The influences of elevated carbon dioxide on trichome densities and trichome molecular pathways

By

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The influences of elevated carbon dioxide on trichome densities and trichome molecular pathways

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Abstract

Leaf hairs (trichomes; Fig. 1) are small and rigid epidermal structures that serve in herbivore defense, temperature regulation, boundary layer fortification, and UV-B protection, and can even act as mechanosensory switches indicating insect herbivore presence (Fig. 2). As such, leaf trichomes have impacts on overall plant physiology, photosynthetic efficiency, fitness, and plant-environment interactions. It has been found that leaf trichomes shift in density on the leaf surface when grown at elevated [CO_2] in a number of species (Fig. 3). Elevated [CO_2] *decreases* trichome densities by as much as 60% in some species (wheat, *Arabidopsis*) and increases densities by as much as 57% in other species (*Brassica rapa, Medicago truncatula*). However, the responses of trichomes to elevated [CO_2] remain critically understudied, and little is known about the molecular/developmental mechanisms driving these responses. As trichomes are physiologically important structures for numerous food crop species (e.g. wheat, soybean) and critical ecological species, it is imperative that further research be dedicated to understanding the implications of shifting trichome densities in an elevated [CO_2] environment of the future. The goal of this dissertation is to further understand the phenotypic responses driving trichome density shifts at elevated [CO_2], and molecular mechanisms that potentially underlie trichome density shifts at elevated [CO_2].

Our first aim sought to quantify trichome density responses to elevated [CO_2] across the entire *Arabidopsis* leaf, and not a smaller subsection of the leaf as previous publications have. Furthermore, we analyzed multiple leaves spanning whole-plant development, to observe how trichome densities respond as the plant develops in an elevated [CO_2] environment. Finally, we were able to relate full-leaf trichome numbers with leaf area and trichome density under elevated [CO_2], to tease apart the relationships between the trichome initiation and trichome densities at

elevated [CO_2]. Here we show that there trichome number shifts nor leaf area shifts exclusively underlie trichome density shifts under elevated [CO_2], dependent upon the genotype. We found significant genotypic variation in trichome density, trichome number, and leaf area responses to elevated [CO_2]. Furthermore, we found significant variation in elevated [CO_2] response between developmental stages, in regards to trichome density, number, and leaf area.

Our second aim was to determine the effect of elevated [CO_2] on trichome patterning across the leaf surface, which remains wholly unstudied. Previous to our work, no publications have investigated interactions between trichome patterning and environmental perturbation, therefore our phenotyping work here is novel. We show that elevated [CO_2] can significantly shift the patterning of trichomes across the leaf surface. Furthermore, we show evidence that elevated [CO_2] may alter trichome-to-trichome spatial relationships. Finally, we find that the impacts of elevated [CO_2] on trichome patterning was highly variable across *Arabidopsis* lines, as well as across developmental stages within lines.

There exists little published on potential molecular pathways underpinning the response of trichome densities to elevated [CO_2]. As such, our final aim was to present potential molecular/developmental mechanisms behind elevated [CO_2]-trichome responses, grounded heavily in trichome genetics, and elevated [CO_2] literature. Furthermore, we sought to investigate differential gene expression in growing primordial leaves across current and elevated [CO_2], to provide candidate genes that may be involved in the response of trichome densities to elevated [CO_2]. Most importantly, we show that unlike other environmental perturbations,

elevated [CO_2] may not directly affect core trichome gene transcription to alter trichome numbers, and we provide candidate genes for future research avenues.

Our research advances our understanding of the phenotypic impacts of elevated [CO_2], and the magnitude and variation in the density and patterning of important leaf micro-structures. Through extensive phenotyping we are able to provide a novel and detailed picture of trichome density and trichome patterning responses under elevated [CO_2]. As trichomes mitigate herbivory damage, improve water use efficiency, affect fungal infection rates, and increase photosynthetic efficiency, we believe that a deep understanding of how trichome densities may respond to environmental change is an important addition to the field. Finally, we construct a molecular framework and provide novel results concerning potential molecular pathways underlying elevated [CO_2] driven trichome density shifts.

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Chapter 1

The impacts of elevated carbon dioxide on trichome production and density in *Arabidopsis* genotypes

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Abstract

Trichomes (leaf hairs) are known to reduce leaf water loss, enhance herbivory defense, and deflect excess light, which increases plant productivity and survival. Trichome densities of leaves grown in an elevated [CO_2] environment have been shown to increase as high as 57% higher than ambient trichome densities in certain species such as *Brassica rapa*. Conversely, some species— such as wheat—reduce trichome densities by as much as 60% when grown in elevated [CO_2]. Previous experiments only looked at a small subsection of the leaf, which makes interpretations of these results and application to future molecular data from *Arabidopsis* leaves difficult. Our work is novel in that we sought to quantify trichome density responses to elevated [CO_2] across the entire *Arabidopsis* leaf, and not a smaller subsection of the leaf. Furthermore, we analyzed multiple leaves spanning whole-plant development, to observe how trichome densities respond as the plant develops in an elevated [CO_2] environment. Finally, we were able to relate full-leaf trichome numbers with leaf area under elevated [CO_2], to tease apart the relationships between the two variables and how this relationship might change under elevated [CO_2]. Here we show that there is significant genotypic variation in trichome density, trichome number, and leaf area responses to elevated [CO_2]. Furthermore, we found significant variation in elevated [CO_2] response between developmental stages, in regards to trichome density, number, and leaf area. As such, we conclude that neither trichome number shifts nor leaf area shifts exclusively underlie trichome density shifts under elevated [CO_2], dependent upon the genotype.

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Introduction

Global atmospheric carbon dioxide concentration ([CO_2]) is rising rapidly, having increased by almost 50% between the pre-industrial (270 ppm) and modern (402 ppm) periods, with expectations that it will double by the end of this century (NOAA, 2016). Elevated [CO_2] can affect plants across the ecosystem, physiological, and molecular levels through, for example, secondary compound production, alteration of leaf gas exchange, and whole-plant development (Becklin, 2016; Bidart-Bouzat, Mithen, & Berenbaum, 2005; Dong, Gruda, Lam, Li, & Duan, 2018; Masle, 2000; Medeiros & Ward, 2013; Teng et al., 2006). Elevated [CO_2] can also lead to differential production of leaf surface micro-structures, including stomata (pores required for gas exchange) and trichomes (leaf hairs) (Bidart-Bouzat, 2004; Bidart-Bouzat et al., 2005; Haus, Li, Chitwood, & Jacobs, 2018; Karowe & Grubb, 2011; Lake & Wade, 2009; Masle, 2000).

Trichomes are known to reduce leaf water loss, enhance herbivory defense, and deflect excess light, which increases plant productivity and survival (Johnson, 1975; Mauricio, 1998). Elevated [CO_2] has been shown to increase trichome densities by as much as 57% some species, such as *Brassica rapa*. Conversely, some species— such as wheat—reduce trichome densities by as much as 60% when grown in elevated [CO_2] (Guo et al., 2014; Guo et al., 2013; Karowe & Grubb, 2011; Masle, 2000). Environmental perturbations which lead to shifts away from locally adaptive trichome densities could lead to increased susceptibility to increased fungal infection, excessive energy investment, overheating, and herbivory (Calo, García, Gotor, & Romero, 2006; Kergunteuil, Descombes, Glauser, Pellissier, & Rasmann, 2018; Kim, 2019; Løe, Toräng, Gaudeul, & Ågren, 2007; Manetas, 2003; Sletvold, 2010).

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Maintaining an optimal trichome density may involve a fine balance between maximizing trichome density to inhibit herbivory and alleviate the impacts of environmental stressors (e.g. UV-B), while simultaneously preventing the impacts of overproduction of trichomes, including fungal infections and excessive energy expenditure (Calo et al., 2006; Imboden, Afton, & Trail, 2018; Kergunteuil et al., 2018; Kim, 2019; Løe et al., 2007; Nguyen, Dehne, & Steiner, 2016; Sletvold, 2010). Such a balance between too few and too many trichomes is important to consider with regards to shifting trichome densities at elevated [CO_2]. Trichomes of maize, *Brachypodium distachyon*, and barley act as entry points for *Fusarium* infection, with fungal hyphae gaining entry into the leaf through trichome base socket cells (Imboden et al., 2018; Nguyen et al., 2016; Peraldi, Beccari, Steed, & Nicholson, 2011). In addition, a glabrous *Arabidopsis* trichome mutant (*gl1*) showed increased resistance to the necrotrophic fungus *Botrytis cinereal* compared to wildtype, while a trichome overproducing mutant (*try*) was more susceptible to fungal infection (Calo et al., 2006). Furthermore, trichomes can be energetically costly to produce, whereby trichome-dense genotypes of *A. lyrata* grown in the absence of herbivory exhibited decreased fitness and performance compared to plants exposed to herbivory where trichomes were beneficial (Sletvold, 2010). Therefore, excessive trichome density may not be entirely beneficial, as increased trichome densities carry increased risk of detrimental fungal infections, increased fitness costs, and possibly other yet unknown outcomes.

Elevated [CO_2] significantly *decreases* trichome densities in *Arabidopsis* genotypes Cvi and Col-0 (approximately 34.5% and 51.2% decreases, respectively) (Bidart-Bouzat, 2004; Bidart-Bouzat et al., 2005; Lake & Wade, 2009; Masle, 2000). The other genotypes examined— Ler, Zn-0, Edi, Wu-0, and Can-0—were found to be non-responsive in trichome densities under elevated [CO_2]

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(Lake, JA and Wade, RN unpublished, 2009; Lake and Wade 2009). While *Arabidopsis* exhibits genotypic variation, *Medicago truncatula* exhibits variation among whole-plant developmental stages. Elevated [CO_2] increased the densities of non-glandular trichomes on both young and mature *M. truncatula* leaves (approximately 26% and 13% density increase respectively), but [CO_2] enrichment increased glandular trichomes only on *mature* leaves (approximate 42% increase) (Guo et al., 2014).

Arabidopsis, wheat, *B. rapa*, and *M. truncatula* have shown genotypic variation in the response of trichome densities to elevated [CO_2], and in some cases, variation in response among developmental stages (Bidart-Bouzat, 2004; Bidart-Bouzat et al., 2005; Guo et al., 2014; Lake & Wade, 2009; Masle, 2000). Therefore, through newly developed novel techniques, we addressed the following questions:

1. What is the genotypic variation in trichome density response across *Arabidopsis* ecotypes under elevated [CO_2]?
2. Are shifts in trichome numbers or shifts in leaf area driving trichome density shifts under elevated [CO_2]?
3. Is there variation in the response of trichomes to elevated [CO_2] across whole-plant development?

We hypothesized that trichome density shifts at elevated [CO_2] will be correlated with significant shifts in leaf area. Shifts in leaf area under elevated [CO_2] have been found across a number of species, but trends remain unclear (Ferris, Sabatti, Miglietta, Mills, & Taylor, 2001; Reddy & Zhao, 2005; Taylor, Ceulemans, Ferris, Gardner, & Shao, 2001). Therefore, we predict an

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indirect effect of elevated [CO_2] on trichome densities through shifted leaf area, instead of a direct effect through shifting trichome numbers. Our work is novel in that we sought to quantify trichome density responses to elevated [CO_2] across the entire *Arabidopsis* leaf, and not a smaller subsection of the leaf. Furthermore, we analyzed multiple leaves spanning whole-plant development, to observe how trichome densities respond as the plant develops in an elevated [CO_2] environment. Finally, we were able to relate full-leaf trichome numbers with leaf area under elevated [CO_2], to tease apart the relationships between the two variables and how this relationship might change under elevated [CO_2].

Materials and Methods

Genotypes

We used the following *Arabidopsis* ecotypes for this work: Col (United States; CS22625), Ler (Germany; CS20), Kondara (Tadjikistan; CS916), Cvi (Cape Verde Islands; CS1096), and Ws (Russia; CS915). Furthermore, we also used two genotypes produced from the same parental lines: genotype SG (Selected Genotype)—selected for high fitness under elevated [CO_2]—and related CG (Control Genotype) (Ward, Antonovics, & Strain, 2000). Ecotypes were selected based on relationship to Col and Ler, to prevent usage of a set of ecotypes all closely related to either the responsive Col or non-responsive Ler. Finally, we selected for variation in source location across the ecotypes. Ecotype seeds were ordered from the Arabidopsis Biological Resource Center (The Ohio State University).

Plant growth conditions

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We controlled plant growth conditions with a walk-in plant growth chamber (Conviron BDW80, Winnipeg, Manitoba). To simulate current ambient CO_2 concentrations one chamber was kept at 400 $\pm$ 10 ppm [CO_2], while a second chamber was maintained at 800 $\pm$ 10 ppm [CO_2] to mock predicted [CO_2]. We simulated daytime with 1 hour of approximately 125 µmol light (mock dawn), followed by 10 hours of approximately 350 µmol light; mock daytime concluded with 1 hour of approximately 125 µmol light (mock dusk). Daytime temperature ramped from 18°C at mock dusk to a maximum of 24°C at the 7th hour of light, followed by a descent to 23°C ending at the end of simulated dusk. The average daytime temperature was 22°C. Mock dusk is followed by a dark period of 12 hours, with a temperature decline from 23°C at the end of mock dusk to a low of 18°C over the 12-hour simulated nighttime period. Humidity was maintained at 60% and 90% during the 12-hour light and 12-hour dark period respectively. Approximately every three days we filled the bottom-watering trays containing the plants to a point of two centimeters on the tray with deionized water. Every other watering event, we fertilized via addition of half-strength Hoagland's solution to the tray.

Imaging

We collected full images of *Arabidopsis* leaves with upright light microscope (Nikon AZ100). Utilizing the stitching function, we collected approximately 10 to 15 images across each leaf, and for each leaf we stitched the images together to produce one large, detailed image of the leaf at magnifications that provided the highest quality for trichome identification. We then electronically tagged each trichome in the digital leaf images were then ImageJ/FIJI, and trichome coordinates were exported into CSV format (Abramoff, Magelhaes, & Ram, 2004; Schindelin et al., 2012). ImageJ/FIJI was used to create a scale (pixel/cm) for each microscope

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image, using a ruler with a set distance in each frame of the image. We than obtained a binary image of the leaf shape with Adobe Photoshop. We uploaded the CSV containing the trichome coordinates, the PNG of the binary leaf image, and the value from the ImageJ pixel/cm scale to R for downstream analyses, described below.

Density measurement software

The R code used in this experiment was developed in our laboratory and is based off geographic spatial statistics packages. The R code we developed here treats the leaf as a geographic space, and the trichomes as points falling within the geographic space of the leaf, allowing us to use geographic statistics software products OsGeo4w and GDAL. We were able to collect full-leaf trichome densities and numbers, a novel addition to the field. The code was used to determine leaf area, number of trichomes, and whole leaf trichome densities of the previously described leaf images.

Sampling

To observe how trichome densities respond across whole-plant developmental time, sampled leaves were selected to fall into three categories: juvenile, juvenile-mature transitional, and mature leaves. We selected consecutive leaf pairs (e.g. leaves 3 and 4, 11 and 12, 21 and 22) for harvest, harvesting leaves from the rosette once the plant had flowering bolt length of approximately 3 inches. We harvested post-bolting to ensure that all leaves we harvested had exited the trichome initiation state that occurs only in early leaf development. Leaves were then

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pressed between glass slides, to prevent inaccurate measurements of curled leaves. We sampled approximately 10-20 leaves (n) for each treatment-genotype-leaf stage combination.

Statistical Analysis

Each variable was averaged across the two leaves within each pair, creating what is heretofore referred to as a "leaf stage". General differences in responses to elevated [CO_2] across *Arabidopsis* genotypes and leaf stages was examined via linear mixed-effects model ANOVA, using formula 1, in which X is the dependent variable (trichome number, leaf area, or trichome density).

(Formula 1) X ~ [CO_2] treatment * Genotype * Leaf Stage + (1 + Leaf Stage | Plant Replicate)

The residuals of each variable were tested for normality with the Shapiro-Wilk's test of normality and transformed with Tukey's Ladder of Power transformation accordingly. The Specific differences between 400 ppm [CO_2] and 800 ppm [CO_2] in each individual genotype/stage combination were investigated via least square means post-hoc testing with a Tukey adjustment, following the mixed-model ANOVA testing when interactions were significant in the preceding ANOVA. All statistical tests were performed in R. Linear mixed model ANOVAs were performed with the *lme4* package, and post-hoc tests were performed with the *emmeans* package.

Results

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Trichome density, trichome number, and leaf area

To determine the underlying phenotype shift driving trichome density shifts at elevated [CO_2], we looked at the differences in trichome number, leaf area, and trichome density at ambient and elevated [CO_2] among different *Arabidopsis* genotypes. Furthermore, we looked at these variables in ambient and elevated [CO_2] across whole-plant development, to test for a developmental component to the effects of elevated [CO_2] on trichome densities (Table 1).

There was strong genotypic variation in elevated [CO_2] response across the *Arabidopsis* genotypes measured. We found a significant three-way interaction between elevated [CO_2], genetic genotype, and leaf stage for all traits (trichome density: $P < 0.001$, trichome count: $P < 0.001$, leaf area: $P < 0.001$) in the overall ANOVAs (Table 1). As such, we found that elevated [CO_2] affected trichome count and leaf area, with a strong relationship with genotype and developmental stages within genotypes (Figs. 4, 5, and 6). Trichome densities and trichome numbers generally increased or did not significantly respond under elevated [CO_2] (described in detail below), with the exceptions being a reduction in trichome number and trichome density in Cvi stage 3 ($P = 0.024$ and $P = 0.043$, respectively; Table 2). Furthermore, all genotypes showed a significant response in trichome density in at least one stage of development, but shifts in trichome number and leaf area were less ubiquitous (Figs. 4, 5, and 6, Table 2).

We measured whole-leaf trichome numbers and leaf areas on the same set of leaves so we could determine if significant shifts in one of these phenotypes at elevated [CO_2] led to significant alterations to trichome densities at elevated [CO_2]. The relationship between trichome number

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shifts and leaf area shifts to changes in trichome densities at elevated [CO_2] depended strongly on genotype and leaf stage (Table 2).

In some cases, a significant trichome density response to elevated [CO_2] observed with a significant shift in trichome number, but not in leaf area (Figs. 4 and 6): these cases included Col stage 2 (trichome density, trichome number and leaf area p-values were P = 0.0368, P = 0.00114, and P = 0.309 respectively; Table 2) and Cvi stage 3 (trichome density, trichome number and leaf area p-values were P = 0.0427, P = 0.024, and P = 0.364 respectively; Table 2).

Conversely, there were instances in which elevated [CO_2] drove a shift in leaf area, which corresponded to a shift in trichome density without a shift in trichome number (Figs. 5 and 6). **This response was more widespread than the trichome number response described previously**, including Kon stage 2 (trichome density, trichome number and leaf area p values were P = 0.00335, P = 0.275, and P < 0.001 respectively; Table 2), Kon stage 3 (trichome density, trichome number and leaf area p values were P < 0.001, P = 0.42, and P < 0.001 respectively; Table 2), Ws stage 2 (trichome density, trichome number and leaf area p values were P = 0.0044, P = 0.742, and P < 0.001 respectively; Table 2), Ws stage 3 (trichome density, trichome number and leaf area p values were P = 0.0234, P = 0.81, and P = 0.00133 respectively; Table 2), and SG stage 2 (trichome density, trichome number and leaf area p values were P = 0.00675, P = 0.802, and P = 0.0028 respectively; Table 2).

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Across the genotypes we measured, no genotype showed a consistent response across the developmental stages we measured (Figs. 4, 5, and 6, Table 2). Ws and Kon showed the same response in the second and third developmental stages, but the first stage in both genotypes showed no significant response under elevated [CO_2] (Table 2).

Discussion

Trichomes (leaf hairs) are known to reduce leaf water loss, enhance herbivory defense, and deflect excess light, which increases plant productivity and survival (Calo et al., 2006; Kergunteuil et al., 2018; Kim, 2019; Løe et al., 2007; Manetas, 2003; Sletvold, 2010). The main goal of this study was to investigate the effects of elevated [CO_2] on important phenotypic trichome traits (trichome count, leaf area, and trichome density) across *Arabidopsis* genotypes, with novel full-leaf and whole-plant developmental aspects. When considering trichome density shifts at elevated [CO_2], one possibility was that shifts in leaf area at elevated [CO_2] was shifting the amount of leaf material separating trichomes from one another, thereby shifting trichome densities. The other possibility was that elevated [CO_2] increases the amount of trichomes initiating across the leaf, thereby increasing trichome numbers and trichome densities, without shifts in leaf area. Surprisingly, we observed instances of both scenarios—as well as combinations of the two scenarios—when we related trichome density, trichome number, and leaf area responses at elevated [CO_2] (Table 2). We found strong genotypic variation across *Arabidopsis* genotypes in trichome density, trichome number, and leaf area responses to elevated [CO_2] (Table 1). The underlying phenotypic response driving altered trichome densities at elevated [CO_2] varied strongly between genotypes and developmental stages (Tables 1 and 2).

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In certain genotypes, the response driving trichome density shifts was clear, such as Col (stage 2) in which elevated [CO_2] significantly increased trichome density through increased trichome numbers (Figs. 4 and 6, Tables 1 and 2). Conversely, Kon (stages 2 and 3) significantly decreased leaf area at elevated [CO_2], forcing trichomes into a smaller physical space, thereby increasing trichome density not through increased trichome numbers but through decreased physical space for dispersion (Figs. 5 and 6, Tables 1 and 2). Finally, we observed cases in which the two scenarios played out in tandem, but in a more subtle manner than the examples previously provided. In scenarios such as this, trichome number and leaf area may be shifting in opposing directions—moderately increasing trichome numbers and moderately decreasing leaf area—to drive a significant increase in trichome density (Figs. 4, 5 and 6, Tables 1 and 2). As such, the underlying phenotype shift driving alterations in trichome densities at elevated [CO_2]—leaf area, trichome number, or a combination of the two—depends significantly on genotype and developmental stage, with no single, direct answer to the question we posed.

We hypothesized that trichome densities would be indirectly shifted at elevated [CO_2], through alterations in individual leaf area, given that leaf area shifts at elevated [CO_2] have been shown in the literature (Ferris et al., 2001; Reddy & Zhao, 2005; Taylor et al., 2001). We found that, though this is the case for some genotypes/developmental stages, there was a direct effect of elevated [CO_2] on trichome numbers in some genotypes/developmental stages that we did not expect.

Incongruity with older literature

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Past research has reported genotypes only displaying trichome density decreases or non-responsiveness across *Arabidopsis* genotypes (Bidart-Bouzat, 2004; Bidart-Bouzat et al., 2005; Lake & Wade, 2009). Here, we observed trichome density increases under elevated [CO_2], with increases in *Arabidopsis* genotypes that were previously reported as decreasing trichome densities under elevated [CO_2] (e.g. Col; Fig. 6, Table 2) (Bidart-Bouzat, 2004; Bidart-Bouzat et al., 2005; Lake & Wade, 2009). The reason our results are incongruous with older literature is most likely due to the scale of our measurements. Past literature has sampled from the central portion of the leaf, while we were able to measure trichome variables across the entire *Arabidopsis* leaf (Bidart-Bouzat, 2004; Bidart-Bouzat et al., 2005; Lake & Wade, 2009). As trichome numbers and trichome densities may not shift uniformly across the leaf, sampling from a small portion of the leaf may not represent the actual elevated [CO_2] response of the leaf.

Ecological perspective

The variety of responses we observed in trichome count, leaf area, and trichome density (Figs. 4, 5, and 6, Table 2) to elevated [CO_2] may have implications for the ecological and physiological roles of trichomes in an elevated [CO_2] environment (Johnson, 1975; Mauricio, 1998). As we observed the response of trichome densities to elevated [CO_2] in a single generation, we cannot explore the adaptive nature of these responses here. However, we observed shifts in trichome densities and trichome numbers at elevated [CO_2] in the single generation we observed, and if this occurred in the field we could see deviations from the aforementioned local trichome density optimum (Calo et al., 2006; Imboden et al., 2018; Kergunteuil et al., 2018; Kim, 2019; Løe et al., 2007; Nguyen et al., 2016; Sletvold, 2010).

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Across-development leaf stage response variation

Within most genotypes, we observed significant variation in the elevated [CO_2] response of trichome density, trichome number, and leaf area across the three leaf stages (Figs. 4, 5, and 6, Tables 1 and 2). The diversity of responses across stages within each genotype may indicate that elevated [CO_2] impacts trichome density through whole-plant related mechanisms, creating variation in responses for individual leaves as the plant develops. For example, the time a plant takes to reach the flowering stage is known to shift at elevated [CO_2], and both accelerations towards floral initiation and delays of floral initiation have been observed in numerous species at elevated [CO_2] (C. J. Springer, Orozco, R.A., Kelly, J.K., Ward, J.K., 2008; C. J. Springer & Ward, 2007). Trichome initiation and floral initiation overlap in several key pathways, including *microRNA156* (*miR156*) (Matias-Hernandez, 2016; Xu et al., 2016; Xue et al., 2014; Yu et al., 2010). *miR156* is highest in young *Arabidopsis* seedlings and declines as plants age and approaches flowering, during which time levels of *miR156*-silenced *SQUAMOSA PROMOTER BINDING-LIKE*S (*SPLs*) increase (Yu et al., 2010). *mir156*-regulated *SPLs (*e.g. *SPL9*) are responsible for the transition to flowering, and upregulate the negative trichome regulator *TRYPTYCHON* (*TRY*) (Yu et al., 2010). An impact of elevated [CO_2] on such genotypically-variable whole-plant developmental pathways (e.g. time to flower) that are pleiotropic with trichome production could potentially cause the genotypic and between-stage variation we saw (Tables 1 and 2). We recommend analysis of trichome genes across whole-plant developmental at elevated [CO_2] to test for differences in trichome gene expression that correlate with shifts in *miR156* expression.

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Molecular implications

Future research must look to understand the underlying molecular pathway(s) triggering trichome number and trichome density shifts under elevated [CO_2]. As there are numerous potential physiological and ecological consequences of trichome density shifts, we believe it to be an important research avenue. Considering that trichome numbers were observed to shift in certain genotypes/stages (Fig. 4, Table 2), core trichome genes may be implicated in the shifts in trichome numbers under elevated [CO_2]. Other environmental perturbations alter expression of key trichome genes to shift trichome densities. Drought conditions, for example, have been shown to increase the trichome densities of *Caragana korshinskii* (Peashrub) through up-regulation of core trichome positive regulators *TRANSPARENT TESTA GLABRA1* (*TTG1*) and *GLABROUS2* (*GL2*), as well as down-regulation of negative regulator *CAPRICE* (*CPC*) (Ning et al., 2016). Furthermore, UV-B induced increases in trichome densities were mediated through an increase in core trichome gene *GLABROUS3* (*GL3*) expression in *Arabidopsis* (Yan, Pan, An, Gan, & Feng, 2012). Therefore, we recommend that future research investigates the effect of elevated [CO_2] on core trichome genes, as past work has shown that environmental perturbations act through key trichome genes to alter trichome density.

Conclusions

Here we showed that there is significant genotypic variation in trichome density, trichome number, and leaf area responses to elevated [CO_2]. Furthermore, we found significant variation in elevated [CO_2] response between developmental stages, in regards to trichome density, number, and leaf area. As such, we conclude that trichome number shifts nor leaf area shifts

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exclusively underlie trichome density shifts under elevated [CO_2], dependent upon the genotype. We suggest that future research investigate the molecular underpinnings of shifting trichome densities and numbers, and what pathway(s) and genetic variability underlies the response variations we observed here. As trichomes play a number of physiologically and ecologically important roles, understanding how trichome densities may respond to a future higher [CO_2] climate is vital, and here we provided novel insights into genotypic and developmental variation.

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Chapter 2

Genotypic and developmental variation in the response of trichome patterns to elevated carbon dioxide in *Arabidopsis*

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Abstract

Elevated carbon dioxide has been shown to shift trichome densities in multiple species, which could affect herbivory damage, water use efficiency, fungal infection rates, and photosynthetic efficiency. In addition to trichome density, the distribution of trichomes across the leaf surface is most likely an important factor in the physiological roles of trichomes. While we previously showed that elevated [CO_2] can affect trichome densities and trichome numbers (see chapter 1), the effect of elevated [CO_2] on trichome patterning across the leaf surface remains wholly unstudied. We developed methods for quantifying full leaf trichome patterns, as well as creating composite patterns for high-throughput phenotyping of sets of *Arabidopsis* leaves. Previous to our work, no publications have investigated interactions between trichome patterning and environmental perturbation, therefore our phenotyping work described below is novel. Here, we show that elevated [CO_2] shifts patterning of trichomes across the leaf surface. Furthermore, we show evidence that elevated [CO_2] may alter trichome-to-trichome spatial relationships. The impacts of elevated [CO_2] on trichome patterning was highly variable across *Arabidopsis* lines, as well as across developmental stages within lines. The shifts in trichome patterning we observed at elevated [CO_2] could have implications for the physiological roles trichomes play.

Introduction

The atmospheric CO_2 concentration ($[CO_2]$) has risen from 270 ppm at the beginning of the industrial revolution to more than 400 ppm currently (NOAA, 2016). $[CO_2]$ is predicted to reach 800-1000 ppm on a global scale by the end of this century. Elevated $[CO_2]$ has a range of physiological impacts on varying plant species, including increased carbon:nitrogen ratios, which is known to impact herbivory (Lincoln, Couvet, & Sionit, 1986). Furthermore, shifts in time to flower have been observed, which could severely alter plant/pollinator relationships (C. J. Springer & Ward, 2007). At the leaf level, stomatal densities—pores vital to carbon dioxide intake—often decrease at elevated $[CO_2]$ (Woodward & Kelly, 1995).

Similar to shifts in stomatal densities, elevated $[CO_2]$ has been shown to influence trichome densities in a number of species. Increases in trichome densities have been observed in *B. rapa* (Oilseed rape; Ambient: 379 ppm, Elevated: 744 ppm) and *Medicago truncatula* (Barrelclover; Ambient: 390 ppm, Elevated: 750 ppm) at elevated $[CO_2]$ (Guo et al., 2013; Karowe & Grubb, 2011). On the contrary, elevated $[CO_2]$ significantly decreases trichome densities in *Triticum aestivum* (Common wheat; Ambient: 350-420 ppm, Elevated: 900 ppm) and in some lines of *Arabidopsis thaliana* (Ambient: 360-400 ppm, Elevated: 720-800 ppm) (Bidart-Bouzat, Mithen, & Berenbaum, 2005; Lake & Wade, 2009; Masle, 2000). Furthermore, *Arabidopsis* displays natural variation in elevated $[CO_2]$ responses, depending on the ecotype. Two experimental studies to date have investigated elevated $[CO_2]$ effects on *Arabidopsis* trichome densities, with inclusion of ecotypic responses. Trichome densities of the Can-0 (Canary Islands) and Edi-0

(Edinburgh, Scotland) lines were unaffected (Lake and Wade, unpublished, 2009; Lake and Wade 2009). On the other hand, Cvi (Cape Verde Islands) and Col-0 (Columbia, Missouri, U.S.) showed significant trichome density decreases under elevated [CO_2] (720-800 ppm) (Bidart-Bouzat et al., 2005; Lake & Wade, 2009).

Trichomes enhance defense, scatter excess light, reduce leaf surface temperature, and increase the boundary layer between the leaf surface and outside air movement, reducing water loss through transpiration (Ehleringer & Björkman, 1978; Johnson, 1975; Konrad, Burkhardt, Ebner, & Roth-Nebelsick, 2015). Due to the close relationship between water lost and carbon gained, trichomes also play a role in carbon cycling at the leaf and ecosystem levels (Bickford, 2016). If trichome densities are shifted away from locally adaptive trichome densities in a future elevated [CO_2] environment, consequences could result affecting herbivory damage, water use efficiency (WUE), fungal infection rates, and photosynthetic efficiency (Calo, García, Gotor, & Romero, 2006; Kergunteuil, Descombes, Glauser, Pellissier, & Rasmann, 2018; Kim, 2019; Løe, Toräng, Gaudeul, & Ågren, 2007; Manetas, 2003; Sletvold, 2010).

In addition to trichome density, the heterogeneity of trichome distribution across the leaf surface is most likely an important factor in the physiological roles of trichomes. For example, a large gap in the trichome pattern could leave leaf tissue more vulnerable to insect herbivory compared to a leaf with a more evenly distributed trichome pattern. While we previously showed that elevated [CO_2] can affect trichome densities and trichome numbers (see chapter 1), the effect of elevated [CO_2] on trichome patterning across the leaf surface remains wholly unstudied.

There must presumably be a somewhat even distribution of trichomes *across* the leaf (or stem) in order to allow adaptive trichome functionality. As such, the trichome initiation pathway directly controls trichome patterning across the leaf (Hülskamp, 2004). Trichome initiation begins at the distal tip and proceeds toward the stem in leaf primordia no less than 100 μm long (Larkin, Young, Prigge, & Marks, 1996). As the primordial leaf lengthens, trichome initiation continues at the base, while previously initiated trichomes on the non-basal leaf area reach maturity. Finally, the time window in which trichomes initiate on the primordial leaf closes and the number of trichomes on a leaf is concrete, and cellular division and growth in pavement cells continue, increasing the distances between trichomes (Larkin, Brown, & Schiefelbein, 2003).

As trichomes form on the primordial leaf surface, a random pattern model fails to predict the resulting pattern, as a random model overestimates the amount of trichomes that are directly adjacent to another trichome (Larkin et al., 2003). The distinctive non-random pattern of trichome initiation is in-part due to a lateral inhibition mechanism, in which a developing trichome inhibits neighboring protodermal cells from entering into the trichome initiation pathway. The molecular components involved in the lateral inhibition mechanism are also the core components involved in trichome initiation. As trichome initiation and trichome patterning are intimately connected through the core molecular components of trichome initiation, a shift in trichome numbers and densities may also lead to shifts in trichome patterning. Yet the impact of elevated [CO_2] on the full-leaf patterning of trichomes remains unstudied. As such, we address the following questions below:

1. Can elevated [CO_2] alter trichome patterning across the leaf surface?

2. Does trichome patterning differentially respond to elevated [CO_2] in leaves spread across developmental time?

Until this point, methods were not available to quantify full leaf trichome patterns. We developed methods for quantifying full leaf trichome patterns, as well as creating composite patterns for high-throughput phenotyping of sets of *Arabidopsis* leaves. As such, our work offers not only observations on the interactions between trichome patterning and elevated [CO_2], but methods for observing general trichome patterning trends in *Arabidopsis*. Furthermore, previous to our work, no publications have investigated interactions between trichome patterning and environmental perturbation, therefore our phenotyping work described below is novel. Investigating shifts in trichome patterns may indicate potential molecular mechanisms underlying trichome density shifts at elevated [CO_2], as the mechanisms controlling trichome patterning are well studied. As many trichome genes play dual roles in trichome initiation and trichome patterning, we hypothesize that elevated [CO_2] has the capacity to shift trichome patterning, as we previously observed shifts in trichome numbers at elevated [CO_2], and there is an intimate molecular tie between trichome number/initiation and trichome patterning (Balkunde, Pesch, & Hülskamp, 2010; Digiuni et al., 2008; Zhang, 2018).

Materials and Methods

Ecotypes/lines

Ecotypes used were Col (United States; CS22625), Ler (Germany; CS20), Kondara (Tadjikistan; CS916), Cvi (Cape Verde Islands; CS1096), and Ws (Russia; CS915). We selected ecotypes to have variation in previously reported elevated [CO_2] responses, variation in source location, and variation in relationship to Col and Ler; Col was previously reported decrease trichome densities

under elevated [CO_2], while Ler was previously reported to not respond in trichome densities under elevated [CO_2] (Bidart-Bouzat et al., 2005; Lake & Wade, 2009). We also used genotype SG (Selected Genotype)—selected for high fitness under elevated [CO_2]—and related CG (Control Genotype), both produced from the same parental lines (Ward, Antonovics, & Strain, 2000).

Ecotype seeds were ordered from the Arabidopsis Biological Resource Center (The Ohio State University) and grown up for seed. Relationship among ecotypes was determined using *Arabidopsis* ecotype SNP/indel data (1001 Genomes Project) and the R package SNPRelate (Zheng et al., 2012). Sequence data were produced by the Weigel laboratory at the Max Planck Institute for Developmental Biology.

Plant growth conditions

CO_2 concentrations were kept at 400 $\pm$ 10 ppm or 800 $\pm$ 10 ppm, for ambient and elevated [CO_2] treatments respectively. Daytime was simulated with 10 hours of approximately 350 µmol light, with 1 hour of approximately 125 µmol light at the beginning and end of the 10 hours to simulate dawn and dusk. The average daytime temperature was 22°C, with a ramp from 18°C to 24°C at the 7^{th} daytime hour, then a descent to 23°C ending at the end of simulated dusk. Nighttime temperatures begin with 23°C at the end of dusk and fall to 18°C over the 12-hour simulated nighttime period. Daytime and nighttime humidity were 60% and 90% respectively. Growth conditions were tightly controlled with a walk-in plant growth chamber (Conviron BDW80, Winnipeg, Manitoba). Plants were bottom watered, and watering trays were filled to

two centimeters approximately every three days. Plants were fertilized with half-strength Hoagland's solution every other watering event.

Imaging

Full leaf images were taken with an upright light microscope (Nikon AZ100) using the stitching function to account for the size of the full leaf, collecting approximately 10 to 15 images for each stitched final image. Trichomes were then tagged with ImageJ/FIJI, and trichome coordinates were exported into CSV format. ImageJ/FIJI was also used to create a scale (pixel/cm) for each microscope image (Abramoff, Magelhaes, & Ram, 2004; Schindelin et al., 2012). The leaf image was then edited in ImageJ/FIJI and Adobe Photoshop to obtain a binary image of the leaf shape. The CSV containing the trichome coordinates, the PNG of the binary leaf image, and the value from the ImageJ pixel/cm scale were then uploaded to R.

Density and patterning measurement software

The R code used in this analysis was developed in our laboratory and is based off geographic spatial statistics packages. The code treats the leaf as a geographic space, and the trichomes as points falling within the geographic space of the leaf. As such, we utilized previously collected leaf images (see chapter 1), and were able to calculate trichome density, trichome number, leaf area, and patterning statistics all on the same collection of leaves.

Patterning software

In order to analyze how patterning shifts under elevated [CO_2], we devised a way to obtain an "average" or "composite" pattern from many sampled leaves within a treatment. As patterning software and R packages cannot handle differently shaped windows (e.g. leaves with slightly different shapes, widths, etc.), we had to create a method to control for this variability in leaf shape. Each leaf shape, obtained using the method described above, was divided using a 10 x 10 grid. Each cell of the grid is given a unique alphanumeric identifier. The area of each cell in the 10 x 10 grid scaled with leaf size, so the area of each cell could be used to control for differences in leaf area between individual leaves. Each cell in the leaf grid was given a unique location-based identifier, so the mean of trichome densities across sampled leaves could be obtained for each unique cell (Fig. 9). The mathematical center of the leaf was determined, and any cell overlapping with the center line of the leaf was identified as a "center" cell along with the letter from the alphanumeric identifier of that cell. Edge cells were identified as those with areas less than the full area of the cell; cells that are clipped by the leaf window. Cells on the leaf tip were identified as having the maximum letter value of the alphanumeric labels and were labelled "tip" along with the numerical value from their respective alphanumeric label. Finally, cells that were not "edge", "tip", or "center" are termed "blade" cells. Blade cells were labelled according to their orientation to the center cell of their row in the leaf grid. If a cell is directly to the left of the center cell, it was labelled as "-1". Combined with the letter from the alphanumeric label, this cell would have had a full final label of "B blade -1".

Within each set of leaves (unique treatment x line x leaf stage combinations), the average trichome density was calculated for each cell identifier (e.g. cell B blade -1). This resulted in one trichome density value per each possible unique cell identifier.

To determine the average leaf shape of leaves within a set, binary leaf images were uploaded to R. With the leaf shape oriented parallel to the Y-axis, horizontal lines were drawn across the leaf shape, with the number of lines drawn determined by the length of the leaf. The lengths of the lines were then related to their position in relation to the Y-axis, and this relationship was used to create an average leaf image. This average leaf image was then gridded and labelled identical to the above, and then the previously obtained average densities of each cell type and the cell labels and areas on the new leaf image were used to calculate the number of trichomes per cell. Within each cell, the number of trichomes assigned to that cell were randomly placed. Then, utilizing averages across sampled leaves, distance from centroid of the cell and angle in relationship to the centroid were used to shift the randomly placed points into a more consistent position. To compare two composites (400 ppm [CO_2] versus 800 ppm [CO_2] grown leaves), the R function solutionset from the package spatstat was used (Baddely, Rubak, & Turner, 2015).

Trichome distance from leaf tip

We measured the distance of trichomes from the leaf tip, to determine if elevated [CO_2] may affect the timing of trichome initiation across the expansion of the primordial leaf. A shift in the average distance of trichomes to the leaf tip could indicate a shift in the period during which trichomes can initiate, as a trichome initiates at the base of the leaf and matures as the trichome is pushed toward the leaf tip during leaf expansion. Therefore, an alteration in either the developmental timing of a leaf or an alteration to the time period in which trichomes are able to initiate could affect trichome patterning across the leaf, specifically initiating more or less

trichomes at the tip of the leaf compared to the base (Yang & Ye, 2012). To determine if elevated [CO_2] significantly affects the distribution of trichomes from the leaf base to the leaf tip, which may have implications for the timing of trichome initiation, we measured the distance of each trichome to the tip of the leaf, and averaged this measurement for each leaf. The distance of trichomes to the tip were normalized by dividing measurements by the length of the leaf.

Trichome-to-trichome relationships

To examine the effects of elevated [CO_2] on the spatial relationships between trichomes, we examined the small-scale trichome densities surrounding each trichome with discs of two different areas. As trichome patterning is determined through a cell-to-cell mobile signal, a change in this signal would be apparent through a shift in the local trichome densities surrounding each trichome. To investigate the relationship *between* trichomes at ambient and elevated [CO_2], we used a common spatial method, in which we measured trichome density surrounding each trichome in two differently sized circles, with the trichome of focus acting as the circle center. The radius of the larger circle was 0.13 cm, and the radius of the smaller circle was two-thirds of the larger circle (0.086 cm). The radius of the larger circle was selected as it was the average minimum distance from trichome to trichome in the commonly used *Arabidopsis* line Col at 400 ppm [CO_2].

Sampling

Leaves were selected to fall into three categories: juvenile, juvenile-mature transitional, and mature leaves. Leaf numbers harvested were kept consistent within lines, regardless of treatment.

Consecutive leaf pairs (e.g. leaves 3 and 4, 11 and 12, 21 and 22) were selected for harvest. Selected leaves were harvested from the rosette once the plant had bolted, with a bolt length of approximately 3 inches. Post-bolt harvesting ensures that many of the leaves have ceased expanding, and that **all** leaves harvested have exited the trichome initiation state. Once harvested, leaves were pressed between two glass microscope slides under weight for approximately one day, to prevent inaccurate measurements of curled leaves. Sample size (*n*) for each treatment-line-leaf stage combination was between 10-20.

Statistical Analysis

Each variable was averaged across the two leaves within each pair, creating what is heretofore referred to as a "leaf stage". Normality of ANOVA residuals were tested with Shapiro-Wilks tests, and variables were transformed with Tukey's ladder of power transformation, to meet ANOVA assumptions. General variation in elevated [CO_2] response between *Arabidopsis* genotypes and variation in response between leaf stages within individual genotypes were examined via linear mixed model ANOVAs, employing the model in formula 1; X represents the various patterning variables previously described.

(Formula 1) X ~ [CO_2] treatment * Genotype * Leaf Stage + (1 + Leaf Stage | Plant Replicate)

Significant three-way ANOVA interactions were followed by post-hoc testing, with Tukey adjustment, to detect specific differences in elevated [CO_2] response in each individual stage in a genotype (e.g. difference in distance of trichomes from the leaf tip between 400 and 800 ppm [CO_2] in stage 1 of Ler). All statistical tests were performed in R.

Results

Comparing composite trichome patterns for each line/stage combination under ambient and elevated [CO_2] we determined more concrete ways to define shifting trichome patterns under elevated [CO_2] (Fig. 10). Namely, elevated [CO_2] identifiably altered distribution of trichomes *across* the leaf, and the spatial relationships trichomes held with neighboring trichomes.

Trichome distance from leaf tip

Elevated [CO_2], within certain lines, drove a shift in the average distance of trichomes from the tip of the leaf (Fig. 11; Tables 3 and 4). There was a significant interaction between [CO_2], line, and leaf stage for distance of trichomes from the leaf tip ($P < 0.001$). Kondara (stage 2; $P = 0.0412$) displayed a significant increase in the ratio of trichomes nearer to the leaf tip, while Ler (stage 2; $P < 0.001$) and Ws (stage 3; $P = 0.0128$) displayed decreases (Table 4). These significant shifts in trichome distribution were confirmed with composite heatmaps, and 50% base:50% tip division testing.

Local trichome densities

There was a significant interaction between [CO_2], line, and leaf stage in both the larger and smaller observation area trichome densities ($P = 0.0224$ and $P = 0.0425$, respectively) (Table 3; Figs. 12 and 13). Several genotypes/stages had distinct shifts in local trichome densities; Col (stage 2) significantly increased (Tables 3 and 4) trichome densities in a large area surrounding

individual trichomes (P = 0.00381; Table 4), but did not significantly alter trichome densities in a smaller area around individual trichomes (P = 0.255; Table 4). Col (stage 3) had a similar increase in trichome density in the larger observation area (P = 0.0164; Table 4)—but in contrast to Col (stage 2)—we observed an *increase* in trichome density in the smaller observation area (P < 0.001; Table 4).

Discussion

Trichome patterning is an understudied phenotype, and the responses of trichome patterning to environmental perturbations, including elevated [CO_2], is wholly unrealized. In our study, we developed novel methods to investigate: the possibility of a trichome patterning response to elevated [CO_2], the genotypic variation in trichome patterning response to elevated [CO_2], and the impacts of elevated [CO_2] on trichome patterning across whole-plant development (Tables 3 and 4). We found a significant interaction between elevated [CO_2], genotype, and whole-plant developmental stage across all patterning variables we observed, which indicates elevated [CO_2] affects trichome patterning, with significant genotypic variation (Table 3). Below, we use these findings to hypothesize on the potential molecular underpinnings of trichome patterning shifts at elevated [CO_2], and potential ecological ramifications of shifts in trichome patterning.

Across all *Arabidopsis* genotypes we investigated, elevated [CO_2] significantly affected leaf trichome patterning at one or more points in whole-plant development (depending on genotype; Figs. 11, 12, and 13, Table 4). Some lines—such as Kon—were strongly responsive, with patterning responses to elevated [CO_2] across all three developmental stages (Figs. 11, 12, and

13; Table 4. Conversely, Cvi was generally the most unresponsive genotype, with significant responses in only one patterning variable at only one stage (stage 2; Figs. 11, 12, and 13; Table 4). As such, we found high genotypic variation in elevated [CO_2] response across all of the trichome patterning variables that we measured (Table 3).

Whole-plant development response

Within *Arabidopsis* lines, we observed strong variation in trichome patterning responses to elevated [CO_2] across developmental stages (Tables 3 and 4). There was no genotype in which we observed a similar response of patterning to elevated [CO_2] across all three developmental stages (Figs. 11, 12, and 13), indicating developmental stage of the plant plays an important role in trichome patterning responses to elevated [CO_2]. We hypothesize that whole-plant developmental pathways (i.e. flowering) that are pleiotropic with trichome initiation/patterning may be responding at elevated [CO_2] and potentially driving differences in elevated [CO_2]-trichome responses among developmental stages.

Leaf-level molecular aspects

To gain insight into the potential effects of elevated [CO_2] on trichome-to-trichome communication (e.g. negative regulators of trichome initiation), we observed several different patterns in trichome densities surrounding a given trichome. One of the more distinctive responses was Col (stage 3), in which elevated [CO_2] produced an increase in trichome density in the area surrounding each trichome, but when a smaller individual area was investigated the local trichome density was found to *not respond* under elevated [CO_2] (Figs. 12 and 13). This could

potentially indicate a maintenance of the negative trichome regulators (e.g. TRY, CPC) involved in trichome patterning, which could maintain the zone of inhibition around each trichome although overall trichome density is increasing (Balkunde et al., 2010; Bouyer et al., 2008; Pesch & Hülskamp, 2011). Furthermore, some genotypes/stages exhibited a similar response but in the opposite direction. For example, in Col (stage 2) we observed an increase in trichome densities in the smaller observation areas were found (Fig. 13). This result suggests a relaxation of negative trichome regulation and potentially a decrease in mobile trichome regulator proteins, which is opposite of the response observed in Col (stage 3).

Elevated [CO_2] may affect the timing of trichome initiation during primordial leaf development. If trichomes developed later in the trichome initiation time window, we would expect to see an increased trichome density near the basal portion of the leaf, as observed in Kon (stage 2; Fig. 11), since trichomes initiate at the base of the developing leaf (Larkin et al., 1996). If, instead, elevated [CO_2] triggered an increase in trichome initiation very early in leaf development, we would expect to see an increase in the density of trichomes on the tip of the leaf, as was observed in Ler (Stage 2; Fig. 11), for example (Larkin et al., 1996). As we observed unbalanced responses to elevated [CO_2] between the tip and the base of the leaf, we therefore conclude that elevated [CO_2] may affect the *timing* of trichome initiation during leaf development. Elevated [CO_2] could perhaps increase the expression of trichome initiation genes early in leaf development, to produce the result we saw in Ler (stage 2; Fig. 11), or perhaps elevated [CO_2] could cause the trichome initiation time frame to begin earlier in leaf development (Larkin et al., 1996; Yu et al., 2010). We recommend an investigation into the expression of trichome genes

across the development of a single leaf, to investigate if elevated [CO_2] can affect the expression of trichome genes during the trichome initiation window.

Genotypic variation

We observed high variability in trichome density, number, and patterning responses to elevated [CO_2] across *Arabidopsis* lines, as well as between developmental stages within lines (Tables 3 and 4). This level of genotypic variation indicates the potential for different molecular pathways to be acting to influence trichome densities and patterning in response to elevated [CO_2]. There are multiple pathways that are known to be pleiotropic with trichome initiation/patterning and which have genotypic variability in elevated [CO_2] response. Flowering genes are known to regulate trichome negative regulators, and there is strong variability in the response of flowering to elevated [CO_2] across *Arabidopsis* ecotypes (Matias-Hernandez, 2016; C. J. Springer, Orozco, R.A., Kelly, J.K., Ward, J.K., 2008; C. J. Springer & Ward, 2007; Xu et al., 2016; Xue et al., 2014; Yu et al., 2010). Similarly, stomatal densities are known to respond under elevated [CO_2], which is dependent upon genotype, and stomatal initiation and trichome initiation/patterning are known to be pleiotropic (Caldera, De Costa, Woodward, Lake, & Ranwala, 2016; Yan et al., 2014). Therefore, there are multiple molecular pathways that are sensitive to elevated [CO_2] and that are pleiotropic with trichome patterning. Thus, genetic variation in these pathways could explain the genotypic variability we observed across *Arabidopsis* genotypes (Figs. 11, 12, and 13, Tables 3 and 4).

Ecological perspective

There exists only a small amount of literature concerning the effects of trichome patterning on the physiological roles of trichomes, and these sources mostly deal with a trichome/trichomeless dichotomy (Løe et al., 2007). As the consequences of generally shifted trichome patterns are wholly unrealized, as are the consequences of altered trichome patterns under elevated [CO_2]. We predict that an alteration in trichome patterning under elevated [CO_2] would lead to consequences for the physiological roles trichomes play. For example, an increase in trichome clustering could lead to areas of increased trichome densities, but would also leave large gaps in trichome coverage across the leaf. These gaps would increase the amount of tissue unprotected from herbivory, potentially increasing herbivore damage to the leaf. Similar consequences to water conservation, fungal infection, and UV-B damage may also result from shifted trichome patterning (Calo et al., 2006; Kergunteuil et al., 2018; Kim, 2019; Løe et al., 2007; Manetas, 2003; Sletvold, 2010). Shifts in trichome patterning under elevated [CO_2], in addition to the potential consequences arising from shifts in trichome density, could have ramifications for leaf physiology and whole-plant fitness. We recommend future research focus on the ecological consequences of altered trichome patterning under elevated [CO_2], utilizing the novel methods we used here for trichome pattern quantification.

Conclusions

Here, we showed that elevated [CO_2] shifts trichome numbers and densities, as well as patterning of trichomes across the leaf surface. Furthermore, we showed evidence that elevated [CO_2] may alter trichome-to-trichome spatial relationships, possibly through the negative regulators controlling their initiation. The impacts of elevated [CO_2] on trichome patterning was highly variable across *Arabidopsis* lines, as well as across developmental stages within lines. From our

study, we conclude that elevated [CO_2] can have varying impacts on trichome patterning, and this may be an indication that there are a number of pathways influencing the response of trichome initiation to elevated [CO_2] across *Arabidopsis* lines and developmental stages.

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Chapter 3

Molecular mechanisms potentially underlying the responses of trichome density to elevated [CO_2] in *Arabidopsis*

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Note to Dissertation Committee: We previously published this chapter in "Photosynthesis, Respiration, and Climate Change" (Springer, 2019). For this dissertation, we have added novel RNA-sequencing results from our study concerning the impacts of elevated [CO_2] on gene expression during early leaf development. Results from our RNA-sequencing work provide more concrete future directions for trichome-elevated [CO_2] research, as well as providing the first published data concerning trichome gene expression across early leaf development.

Abstract

Leaf hairs (trichomes) are small and rigid epidermal structures that serve in herbivore defense, temperature regulation, boundary layer fortification, and UV-B protection, and can even act as mechanosensory switches indicating insect herbivore presence (Zhou et al. 2016; Xiao et al. 2017). As such, leaf trichomes have impacts on overall plant physiology, photosynthetic efficiency, fitness, and plant-environment interactions (Calo et al. 2006; Løe et al. 2007; Sletvold 2010; Nguyen et al. 2016; Xiao et al. 2017; Imboden et al. 2018; Kergunteuil et al. 2018; Kim 2019). Elevated [CO_2] affects plants across multiple scales, ranging from the molecular and physiological levels to the ecosystem level (Masle 2000; Bidart-Bouzat et al. 2005; Teng et al. 2006; Medeiros and Ward 2013; Becklin 2016; Dong et al. 2018). It has been noted that leaf trichomes shift in density on the leaf surface when grown at elevated [CO_2] in a number of species (Masle 2000; Bidart-Bouzat et al. 2005; Lake and Wade 2009; Karowe and Grubb 2011; Guo et al. 2013; Guo et al. 2014). Elevated [CO_2] *decreases* trichome densities by as much as 60% in some species (wheat, *Arabidopsis*) and increases densities by as much as 57% in other species (*Brassica rapa, Medicago truncatula*) (Karowe and Grubb 2011; Guo et al. 2013; Guo et al. 2014). However, the responses of trichomes to elevated [CO_2] remain critically understudied, and little is known about the molecular/developmental mechanisms driving these responses. As trichomes are physiologically important structures for numerous food crop species (e.g. wheat, soybean) and critical ecological species, it is imperative that further research be dedicated to understanding the implications of shifting trichome densities in an elevated [CO_2] environment of the future.

Here we review the ecological roles of trichomes, describe what is known about elevated [CO_2]-trichome phenotypic responses, provide a relevant background of trichome genetics, and present

potential molecular/developmental mechanisms behind elevated [CO_2]-trichome responses. Finally, we propose future research directions to stimulate future elevated [CO_2]-trichome research in order to uncover the mechanisms that control altered trichome densities at elevated [CO_2].

Introduction

The atmospheric concentration of carbon dioxide ([CO_2]) currently resides at approximately 410 ppm, having risen from a pre-industrial value (approximately 200 years ago) of 270 ppm (NOAA 2016). Atmospheric [CO_2] is projected to reach 800-1000 ppm by the end of this century if CO_2 emissions are not curtailed (NOAA 2016). Elevated [CO_2] affects plants across multiple scales, ranging from the ecosystem level to the physiological and molecular levels, whereby elevated [CO_2] can alter leaf gas exchange, plant nutritional quality, growth, development, and production of secondary defense compounds (Masle 2000; Bidart-Bouzat et al. 2005; Teng et al. 2006; Medeiros and Ward 2013; Becklin 2016; Dong et al. 2018). Elevated [CO_2] may also influence the production and density of micro-structures on the leaf surface—such as stomata (pores required for gas exchange) and trichomes (leaf hairs) (Masle 2000; Bidart-Bouzat 2004; Bidart-Bouzat et al. 2005; Lake and Wade 2009; Karowe and Grubb 2011; Haus et al. 2018). Such changes can have major effects on plant carbon assimilation, water use, and defense. Stomata densities (number of stomata normalized by leaf area) and/or indices (percentage of stomata to epidermal cells) shift in a large number of plant species grown at elevated [CO_2] and under other climatic shifts (Woodward and Kelly 1995; Teng et al. 2006). Wide-scale attention has been paid to understanding the mechanisms that control stomatal density shifts at elevated [CO_2], ranging from changes in cuticular wax composition to mobile signals transported through the phloem (Gray et al. 2000; Lake et al. 2001; Ainsworth and Rogers 2007; Lake and Woodward 2008; Haus et al. 2018; See Xu et al., 2016b for an excellent review on stomata and elevated [CO_2]).

Relevant to this review, trichomes have also been shown to shift in density at elevated [CO_2], along with stomata densities (Bidart-Bouzat et al. 2005; Lake and Wade 2009). However, altered trichome production at elevated [CO_2] and the mechanism(s) controlling these responses remain critically understudied. Generally, the ecological and physiological roles of trichomes have been well characterized, as has the genetic and developmental pathways behind trichome initiation (Pesch and Hülskamp 2009; Balkunde et al. 2010; Bickford 2016; Xiao et al. 2017). This past work provides an excellent foundation for trichome research that is aimed at understanding the implications of elevated [CO_2] on trichome production and provides great insights for enhancing our ability to elucidate the molecular mechanisms driving trichome density shifts at elevated [CO_2].

In a review of the literature, we found that elevated [CO_2] reduces trichome densities by as much as 60% in some species (e.g. wheat) and promotes trichome density increases as high as 57% in other species (e.g. *Brassica rapa*) (Masle 2000; Karowe and Grubb 2011; Guo et al. 2013; Guo et al. 2014). These altered trichome densities that may deviate from locally adaptive trichome densities could potentially alter plant fitness, herbivory damage, water use efficiency (WUE), fungal infection rates, and photosynthetic efficiency (Manetas 2003; Calo et al. 2006; Løe et al. 2007; Sletvold 2010; Kergunteuil et al. 2018; Kim 2019). Therefore, trichome density shifts may have large implications for the responses of native species and food crops (e.g. wheat, soybean) to future global climate change, and further research will be necessary for understanding the underlying mechanisms of shifting trichome densities in an elevated [CO_2] future.

In this review we describe (1) the multiple roles of trichomes, (2) trichome responses to elevated [CO_2], (3) molecular mechanisms of trichome production, (4) potential mechanisms for shifted trichome densities at elevated [CO_2], and (5) directions for future research. We have culled information on numerous trichome genes, and potential connections between elevated [CO_2], trichome densities/patterning, and other elevated [CO_2]-responsive physiological/developmental processes as the basis for this review. Moreover, the purpose of this review is to unite multiple fields in an effort to reveal a framework of potential molecular mechanisms that control the responses of trichome productivity to rising [CO_2].

The multiple roles of trichomes

Trichomes appear in a host of different forms, ranging from single-cell rigid hairs, as in *Arabidopsis thaliana* (Fig. 1a) to fluid-secreting (glandular), multicellular structures such as those found in tomato (*Solanum lycopersicum*). With highly varied functions and structural morphologies, trichomes can be found on all aerial organs of the plant, across the majority of plant species (Werker 2000). In this review, we primarily focus on leaf trichomes since they have the largest implications for plant water use and carbon assimilation. Leaf trichomes have impacts on overall plant physiology, photosynthetic efficiency, fitness, and plant-environment interactions (Xiao et al. 2017). More specifically, leaf trichomes act in herbivore defense, temperature regulation, boundary layer fortification, and UV-B protection. Further, there have been shown to be trade-offs between trichome production and fitness/performance, and elevated [CO_2] may potentially offset this balance (Hanley 2007; Sletvold 2010). Below we provide a more thorough description of the many roles that trichomes play in plant physiology, defense, and overall survival.

Herbivore defense

One primary physiological role of non-glandular trichomes is mechanical defense of the leaf. Sharp branch tips on trichomes (Fig. 1b) deter large herbivores and can injure smaller insects or trap them until starvation or predation occurs (Johnson 1975; Krimmel and Pearse 2013). In glandular trichomes, defensive capabilities such as those present on *Solanum lycopersicum* (cultivated tomato) rely on noxious compounds produced from the tip of the trichome as a means of chemical defense against insect herbivores (Glas et al. 2012; Bergau et al. 2015). Trichomes have also recently been shown to act as mechanosensory switches whereby a large insect herbivore can buckle a trichome, triggering a shift in leaf pH in the area surrounding the trichome, potentially initiating secondary metabolite defense mechanisms (Zhou et al. 2016).

A lack or reduced level of trichome-based herbivore defenses may be detrimental to plant survivorship, which is especially important when considering potential shifts in trichome densities at elevated [CO_2]. In natural *Arabidopsis lyrata* populations from across Sweden, glabrous (lacking trichomes) individuals consistently exhibited increased herbivore damage compared to trichome-producing individuals (Løe et al. 2007). The difference in herbivory was even more pronounced for mixed populations with both glabrous and trichome-producing individuals, whereby there was selection for higher trichome densities in mixed populations (Løe et al. 2007).

Temperature regulation

Trichomes aid in temperature regulation on the leaf surface, which primarily occurs through reflectance of solar radiation. Pubescent lines of *Encelia farinosa*, for example, were shown to regulate leaf temperature through trichome-mediated light reflectance, whereas glabrous *Encelia frutescens* maintained leaf temperature through heat-dissipating transpiration (Ehleringer 1988). Although *E. farinosa* and *E. frutescens* modulate leaf temperature through two different physiological mechanisms, leaf temperatures were found to be highly similar between the two species—signifying that trichome production is one adaptive pathway that can replace other physiological responses (Ehleringer 1988). There is also wide intraspecific variation in trichome densities, and this density variation can lead to differences in the mechanisms used for leaf temperature modulation. For example, within *E. farinose,* a wide variation in trichome densities exists whereby less pubescent *E. farinosa* individuals increase transpiration to modulate leaf temperatures, while more pubescent individuals reflect excess light via denser trichome mats (Ehleringer and Björkman 1978; Ehleringer and Mooney 1978; Bickford 2016).

Conversely, extremely trichome dense leaves may experience increased overheating. For example, dense pubescence in *Euphorbia wallichii*, native to the Himalaya-Hengduan Mountains, absorbed high amounts of solar radiation during times of peak radiation, leading to reduced heat loss and an increased likelihood of overheating (Peng et al. 2015). Others have hypothesized that the connection between dense pubescence and increased leaf temperature may be adaptive to pubescent arctic species, but this hypothesis requires further investigation (Peng et al. 2015).

Boundary layer fortification

In addition to regulating temperature and thereby water loss, trichomes also reduce water loss through transpiration via fortification of the boundary layer between the leaf surface and the ambient outside air (Konrad et al. 2015). The thickness of the boundary layer, fortified by trichome presence, scales with resistance to water diffusion (Amada et al. 2017). The contributions of trichomes to improved water use efficiency (WUE; carbon gain per water loss) vary significantly among species. In *Metrosideros polymorpha*, only 9% of resistance to water flux can be attributed to trichome resistance (Amada et al. 2017). Conversely, *S. lycopersicum* trichome densities are significantly positively correlated with WUE and significantly negatively correlated with stomatal conductance (g_s) (Galdon-Armero et al. 2018). Further, the ratio of trichomes to stomata (T/S) on the leaf surface also positively correlates with WUE in tomato (Galdon-Armero et al. 2018). T/S, therefore, may be viewed as a measurement to interconnect stomatal densities, trichome densities, and gas exchange (Galdon-Armero et al. 2018). In addition to T/S, whole leaf characteristics also play a role in determining the magnitude to which trichomes can control water loss. Small leaves subjected to low wind speeds, for example, had high contributions of trichome layers to water flux resistance (Schreuder et al. 2001). Through modelling, the relationship between leaf size and shape, wind speed and the effects of trichome characteristics and densities is useful for determining whether trichomes play a significant role in boundary layer resistance in a given scenario, and understanding these relationships may prove important in a changing climate (Schreuder et al. 2001).

UV-B & photosystem II protection

Trichomes protect the leaf photosystems from excess light via a papillae coating on the trichome surface (Fig. 1d) and through phenolic compounds, which individually lower internal leaf

temperatures and prevent UV-B damage to photosystem II (PSII) (Johnson 1975; Ehleringer and Björkman 1978; Manetas 2003). When trichomes were experimentally removed from photosynthetic stems of *Verbascum speciosum*, these hairless stems exhibited significant decreases in photosystem II yield (23% decrease overall) and electron transport rates (14% decrease overall) when exposed to UV-B (Manetas 2003). Increased UV-B exposure, in conjunction with lowered trichome densities, has other physiological effects in addition to impaired photosynthesis. For example, *Arabidopsis* mutants with reduced or absent trichomes exhibited increased effects of UV-B, such as shifts in flowering time due to the impacts of stress on plant development (Yan et al. 2012). Furthermore, a trichome mutant (*gl1*) with reduced trichome densities displayed a 62% delay in flowering time when exposed to elevated UV-B, while the control (Col-0) and a trichome over-producing mutant (*try82*) displayed only 41% and 35% delays in flowering time, respectively (Yan et al. 2012). Thus, altered leaf trichome densities not only shift the micromorphology of the leaf, but can influence the entire development of the plant under high levels of stress.

Environmental adaptation

Maintaining an optimal trichome density for the enhancement of plant fitness may involve a fine balance between maximizing trichome density to inhibit herbivory and alleviate the impacts of environmental stressors (e.g. UV-B), while simultaneously preventing the impacts of overproduction of trichomes including fungal infections and excessive energy expenditure (Fig. 2) (Calo et al. 2006; Løe et al. 2007; Sletvold 2010; Nguyen et al. 2016; Imboden et al. 2018; Kergunteuil et al. 2018; Kim 2019). Such a balance between too few and too many trichomes is important to consider with regards to shifting trichome densities at elevated [CO_2]. Trichomes of

maize, *Brachypodium distachyon*, and barley act as entry points for *Fusarium* infection, with fungal hyphae gaining entry into the leaf through trichome base socket cells (Fig. 1c) (Peraldi et al. 2011; Nguyen et al. 2016; Imboden et al. 2018). In addition, a glabrous *Arabidopsis* trichome mutant (*gl1*) showed increased resistance to the necrotrophic fungus *Botrytis cinereal* compared to wildtype, while a trichome overproducing mutant (*try*) was more susceptible to fungal infection (Calo et al. 2006). Therefore, excessive trichome density may not be entirely beneficial, as increased trichome densities carry increased risk of detrimental fungal infections, and possibly other yet unknown outcomes.

Trichomes can be energetically costly to produce, whereby trichome-dense lines of *A. lyrata* grown in the absence of herbivory exhibited decreased fitness and performance compared to plants exposed to herbivory where trichomes were beneficial (Sletvold 2010). A similar observation was made in *A. thaliana* whereby trichome density was found to be significantly negatively correlated with fruit number, suggesting fitness implications as a result of excessive trichome production (Mauricio 1998). Across an elevational gradient in the Swiss Alps, plant communities located at the lowest and highest elevations were the most trichome dense, while mid-elevation communities had the lowest overall trichome densities (Kergunteuil et al. 2018). This observation may be explained by the fact that high elevation sites are adapted to higher UV-B exposure, while low elevation sites may be subjected to higher herbivory pressure (Kergunteuil et al. 2018). Thus, mid-elevation communities in this case may have experienced both relaxed herbivory and UV stress that could have contributed to lower energetic costs devoted to trichome production (Kergunteuil et al. 2018). Therefore, the fitness/performance costs of trichome production is context dependent, influenced by plant species, micro-habitat,

and environmental pressures, and likely the interaction of these factors (Hanley 2007; Sletvold 2010).

Taken together, the above studies indicate that trichomes play physiological and ecological roles across many species, and that selective pressures may "fine-tune" levels of trichome densities to match local abiotic/biotic pressures (e.g. risk of fungal infection, UV-B levels, herbivory) (Løe et al. 2007; Kergunteuil et al. 2018). Therefore, we must understand and better predict how trichome densities may be altered in response to future climate change, including rising atmospheric [CO_2]. Since many major crop species rely on trichome-dense leaves to reduce environmental stress, a shift in trichome density in future global climate scenarios could pose a challenge to crop productivity and food production. In more natural ecological settings, if trichome densities are shifted outside of adaptive realms, a plant may be more susceptible to overheating, herbivory, excessive energy investment, or increased fungal infection. In addition to shifting trichome densities, elevated [CO_2] may reduce stomatal densities and stomatal apertures, which can reduce the cooling ability of the leaf through transpiration limitation, further contributing to potential heat stress on leaves through an interactive effect through alterations on both micro-structures (Engineer et al. 2016).

The potential impacts of altered trichome densities on crop and native species physiology, survival, and yield in future environments are poorly understood and therefore we cannot fully predict plant responses in the future without this understanding. Future work must address the potential for elevated [CO_2] to disrupt past adaptive trichome densities of a variety of species, and how such disruptions may affect plant physiology, defense, and overall fitness. While the

roles of trichomes are well known, as are the consequences of trichome density shifts—through leaf manipulation and mutant analyses—we know little about the effects of elevated [CO_2] on trichome initiation and densities and the mechanisms that control these responses. Therefore, we take this opportunity to summarize the current state of this field and describe how to motivate research to move this field forward in order to improve predictions of the impacts of rising [CO_2] on future plants that will be growing and adapting to elevated [CO_2] environments on a global scale.

Trichome responses to elevated [CO_2]

At elevated [CO_2] (720-900 ppm [CO_2]), trichome density responses occur across a spectrum, with some species exhibiting significant decreases in trichome density while others show significantly higher trichome densities when compared to plants grown in ambient [CO_2] (350-400 ppm [CO_2]) (Fig. 3) (Masle 2000; Bidart-Bouzat 2004; Bidart-Bouzat et al. 2005; Lake and Wade 2009; Karowe and Grubb 2011; Guo et al. 2013; Guo et al. 2014). Responses of leaf trichome densities in plants grown at elevated [CO_2] have been characterized primarily in the model species *Arabidopsis thaliana*. *Arabidopsis* lines Cvi and Col-0 experience significant trichome density *decreases* in response to elevated [CO_2] (approximately 34.5% and 51.2% decreases, respectively) (Masle 2000; Bidart-Bouzat 2004; Bidart-Bouzat et al. 2005; Lake and Wade 2009). The other lines examined were found to be non-responsive (Ler, Zn-0, Edi, Wu-0, Can-0) in trichome densities at elevated [CO_2] (Lake, JA and Wade, RN unpublished, 2009; Lake and Wade 2009). Similar to *Arabidopsis*, wheat (*Triticum aestivum* L.) shows intraspecific variation in trichome density responses to elevated [CO_2] (900 ppm [CO_2]) (Masle 2000). Trichome densities of the Hartog cultivar decreased significantly at elevated [CO_2], with an

approximate 60% decrease compared to plants grown at ambient conditions. However, it is worth noting that this significant trichome density response was only observed in *non-vernalized* Hartog plants. Furthermore, the decrease response observed in wheat was line dependent, with no response observed in the Birch cultivar (Masle 2000). Therefore, the effects of elevated [CO_2] on trichome density are far reaching, including important crop species, with the significance of the trichome density decrease partially dependent upon other environmental factors.

Along with the above examples of trichome reductions, some species exhibited significant *increases* in trichome densities at elevated [CO_2]. For example, elevated [CO_2] increases trichome densities in *Brassica rapa* (approximate 57% increase; 744 ppm [CO_2]) and *Medicago truncatula* (up to an approximate 42% increase; 750 ppm [CO_2]) (Karowe and Grubb 2011; Guo et al. 2013; Guo et al. 2014). Elevated [CO_2] increased the densities of non-glandular trichomes on both young and mature *M. truncatula* (cv. Jemalong) leaves (approximately 26% and 13% density increase respectively), but [CO_2] enrichment increased glandular trichomes only on *mature* leaves (approximate 42% increase) (Guo et al. 2014). As such, the four species studied up to this point in regard to the impacts of [CO_2] enrichment on trichome densities—*Arabidopsis*, wheat, *B. rapa*, and *M. truncatula*—all have shown a degree of intraspecific variation. Furthermore, the trichome density responses of these species at elevated [CO_2] also show variation dependent upon either trichome types, leaf stages, and/or environmental factors experienced by the plants. Overall, there is too little research to determine whether increases or decreases are most common among and within species; more phenotypic data is needed.

As described above, in the limited work available, elevated [CO_2] has substantial effects on trichome densities among many species, but the molecular mechanisms underlying shifts in trichome densities at elevated [CO_2] remain unclear. Fortunately, a wealth of molecular information concerning the initiation and patterning of trichomes is available and will be key for understanding trichome responses to elevated [CO_2]. Next in this review, we cover the relevant molecular pathways of trichome production that may pertain to elevated [CO_2], and describe how these pathways are affected by various environmental perturbations (e.g. drought). We then describe potential molecular mechanisms driving responses of trichome densities to [CO_2] enrichment, utilizing the strong foundation of knowledge on the molecular/developmental basis of trichome initiation and production.

Molecular mechanisms of trichome initiation and patterning

Prior to discussing the potential mechanisms that may underly altered trichome production at elevated [CO_2], we first provide a brief introduction on trichome genetics as is relevant to understanding the impacts of elevated [CO_2] (for more details see excellent work by Yang and Ye 2012, and Pattanaik et al. 2014 for reviews of this field; see Pesch et al. 2015 for a relevant update to this field). We will focus on the core genetic mechanisms of trichome production that are most likely to be relevant to elevated [CO_2] effects on trichome densities. The molecular pathways driving trichome initiation in the leaf have been well-elucidated over the past 30 years and trichomes have long served as models for single-cell development and initiation, providing an excellent molecular/developmental foundation for understanding how elevated [CO_2] may intercept trichome pathways (Hülskamp 2004).

Trichome initiation

Here we outline the trichome initiation pathway, beginning with young protodermal cells in the developing leaf primordia, leading up to the point at which the trichome cell begins to protrude from the leaf. Trichome initiation begins with accumulation of transcription factors (TF) GLABROUS1 (GL1) and GLABROUS3 (GL3) in the protodermal cells of early developing leaves (Larkin et al. 1993; Payne et al. 2000; Balkunde et al. 2010). Trichome initiation, and the subsequent resulting trichome pattern, are *de novo*, in that all young protodermal cells on the leaf surface are potentially able to enter into the trichome initiation pathway, ubiquitously expressing *GL1* and *GL3* (Schnittger et al. 1999; Larkin et al. 2003; Zhang 2003; Pesch and Hülskamp 2009). Later in leaf development, *GL1* and *GL3* expression is restricted to trichome initials—cells that have entered the early stages of the trichome morphogenesis pathway (Schnittger et al. 1999; Zhang 2003; Kryvych et al. 2008). Due to stochastic variation in expression among protodermal cells, some cells have increased concentrations of the trichome initiation positive regulators (Larkin et al. 2003). These cells will potentially commit to the trichome pathway, and subsequently inhibit neighboring cells from entering the trichome pathway (Larkin et al. 2003; Hülskamp 2004). GL1-GL3 binding creates the primary building block for the trichome multimer (Fig. 8a), while the physical GL1-GL3 protein interaction is stabilized by the binding of TRANSPARENT TESTA GLABRA1 (TTG1) to GL3 to create a multimeric complex (Fig. 8b; Fig. 8c.1) (Payne et al. 2000; Larkin et al. 2003; Hülskamp 2004; Ramsay and Glover 2005; Pesch and Hülskamp 2009; Yoshida et al. 2009; Balkunde et al. 2010; Tsuji 2013). The GL1/GL3/TTG1 multimer is necessary for the activation of downstream trichome morphogenesis genes, such as those controlling the cell expansion necessary to create the distinct protuberance of the trichome from the leaf (Fig. 8c.1) (Szymanski and Marks 1998; Walker et al.

2000; Johnson et al. 2002; Hülskamp 2004; Wang 2008; Balkunde et al. 2010; Yang and Ye 2012). A cell is locked into the trichome fate once endoreduplication begins, controlled by the cyclin-dependent protein kinase inhibitor *SIAMESE* (*SIM*), the transcription of which is regulated by the GL1/GL3/TTG1 multimer (Walker et al. 2000; Yang and Ye 2012). Furthermore, the GL1/GL3/TTG1 multimer promotes expression of homeodomain transcription factor *GLABROUS2* (*GL2*), mutants of which show defects in post-initiation trichome structure (Johnson et al. 2002; Ohashi et al. 2002; Hülskamp 2004; Wang 2008; Balkunde et al. 2010).

Incongruous with older literature, the protein complex controlling trichome initiation is not strictly a trimer complex (e.g. GL1-GL3-TTG1), and many organizations of the complex are possible (Fig. 8b) (Pesch et al. 2015; Zhang 2018). The GL1-binding domain of GL3 also binds proteins controlling anthocyanin biosynthesis (PRODUCTION OF ANTHOCYANIN PIGMENT 1 [PAP1]) and root hair initiation (WEREWOLF [WER]) (Zhang 2003; Ramsay and Glover 2005). Furthermore, proteins related to phytohormone signal transduction (JAZs, DELLAs) and proteasome protein degradation (UBIQUITIN PROTEIN LIGASE 3 [UPL3]) heterodimerize with GL3 at the C-terminal domain (Qi et al. 2011; Patra et al. 2013; Qi et al. 2014). GL3 homodimerization coupled with the range of GL3-heterodimerizing myb proteins results in numerous potential organizations of the multimer complex (Payne et al. 2000; Zhang 2003; Ramsay and Glover 2005; Qi et al. 2011; Patra et al. 2013; Qi et al. 2014; Pesch et al. 2015; Zhang 2018). Such numerous organizations of the multimer allow for a large swathe of different gene targets, and the multimer promotes different biological processes dependent upon multimer composition. Later in this review, we delve into how elevated [CO_2] may impact overlapping pathways (e.g. trichome initiation and anthocyanin biosynthesis), affecting

organization of the multimer with subsequent effects on trichome initiation and other pathways utilizing components of the multimer.

Negative regulation and patterning

A trichome patterning mechanism is intertwined intimately with the trichome initiation pathway, as there must presumably be a defined distribution of trichomes *across* the leaf (or stem) in order to allow adaptive trichome functionality (Hülskamp 2004). Furthermore, as trichomes form across the leaf surface, they are less often found directly next to one another than a random pattern model would predict (Larkin et al. 2003). Any impacts of elevated [CO_2] on this patterning mechanism could potentially alter trichome functioning, therefore we briefly delve into the mechanics of trichome patterning below.

In a mechanism presumably to reduce trichome clumping, trichome precursor cells produce mobile regulators, TRIPTYCHON (TRY) and CAPRICE (CPC), to prevent neighboring cells from entering the trichome precursor state (Hülskamp et al. 1994; Wada et al. 1997; Wang and Chen 2014). TRY and CPC proteins exit the source cell and enter surrounding protodermal cells (Fig. 8c.2) (Wang and Chen 2014). Thoroughly compromised protodermal cells then fail to enter into trichome initiation, as negative regulators compete with GL1 for binding to GL3 that prevents the formation of the trimeric complex (Hülskamp 2004). TRY/CPC are R3-MYB proteins lacking DNA binding domains, and therefore replacement of GL1 with TRY or CPC in the multimeric complex creates an inactive complex incapable of DNA-binding (Fig. 8a) (Hülskamp 2004; Wang and Chen 2014). As such, *GL2* and *SIM* transcription—and the

transition from protodermal cell to trichome precursor—are prevented by deactivation of the GL1/GL3/TTG1 multimer (Payne et al. 2000; Wang and Chen 2014).

Arabidopsis trichome formation is initiated in the protodermal cells of developing leaves, following the intracellular stochastic buildup of a multimeric protein complex. This complex has often been proposed as a three-protein complex (For excellent reviews see: (Balkunde, Pesch, & Hülskamp, 2010; Pesch & Hülskamp, 2009; Ramsay & Glover, 2005), producing negative regulators that block the formation of the trimer. However, research now indicates a more complex system of competition among an array of complexes, both multimeric and dimeric (Pesch et al., 2015; Zhang, 2018). Throughout this review, a mixed model theory will be used, in accordance with the most recent molecular trichome studies. The newest dimer concept will be used to address the production of trichome negative regulators, but this concept does not address positive regulator production. Therefore, the trimer/multimer concept will be used for positive regulator production, as current data indicates.

Antagonistic dimer complexes, comprised of members of the multimer, promote either *TRY* or *CPC*, depending on the protein composition of the promoting dimer (Hülskamp 2004; Pesch et al. 2015; Zhang 2018). The GL1-GL3 dimer promotes *CPC*, while the formation of the GL1-GL3 dimer is blocked by TTG1. TTG1 binds with GL3 (TTG1-GL3) to promote *TRY*, while the formation of this dimer is blocked by GL1. Therefore, GL1 is capable of downregulating *TRY* expression, while TTG1 is capable of downregulating *CPC* (Fig. 8c.2) (Hülskamp 2004; Pesch et al. 2015; Zhang 2018).

Further contributing to the patterning mechanism, TTG1 is a mobile protein, and when bound to the non-mobile GL1-GL3 complex, becomes trapped in the cell in which this complex is contained (Fig. 8c.3) (Bouyer et al. 2008; Balkunde et al. 2010). Therefore, TTG1 is more likely to become trapped in cells with stochastically higher amounts of GL3, depleting TTG1 in the surrounding cells. This trapping-depletion mechanism adds further stochasticity to trichome initiation, dependent upon the stochasticity of initial GL3 concentrations in the protodermal cells (Bouyer et al. 2008; Balkunde et al. 2010).

Phytohormones and trichome initiation

Trichome densities are in part determined by a subset of intertwined endogenous phytohormones, and as discussed later, concentrations of these hormones are affected at elevated [CO_2] (Traw and Bergelson 2003; Maes et al. 2008; Pattanaik et al. 2014). Jasmonic acid (JA) and gibberellic acid (GA) act in concert to promote the transcriptional functions of the GL1 and GL3 proteins. GL1 and GL3 DNA-binding sites are bound by antagonistic JAZ and DELLA proteins, blocking GL1/GL3 transcriptional functions. These transcription-blocking JAZ and DELLA proteins degrade following JA and GA application, respectively (Qi et al. 2014). Additionally, JA directly regulates 'default' *GL3* expression, positively controlling trichome densities in wild-type plants (Yoshida et al. 2009). Therefore, JA and GA act synergistically to promote trichome initiation and wild-type trichome densities.

The positive regulatory actions of JA/GA are tempered via salicylic acid (SA). SA applied exogenously to the leaf decreases trichome formation (Traw and Bergelson 2003). In support of this, trichome density of leaves post JA application increased 31.4%, but trichome densities decreased 0.9% with application of JA and SA in tandem (*Arabidopsis* ecotype Ler) (Traw and Bergelson 2003). Similarly, GA application alone increased trichome number 72%, while GA application coupled with SA application increased trichome number by only 29.6% in *Arabidopsis* (Traw and Bergelson 2003). The effect of phytohormones on trichome density is dependent upon leaf stage; *GL1* and *GL3* were highly induced by GA and JA in leaf seven of *Arabidopsis*, but much less so in leaf three (Maes et al. 2008). Finally, GA and JA affect negative regulators of trichome initiation as well. *TRY* expression is significantly reduced with application of JA and GA in *Arabidopsis*, while *CPC* expression is significantly induced via JA and GA (Maes et al. 2008). Therefore, wild-type trichome density is in part determined by a balance between GA, JA, and SA in developing leaves that is currently not well understood. As discussed later, elevated [CO_2] affects levels of these phytohormones, and it is tempting to predict a role of phytohormone shifts in the responses of trichome densities to elevated [CO_2].

Root hair-trichome pleiotropy

Root hair initiation—which is known to be altered at elevated [CO_2]—and trichome initiation share core genetic machinery (*TTG1*, *GL3*, *GL2*, etc.), save for *GL1*. In lieu of GL1, WER binds to GL3 in the protein multimer controlling root hair initiation (WER-GL3-TTG1) (Schiefelbein 2003). Despite these overlaps, this genetic machinery plays opposite roles in the initiation of the two structures (Schiefelbein 2003). Namely, positive regulators of trichome initiation (e.g. *GL2*,

GL3) serve as negative regulators of root hair initiation, and vice versa (e.g. negative trichome regulator *TRY* is a positive regulator of root hair initiation).

Niu et al. found that positive regulators of root hair initiation *CPC* and *TRY* were upregulated in the root at elevated [CO_2], while negative regulators of root hair initiation *TTG1*, *GL2*, and *GL3* were downregulated in the root (Niu 2011). Such shifts in core root hair genes lead to increased root hair numbers at elevated [CO_2] (Yue et al. 2009; Niu 2011). As such, although observed in a separate organ of the plant, there is evidence for elevated [CO_2] affecting core trichome machinery (*CPC*, *TRY*, *TTG1*, *GL2*, *GL3*), which may have implications for trichome production as well.

Molecular mechanisms for environmental perturbations of trichome production

While the molecular mechanisms driving trichome density shifts at elevated [CO_2] remain unknown, the mechanisms driving trichome density shifts under several other environmental perturbations have been better described. Drought and excess UV-B have been shown to increase trichome densities in leaves that are initiated subsequent to application of these stressors, in both *Arabidopsis* and *Caragana korshinskii* (Peashrub) (Traw and Bergelson 2003; Yan et al. 2012; Ning et al. 2016). Across experimental field sites in the Loess Plateau (China), drought stressed *C. korshinskii* plants showed an upregulation in core trichome genes *GL2* and *TTG1* (positive regulators), as well as an upregulation in phytohormone GA (Ning et al. 2016). Furthermore, *CPC*—a core negative regulator of trichome initiation—was downregulated in drought-stressed *C. korshinskii* (Ning et al. 2016). Similarly, the core trichome gene *GL3* was shown to be

upregulated in UV-B responsive *Arabidopsis* mutants exposed to excess UV-B, which in turn increased trichome densities (Yan et al. 2012).

Clearly, there is indication in the literature that shifts in trichome densities in response to environmental perturbations can be traced back to core trichome genes. In the sections that follow, we will use these known molecular mechanisms that produce altered trichome production under well-studied stressors (e.g. drought) to provide a foundation for better understanding the possible mechanisms that may be driving altered trichome production at elevated [CO_2]. Being that this field is relatively new, we propose paths for short-term and long-term investigations in the hopes that more attention is paid to this critical research area in the near future.

Potential mechanisms for altered trichome densities at elevated [CO_2]

While the underlying mechanisms driving altered trichome density at elevated [CO_2] are currently unknown, the molecular trichome literature provides a trove of potential mechanisms for explaining the impacts of elevated [CO_2] on trichome production. In this review, we describe potential mechanisms driving shifts in trichome densities at elevated [CO_2] and outline potential research avenues that would further distinguish these possible mechanisms. We outline known elevated [CO_2] responses outside of trichome initiation, and how these responses may be intertwined with trichome initiation, either in developmental signaling (see mechanism A. phytohormone shifts and mechanism B. cuticle and signal transmission) or elevated [CO_2] responsive-pathway overlaps (see mechanism C. flowering and trichome pleiotropy). By combining information from numerous publications, we determined overlaps between known

elevated [CO_2]-responsive pathways and the trichome initiation pathway (Figure 14a offers a general overview of how elevated [CO_2] connects to overarching physiological/developmental categories, and figure 15b offers a closer look at the genes within these physiological and developmental categories and potential pleiotropies). This was conducted using numerous literature sources to identify pleiotropies and overlaps in these pathways and constructed using a database of trichome genes (keyword-coded citation database available upon request), and displayed with the R package GOplot (Lolle et al. 1997; Tsuji 2013; Yan et al. 2014; Walter 2015; Matias-Hernandez 2016). We use the information shown in figure 14—such as the connection between anthocyanin genes (e.g. *PAP1*), trichome genes (e.g. *GL3*), and elevated [CO_2]—to formulate the potential mechanisms we highlight below (Fig. 14b). A majority of our hypotheses concern overlap between trichome initiation and other pathways, both at the whole-plant and leaf-levels. As shown in figure 14, there are numerous connections between anthocyanin, flowering, stomata production, and trichome production; a number of these overlapping pathways are known to be elevated [CO_2]-responsive. We draw from the growing elevated [CO_2] literature to extrapolate possible mechanisms from known elevated [CO_2]-responsive pathways (e.g. stomata).

Mechanism A. Phytohormone concentration shifts

Trichome density shifts at elevated [CO_2] may be tied into a larger [CO_2]-response cascade, rather than a direct mechanism targeting trichome density (Fig. 15a). Phytohormones influence most plant physiological processes and are known to shift in concentration at elevated [CO_2] (Ivakov and Persson 2013; Matias-Hernandez 2016). In 2016, Sun et al. briefly acknowledged phytohormone concentration shifts as a possible mechanism behind trichome density shifts at

elevated [CO_2] (Sun et al. 2016). SA concentration was shown to increase by 70% and JA concentration decreased by 35% in plants grown at elevated [CO_2] (750 ppm [CO_2]) (Zavala et al. 2012; Sun et al. 2013). As SA is a potent inhibitor of trichome initiation and JA is a positive regulator of trichome initiation, the observed shifts at elevated [CO_2] could potentially decrease *GL3* transcription and thus trichome initiation itself (Fig. 15a) (Teng et al. 2006; Zavala et al. 2012; Sun et al. 2013; Sun et al. 2016). Yet, further work is required to determine if *GL3* is downregulated at elevated [CO_2], and whether phytohormones might be the mediators of such a response (Teng et al. 2006; Sun et al. 2013; Sun et al. 2016). On the contrary, GA is a required positive regulator of *GL3* and *GL1* transcription, and is *upregulated* (55% increase) at elevated [CO_2] (700 ppm [CO_2]) (Chien and Sussex 1996; Perazza et al. 1998; Traw and Bergelson 2003; Teng et al. 2006; Tian et al. 2016). Increased GA may fail to rescue trichome production at elevated [CO_2], as GA requires the presence of elevated [CO_2]-attenuated JA for trichome initiation (Qi et al. 2014). Finally, as JA and GA affect the expression of core trichome negative regulators differentially (i.e. JA and GA upregulate *CPC* but downregulate *TRY*), further understanding of the shifts in complex ratios of phytohormones and core trichome genes at elevated [CO_2] is needed (Maes et al. 2008).

Phytohormone concentration shifts at elevated [CO_2] are also thought to be tied to increased carbohydrate reserves, and therefore altered trichome density may be potentially traced upstream to whole-plant carbohydrate status (Teng et al. 2006). To test the role of phytohormones, we recommend experiments to measure phytohormone ratios at elevated [CO_2] (e.g. SA:JA:GA) with concurrent measures of gene expression in core trichome genes and other potentially related

factors such as plant carbohydrate status). Furthermore, more work is needed to better understand how elevated [CO_2] effects on phytohormones and trichomes may alter herbivory. Although elevated [CO_2] increases the defense hormone SA, the reduction of JA and ethylene (ET) at elevated [CO_2] decreases the time it takes for aphids to reach the phloem, increasing aphid population abundance and adult aphid mass (Sun et al. 2013; Sun et al. 2016). As such, further study of how the combined effects of elevated [CO_2] on phytohormones *and* defensive trichome densities affect herbivore damage will be important.

Mechanism B. Cuticle and signal transmission

Alternative to changes in the trichome-altering signal itself (e.g. mechanism A, Phytohormone concentration shifts), elevated [CO_2] could alter the *efficiency* to which a trichome-influencing signal spreads across the leaf (Fig. 15c). Since trichome initiation occurs within a specific timeframe, the speed or distance to which a trichome signal can reach surrounding competent protodermal cells may in part determine the final outcome of trichome density across a leaf (Lloyd et al. 1994; Larkin et al. 2003). The uppermost wax layer of the leaf cuticle may act as a conduit for signals which alter trichome and stomata density, and changes in cuticle wax composition are known to shift the densities of leaf structures, including trichomes and stomata (Gray et al. 2000; Bird and Gray 2002). *HIGH CARBON DIOXIDE* (*HIC*), primarily expressed in guard cells, contributes to cuticle wax constituency through biosynthesis of long chain fatty acids (VLCFA) (Engineer et al. 2016). In *HIC Arabidopsis* mutants grown at elevated [CO_2], stomatal densities significantly increased, compared to the non-responsive wild-type background (ecotype C24) (Gray et al. 2000). Bird and Gray (2002) offer two hypotheses for how *HIC* prevents increases in wildtype stomatal density at elevated [CO_2]: VLCFAs temper stomatal

densities by either (1) aiding in the diffusion of an unidentified stomata inhibitor across the surface of the leaf, or (2) directly inhibiting stomatal initiation itself (Bird and Gray 2002). Trichome production partially relies on a gene highly homologous to HIC— *FIDDLEHEAD* (*FDH*/*KCS10*), also a member of the 21-gene 3-ketoacyl-CoA synthase (KCS) family —with both genes involved in the synthesis of VLCFA elongases (Lolle et al. 1997; Yephremov 1999; Gray et al. 2000; Joubes et al. 2008). *FDH* mutation in *Arabidopsis* decreases leaf trichome densities approximately 50% compared to wild-type (ecotype Ler) (Yephremov 1999; Bird and Gray 2002). Furthermore, *FDH* is expressed primarily in trichomes and is required in the early stages of trichome formation, with core trichome proteins GL1 and GL3 targeting the *FDH* promotor (Gray et al. 2000; Bird and Gray 2002; Morohashi and Grotewold 2009; Hegebarth et al. 2016). Due to the role of *FDH* in trichome initiation, and the close relationship between *FDH* and *HIC*, it is tempting to infer that *FDH* and wax constituency might play a role in alterations of trichome initiation at elevated [CO_2] (Fig. 15c). If this mechanism were in fact driving trichome density shifts at elevated [CO_2], further research may reveal exciting insights into the role of cuticle wax as a signaling mechanism. Furthermore, as the primary role of the waxy cuticle is to reduce water loss through the leaf epidermis, elevated [CO_2] may affect water loss through both shifts in cuticle wax composition *and* trichome densities (Riederer and Schreiber 2001). Therefore, more work is required to connect wax composition, trichome densities, and stomatal densities at elevated [CO_2], and how these possibly intertwined responses may interact to affect leaf and whole-plant water loss.

Mechanism C. Flowering and trichome pleiotropy

The following potential mechanism for shifting trichome densities at elevated [CO_2] takes a more whole-plant approach to the potential molecular mechanism underlying altered trichome densities at elevated [CO_2]. Here we consider a mechanism where trichome density shifts may be responding to alterations in whole-plant developmental programs that are sensitive to elevated [CO_2] (Fig. 16b). The following potential mechanism is derived from known overlaps between floral initiation and trichome initiation, along with well-characterized responses of floral initiation to elevated [CO_2].

The time a plant takes to reach the flowering stage is known to shift at elevated [CO_2], and both accelerations towards floral initiation and delays of floral initiation have been observed in numerous species at elevated [CO_2] (Springer and Ward 2007; Springer 2008). Trichome initiation and floral initiation overlap in several key pathways, including *microRNA156* (*miR156*) (Yu et al. 2010; Xue et al. 2014; Matias-Hernandez 2016; Xu et al. 2016a). *miR156* is a small non-coding RNA, responsible for silencing genes involved in several developmental processes including flowering. *miR156* is highest in young *Arabidopsis* seedlings and declines as plants age and approaches flowering, during which time levels of *miR156*-silenced *SQUAMOSA PROMOTER BINDING-LIKE*S (*SPLs*) increase (Yu et al. 2010). *mir156*-regulated *SPLs (*e.g. *SPL9*) are responsible for the transition to flowering, and relevant to this review, upregulate the negative trichome regulators *TRY* and *TRICHOMELESS1* (*TCL1*) (Yu et al. 2010). Furthermore, SPL4 and SPL5 recruit TTG1, disrupting the formation of the multimer promoter complex and reducing *GL2* transcription (Ioannidi et al. 2016).

miR156 and associated *SPLs* not only control the floral transition, but also play a large role in trichome initiation, and therefore it is tempting to consider that trichome responses at elevated [CO_2] are an indirect result of over-riding developmental pathways that are commonly sensitive to elevated [CO_2] (e.g. flowering time). Elevated [CO_2] has been shown to affect *miR156* and *SPLs* transcription, but the effect on trichome initiation is wholly unknown. *miR156* expression in *Arabidopsis* (Col-0) rosette tissue decreases at elevated [CO_2], and Col-0 tends to flower earlier at elevated [CO_2], which may be attributable to an accelerated decrease in *miR156* at elevated [CO_2] (Van Der Kooij and De Kok 1996; Springer and Ward 2007; May et al. 2013). In addition to phytohormone shifts, Sun et al. briefly proposed *miR156* as potentially playing a role in shifting trichome densities at elevated [CO_2], although no further work has been published to date (Sun et al. 2016). As such, an accelerated decline of *miR156* at elevated [CO_2] could potentially increase *SPLs*, *TRY,* and *TCL1* transcription in late rosette leaves, disrupting formation of the multimer complex (Fig. 15b) (Van Der Kooij and De Kok 1996; Springer and Ward 2007; Yu et al. 2010; May et al. 2013; Ioannidi et al. 2016; Matias-Hernandez 2016; Sun et al. 2016; Xu et al. 2016a). If this mechanism is in fact relevant, the interconnection between developmental speed (e.g. time to flower) and trichome production would be an important direction for elevated CO_2 studies in the future. As such, we recommend extensive phenotyping of the trichome response across all stages of development in *Arabidopsis* to test for a potential correlation between the time in the overall life cycle that that a leaf is initiated (e.g. early in the plant life cycle versus just prior to flowering) and the trichome response of a given leaf to elevated [CO_2]. Furthermore, we recommend analysis of trichome genes across whole-plant developmental at elevated [CO_2] to test for differences in trichome gene expression that correlate with shifts in *miR156* expression.

Other potential mechanisms

Anthocyanin and trichome pleiotropy

Leaf-level stress responses could incidentally affect trichome initiation at elevated [CO_2] through pleiotropic overlaps with the trichome production pathway. Anthocyanin response to elevated [CO_2] may decrease trichome densities through interactions between the trichome and anthocyanin pathways. Anthocyanin production is controlled by a complex similar to that of the GL1-GL3-TTG1 complex, with an anthocyanin specific MYB protein, PAP1, in lieu of the trichome-specific GL1; furthermore, numerous other trichome genes are pleiotropic with anthocyanin production (e.g. *miR156*, *CPC*, *GL2*) (Zhang 2003; Maes et al. 2008; Zhu et al. 2009; Gou 2011; Shi and Xie 2014; Pesch et al. 2015; Wang 2015). *PAP1* expression and anthocyanin concentrations increase in *Arabidopsis* (Col-0) at elevated [CO_2] (Li 2008; Takatani 2014). Therefore, protein stoichiometries between the trichome and anthocyanin pathways could be upended at elevated [CO_2] as PAP1 concentrations increase. Shared components between the two pathways, such as the GL3-TTG1 dimer, could potentially be bound by increased PAP1 concentrations at elevated [CO_2] and removed from the available protein pool (Zhang 2003; Li 2008; Takatani 2014). PAP1-GL3-TTG1 multimers may result in reduced GL3-TTG1 dimers available for GL1 binding and trichome initiation.

Conversely, *PAP1* over-expressors (*pap1-D*) do not display noticeable disruptions in trichome production; but otherwise there is little information regarding potential trade-offs between anthocyanin production and trichome initiation and therefore this area requires further

investigation (Tohge et al. 2005). Testing for this potential mechanism would require measurement (e.g. a series of pulldown experiments) of ratios between anthocyanin-related multimers (e.g. PAP1-GL3-TTG1, PAP1-GL3) compared to trichome-related multimers (e.g. GL1-GL3-TTG1, GL1-GL3). We therefore recommend future experiments investigate protein behavior at elevated [CO_2] with an emphasis on trichome and anthocyanin multimer protein components.

Effects of elevated [CO_2] on cell division

We also briefly consider that elevated [CO_2] may alter the *time frame* in which trichomes can initiate during leaf development, in turn affecting final trichome density through shifts strictly in leaf development. The basal area of the developing leaf is the primary site of trichome initiation and is the main portion of the leaf expressing the core trichome genes (Balkunde et al. 2010). Elevated [CO_2] increases the rate of cell division in the basal portion of the leaf (shown in *Populus*) and can decrease the time a cell spends in the division and elongation zones (shown in wheat) (Masle 2000; Ferris et al. 2001). As mentioned previously, trichome initiation can only occur within a specific time frame (Larkin et al. 2003). Therefore, it is tempting to think that there could be a relationship between basal cell division rate and trichome density. Yet, this relationship remains untested. Furthermore, the effects of elevated [CO_2] on cell division, trichome gene expression, and other related traits (e.g. anthocyanin) are almost wholly unrealized, and more work is required to determine whether increased basal cell division rates could affect trichome densities at elevated [CO_2].

Directions for future research

Teasing apart the relationships between elevated [CO_2] and trichomes is still an almost wholly unrealized field of study. Future work (Table 5) must research the relationships between elevated [CO_2] and trichomes, as trichomes play important physiological and defense roles in crop and wild species. Therefore, we recommend further development of specific trichome-elevated [CO_2] research tracts that relate to the specific mechanisms proposed above, as well as new approaches for the overall field as described below.

Full-leaf trichome patterning

Extensive phenotyping is still needed to further understand the genetic underpinnings of the elevated [CO_2] trichome response. In addition to trichome density as is commonly measured, we recommend a complete phenotyping of trichome *patterning* across the entire leaf surface in response to elevated [CO_2]. Also, past work generally addressed elevated [CO_2]-trichome density shifts in small subsections of the leaf, but work has yet to be done on whole-leaf trichome densities with attention paid to how elevated [CO_2] affects trichome patterning (Bidart-Bouzat et al. 2005; Lake and Wade 2009). As many trichome genes (e.g. *TRY*, *CPC*, *TTG1*) play dual roles in trichome initiation and trichome patterning, an elevated [CO_2] effect on trichome initiation through core genes may affect both trichome initiation and trichome patterning (Digiuni et al. 2008; Balkunde et al. 2010; Zhang 2018). As a majority of the potential mechanisms we highlighted above (Fig. 15) involve potential shifts in expression of core trichome-patterning genes elevated [CO_2] could theoretically affect both trichome density *and* patterning. For example, mechanism A would lead to trichome density shifts through a potential change in *GL3* and *GL1*, and *CPC* expression (Fig. 15a); mechanism C on the other hand would drive alterations in *TRY* and *TTG1* expression (Fig. 15b). In both of these examples, patterning would

be affected, as the genes shifting in expression belong to both the trichome density and trichome patterning pathways. Therefore, more work is needed to investigate the effects of elevated [CO_2] on trichome patterning, and whether shifts in trichome densities are tethered to shifts in leaf trichome patterning.

To test if there is alteration in the quality of the trichome initiation signal itself, we believe future experiments should not only observe signal quantity (e.g. phytohormone concentrations, core gene expression), but also measure qualities of the signal (e.g. protein location). For example, we encourage the use of green fluorescent protein (GFP) to observe if elevated [CO_2] may affect localization of trichome proteins, such as mobile negative regulators TRY and CPC. Measuring the dispersion distance of negative regulators from the source trichome, with new advances in molecular plant microscopy, would be an interesting method for detecting the influence of elevated [CO_2] on trichome initiation and patterning.

Differently aged leaves

We recommend more analyses of trichome responses at the leaf level across various stages of plant development in order to determine whether altered whole-plant development could play a role in the trichome density responses to elevated [CO_2]. In previous elevated [CO_2]-trichome research, only mature adult leaves have been analyzed and therefore there are gaps in our understanding, such as how trichome densities of different leaf stages (e.g. juvenile versus adult leaf) respond to elevated [CO_2] (Bidart-Bouzat et al. 2005; Lake and Wade 2009). To fully understand potential mechanisms of trichome shifts at elevated [CO_2], further and more detailed

phenotyping is necessary. Additionally, related research only investigates a single leaf pair from the rosette, therefore we recommend future research phenotype elevated [CO_2]-trichome density shifts across the spectrum of juvenile to adult leaf transition, with a focus on *Arabidopsis thaliana*. (Bidart-Bouzat et al. 2005; Lake and Wade 2009).

In the responses of stomatal densities to elevated [CO_2]—which are similar to the phenotypic responses of trichome densities at elevated [CO_2]—there is precedence for a signaling system from mature to developing leaves. At elevated [CO_2], a mobile signal from mature to developing leaves may alter stomatal densities of developing leaves according to the environment under which the mature leaf developed (Lake et al. 2001; Haus et al. 2018). Coupe et al. (2006) hypothesized that the mobile signal may be related to sugar and hormonal signaling, and some evidence may indicate microRNAs as a potential signal (Coupe et al. 2006). Similar to the potential roles of carbohydrates in the mobile signal altering stomatal densities, Guo et al. attributes the elevated [CO_2]-increased *M. truncatula* trichome densities to elevated primary metabolism at elevated [CO_2], as the increased pool of resources can be routed to structural defenses (Guo et al. 2014). On the contrary, *Arabidopsis* line Col-0 also sharply increases primary metabolism at elevated [CO_2], but *decreases* trichome densities with increased [CO_2] (Bidart-Bouzat et al. 2005; Teng et al. 2006; Lake and Wade 2009; Noguchi et al. 2015). Therefore, more work needs to be done on the connection between primary metabolism, elevated [CO_2], and trichome production across species. Furthermore, we recommend future research utilize the dual-compartment CO_2 chambers from Haus et al. 2018 to investigate whether the trichome density-altering signal is mobile from mature to developing leaves, similar to the signal altering stomatal densities at elevated [CO_2] (Haus et al. 2018).

Ecological and physiological implications

The implications of elevated [CO_2] induced trichome density shifts is almost wholly unrealized. As mentioned in the subsection "Trichome density and environmental adaptation", a disruption of trichome densities from the locally fine-tuned levels of trichome densities could, hypothetically, alter herbivory damage, WUE, fungal infection rates, and photosynthetic efficiency, as well as overall fitness. We recommend thorough investigations into the impacts of elevated [CO_2] on the potential consequences of trichome density shifts, such as fungal infection rates, across lines that differ in their trichome responses to elevated [CO_2]. As most of our potential mechanisms described above indicate trade-offs between trichomes and other physiologically important functions, we believe it is important to utilize lines in which, for example, anthocyanin concentrations and trichome densities both shift at elevated [CO_2], compared with lines that only respond in one of these traits (and control lines that do not respond in either trait). We currently understand very little regarding the potential implications of shifted trichome densities at elevated [CO_2], but further investigations are critical since shifting trichome densities could scale up to affect plant community composition and plant-herbivore interactions.

RNA-sequencing

To gain further insight into potential mechanisms underlying trichome density shifts at elevated [CO_2], we analyzed gene expression across early leaf development. We selected leaves from Arabidopsis ecotype Col, which we found to increase trichome numbers under elevated [CO_2] (see chapter 1). Via RNA-sequencing, we examined gene expression in four stages of early leaf

development—selected to cover the range of the trichome initiation window (see methods at end of chapter)—at ambient and elevated [CO_2]. Broadly, we found that the developing leaf became more responsive to elevated [CO_2] as development progressed. As development progressed, the number of DEGs and enriched GO terms increased with time (Figs. 16 and 17).

Core trichome genes

We found that core trichome genes—such as those comprising the multimeric complex (Fig. 8)—were unresponsive under elevated [CO_2] (Fig. 18). Furthermore, only one stage (stage four) had a trichome initiation-related GO term differentially expressed.

The lack of response in core trichome genes under elevated [CO_2] is interesting, as other known environmental perturbations are known to drive trichome density shifts through core trichome genes. Excess UV-B, for example, drove an increase in the trichome densities of Arabidopsis mutants through upregulation of the core trichome regulator *GL3* (Yan, Pan, An, Gan, & Feng, 2012). Therefore, elevated [CO_2] may be altering trichome densities through a mechanism different than other environmental perturbations that affect trichome densities. Furthermore, certain trichome genes displayed in figures 8 and 18 are essential for trichome initiation, and one would assume that to shift trichome numbers, elevated [CO_2] would have to affect one or more of the essential trichome genes.

SPL9 and trichome negative regulators

As described above, *SPL9* is a member of the flowering pathway that is also a negative regulator of trichome initiation, through upregulation of *TRY* (Matias-Hernandez, 2016). At stage four of leaf development, we observed a significant decrease in *SPL9* transcription at elevated [CO_2] (Fig. 19). A decrease in *SPL9* and downstream negative regulators of trichome initiation would result in the trichome number increase previously reported for Col (mid-development leaves; see chapter 1). Yet, such a decrease in *SPL9* would presumably result in a decrease in transcription of negative trichome regulator *TRY*, but such a decrease was not seen (Fig. 18). There may be a delayed decrease in *TRY* later in leaf development, following the decrease in *SPL9*. As the significant decrease in *SPL9* transcription was observed in the last stage of leaf development we observed, stage four, we recommend future work extend observations into later stages of leaf development to investigate potentially delayed responses of trichome genes to the *SPL9* decrease at elevated [CO_2].

Anthocyanin genes and anthocyanin-trichome pleiotropy

As previously discussed, anthocyanin synthesis and trichome initiation share a number of core genes. Anthocyanin synthesis and trichome initiation share similar core protein complexes, with a one protein substitution: the anthocyanin complex contains PAP1 (MYB75) while the trichome complex contains GL1 (Fig. 8b) (Zhang 2003; Maes et al. 2008; Zhu et al. 2009; Gou 2011; Shi and Xie 2014; Pesch et al. 2015; Wang 2015). Under elevated [CO_2], transcription of the core anthocyanin gene *PAP1* significantly decreased, primarily at stage four of leaf development (Fig. 20). Therefore, it is plausible that a decrease in *PAP1* transcription and subsequent decrease in the PAP1 protein pool at elevated [CO_2] would increase the amount of GL3-TTG1 protein dimers available for GL1 binding. An increase in GL1-GL3-TTG1 multimers could lead to the

increase in trichome numbers we observed in Col. This finding offers an exciting avenue of future proteomic research, investigating the available pool of GL3-TTG1 dimers and the amount of active GL1-GL3-TTG1 multimers at elevated [CO_2], and how this could correspond to an increase in trichome numbers at elevated [CO_2].

Here we found that, in the developmental stages we observed, core trichome genes were unresponsive to elevated [CO_2]. There was a significant decrease in *SPL9* transcription as well as a decrease in *PAP1* transcription, both of which could lead to the trichome number increase at elevated [CO_2] we previously observed in *Arabidopsis* line Col (see chapter 1). Our findings are exciting as we showed that unlike other environmental perturbations, elevated [CO_2] may not directly affect core trichome gene transcription to alter trichome numbers. We also found candidate genes—*PAP1* and *SPL9*—which respond under elevated [CO_2], and could be underlying the trichome number increase. We recommend future research investigate a broader spectrum of early leaf development, as well as investigating proteomics and trichome-related protein complex ratios at elevated [CO_2]. We hope our work here drives increased research into the underpinnings of shifting trichome densities and patterns at elevated [CO_2], and provides a strong framework for future investigations.

Conclusions

In summary, trichomes play physiologically important roles in herbivore defense, temperature regulation, boundary layer fortification, and UV-B protection, which can impact overall plant physiology, photosynthetic efficiency, fitness, and plant-environment interactions (Xiao et al.

2017). Studies have shown effects of elevated [CO_2] on trichome density, with significant increases in some species (e.g. *B. rapa*) and significant decreases in others (e.g. wheat) (Masle 2000; Karowe and Grubb 2011; Guo et al. 2013; Guo et al. 2014). Yet the molecular mechanisms driving trichome density shifts at elevated [CO_2] remain elusive. The potential mechanisms we highlighted above included broad responses to elevated [CO_2] affecting trichome densities (mechanism A. phytohormone shifts), leaf-scale changes affecting trichome initiation signaling (mechanism B. cuticle and signal transmission) and overlaps between elevated [CO_2]-responsive pathways and the trichome pathway (mechanism C. flowering and trichome pleiotropy). Further work must be done on the relationship between trichome production and elevated [CO_2], and the potential molecular mechanisms that drive trichome density shifts at elevated [CO_2], as described above. We recommend further trichome phenotyping of leaves at elevated [CO_2], and expansive genotyping of responsive and non-responsive individuals to further elucidate potential mechanisms. An understanding of these molecular mechanisms is crucial to predicting how wild and crop species may respond to a changing climate and will be a factor for helping us to best improve crop performance in preparation for future climate change.

RNA-sequencing methods

Plant growth conditions

For our RNA-sequencing experiment, *Arabidopsis* line Col (CS2265) was selected, as Col trichome densities have been shown in the literature to be responsive under elevated [CO_2], and we previously showed that mid-development Col leaves experience increased trichome numbers

at elevated [CO_2] (see chapter 1). Col seeds were purchased from the Arabidopsis Biological Resource Center (The Ohio State University) and grown for seed.

For ambient and elevated [CO_2] treatments, CO_2 concentrations were kept at 400 $\pm$ 10 ppm or 800 $\pm$ 10 ppm respectively. Daytime was simulated with 10 hours of approximately 350 μmol light, with an additional 1 hour of approximately 125 μmol light at the beginning of the ten hour period to simulate dawn and at the end of the 10 hours dusk, leading to a total of 12 hours of mock daytime. The average daytime temperature was 22°C, beginning at 18°C at simulated dawn, rising to a maximum of 24°C at the 7th daytime hour, then a descent to 23°Cat simulated dusk. At the end of simulated dusk nighttime temperatures began with 23°C at the end of dusk, and fell to 18°Cat the beginning of mock dawn. Humidity was maintained at 60% during the 12 hour light period and 90% during the 12 hour dark period. We tightly controlled growth conditions with a walk-in plant growth chamber (Conviron BDW80, Winnipeg, Manitoba). We bottom watered growing plants, and watering trays every 3 days with a water level of approximately 2 centimeters. Every other watering event we added half-strength Hoagland's fertilizer solution to the tray.

Tissue sampling for RNA-sequencing

The meristem region of the growing plant was sampled with a disposable biopsy punch and immediately stored in aliquots of RNAlater (Ambion) in 4°C for at least 24 hours. Meristem samples were quickly micro-dissected under an upright light microscope (Nikon AZ100), removing the target developing leaf. The developmental stage (1-4) of micro-dissected leaves was determined using multiple parameters: age of the plant, size (μm) of developing leaf, and

trichome maturation. Trichome maturation begins at the tip of the leaf, and as the plant ages, continues towards the basal portion of the leaf (Fig. 21). Younger leaves (e.g. stage 1-2) only have mature trichomes on the tip of the leaf, while more developed leaves, as with stages 3 and 4, have mature trichomes over half or most of the leaf, respectively (Fig. 22). Mature trichomes were defined as those with multiple well-developed branches. Micro-dissected leaves were temporarily placed on liquid nitrogen, and stored in a -80F freezer until the RNA isolation step. The leaf that was harvested from each plant was the tenth leaf to emerge from the meristem. We selected the tenth leaf as this leaf was generally the second adult leaf to emerge (data not shown), as juvenile leaf trichomes densities were found to be unresponsive to elevated [CO_2], and later stage adult leaves were found to also shift leaf area under elevated [CO_2]. We previously found mid-development Col leaves were only responsive in trichome number under elevated [CO_2] (see chapter 1), which allows us a simple response system to investigate through RNA-sequencing.

RNA-sequencing and analysis

Micro-dissected leaves for each leaf development stage were pooled to provide enough RNA for accurate RNA-sequencing. Number of leaves per pooled sample were as follows: approximately 20 leaves per stage 1 sample, approximately 15 leaves per stage 2 sample, approximately 10 leaves per stage 3 sample, approximately 5 leaves per stage 4 sample. RNA was isolated from pooled leaf samples using an RNeasy Mini Kit (Qiagen) after homogenization with a bead homogenizer (Benchmark) and QIAshredder columns (Qiagen). Library preparation and RNA-sequencing was performed at the University of Kansas Genome Sequencing Core (Lawrence, KS) on an Illumina MiSeq with single end reads.

Alignment to the *Arabidopsis* genome (TAIR10) and transcript quantification were performed with CLC Genomics Workbench (Qiagen). Downstream analyses were performed in R; differential expression was calculated with the Bioconductor package DESeq2 (Love, Huber, & Anders, 2014).

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Conclusions

Global atmospheric carbon dioxide concentration ([CO_2]) is rising rapidly, having increased by almost 50% between the pre-industrial (270 ppm) and modern (402 ppm) periods, with expectations that it will double by the end of this century. Elevated [CO_2] can lead to differential production of leaf surface micro-structures, including stomata (pores required for gas exchange) and trichomes (leaf hairs). Trichomes reduce leaf water loss, enhance herbivory defense, and deflect excess light, which increases plant productivity and survival. Trichome densities of leaves grown in an elevated [CO_2] environment have been shown to increase as high as 57% higher than ambient trichome densities in certain species such as *Brassica rapa*. Conversely, some species— such as wheat and *Arabidopsis thaliana*—reduce trichome densities by as much as 60% when grown in elevated [CO_2]. Environmental perturbations which lead to shifts away from locally adaptive trichome densities could lead to increased susceptibility to increased fungal infection, excessive energy investment, overheating, and herbivory.

One major objective of our research was to determine if elevated [CO_2] affects trichome densities across the whole leaf, as the small pool of published results on the relationship between elevated [CO_2] and trichome densities only investigates small portions of the leaf. Furthermore, we wanted to understand whether trichome density shifts were driven indirectly by shifts in leaf area, or directly via shifts in the amount of trichomes on the leaf. From our work, we draw the following major conclusions:

1. We found strong genotypic variation across *Arabidopsis* genotypes in trichome density, trichome number, and leaf area responses to elevated [CO_2]. Trichome densities and trichome numbers generally increased or did not significantly respond under elevated [CO_2]. Furthermore, all genotypes showed a significant response in trichome density in at

least one stage of development, but shifts in trichome number and leaf area were less ubiquitous.

2. Within most genotypes, we observed significant variation in the elevated [CO_2] response of trichome density, trichome number, and leaf area across three leaf stages. The diversity of responses across stages within each genotype may indicate that elevated [CO_2] impacts trichome density through whole-plant related mechanisms.
3. We sought to find the underlying phenotypic response—whether it be shifting leaf area or shifting trichome numbers—that drives altered trichome densities at elevated [CO_2] in *Arabidopsis*. One possibility was that trichome densities were shifting at elevated [CO_2] was shifting leaf area, which in turn would increase or decrease the amount of leaf material separating trichomes from one another. The other possibility was that elevated [CO_2] increases the amount of trichomes initiating across the leaf, thereby increasing trichome numbers and trichome densities. Surprisingly, we observed instances of both scenarios when we related trichome density, trichome number, and leaf area responses at elevated [CO_2].

A second objective of our work was to investigate the effects of elevated [CO_2] on trichome patterning across the leaf. Trichome patterning molecular pathways are well understood, but due to a lack of phenotyping methods, little work has been done on the effects of environmental perturbations on trichome patterning. As such, we sought to determine the impacts of elevated [CO_2] on trichome patterning, utilizing a novel phenotyping method we developed. We found the following:

1. We found high genotypic variation in elevated [CO_2] response across all of the trichome patterning variables that we measured. Across all *Arabidopsis* genotypes we investigated, elevated [CO_2] significantly affected leaf trichome patterning at one or more points in whole-plant development (depending on genotype).
2. Elevated [CO_2] may affect the *timing* of trichome initiation during leaf development. We observed unbalanced responses to elevated [CO_2] between the tip and the base of the leaf in some *Arabidopsis* genotypes. As trichomes initiate at the base of the leaf and reach their final position through expansion of the leaf, differences in trichome initiation at elevated [CO_2] could lead to a shift in the dispersion of trichomes from the leaf base to the tip, as we observed in some instances here.
3. Potentially indicating an effect of elevated [CO_2] on trichome-to-trichome communication (e.g. negative regulators of trichome initiation), we observed several different patterns in trichome densities surrounding a given trichome. This could potentially indicate differential production or efficacy of the negative trichome regulators involved in trichome patterning, which could alter the zone of inhibition around each trichome at elevated [CO_2].

Finally, we sought to determine potential hypotheses for the underlying molecular mechanism(s) driving differential trichome production at elevated [CO_2] in *Arabidopsis*. As such:

1. We reviewed the ecological roles of trichomes, described what is known about elevated [CO_2]-trichome phenotypic responses, and provided a relevant background of trichome genetics.

2. We presented potential molecular/developmental mechanisms behind elevated [CO_2]-trichome responses. Finally, we proposed future research directions to stimulate future elevated [CO_2]-trichome research in order to uncover the mechanisms that control altered trichome densities at elevated [CO_2].
3. We showed that unlike other environmental perturbations, elevated [CO_2] may not directly affect core trichome gene transcription to alter trichome numbers, through an RNA-sequencing experiment we conducted. Furthermore, we found candidate genes—*PAP1* and *SPL9*—which respond under elevated [CO_2], and could be underlying the trichome number increase.

Our work contributes to a growing body of knowledge concerning the effects of rising carbon dioxide on plant growth and development. Our research advances our understanding of the phenotypic impacts of elevated [CO_2], and the magnitude and variation in the density and patterning of important leaf micro-structures. Through extensive phenotyping we were able to provide a novel and detailed picture of trichome density and trichome patterning responses under elevated [CO_2]. As trichomes mitigate herbivory damage, improve water use efficiency, affect fungal infection rates, and increase photosynthetic efficiency, we believe that a deep understanding of how trichome densities may respond to environmental change is an important addition to the field. Finally, provided a molecular framework and novel results concerning potential molecular pathways underlying elevated [CO_2] driven trichome density shifts.

Figures

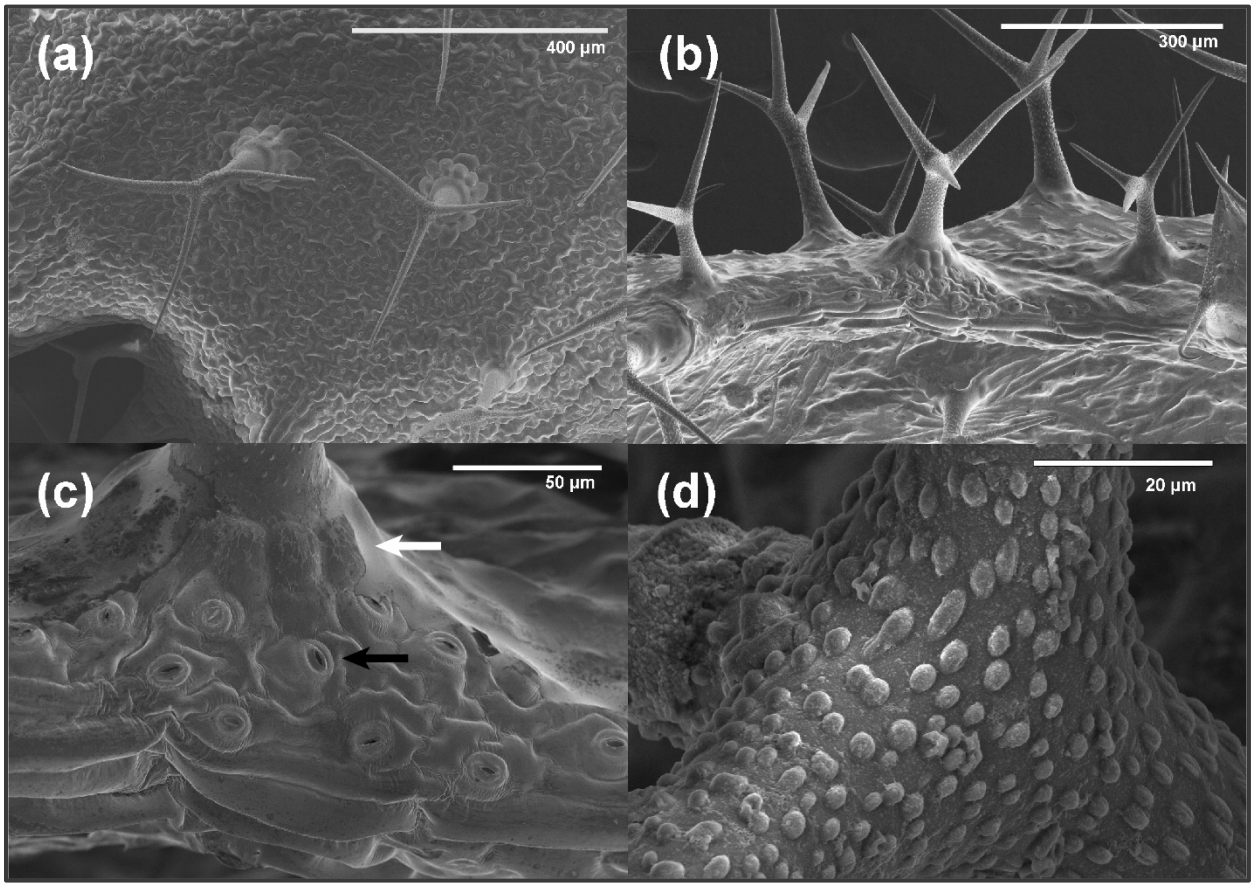

Fig. 1 (a-b): Important in physiology, defense, and overall plant survival, trichomes are scattered across the adult leaf surface. (c): Base of trichome, with trichome socket cell (white arrow) and stomate (black arrow). (d): Meeting point of three trichome branches, coated in papaillae (Credits: Fischer, J.M., Rosa-Molinar, E, Kilcrease, J.)

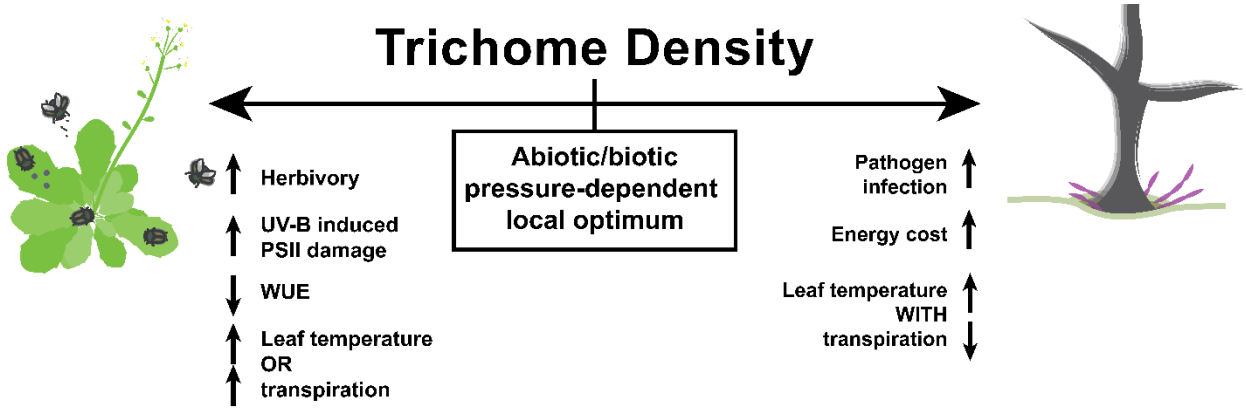

Fig. 2 Reductions in leaf trichome densities may subject a plant to increased intensity of herbivore damage, increased UV-B damage, decreased WUE, and leaf temperature/transpiration. Conversely, increased trichome densities may lead to higher pathogen infection, increased energy diversions to trichome growth, and either an increase in leaf temperature or a decrease in transpiration (Figure by J.M. Fischer using publications from Mauricio 1998; Calo et al. 2006; Hanley 2007; Løe et al. 2007; Sletvold 2010; Peraldi et al. 2011; Nguyen et al. 2016; Imboden et al. 2018; Kergunteuil et al. 2018; Kim 2019)

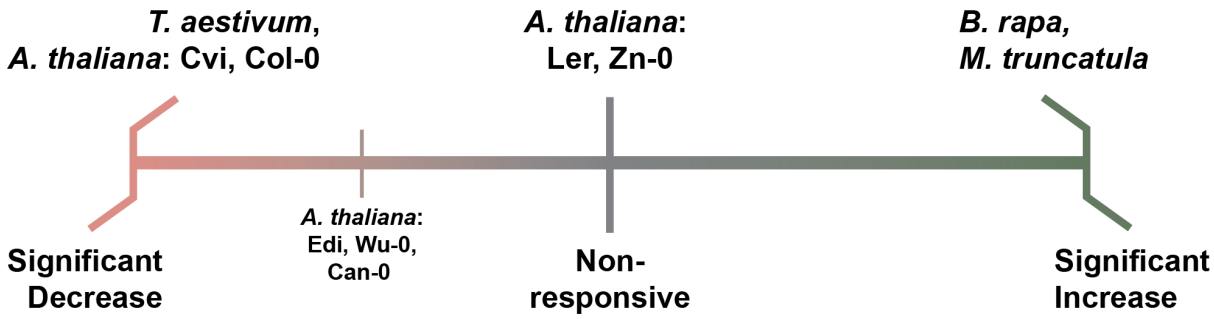

Fig. 3 Trichome density shifts among various species and *Arabidopsis* ecotypes grown at elevated [CO_2] (720-900 ppm [CO_2]) compared to current [CO_2] (350-400 ppm [CO_2]) (Figure based on work by Masle 2000; Bidart-Bouzat 2004; Bidart-Bouzat et al. 2005; Lake and Wade 2009; Karowe and Grubb 2011; Guo et al. 2013; Guo et al. 2014)

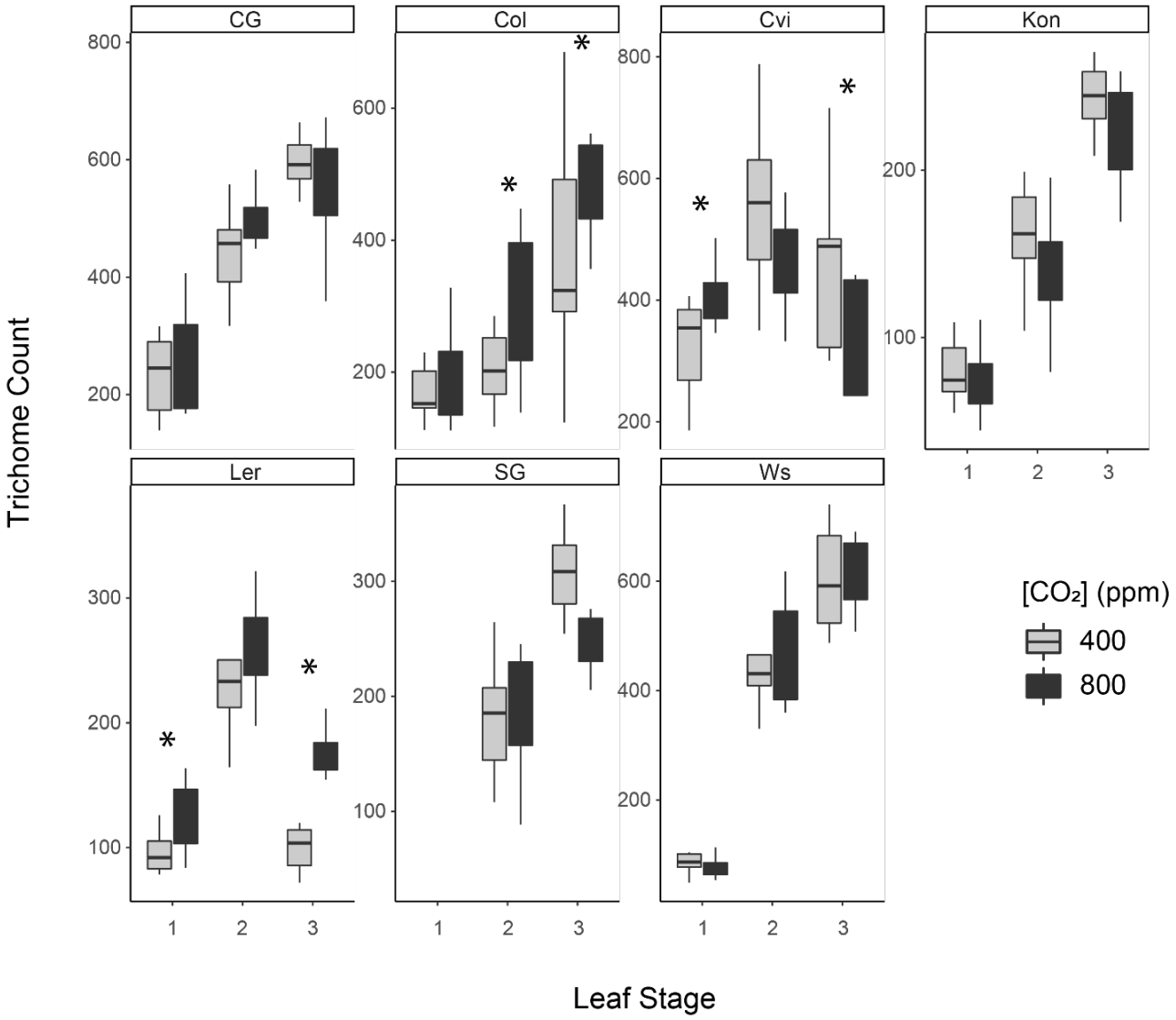

Fig. 4: Number of trichomes across the full leaf at current and elevated [CO_2] (400 ppm and 800 ppm respectively), measured across *Arabidopsis* genotypes and developmental leaf stages. Asterisks indicate a significant difference in trichome count between current and elevated [CO_2] in the indicated developmental stage of the indicated genotype. Significance was tested with post-hoc means testing (Tukey adjusted; Table 2)

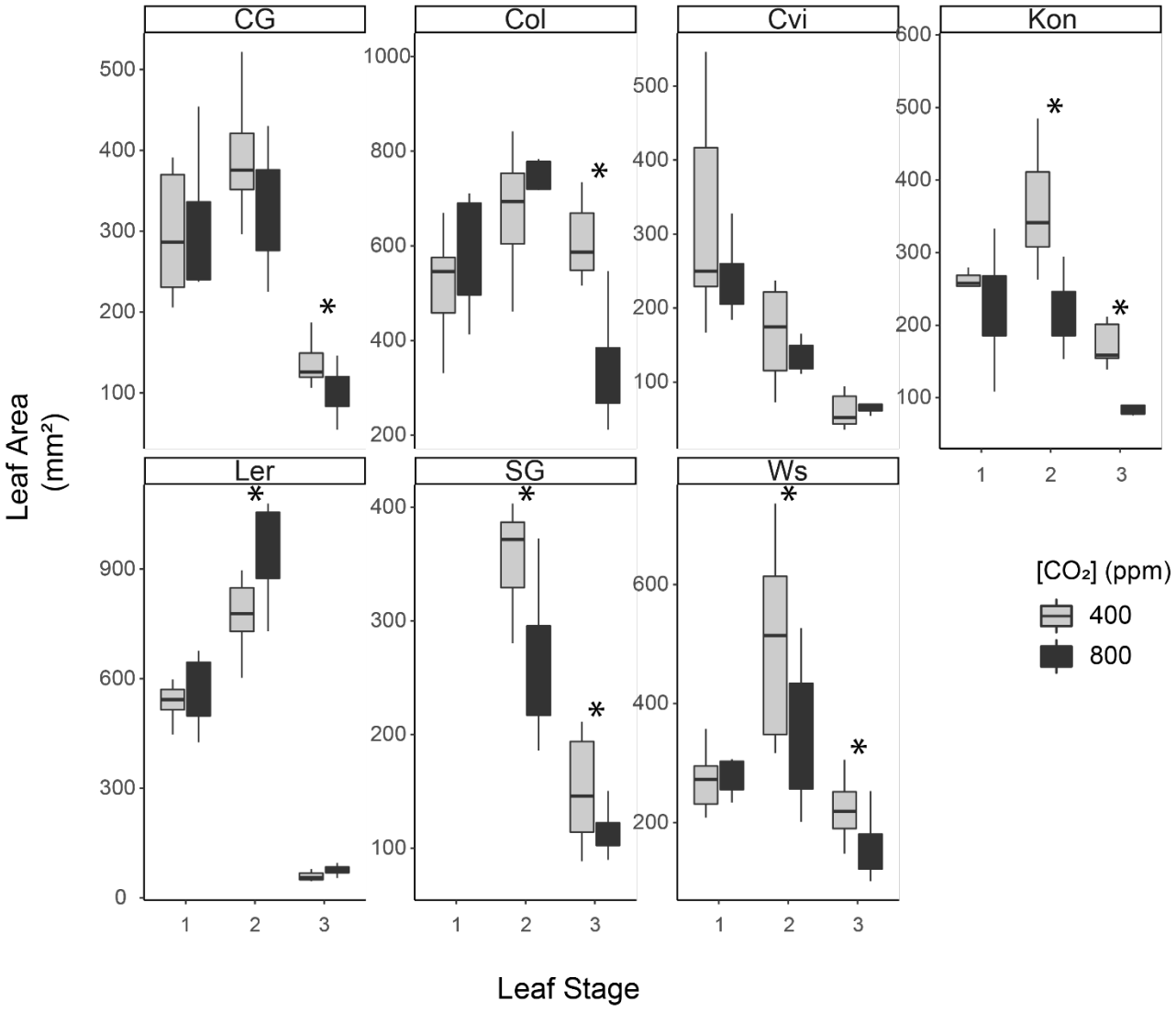

Fig. 5: Area of leaves harvest from plants grown at 400 ppm (current) and 800 ppm (elevated) [CO_2]. Asterisks indicate a significant difference in leaf area in that stage and genotype at elevated [CO_2] compared to current [CO_2]; significance tested via post-hoc means testing (Table 2)

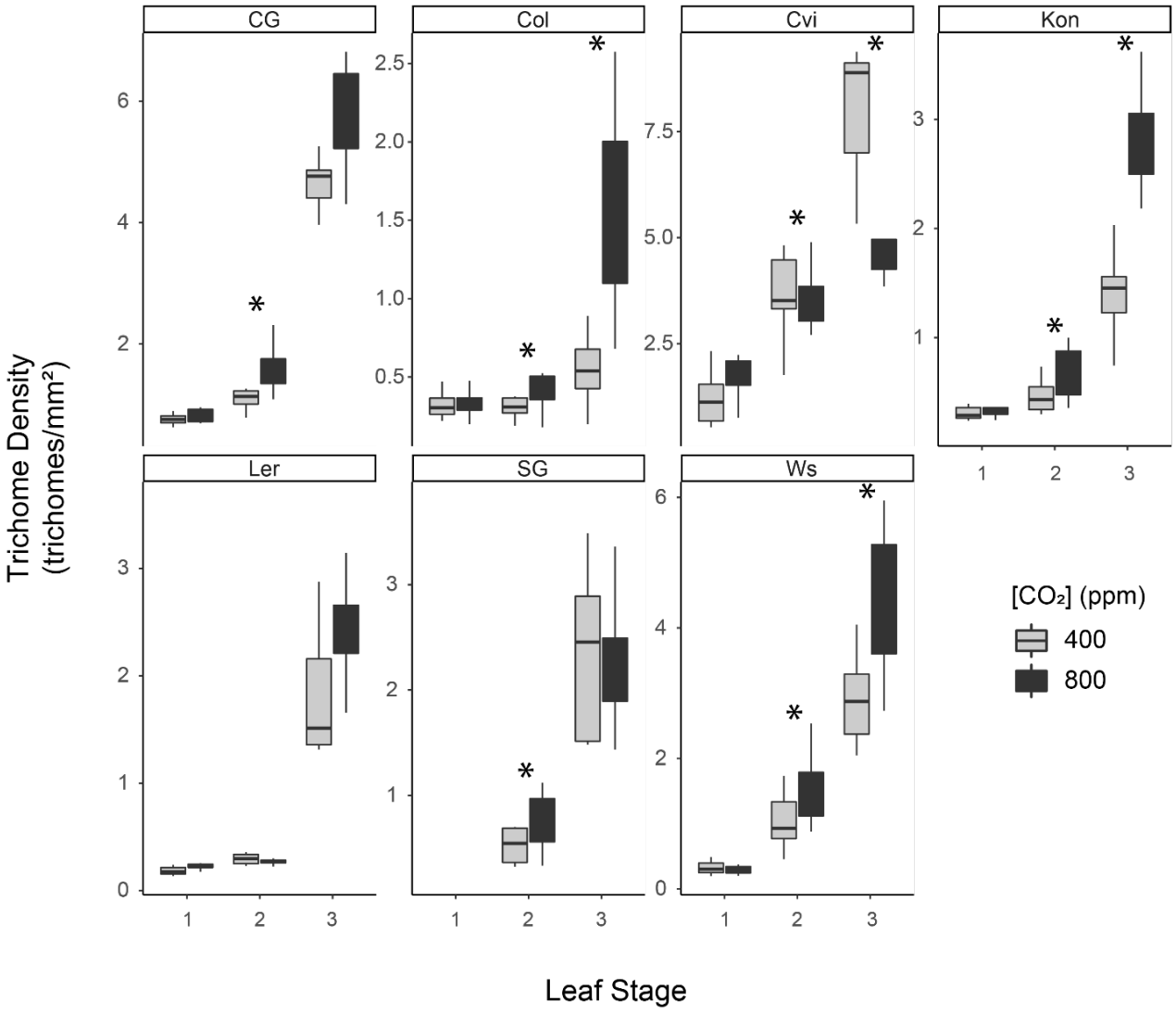

Fig. 6: Full-leaf trichome densities at current (400 ppm) and elevated (800 ppm) [CO_2] across Arabidopsis genotypes and developmental leaf stages. Asterisks indicate a significant difference in trichome density of leaves grown at elevated [CO_2] compared to leaves grown at current [CO_2]; significance tested via post-hoc means testing (Table 2)

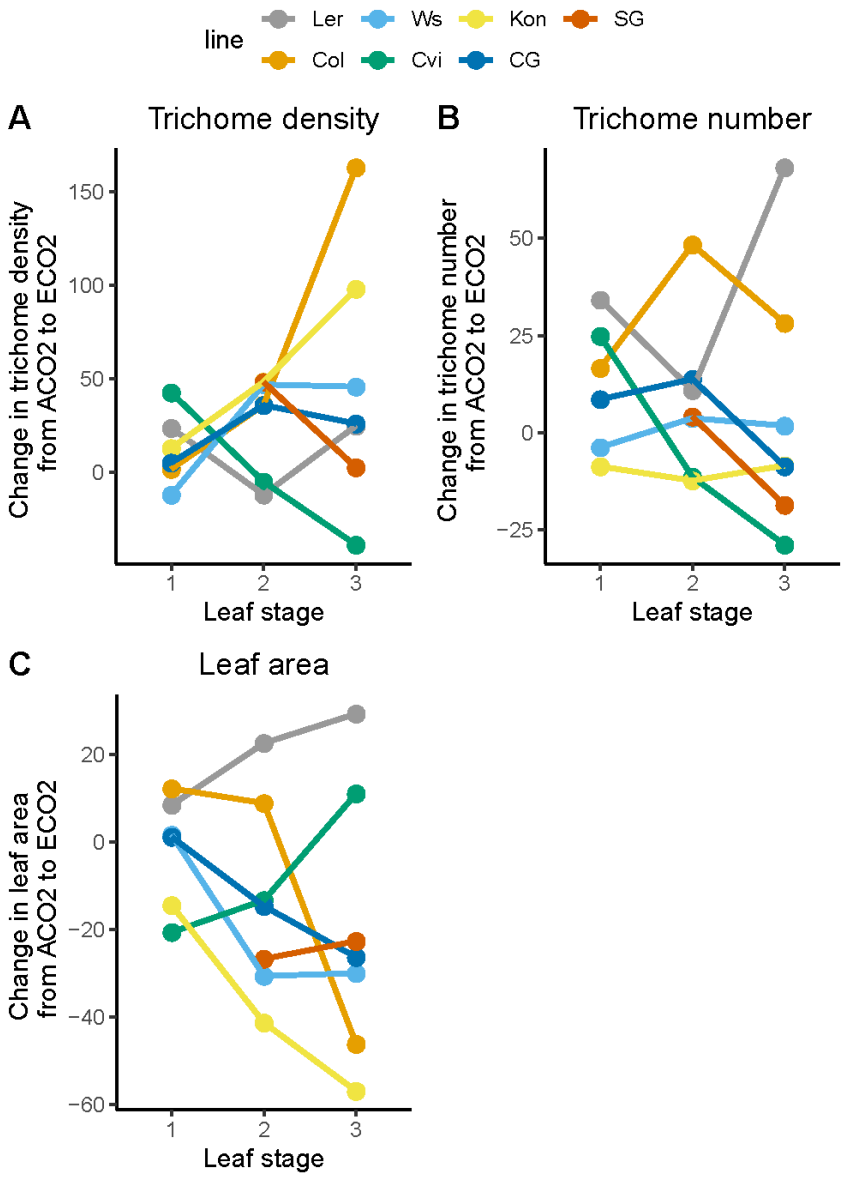

Fig. 7: Percent difference across genotypes between ambient [CO_2] and elevated [CO_2] for (a) trichome density, (b) trichome number, and (c) leaf area

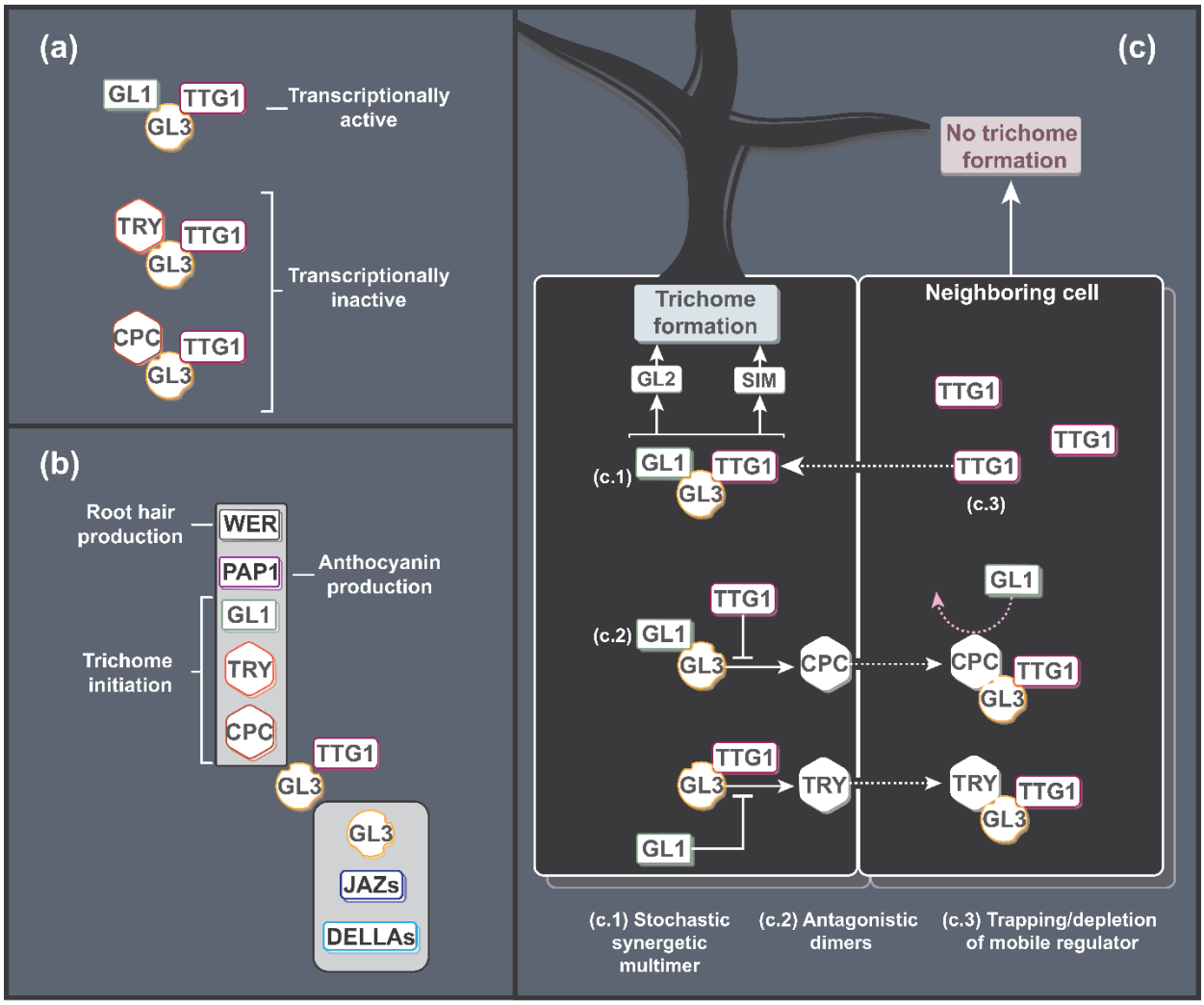

Fig. 8: The molecular pathways driving trichome production in *Arabidopsis* have been well studied. (a) A transcriptionally active complex containing GL1, GL3, and potential accessory protein TTG1 is required for trichome initiation. Negative regulators TRY and CPC form transcriptionally inactive complexes via blocking the formation of the GL1-GL3-TTG1 regulating complex. (b) A high number of potential multimer complex organizations results from GL3 binding a range of proteins across three GL3 protein binding domains. (c) *GL2* and *SIM* transcription is promoted by the GL1-GL3-TTG1 multimer, and GL2 and SIM subsequently promote trichome initiation (c.1). TTG1 is trapped in cells with stochastically increased concentrations of GL3, which depletes cell-to-cell mobile TTG1 from surrounding cells (c.3). TRY and CPC are promoted by antagonistic dimers formed from members of the synergetic positive regulating multimer. To prevent the formation of the synergetic multimer and to inhibit trichome initiation, TRY and CPC enter surrounding cells (c.2). (Figure created by J.M. Fischer utilizing work by Wada et al. 1997; Hülskamp et al. 1994; Szymanski and Marks 1998; Payne et al. 2000; Walker et al. 2000; Johnson et al. 2002; Larkin et al. 2003; Zhang 2003; Hülskamp 2004; Ramsay and Glover 2005; Bouyer et al. 2008; Wang 2008; Pesch and Hülskamp 2009; Yoshida et al. 2009; Balkunde et al. 2010; Qi et al. 2011; Yang and Ye 2012; Patra et al. 2013; Tsuji 2013; Qi et al. 2014; Wang and Chen 2014; Pesch et al. 2015; Zhang 2018)

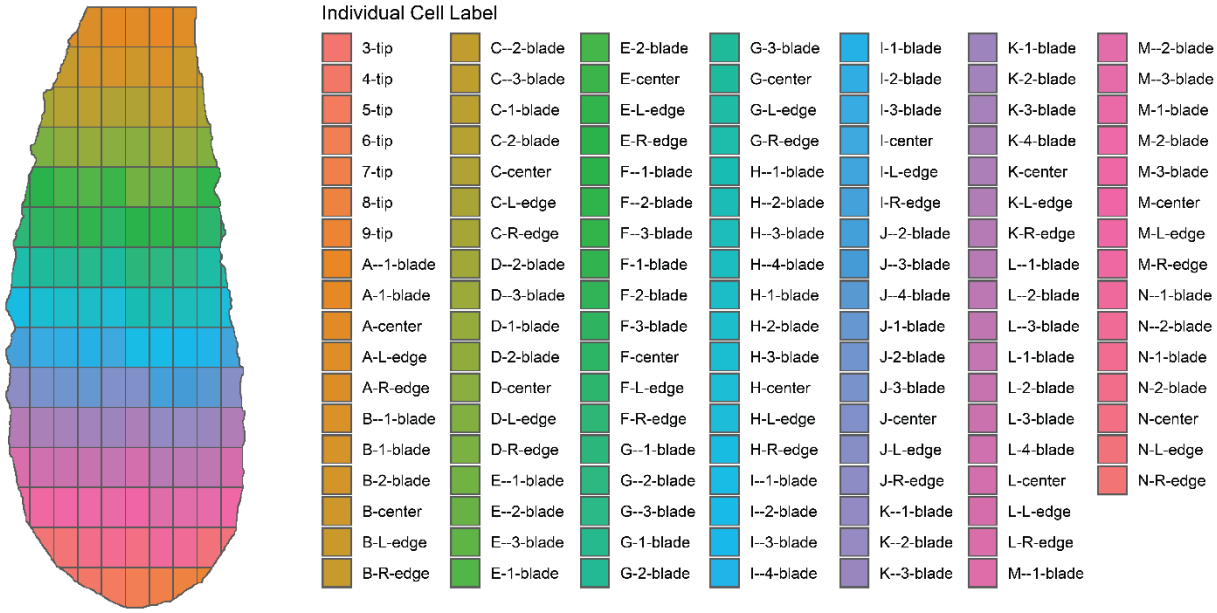

Fig. 9: A gridded Col leaf, labelled with unique grid cell identifiers. The trichome density of each cell is noted, and the average density for each unique identifier is averaged across leaves.

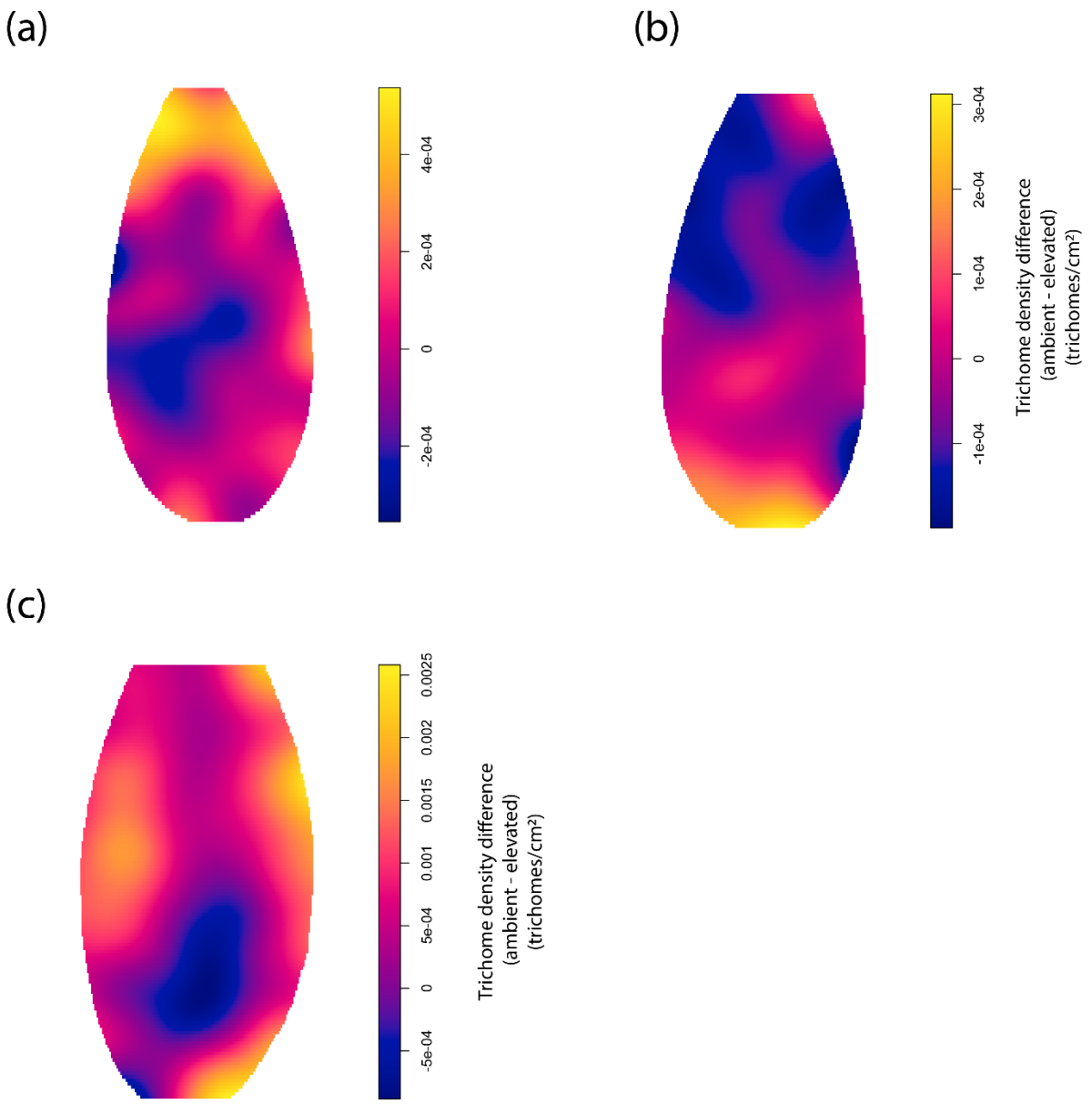

Fig. 10: Composite leaves. Composites are created from each treatment x line x developmental stage combination. For each line x development combination, the two composites from the 400 ppm and 800 ppm [CO_2] treatments are compared using R spatstat function solutionset. Warmer colors indicate areas in which 400 ppm [CO_2] grown leaf composites had a higher trichome density than the 800 ppm [CO_2] grown counterparts. Cooler colors indicate areas in which 800 ppm [CO_2] grown leaves had higher trichome densities. (a) Elevated [CO_2] may increase trichome densities on the leaf tips of Kon (stage 2). (b) Elevated [CO_2] potentially decreases the trichome densities on the tips of Ler (stage 2) leaves. (c) Elevated [CO_2] may alter trichome-to-trichome communication in Ler (stage 3) leaves, altering the clumping of trichomes on the leaf surface

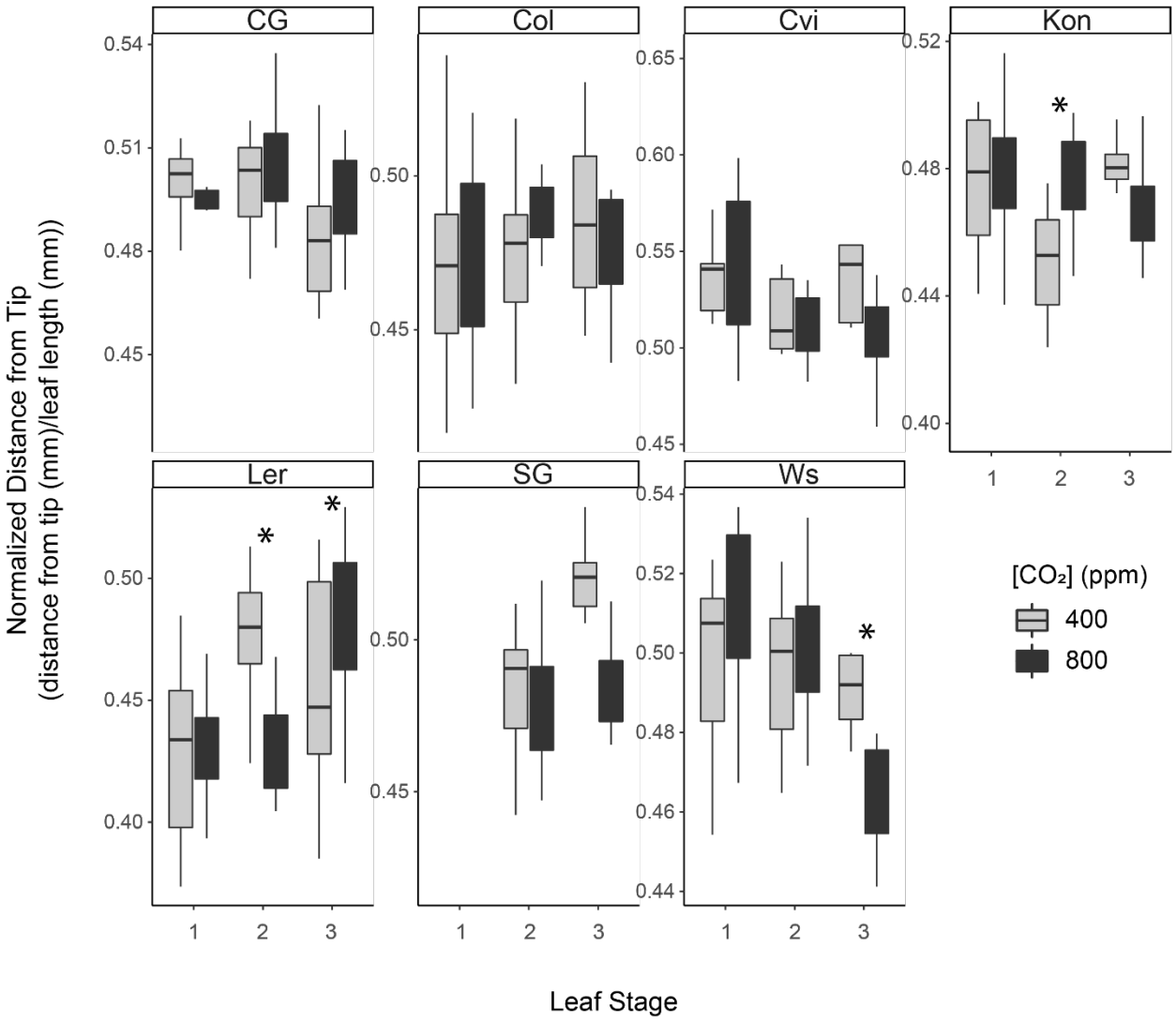

Fig. 11: The distances of trichomes from the leaf tip across *Arabidopsis* genotypes and across developmental stages. Asterisks indicate a significant difference in the distances of trichomes from the leaf tip at 800 ppm [CO_2] compared to 400 ppm [CO_2] grown leaves; significance determined from post-hoc means testing (Table 4)

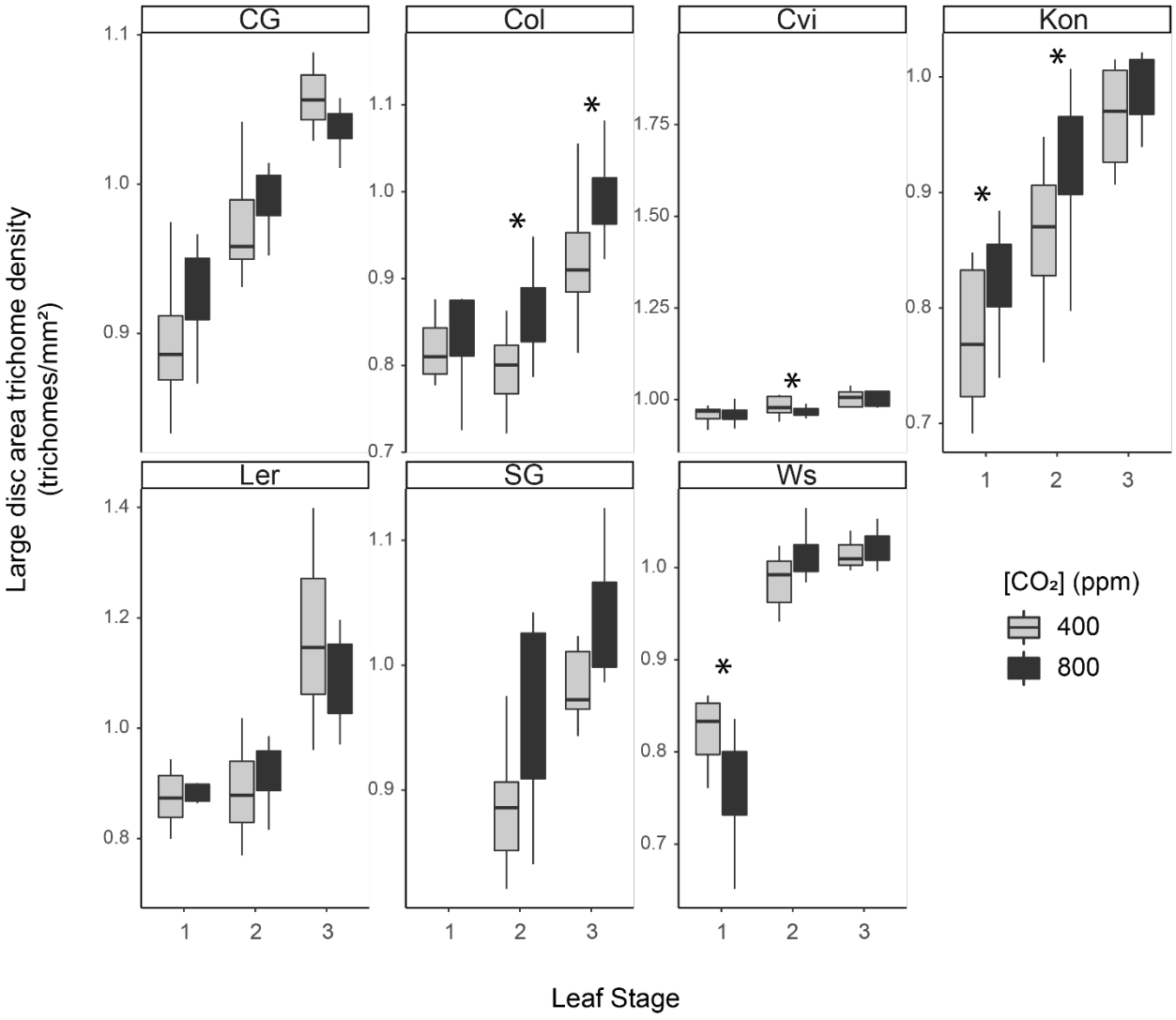

Fig. 12: Trichome densities in a large circular area surrounding individual trichomes, measured at current [CO_2] (400 ppm) and elevated [CO_2] (800 ppm). Asterisks indicate a significant difference between current and elevated [CO_2] grown plants, tested via post-hoc means testing (Table 4)

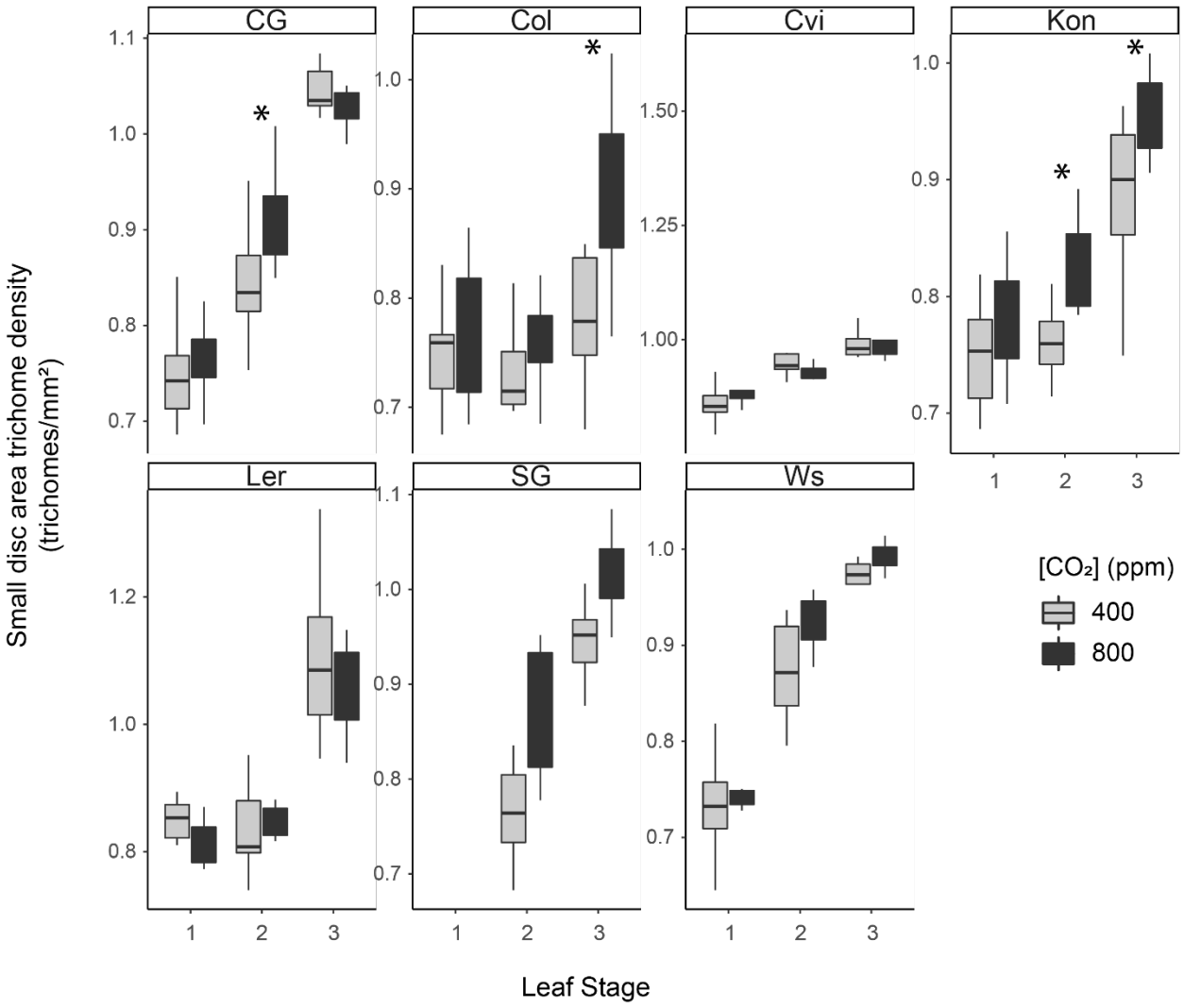

Fig. 13: Trichome densities in a smaller (compared to the area of observation used previously; Fig. 13) circular area surrounding individual trichomes, at 400 ppm [CO_2] and 800 ppm [CO_2]. Asterisks indicate a significant difference between the variable at current and elevated [CO_2]; significance tested via post-hoc means testing (Table 4)

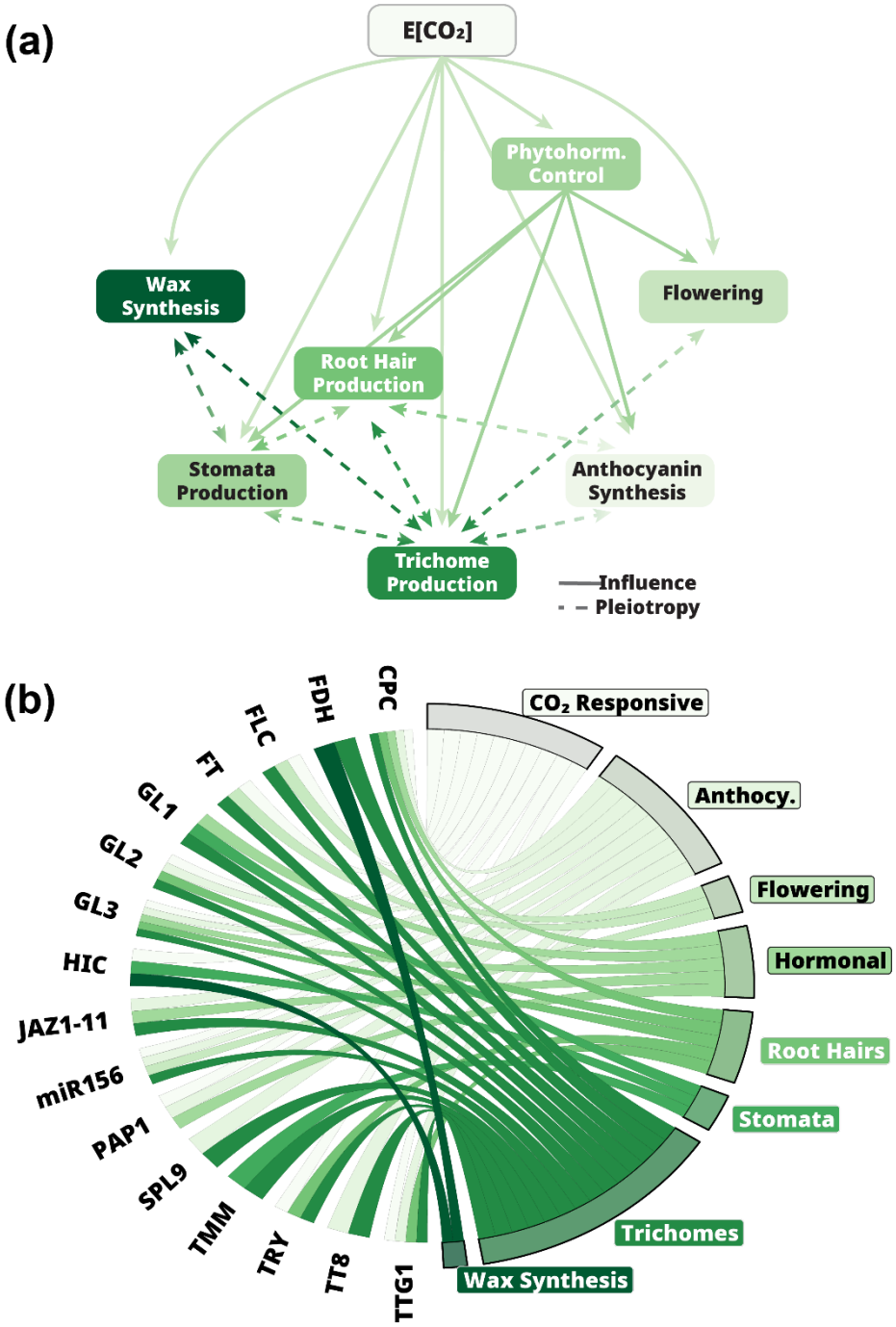

Fig. 14: (a) Multiple pathways overlap with trichome production, either through a pathway directly controlling trichome production, or through pleiotropy. Dashed lines indicate pleiotropy between the trichome pathway and another pathway, while solid lines indicate control over the connected pathway. (b) Pleiotropy of various genes controlling the initiation of trichomes. On the left-hand side are genes related to trichome initiation, while in the boxes on the right-hand side are biological roles the genes play. "Elevated [CO_2] responsive" refers to genes that have been found to respond to elevated [CO_2] within any organ or time point of plant growth. Figure created using R package GOplot (The figure was created by J.M. Fischer, based on results from

Lolle et al. 1997; Wada et al. 1997; Szymanski and Marks 1998; Yephremov 1999; Gray et al. 2000; Payne et al. 2000; Walker et al. 2000; Bird and Gray 2002; Johnson et al. 2002; Ohashi et al. 2002; Schiefelbein 2003; Traw and Bergelson 2003; Zhang 2003; Li 2008; Maes et al. 2008; Springer 2008; Yoshida et al. 2009; Yu et al. 2010; Gou 2011; Niu 2011; Qi et al. 2011; Yan et al. 2012; May et al. 2013; Tsuji 2013; Qi et al. 2014; Shi and Xie 2014; Wang and Chen 2014; Xue et al. 2014; Yan et al. 2014; Pesch et al. 2015; Walter 2015; Wang 2015; Engineer et al. 2016; Hegebarth et al. 2016; Matias-Hernandez 2016)

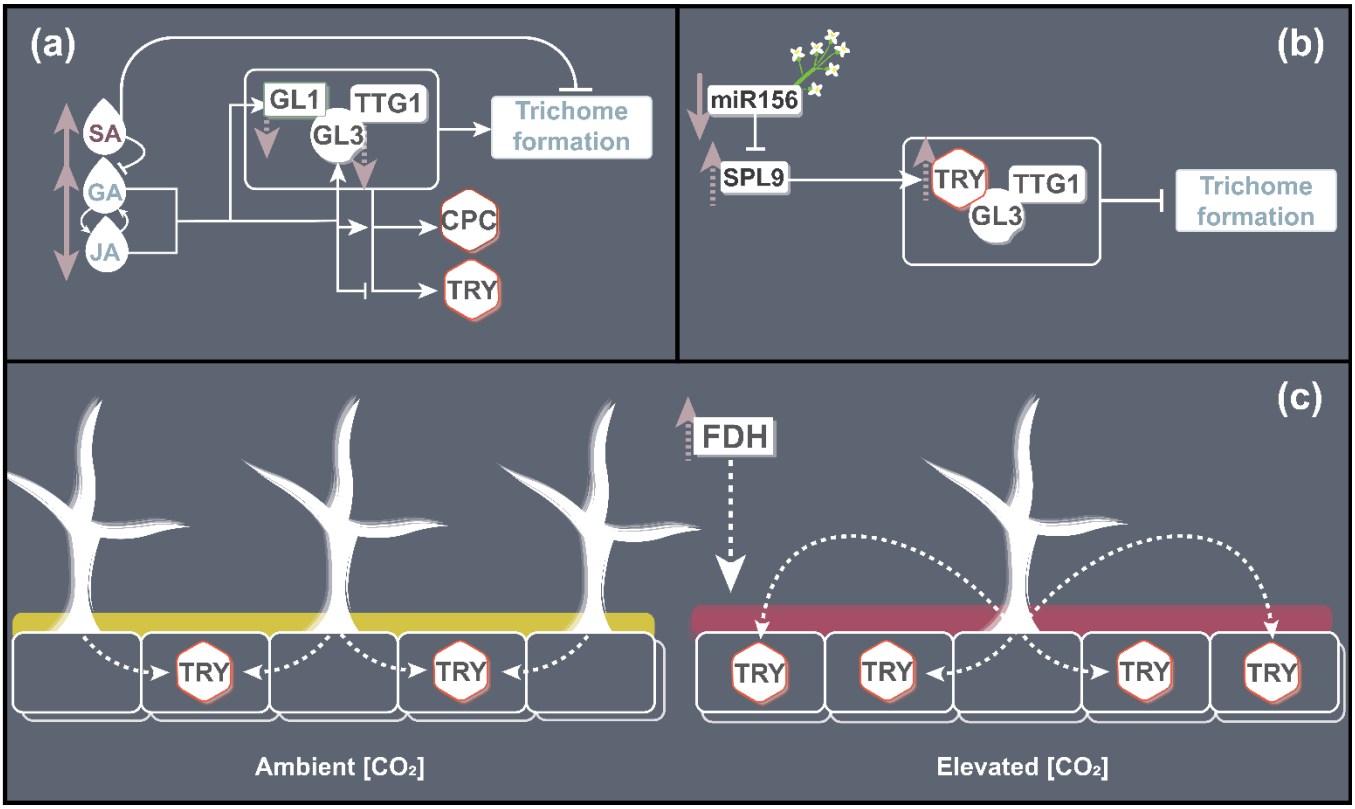

Fig. 15: Here we use trichome density decreases as the outcome of each potential mechanism for efficiency, and present potential mechanisms behind shifts in trichome densities under elevated [CO_2]. Solid lines indicate known connections/responses pulled from the literature; dashed lines indicate predicted connections/responses. Arrows either pointing up or down next to genes/proteins indicates an up or down regulation, respectively, at elevated [CO_2]. (a) Shifting concentrations of phytohormones alter transcription of key trichome genes, decreasing trichome numbers (Sun, Guo, & Ge, 2016; Sun, Guo, Zhu-Salzman, & Ge, 2013; Teng et al., 2006; Zavala, Nabity, & DeLucia, 2012). (b) Flowering regulators *miR156* and *SPL9* respond at elevated [CO_2] in a manner that may increase transcription of core negative trichome regulator TRY, decreasing trichome numbers as TRY increases at elevated [CO_2] (Ioannidi et al., 2016; Matias-Hernandez, 2016; May et al., 2013; Springer & Ward, 2007; Sun et al., 2016; Van Der Kooij & De Kok, 1996; Xu et al., 2016; Yu et al., 2010). (c) Similar to the relationship between wax gene *HIC* and stomatal densities at elevated [CO_2], *FDH* is a wax gene known to affect trichome densities. Here we hypothesize that if *FDH* is elevated [CO_2]-responsive similar to the *FDH* relative *HIC*, shifts in wax constituency may cause differential signal transmission across cuticular wax at elevated [CO_2]. Although there are numerous possibilities for wax-mobile signals, in this example we show TRY as the signal with elevated [CO_2] increasing the distance TRY can travel across the cuticle. (Bird & Gray, 2002; Gray et al., 2000; Haus, Li, Chitwood, & Jacobs, 2018; Hegebarth, Buschhaus, Wu, Bird, & Jetter, 2016; Morohashi & Grotewold, 2009; Shepherd & Griffiths, 2006; Yephremov, 1999). Figure from J.M. Fischer

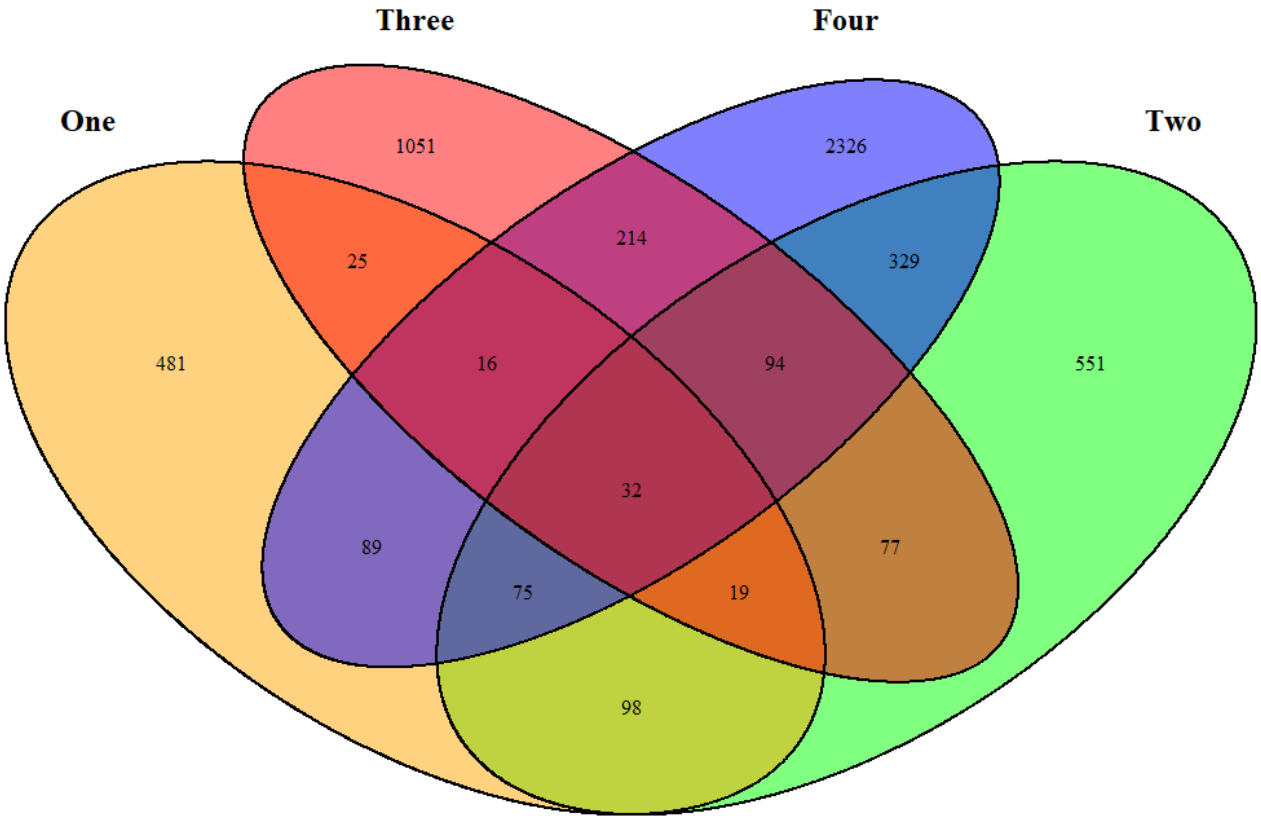

Fig. 16: Venn diagram of significant DEGs across four stages of leaf development, compared between ambient and elevated [CO_2]. Top labels indicate the stage of the developing leaf. Generally, the amount of DEGs increased as the leaf developed.

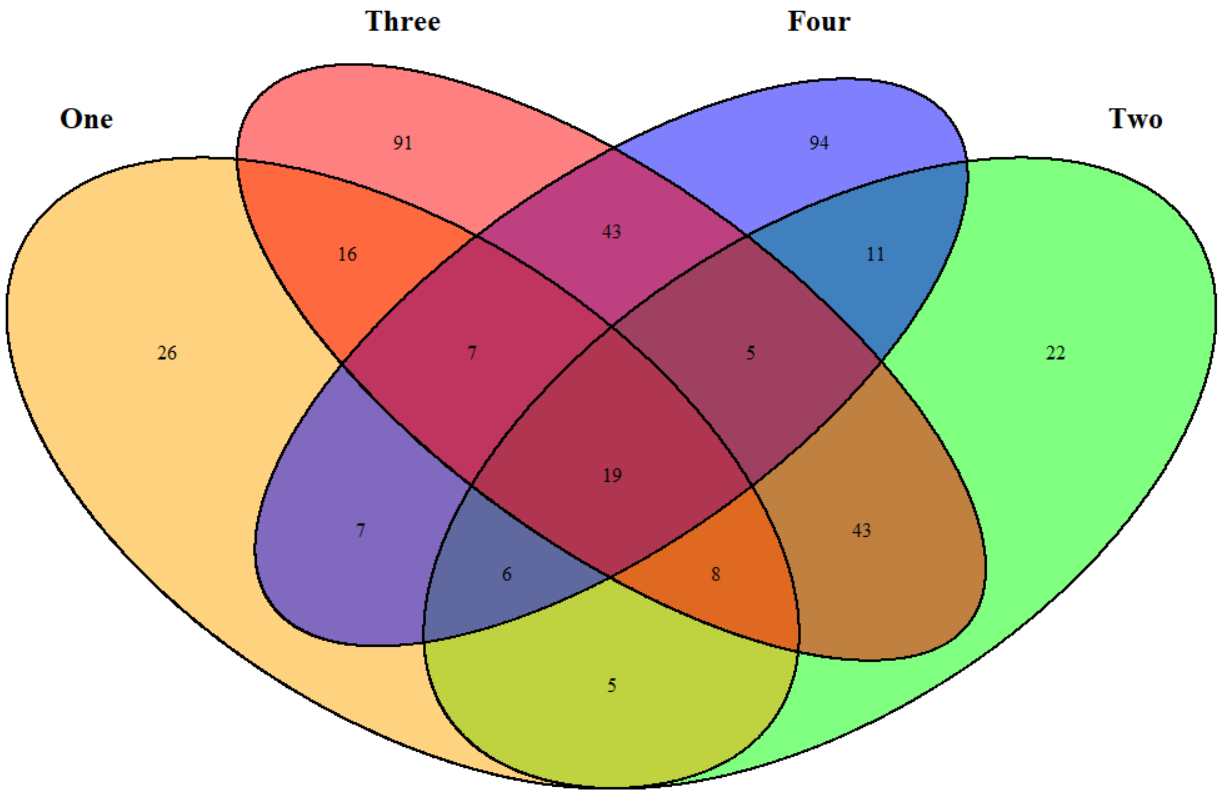

Fig. 17: Venn diagram of GO terms associated with DEGs across four stages of leaf development, compared between ambient and elevated [CO_2]. Top labels indicate the stage of the developing leaf. Similar to DEGs (Fig. 7), the amount of DEG GO terms increased as the leaf developed

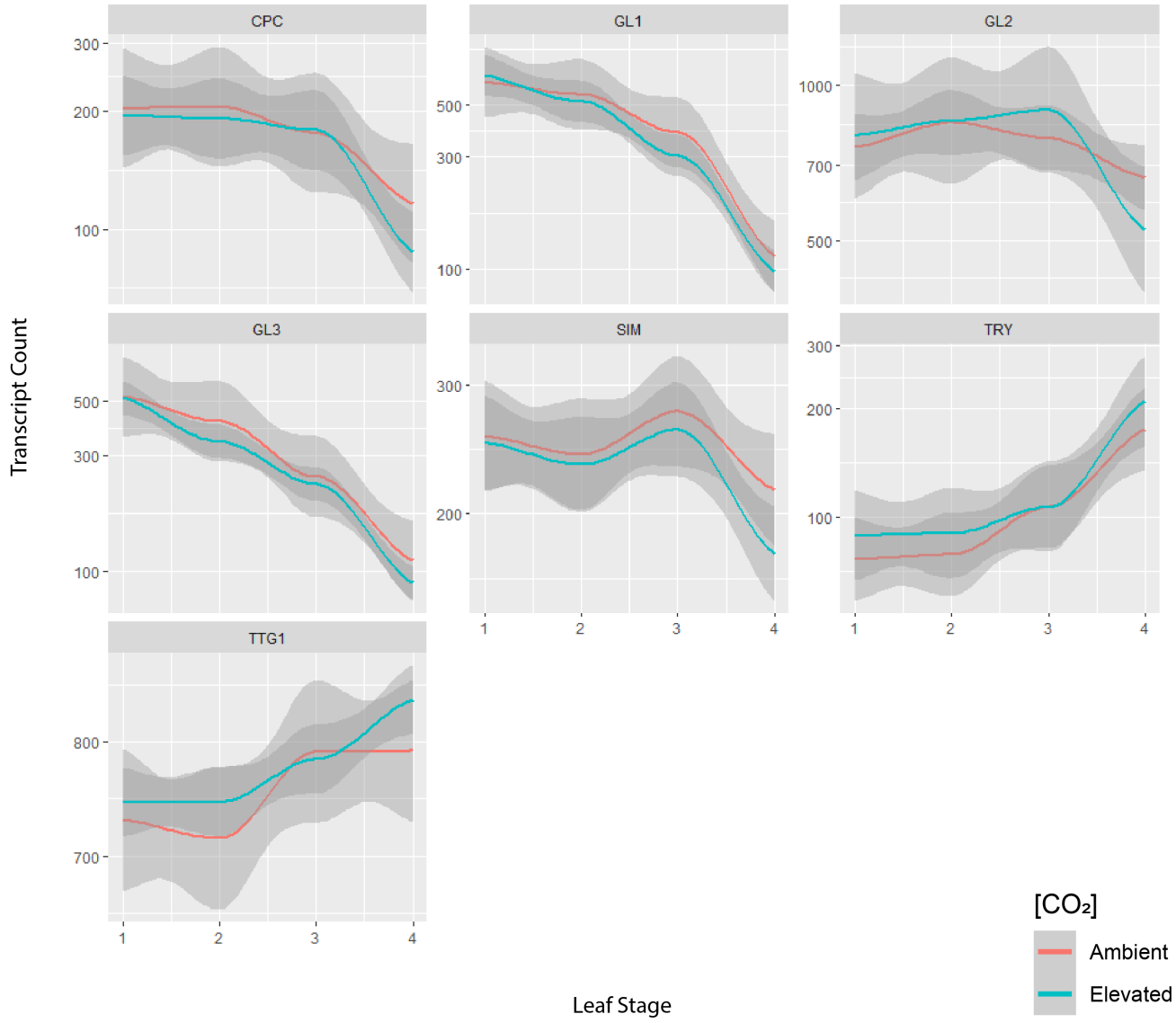

Fig. 18: Expression of core trichome genes across time, under ambient and elevated [CO_2]. Most notably, none of these seven genes, necessary for trichome initiation, were differentially transcribed at elevated [CO_2]

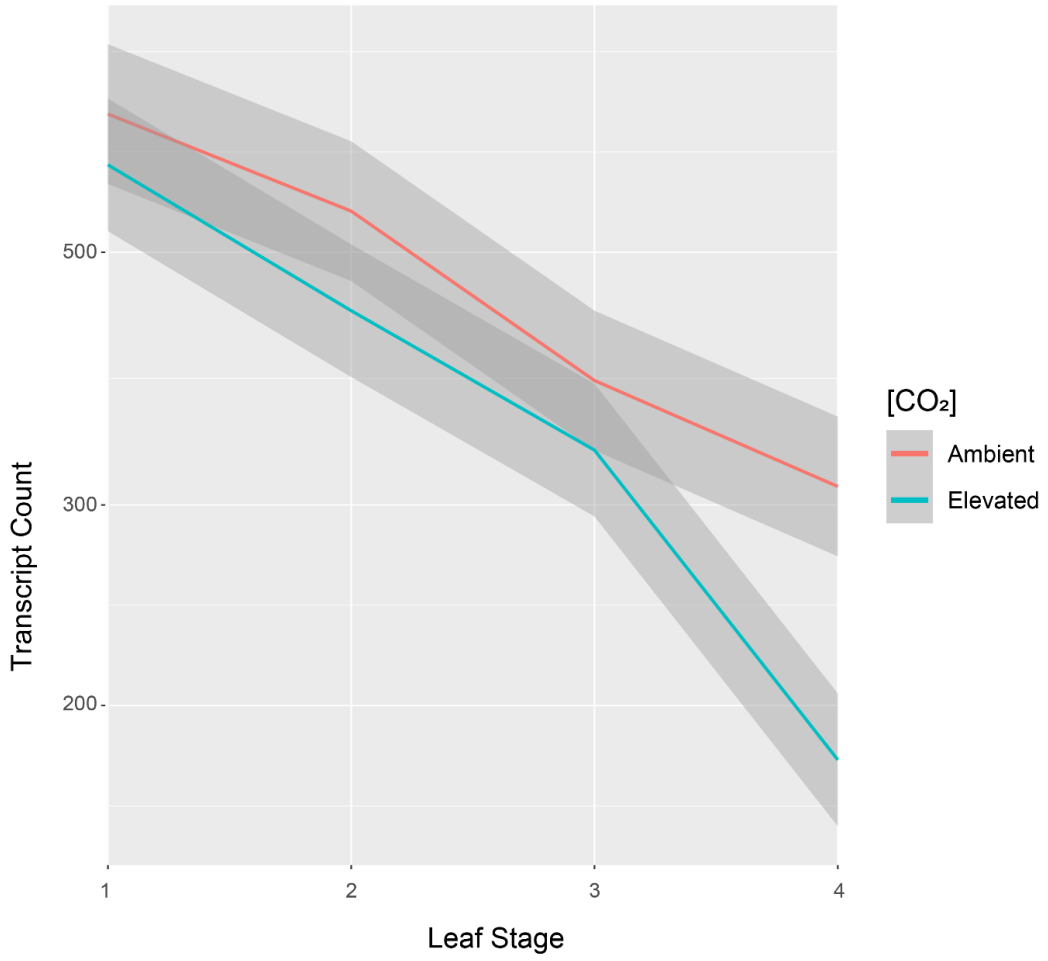

Fig. 19: *SPL9* transcription across early development of the leaf, at ambient and elevated [CO_2]. *SPL9* transcription was significantly reduced at stage four of leaf development under elevated [CO_2]. See text for details on how this could potentially drive increases in trichome numbers across the leaf

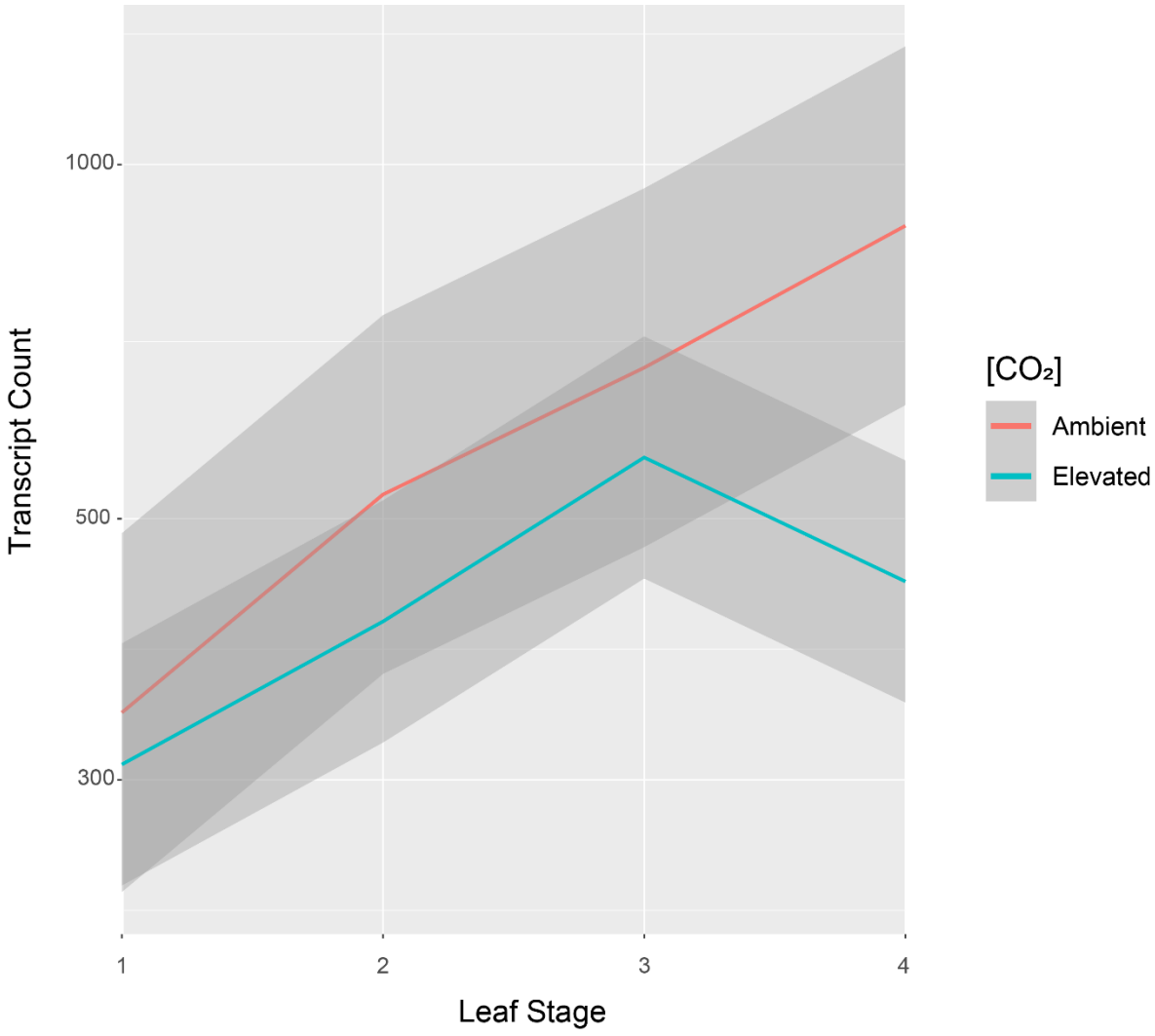

Fig. 20: *PAP1* transcription across early development of the leaf, at ambient and elevated [CO_2]. *PAP1* transcription was significantly reduced at stage four of leaf development under elevated [CO_2]. PAP1 is an anthocyanin protein which binds to the GL3-TTG1 dimer, which is also utilized in the trichome initiation pathway. See text for details on how this could potentially drive increases in trichome numbers across the leaf

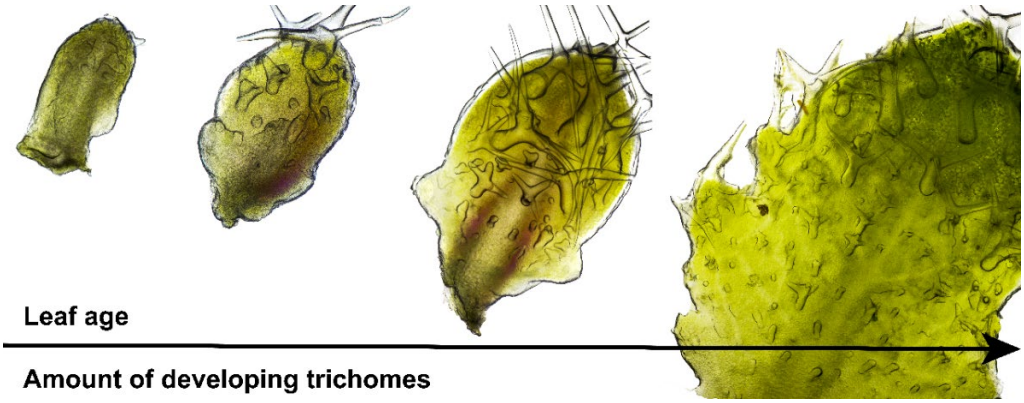

Fig. 21: As the primordial leaf develops, trichomes initiate at the base of the leaf. Once initiated, trichomes are separated and pushed to other portions of the leaf through leaf expansion and cell division

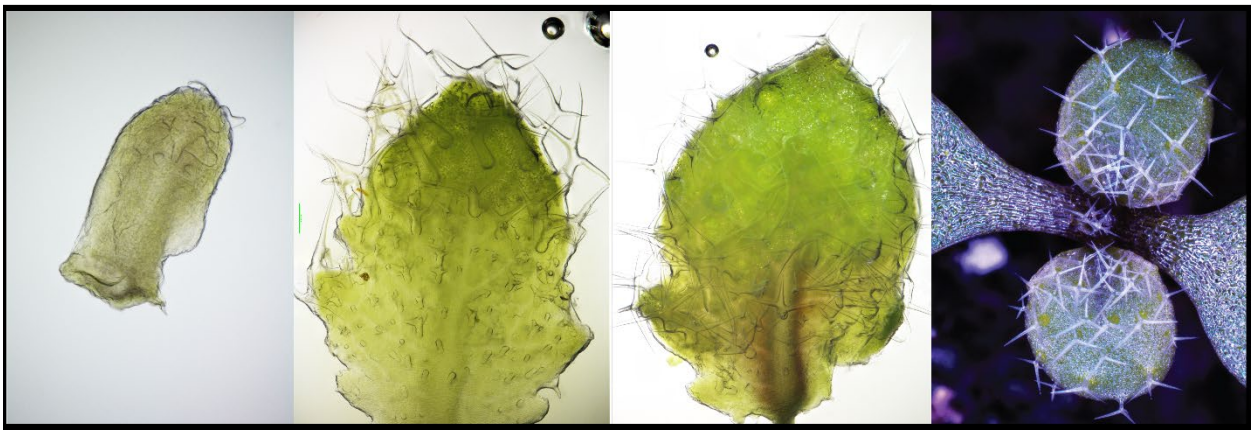

Fig. 22: Harvesting stages one through four (left to right) of the developing leaf, harvested for RNA-sequencing.

Tables

Table 1: General linear mixed-model ANOVA results for trichome density, trichome count, and leaf area with interaction between [CO_2], *Arabidopsis* genotype, and leaf stage. Variables were Tukey transformed prior to ANOVA analysis. See text for model

Trichome Density			
	Chisq	Df	P
[CO_2] Treatment:Genotype:Leaf Stage	60.97805	12	1.50E-08
Trichome Count			
	Chisq	Df	P
[CO_2] Treatment:Genotype:Leaf Stage	36.70576	12	0.000249
Leaf Area			
	Chisq	Df	P
[CO_2] Treatment:Genotype:Leaf Stage	53.50887	12	3.34E-07

Table 2: Specific comparisons of trichome density, trichome count, and leaf area between ambient and elevated [CO_2] treatments, within each genotype x leaf stage combination. Specific comparisons were examined with post-hoc means testing following a significant interaction (Table 1), with a Tukey adjustment

Variable	Genotype	Leaf Stage	Estimate (400 - 800)	SE	t Ratio	P
Leaf Area	CG	1	-0.00325	0.05728	-0.05676	0.955
Leaf Area	CG	2	0.078424	0.047618	1.646922	0.101
Leaf Area	CG	3	0.129365	0.047618	2.716705	0.00694
Trichome Density	CG	1	-0.00738	0.019917	-0.37077	0.711
Trichome Density	CG	2	-0.03731	0.016571	-2.25178	0.025
Trichome Density	CG	3	-0.0232	0.016571	-1.40011	0.162
Trichome Count	CG	1	-0.06461	0.127994	-0.50482	0.614
Trichome Count	CG	2	-0.16006	0.106544	-1.50233	0.134
Trichome Count	CG	3	0.118131	0.106544	1.10875	0.268
Leaf Area	Col	1	-0.06159	0.049943	-1.23313	0.218
Leaf Area	Col	2	-0.05092	0.049943	-1.01951	0.309
Leaf Area	Col	3	0.327725	0.049943	6.562026	2.07E-10
Trichome Density	Col	1	-0.00152	0.01738	-0.08735	0.93
Trichome Density	Col	2	-0.03644	0.01738	-2.09696	0.0368
Trichome Density	Col	3	-0.12631	0.01738	-7.26782	2.69E-12
Trichome Count	Col	1	-0.12098	0.111744	-1.08266	0.28
Trichome Count	Col	2	-0.36667	0.111744	-3.2813	0.00114
Trichome Count	Col	3	-0.35649	0.111744	-3.19019	0.00156
Leaf Area	Cvi	1	0.091425	0.052644	1.736663	0.0834
Leaf Area	Cvi	2	0.04496	0.054373	0.826872	0.409
Leaf Area	Cvi	3	-0.06518	0.071655	-0.90964	0.364
Trichome Density	Cvi	1	-0.05354	0.01832	-2.92235	0.00371
Trichome Density	Cvi	2	0.00306	0.01892	0.161722	0.872
Trichome Density	Cvi	3	0.050705	0.024929	2.033954	0.0427
Trichome Count	Cvi	1	-0.25784	0.117789	-2.18897	0.0293
Trichome Count	Cvi	2	0.143757	0.121653	1.181703	0.238
Trichome Count	Cvi	3	0.363222	0.160234	2.266828	0.024

Leaf Area	Kon	1	0.083964	0.049943	1.681215	0.0937
Leaf Area	Kon	2	0.245209	0.049943	4.909805	1.44E-06
Leaf Area	Kon	3	0.328174	0.049943	6.571015	1.96E-10
Trichome Density	Kon	1	-0.01292	0.01738	-0.74315	0.458
Trichome Density	Kon	2	-0.05136	0.01738	-2.95493	0.00335
Trichome Density	Kon	3	-0.08328	0.01738	-4.79166	2.51E-06
Trichome Count	Kon	1	0.073162	0.111744	0.654724	0.513
Trichome Count	Kon	2	0.122314	0.111744	1.094587	0.275
Trichome Count	Kon	3	0.090201	0.111744	0.807208	0.42
Leaf Area	Ler	1	-0.03736	0.049943	-0.74811	0.455
Leaf Area	Ler	2	-0.1156	0.049943	-2.31473	0.0212
Leaf Area	Ler	3	-0.09493	0.049943	-1.90074	0.0582
Trichome Density	Ler	1	-0.03348	0.01738	-1.92616	0.0549
Trichome Density	Ler	2	0.014785	0.01738	0.850708	0.396
Trichome Density	Ler	3	-0.03164	0.01738	-1.8204	0.0696
Trichome Count	Ler	1	-0.2278	0.111744	-2.03855	0.0423
Trichome Count	Ler	2	-0.10295	0.111744	-0.92131	0.358
Trichome Count	Ler	3	-0.45978	0.111744	-4.11455	4.91E-05
Leaf Area	SG	1	-0.12175	0.067103	-1.81433	0.0705
Leaf Area	SG	2	0.154824	0.051401	3.01205	0.0028
Leaf Area	SG	3	0.102004	0.051406	1.984268	0.0481
Trichome Density	SG	1	-0.06002	0.023345	-2.57088	0.0106
Trichome Density	SG	2	-0.04875	0.017883	-2.72591	0.00675
Trichome Density	SG	3	-0.00439	0.017886	-0.24535	0.806
Trichome Count	SG	1	-0.52438	0.150062	-3.49444	0.000538
Trichome Count	SG	2	-0.02893	0.114995	-0.25156	0.802
Trichome Count	SG	3	0.211738	0.114986	1.841432	0.0665
Leaf Area	Ws	1	-0.00717	0.049943	-0.14363	0.886
Leaf Area	Ws	2	0.183083	0.049943	3.665856	0.000287
Leaf Area	Ws	3	0.161734	0.049943	3.238385	0.00133
Trichome Density	Ws	1	0.013515	0.01738	0.777626	0.437
Trichome Density	Ws	2	-0.04979	0.01738	-2.86471	0.00444
Trichome Density	Ws	3	-0.03957	0.01738	-2.27683	0.0234

Trichome Count	Ws	1	0.049883	0.111744	0.446405	0.656
Trichome Count	Ws	2	-0.03682	0.111744	-0.32949	0.742
Trichome Count	Ws	3	-0.02694	0.111744	-0.24107	0.81

Table 3: ANOVA results for three patterning statistics, with interactions between [CO_2], *Arabidopsis* genotype, and developmental leaf stage. A linear mixed-model ANOVA was used (model is given in text), treating plant replicate as a random effect

Larger observation area, trichome density			
	Chisq	Df	P
[CO_2] Treatment:Genotype:Leaf Stage	20.82057	10	0.02238
Smaller observation area, trichome density			
	Chisq	Df	P
[CO_2] Treatment:Genotype:Leaf Stage	18.82984	10	0.042478
Trichome distance from leaf tip			
	Chisq	Df	P
[CO_2] Treatment:Genotype:Leaf Stage	36.96671	10	5.73E-05

Table 4: Post-hoc means testing (Tukey adjusted) results comparing the trichome densities in a large circular area surrounding each trichome, in 400 and 800 ppm [CO_2] treatments. Statistical tests were executed in R following a significant interaction result in the ANOVAs (Table 3)

Variables	Genotype	Leaf Stage	Estimate (400 -800)	SE	df	T ratio	P
Distance from tip	CG	1	1.00E-02	0.012849	325.4106	0.780259	0.435806
Distance from tip	CG	2	-4.93E-03	0.010688	316.3547	-0.46154	0.64473
Distance from tip	CG	3	-1.35E-02	0.010688	316.3547	-1.26512	0.20676
Larger obs. Area	CG	1	-0.03028	0.028254	323.0853	-1.07155	0.284721
Larger obs. Area	CG	2	-0.02063	0.023371	316.065	-0.88273	0.378052
Larger obs. Area	CG	3	0.01593	0.023371	316.065	0.681624	0.495976
Smaller obs. Area	CG	1	-0.03569	0.04138	325.3765	-0.86252	0.389037
Smaller obs. Area	CG	2	-0.08835	0.034422	316.3169	-2.56673	0.010726
Smaller obs. Area	CG	3	0.014923	0.034422	316.3169	0.43354	0.664918
Distance from tip	Col	1	-6.43E-03	0.01121	316.3547	-0.5737	0.566576
Distance from tip	Col	2	-7.80E-03	0.01121	316.3547	-0.69582	0.487054
Distance from tip	Col	3	5.26E-03	0.01121	316.3547	0.469568	0.638987
Larger obs. Area	Col	1	-0.03853	0.024512	316.065	-1.57177	0.117005
Larger obs. Area	Col	2	-0.07147	0.024512	316.065	-2.91552	0.003805
Larger obs. Area	Col	3	-0.05913	0.024512	316.065	-2.41243	0.016416
Smaller obs. Area	Col	1	-0.04375	0.036102	316.3169	-1.21171	0.226527
Smaller obs. Area	Col	2	-0.04115	0.036102	316.3169	-1.13973	0.255261
Smaller obs. Area	Col	3	-0.15516	0.036102	316.3169	-4.29791	2.3E-05
Distance from tip	Cvi	1	-7.62E-03	0.011816	316.3547	-0.64463	0.519637
Distance from tip	Cvi	2	1.69E-02	0.012204	318.7119	1.385735	0.166797
Distance from tip	Cvi	3	2.72E-02	0.016078	325.6224	1.69288	0.091435
Larger obs. Area	Cvi	1	-0.02529	0.025838	316.065	-0.97882	0.328418
Larger obs. Area	Cvi	2	0.055324	0.026684	318.6398	2.073301	0.038948
Larger obs. Area	Cvi	3	-0.01002	0.035148	325.2116	-0.28514	0.775719
Smaller obs. Area	Cvi	1	-0.05934	0.038055	316.3169	-1.55934	0.119917
Smaller obs. Area	Cvi	2	0.06582	0.039304	318.5176	1.67465	0.094984
Smaller obs. Area	Cvi	3	-0.00024	0.051783	325.5891	-0.00463	0.996306
Distance from tip	Kon	1	-8.44E-03	0.01121	316.3547	-0.75295	0.452039
Distance from tip	Kon	2	-2.30E-02	0.01121	316.3547	-2.04976	0.041212
Distance from tip	Kon	3	1.57E-02	0.01121	316.3547	1.403692	0.161391
Larger obs. Area	Kon	1	-0.04993	0.024512	316.065	-2.03691	0.042492
Larger obs. Area	Kon	2	-0.06511	0.024512	316.065	-2.65611	0.008306
Larger obs. Area	Kon	3	-0.01898	0.024512	316.065	-0.7742	0.439391
Smaller obs. Area	Kon	1	-0.05279	0.036102	316.3169	-1.46227	0.144661
Smaller obs. Area	Kon	2	-0.07266	0.036102	316.3169	-2.01259	0.045005
Smaller obs. Area	Kon	3	-0.10022	0.036102	316.3169	-2.77596	0.005832
Distance from tip	Ler	1	-1.97E-05	0.01121	316.3547	-0.00176	0.998601
Distance from tip	Ler	2	4.31E-02	0.01121	316.3547	3.84286	0.000147

Distance from tip	Ler	3	-2.81E-02	0.01121	316.3547	-2.50773	0.012651
Larger obs. Area	Ler	1	0.000455	0.024512	316.065	0.018569	0.985196
Larger obs. Area	Ler	2	-0.03251	0.024512	316.065	-1.32623	0.185722
Larger obs. Area	Ler	3	0.04254	0.024512	316.065	1.735476	0.083631
Smaller obs. Area	Ler	1	0.052173	0.036102	316.3169	1.445146	0.149407
Smaller obs. Area	Ler	2	-0.02647	0.036102	316.3169	-0.73308	0.464052
Smaller obs. Area	Ler	3	0.037072	0.036102	316.3169	1.026869	0.305267
Distance from tip	SG	2	2.49E-03	0.011535	318.5346	0.215694	0.829364
Distance from tip	SG	3	3.39E-02	0.011536	318.6544	2.940729	0.003514
Larger obs. Area	SG	2	-0.06486	0.025221	318.3872	-2.57145	0.010581
Larger obs. Area	SG	3	-0.03892	0.025224	318.4935	-1.54282	0.123867
Smaller obs. Area	SG	2	-0.15907	0.037152	318.386	-4.28164	2.46E-05
Smaller obs. Area	SG	3	-0.06802	0.037154	318.5534	-1.83084	0.068058
Distance from tip	Ws	1	-1.36E-02	0.01121	316.3547	-1.21136	0.226662
Distance from tip	Ws	2	-3.53E-03	0.01121	316.3547	-0.31465	0.753237
Distance from tip	Ws	3	2.81E-02	0.01121	316.3547	2.50258	0.012833
Larger obs. Area	Ws	1	0.082028	0.024512	316.065	3.346433	0.000917
Larger obs. Area	Ws	2	-0.01256	0.024512	316.065	-0.51221	0.608861
Larger obs. Area	Ws	3	-0.01063	0.024512	316.065	-0.4335	0.664944
Smaller obs. Area	Ws	1	-0.01867	0.036102	316.3169	-0.51715	0.605413
Smaller obs. Area	Ws	2	-0.05785	0.036102	316.3169	-1.60248	0.110048
Smaller obs. Area	Ws	3	-0.03235	0.036102	316.3169	-0.89612	0.370868

Table 5: Avenues of proposed research for future advances in the elevated [CO_2]-trichome field (see text for more details)

Future Research Avenues	**Related Resources**
Mobile signal from mature-to-developing leaves	(Haus et al. 2018)
Cuticle wax composition at elevated [CO_2]	
Proteomics of developing leaves at elevated [CO_2]; core trichome protein ratios	(Zhang 2018)
Single cell analyses across time; how elevated [CO_2]-accelerated basal cellular division/development may affect trichome densities	(Masle 2000; Ferris et al. 2001)
RNA-sequencing analyses throughout leaf development for plants grown in ambient and elevated [CO_2]	
Effects of elevated [CO_2] on phytohormone ratios (JA:GA:SA) in a single leaf	(Traw and Bergelson 2003; Yoshida et al. 2009)
Ecophysiological impacts of trichome shifts at elevated [CO_2], including: • WUE • Photosynthetic efficiency • UV-B reflectance • Herbivory • Leaf temperature • Effects on herbivore communities • Effects on intraspecific and interspecific competition • Crop output	(Sun et al. 2016)