treatment led to the up- and down-regulation of 1,337 and 1,446 genes,

349 respectively. To investigate the extent to which these genes have a photosynthetic or respiratory

350 function, we first examined expression of genes involved in photosynthesis, glycolysis, TCA and

351 mitochondrial electron transport using MapMan pathway annotations (Fig. S2-4) (Thimm et al., 2004).

352 This qualitative analysis revealed that the expression of only a small number of

353 photosynthetic/respiratory genes were affected. To extract a list of high-confidence differentially

354 expressed respiration-related genes, we manually curated a list of rice loci with homology to

355 Arabidopsis respiration genes (Data Set S3). Using this list, we found that eight genes were

356 differentially expressed at high temperature, with two genes downregulated more than 2-fold: *aox* and

357 ATP-dependent *phosphofructokinase* (Table 1). The seemingly conflicting result of an initial increase

358 in *aox* from the qPCR results, but a decline in *aox* during the same period from the RNA-seq results,

359 can be explained by our qPCR primers targeting the *aox1a* isoform while the RNA-seq identified a

360 decline in the *aox1c* isoform (Data Set S3).

361   It is interesting that in addition to the increase in *aox* and *ucp* gene expression, the expression

362 of an external NAD(P)H dehydrogenase also increased, while that of Complex II decreased

363 significantly (Table 1). Together these changes suggest that an increase in non-phosphorylating

364 electron transport occurred in response to exposure to higher *T*, at the expense of electron transport

365 coupled to ATP synthesis, at least in the short term. The increase in external NAD(P)H dehydrogenase

366 gene expression may also indicate an increased need for mitochondrial oxidation of excess reductant

367 produced in the chloroplast at higher *T*.

368   Given the relatively small effect of the heat treatment on the expression of respiration- or

369 photosynthesis-related genes, we next performed Gene Ontology enrichment analysis. This revealed a

370 notable enrichment for genes involved in primary metabolism (eg GO:0044238) and response to
371 abiotic stimuli (eg GO:0050896), as well as in many biosynthetic pathways (Fig 4).

372   Protein abundance (expressed on a leaf area basis) of key mitochondrial electron transport
373 components – CYTOCHROME C OXIDASE (COX) subunit II, ALTERNATIVE OXIDASE (AOX)
374 and UNCOUPLING PROTEIN (UCP) – were determined by Western blots in PE leaves 6 h and one-
375 day after T-transfer, and in newly developed (ND) leaves that formed under each prevailing growth T
376 (Fig. 5, Fig. S4). The cold (20/25°C) and heat (40/35°C) treatments did not affect the total protein
377 concentration of leaves, at any time after T-transfer (Table S4). As was the case for gene expression,
378 there was a significant decline in COX subunit II protein abundance after 24 h. COX subunit II also
379 declined in abundance in ND leaves when grown at 40/35°C compared to 25/20°C (Fig. 5a). We
380 assume the changes observed in COX subunit II reflect changes in abundance of the entire complex.
381 The abundance of AOX and UCP protein did not vary in response to growth T or duration of exposure
382 to heat for either PE or ND leaves, despite the initial spike in *aox* and *ucp* gene expression after transfer
383 to 40/35°C (Fig. 1). Interestingly two bands of AOX that varied relative to one another with
384 temperature treatment were evident in the Western blot (Fig. S5). This is consistent with the
385 fluctuations in expression of different *aox* genes noted above. Patterns of protein abundance were
386 similar when the analysis was standardised to dry mass or porin abundance (Fig. S6). Porin is a voltage-
387 dependent channel protein located at the outer membrane of mitochondria and is widely used as a
388 proxy for mitochondrial surface area due to its stability under a wide range of environmental conditions
389 (Noguchi, Taylor, Millar, Lambers, & Day, 2005; Shane et al., 2004). Thus, the matching results using
390 leaf area, dry mass or porin abundance indicate that the decline in COX abundance with increased T
391 was not a result of changes in Leaf Mass to Area ratio (LMA) or reduced mitochondria per unit area
392 of leaf. There was a trend for the abundance of Rubisco to decline with the amount of time a leaf
393 developed under 40/35°C (Fig. 5d), although there were no statistically significant T or developmental
394 stage effect.

395   LMA and starch, glucose, fructose and sucrose ~~contents~~ concentrations were measured in PE
396 leaves one and seven days after T-transfer, and in ND leaves at the prevailing temperature (Table 2).
397 LMA did not change significantly in response to T, for either transferred PE leaves or ND leaves,
398 similar to previous observations in rice over a similar T range (Nagai & Makino, 2009). However, ND
399 leaves did exhibit significantly greater LMA than PE transferred leaves, suggesting an effect of leaf
400 rank on LMA. Transferred PE and subsequently formed ND leaves exhibited consistently lower starch
401 ~~content~~ concentrations with increasing T and significantly lower starch with extended duration of
402 development at the prevailing T. Unlike the dynamic responses of leaf starch ~~content~~ concentrations

403   to T change, ~~contents~~ concentrations of soluble sugars were remarkably stable across both PE and ND

404   leaves, in terms of both T-regime and exposure time. Negative correlations between R_{dark} and soluble

405   sugars were observed among leaves within each individual T treatment but not among the three T

406   treatments (Table S5).

407

408   *CO_2 flux in responses to T*

409   How molecular changes altered the physiological performance of rice carbon metabolism at differing

410   growth T was investigated through gas-exchange measurements. Rates of A_n and R_{dark} are here

411   presented on a dry mass (DM) basis, noting that the patterns are similar when expressed on a leaf area

412   basis (Fig S7), reflecting the fact that growth T had no significant effect on LMA (Table 2).   A

413   significant change in both A_n and R_{dark} (using mid-sections of leaves placed in Licor 6400 3 x 2 cm

414   chambers) occurred within the first 24 h of transfer to a 40/35°C T-regime for PE leaves, with A_n

415   falling and R_{dark} increasing when measured at the prevailing growth T (Fig. 6). This was followed by

416   stabilisation at the new rate over the subsequent six days. By contrast, A_n and R_{dark} remained relatively

417   constant at both 30/25°C and 25/20°C over a seven-day period monitoring period. Interestingly, rates

418   of R_{dark} for the 30/25°C treated plants decreased from day 3 to 7, compared to the first three days,

419   resulting in slightly lower rates of R_{dark} than for the 25/20°C treated plants by day 7. This possibly

420   reflects temperature-dependent differences in leaf senescence rates. ~~Interestingly~~As it could not be

421   controlled, relative humidity in the 40/35°C glasshouse room (Fig. S8) was substantially lower than

422   the other two rooms, leading to reduced humidity during gas-exchange measurements (Fig. 6c). As a

423   consequence,~~,~~ the vapour pressure deficit between the leaf and surrounding air (VPD$_{Leaf}$) increased

424   ~~consistently~~ over the first seven days in PE leaves transferred to 40/35°C, resulting in a dramatic

425   difference by day seven (Fig. 6de). Th~~is, and the associated reduction in~~e higher VPD$_{Leaf}$ coincided

426   with lower *~~stomatal conductance~~*g$_s$ ~~of the~~in 40/35°C treated leaves at days three, five and seven, and

427   lower intercellular to ambient CO_2 concentration ratios (C_i/C_a) at days five and seven (Fig. 6e, f~~d~~).~~,~~

428   However, for the first two days post transfer~~,~~ both~~the~~ VPD$_{Leaf}$~~vapour pressure deficit~~ and g_s~~stomatal~~

429   ~~conductance was~~were similar between the three growth Ts, ~~and~~ Ttherefore, the ~~did not explain the~~

430   ~~initial 48 h~~decline in A_n and changes in transcript abundance ~~at~~within one day of transfer to 40/35°C

431   were not attributable to water relations.~~likely contributing to the longer-term limitation in~~

432   ~~photosynthesis at this warmer temperature in PE leaves~~. Over the longer-term, water relations may

433   have contributed to a slight reduction in C_i/C_a, but not enough to influence A_n, with A_n being stable

434   from one to seven days after transfer irrespective of changes in g_s and C_i/C_a (Fig. 6b). The changes in

435   g_s were not substantive enough to change leaf T, which was stable over the seven days, with both air

T and leaf *T* deviating by less than 2°C from the set room *T* (Fig. S8). ~~However, for the first two days post transfer, the vapour pressure deficit and stomatal conductance was similar between the three growth *T*s, and therefore did not explain the initial 48 h decline in *A*n at 40/35°C.~~

Short-term temperature response curves of entire ND leaves that formed at each prevailing growth *T* regime were quantified over a 20 to 60°C range using the Walz large leaf chamber (Fig. 7; refer to Figure S9~~8~~ for area-based rates and Table S6 for quadratic equations fit to curves). Over most of the range of measuring *T*s, leaves developed at 25/20°C exhibited higher rates of R_{dark} than those developed under the other two *T*-regimes. Rates were lowest in leaves developed at 40/35°C (Fig. 7a). When normalised to rates at 30°C, differences in R_{dark} were less pronounced (Fig. 7b), indicating that while R_{dark} at a given measuring *T* was affected by growth *T*, the general shape of the R_{dark}-*T* curves remained largely similar across the three treatments. These observations are consistent with a Type II (changes in baseline) rather than Type I (changes in Q_{10}, the increase in R_{dark} with a 10°C increase in *T*) respiratory acclimation response (Atkin & Tjoelker, 2003). Importantly, while respiratory thermal acclimation occurred, it was not sufficient to result in R_{dark} being homeostatic across the three growth *T* treatments. As a result, R_{dark} measured at the growth *T* was significantly ~~faster~~ greater in the leaves developed under hot conditions than under the other two treatments (Table 3). Growth *T* also had a significant effect on the measuring *T* at which R_{dark} and A_{n} reached their maximum rates, with leaves developed under high *T* exhibiting higher *T*-maxima than control 30/25°C leaves (Table 3). When measured at the prevailing growth *T* of each treatment, mass-based rates of light-saturated A_{n} were stable (i.e. homeostatic), further supporting the occurrence of strong thermal acclimation of A_{n} in ND leaves (Fig. 7), contrary to PE leaves (Fig. 6). The temperature at which PSII lost functionality (T_{crit}) ~~also increased~~ tended to increase with growth *T* (being 3.8°C higher in the hot-grown plants compared to those grown at 25/20°C), although the differences were not statistically significant at *p*<0.05 (Table 3). The high degree of thermal acclimation exhibited by photosynthesis resulted in the ratio of R_{dark} to A_{n} being lowest in the hot-grown plants, particularly at high measuring *T* (Fig. 8a); at a measuring *T* of 40°C, hot-acclimated plants exhibited R_{dark}/A_{n} ratios that were 50% lower than those measured for their cold-grown counterparts. Further evidence that rice acclimated to heat is seen in the fact that leaf elongation rates – taken over the day and night period – were faster for the 40/35°C grown plants at all times (Fig. 8b). Interestingly, A_{n} of PE leaves ranged from 1.2 to 1.8 μmol g⁻¹ DM s⁻¹ (Fig. 6), substantially faster than the 0.6 μmol g⁻¹ DM s⁻¹ in ND leaves (Fig. 7). The former were obtained from measurements on mid-leaf sections placed in a 6 cm² chamber, while the latter were obtained from whole leaves placed in a 14 x 10 cm Walz chamber. The lower A_{n} rates in the latter might reflect a

468　lower proportion of mesophyll cells per unit of area or DM across whole blades compared to the mid-
469　blade section.

470

471　**Discussion**

472

473　Our study investigated the response of photosynthetic and respiratory metabolism to short- and long-
474　term changes in growth T – the highest of which is indicative of heat-wave Ts – to explore: (1) the
475　extent of thermal acclimation of photosynthesis and respiration; and, (2) what underlying changes in
476　gene expression and protein abundance occur during the acclimation process. The results demonstrate
477　that the process of acclimation begins with abrupt changes in gene expression in PE leaves within the
478　first 24 h of heat exposure, followed by a return to homeostatic gene expression (Fig. 1). Importantly,
479　the abundance of the key energy-conserving respiratory protein, COX, declines in abundance when
480　pre-existing leaves are heat-treated for 24 hours, with this phenotype being maintained in newly-
481　developed leaves formed at 40/35°C (Fig. 5). This decline in COX was linked to a slight decline in
482　overall rates of R_{dark} (Fig. 7). The results support the hypothesis that acclimation of photosynthesis
483　and dark respiration are asynchronous in rice, but contrary to observations in non-crop species
484　(Campbell et al., 2007), light-saturated A_n acclimated to a greater extent than R_{dark} (Fig. 7; Table 3).
485　This ability to maintain photosynthetic carbon gain at 40°C is likely to be of crucial importance in
486　helping rice maintain growth during heat-wave conditions.

487

488　*Acclimation to changes in T are rapid and involve a multitude of genes*
489　There was a substantial change in the gene expression profile of rice leaves shifted from 30°C to 40°C
490　within the first 24 h of transfer (Figs. 1, 2, 3, and 4). As might be expected, the largest number of gene
491　expression perturbations were in primary and cellular metabolic processes (Fig. 4). This extensive
492　metabolic response aligns with the instability in R_{dark} and A_n fluxes over the initial 24 h post T-transfer
493　(Fig. 6), which would have contributed to a metabolic imbalance through changes in assimilate supply
494　and demand. Interestingly, the most responsive genes to the initial exposure to heat among upregulated
495　genes were genes involved in biosynthetic processes (Fig. 4) suggesting a stimulation of growth. This
496　is supported by the longer-term increase in leaf elongation rates observed in the 40/35°C grown plants.
497　　　　　　When analysed in more detail, we observed that heat to induced genes linked to energy
498　dissipation (*aox* and *ucp*) over the first 24 h of 40°C heat exposure (Fig. 1, Table 1). AOX and UCP
499　are involved in the diversion of electrons for formation of proton gradients and subsequent ATP
500　synthesis (Krauss, Zhang, & Lowell, 2005; Vanlerberghe, 2013). Past work has shown that

501   overexpressing *aox* in young rice seedlings imparts a benefit on growth under a *T* of 37°C for eight

502   days, which was attributed to a reduction of excessive proton motive force and reactive oxygen species

503   (Murakami & Toriyama, 2008). Given that AOX and UCP both divert electrons away from ATP

504   synthesis under conditions of high reductant supply, the rapid upregulation of these genes following

505   the initial changes in growth *T* – with rapid stimulation of R_{dark} and presumably greater reduction of

506   ubiquinone pools (UQ) – indicates that there may have been a temporary imbalance between NAD(P)H

507   supply and demand for ATP. The initial increases in *aox* and *ucp* gene expression (Fig. 1) did not

508   translate into increased total AOX and UCP protein abundance (Fig. 5). However, qPCR results

509   indicate upregulation of the *aox1a* isoform, responsive to abiotic stress in Arabidopsis mitochondria

510   (Clifton, Millar, & Whelan, 2006; Shapiguzov et al., 2019), while over the same period RNA-seq

511   analysis indicated a significant decline in a separate *aox1c* isoform. It is possible that the AOX1C

512   isoform is less tolerant of high temperatures and therefore is partially replaced by the AOX1a isoform.

513   In this context it is interesting that in Arabidopsis AOX1a is the major stress-inducible isoform. Since

514   AOX operates as a non-covalently linked dimer (Siedow & Umbach, 2000), the change in the relative

515   expressions of *aox1a* and *1c* isoforms may also indicate a change in the conformation of the AOX

516   dimer, with a different mix of homo- and hetero-dimers in response to heat. This suggests that AOX

517   may have shifted to a more heat-tolerant conformation at 40/35°C, at least when the initial shock was

518   imposed. This is an illustration that enzyme isoforms can be an important part of abiotic stress

519   responses that can be easily overlooked when only considering total protein abundance.

520   The limited gene induction when leaves were transferred from 30 to 25°C (Fig. 3c) suggests

521   that a shift to this colder growth *T* did not significantly perturb metabolic processes in rice leaves,

522   consistent with the limited PE leaf response of R_{dark} or A_n when exposed to the cold (Fig. 6). However,

523   cold-responsive transcriptional regulators and associated changes in metabolism expected from cold

524   exposure (Zhu, Dong, & Zhu, 2007) must have been triggered by the colder *T*s. Regulatory adjustments

525   did indeed occur in ND rather than PE leaves, with R_{dark} at a given *T* being higher in the cold-grown

526   ND leaves (Fig. 7), and homeostasis of A_n being reached in ND leaves when measured at the prevailing

527   growth *T* (Table 3, Fig. 7).

528

529   *The most evident longer-term acclimation response is reduced COX abundance*

530   The clearest biochemical response to increasing growth *T*, both in PE and ND leaves, was a decline in

531   the abundance of COX (Fig. 5). A decline in COX has been reported for rice roots when grown at

532   25°C relative to 15°C (Kurimoto, Millar, Lambers, Day, & Noguchi, 2004). Conversely, COX content

533   increased in *Arabidopsis thaliana* leaves grown at 5°C relative to 21°C (Armstrong et al., 2008). In

534   all these cases, COX protein content and rates of respiration at a common measuring T (including in

535   our study; Fig. 5 and Fig. 7) decreased when plants grew at hotter T, suggesting that thermal

536   acclimation results in changes not only in overall rates of respiration but also in the capacity to produce

537   ATP. The acclimation response was rapid as COX declined in abundance by 24 h after 40°C T transfer

538   in PE leaves (Fig. 5).

539   The decline in COX abundance with hotter growth T is intriguing. If COX activity became

540   rate-limiting, it is likely that more ROS would be produced as the UQ pool would quickly become

541   over-reduced. However, other reports suggest that the UQ redox state is relatively stable, including

542   during changes in T, despite ~~faster~~ higher R_{dark} (Covey-Crump et al., 2007; Wagner & Wagner, 1995).

543   If we assume that UQ redox poise was also stable during the ~~faster~~ greater R_{dark} at the hottest growth

544   T in our experiments, there are two possible explanations. (1) The absolute flux of electrons through

545   COX actually increased despite the decrease in protein abundance. This could be due to COX capacity

546   being far greater than the capacity of the overall mETC. But since increasing Ts stimulates the relative

547   activity of enzymes (Copeland, 2000), it is possible that the smaller amount of COX protein had higher

548   activity. In other words, the plants could make do with less COX at hotter T. (2) Alternatively,

549   activation of AOX at the higher T may have occurred to supplement COX activity thereby preventing

550   overload of the UQ pool. Measuring T-dependent in vivo ^{18}O fluxes through COX and AOX, as well

551   as leaf ATP content is required to determine terminal oxidase activity and ATP synthesis.

552   Understanding what, if any, biological benefit arises from synthesising less COX at warmer growth T

553   is another important consideration. Alternatively, a reduction in COX might be a consequence of heat

554   directly interfering with its synthesis. In support of this, a recent report shows that COX abundance

555   and capacity in Arabidopsis is significantly reduced by knocking out a HSP70 isoform, suggesting that

556   heat in some way interacts with COX formation (Wei et al., 2019).

557

558   *Acclimation of R_{dark} and A_n is asynchronous in rice*

559   The R_{dark}/A_n ratio increased with short-term increases in measuring T (Fig. 8), reflecting the fact that

560   R_{dark} is more temperature dependent than is A_n. R_{dark}/A_n ratios were similar in 25 and 30°C grown leaves,

561   when measured at the prevailing growth T of each treatment (i.e. R_{dark}/A_n was homeostatic). Thus, the

562   acclimation process led to the balance between carbon gain and release being maintained across this

563   moderate range of growth Ts (Fig. 8). Acclimation was not, however, sufficient to maintain

564   homeostasis of R_{dark}/A_n in 40°C grown plants (Fig. 8). Similar results of R_{dark}/A_n ratios in leaves and

565   whole plants remaining relatively stable over moderate but not extremely high T have been reported

566   (Atkin, Scheurwater, & Pons, 2006, 2007; Campbell et al., 2007; Drake et al., 2016; Loveys et al.,

2003). Different to past studies, our findings in rice show that homeostasis of R_{dark}/A_n is largely the result of maintenance of A_n more than through a marked reduction in rates of R_{dark}. Our results categorically show A_n acclimates to a greater extent than R_{dark} in rice, supporting previous studies of rice that collectively point to greater A_n than R_{dark} acclimation capacity (Bahuguna et al., 2017; Glaubitz et al., 2014; Kurimoto, Millar, et al., 2004; Mohammed et al., 2013; Nagai & Makino, 2009; Yamori et al., 2010), and field studies that infer limited rice R_{dark} acclimation capacity (Peng et al., 2004; Welch et al., 2010). However, for many plant functional types, including temperate grasses, the opposite occurs; R_{dark} acclimates to a greater extent than A_n (Campbell et al., 2007; Ow, Griffin, Whitehead, Walcroft, & Turnbull, 2008; Way & Oren, 2010; Way & Sage, 2008; Yamori et al., 2005). In this context, it should be noted that the previous studies are of species from temperate rather than tropical habitats, raising the question of whether, beyond rice, tropical grasses generally have asynchronous acclimation favouring A_n. The homeostasis of A_n and superior LER of hot-grown ND rice leaves was more remarkable when viewed alongside evidence that prolonged exposure to drier air was closing stomata and presenting slight reductions in CO_2 availability, at least in PE leaves (Fig. 6). There is evidence that stomata close following a T-dependent increase in VPD_{Leaf}, with the mechanism yet uncharacterised but likely involving guard cell sensing of water potential below the epidermis (Peak & Mott, 2010; Shope, Peak, & Mott, 2008). It seems that declining VPD_{Leaf} triggers stomatal closure in rice, even with unlimited root water supply.

As noted earlier, in recent years, rice yields have declined in response to increased daily mean Ts, with the decline being more strongly correlated with increasing night rather than day Ts (Peng et al., 2004; Welch et al., 2010). Our finding that A_n is homeostatic across growth T, whereas R_n is not (Table 3) – underpinned by greater acclimation of photosynthesis than respiration – suggests that one reason why yields are declining with increasing night temperatures is because high temperatures stimulate respiratory CO_2 release. This would have a negative effect on daily net carbon gain, and thus the ability to accumulate biomass in the lead up to anthesis.

Potential implications of rice leaf acclimation and starch ~~content~~ concentration on crop yield

We found that soluble sugar ~~contents~~ concentrations of rice leaves were remarkably stable, irrespective of growth T or developmental time at each growth T (Table 2). Maintaining soluble sugar homeostasis is an important physiological requirement for many plant species, achieved through balancing CO_2 uptake and release in source leaves with sugar export to sink tissues (Rolland, Moore, & Sheen, 2002). Homeostasis of sucrose concentrations in rice leaves has been observed even when carbon demand by sink tissues is limited [e.g. reduced partitioning of sugars to grain (Wang et al., 2008)]. In our study, homeostasis of soluble sugar concentrations occurred even at 40°C, where rates of R_{dark} where

601   significantly higher than in plants at the cooler growth Ts. Associated with the maintenance of sugar

602   concentrations was a T-dependent decline in starch ~~content~~concentration, both in PE and ND leaves

603   (Table 2). For PE leaves exposed to 40°C, assimilate supply declined, particularly for 40°C transferred

604   leaves, due to a marked increase in R_{dark} and a decline in A_n (Fig. 6). Starch content also significantly

605   declined with developmental duration under high T, contrary to soluble sugar concentrations (Table

606   2). It seems likely, therefore, that the reason soluble sugars did not significantly decline at warmer T

607   for PE leaves – even though assimilate supply fell – was a greater draw-down in the starch pool to

608   maintain soluble sugar ~~contents~~ concentrations (i.e. a reliance on stored assimilate). Other studies [e.g.

609   on the temperate tree *Populus tremula* (Hüve et al., 2012)] have highlighted the importance of starch

610   degradation in maintaining soluble sugar concentrations, particularly under conditions that stimulate

611   CO_2 release by respiration. Interestingly, in our study, ND leaves exhibited reduced starch ~~content~~

612   concentrations while also maintaining assimilate supply; one explanation for this might be that the

613   decline in starch and maintenance of sugars of ND leaves was linked to the increased leaf elongation

614   rates we observed for 40°C ND leaves (Fig. 8b), with increased growth (i.e. sink demand) necessitating

615   a greater supply of sugars mediated by the starch pool (Stitt & Zeeman, 2012).

616   The decline in starch ~~content~~ concentrations for PE and ND leaves at 40°C (Table 2) has

617   interesting implications for rice development and yield. Starch is stored in the stems in the late

618   vegetative stage of rice, and accounts for a large proportion of the carbon accumulated in seeds, a

619   process that is detrimentally affected by heat stress (Blum, Sinmena, Mayer, Golan, & Shpiler, 1994;

620   Impa et al., 2018; Morita & Nakano, 2011; Yang & Zhang, 2005). Other studies using the IR64 cultivar

621   exposed to hot night temperatures have shown an increase in R_{dark} and associated cost to vegetative

622   growth and starch content of panicles, ultimately reducing yield (Bahuguna et al., 2017; Glaubitz et

623   al., 2014). The reduced storage of starch in leaves with increasing T that we observed at the vegetative

624   stage – assuming it did not reflect diversion of starch to stems – would suggest reduced potential for

625   the storage of starch in stems and a penalty to yield of rice growing in warmer environments. This

626   would be particularly true for rice plants exposed to transient extreme T – such as during heat waves –

627   as we postulate the reduction in starch for PE leaves was due to a reduction in assimilate acquisition

628   due to stimulated R_{dark} and supressed A_n. However, ND leaves did show reduced starch

629   ~~content~~concentration, not as a result of reduced assimilate acquisition, but most likely associated with

630   an increase in growth rates (Table 2; Fig. 8). Thus, it is likely that rice will experience different

631   limitations on yield depending on the duration of thermal changes, with shorter-term exposure to rising

632   T – over a period in which tissue cannot develop anew – likely leading to a greater suppression of yield

633   than leaves developed under the prevailing growth T. Rice may even experience increased yield with

634 sustained mild warming of both night and particularly day *T*. However, yield potential is dependent on

635 whether heat-dependent changes in growth at the vegetative stage of rice positively contributes to yield,

636 which may be true (Glaubitz et al., 2014; Scafaro et al., 2018), and not simply accelerate development

637 and shorten the time to flowering.

638

639 *Conclusions*

640 Overall, the results we present here demonstrate that both leaf respiration and photosynthesis can

641 acclimate in rice but the extent of acclimation is asynchronous and dependent on the timeframe of *T*

642 exposure. Warmer growth *T* of 40°C relative to 25°C will have a greater impact on rice CO_2 flux,

643 metabolic pathways, starch concentrationcontent and ultimately growth. Consequently, rice growing

644 in a warmer climate with more extreme heating events will likely experience *T*-dependent alterations

645 in growth and yield. The duration and intensity of *T* changes, together with complex interactions

646 between assimilate acquisition, storage and utilisation will determine if this warmer environment will

647 be beneficial or detrimental to rice productivity over the coming decades. We suggest that enhancing

648 the acclimation capacity of R_{dark} for rice at warmer growth *T* – potentially through COX, AOX and

649 UCP regulation – could be a key target for improving rice productivity in a warmer world.

650

651 **Acknowledgements**

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658 **References**

659

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Table 1. Differential expression of respiration genes in leaves after exposure to *T* of 40°C relative to 30°C. Differential expression defined as FDR < 0.05, marked as '*'. Electron transport chain (ETC), Pentose Phosphate Pathway (PPP). No TCA cycle genes were differentially expressed.

Pathway	Gene_name	locus	Log2 fold-change	
			2 hours	6 hours
ETC	Complex II (Succinate dehydrogenase)	LOC_Os08g02640	-0.55*	-0.58*
ETC	External NAD(P)H dehydrogenase	LOC_Os06g47000	0.72*	0.44
ETC	Uncoupling protein	LOC_Os11g48040	0.81*	0.46
ETC	Alternative oxidase	LOC_Os02g47200	-0.99*	-1.91*
glycolysis	ATP-dependent phosphofructokinase	LOC_Os01g53680	-0.11	-1.13*
glycolysis	Phosphoglycerate kinase	LOC_Os02g07260	0.78*	0.39
glycolysis	Enolase	LOC_Os10g08550	0.61*	0.34
PPP	Ribulose 5-phosphate 3-epimerase	LOC_Os09g32810	0.53*	0.87*

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Table 2. Leaf mass per unit area (LMA), starch and soluble sugars of pre-existing (PE) leaves transferred from 30/25°C to 25/20°C or 40/35°C for one and seven days, and leaves newly-developed (ND) at the prevailing T. Data represents mean of three or four separate leaves from separate previously unsampled plants ± SE. The F-values and P-values of a two-way ANOVA comparing T, developmental stage (D) and any interaction ($T \times D$) are reported with asterisks indicating significance at $P<0.05$.

		LMA (g m^{-2})	Starch (mg g^{-1} DM)	Soluble sugar (mg g^{-1} DM)
25/20°C	Day 1	20 ± 3	11.3 ± 1.9	13.5 ± 0.2
	Day 7	19 ± 2	5.5 ± 0.3	11.2 ± 0.1
	ND	30 ± 2	14.4 ± 3.0	11.1 ± 0.2
30/25°C	Day 1	19 ± 2	14.9 ± 2.1	13.0 ± 0.5
	Day 7	21 ± 2	4.9 ± 1.0	10.5 ± 0.2
	ND	28 ± 0.4	9.6 ± 1.6	11.0 ± 0.6
40/35°C	Day 1	18 ± 2	8.5 ± 0.4	11.9 ± 0.2
	Day 7	23 ± 2	3.5 ± 0.3	10.9 ± 0.3
	ND	29 ± 1	6.7 ± 0.5	11.6 ± 0.3
$T \times D$		$F=0.7, P=0.6$	$F=1.7, P=0.14$	$F=0.01, P=0.99$
D		$F=28, P<0.001*$	$F=13, P<0.001*$	$F=0.14, P=0.94$
T		$F=0.1, P=0.9$	$F=4.2, P=0.03*$	$F=0.01, P=0.99$

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Table 3. Summary of key photosynthetic and respiratory parameters generated from temperature-response curves. Parameters are: leaf mass per area; the temperature at which R_{dark} and A_n exhibited maximum rates (T_{max} and T_{opt}, respectively); the maximum rates of R_{dark} and A_n reached (R_{max} and A_{opt}, respectively); rates of R_{dark} and A_n at the prevailing growth temperature; and, the temperature at which PSII lost functionality as determined by an increase in basal fluorescence (T_{crit}). Data represents means of three or four separate leaves from separate plants ± SE and statistical data (F-value and P-value) based on one-way ANOVA of temperature treatment effect. Superscript letters show significant differences between the T treatments according to a Tukey test.

	25/20°C	30/25°C	40/30°C	F-value	P-value
LMA (g m^{-2})	33±2	30±2	35±3	1.4	0.31
T_{max} (°C)	51± 1[a]	54 ± 1[a,b]	55± 1[b]	4.7	0.04*
T_{opt} (°C)	29± 1[a]	31± 1[a,b]	33 ± 0.3[b]	6.1	0.04*
R_{max} (µmol g^{-1} DM s^{-1} ×10^{-3})	120 ± 5	117 ± 6	121 ± 2	0.16	0.86
A_{opt} (µmol g^{-1} DM s^{-1})	0.65 ± 0.05	0.67± 0.02	0.69 ± 0.04	0.28	0.76

R_{dark} (µmol g^{-1} DM s^{-1} ×10^{-3})	24±3 [a]	27±3 [a]	57±2 [b]	35	<0.001*
A_n (µmol m^{-2} s^{-1})	0.62 ± 0.04	0.67 ± 0.02	0.65 ± 0.04	0.52	0.62
T_{crit} (°C)	46.0 ± 0.6	46.9 ± 0.9	49.8 ± 1.5	3.726	0.089

Figure Legends

Figure 1. Quantitative PCR analysis of gene expression over the first 168 hours (7 days) after transfer of leaves from 30/25°C to 25/20°C or 40/35°C. Genes analysed were: the respiratory cytochrome *c* complex (*cox*) subunit II, alternative oxidase complex (*aox*) and uncoupling protein (*ucp*); the photosynthetic genes ferredoxin NADH reductase (*fnr*) and phosphoribulose kinase (*prk*); and the sugar metabolism gene sucrose phosphatase synthase (*sps*). Gene expression was revitalised at each time-point to the non-transferred 30/25°C control.

Figure 2. Principal component analysis of normalised RNA-seq expression values for each sample following temperature treatment for (a) 2 hours and (b) 6 hours. Samples are coloured by treatment, day/night temperatures of 30/25°C (control), 40/35°C (hot), and 25/20°C (cold). The y-axis is principle

1003 component 1 (PC1) and the x axis is principle component 2 (PC2); the percent of variation explained

1004 by each axis is indicated. RNA-seq libraries were normalised using *edgeR* ("TMM" method) and *voom*

1005 transformation, scaled by unit variance and clustered using singular value decomposition.

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1007 **Figure 3.** Identification of genes differentially expressed during temperature treatments. (a, b)

1008 Common and time point specific differentially expressed genes under heat treatment (40/35°C). The

1009 overlap between genes differentially expressed at 2 and 6 h under heat treatment for (a) upregulated

1010 genes and (b) downregulated genes. '*' indicates significant overlap p << 0.001, fisher's one-tailed

1011 exact test (hypergeometric). (c) Hierarchal clustering of differentially expressed genes. For each time

1012 point (2, 6 and 24 h) differentially expressed genes were determined for both the hot (40/35°C) and

1013 cold (25/20°C) temperature treatments relative to the 30/25°C control conditions (FDR < 0.05). For

1014 each differentially expressed gene, the relative fold-change under each condition over the time series

1015 is then displayed on a log2 scale: red = upregulated, blue = downregulated.

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1017 **Figure 4.** Gene ontology (GO) term enrichment among genes differentially Upregulated (a) or

1018 downregulated (b) genes after 2 h at 40°C. Ontological annotations downloaded from MSU and

1019 ontology enrichment tests performed with topGO in R using the Fisher standard test (on tailed fisher's

1020 exact test/ hypergeometric test) with post hoc p value correction for multiple testing using the

1021 Benjamini & Hochberg method.

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1023 **Figure 5.** Abundance of mitochondrial electron transport chain proteins and Rubisco determined by

1024 Western blot analysis for rice leaves sampled at different developmental stages of; PE leaves six and

1025 24 h after *T* transfer to 25/20°C or 40/35°C, and leaves newly developed (ND) post *T*-transfer. (a)

1026 Abundance of CYTOCHROME C OXIDASE (COX) subunit II, (b) ALTERNATIVE OXIDASE

1027 (AOX), (c) UNCOUPLING PROTEIN (UCP) and (d) Rubisco large subunit on a leaf area basis with

1028 data normalised by adjusting the largest value in each dataset to 100. Data represent mean ± SE of four

1029 independent western blots, with each blot representing leaf tissue from a separate plant. The *P*-values

1030 of a two-way ANOVA comparing temperature (*T*), developmental stage (*D*) and the interaction

1031 between the two (*T×D*) are reported on each graph. Representative blots are presented in Figure S5.

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1033 **Figure 6.** Rates of dry mass (DM) based dark respiration (R_{dark}; a), net photosynthesis (A_n; b), Relative

1034 humidity (RH; c), vapour pressure deficit between the leaf and surrounding air (VPD_{Leaf}; de), and

1035 stomatal conductance (g_s; ed), and ratio of intercellular to ambient CO_2 concentrations (Ci/Ca; f)

1036   measured at the respective day-time growth temperature of each treatment just prior to (day 0), and 1,

1037   2, 3, 5 and 7-days after transfer of control 30/25°C day/night grown leaves to either 25/20°C, 40/35°C

1038   or maintained at 30/25°C. Values are means of four biological replicates ± SE.

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1040   **Figure 7.** Temperature-response curves (a, b) of dark respiration (R_{dark}) and (c, d) net photosynthesis

1041   (A_n), on a dry mass (DM) basis. Values are absolute (a, c) or normalised to values at 30°C (b, d).

1042   Measurements were made on whole newly-developed (ND) leaves growing for 21 d at day/night

1043   temperatures of 25/20°C, 30/25°C or 40/35°C. Curves fitted to R_{dark} and A_n are quadratic functions.

1044   Calculated acclimation parameters from the curves are presented in Table 3. Rates were recorded every

1045   30 sec as leaves were heated at 1°C per minute. Filled area represent standard error of three to four

1046   biological replicates.

1047

1048   **Figure 8**. The percentage of dark respiration (R_{dark}) relative to light-saturated net assimilation (A_n) (a),

1049   and leaf elongation rates (LER) over a 24 h day/night cycle (b), for ND leaves growing for 21 d at

1050   day/night temperatures of 25/20°C, 30/25°C or 40/35°C. For the R_{dark}/A_n ratio values are calculated

1051   from the absolute means presented in Figure 7. For LER the dark (night) period of the 24 h cycle is

1052   shaded in grey and values are the means ± SE of four plant replicates.

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