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Running title: *CGR2* and *CGR3* Alters Photosynthesis, and Plant Growth

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Pectin Methylesterification Impacts the Relationship Between Photosynthesis and Plant Growth in *Arabidopsis thaliana*

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High levels of methylesterification of pectin allow for enhanced cell expansion, partitioning of resources to leaf growth and increased leaf area for photosynthesis, which in turn enhance plant growth

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S.M.W. carried out much of the research, analyzed the data and wrote the manuscript, S.-J.K. and L.R. characterized the mutants and performed cell wall component analysis and part of anatomical studies. F.C.A. provided technical help, S.M.W. and T.D.S and F.B. conceived the experiments and T.D.S and F.B. supervised the research and edited the manuscript.

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Abstract

Photosynthesis occurs in mesophyll cells of specialized organs such as leaves. The rigid cell wall encapsulating photosynthetic cells controls the expansion and distribution of cells within photosynthetic tissues. The relationship between photosynthesis and plant growth is affected by leaf area. However, the underlying genetic mechanisms affecting carbon partitioning to different aspects of leaf growth is not known. To fill this gap, we analyzed *Arabidopsis thaliana* plants with altered levels of pectin methylesterification, which is known to modulate cell wall plasticity and plant growth. Pectin methylesterification levels were varied through manipulation of *cotton Golgi-related* (*CGR*) *2* or *3* genes encoding two functionally redundant pectin methyltransferases. Increased levels of methylesterification in a line overexpressing *CGR2* (CGR2OX) resulted in highly expanded leaves with enhanced intercellular air spaces; reduced methylesterification in a mutant lacking both *CGR*-genes (*cgr2/3)* resulted in thin but dense leaf mesophyll that limited CO_2 diffusion to chloroplasts. Leaf, root, and plant dry weight were enhanced in CGR2OX but decreased in *cgr2/3*. Differences in growth between wild type and the *CGR*-mutants can be explained by carbon partitioning but not by variations in area-based photosynthesis. Therefore, photosynthesis drives growth through alterations in carbon partitioning to new leaf area growth and leaf mass per unit leaf area (LMA); however, *CGR*-mediated pectin methylesterification acts as a primary factor in this relationship through modulation of the expansion and positioning of the cells in leaves, which in turn drive carbon partitioning by generating dynamic carbon demands in leaf area growth and LMA.

INTRODUCTION

Photosynthesis is the major source of carbon (C) for plant growth. However, there is no clear correlation between area-based photosynthesis and plant growth and studies have shown that the relationship between photosynthesis and plant growth is affected by leaf area growth parameters. A strong positive correlation exists between relative growth rate and leaf growth parameters such as leaf area per unit leaf mass (specific leaf area - SLA), leaf area per unit plant mass (leaf area ratio), and investment of C in leaf growth (leaf mass ratio) (Shipley, 2002; Lambers et al., 2008; Poorter et al., 2009). In fact, modeled partitioning coefficients obtained from the *Arabidopsis* Leaf Area Growth Model showed that plant growth is more sensitive to changes in C partitioning to leaf 'thickening' and leaf area growth than to changes to area-based photosynthesis (Weraduwage et al., 2015). The term 'thickening' was used to indicate increased mass per unit leaf area (LMA) whether that results from increased thickness in the normal sense of more depth to the leaf between adaxial and abaxial surfaces or from increased cell density. LMA is the reciprocal of SLA. While LMA has been shown to positively and linearly correlate with investment to leaves in the form of anatomical and chemical composition changes, SLA shows a hyperbolic relationship with such changes (Poorter et al., 2009). Therefore, we will mainly focus on LMA throughout this work. Weraduwage et al. (2015) concluded that even though photosynthetic C is required for growth, the degree of growth is determined by the amount of C partitioned to leaf area growth and LMA (Weraduwage et al., 2015). However, how and to what degree this variation in leaf expansion and LMA is affected by cell wall properties of leaf mesophyll cells is not known. In addition, the underlying mechanisms that drive C partitioning to favor either leaf area growth or LMA, and the genes that can influence changes in C demands towards these two processes are yet to be found.

Leaves are the major photosynthetic organs in plants and regulation of cell expansion and growth is crucial for the development of leaf architecture. Leaf growth can occur in the form of leaf area or LMA (Lambers et al., 2008; Weraduwage et al., 2015). The size and shape of cells in the leaf mesophyll can have a significant impact on: (1) projected or effective leaf surface area, which is the leaf area capable of intercepting light, (2) leaf mass per unit leaf area or LMA, (3) CO_2 conductance from the external environment through intercellular air spaces into chloroplasts in mesophyll cells, and (4) on the surface area of mesophyll cells and chloroplasts exposed to

intercellular airspaces (Honda and Fisher, 1978; Evans et al., 1994; Galmés et al., 2013). The plant cell wall controls expansion and positioning of cells of photosynthetic tissues and therefore, changes in cell wall properties will have a direct impact on above properties. Although there is evidence for changes in leaf architecture as a result of alterations in genes that affect in synthesis and properties of the cell wall (Burton et al., 2000; Kim and Delaney, 2002; Pilling et al., 2004; Wolf et al., 2009; Kim et al., 2015), an in-depth analysis of affects on leaf gas exchange and overall plant growth under such circumstances has not yet been conducted.

The plant cell wall is composed mainly of polysaccharides, and among these, pectin is considered a critical element that controls cell wall elasticity and expansion. Pectin is synthesized in the Golgi apparatus and secreted as a highly methylesterified form. In the cell wall the levels of pectin methylesterification vary and are controlled by methyltransferases and methylesterases, which catalyze methylation and demethylation of galacturonic acid in the pectin backbone (Wolf et al., 2009; Kim et al., 2015). Removal of methyl groups allows carboxyl groups of galacturonic acid to form Ca^{2+} and Mg^{2+}-mediated intermolecular linkages that lead to hardening of pectin (Burton et al., 2000; Heldt and Piechulla, 2010; Kim et al., 2015). Indeed, overexpression of pectin methylesterase inhibitors results in longer root cells (Lionetti et al., 2007). Therefore, the degree of methylation and de-methylation of pectin determines the delicate balance between cell wall extensibility and rigidity with consequent effects on growth and shape of cells (Burton et al., 2000; Pilling et al., 2004; Lionetti et al., 2007; Wolf et al., 2009; Xiao and Anderson, 2013; Kim et al., 2015). Consistent with this notion, over-expression of pectin methyltransferases, *CGR2* or *CGR3*, led to enhanced rosette size and fresh weight in *Arabidopsis* (Kim et al., 2015). However, a double knockout of *CGR2* and *CGR3* genes, that was named *cgr2/3*, showed reduced cell expansion and overall plant growth; both phenotypes correlated with reduced levels of pectin methylesterification compared to wild type (Kim et al., 2015). To our knowledge, the effect of pectin methylation on leaf growth parameters, photosynthesis and respiration through its effect on leaf architecture has not been studied.

The goal of this study was to use the *Arabidopsis* model system developed by Kim et al. (2015) with altered levels of pectin methyltransferases, *CGR2* or *CGR3*, which are single gene modifiers found to positively or negatively affect cell expansion in function of their cellular availability, to

investigate how changes in cell wall plasticity affect C assimilation, leaf respiration and overall plant growth through effects on leaf area growth and mesophyll structure. We examined whether the changes in overall plant growth in *Arabidopsis* with altered *CGR2* and *CGR3* could result from changes in leaf area growth and LMA or changes in area-based photosynthesis to explore the relationship between photosynthesis and growth. Based on the differences in rosette size reported previously (Kim et al., 2015), we hypothesized that area-based photosynthesis rates would be lower in a *CGR2* overexpressor (CGR2OX) but larger in *cgr2/3*. As *CGR2* and *CGR3* affect pectin methylation and cell expansion in leaves, we also hypothesized that plant growth would be enhanced in the CGR2OX line and reduced in the *cgr2/3* mutant mainly as a result of altered LMA. Indeed, through analysis of leaf and plant growth, gas exchange, and growth modelling, we found that *CGR*2 and *CGR*3 have significant impacts on photosynthesis and respiration through alteration of mesophyll structure and LMA. Plant growth was enhanced in the CGR2OX line but reduced in the *cgr2/3* mutant. This variation in plant growth was as a result of differences in C partitioning and not as a result of changes in photosynthetic rate per unit leaf area. These observations support a novel model that changes in cell wall plasticity due to changes in pectin composition influences C partitioning between leaf area growth and LMA thereby regulating the relationship between photosynthesis and plant growth.

MATERIALS AND METHODS

Growth Analysis

Wild type *Arabidopsis thaliana* (Col-0), *cgr2/3* (loss of function double mutant line of *CGR2* and *CGR3*), CGR2OX (*CGR2* overexpression line), and cgr2com (*cgr2/3* complemented by *CGR2*) (Kim et al., 2015) were used in the study. Gene accession numbers for *CGR2* and *CGR3* are At3g49720 and At5g65810, respectively. Plants were grown hydroponically in ½ strength Hoagland's solution, under a light intensity of 120 μmol m^{-2} s^{-1} and an 8 h photoperiod, daytime and nighttime temperature of 22°C and 20°C respectively, and 60% relative humidity. The hydroponics system was housed in a GC-20, Bigfoot series growth chamber (BioChambers Inc., Winnipeg, MB, Canada).

Plant growth parameters including projected and total leaf area, leaf, root, and inflorescence dry weights, relative growth rates, SLA, LMA, leaf area ratio, and leaf, root, and inflorescence mass

ratios were measured as described in Weraduwage et al. (2015). When calculating SLA, LMA, leaf area ratio, and leaf, root, and inflorescence mass ratios, the total leaf area, and organ and plant mass corresponding to each harvest was used: SLA (m^2 kg^{-1}) = total leaf area/leaf mass; LMA (kg m^{-2}) = leaf mass/total leaf area; leaf area ratio (g g^{-1}) = total leaf area/total plant mass; leaf, root or inflorescence mass ratio (g g^{-1}) = leaf, root or inflorescence mass/total plant mass. Growth measurements were obtained throughout the life cycle of the plant at 2-3 week intervals at 29, 49, 63, and 82 days after seeding (DAS). The 100-seed weight prior to stratification and sowing, cotyledon area and hypocotyl length was also measured.

Determination of Leaf Thickness, Mesophyll Cell Density, S_{mes}, S_c, and Chloroplast Size and Abundance

Leaves of similar age from 34 and 51 day old rosettes were used for anatomical measurements. Leaves were harvested and processed for leaf cross-sectioning as described in Weraduwage et al., 2015, at the Center for Advanced Microscopy, Michigan State University. Thin (500 nm) leaf sections (PTXL ultramicrotome, RMC, Boeckeler Instruments, Tucson, AZ) were observed under an Olympus SpectralView FV1000, Confocal Laser Scanning Microscope (Olympus, NJ, USA) and the distance between the adaxial and abaxial leaf surfaces was measured to determine leaf thickness.

Micrographs of leaf cross sections were used to determine the number of palisade and spongy parenchyma cells, the total number of cells, and the number of mesophyll cell layers in a 20,000 μm^2 area of a leaf cross section. This data was later expressed per mm^2. ImageJ (http://imagej.nih.gov/ij/) software was utilized to measure the total area of intercellular air spaces, total length of mesophyll cells, total length of mesophyll cells exposed to intercellular air spaces (L_{mes}), and the total length of chloroplasts exposed to intercellular air spaces (L_c). The latter three measurements were utilized to determine the surface are of mesophyll cells exposed to intercellular air spaces (S_{mes}) and the surface area of chloroplast exposed to intercellular air spaces (S_c) per unit leaf area as described in Evans et al. (1994).

A series of z-stack images of leaves of 34 day old plants was acquired using an inverted laser scanning confocal microscope (Zeiss LSM510 META, http://www.zeiss.com). Chloroplasts were

imaged using an ECPlan-Neofluar 40X/1.30 oil M27 objective at a 512x512 pixel resolution using an excitation wavelength of 514 nm and an emission wavelength of 673 nm. The different planes acquired were then projected on the same image and transformed in binary using Image J. The resulting number of chloroplasts per unit area and average chloroplast size was measured using ImageJ software.

Gas Exchange Measurements, $^{14}CO_2$ Feeding Experiments, and Analysis of Leaf ^{13}C Discrimination

Whole rosette photosynthesis and nighttime dark respiration was measured at 2-3 week intervals at 29, 49, 63, 82 days after seeding (DAS). A custom-built rosette-gas-exchange chamber connected to a LI-COR 6400 portable gas exchange system (LI- COR Biosciences, Inc., Lincoln, NE) was used to obtain photosynthesis and respiratory measurements. Conditions in the *Arabidopsis* rosette gas-exchange chamber during photosynthesis measurements were: a leaf temperature of 22^{o}C, [CO_2] of 400 ppm, 120 μmol m^{-2} s^{-1} light intensity. During nighttime respiration measurements the leaf temperature was maintained at 20^{o}C. Flow rate was set at 500 μmol s^{-1} when making measurements from 49 and 63 day old plants. For 29 and 82 day old plants, flow rate was set at 300, and 700 μmol s^{-1}, respectively in order to better control humidity.

After the initial measurements of photosynthesis, 29 day old *Arabidopsis* rosettes were fed with $^{14}CO_2$ for 10 minutes. After a 2 h chase period, leaf and root tissue were harvested separately into scintillation vials. Radioactivity was determined as disintegrations per minute (DPM) by liquid scintillation counting. The DPM recorded from roots and rosettes and the total DPM was used to calculate the proportion of C present in these organs.

The content ratio of $^{13}CO_2$ to $^{12}CO_2$ in *Arabidopsis* freeze-dried leaf material was measured at the Stable Isotope Ratio Facility for Environmental Research, University of Utah by mass spectrometry. This data was used to calculate the $\delta^{13}C_{VPDB}$ value which is the ^{13}C to ^{12}C isotopic ratio of leaves relative to the ^{13}C to ^{12}C isotopic ratio of the Vienna-Pee-Dee Belemnite (VPDB) standard (Farquhar et al., 1982). The $\delta^{13}C_{VPDB}$ values were used to calculate the CO_2 partial pressure at rubisco using equation 12 given in Farquhar et al. (1982). Values for the various

227 factors are as follows: δ value of CO_2 in the air at present = - 8.4‰ (http://www.esrl.noaa.gov),
228 partial pressure of CO_2 in the air = 39 Pa, ^{13}C discrimination associated with the diffusion of CO_2
229 through air = 4.4‰, ^{13}C discrimination in the carboxylation reaction = 27‰ (Farquhar et al.,
230 1989).
231
232 **Analysis of Cell Wall Polysaccharides in Leaves**
233 Preparation of alcohol-insoluble residues (AIR), uronic acid assays and methyl ester assays were
234 performed based on the methodology described in Kim et al. (2015). The matrix polysaccharide
235 (hemicellulose) and crystalline cellulose composition, and soluble sugar content in leaves were
236 measured at the Great Lakes Bioenergy Research Center Cell-Wall Facility, Michigan State
237 University (East Lansing, MI) as described in Kim et al. (2015).
238
239 **Parameterization of the *Arabidopsis* Leaf Area Growth Model**
240 Development, parameterization and execution of the *Arabidopsis* Leaf Area Growth Model were
241 described in detail in Weraduwage et al. (2015). This model is a tool to determine partitioning
242 coefficients of C that could give rise to the growth patterns observed in different plants
243 (Weraduwage et al., 2015). In the model, plant growth is divided into four growth stages: (1)
244 heterotrophic phase (1-4 DAS), (2) early vegetative phase, (3) late vegetative phase, and the (4)
245 reproductive phase. Ninety days of plant growth is simulated in the model using a fixed time step
246 of 1 h and the photoperiod is set at 8 h. As described in detail in Weraduwage et al. (2015), the
247 following was used as inputs of the model: weight of storage reserves, ratio of mobilized storage
248 reserves to stored reserves, initial leaf area, initial leaf mass, projected to total leaf area ratio, net
249 photosynthesis rate per unit leaf area, proportion of C stored as starch during the day, leaf
250 maintenance and growth respiratory coefficients, photoperiod, lengths of growth phases.
251
252 Values for area-based photosynthesis obtained during the four harvests were fitted with a
253 polynomial 3rd order equation to extrapolate area-based photosynthesis through the entire 90
254 days of the life cycle. Similarly, measured projected to total leaf area ratios during the four
255 harvests was fitted with a polynomial 2nd order equation to extrapolate the values over 90 days.
256 These extrapolated values were used in the model as input photosynthesis values and projected to
257 total leaf area ratios over the life span. The model also takes both growth and maintenance

respiration into consideration. The model considers maintenance respiration of a plant/particular organ to be proportional to the corresponding dry mass and growth respiration to be proportional to the growth rate (De Vries et al., 1974; Amthor, 1984; Thomas et al., 1993; Lambers et al., 2008). The model has set values for maintenance coefficients (amount of C respired to maintain the existing mass of the plant or specific organ, μmol C g^{-1} s^{-1}), and for growth coefficients (amount of C respired per unit increase in mass, g g^{-1}) based on values provided in the literature (Amthor, 1984; Mariko, 1988) and assumes that growth respiration coefficients of leaf growth in terms of area and LMA are the same throughout the life cycle. The net amount of C available for growth and maintenance processes of the plant is determined as the product of area-based photosynthetic rate and the projected leaf area. Next, C consumed in maintenance respiration and exudation is subtracted to determine the net assimilation rate, which provides the amount of C available for growth. The model then simulates the partitioning of C available for growth to leaf area growth, growth in terms of LMA, and root and inflorescence growth based on the set partitioning coefficients which are adjustable to facilitate fitting of the data. Partitioning coefficients vary based on the growth phases. The increase in mass during growth in terms of leaf area and LMA, and root and inflorescence growth is calculated by subtracting the C consumed in growth respiration from the total C allocated for growth of that particular organ. Accordingly, the model simulates the increase in leaf area and mass, and the mass of the inflorescence and roots. The model calculated SLA, LMA, and leaf, root and inflorescence mass ratios over time based on the equations given above.

In order to fit the modeled data to measured data, 16 partitioning coefficient parameters (partitioning to the inflorescence, roots, leaf area and LMA, for each of the four growth phases: germination phase, early and late vegetative phases, and reproductive phase), were adjusted so that the modeled and measured leaf area, mass of inflorescence, root, and leaf matched (Weraduwage et al., 2015). C partitioning data obtained by feeding *Arabidopsis* rosettes at 29 DAS with $^{14}CO_2$ were used as initial estimates which were fine tuned to fit the data. Sensitivity of the model to changes in the inputs was tested in Col-0 by simulating an increase or decrease of 1% in C partitioned to growth processes, and other model inputs, and noting the resulting response in output. The model was considered sensitive, in cases where more than 1% variation occurred in model outputs as a consequence of changing a particular input.

Statistical Analyses of Experimental Data

Four to five biological replicates per line were used to collect experimental data at a given time point. Statistical analyses were carried out using SPSS 22 (IBM Corporation, NY, NY). The effects of altered *CGR2* and *CGR3* expression on plant growth and gas exchange were compared using a univariate general linear model and the differences between means were tested by subjecting data to one-way ANOVA at $\alpha = 0.05$, followed by a Least Significant Difference Test. Measured data presented in figures represent the mean ± standard error (SE).

RESULTS

CGR2OX and *cgr2/3* Mutant Lines Retained Altered Cell Wall Composition

Cell wall composition of Col-0, cgr2com, CGR2OX and *cgr2/3* was analyzed in order to determine whether the previously observed changes in methylesterification and modification of cell-wall polysaccharides by Kim et al. (2015) was retained in the mutant lines. Similar to what was seen by Kim et al. (2015), crystalline cellulose content was lower in *cgr2/3* mutant line and it was greater in CGR2OX than that in Col-0 (**Supplemental Fig. S1A**). In addition, neutral sugars such as fucose, arabinose, and glucose content was significantly higher in *cgr2/3* than Col-0 as was shown by Kim et al. 2015 (**Supplemental Fig. S1B**). The uronic acid content and the amount of methylesters per uronic acid were greater in CGR2OX compared to wild type (Col-0); the uronic acid content was lower in *cgr2/3* (**Supplemental Fig. S1C-D**). These data support that the cell wall composition, including pectin and the degree of methylesterification is altered in the CGR2OX and *cgr2/3* mutant lines in response to altered *CGR2* and *CGR3* gene expression, as previously reported (Kim et al., 2015).

Enhanced Pectin Methylesterification Increases Leaf Area while a Decrease in Pectin Methylesterification Reduces Leaf Area.

Hypocotyl length, and cotyledon and leaf areas were measured in order determine how altered expression of *CGR2* and *CGR3* affects early seedling growth, and expansion of the leaf blade. Similar to earlier results (Kim et al., 2015), hypocotyls of CGR2OX were significantly longer than wild type (Col-0), while those of *cgr2/3* were shorter (**Supplemental Figs. S2A and S3A**). Cotyledon area was also smaller in *cgr2/3* than Col-0 (**Supplemental Fig. S3A**). The CGR2OX mutant produced rosettes with greater projected and total leaf area compared to Col-0 throughout

the life cycle (**Fig. 1, Supplemental Fig. S2, B and C**). In contrast, a significant reduction in projected and total leaf area was observed in *cgr2/3*. The degree of leaf overlap measured by the ratio between projected and total leaf area was not different between the transgenic lines and Col-0 (**Figure 1**). Thus, functional availability of *CGR2* and *CGR3* has a significant impact on leaf area growth.

Genetic Depletion of *CGR2* and *CGR3* Results in Thin Leaves with Small, Densely Packed Mesophyll Cells

Leaf area and thickness are generally negatively correlated (Lambers et al., 2008; Weraduwage et al., 2015). In order to determine whether changes in leaf area in CGR2OX and *cgr2*/3 were accompanied by changes in leaf thickness, we measured leaf thickness. Interestingly, leaves of *cgr2/3* were both thin and of small leaf area (**Fig. 2, A and B**); this trend was observed throughout the life cycle (data not shown). During early vegetative growth (34 DAS), CGR2OX produced thinner leaves with a tendency toward more area than Col-0. Nonetheless, the CGR2OX leaves were thicker than those of *cgr2/3*.

Next, we determined whether *CGR2* and *CGR*- mediated pectin methylesterification was correlated with changes in mesophyll architecture. CGR2OX had larger palisade cells and 3-4 mesophyll cell layers compared to Col-0, which produced 4-5 layers of comparatively smaller palisade cells (**Fig. 2A**). However, the mesophyll cells of *cgr2/3* were significantly smaller and more numerous than in Col-0 (**Fig. 2A**) which lead to a marked reduction in intercellular airspaces in the leaf mesophyll (**Fig. 2C**). Consequently, a significant negative correlation was observed between the number of cells and intercellular air spaces in the leaf mesophyll (**Fig. 2C**). Enhanced cell density and the reduction in intercellular air spaces in *cgr2/3* compared to the other *Arabidopsis* lines also led to a significant reduction in mesophyll cell surface area (S_{mes}) and chloroplast surface area (S_c) exposed to intercellular air spaces per unit leaf area (**Fig. 2, D and E**).

Interestingly also, the number of chloroplasts per unit area of a leaf cross section was lower in CGR2OX and greater in *cgr2/3* compared to Col-0 (**Fig. 3A**). However, the average size of chloroplasts was smaller in all transgenic lines compared to Col-0 (**Fig. 3B**). The data indicate

that the reduction in S_c in *cgr2/3* was not as a result of a reduction in chloroplast area per unit leaf area, but was linked to the significant reduction in intercellular air spaces in the leaf mesophyll. Therefore, our results show that variations in the levels of methylesterification of the cell wall significantly impact leaf thickness, cell density, and mesophyll architecture.

Changes in Mesophyll Architecture due to Alterations in *CGR*-Expression Affects Area-Based Photosynthesis and Respiration

We next aimed to establish whether changes in mesophyll architecture as well as projected and total leaf areas affect net C assimilation in mutant lines with altered *CGR*-expression. To do so, we measured area- and plant-based photosynthesis and respiration. During early vegetative growth, at around 29 DAS, area-based photosynthesis in *cgr2/3* was significantly higher than the other lines (**Fig. 4A**). Afterwards, *cgr2/3* showed lower area-based photosynthesis rates compared to Col-0 throughout the vegetative growth phase (**Fig. 4A**). Area-based photosynthesis in CGR2OX was lower than Col-0 around 49 DAS, but, remained higher compared to *cgr2/3*. We also established that area-based respiration in *cgr2/3* was 2-5 times greater than Col-0 (**Fig. 4B**). Thus, photosynthesis to respiration ratio remained significantly smaller in *cgr2/3* throughout the life cycle (**Fig. 5A**). Although, not statistically significant at the 5% level, CGR2OX showed a trend towards lower area-based respiration (**Fig. 4B**) and therefore, its photosynthesis to respiration ratio appeared to be greater in young rosette leaves at 29 DAS (**Fig. 5A**). Increased area-based respiration in *cgr2/3* and lower area-based respiration in CGR2OX, correlated well with the differences in the number of mesophyll cells in these lines (**Fig. 2C**). In general, plant-based photosynthesis in CGR2OX was similar to Col-0 with an upward trend, even though, area-based photosynthesis was smaller (**Fig. 4, A and C**); this was mainly due to the larger projected leaf area in CGR2OX (**Fig. 1A**). However, owing to its smaller projected leaf area, plant-based photosynthesis in *cgr2/3* was much lower than Col-0 (**Fig. 4C**). Plant-based respiration was greater in CGR2OX, and smaller in *cgr2/3* than in Col-0 (**Fig. 4D**), which could be explained by differences in total leaf area (**Fig. 1B**).

Daily C gain in rosettes was greater in CGR2OX compared to Col-0 when measured at 29 and 49 DAS, while it was significantly smaller in *cgr2/3* (**Fig. 5B**). The upward trend in daily C gain observed in CGR2OX was a result of its higher plant-based photosynthetic rates due to greater

projected leaf area (**Figs. 1A and 4C**). The decrease in C gain in *cgr2/3* resulted from reduced plant-based photosynthesis due to the smaller projected leaf area and smaller photosynthesis: respiration ratio compared to CGR2OX (**Figs. 1A, 4C and 5A)**. Together these results support the hypothesis that by controlling leaf area and mesophyll architecture, pectin methylesterification is a critical factor for regulation of whole plant rates of photosynthesis and respiration.

Suppression of Pectin Methylesterification Leads to a Reduction in CO_2 Availability for Photosynthesis

The differences in chloroplast number and size could not account for the large differences observed in area-based photosynthesis between *cgr2/3* and the other three *Arabidopsis* lines (**Fig. 3, A and B**). Therefore, in order to investigate whether lower area-based photosynthesis in *cgr2/3* at harvests 2, 3, and 4 was a result of a reduction in CO_2 availability owing to altered mesophyll architecture, the degree of ^{13}C discrimination was analyzed in leaf tissue of the four *Arabidopsis* lines. The ^{13}C content of *cgr2/3* was greater consistent with less discrimination against $^{13}CO_2$ during photosynthesis compared to the other lines (**Fig. 6, A-C**). The average CO_2 partial pressure at rubisco calculated using the $\delta^{13}C_{VPDB}$ values showed CO_2 availability to be significantly lower in *cgr2/3* (**Fig. 6, D-F**). These data are in agreement with the hypothesis that there is a greater resistance to CO_2 diffusion into chloroplasts of *cgr2/3* owing to lower surface areas of mesophyll cells and chloroplasts facing intercellular air spaces per unit leaf area as a result of enhanced cell density and reduced intercellular air spaces (**Fig. 2, C-D**). Therefore, reduction in *CGR2* and *CGR3* availability appears to have a strong negative impact on area-based photosynthesis owing to reduction in CO_2 availability to chloroplasts, most likely as a result of altered mesophyll architecture.

A Decrease in Pectin Methylesterification Increased LMA, and Reduced Plant Growth and Relative Growth Rates.

We next analyzed the effects of altered expression of *CGR2* and *CGR3* on plant growth by measuring leaf growth parameters, plant biomass, and relative growth rates. We found that LMA was higher in *cgr2/3* during the early to late vegetative phases around 29 to 63 DAS (**Fig. 7A**). In addition, LMA in CGR2OX remained significantly lower than in *cgr2/3*. Leaf area per unit plant

mass (leaf area ratio) and leaf mass ratio (mass of leaf per unit plant mass) were significantly smaller in *cgr2/3* than other lines (**Fig. 7, B and C**). In contrast, root mass ratio (mass of root per unit plant mass) was larger in *cgr2/3* than other lines (**Fig. 7D**).

Interestingly, CGR2OX maintained the greatest leaf, root and plant biomass throughout the life cycle (**Fig. 8, A-D**). In contrast, *cgr2/3* plants exhibited a significant reduction in leaf, root and plant dry weight throughout the life cycle. Collectively, these data reveal that alterations in *CGR2* and *CGR3* expression can have a significant effect not only on leaf growth, but also on overall plant growth. Therefore, while an increase in pectin methylesterification enhances plant biomass, a decrease in pectin methylesterification reduces it. Modeled relative growth rates revealed that both area-based (**Fig. 9A**) and mass-based (**Fig. 9B**) relative growth rates were higher in CGR2OX and smaller in *cgr2/3* than Col-0 during most part of the life cycle. In summary, alterations in *CGR2* and *CGR3* has a significant impact on important leaf growth parameters such as LMA, and biomass partitioning to leaf growth, and also affects plant growth in terms of biomass accumulation and relative growth rates. LMA showed a negative correlation with plant growth and relative growth rates.

Enhanced Pectin Methylesterification and C Partitioning to Leaf Area Growth

The *Arabidopsis* Leaf Area Growth Model parameterized with measurements obtained from the four *Arabidopsis* lines was used to estimate how much C was partitioned to new leaf area growth and LMA, as a proportion of net assimilated C that is available for growth. These values, referred to as partitioning coefficients (**Table 1**), would allow determination of whether changes in *CGR2* and *CGR3* availability could affect C partitioning to growth or respiratory processes. The proportions of C partitioned to leaf growth in total (C partitioned to area + LMA), and to root growth found by $^{14}CO_2$ feeding experiments at 29 DAS were used as an initial estimate when setting the partitioning coefficients to leaf growth for the early vegetative growth phase (**Supplemental Fig. S4, Table 1**). Partition coefficients obtained from growth modelling for CGR2OX revealed that, compared to the other lines, the overexpression line of *CGR2* partitioned more of the C available for leaf growth towards leaf area growth and less towards LMA, especially during the early vegetative phase (**Table 1**). In contrast, we found that in *cgr2/3* a significantly greater proportion of C was allocated to LMA rather than to leaf area growth

(**Table 1**). The differences in C partitioning to leaf area growth and LMA between Col-0 and CGR2OX and *cgr2/3* were most prominent during early vegetative phases of growth.

The patterns of C partitioning in transgenic lines seen in **Table 1** were further investigated by determining the amounts of C being partitioned to specific growth processes as well as respiratory processes as a proportion of the total available C in the plant (**Fig. 10**). CGR2OX, still partitioned a greater proportion of total available C to leaf area growth and a lesser proportion to LMA compared to the other lines; *cgr2/3* showed an opposite trend to that seen in CGR2OX (**Fig. 10**). The maintenance respiratory cost increased as the plant aged. Towards the end of its life cycle (82 DAS), close to 75% of assimilated C was consumed in maintenance respiration (**Fig. 10**). While the maintenance costs in CGR2OX was somewhat larger than Col-0 towards the end of the life cycle, a significant enhancement in the proportion of C spent in maintenance respiration could be seen in *cgr2/3* throughout the life cycle (**Fig. 10**). Collectively, data provide evidence to support that alteration in *CGR2* and *CGR3* expression has a significant impact on determining the proportions of C partitioned to new leaf area growth versus LMA, and to maintenance respiration in plants.

Growth in CGR2OX and *cgr2/3* is Affected More by Alterations in C Partitioning than by Changes in Area-based Photosynthesis

In order to determine whether the alterations in growth observed in CGR2OX and *cgr2/3* were a result of alterations in area-based photosynthesis (**Fig. 4A**) or partitioning coefficients to leaf area growth and LMA growth (**Table 1**), the values for these parameters in the growth model for Col-0 were substituted separately by corresponding values from the two transgenic lines. Interestingly, by keeping the partitioning coefficients the same, substitution of area-based photosynthesis rates for 1-90 days with those of CGR2OX resulted in a small reduction in modeled growth in Col-0 whereas *cgr2/3* caused a large increase in growth (gray lines in **Fig. 11**).

Table 1: Comparison of modeled partition coefficients during different growth phases for *Arabidopsis* lines with altered pectin methyltransferase expression. Partitioning coefficients for partitioning of net assimilated C to inflorescence and root growth, LMA, and leaf area growth during the early and late vegetative phases and the reproductive phase for Col-0, cgr2com, CGR2OX, and *cgr2/3* were obtained from the *Arabidopsis* Leaf Area Growth Model. The durations of the early and late vegetative phases and the reproductive phase for each line is as follows: for Col-0, 5 -51 DAS, 52 - 74 DAS and 75 - 90 DAS; for cgr2com, 5 - 48 DAS, 49 - 70 DAS and 71 - 90 DAS; for CGR2OX, 5 -49 DAS, 50 - 72 DAS and 73 - 90 DAS; for *cgr2/3*, assuming flower initiation started at 82DAS, 5 - 55 DAS, 56 - 81 DAS and 82 - 90 DAS.

***Arabidopsis* line**	**Organ/process to which C is partitioned**	**Partitioning coefficients**		
		Early vegetative growth phase	Late vegetative growth phase	Reproductive growth phase
Col-0	Inflorescence growth	0.00	0.02	0.30
	Root growth	0.06	0.13	0.12
	LMA	0.17	0.10	0.10
	Leaf area growth	0.77	0.75	0.48
cgr2com	Inflorescence growth	0.00	0.03	0.40
	Root growth	0.03	0.12	0.12
	LMA	0.19	0.10	0.10
	Leaf area growth	0.78	0.75	0.38
CGR2OX	Inflorescence growth	0.00	0.03	0.30
	Root growth	0.04	0.15	0.15
	LMA	0.16	0.11	0.10
	Leaf area growth	0.80	0.71	0.45
cgr2/3	Inflorescence growth	0.00	0.00	0.10
	Root growth	0.10	0.25	0.25
	LMA	0.24	0.18	0.10
	Leaf area growth	0.66	0.57	0.55

However, by keeping area-based photosynthesis the same, substituting the partitioning coefficients of Col-0 with that of CGR2OX had a strikingly large positive effect on modeled growth; *cgr2/3* partitioning coefficients had a significantly large negative effect on growth which mirrored the observed differences in growth in the *CGR2* overexpression line and the *CGR2* and *CGR3* double mutant lines (orange lines in **Fig. 11**). These observed changes in growth could be achieved by substituting the partitioning coefficients of only the early vegetative growth phase (data not shown). The data show that the observed changes plant growth in CGR2OX and *cgr2/3* were not a result of differences in photosynthetic rates, but a result of differences in C partitioning to growth in terms of leaf area and LMA especially during early vegetative growth. Thus, our data support the hypothesis that the magnitude of plant growth is determined by how photosynthetic carbon is partitioned to new leaf area growth and LMA.

DISCUSSION

In this study, we investigated how modification of cell wall plasticity via the genetic manipulation of pectin methyltransferases, *CGR2* and *CGR3*, affects C assimilation, leaf respiration and overall plant growth through effects on leaf area growth and mesophyll structure. We also wanted to further understand the relationship between photosynthesis and plant growth by determining whether the changes in overall plant growth in *Arabidopsis* with altered cellular availability of *CGR2* and *CGR3* were a result of changes in growth in terms of leaf area and LMA or due to changes in area-based photosynthesis. We found that overexpression of *CGR2* has a significant positive effect on leaf area and plant growth. On the other hand, suppression of *CGR2* and *CGR3* expression caused a marked increase in LMA and a reduction in leaf area and plant growth. Altered expression of *CGR2* and *CGR3* also had a significant impact on photosynthesis and respiration through altered mesophyll architecture. The differences in plant growth in *Arabidopsis* with altered *CGR2* and *CGR3* levels were linked to changes in C partitioning to leaf area growth and LMA rather than altered area-based photosynthesis rates. These findings establish that *CGR2* and *CGR3* mediated pectin methylesterification affects mesophyll architecture, C accumulation, C partitioning, leaf and plant growth, and the relationship between photosynthesis and plant growth.

514 ***CGR2* and *CGR3* Directly Affect Leaf Mass per Unit Area by Altering Both Leaf Thickness**
515 **and the Mass of Leaf per Unit Volume of Leaf Tissue**
516 Often, thicker leaves are associated with larger LMA due to an increase in the number of cell
517 layers, which can increase leaf mass per unit leaf area (Lambers et al., 2008; Poorter et al., 2009;
518 Villar et al., 2013; Weraduwage et al., 2015). However, a negative correlation exists between
519 leaf mass density or LMD (mass of leaf per unit volume of leaf tissue) and leaf thickness, and
520 both parameters can affect LMA as follows; LMD (kg m^{-3}) = leaf mass / (leaf area x leaf
521 thickness); LMA (kg m^{-2}) = LMD x leaf thickness (Witkowski and Lamont, 1991; Lambers et
522 al., 2008; Poorter et al., 2009; Villar et al., 2013). Therefore, leaf 'thickening' or an increase in
523 LMA can occur due to an increase in leaf thickness or LMD. While thicker leaves have been
524 shown to be associated with larger mesophyll cells (elongated, especially in the depth direction)
525 and larger air spaces, LMD has been associated with smaller proportions of intercellular air
526 spaces and smaller cells (Tsuge et al., 1996; Poorter et al., 2009; Villar et al., 2013). In addition
527 to being affected by anatomical changes, LMA and LMD can also be determined by variations in
528 cellular and chemical composition (Cunningham et al., 1999; Lambers et al., 2008). For
529 example, plants with slow growth rates possess a larger number of smaller cells with thicker cell
530 walls, and a greater proportion of lignin and other cell wall polysaccharides per unit leaf area that
531 can increase LMD and LMA (Cunningham et al., 1999; Lambers et al., 2008). A key aspect of
532 cellular mass is formed by organelles and plants with higher growth rates may possess large cells
533 with a lower number of chloroplasts that result in lower LMA (Lambers et al., 2008).
534
535 Interestingly, while both CGR2OX and *cgr2/3* had thinner leaves compared to Col-0, *cgr2/3*
536 showed a significant increase in LMA. Such increase in *cgr2/3* can be attributed to increased
537 LMD as a result of enhanced cell density and reduction of intercellular air spaces in the leaf
538 mesophyll. Smaller cells can lead to concentration of cellular metabolites, which can also lead to
539 increased LMD. In addition, *cgr2/3* also possessed a larger number of chloroplasts/leaf area. The
540 proportions of cell wall components per unit leaf area are also likely greater in *cgr2/3* compared
541 to the other backgrounds. Therefore, collectively the data clearly show that although *cgr2/3* had
542 thinner leaves, enhanced LMD led to greater leaf mass per unit area (or lower SLA) in *cgr2/3*.
543 Conversely, LMA in CGR2OX was significantly smaller than *cgr2/3*. Leaves of CGR2OX were
544 also thicker than *cgr2/3* and contained larger intercellular airspaces, and larger cells with a lower

number of chloroplasts. Therefore, our results support that variation in LMA between CGR2OX and *cgr2/3* resulted from the combination of increased leaf thickness and reduced leaf mass density.

The significant alterations in mesophyll architecture observed during the present study are linked to changes in *CGR2* and *CGR3* expression. Based on the model that reduced methylation of pectin may enhance Ca^{2+} mediated cross-linking of galacturonic acid in cell walls thereby preventing normal cell expansion in *cgr2/3* (Burton et al., 2000; Wolf et al., 2009; Kim et al., 2015), we proposed that the larger number of smaller mesophyll cells observed during the present study is likely linked to the hardening of the cell wall and consequent restriction of cell wall expansion. This in turn is likely to cause an increase of cell number/leaf area in *cgr2/3* compared to Col-0. In addition, cross-linking between galacturonic acid and Ca^{2+} in cell wall pectin is required for cellular adhesion (Caffall and Mohnen, 2009). Also a reduction in galacturonic acid-Ca^{2+} complex formation in *Lycopersicon esculentum* resulted in large air spaces in the fruit pericarp caused by dramatic reductions in the middle-lamellae-mediated cell to cell adhesion (Caffall and Mohnen, 2009). The observed decrease in air spaces in *cgr2/3* and the dense distribution of the cells (hence thinner leaves) is likely due to enhanced cell-to-cell adhesion as a result of the lower degree of methylesterified pectin in *cgr2/3* compared to Col-0. In contrast, enhanced methylation of pectin promotes cell expansion (Kim et al. 2015); this resulted in the observed presence of wider palisade cells and a fewer number of cell layers (hence thinner leaves) in CGR2OX than Col-0. The number of cells and area of airspaces in CGR2OX differed only marginally from Col-0. However, CGR2OX did have a smaller number of smaller chloroplasts compared to Col-0, which may be an indication of lower LMA. Whether overexpression of *CGR2* has a dosage effect and whether overexpression of both *CGR2* and *CGR3* would result in larger and thicker leaves with lower cell density remains to be investigated.

Thinner leaves usually correlate with larger SLA or smaller LMA (Lambers et al., 2008; Weraduwage et al., 2015). However, based on the negative correlation between LMD and leaf thickness, it is likely that LMD would start to increase should leaf thickness decrease below a certain optimum, thereby resulting in an increase in LMA. This was demonstrated in *cgr2/3*,

where suppression of *CGR2* and *CGR3* resulted in leaves that were too thin, leading to higher cell density and LMA. This can have significant negative effects on leaf gas exchange properties and daily C gain as discussed below. The above phenotype was rescued by complementing with *CGR2* as seen in cgr2com or by overexpressing *CGR2* as in CGR2OX. Thus, the degree of *CGR2* and *CGR3* mediated pectin methyl esterification can directly affect LMA by altering both leaf thickness and leaf mass density.

CGR2 and *CGR3* Affect Area-Based Respiration and Daily C Gain by Altering LMD and LMA

In general, leaves growing under high light develop thicker leaves with multiple cell layers and higher LMA (lower SLA) and incurs higher area-based respiration rates corresponding to their greater mass per unit leaf area (Mitchell et al., 1999; Lambers et al., 2008). In contrast, plants grown under low light have lower LMA (higher SLA) which optimizes light capture and lowers area-based respiration rates (Lambers et al., 2008). This assists in maximizing daily C gain and compensates for lower area-based photosynthesis rates when growing under low light conditions (Lambers et al., 2008). Although *cgr2/3* produced thinner leaves, the marked enhancement in area-based respiration positively correlated with enhanced leaf cell density and higher LMA. Higher area-based respiratory rates led to smaller photosynthesis to respiration ratios, and when coupled with smaller projected leaf area, they had a significant negative impact on daily carbon gain. Thus, pectin methylesterification affects area-based respiration rates through alterations in leaf cell density, LMD, and LMA and consequently affect photosynthetic efficiency in plants.

CGR2 and *CGR3* Directly Affect Area-Based Photosynthesis by Altering Mesophyll Architecture

Diffusion of CO_2 into chloroplasts occurs along the path which poses the least resistance through the cell wall, plasma membrane, chloroplast envelope and into the chloroplast stroma (Terashima et al., 2006). Therefore, S_c represents the active area through which CO_2 diffuses in to the chloroplast stroma (Terashima et al., 2006). A decrease in S_c leads to a reduction in CO_2 concentration at rubisco and therefore to a reduction in the carboxylation to oxygenation ratio (Terashima et al., 2006). Parameters that affect CO_2 diffusion into the chloroplast stroma are thickness of the mesophyll cell wall, internal conductance to CO_2 diffusion, and stomatal

conductance (Terashima et al., 2006). S_c has been shown to be positively correlated with internal conductance, while the latter is negatively correlated with cell wall thickness (Terashima et al., 2006).

In contrast to our original hypothesis, area-based photosynthesis rates were significantly lower in *cgr2/3* for most of the life cycle, even though this mutant possessed smaller projected leaf area and denser leaves. The present study revealed that this reduction in area based photosynthesis in *cgr2/3* was not a result of differences in chloroplast number and size, but, mainly a result of a reduction in CO_2 partial pressure at rubisco due to restrictions imposed on CO_2 diffusion by increased cell density and reduced airspaces in the leaf mesophyll which lowered S_c. Preliminary investigations revealed that stomatal conductance is not negatively impacted in the mutant lines examined during the present study (data not shown). Therefore, the observed reduction in area-based photosynthetic rates is most probably due to a cumulative effect of lower S_c, higher internal resistance and greater cell wall thickness per unit area. The greater area-based photosynthesis rates in *cgr2/3* observed during early stages of vegetative development may have been a result of leaves and cell walls being comparatively thinner in young plants. S_c values and CO_2 partial pressure at rubisco in *cgr2/3* was restored to the levels of Col-0 in cgr2com owing to alterations brought about by *CGR2* expression in its leaf anatomy: thicker leaves with less dense mesophyll, more intercellular air spaces. S_c and CO_2 partial pressure in CGR2OX was similar to that of Col-0. The lower area-based photosynthesis in CGR2OX may have resulted from thinner but larger leaves which may have resulted in less photosynthetic machinery per unit leaf area. Correspondingly, chloroplast number and size was lower in CGR2OX. These data emphasize the importance of pectin methylesterification in fine tuning cell expansion and size and cell-to-cell adhesion qualities in building a leaf mesophyll optimized for CO_2 exchange. While thin leaves are associated with lower LMA (or enhanced SLA), it is important to maintain an optimum thickness in order to maintain sufficient intercellular air spaces, which would maintain high internal conductance, S_{mes} and S_c and therefore higher carboxylation rates.

Pectin Methylesterification Mediates the Relationship Between Photosynthesis and Plant Growth Through Alterations in C Partitioning to Leaf Area Growth and Thickening

As hypothesized, suppression of *CGR2* and *CGR3* mediated pectin methylesterification had a profound negative effect on leaf area growth while overexpression of *CGR2* significantly enhanced leaf area growth. Suppression of *CGR2* and *CGR3* also led to a reduction in plant dry weight. Overexpression of *CGR2* enhanced plant dry weight. Growth modelling revealed that the observed variations in growth were not caused by changes in area-based photosynthesis. In fact, high rates of area-based photosynthesis measured in *cgr2/3* during early growth were found to be sufficient to drive growth higher than that seen in Col-0. Thus, growth had the opposite trend (CGROX>Col-0> *cgr2/3*) compared with area-based photosynthetic rate (*cgr2/3*>Col-0>CGROX). In order to account for enhanced overall plant growth, the amount of net assimilated C available for growth has to increase which will depend on the total amount of C assimilated and the amount of C respired to support maintenance processes (Weraduwage et al., 2015). Our study showed that *CGR2* and *CGR3*, through pectin methylesterification, is capable of affecting total C assimilation by directly altering leaf area growth and by altering maintenance respiratory costs through direct effects on LMA. We also showed that although *CGR2* and *CGR3* affect area-based photosynthetic rates, it is not a major factor affecting overall plant growth.

A reduction in pectin methylesterification by reduced expression of *CGR2* and *CGR3* seem to enhance cell wall hardening and cell-to-cell adhesion through increased cross-linking between galacturonic acid and Ca^{2+}, favoring an increase in LMA against cell expansion and posing a greater demand for C for LMA growth compared to area growth (**Fig. 12A**). Hence less C will be utilized in area growth of new leaves and more C will be utilized in increasing LMA leading to a smaller projected leaf area (**Fig. 12A**). Smaller projected leaf area yields lower whole plant C assimilation. In contrast, an increase in pectin methylesterification via enhanced expression of *CGR2* and CGR3 seem to reduce cell wall hardening and cell-to-cell adhesion and thereby favoring cell expansion and area growth against increases in LMA and posing a greater demand for C for leaf area growth compared to that for LMA growth (**Fig. 12B**). Hence more C will be utilized in area growth of new leaves and a lower proportion C will be utilized in LMA growth leading to larger projected leaf area and enhanced whole plant C assimilation (**Fig. 12B**). Suppression of *CGR2* and *CGR3* increases respiratory costs through higher LMA resulting in

lower photosynthesis: respiration ratios, lower daily C gain, reduced net assimilated C available for growth and ultimately reduced plant growth (**Fig. 12A**); overexpression of *CGR2* yields opposite physiological effects (**Fig. 12B**).

There may be other genes that may also impact partition of carbon toward leaf area growth or LMA. One example is the xyloglucan endotransglucosylase/ hydrolase (*XTH*) gene family. Reduced expression of *XTH8* and *XTH31* gene expression led to reduced leaf width, length, and leaf area (Miura and Hasegawa, 2010). The leaf mesophyll of these mutant lines consisted of a large number of small mesophyll cells per unit area (Miura and Hasegawa, 2010). Suppression of *XTH21* led to a reduction in cellulose, and xyloglucan content with subsequent reductions in both leaf and plant size (Liu et al., 2007). One of the major roles of XTH enzymes is cleavage of cross-linkages between cellulose and hemicellulose microfibrils which promotes wall loosening and cell expansion. This may explain the above leaf phenotypes in *xth8*, *xth21*, and *xth31* mutant lines (Liu et al., 2007; Miura and Hasegawa, 2010). Thus, through its effects on cell wall properties, *XTH* genes may be able to alter C partitioning to leaf area and thickening and thereby affect overall plant growth similar to the role played by *CGR* genes.

In summary, results from the present study are in agreement with findings from Weraduwage et al. (2015) that optimization of leaf area growth and plant growth can be achieved by increased partitioning of C to leaf area growth with concomitant reduction in partitioning to growth in terms of LMA especially during the early vegetative growth phase (Weraduwage et al., 2015) and that photosynthesis drives growth through alterations in C partitioning to leaf area and LMA growth. In the present study we found that *CGR2* and *CGR3* directly affect this relationship by altering the degree of cell expansion and/or adhesion thereby driving C partitioning by generating C demands for leaf area growth and for growth in terms of LMA.

CONCLUSION

Our study supports the model that qualitative and quantitative changes in pectin in the cell wall have a significant impact on area-based photosynthesis and respiration through alterations in leaf thickness, mesophyll cell density, and area of the leaf blade. Such changes in mesophyll architecture directly altered LMA and consequently area-based respiration. *CGR*2 and *CGR*3

affected area-based photosynthesis by altering CO_2 availability at rubisco. These genes could affect plant-based photosynthesis by enabling enhanced light capture through larger leaf area. These data emphasizes the importance of pectin methylesterification in fine-tuning cell expansion and size, and adhesion qualities when building an optimum leaf to maximize photosynthesis under different environmental conditions. While thin leaves are associated with reduced LMA, it is important to maintain an optimum thickness in order to maintain sufficient intercellular air spaces that would maintain high internal conductance as reflected in S_{mes} and S_c and therefore higher carboxylation rates. *CGR2* and *CGR3,* which directly alter the size of the leaf blade and mesophyll architecture, may find applications in the future to improve light interception in crop plant canopies.

Overexpression of *CGR2* has had a significant positive effect on leaf area and plant growth. Suppression of *CGR2* and *CGR3* caused a marked increase in LMA, and a reduction in leaf area and plant growth. These differences resulted through changes in C partitioning to leaf area growth and growth in terms of LMA and not as a result of altered area-based photosynthesis rates. Therefore, photosynthesis drives plant growth through alterations in C partitioning to new leaf area growth and LMA growth, and the present study discovered that pectin methylesterification affects this relationship by directly altering the degree of cell expansion and positioning in the leaves to create dynamic carbon demands in leaf area growth and leaf mass thereby driving carbon partitioning for these processes. Collectively, these results uncover an unexpected connection between cell wall composition and photosynthesis and support the novel model that through its role in modulation of organ development, the cell wall plasticity is a key factor influencing photosynthetic processes in land plants.

SUPPLEMENTAL DATA

Supplemental Figure S1. Cell wall composition in *Arabidopsis* with altered *CGR2* and *CGR3* expression.

Supplemental Figure S2. Hypocotyl and rosette size in *Arabidopsis* with altered *CGR2* and *CGR3* expression.

Supplemental Figure S3. Early growth phenotypes in *Arabidopsis* with altered *CGR2* and *CGR3* expression.

Supplemental Figure S4. Partitioning of photosynthetic C to shoots and roots in *Arabidopsis* with altered *CGR2* and *CGR3* expression.

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FIGURE LEGENDS

Figure 1. Projected and total leaf area over time in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) Projected leaf area and **(B)** total leaf area at 4 different harvests (H) is presented for Col-0, cgr2com, CGR2OX, and *cgr2/3*. H1, H2, H3, and H4 correspond to 29, 49, 63, and 82 DAS, respectively. For clarity, data for H1 is presented as inserts in **A** and **B**. Values represent the mean ± SE and n = 5 plants per line. Statistical differences at $\alpha = 0.05$ are marked with lower case letters.

Figure 2. Leaf thickness and anatomy in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) Representative micrographs of leaf cross sections, **(B)** leaf thickness, **(C)** the relationship between the number of mesophyll cells and size of the intercellular air spaces in the leaf mesophyll, **(D)** the surface area of mesophyll cells exposed to intercellular air spaces per unit leaf area (S_{mes}), and **(E)** the surface area of chloroplasts exposed to intercellular air spaces per unit leaf area (S_c) are presented for Col-0, cgr2com, CGR2OX, and *cgr2/3*. In **(A)**, leaf thickness is denoted by red double arrows. Data were obtained from 34-day old leaves. In **(B)**, **(D)**, and **(E)** values represent the mean ± SE and n = 4 plants per line. In **(C)** n = 4 plants per line were used to obtain the mean values for the area of air spaces as a % of area of leaf cross section and the number of mesophyll cells per mm^2 of leaf cross section. Statistical differences at $\alpha = 0.05$ are marked with lower case letters.

Figure 3. Abundance and size of chloroplasts in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) The number of chloroplasts per unit area of leaf cross section and **(B)** size of chloroplasts in 34-day old leaves is shown for Col-0, cgr2com, CGR2OX, and *cgr2/3*. Values represent the mean ± SE and n = 12. Statistical differences at $\alpha = 0.05$ are marked with lower case letters.

Figure 4. Photosynthesis and respiration overtime in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) Area-based photosynthesis, **(B)** area-based nighttime respiration, **(C)** photosynthesis on a whole plant basis, and **(D)** nighttime respiration on a whole plant basis at 4 different harvests (H) is shown for Col-0, cgr2com, CGR2OX, and *cgr2/3*. Photosynthesis (C gain) is shown as a positive number **(A and C)** while respiration (C loss) is shown as a negative number **(B and D)**. H1, H2, H3 and H4 correspond to 29, 49, 63 and 82 DAS, respectively. Values represent the mean ± SE and n = 5 plants per line. Statistical differences at $\alpha = 0.05$ are marked with lower case letters.

Figure 5. The daily C gain in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) Photosynthesis to respiration ratio at 4 different harvests (H) and **(B)** the daily C gain at 2 different harvests for Col-0, cgr2com, CGR2OX, and *cgr2/3* is given. H1, H2, H3 and H4 correspond to 29, 49, 63 and 82 DAS, respectively. Values represent the mean ± SE and n = 5 plants per line. Statistical differences at $\alpha = 0.05$ are marked with lower case letters.

Figure 6. The degree of resistance to CO_2 diffusion through the leaf mesophyll overtime in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A-C) The $\delta^{13}C_{VPDB}$ value calculated as the ratio of ^{13}C to ^{12}C isotopes in leaf tissue relative to a Vienna-Pee-Dee Belemnite standard (VPDB), and **(D-F)** CO_2 partial pressure at rubisco at 3 different harvests (H) is shown for Col-0, cgr2com, CGR2OX, and *cgr2/3*. H2, H3, and H4 correspond to 49, 63 and 82 DAS, respectively. Values represent the mean ± SE and n = 5 plants per line. Statistical differences at $\alpha = 0.05$ are marked with lower case letters.

Figure 7. Variations in leaf growth parameters overtime in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) Leaf mass per unit leaf area or LMA, **(B)** leaf area ratio, **(C)** leaf mass ratio, and **(D)** root mass ratio at 4 different harvests (H) for Col-0, cgr2com, CGR2OX, and *cgr2/3* is presented. H1, H2, H3 and H4 correspond to 29, 49, 63 and 82 DAS, respectively. Values represent the mean and n = 5 plants per line. Statistical differences at $\alpha = 0.05$ are marked with lower case letters.

Figure 8. Variations in dry weight overtime in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) Leaf, **(B)** root, **(C)** inflorescence, and **(D)** whole plant dry weight at 4 different harvests (H) for Col-0, cgr2com, CGR2OX, and *cgr2/3* is given. H1, H2, H3 and H4 correspond to 29, 49, 63 and 82 DAS, respectively. Values represent the mean ± SE and n = 5 plants per line. Statistical differences at $\alpha = 0.05$ are marked with lower case letters.

Figure 9. Modeled relative growth rates overtime in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) Area-based relative growth rates and **(B)** mass-based relative growth rates simulated from day 5-84 using the *Arabidopsis* Leaf Area Growth Model fitted with data obtained from Col-0, cgr2com, CGR2OX, and *cgr2/3* is presented.

Figure 10. Modeled C partitioning to growth and respiration in *Arabidopsis* with altered *CGR2* and *CGR3* expression. C partitioned to support growth in terms of leaf area and LMA, and inflorescence growth, root growth, and maintenance and growth respiration is presented as percentages of the daily available C at 29, 49, 63 and 82 DAS. Data was derived from the *Arabidopsis* Leaf Area Growth Model fitted with data obtained from Col-0, cgr2com, CGR2OX, and *cgr2/3*.

Figure 11. Comparison of the effects of altered area-based photosynthesis and C partitioning coefficients on plant growth in *Arabidopsis* with altered *CGR2* and *CGR3* expression. Modeled effects on leaf area and leaf, root, and plant dry weights when either photosynthetic rates or partitioning coefficients spanning the entire growth cycle for Col-0 is replaced by that of **(A)** CGR2OX and **(B)** *cgr2/3* are presented. In **(A)** and **(B)**, blue lines represent modeled growth for Col-0 and red squares represent measured data points (mean ± SD)

for Col-0 at 29, 49, 63, and 82 DAS. Grey lines represent modeled data for Col-0 when its area-based photosynthesis rates are replaced by that of either CGR2OX **(A)** or cgr2/3 **(B)**. Orange lines represent modeled data for Col-0 when its partitioning coefficients **(Table 1)** are replaced by that of either CGR2OX **(A)** or cgr2/3 **(B)** without making any changes to area-based photosynthesis rates of Col-0. In (**A)**, asterisks represent measured data for CGR2OX at 63 and 82 DAS. In **(B)**, the dotted line indicates the upper limit of measured data for cgr2/3.

Figure 12. *CGR2* and *CGR3* mediated pectin methylesterification regulates the relationship between photosynthesis and plant growth. A schematic diagram outlining how **(A)** suppression or **(B)** overexpression of *CGR2* and *CGR3* gene expression affects the relationship between photosynthesis and plant growth in *Arabidopsis thaliana* is presented. Suppression of *CGR2* and *CGR3* reduces the degree of pectin methylation which leads to an increase in cell-to-cell adhesion, cation mediated cross linking of galacturonic acid and consequent hardening of cell walls. *CGR2* overexpression increases the degree of pectin methylation which allows cell expansion through reduced cell-to-cell adhesion and cation mediated cross-linking. We propose that pectin methyltransferase enzyme, through its ability to directly alter cell expansion, determines the amount of C partitioned to leaf area growth vs. growth in terms of leaf mass per unit area (LMA). For example, while more C is partitioned to growth in terms of LMA in the *CGR2* and *CGR3* double knockout mutant, in the *CGR2* overexpression line more C is partitioned to leaf area growth. An increase in LMA leads to enhanced area-based respiration and a reduction in leaf area contribute to a reduction in whole plant photosynthesis. Collectively, this results in a reduction in C available for growth and consequently a decrease in overall plant growth; an opposite trend is seen in CGR2OX. Thus, *CGR2* and *CGR3* through their ability to alter the degree of methylated pectin in the cell wall of the mesophyll cells, determines how photosynthate is utilized to grow the plant. In other words, *CGR2* and *CGR3* mediated pectin methylesterification affects the relationship between photosynthesis and plant growth by regulating the proportions of C that is partitioned to leaf area growth and LMA. Final overall growth mainly depends on the expression patterns of *CGR2* and *CGR3* and how much C is partitioned to area growth and LMA and not on area-based photosynthesis. Abbreviations: PME – pectin methylesterase, PMEI – PME inhibitor, PMT – pectin methyltransferase. CH_3OH is methanol.

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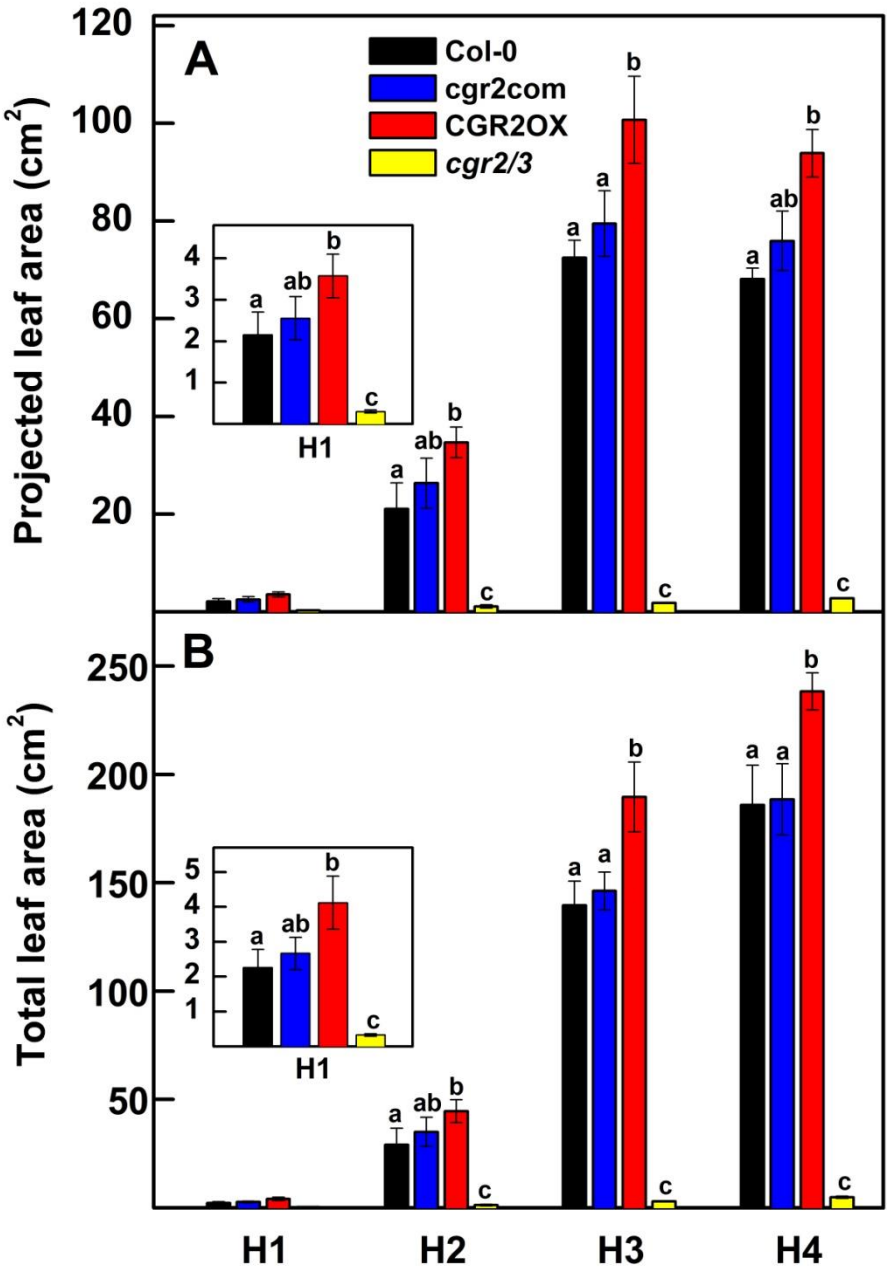

Figure 1. Projected and total leaf area over time in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) Projected leaf area and **(B)** total leaf area at 4 different harvests (H) is presented for Col-0, cgr2com, CGR2OX, and *cgr2/3*. H1, H2, H3, and H4 correspond to 29, 49, 63, and 82 DAS, respectively. For clarity, data for H1 is presented as inserts in **A** and **B**. Values represent the mean ± SE and n = 5 plants per line. Statistical differences at $\alpha = 0.05$ are marked with lower case letters.

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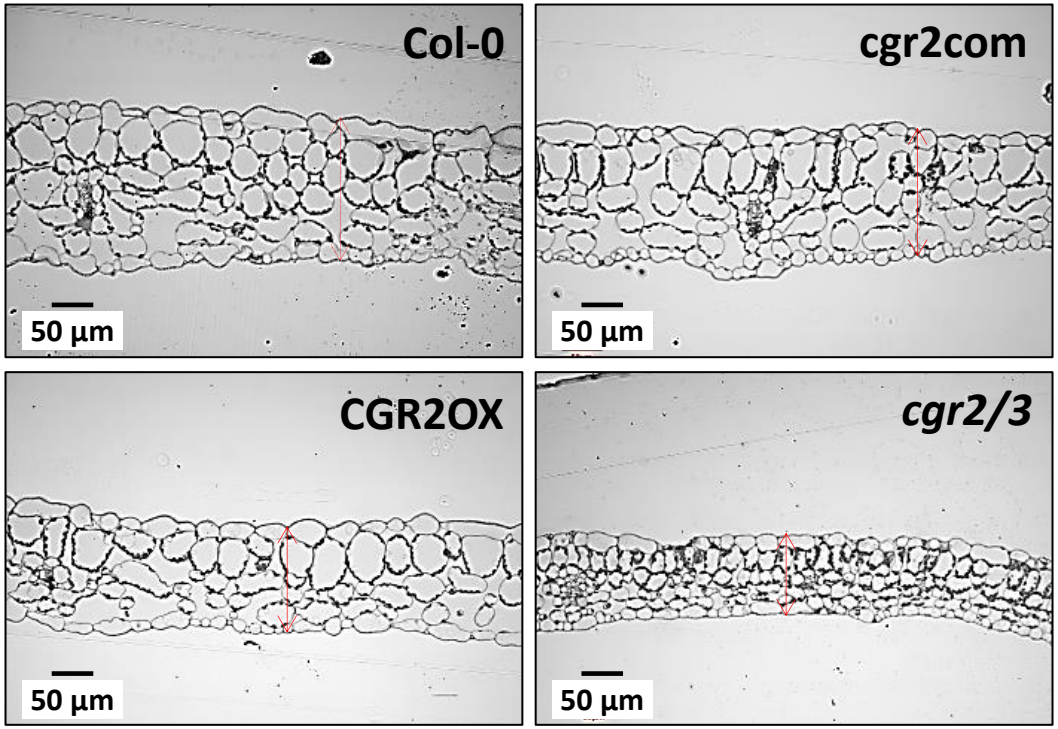


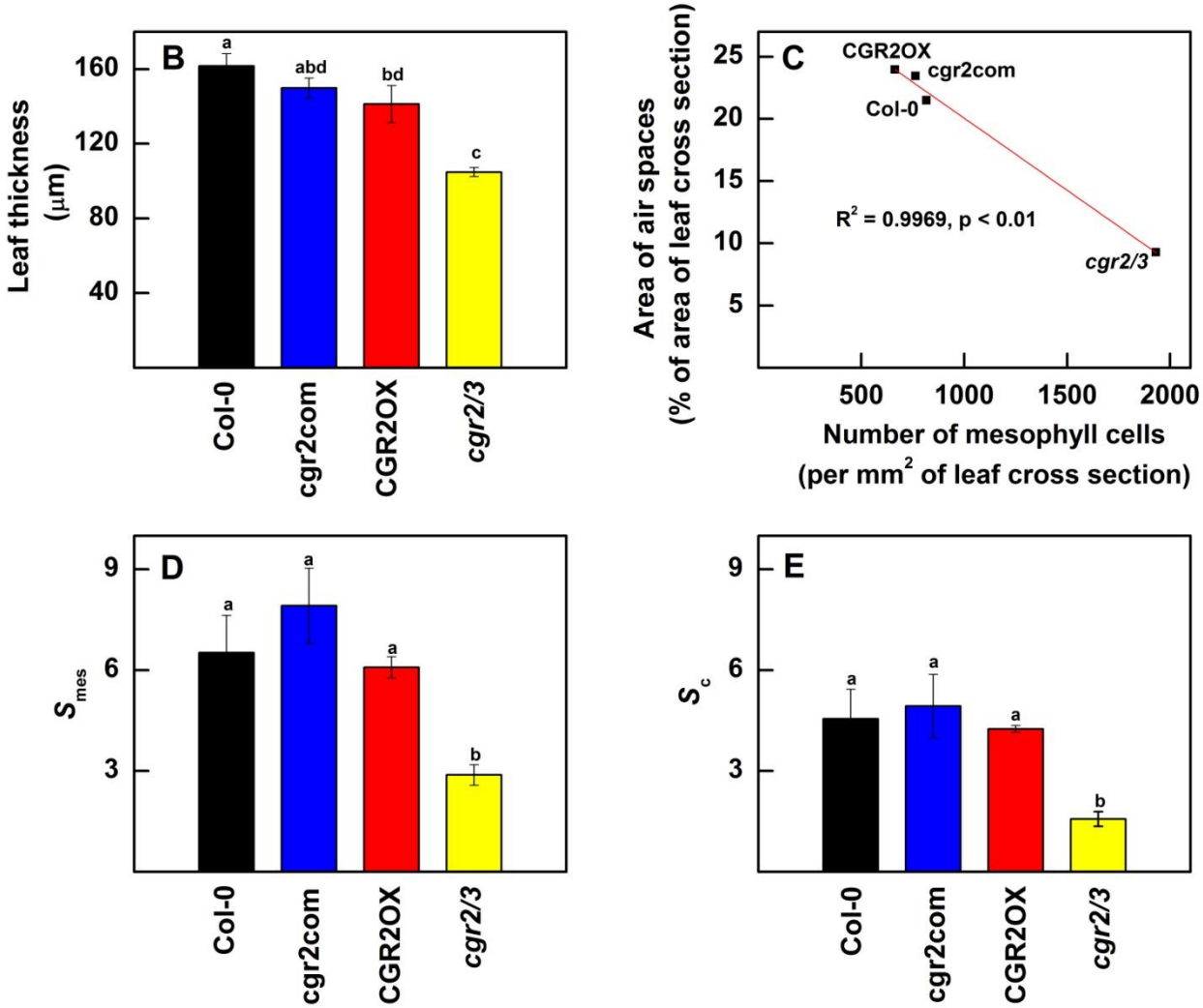


Figure 2. Leaf thickness and anatomy in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) Representative micrographs of leaf cross sections, **(B**) leaf thickness, **(C)** the relationship between the number of mesophyll cells and size of the intercellular air spaces in the leaf mesophyll, **(D)** the surface area of mesophyll cells exposed to intercellular air spaces per unit leaf area (S_{mes}), and **(E)** the surface area of chloroplasts exposed to intercellular air spaces per unit leaf area (S_c) are presented for Col-0, cgr2com, CGR2OX, and *cgr2/3*. In **(A)**, leaf thickness is denoted by red double arrows. Data were obtained from 34-day old leaves. In **(B)**, **(D)**, and **(E)** values represent the mean ± SE and n = 4 plants per line. In **(C)** n = 4 plants per line were used to obtain the mean values for the area of air spaces as a % of area of leaf cross section and the number of mesophyll cells per mm^2 of leaf cross section. Statistical differences at $\alpha = 0.05$ are marked with lower case letters.

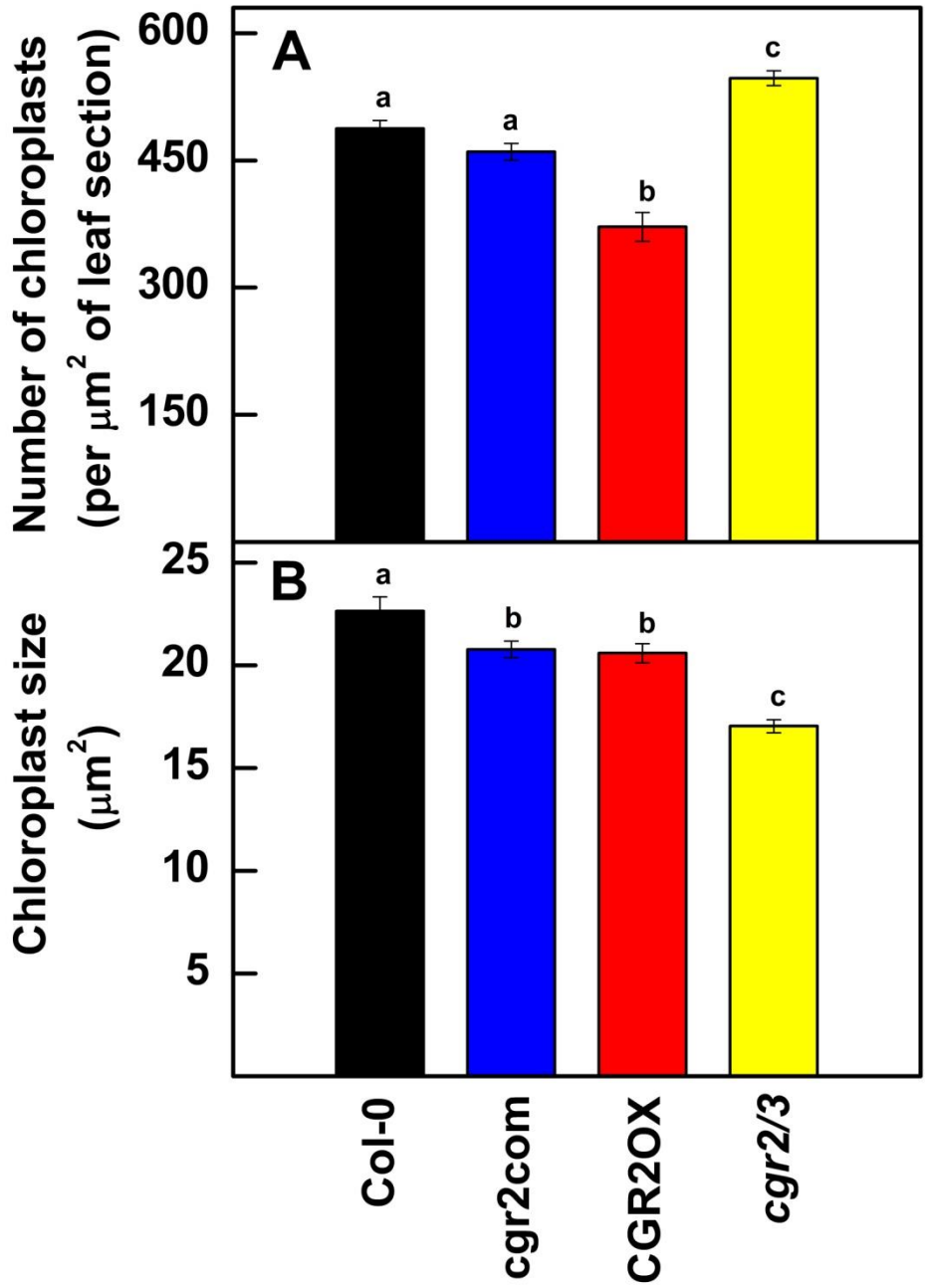


Figure 3. Abundance and size of chloroplasts in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) The number of chloroplasts per unit area of leaf cross section and **(B)** size of chloroplasts in 34-day old leaves is shown for Col-0, cgr2com, CGR2OX, and *cgr2/3*. Values represent the mean ± SE and n = 12. Statistical differences at $\alpha = 0.05$ are marked with lower case letters.

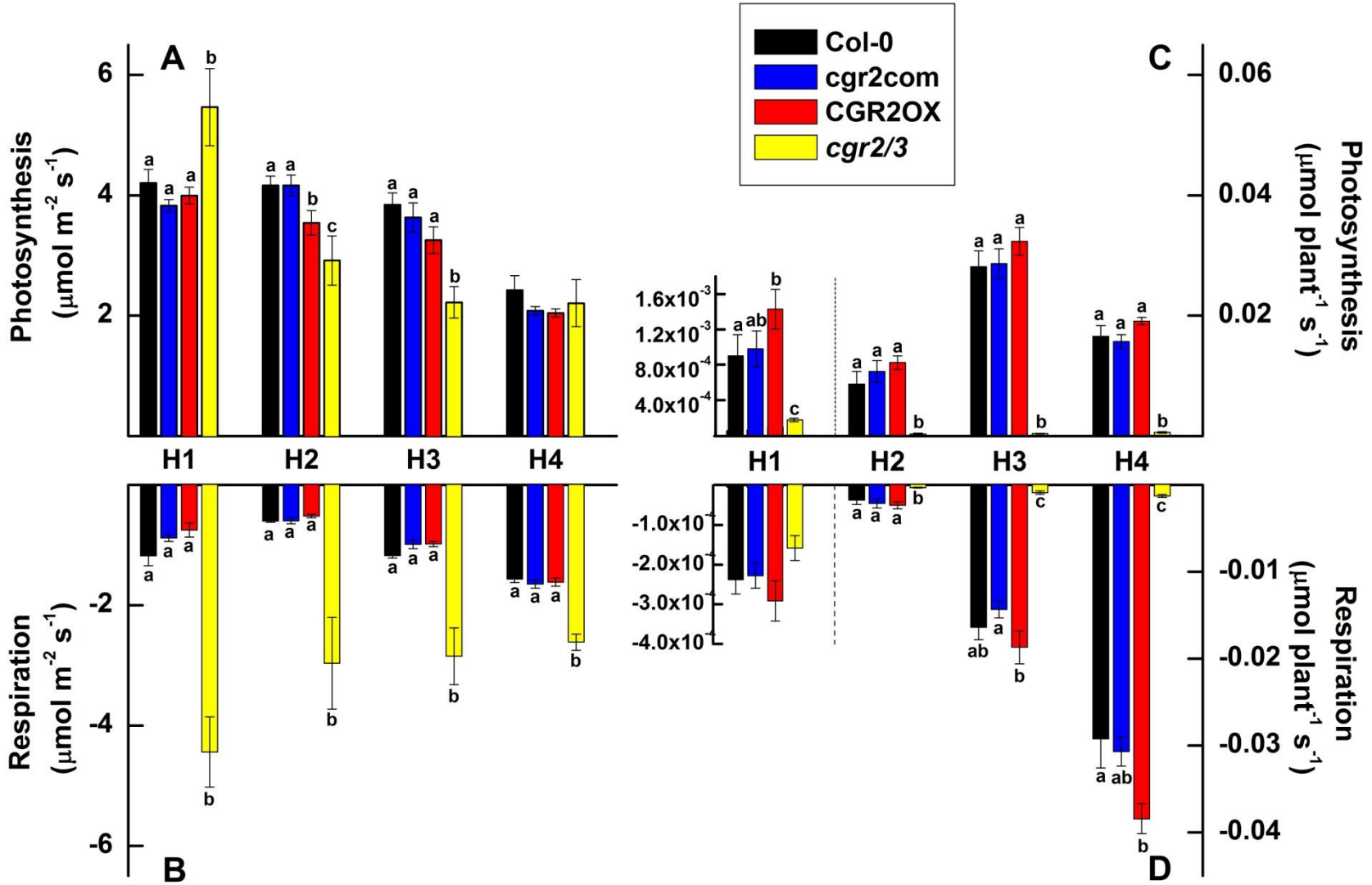


Figure 4. Photosynthesis and respiration overtime in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) Area-based photosynthesis, **(B)** area-based nighttime respiration, **(C)** photosynthesis on a whole plant basis, and **(D)** nighttime respiration on a whole plant basis at 4 different harvests (H) is shown for Col-0, cgr2com, CGR2OX, and *cgr2/3*. Photosynthesis (C gain) is shown as a positive number **(A and C)** while respiration (C loss) is shown as a negative number **(B and D)**. H1, H2, H3 and H4 correspond to 29, 42, 61 and 82 DAS, respectively. Values represent the mean ± SE and n = 5 plants per line. Statistical differences at $\alpha = 0.05$ are marked with lower case letters.

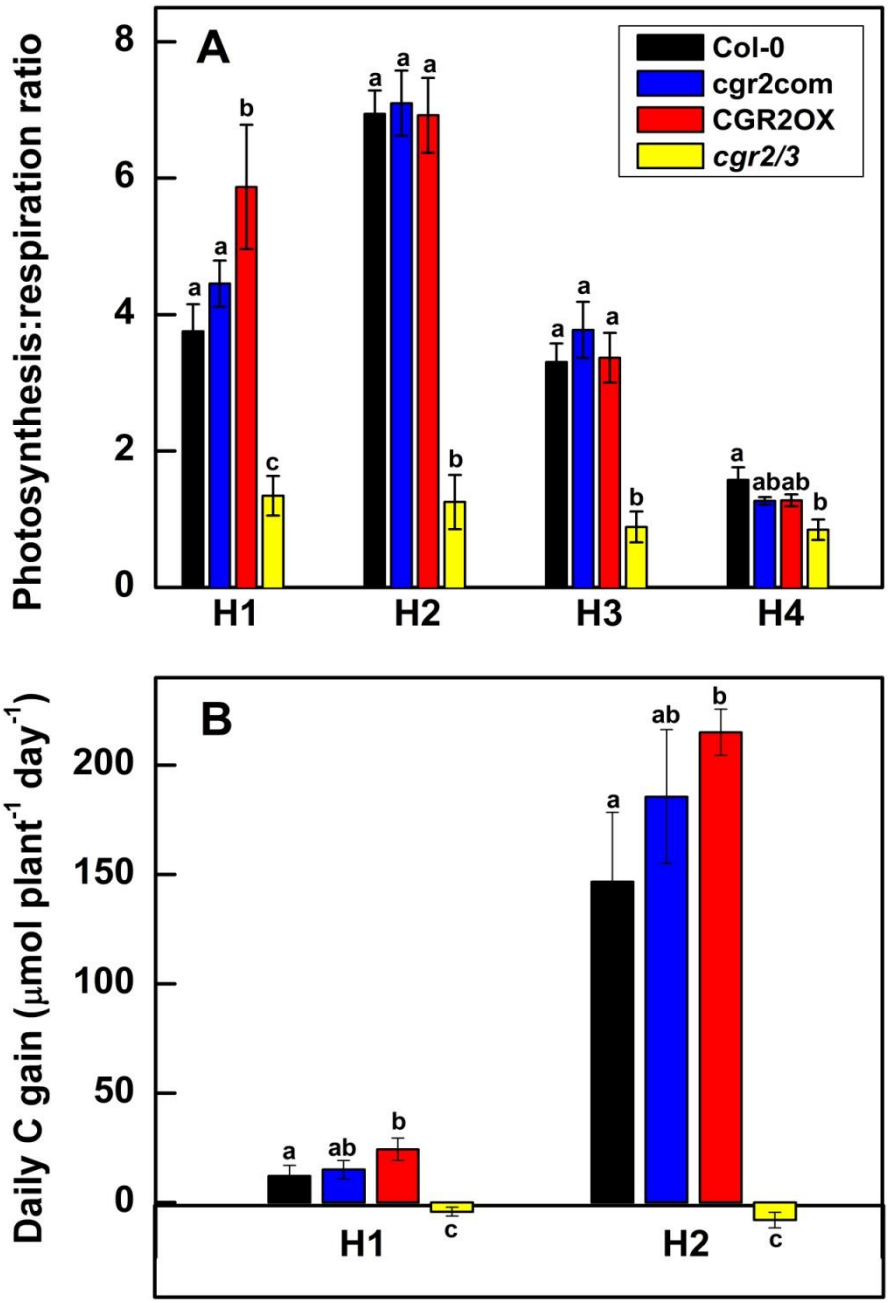


Figure 5. The daily C gain in *Arabidopsis* with altered *CGR2* and *CGR3* expression. **(A)** Photosynthesis to respiration ratio at 4 different harvests (H) and **(B)** the daily C gain at 2 different harvests for Col-0, cgr2com, CGR2OX, and *cgr2/3* is given. H1, H2, H3 and H4 correspond to 29, 49, 63 and 82 DAS, respectively. Values represent the mean ± SE and n = 5 plants per line. Statistical differences at $\alpha = 0.05$ are marked with lower case letters.

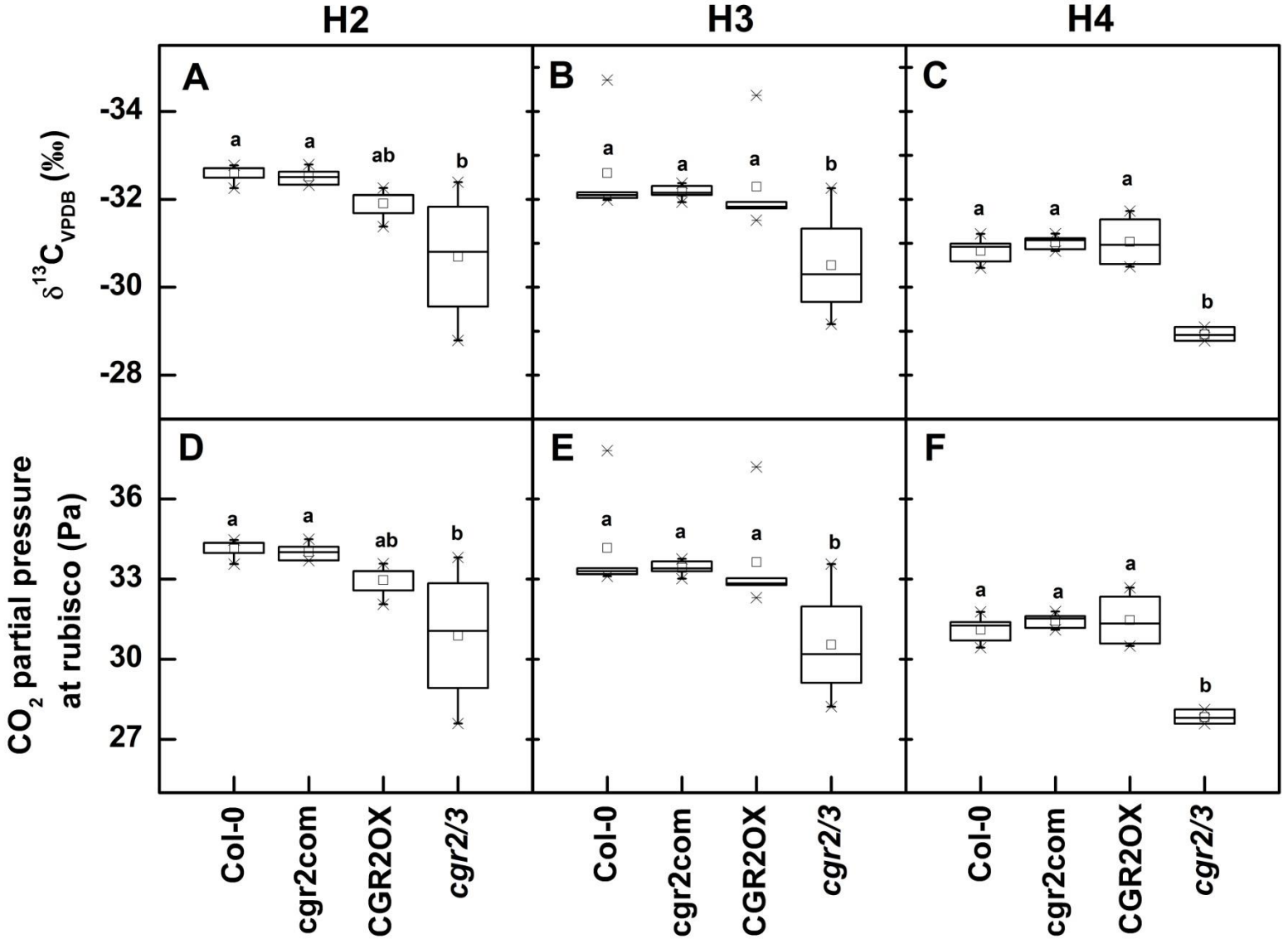


Figure 6. The degree of resistance to CO_2 diffusion through the leaf mesophyll overtime in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A-C) The $\delta^{13}C_{VPDB}$ value calculated as the ratio of ^{13}C to ^{12}C isotopes in leaf tissue relative to a Vienna-Pee-Dee Belemnite standard (VPDB), and **(D-F)** CO_2 partial pressure at rubisco at 3 different harvests (H) is shown for Col-0, cgr2com, CGR2OX, and *cgr2/3*. H2, H3, and H4 correspond to 49, 63, and 82 DAS, respectively. Values represent the mean ± SE and n = 5 plants per line. Statistical differences at $\alpha = 0.05$ are marked with lower case letters.

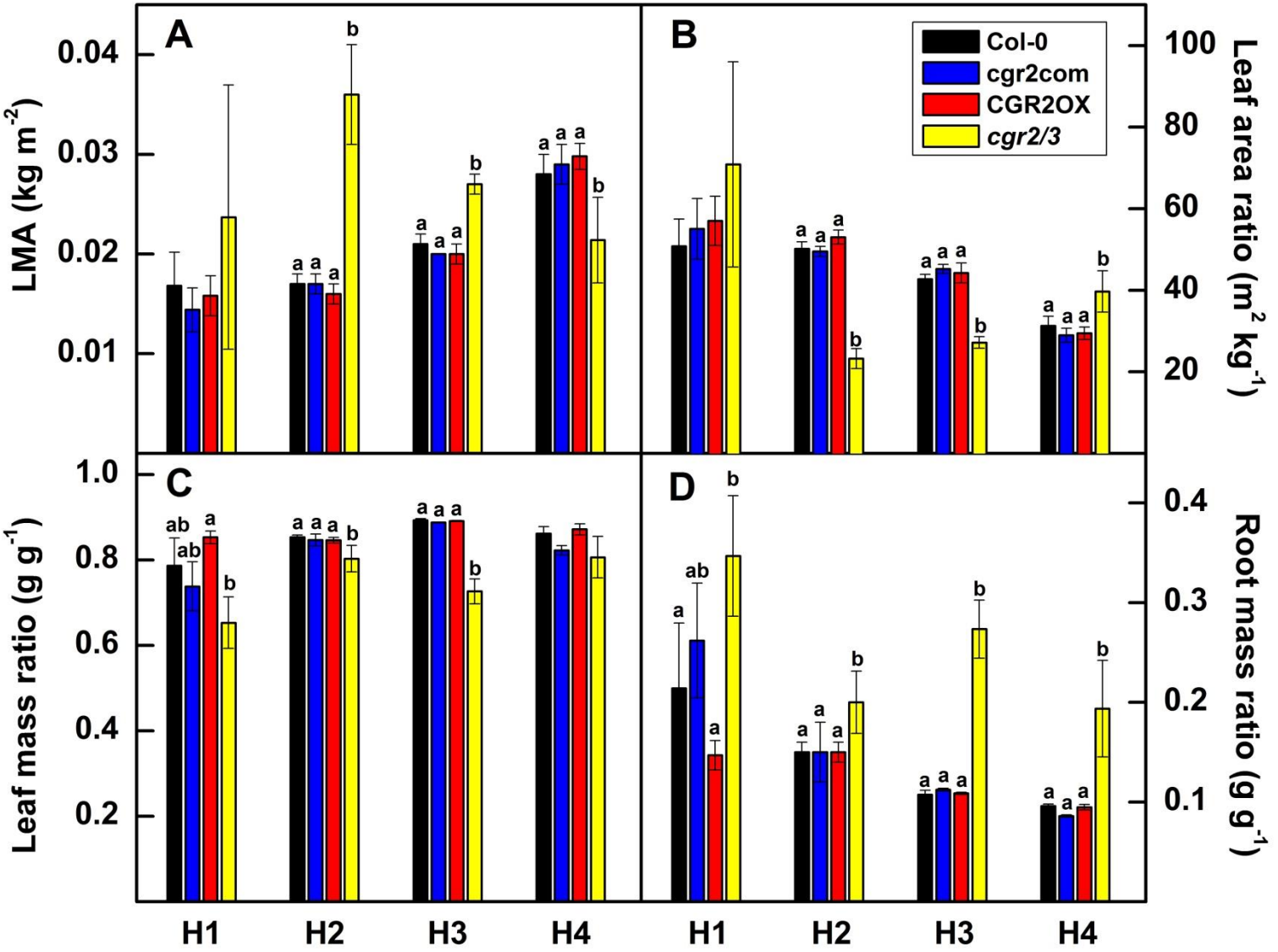


Figure 7. Variations in leaf growth parameters overtime in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) Leaf mass per unit leaf area or LMA, **(B)** leaf area ratio, **(C)** leaf mass ratio, and **(D)** root mass ratio at 4 different harvests (H) for Col-0, cgr2com, CGR2OX, and *cgr2/3* is presented. H1, H2, H3 and H4 correspond to 29, 49, 63 and 82 DAS, respectively. Values represent the mean and ± SD (n = 5 plants per line). Statistically distinct groups at $\alpha = 0.05$ are marked with lower case letters.

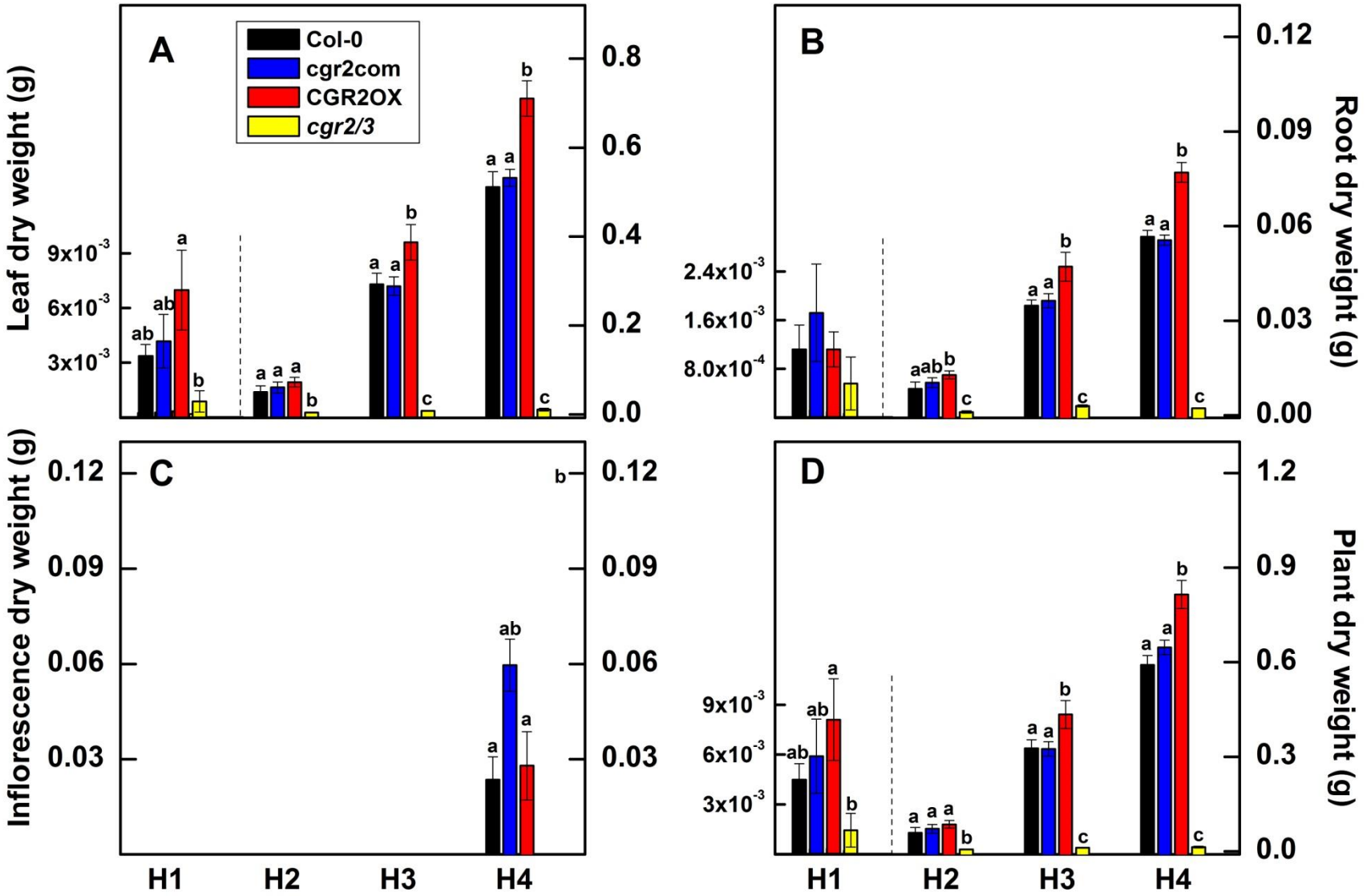


Figure 8. Variations in dry weight overtime in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) Leaf, **(B)** root, **(C)** inflorescence, and **(D)** whole plant dry weight at 4 different harvests (H) for Col-0, cgr2com, CGR2OX, and *cgr2/3* is given. H1, H2, H3 and H4 correspond to 29, 49, 63 and 82 DAS, respectively. Values represent the mean ± SE and n = 5 plants per line. Statistical differences at $\alpha = 0.05$ are marked with lowercase letters.

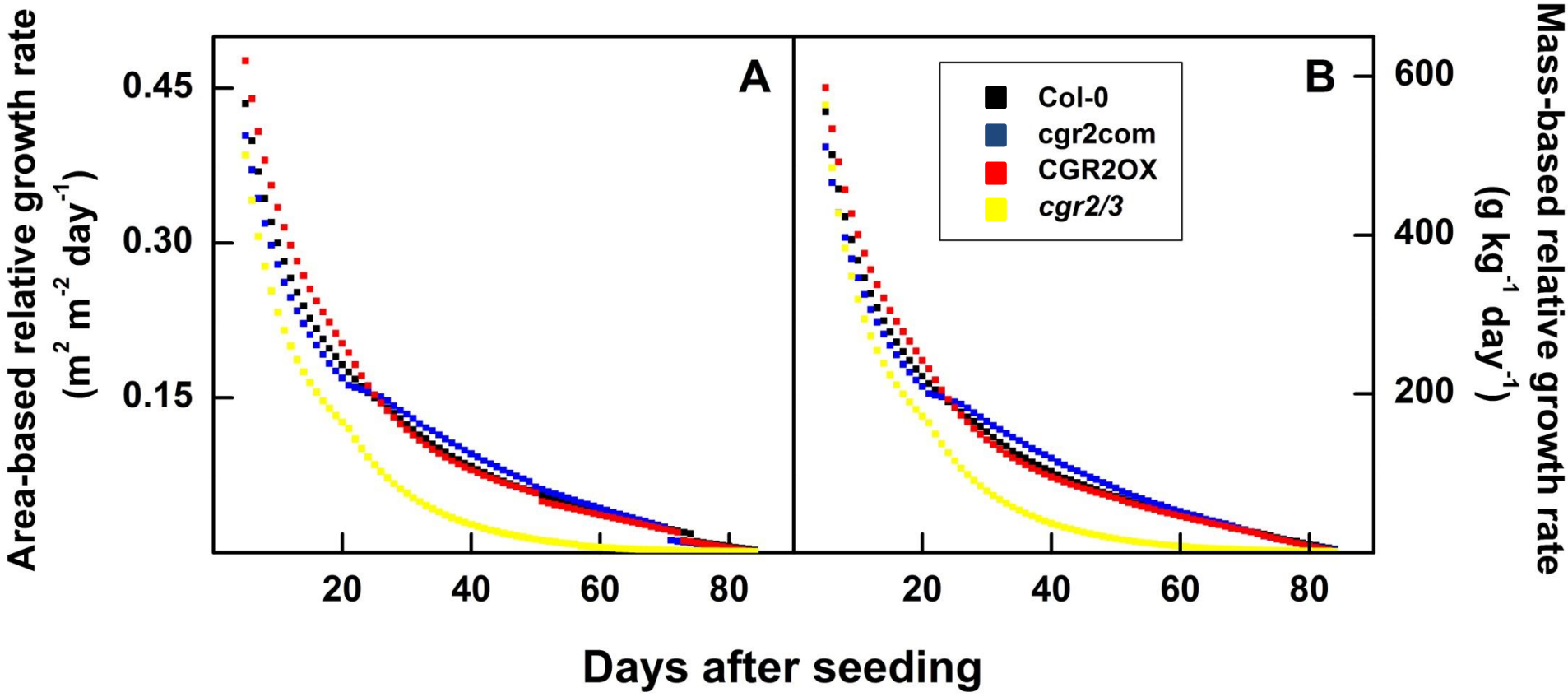


Figure 9. Modeled relative growth rates overtime in *Arabidopsis* with altered *CGR2* and *CGR3* expression. (A) Area-based relative growth rates and **(B)** mass-based relative growth rates simulated from day 5-84 using the *Arabidopsis* Leaf Area Growth Model fitted with data obtained from Col-0, cgr2com, CGR2OX, and *cgr2/3* is presented.

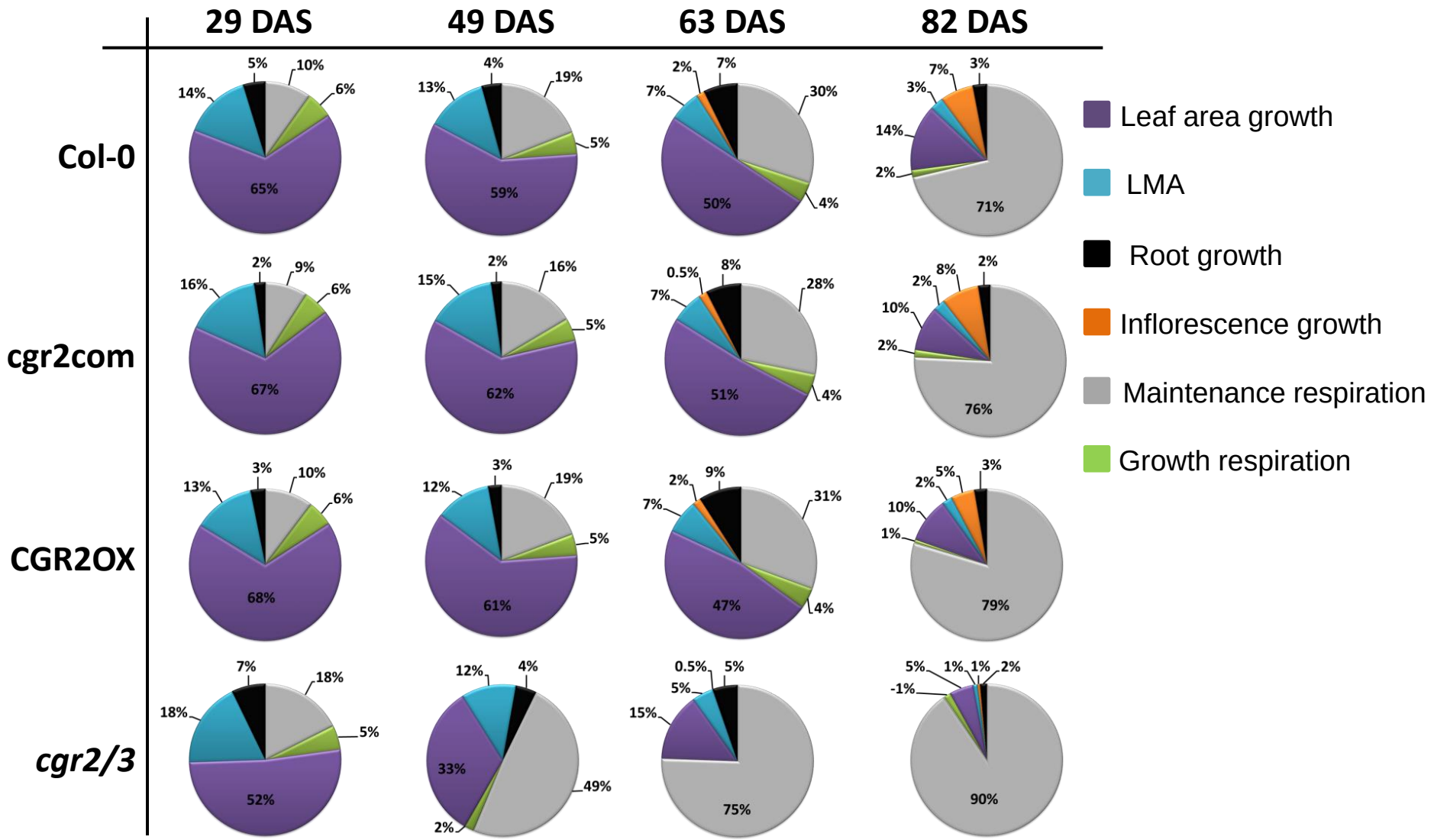
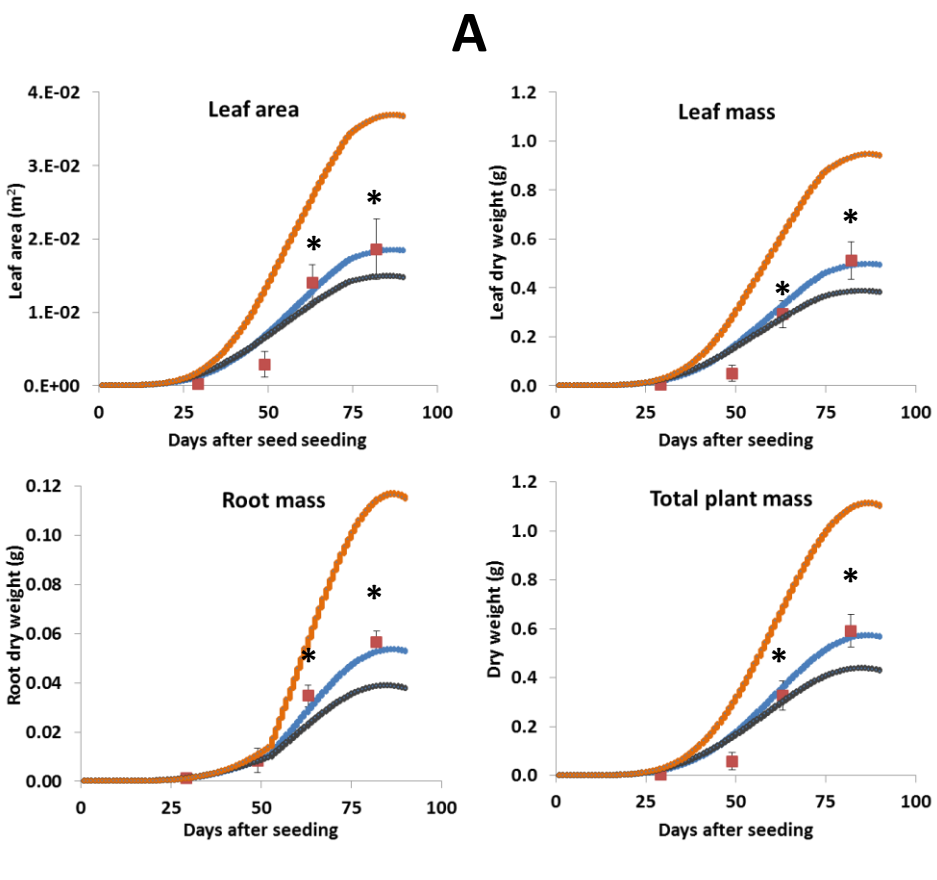
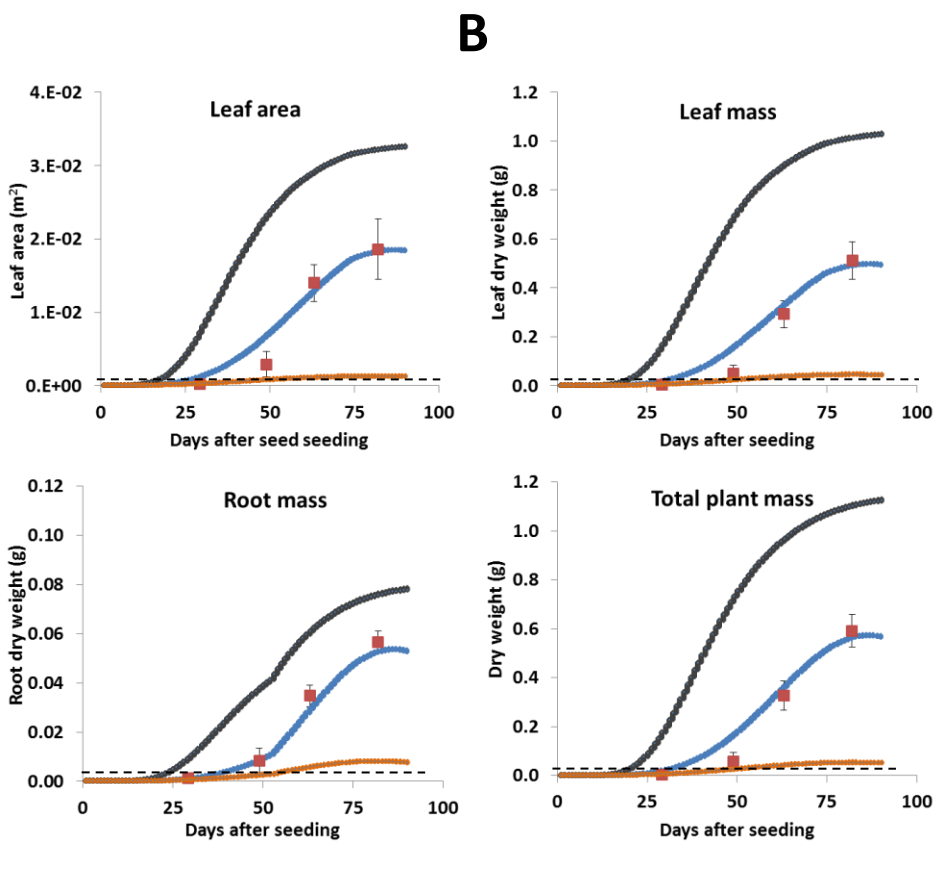


Figure 10. Modeled C partitioning to growth and respiration in *Arabidopsis* with altered *CGR2* and *CGR3* expression. C partitioned to support growth in terms of leaf area and LMA, and inflorescence growth, root growth, and maintenance and growth respiration is presented as percentages of the daily available C at 29, 49, 63 and 82 DAS. Data was derived from the *Arabidopsis* Leaf Area Growth Model fitted with data obtained from Col-0, cgr2com, CGR2OX, and *cgr2/3*.

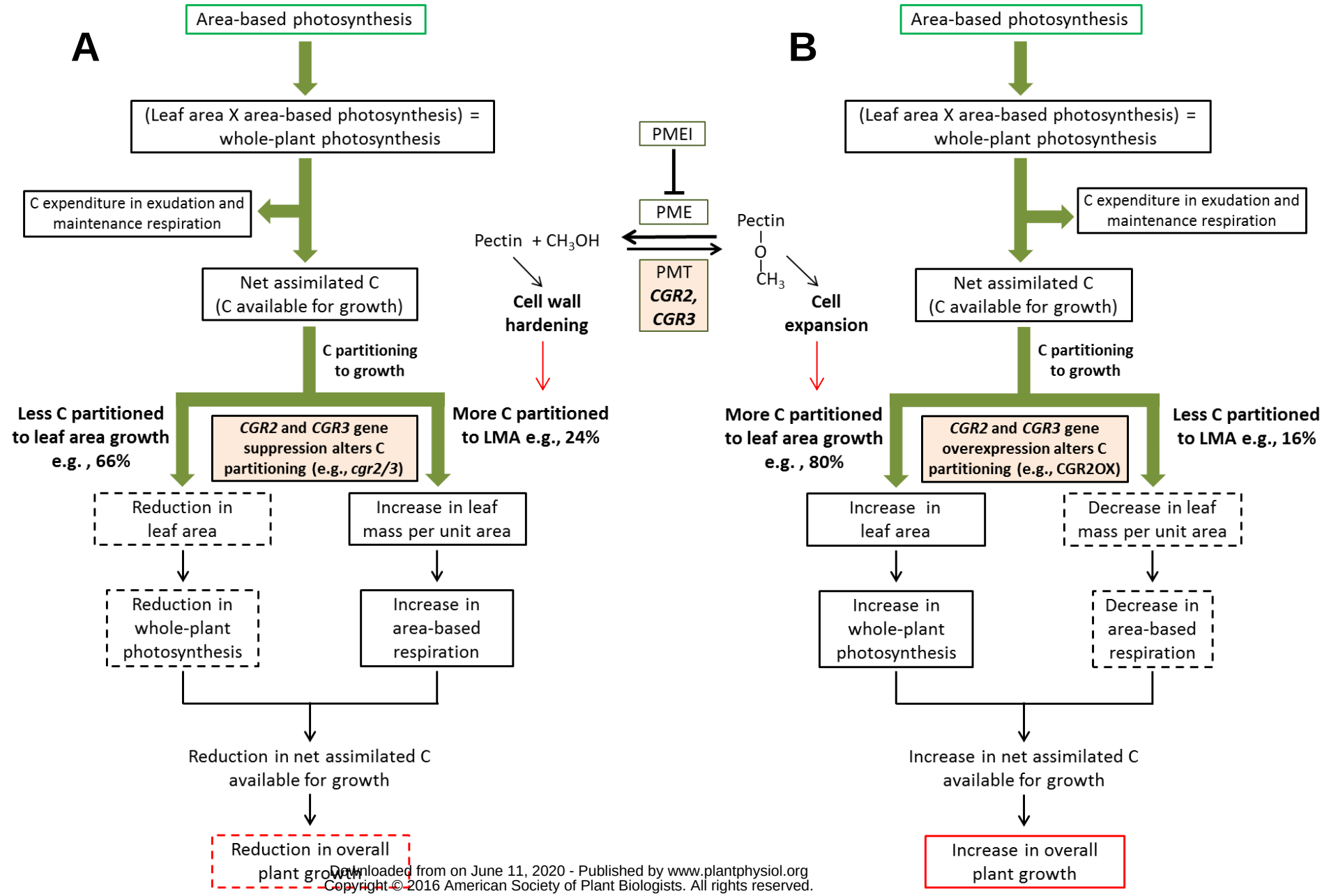


Modeled effects on growth when either photosynthetic rates or partitioning coefficients for Col-0 is replaced by that of CGR2OX



Modeled effects on growth when either photosynthetic rates or partitioning coefficients for Col-0 is replaced by that of *cgr2/3*

Figure 11. Comparison of the effects of altered area-based photosynthesis and C partitioning coefficients on plant growth in *Arabidopsis* with altered *CGR2* and *CGR3* expression. Modeled effects on leaf area and leaf, root, and plant dry weights when either photosynthetic rates or partitioning coefficients spanning the entire growth cycle for Col-0 is replaced by that of **(A)** CGR2OX and **(B)** *cgr2/3* are presented. In **(A)** and **(B)**, blue lines represent modeled growth for Col-0 and red squares represent measured data points (mean ± SD) for Col-0 at 29, 49, 63, and 82 DAS. Grey lines represent modeled data for Col-0 when its area-based photosynthesis rates are replaced by that of either CGR2OX **(A)** or cgr2/3 **(B)**. Orange lines represent modeled data for Col-0 when its partitioning coefficients **(Table 1)** are replaced by that of either CGR2OX **(A)** or cgr2/3 **(B)** without making any changes to area-based photosynthesis rates of Col-0. In (**A)**, asterisks represent measured data for CGR2OX at 63 and 82 DAS. In **(B)**, the dotted line indicates the upper limit of measured data for cgr2/3.



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