Plant, Cell & Environment

Plant, Cell and Environment (2014) **37**, 2542–2552 doi: 10.1111/pce.12323

Original Article

Developmental stage specificity of transcriptional, biochemical and CO_2 efflux responses of leaf dark respiration to growth of *Arabidopsis thaliana* at elevated [CO_2]

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ABSTRACT

Plant respiration responses to elevated growth [CO_2] are key uncertainties in predicting future crop and ecosystem function. In particular, the effects of elevated growth [CO_2] on respiration over leaf development are poorly understood. This study tested the prediction that, due to greater whole plant photoassimilate availability and growth, elevated [CO_2] induces transcriptional reprogramming and a stimulation of nighttime respiration in leaf primordia, expanding leaves and mature leaves of *Arabidopsis thaliana*. In primordia, elevated [CO_2] altered transcript abundance, but not for genes encoding respiratory proteins. In expanding leaves, elevated [CO_2] induced greater glucose content and transcript abundance for some respiratory genes, but did not alter respiratory CO_2 efflux. In mature leaves, elevated [CO_2] led to greater glucose, sucrose and starch content, plus greater transcript abundance for many components of the respiratory pathway, and greater respiratory CO_2 efflux. Therefore, growth at elevated [CO_2] stimulated dark respiration only after leaves transitioned from carbon sinks into carbon sources. This coincided with greater photoassimilate production by mature leaves under elevated [CO_2] and peak respiratory transcriptional responses. It remains to be determined if biochemical and transcriptional responses to elevated [CO_2] in primordial and expanding leaves are essential prerequisites for subsequent alterations of respiratory metabolism in mature leaves.

Key-words: climate change; gene expression; genomics; leaf respiration; metabolism.

INTRODUCTION

Elevated atmospheric [CO_2] generally increases photosynthetic CO_2 uptake in C_3 plants, causing stimulations in plant biomass and yield (Ainsworth & Long 2005). However, consensus has not been reached on how and why dark respiration responds to elevated [CO_2] (Amthor 1991; Poorter *et al.* 1992; Drake *et al.* 1999; Griffin *et al.* 2001; Gifford 2003; Davey *et al.* 2004; Gonzalez-Meler *et al.* 2004). Respiration provides the carbon skeletons and energy needed for tissue growth and maintenance processes during the dark cycle and as a by-product can re-release 30–80% of daily CO_2 uptake (Amthor 2000). Therefore, accurately modelling the impact of growth [CO_2] on plant and ecosystem carbon balance requires a mechanistic understanding of respiration (Atkin *et al.* 2010).

Although many studies have examined the biochemical and molecular basis for photosynthetic responses to elevated [CO_2] (Webber *et al.* 1994; Moore *et al.* 1999; Isopp *et al.* 2000; Rolland *et al.* 2002; Ainsworth & Rogers 2007), much less is known about the mechanisms of respiratory responses to elevated [CO_2]. Increased photosynthetic rates in elevated [CO_2] make greater amounts of carbohydrate substrate available for respiration (Ainsworth & Long 2005). Transcriptional reprogramming of genes coding for respiratory machinery in response to elevated [CO_2] in mature leaves has been observed across three functional groups of C_3 herbaceous species, including a dicot (*Arabidopsis thaliana*; Markelz *et al.* 2014), a monocot (*Oryza sativa*; Fukayama *et al.* 2011) and a legume (*Glycine max*; Ainsworth *et al.* 2006; Leakey *et al.* 2009). This suggests that there is a conserved leaf transcriptional response to stimulation of carbon availability, with significant consequences for whole plant carbon balance. The transcriptional up-regulation of the respiratory pathway in elevated [CO_2] treatments coincided with greater rates of leaf dark respiration in *G. max* (Leakey *et al.* 2009) and *A. thaliana* (Markelz *et al.* 2014). These transcriptional and physiological responses are consistent with the observation of greater mitochondrial numbers per cell in mature leaves of plants grown in elevated [CO_2] across a wide variety of C_3 species (Griffin *et al.* 2001; Wang *et al.* 2004). However, inconsistency in the response of dark respiration to plant growth at elevated [CO_2] has been widely reported and is likely attributable to challenges associated with measuring small respiratory fluxes (Jahnke 2001; Gonzalez-Meler *et al.* 2004), lack of a universal basis on which to meaningfully

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express rates of respiration (e.g. per unit leaf area or mass), variation in plant nitrogen status (Markelz *et al*. 2014), as well as additional biological factors as yet unidentified.

The influence of tissue developmental status on dark respiration responses to elevated [CO_2] has the potential to be significant, but is poorly understood (Hrubec *et al*. 1985; Thomas & Griffin 1994). In *A. thaliana*, transcript abundance for components of the respiratory pathway, along with numbers of mitochondria per mesophyll cell, increases as cells expand and leaves mature (Preuten *et al*. 2010; Skirycz *et al*. 2010; Carrie *et al*. 2013). Within hours of exposure of mature *A. thaliana* leaves to elevated [CO_2], changes in sugar pools and gene expression have been observed in younger expanding leaves that were not exposed to elevated [CO_2] (Coupe *et al*. 2006). This response suggests that systemic signalling of mature tissue carbon status can affect transcriptional reprogramming in developing tissues. Greater numbers of mitochondria and chloroplasts per cell were observed in very early stages of leaf development in wheat grown at elevated [CO_2] (Robertson *et al*. 1995). In *G. max*, significant transcriptional responses of respiration genes to elevated [CO_2] were observed in expanding leaves, but respiration rates were not measured (Ainsworth *et al*. 2006). Evidence exists for leaf tissue age being an important factor for respiratory acclimation to other environmental variables. For example, young *A. thaliana* leaves showed little evidence of temperature acclimation of respiration (Armstrong *et al*. 2006). Yet it remains unclear if transcriptional modifications driven by elevated [CO_2] occurring early in leaf development increase respiratory capacity at that time, or later in leaf development.

A well-described developmental event in leaves is the shift from net import of photoassimilate to net export; often termed the sink to source transition (Ho 1988; Turgeon 1989). Source–sink relationships are important to the overall plant growth responses to elevated [CO_2] (Rogers *et al*. 1996; Isopp *et al*. 2000). The respiratory demands on growing tissues are high because of the demand to provide the carbon skeletons and reducing energy needed for building new tissue and maintaining existing tissue (Lambers *et al*. 2008). Consistent with this high demand for carbon, the leaves of several species have been shown to grow faster in elevated [CO_2] versus ambient [CO_2] (Kriedemann & Wong 1984; Ferris *et al*. 2001). Nevertheless, greater leaf growth in elevated [CO_2] is not always observed (McGrath *et al*. 2010). It has also been suggested that greater respiration in mature leaves at elevated CO_2 might result from greater demand for energy to drive phloem loading (Korner *et al*. 1995; Komor 2000; Leakey *et al*. 2009), an activity found in source leaves that can consume ~30% of nighttime energy supply (Bouma *et al*. 1995).

This study is a time-course experiment that followed a leaf cohort across three key stages of leaf development (primordia, expansion and mature) when *A. thaliana* was grown at ambient [CO_2] and elevated [CO_2]. These time points were specifically chosen as important stages in the transition of leaves from being net carbon importers to net carbon exporters and corresponding to previous leaf developmental studies (Skirycz *et al*. 2010). By integrating physiological, biochemical and transcriptional data, the experiment tested the prediction that elevated [CO_2] would induce transcriptional reprogramming and a stimulation of respiration during all three key stages in leaf development beginning with the primordia and continuing through leaf expansion to maturity.

MATERIALS AND METHODS

Plant growth conditions

A. thaliana (Col-0) seeds were soaked in deionized water for 15 min and planted directly on sterilized LC1 Sunshine Mix (Sun Gro Horticulture, Manitoba, Canada) in 216 cm^3 pots. Planted pots were cold treated in the dark at 4 °C for 48 h prior to being moved into growth chambers. Plants were grown in two identical Conviron (PGR14, Winnipeg, Canada) growth chambers that provided 10/14 h day/night cycle at 21 °C/18 °C, 70% relative humidity (RH) and 300 μmol m^{-2} s^{-1} of photosynthetically active radiation. Each individual pot was covered with an upside-down Petri dish to raise local RH to encourage germination and removed 7 d after germination (DAG). Ambient [CO_2] (370 ppm) or elevated [CO_2] (750 ppm) was achieved using a custom-retrofitted growth chamber CO_2 scrubbing and delivery system described in detail in Markelz *et al*. (2014). Trays of 32 pots were rotated within chambers and between trays every other day. Trays and [CO_2] treatment were rotated between chambers every 5 d to avoid chamber bias. Pots were watered weekly by adding 1 L of 40% Long Ashton solution containing 6 mM NH_4NO_3 (Sigma, St. Louis, MO, USA) to each tray of pots. Preliminary apical meristem dissections coupled with time lapse photography indicated that leaf 10 started to form at least 8 d after CO_2 treatments began and would be the youngest mature leaf 30 DAG.

To avoid measurement artefacts associated with using open-path gas analysers to measure small respiratory fluxes of CO_2 (Jahnke 2001; Gifford 2003), midnight dark respiratory CO_2 efflux was measured using a custom-designed closed gas exchange system described in detail in Markelz *et al*. (2014). In brief, the closed system consisted of a LI-840 infrared gas analyser (Li-Cor, Lincoln, NE, USA) connected to a brushless DC pump (Brailsford, Antrim, NH, USA) and a custom-machined nickel-polytetrafluoroethylene (PTFE) (Teflon™) coated leaf chamber by stainless steel tubing. Leaf temperature was maintained at growth temperature (18 °C) with a circulating water bath connected to a water jacket on the outside of the chamber and leaf temperature recorded with a thermocouple. Individual leaves were placed in the leaf chamber and sealed around the base of the petiole with non-stick putty (Qubit Systems, Ontario, Canada). After 2 min, rates of [CO_2] increase over time were recorded with a CR1000 datalogger (Campbell Scientific, Logan, UT, USA). Measurements were performed at subjective midnight (from 6.5 to 7.5 h after dusk) because preliminary data demonstrated that the middle four hours of the dark period had the greatest and most stable rates of CO_2 efflux. Data were collected when leaf 10 was rapidly expanding 23 and 24 DAG and as leaves transitioned into maturity 29, 30 and 31 DAG (n = 10–12). After respiration measurements, leaves were

excised, photographed for leaf area and oven dried at 70 °C for calculation of specific leaf area (SLA). Leaf disks ($n = 8$) were collected from leaf 10 at 23 DAG (0.264 cm^2) and 30 DAG (1.2 cm^2), were wrapped in aluminium foil, immediately frozen in liquid N and stored at −80 °C until carbohydrates and protein were extracted and analysed as described in detail in Ainsworth & Rogers (2007). Midday rates of photosynthetic [CO_2] assimilation were measured 30 DAG at growth conditions [370 or 750 ppm CO_2, 21 °C and 300 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD)] on leaf 10 using a LI-6400 portable infrared gas exchange system ($n = 8$; Li-Cor). Whole plant aboveground tissue was harvested and oven dried for determination of dry biomass on 23 and 30 DAG.

Gene expression

Leaf 10 was harvested at subjective midnight on 16, 23 and 30 DAG. For the leaf primordia time point (16 DAG), whole rosettes were harvested, flash frozen in liquid N_2, placed in 50 mL conical tubes and stored at −80 °C. RNA *later*-ICE (Invitrogen, Grand Island, NY, USA) stabilizing solution was chilled on dry ice before being added in excess to the conical tubes containing the tissue. The solution was allowed to penetrate the tissue overnight at −20 °C following the manufacturer's protocol. The following day, leaf primordial tissue was dissected using precision forceps under a dissection scope. Twenty individual plants were dissected for each replicate ($n = 4$) and the tissue was stored in 1.5 mL tubes at −80 °C. Tissue was ground in liquid N_2 chilled 1.5 mL tubes using a chilled plastic pestle aided by acid sterilized fine sand in the Spectra Total Plant RNA Isolation Kit (Sigma) extraction buffer. At 23 and 30 DAG, leaf 10 was excised at the base of the blade from two individual plants, pooled together to make a single replicate, immediately flash frozen in liquid N and stored at −80 °C. RNA was isolated from 4 replicates per treatment and developmental stage using the Spectra Total Plant RNA Isolation Kit. Prior to cRNA labelling, total RNA quality was checked for all samples by gel electrophoresis, which confirmed intact ribosomal bands without smearing, indicating no RNA degradation. The cRNA labelling, the subsequent steps leading up to hybridization and the scanning of the Genechip *A. thaliana* ATH1 Genome Array (Affymetrix, Santa Clara, CA, USA) were performed following manufacturer's protocols at the University of Illinois Keck Center for Functional Genomics (http://www.biotech.uiuc.edu/). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE56480.

Statistics

All physiological and biochemical data were analysed using an analysis of variance (ANOVA) (PROC GLM, SAS 9.1; SAS Institute, Inc., Cary, NC, USA) where growth [CO_2] and developmental stage were considered fixed effects and a P-value < 0.05 was used as a significance threshold. Analysis of transcriptomic data was restricted to the 12 570 transcripts that were called present in at least three out of four replicate samples from each treatment and individual chips were normalized to median intensity values following the protocol of Leakey *et al*. (2009). The abundance of individual transcripts were tested by ANOVA treating growth [CO_2] and developmental stages as fixed effects with a 0.05 FDR multi-testing correction (JMP Genomics 5.1; SAS Institute, Inc.). Genes that were significant for the main effects of [CO_2] or interactive effect of [CO_2] × Development were visualized on metabolic pathways using MAPMAN functional gene categories for each leaf developmental stage (Thimm *et al*. 2004; Usadel *et al*. 2005).

RESULTS

Biomass, photosynthesis, leaf biochemistry and respiration

The difference in biomass between [CO_2] treatments became significantly greater ($P < 0.05$) during the period up to 30 DAG (+29% stimulation; Fig. 1a). Greater photosynthetic carbon assimilation rates were observed in leaf 10 at maturity (30 DAG) in elevated [CO_2] compared to ambient [CO_2] (+69%; Fig. 1b). Examining the biochemistry of leaf 10 during expansion (23 DAG) revealed that there was a significant stimulation in leaf glucose content (42%) in the elevated [CO_2] treatment (Fig. 2a,b). However, there was no significant difference between CO_2 treatments in fructose or sucrose content (Fig. 2c–f). And, at subjective midnight, there were no detectable levels of starch in leaf 10 in either [CO_2] treatment (Fig. 2g,h). This contrasts sharply with growth at elevated [CO_2] leading to significantly greater glucose (+107%; Fig. 2a,b), sucrose (+76%; Fig. 2e,f) and starch (+90%; Fig. 2g,h) contents in mature leaf tissue (30 DAG). The greater levels of non-structural carbohydrates at elevated [CO_2] contributed to a significant reduction in SLA in the mature leaves (−10%; Fig. 2i). However, there was no effect of elevated [CO_2] on the soluble protein content of either expanding (23 DAG) or fully expanded (30 DAG) leaves (Fig. 2j). The rates of dark respiration at night varied significantly during the transition of leaf 10 from a carbon sink to a carbon source and did so differently in ambient [CO_2] versus elevated [CO_2]. Rates of respiratory CO_2 efflux gradually declined in both [CO_2] treatments as the rates of leaf expansion slowed, leaves matured and reached their final size (Fig. 3). However, when leaves were rapidly expanding, there was no difference in dark respiration rates between ambient and elevated [CO_2] (23 DAG; Fig. 3). Subsequently, rates of respiration declined more slowly, and to a lesser extent, in elevated [CO_2] compared to ambient [CO_2]. As a result, a significant stimulation of respiration progressively developed under elevated [CO_2] from 24 DAG (+13%) to 30 DAG (+25%; Fig. 3).

Transcript profiles

Of the 12 570 transcripts expressed in the sampled leaf tissue, 11 337 were differentially expressed across leaf development,

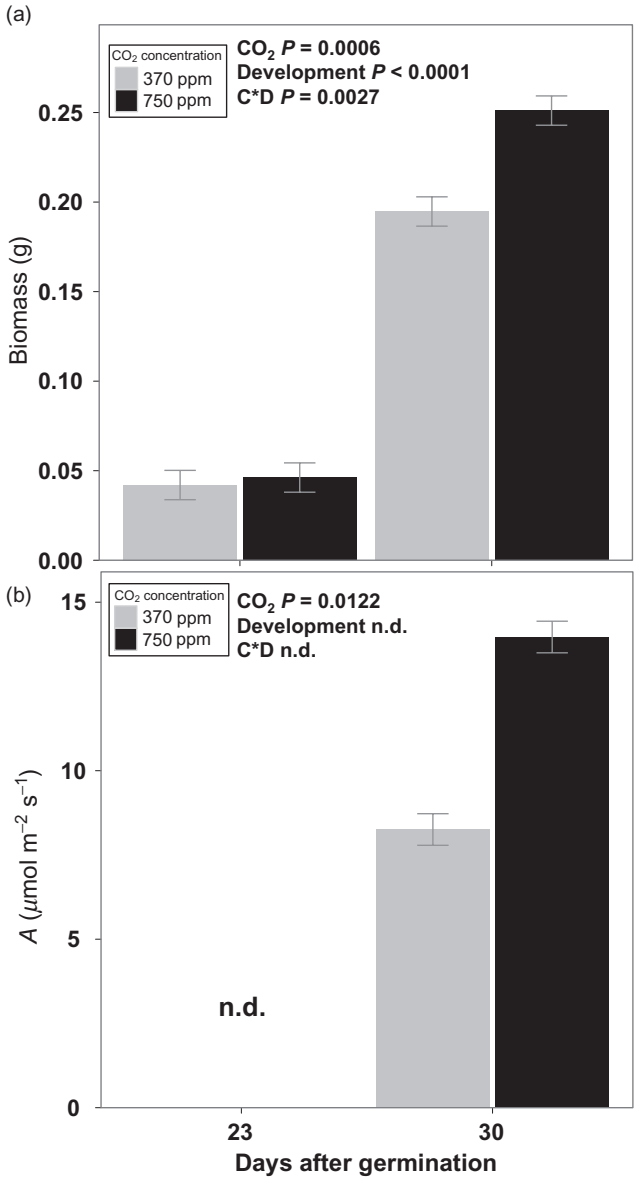


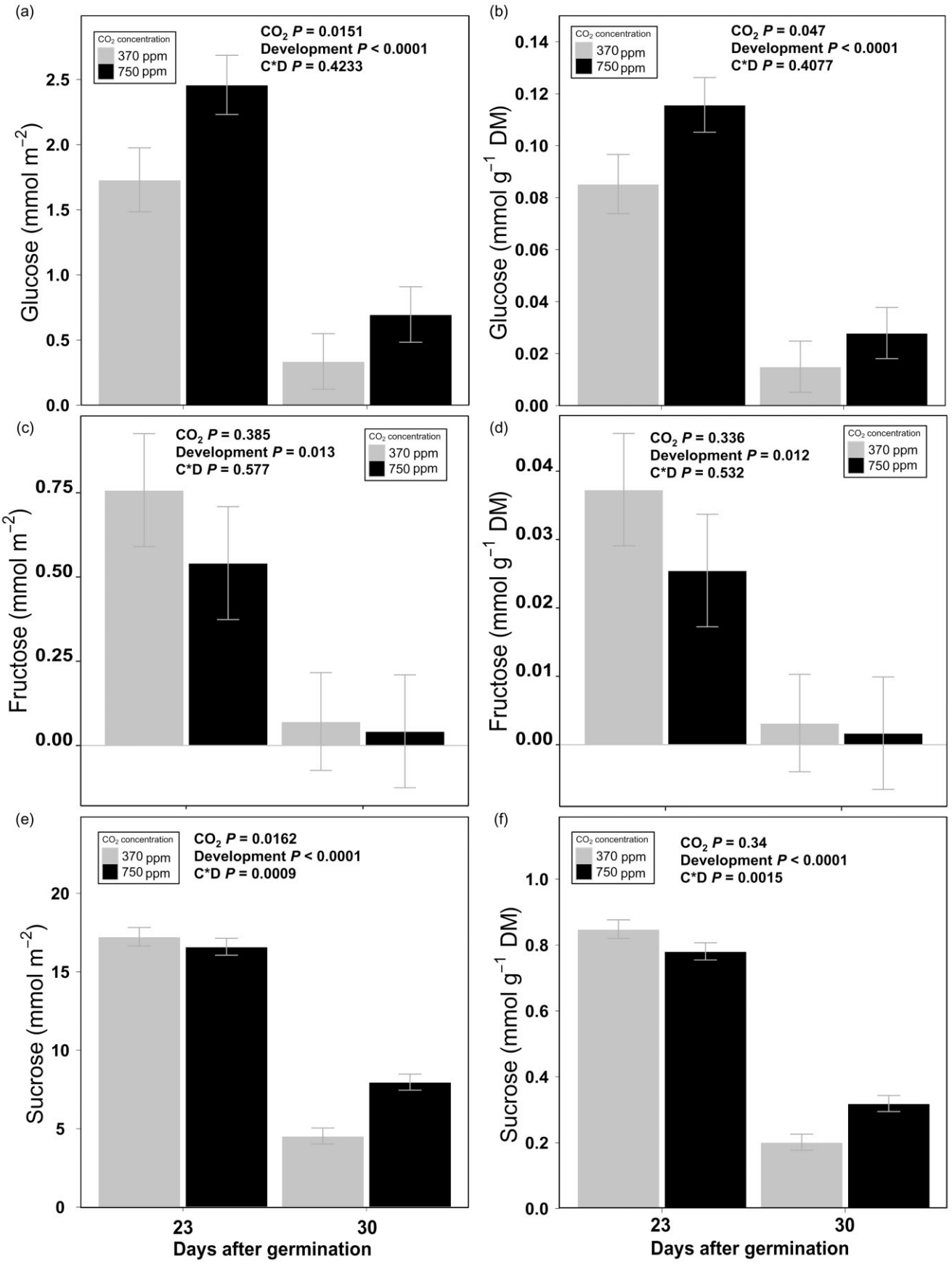
Figure 1. (a) Total aboveground dry biomass and (b) midday net CO_2 assimilation (A) of leaf 10 measured at growth [CO_2], of *Arabidopsis thaliana* grown at ambient [CO_2] (370 ppm) or elevated [CO_2] (750 ppm), at 23 (Expanding) or 30 (Mature) days after germination. A was not determined (n.d.) at 23 d after germination due to the small size of expanding leaves. Values are means (±SD). P-values indicate statistical significance of CO_2, development and $CO_2 \times$ Development interaction effects.

2141 were differentially expressed between ambient and elevated [CO_2] and 1696 had a significant [CO_2] × Developmental stage interaction (Table 1). The number of transcripts for which there was a main effect of [CO_2] treatment or a significant [CO_2] × Development interaction, and for which a pairwise test indicated significant differences in transcript abundance between ambient [CO_2] and elevated [CO_2] within a specific developmental stage, increased over time from 356 in the primordia, to 1359 in expanding leaves, to 1908 in mature leaves (Fig. 4, Supporting Information Table S1). Very few of the [CO_2]-responsive transcripts in the leaf primordia encoded enzymes involved in carbohydrate metabolism, glycolysis, TCA cycle or mitochondrial electron transport (Figs 4 & 5). However, growth at elevated [CO_2] did lead to greater abundance of transcripts encoding respiratory enzymes in expanding leaves (Figs 5 & 6, Supporting Information Table S2). And, this effect was even stronger in mature leaves, where the number of respiration-related transcripts responding to elevated [CO_2] and the average magnitude of the treatment effect on transcript abundance were both greater (Figs 5 & 6, Supporting Information Table S2).

DISCUSSION

This study indicates that the response of respiratory metabolism in *A. thaliana* leaves is strongly dependent upon leaf developmental stage. The progressive strengthening of elevated [CO_2] effects on the transcriptome and carbohydrate pools over the course of leaf development corresponded with the emergence of greater respiratory CO_2 efflux under elevated [CO_2] when leaves were in the later phases of expansion and maturation. The lack of detectable starch pools and relatively high levels of mobile sugars indicate that expanding leaf 10 was primarily a sink tissue 23 DAG (Ho 1988; Turgeon 1989). By contrast, 7 d later, mature leaf 10 had low glucose : sucrose ratios and significant starch storage, indicating that these leaves were significant net sources of carbon for the plant. Taken together, these data suggest that dark respiration rates of leaves are only greater in elevated [CO_2] once they become source leaves. This is consistent with: (1) the hypothesis that greater rates of respiration under elevated [CO_2] in mature leaves are associated with enhanced substrate supply and greater demand for energy to export larger amounts of photoassimilate from source leaves to the rest of the plant (Komor 2000; Leakey *et al*. 2009); and (2) the general model of respiratory capacity being controlled by substrate supply in the long term and energy demand in the short term (Williams & Farrar 1990). Furthermore, these data are also consistent with the finding that leaf sugars, TCA cycle intermediates and dark respiration rates are determined largely by carbon status from the previous light period (Florez-Sarasa *et al*. 2012) and an increase in daytime cyclic TCA flux in elevated [CO_2] (Tcherkez *et al*. 2012). However, disentangling the exact contributions of respiratory energy demand used for growth versus maintenance processes at night is very challenging (Amthor 2000). Further experiments are needed to directly quantify changes in demand for respiratory products at night in elevated [CO_2].

The potential for mechanisms underpinning physiological acclimation processes in plants to be intertwined with tissue developmental programs have long been recognized with respect to elevated [CO_2] and many other abiotic and biotic factors (Chabot & Hicks 1982; Xu *et al*. 1994; Valladares *et al*.


(a)
CO2 concentration
370 ppm
750 ppm
CO2 P = 0.0151
Development P < 0.0001
C*D P = 0.4233
Glucose (mmol m−2)
(b)
CO2 concentration
370 ppm
750 ppm
CO2 P = 0.047
Development P < 0.0001
C*D P = 0.4077
Glucose (mmol g−1 DM)
(c)
CO2 P = 0.385
Development P = 0.013
C*D P = 0.577
CO2 concentration
370 ppm
750 ppm
Fructose (mmol m−2)
(d)
CO2 P = 0.336
Development P = 0.012
C*D P = 0.532
CO2 concentration
370 ppm
750 ppm
Fructose (mmol g−1 DM)
(e)
CO2 concentration
370 ppm
750 ppm
CO2 P = 0.0162
Development P < 0.0001
C*D P = 0.0009
Sucrose (mmol m−2)
(f)
CO2 concentration
370 ppm
750 ppm
CO2 P = 0.34
Development P < 0.0001
C*D P = 0.0015
Sucrose (mmol g−1 DM)
23
30
Days after germination
23
30
Days after germination

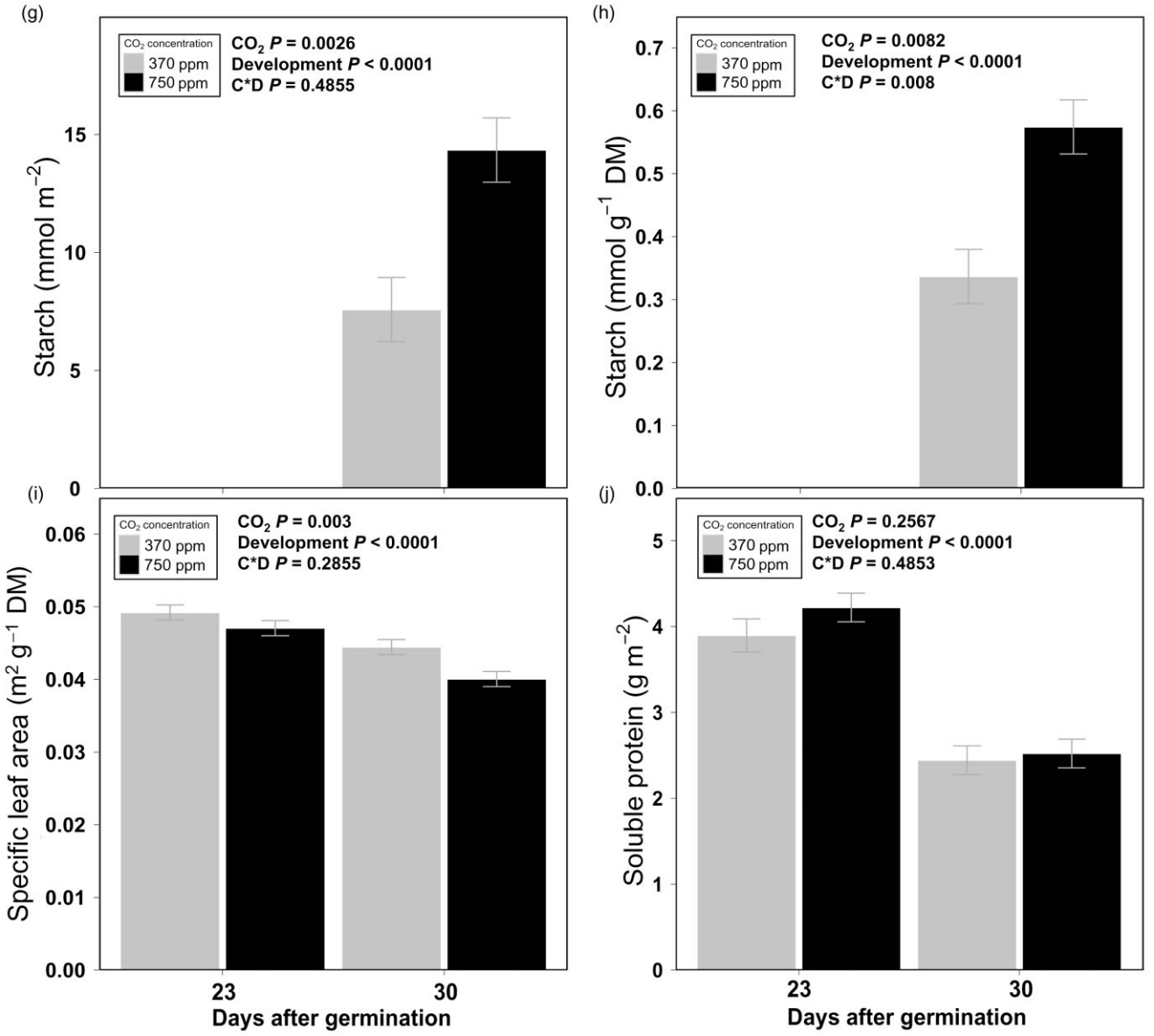


Figure 2. Midnight (a) glucose content per unit leaf area, (b) glucose concentration on a dry mass basis, (c) sucrose content per unit leaf area, (d) sucrose concentration on a dry mass basis, (e) starch content per unit leaf area, (f) starch concentration on a dry mass basis, (g) specific leaf area and (h) leaf soluble protein content per unit leaf area, of leaf 10 of *Arabidopsis thaliana* grown at ambient [CO_2] (370 ppm) or elevated [CO_2] (750 ppm), at 23 (Expanding) or 30 (Mature) days after germination. Values are means (±SD). *P*-values indicate statistical significance of CO_2, development and $CO_2 \times$ Development interaction effects.

2000; Lake *et al*. 2001; Atkin *et al*. 2005). Molecular and biochemical responses to elevated [CO_2] were detected at early stages in leaf development. This did not appear to have an immediate impact on respiratory flux, but might have been involved in pre-conditioning the leaf to later achieve greater respiratory capacity at elevated [CO_2]. The abundance of 356 gene transcripts in primordia was significantly altered by growth at elevated [CO_2]. In expanding leaves that were carbon sinks, growth at elevated [CO_2] resulted in greater leaf glucose content as well as alterations to the transcriptome, which included greater transcript abundance for some genes encoding respiratory machinery. This is consistent with a previous report of systemic signalling from old to young tissue of plants growing at elevated [CO_2] (Coupe *et al*. 2006) as well as growing individual leaflets and individual plants in different CO_2 environments (Sims *et al*. 1998). The expression of genes encoding respiratory machinery increases during leaf expansion and maturation (Preuten *et al*. 2010; Skirycz *et al*. 2010; Carrie *et al*. 2013). This general pattern was observed (Supporting Information Table S1), but growth at elevated [CO_2] resulted in greater abundance of transcripts encoding components of the glycolytic, TCA cycle

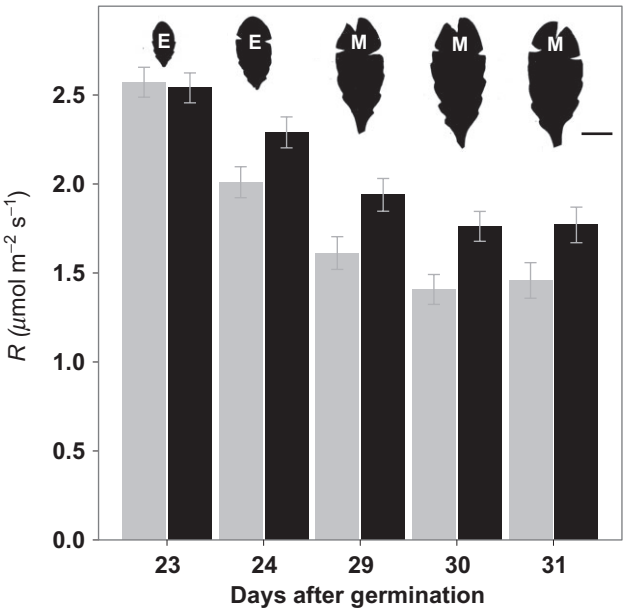


Figure 3. Midnight dark respiration rates (R) of leaf 10 of *Arabidopsis thaliana* grown at ambient [CO_2] (370 ppm) or elevated [CO_2] (750 ppm), at 23, 24 (Expanding) or 29, 30 and 31 (Mature) days after germination. Values are means (±SD). Values are means (±SD). Statistical significance of experimental treatments: CO_2 $P < 0.0001$; Development $P < 0.0001$; $CO_2 \times$ Development $P = 0.1486$. To show relative leaf sizes at each developmental stage, representative leaf areas are shown above each time point. As leaves aged, it was necessary to cut the leaf blades slightly to get them to lie flat for imaging. Scale bar = 1 cm.

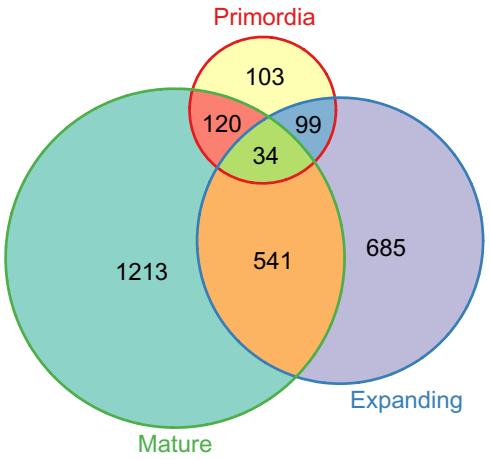


Figure 4. Venn diagram of transcripts significantly responding ($P < 0.05$) to elevated [CO_2] across the three leaf developmental stages (primordial, expanding, mature) of leaf 10 with the size of each circle scaled to the number of genes represented in each category.

and mitochondrial electron transport chain. Many of the transcripts coding for respiratory proteins that were significantly greater in elevated CO_2 in this study, as well as in Markelz *et al.* (2014), were previously identified as having significantly high positive correlations with protein levels (Lee *et al.* 2012). The number of mitochondria per leaf cell is often greater in plants grown at elevated [CO_2] compared with ambient [CO_2] (Griffin *et al.* 2001). Mitochondrial biogenesis is likely to be coordinated in the leaf development programme with gene expression and protein synthesis for components of the TCA cycle and mitochondrial electron transport pathway. However, very little data are currently available on the interplay between transcriptional, post-transcriptional and biochemical regulation of respiration responses to elevated [CO_2] and this should be an interesting future research topic. The regulatory genes identified in this study as responding to elevated [CO_2] (Supporting Information Table S1) provide a list of targets for future studies to explore whether early signalling and transcriptional responses are necessary for greater rates of respiration to be achieved later by mature leaves under elevated [CO_2].

Table 1. The number of transcripts responding significantly ($P < 0.05$) to each of the main effects and/or interactions in the ANOVA model of the 12 570 that were represented in at least 3 biologically replicated microarray chips

Factor in ANOVA model	No. of significant transcripts
Developmental time point	11 337
CO_2 concentration	2 141
$CO_2 \times$ Development interaction	1 696

There has been no consensus for over 20 years on whether respiration increases, decreases or does not change in plants grown at elevated [CO_2] (Amthor 1991; Poorter *et al.* 1992; Drake *et al.* 1999; Griffin *et al.* 2001; Gifford 2003; Davey *et al.* 2004; Gonzalez-Meler *et al.* 2004). Uncertainty in the representation and parameterization of respiratory processes in ecosystem models has been identified as a key source of error when predicting net primary productivity (Wang *et al.* 2012; LeBauer *et al.* 2013). At smaller scales, mathematical representations of source–sink relationships were necessary to accurately model *A. thaliana* rosette growth in elevated CO_2 (Rasse & Tocquin 2006). Variation in the response of respiratory CO_2 efflux to elevated [CO_2] across different leaf developmental stages, in addition to different rates of nitrogen supply (Markelz *et al.* 2014), may partially explain the inconsistent treatment effects previously reported. In addition, evidence is emerging from mature leaves of soybean, rice and *A. thaliana* that transcriptional reprogramming of respiration to support greater rates of dark respiration at night under elevated [CO_2] may be a mechanism conserved across the major functional groups of C_3 herbaceous plants (Ainsworth *et al.* 2006; Leakey *et al.* 2009; Fukayama *et al.* 2011; Markelz *et al.* 2014). If this holds true, it opens up the possibility of identifying the mechanisms of response that need to be incorporated into models of plant and ecosystem functional responses to global environmental change in both agricultural and natural systems (Atkin *et al.* 2010).

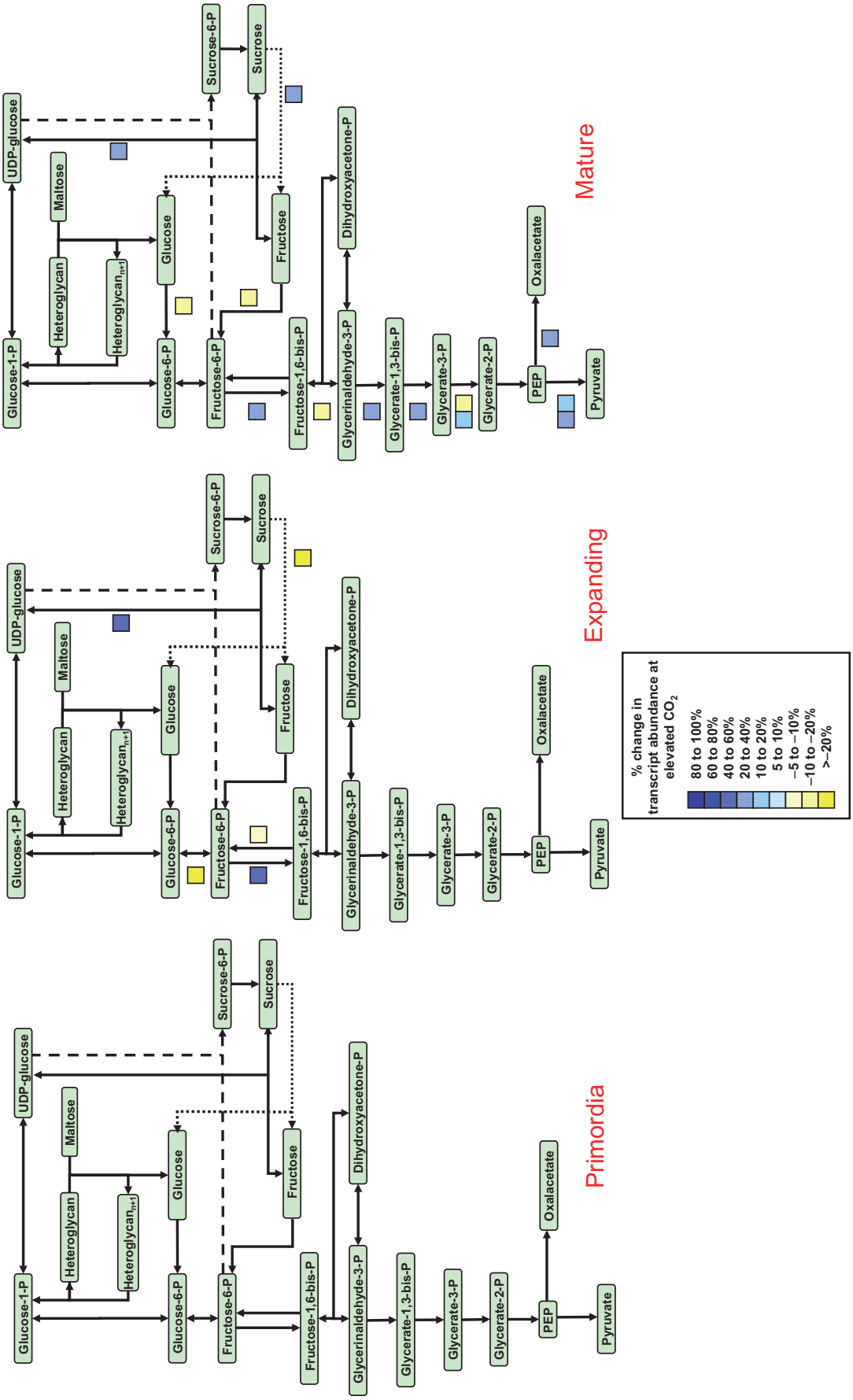


Figure 5. A graphical representation of the abundance at midnight of transcripts encoding components of sugar metabolism and glycolysis pathways in leaf 10 of *Arabidopsis thaliana* grown at ambient [CO_2] (370 ppm) or elevated [CO_2] (750 ppm), at 16 (Primordia), 23 (Expanding) and 30 (Mature) days after germination. Green boxes represent metabolites. Arrows represent reactions. Each blue and yellow box represents the average treatment response (%; see colour scale in inset) of a unique transcript that responded significantly ($P < 0.05$) to elevated [CO_2] in a given leaf developmental stage (details of transcriptional response and gene annotations in Supporting Information Tables S1 & S2).

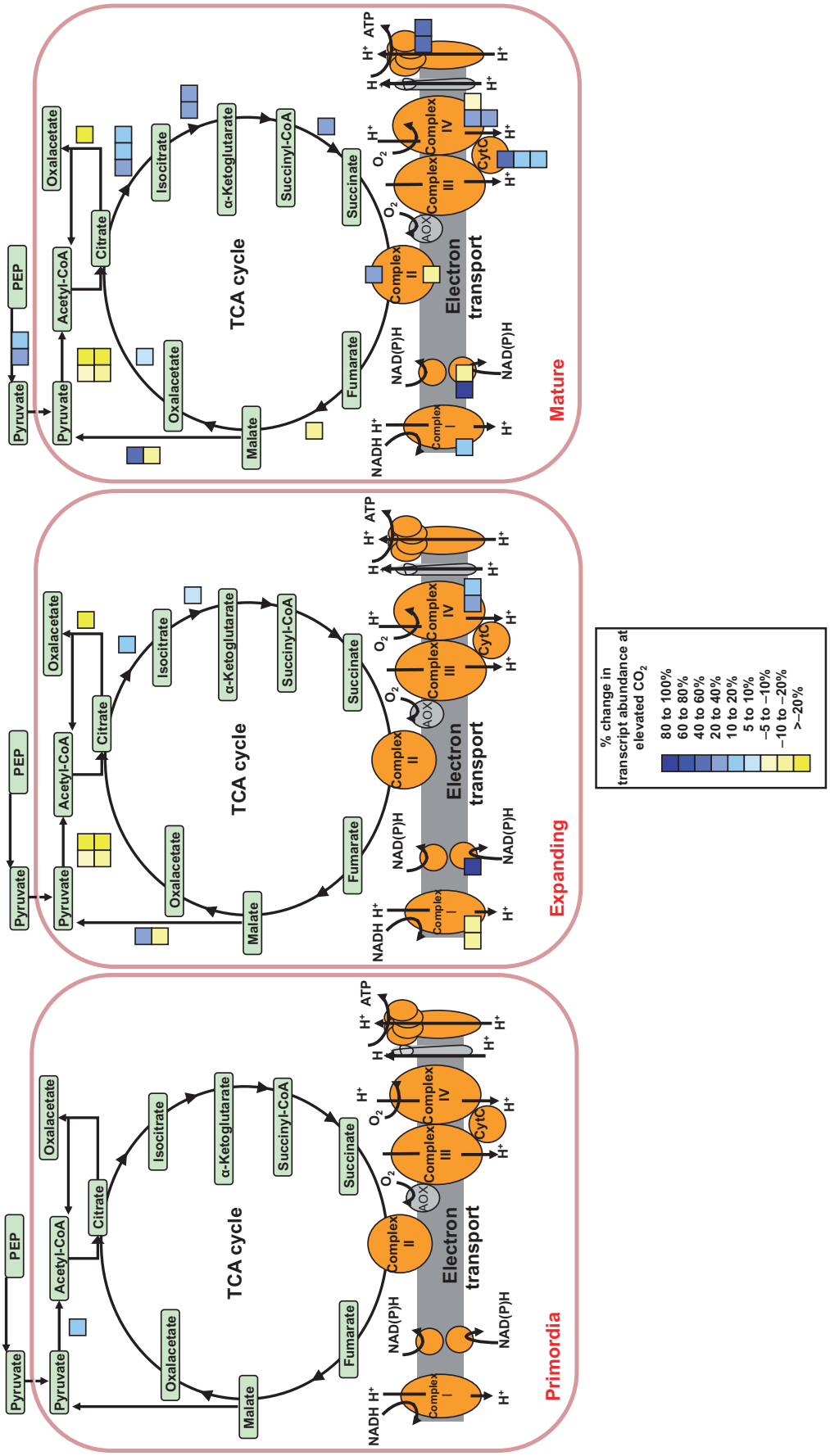


Figure 6. A graphical representation of the abundance at midnight of transcripts encoding components of carbon metabolism pathways in mitochondria in leaf 10 of *Arabidopsis thaliana* grown at ambient [CO_2] (370 ppm) or elevated [CO_2] (750 ppm), at 16 (Primordia), 23 (Expanding) and 30 (Mature) days after germination. Green boxes represent metabolites. Arrows represent reactions. Each blue and yellow box represents the average treatment response (%; see colour scale in insert) of a unique transcript that responded significantly ($P < 0.05$) to elevated [CO_2] in a given leaf developmental stage (details of transcriptional response and gene annotations in Supporting Information Tables S1 & S2).

CONCLUSIONS

This study revealed that the stimulation of leaf respiratory CO_2 efflux by elevated [CO_2] occurs as leaves transition from rapidly expanding sink tissues to mature source tissues in *A. thaliana* leaves. The transcriptional reprogramming of the respiratory machinery in response to elevated [CO_2] begins in expanding tissue, but is most apparent in mature leaves. The transcriptional reprogramming of respiration in expanding tissue occurred prior to treatment effects on rates of respiratory CO_2 efflux, revealing the possibility that additional synthesis of respiratory machinery might be a precursor to greater flux under elevated [CO_2]. These findings have implications for modelling of future plant function and identifying targets for further investigation of the molecular and biochemical mechanisms regulating metabolism in response to global environmental change.

ACKNOWLEDGMENTS

We would like to thank Craig Yendrick, Bob Koester, Kannan Puthuval, Sharon Gray, Tim Mies and Jesse McGrath for helping with sample collection and growth chamber operation. We thank Chris Black, Paul Nabity, Justin McGrath and Lisa Ainsworth for helpful discussion on the manuscript. R.J.C.M. was supported through a NSF Graduate Research Fellowship.

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Received 10 December 2013; received in revised form 7 March 2014; accepted for publication 10 March 2014

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. List of transcripts identified by ANOVA that vary significantly with either CO_2. Developmental stage or $CO_2 \times$ Developmental stage interaction (FDR corrected $P < 0.05$). Probe set IDs, AT locus IDs, functional description, *P*-values, percentage change in elevated [CO_2] versus ambient [CO_2] at primordia, expanding, or mature time points, MAPMAN bin(s) (some transcripts map to multiple bins).

Table S2. List of transcripts significant for the main effects of CO_2 and/or CO_2 by development interaction displayed in Figs 5 and 6. Transcripts are only displayed if the pairwise comparisons at each developmental stage was (FDR corrected $P < 0.05$). Also included are the AT numbers, MAPMAN bin numbers and functional descriptions of each of the genes.