Elevated CO2 impacts on the transcriptome, carbohydrate pools and respiratory flux of Arabidopsis leaves

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### Summary

* Plant respiratory responses to elevated [CO2] are a key uncertainty in future crop productivity and ecosystem function. Transcriptional reprograming, greater mitochondrial numbers and greater respiratory flux have been observed in leaves of diverse C3 species grown at elevated [CO2]. However, the impact of elevated [CO2] on respiration and gene expression over the course of leaf development is unknown.
* This study tested the hypothesis that elevated [CO2] induces transcriptional reprogramming and stimulation of nighttime respiratory flux throughout the three major phases of leaf development: (1) primordia; (2) expanding; and (3) mature.
* Growth at elevated [CO2] caused transcriptional reprogramming of respiration beginning in expanding leaves, but not in leaf primordial, that coincided with increases in leaf glucose concentration in elevated [CO2]. These effects occurred prior in leaf development to starch accumulation or treatment effects on sucrose. However, the stimulation of respiratory flux only occurred after leaves transitioned from expanding (sink) tissues without starch storage to mature (source) tissues with greater concentrations of starch and sucrose in elevated [CO2].
* Stimulation of nighttime respiration by elevated [CO2] is associated with biochemical and transcriptional responses during leaf expansion, but greater respiratory flux occurs only after leaves mature into source tissue with greater pools of locally produced photoassimilate.

**Key Words:** Elevated CO2, leaf respiration, genomics, metabolism, leaf development

### Introduction

Elevated atmospheric [CO2] generally increases photosynthetic CO2 uptake in C3 plants causing stimulations in plant biomass and yield (Ainsworth and Long 2005). However, understanding and modeling the impact of growth [CO2] on plant and ecosystem carbon balance requires a mechanistic understanding of respiration because it provides the carbon skeletons and energy needed for growth and maintenance during the dark cycle, and at the same time releases CO2 (Atkin *et al.* 2010). While many studies have examined the physiological and molecular photosynthetic responses to elevated [CO2] (Moore *et al.* 1999), far fewer have examined the respiratory response in the post-genomics era (Ainsworth *et al.* 2006; Leakey *et al.* 2009; Fukayama *et al.* 2011). Transcriptional reprogramming of genes coding for respiratory machinery in response to elevated [CO2] in mature leaves has been observed across three functional groups of C3 herbaceous species including a dicot (Arabidopsis; Markelz *et al.* in review), a monocot (rice; Fukayama *et al.* 2011) and a legume (soybean; Ainsworth *et al.* 2006; Leakey *et al.* 2009). This suggests that there is a conserved transcriptional response to stimulation of carbon availability, with significant consequences for plant C balance. The transcriptional up-regulation of the respiratory pathway in elevated [CO2] treatments coincided with greater rates of leaf dark respiration in soybean (Leakey *et al.* 2009) and Arabidopsis (Markelz *et al.* in review). These transcriptional and physiological responses are consistent with the observation of greater mitochondrial numbers per cell in mature leaves of plants grown in elevated [CO2] across a large number of C3 species (Griffin *et al.* 2001).

In Arabidopsis, transcript abundance for components of the respiratory pathway, along with numbers of mitochondria per mesophyll cell, increase as cells expand and leaves mature (Skirycz *et al.* 2010; Preutin *et al.* 2010; Carrie *et al.* 2012). Yet it remains unclear if transcriptional modifications driven by elevated [CO2] occur early in leaf development in order to increase overall energy metabolism observed in mature leaves and how these modifications are coordinated with the general leaf developmental program. This possibility is suggested by the observation that within hours of mature Arabidopsis leaves being exposed to elevated [CO2], changes in gene expression and sugar pools have been observed in younger expanding leaves which were not exposed to the elevated [CO2], suggesting systemic signaling of whole plant carbon status can occur (Coupe *et al.* 2006). Additionally, greater numbers of mitochondria and chloroplasts per cell were observed in very early stages of leaf development in wheat grown at elevated [CO2] (Robertson and Leech 1995; Robertson *et al.* 1995). However, the consequences of these cellular responses for respiratory fluxes in leaves at early developmental stages, or subsequently upon maturation, are not known. Significant evidence exists for Arabidopsis leaf tissue age being an important factor for respiratory acclimation to other environmental variables such as temperature where young leaves showed no evidence for temperature acclimation (Armstrong *et al.* 2006).

A well-described metabolic transition in developing leaves is from sink to source where leaves shift from being net importers of photoassimilate to net exporters (Ho 1988; Turgeon 1989). Source-sink relationships have been shown to be very important to the overall plant growth response to elevated [CO2] (Rogers *et al.* 1996; Isopp *et al.* 2000;). The respiratory demands on growing tissue are high, given the demand to provide the carbon skeletons and reducing energy needed for both building and maintaining tissue (Lambers *et al.* 2008). However, it has also been speculated that greater respiration in mature leaves at elevated CO2 results from stimulated demand for energy to drive phloem loading, an activity only found in source leaves. This study is a time-course experiment that followed a leaf cohort across three key stages of leaf development (primordia, expansion, and mature) when Arabidopsis was grown at ambient and elevated [CO2]. These time points were specifically chosen as both key stages in leaf development and as important stages as leaves transition from net energy importers to net energy exporters. By integrating physiological, biochemical, and transcriptional data we tested the hypothesis that elevated [CO2] 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. The rich transcriptional data generated in this study allowed us to ask additional questions regarding whether or not fundamental shifts in gene expression occur in the leaf developmental program in elevated [CO2].

### Materials and Methods

### Plant Growth Conditions

*Arabidopsis thaliana* (Col) seeds were soaked in DI water for 15 minutes and planted directly on sterilized LC1 Sunshine Mix (Sun Gro Horticulture, Canada) in 216 cm3 pots. Planted pots were cold treated at 4 oC for 48 hours prior to being moved into growth chambers. Plants were grown in two identical Conviron (PGR14, Winnepeg, Canada) growth chambers that provided 10/14 hour day/night cycle at 21 °C/18 °C, 70% 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 relative humidity and removed 7 days after germination, at which CO2 fumigation was started. [CO2] concentration was maintained at ambient (370 ppm) or elevated (750 ppm) using a custom retrofitted chamber CO2 scrubbing and delivery system described in detail in Markelz *et al.* (in-review). Trays of 32 pots were rotated within chambers and between trays every other day. Trays and [CO2] treatment were rotated between chambers every five days to avoid chamber bias. Pots were watered weekly by adding 1 L of 40% Long Ashton solution containing 6 mM NH4NO3 to each tray of pots. Preliminary time-lapse photography and apical meristem dissection indicated that leaf 10 started to form at least 8 days after CO2 treatments began and would be the youngest mature leaf 30 DAG.

### Physiology, Biochemistry, Specific Leaf Area, Biomass

In order to avoid significant measurement artifacts identified when using open-path gas analyzers to measure small respiratory fluxes of CO2 (Jahnke 2001; Gifford 2003) midnight dark respiratory CO2 efflux was measured using a custom designed closed gas exchange system (n=8) described in detail in Markelz *et al.* (in review). In brief, the closed system consisted was built around a LI-840 infrared gas analyzer (LICOR, Lincoln Nebraska, USA) connected to a brushless DC pump (Brailsford, NH) and a custom machined nickel-polytetraflouroethylene (PTFE) (TeflonTM) coating with stainless steel tubing. Leaf temperature was maintained at 18 °C with a circulating water bath connected to the a water jacket on the outside of the chamber and leaf temperature recorded with an internal chamber thermocouple. Leaves were placed in the leaf chamber and sealed around the base of the petiole with non-stick putty. After 2 minutes, CO2 increase over time was recorded with a CR1000 datalogger (Campbell Scientific; Logan UT, USA). Rates were measured at subjective midnight as preliminary data collected demonstrated that the middle 4 hours of the dark period to had stable rates. Respiration rates were determined on leaf 10 when leaves were 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 70oC for calculation of Specific Leaf Area (SLA). Leaf disks (n=8) were collected from leaf 10 at 23 DAG (0.264 cm2) and 30 DAG (1.2 cm2), were wrapped in aluminum foil, immediately frozen in liquid N, and stored at -80 oC until carbohydrates and protein were extracted and analyzed as described in Ainsworth *et al.* (2007). Whole aboveground tissue was harvested and oven dried for determination of dry biomass. *In-situ* midday rates of photosynthetic [CO2] assimilation were measured 30 DAG at growth CO2 concentration, at 21 °C and 300 μmol m-2/s-1 PPFD on leaf 10 using an open-path LI-6400 portable infrared gas analyzer (n=8; LICOR, NE, USA).

### 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, placed in 50 mL conical tubes and stored at -80 oC. RNA *later*-ICE (Invitrogen, NY, USA) stabilizing solution was chilled on dry ice before being added in excess to the conical tubes containing the tissue. The tissue was then allowed to be penetrated by the solution overnight at -20 oC following manufactures protocols. The following day, leaf primordial tissue was dissected using precision forceps under a dissection scope. Twenty individual plants were dissected for each replicate and the tissue was stored in 1.5 mL tubes at -80 oC. Tissue was ground in liquid N 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, MO USA) extraction buffer. At 23 and 30 DAG, leaf 10 was excised at the base of the blade from eight individual plants per replicate, immediately flash frozen in liquid N and stored at -80 oC until RNA was isolated using the Spectra Kit. Prior to cRNA labeling, total RNA quality was checked for all samples by gel electrophoresis, which confirmed intact ribosomal bands without smearing. The cRNA labeling, the subsequent steps leading up to hybridization and the scanning of the Genechip Arabidopsis ATH1 Genome Array (Affymetrix, Santa Clara, CA, USA) were performed following manufacturer’s protocols at the University of Illinois Keck Center for Functional Genomics ([www.biotech.uiuc.edu/centers/Keck](http://www.biotech.uiuc.edu/centers/Keck)).

### Statistics

All physiological and biochemical data were analyzed using an ANOVA (PROC GLM, SAS 9.1; SAS Institute, Inc., Cary, NC, USA) where [CO2] and developmental stage were considered fixed effects and a p-value <0.05 was used as a significance threshold. For microarray analysis, individual transcripts were not tested if they were not called present in at least three replicate chips for each [CO2] by development treatment combination. The transcriptional data set (12, 570 transcripts following replicate filtering), were analyzed using an ANOVA following Leakey *et al.* (2009) with a 0.05 FDR multi-testing correction (JMP Genomics 5.1; SAS, Cary, NC, USA) where [CO2] and developmental stage were treated as fixed effects. Genes that were significant for the main effects of [CO2] within each leaf developmental stage were visualized on metabolic pathways using MAPMAN functional gene categories (Thimm *et al.* 2004, Usadel *et al.* 2005). In order to examine patterns in gene expression across the developmental time points to compare with other developmental data sets (e.g. Skirycz *et al.* 2010), a K-means clustering analysis was performed on the 11, 337 significant transcripts from the ANOVA model fit into seven major expression pattern clusters with row scaling so that relative expression differences could be viewed on the same scale for all clusters (JMP Genomics 5.1; SAS, Cary, NC, USA).

### Results

### Biomass, Photosynthesis, Leaf Biochemistry and Respiration

23 days after germination (DAG), elevated [CO2] significantly stimulated total plant biomass (+10%; Figure 1a). The difference in biomass between treatments became greater when the rosettes moved into the exponential growth phase at 30 DAG (+29% stimulation; Figure 1a). The stimulation in final biomass was driven by greater photosynthetic carbon assimilation in mature leaves, such as leaf 10, 30 DAG (+69%, Figure 1b). Examining expanding leaf 10 biochemistry at 23 DAG revealed that there was a significant stimulation in leaf glucose content (42%) in the elevated [CO2] treatment, but there was no significant difference between CO2 treatments in sucrose content and there were no detectable levels of starch in leaf 10 in either [CO2] treatment (Figure 2). This expanding leaf response is in sharp contrast to the stimulation of photosynthesis in elevated [CO2] in mature leaves lead to significantly greater starch (+90%), glucose (+107%), and sucrose (+76%) content at midnight at the mature leaf time-point (30 DAG; Figure 2). These greater levels of non-structural carbohydrates contributed to the significant 10% reduction in specific leaf area in the mature leaves (Figure 2g). Leaf 10 dark respiration rates varied significantly across leaf development where rates were greater for rapidly expanding tissue (23 DAG) with a gradual decline as the leaves slowed expansive growth and transitioned into maturity (29, 30, and 31 DAG; Figure 3). When leaves were rapidly expanding there was no difference in dark respiration rates between ambient and elevated [CO2] (23 DAG; Figure 3), but there was a significant stimulation of respiration in the elevated [CO2] treatment from 24 DAG (+13%) onwards to 29, 30, and 31 DAG (+20-25%; Figure 3).

### Transcript Profiles

Of the 12,570 transcripts that were present in at least three replicate samples from every [CO2] by developmental stage treatment combination, 11,337 were differentially expressed across the three leaf developmental time points (p<0.05), 2,141 were differentially expressed between ambient and elevated [CO2], and 1,696 had a significant [CO2] by developmental stage interaction (Table 1). Examining the three specific developmental stages, the number of transcripts significantly responding to elevated [CO2] increased from 162 in the primordia, to 854 in expanding leaves, to 1384 in mature leaves (Figure 6). A similar pattern was apparent for the effect of elevated [CO2] on transcript abundance for genes encoding components of glycolysis, the TCA cycle and the mitochondrial electron transport chain gradually increased in number and difference between [CO2] treatments as leaves developed (Figures 4, 5, Table S1). Following up on this observation, K-means clustering was performed to identify broad patterns in gene expression across leaf development and the two [CO2] treatments. Simplified visual representations of the seven K-means clusters are shown in Figure 7 with the clusters containing the greatest number of transcripts were clusters 4 (4650 transcripts) and 7 (4227 transcripts) representing 77% of all the significant transcripts in these two clusters (Figure 7, Table S2). Cluster 4 contained transcripts that had relatively low expression during the primordia timepoint, but increased during the expanding and mature timepoint. Transcripts in Cluster 7, on the other hand, had the opposite response with relatively greater expression in the primordia and decreasing in the expanding and mature timepoints. A great majority (88%) of transcripts coding for proteins involved in photosynthesis belong to Cluster 4 with relatively high expression during the expanding and mature timepoint (Figure 7, Table S3). Transcripts that were significant for the main effect of [CO2] and the interaction between [CO2] and developmental stage were represented in each of the 7 clusters, but the relative distribution of the significant transcripts between clusters was skewed (Figure 7) with the greatest number of transcripts responding to CO2 being part of Cluster 4 (Figure 7).

### Discussion

In mature leaf tissue, such as leaf 10 at 30 DAG, the significant stimulation of photosynthetic [CO2] assimilation leads to significant increases in starch, glucose and sucrose concentrations at night, a response that is well documented (Ainsworth and Long 2005; Leakey *et al.* 2009b). The constant stimulation of photosynthetic carbon gain in mature tissue averaged across the rosette in elevated [CO2] is likely the main upstream driver behind the increase in total plant biomass observed at 23 and 30 DAG. Indeed, the increase in respiratory substrate availability in elevated [CO2] led to significant increases in mature leaf dark respiration at 30 and 31 DAG. The stimulation of photosynthetic CO2 assimilation, carbohydrate pools, respiratory flux, and biomass in elevated [CO2] mirrors previous data sets collected in soybean (Ainsworth *et al.* 2006, Leakey *et al.* 2009a) and rice (Fukayama *et al.* 2011). However, the hypothesis that transcriptional reprogramming of respiration and greater respiration rates would occur in the elevated [CO2] treatment during the primordia, expanding and mature time-points was not fully supported.

Respiration rates are generally greater in expanding tissues because demands for energy and carbon skeletons are needed for both growth and maintenance processes instead of just maintenance of mature tissues (Lambers *et al.* 2008). Overall, leaves developing in ambient and elevated CO2 followed this pattern. While respiration rates were significantly greater in the rapidly expanding tissues at 23 DAG compared to mature tissues at 30 DAG, there were no significant differences between ambient and elevated [CO2] during the expanding time point (Figure 3). At the expanding leaf time point 23 DAG there were no detectable levels of starch in either [CO2] treatment. The lack of detectable starch pools and relatively high levels of mobile sugars indicate that expanding leaf 10 was a primarily a sink tissue 23 DAG (Ho 1988; Turgeon 1989). Coinciding with leaf 10 transitioning from expanding sink tissues to mature source tissues was an increasingly significant stimulation in leaf dark respiration in elevated [CO2] (Figure 3).This finding adds significant mechanistic understanding for the role of respiration across leaf developmental gradients and compliments the findings of the importance of whole plant source-sink relationships to accurately model Arabidopsis rosette growth in elevated CO2 (Rasse and Tocquin, 2006).

Similar to the leaf level response, significant differences between ambient and elevated [CO2] for transcripts encoding components of the respiratory machinery were not detected in the primordia and increased both in magnitude and number of differentially expressed transcripts as leaves expanded and matured (Figures 4, 5, 6). Changes in transcript abundance for respiratory components prior to detectable differences in leaf level respiration coupled with the observation of greater numbers of mitochondria per cell across a large number of species grown in elevated CO2 (Griffin *et al.* 2001) suggests that leaves developing in elevated CO2 are being primed to have altered metabolic rates by building more respiratory machinery prior to becoming source tissues. These observations are consistent with what has been observed in expanding versus mature soybean leaves grown at ambient and elevated [CO2] where the mature tissue has a greater overall transcriptional response of metabolism to elevated [CO2] (Ainsworth *et al.* 2006).

Protein abundance for mitochondrial proteins can be highly correlated with transcript abundance across multiple tissue types in Arabidopsis (Lee *et al.* 2012). For example, transcripts that had significantly greater abundance in elevated [CO2] in mature tissue like aconitate hydratase 2 (ACO2), Succinate dehydrogenase 1 (SDH1-1), and succinyl-CoA ligase, were shown by Lee *et al.* (2012) to have significantly high Pearson correlations (r > 80) with protein abundance. The idea of transcriptional priming in elevated [CO2] has additional support from the literature where gene expression changes in expanding tissue were apparent two hours after an elevated [CO2] treatment being applied to mature tissues only and carbohydrate status was implicated as part of the systemic signal between mature and developing leaves (Coupe *et al.* 2006). Indeed, the negative feedback on photosynthetic gene expression when excess glucose accumulates in plant cells through a hexokinase-mediated pathway has been demonstrated to be the mechanism behind photosynthetic acclimation to elevated [CO2] (Moore *et al.* 1999). More recent evidence of shoot-derived glucose mediating target-of-rapamycin (TOR) signaling to control meristem identity in root tissue through glycolytic and mitochondrial pathways demonstrates that glucose has a key role as both a substrate for metabolism and contributing to the degree of transcriptional reprogramming through alterations in carbon supply (Xiong *et al.* 2013). Thus it is likely that the increasing transcriptional response observed because the relative differences in glucose concentration between ambient and elevated [CO2] increased from 23 DAG (+36%) to 30 DAG (+87%; Figure 2).

Further support for the sink to source transition being important for the overall CO2 response comes from patterns of gene expression across the entire transcriptional data set. 62% of all the genes encoding components of photosynthesis, major and minor carbohydrate metabolism, glycolysis, the TCA cycle and mitochondrial electron transport chain are part of Cluster 4 which shows a large increase between the leaf primordia and expanding/mature timepoints in relative expression (Figure 7, Table S3). What is most interesting about these patterns is that 48% of all transcripts significant for a main effect of CO2 were also part of Cluster 4 (Figure 7) suggesting that this pattern of gene expression across leaf development is important for the overall CO2 response to carbon metabolism. Furthermore, SUC2 (At1g22710) the gene encoding the protein controlling active sucrose loading into the phloem (Wipple and Suaer 2012) belongs to Cluster 4. These developmental cluster patterns of the leaf metabolic machinery are largely consistent with those examining a developmental time course of leaf 2 in Arabidopsis (Skirycz *et al.* 2010) with fewer metabolism genes expressed early in leaf development and gradually increasing as leaves matured. Therefore as leaves shift from net energy importers to net energy exporters, the [CO2] effect on the transcriptome strengthens and differences in leaf respiration rates increase (Figures 3 and 6).

### Conclusions

This study revealed that the stimulation of leaf respiratory CO2 efflux by elevated [CO2] occurs as leaves transition from rapidly expanding sink tissues to mature source tissues in Arabidopsis leaves. The transcriptional reprogramming of the respiratory machinery in response to elevated [CO2] started when leaves were expanding and became more apparent as leaves matured. Greater sugar content during early leaf expansion lends further support to the idea that glucose concentration as one component of the signaling mechanisms that regulates gene expression in elevated [CO2]. The transcriptional reprogramming of respiration occurred prior to there being a significant difference between ambient and elevated [CO2] for the leaf level respiration rates suggesting that mitochondrial proteins are actively being made prior to a detectable leaf level response. These findings add significant mechanistic understanding and have implications for whole plant growth modeling in elevated [CO2] by demonstrating that the magnitude leaf respiratory fluxes and transcriptional responses to elevated [CO2] occur depends on whether leaves are primarily importing or exporting energy.

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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** | **Number of Significant Transcripts** |
| Developmental Time Point | 11,337 |
| CO2 Concentration | 2,141 |
| CO2 by Development Interaction | 1,696 |



Figure 1. Total above ground dry biomass (a), midday in-situ CO2 assimilation (A) at growth [CO2] (b). Mean values (+/- standard errors) of physiological parameters of leaf 10 grown in ambient (370 ppm) or elevated (750 ppm) [CO2] at 23 (Expanding) or 30 (Mature) days after germination. CO2 assimilation was not determined (n.d.) in the expanding leaves at 23 DAG due to technical limitations. Also plotted are the p-values from the statistical model each of the parameters.

Macintosh HD:Users:Cody:Documents:Experiments:CO2xLeaf:Paper:New Phyt Submission:Figure 2.pdf

Figure 2 Midnight glucose content (A), midnight glucose concentration (B), midnight sucrose content (C), midnight sucrose concentration (D), midnight starch content (E), midnight starch concentration (F), Specific leaf area (G), and leaf soluble protein content (H). Mean values (+/- standard errors) of physiological parameters of leaf 10 grown in ambient (370 ppm) or elevated (750 ppm) [CO2] at 23 (Expanding) or 30 (Mature) days after germination. Also plotted are the p-values from the statistical model from each of the parameters.



Figure 3 Mean values (+/- standard errors) of midnight dark respiration rates (R) of leaf 10 grown in ambient (grey bars; 370 ppm) or elevated (black bars; 750 ppm) [CO2] at 23 (Expanding; E), 24, 29, 30 (Mature; M), 31 days after germination (DAG). The p-values from the statistical model each of the parameters are: CO2 p < 0.0001; Development p <0.0001; CO2 by 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 A graphical representation of genes encoding components of sugar transformations reactions and glycolysis. Tissue was harvested from leaf 10 at midnight on 16 (Primordia), 23 (Expanding), and 30 (Mature) days after germination. Each blue and yellow box represents the mean value of a unique transcript that responded significantly (P < 0.05) to elevated [CO2] in each leaf developmental stage. Blue is greater transcript abundance for that gene at elevated [CO2] compared to ambient [CO2] and yellow is less transcript abundance for those genes at elevated [CO2]. Details about individual transcripts can be found in Table S1.



Figure 5 A graphical representation of genes encoding components of the TCA cycle and the mitochondrial electron transport chain. Tissue was harvested from leaf 10 at midnight on 16 (Primordia), 23 (Expanding), and 30 (Mature) days after germination. Each blue and yellow box represents the mean value of a unique transcript that responded significantly (P < 0.05) to elevated [CO2] in each leaf developmental stage. Blue is greater transcript abundance for that gene at elevated [CO2] compared to ambient [CO2] and yellow is less transcript abundance for that gene at elevated [CO2]. Details about individual transcripts can be found in Table S1.

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**Figure 6** Venn-diagram of transcripts significantly responding (P < 0.05) to elevated [CO2] across the three leaf developmental with the size of each circle scaled to the number of genes represented in each.



**Figure 7** K-means clustering of significantly differentially expressed transcripts grouped into seven major expression patterns across leaf development (P: primordia; E: expanding; M: mature). The mean expression values for each cluster across development are shown in black. Positive values are relatively higher expression levels and negative values are relatively lower expression values. Also shown is a table with the distribution of significant transcripts responding to the main effect of CO2 or the interaction between CO2 and developmental stage (Dev) across the different clustering patterns.

**Supplementary Table 1:** List of transcripts that were significant for the main effect (p<0.05) of elevated [CO2] within each developmental stage that are displayed in Figures 4 and 5. AT locus IDs, functional description, and percentage change in elevated [CO2] versus ambient [CO2] at primordia (P), expanding (Ex), or mature (M) time-points.

**Supplementary Table 2:** List of the significant transcripts from the ANOVA model and which K-means cluster they belong to (Figure 6) based on expression pattern across leaf development.

**Supplementary Table 3:** The relative distribution of functional gene categories that each of the K-means clusters represents based on MAPMAN functional categories.