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Measuring CO_2 assimilation of *Arabidopsis thaliana* whole plants and seedlings

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ABSTRACT

Photosynthesis is an essential process in plants that synthesizes sugars used for growth and development, highlighting the importance of establishing robust methods to monitor photosynthetic activity. Infrared gas analysis (IRGA) can be used to track photosynthetic rates by measuring plant CO_2 assimilation and release. Although much progress has been made in the development of IRGA technologies, challenges remain when using this technique on small herbaceous plants such as *Arabidopsis thaliana*. The use of whole plant chambers can overcome the difficulties associated with applying bulky leaf clamps to small delicate leaves. However, respiration from the roots and from soil-based microorganisms may skew these gas exchange measurements. Here, we present a simple method to efficiently perform IRGA on *A. thaliana* plants using a whole plant chamber that removes the confounding effects of respiration from roots and soil-based microorganisms from the measurements. We show that this method can be used to detect subtle changes in photosynthetic rates measured at different times of day, under different growth conditions, and between wild-type and plants with deficiencies in the photosynthetic machinery. Furthermore, we show that this method can be used to detect changes in photosynthetic rates even at very young developmental stages such as 10 d-old seedlings. This method contributes to the array of techniques currently used to perform IRGA on *A. thaliana* and can allow for the monitoring of photosynthetic rates of whole plants from young ages.

1. Introduction

Plants are autotrophic organisms that use light energy to convert carbon dioxide and water into sugar and oxygen through the process of photosynthesis. Given the importance of photosynthesis to optimal plant growth and development, careful monitoring of photosynthetic rates is desirable. One of the methods used to monitor the rate of photosynthesis is infrared gas analysis (IRGA), which has been used in multiple plant species to track photosynthetic rates in a variety of tissues (Brazel and Ó'Maoiléidigh, 2019). Open gas exchange systems are composed of a closed chamber in which a plant organ, or the whole plant, is enclosed while a controlled stream of air passes through it. Infrared sensors are used to measure the CO_2 levels in the air stream before it enters the chamber and after it leaves the chamber, allowing for the calculation of the rate of CO_2 assimilation and release by the plant or organ within the chamber.

A number of challenges are presented when applying IRGA to the analysis of photosynthesis of *Arabidopsis thaliana* (Lake, 2004), a model organism used for molecular genetics approaches. Gas exchange analysis using individual leaf chambers is more easily performed when working

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with plants with large, expanded leaves or long petioles. *A. thaliana*, however, has relatively small, delicate leaves with short petioles that can be difficult to isolate and are easily damaged by leaf chambers. Furthermore, leaf and plant age influences photosynthetic rates (Bielczynski et al. 2017) and it may be difficult to consistently choose comparable leaves, especially when comparing mutant and wild-type plants. For example, the *A. thaliana golden-2-like1* (*glk1*) *glk2* mutant, which is deficient in chloroplast development, is extremely small when compared to wild-type counterparts (Fitter et al. 2002). To overcome these challenges, whole plant chambers can be used to measure the photosynthetic rates of entire plants as opposed to sections of individual leaves. Importantly, IRGA of entire plants grown on soil with a whole plant chamber will measure both the gas exchange rates that arise from the plant itself and any other organisms, including microorganisms, present in the soil. For this reason, different measures have been adapted to limit the gas exchange from the soil (plant roots and microorganisms) into the chamber including the use of plastic (Poulson et al. 2002), rubber (Kölling et al. 2015) or clay (Msanne et al. 2011) seals. The plant chamber can also be pressurized to cause the air within the chamber to flow out through the soil and thus prevent diffusion of gasses from within the soil into the chamber (Dodd et al. 2004). However, the release or uptake of CO_2 by and from the soil sample following these mitigations is not well documented in the literature. Furthermore, manipulation of the plants to place plastic, rubber, or clay seals risks damaging the plant, which can induce changes to the photosynthetic rates (Quilliam et al. 2006) while sample-specific controls for soil-based CO_2 flux are not described. Other solutions to this problem include customized self-manufactured IRGAs that are not commercially available (Kollist et al. 2007) and are not accessible for most groups.

There is no standardized plant growth condition for the measurement of CO_2 assimilation rates, making it difficult to directly compare photosynthetic rates reported for *A. thaliana* (Lake, 2004). Reported CO_2 assimilation rates for the ecotype Columbia-0 (Col-0) range from a maximum of ~4 (Dodd et al. 2004) to ~20 (Poulson et al. 2002) μmol m^{-2} s^{-1}, with many studies reporting a CO_2 assimilation rate of ~7 μmol m^{-2} s^{-1} (Jenny et al. 2003; Nelson et al. 2007; Msanne et al. 2011; Conn et al. 2013; Ré et al. 2014). Measurement of CO_2 assimilation in *A. thaliana* plants is of interest because of its usefulness as a model plant for molecular genetics. However, to compare between mutant and wild-type plants, specific parameters should be taken into account, such as the length (Sulpice et al. 2014; Mengin et al. 2017; Xu et al. 2023) and stability (Vialet-Chabrand et al. 2017) of the light period in which plants are grown, as well as the time of day at which plants are measured (Dodd et al. 2004; Kölling et al. 2015), which have been shown to affect CO_2 assimilation rates assessed by IRGA in plants. For instance, whole *A. thaliana* plants measured continuously over multiple days showed a gradual increase in the rate of CO_2 assimilation during light periods (Dodd et al. 2004; Kölling et al. 2015). The effects on photosynthetic rates introduced by the growth regime and time of day could cause challenges when assessing variation in photosynthetic rates between wild-type and mutant plants. Furthermore, many studies employing genome-wide approaches to understanding the topology of the gene regulatory networks (GRNs) underlying photosynthesis use seedling of 10–14 d old plants (Sun et al. 2009; Ahmad et al. 2019; Susila et al. 2023). However, most studies of *A. thaliana* CO_2 assimilation use plants that are at least 4 weeks old, and often older (Poulson et al. 2002; Zhang et al. 2010; Msanne et al. 2011; Ré et al. 2014; Kölling et al. 2015; Liu et al. 2020).

Here, we employ a simple and efficient method to remove the effects of contamination from the soil during IRGA in whole *A. thaliana* plants. We hypothesized that respiration arising from the roots and microorganisms present in the soil could be accounted for by measuring the pot with and then without the apical region of the plant. Subtraction of the readings of the latter from the former would then present the CO_2 assimilation rates solely of the apical region of the plant in question. We validate this method by detecting differences in CO_2 assimilation rates between wild-type Col-0 plants measured at different times of day and in different growth conditions. In addition, we perform IRGA on two mutants with defects in photosynthetic activity. Importantly, our results show and emphasize that growth conditions can influence, and therefore potentially mask or uncover, changes in photosynthetic rates in these mutants. To enable direct comparisons between molecular approaches and physiological measurements of photosynthesis, we also successfully tested this method on 10 d old *A. thaliana* seedlings. This developmental stage that has not previously been measured with IRGA to our knowledge, but this stage is often used to characterize GRNs through genome-wide approaches.

2. Results

2.1. Assessing soil-based respiration after excision of plant from soil

To assess the impact of growth conditions on gross photosynthesis for *A. thaliana*, we measured CO_2 assimilation using IRGA of plants grown in three different daylengths including continuous light (CL), long day (LD), and short day (SD) conditions (Fig. 1a; Table S1). We performed measurements of whole plants in each daylength in the morning (Early) and in the afternoon (Late) (Fig. 1a; see Materials and Methods) using a light-curve program (0–1000 μmol m^{-2} s^{-1} in 250 μmol m^{-2} s^{-1} increments) in a small plant chamber. For reasons outlined in the introduction, we employed a novel "empty pot" (EP) method in which we obtained CO_2 assimilation rates by subtracting the IRGA values for the soil alone from the measurements of the whole plant and the soil (Fig. 1b; see Materials and Methods). We used several genotypes including Col-0 "wild-type" controls, a mutant for *CHLOROPLAST IMPORT APPARATUS 2* (*CIA2*), whose product controls the expression of genes involved in protein import to, and translation in, the chloroplast (Sun et al. 2001, 2009), and a double mutant of *GLK1* and *GLK2*, whose products are involved in chloroplast development (Fitter et al. 2002) (Fig. 1c). Although the mutant genotypes have known deficiencies in photosynthesis, to our knowledge gas exchange rates have not been analyzed.

We initially used an area-based method to normalize the CO_2 assimilation rates where we measured the area of the plant following IRGA (see Materials and Methods) (Fig. 1b; Fig. S1). To test if CO_2 gas exchange occurs in the soil, we first analyzed IRGA data from the "empty pots", where we excised the aerial section of the plant before performing gas exchange measurements (Fig. 1b; Fig. S2). CO_2 assimilation rates of the "empty pots" were negative for all genotypes and growth conditions tested indicating a strong source of respiration from the "empty pots", which was probably a combination of plant root and microorganism respiration (Fig. 1b; Fig. S2). Notably, mean CO_2 levels did not change over time or respond to light intensity, suggesting that the respiration levels were reasonably steady with no, or very few, photosynthetic microorganisms present in the soil. However, there was a high degree of variation for measurements within a single growth condition and genotype suggesting that the respiration levels are unique to each pot (Fig. S2; Table S1). Notably, the "empty pot" respiration was different between genotypes with Col-0 ranging from −6.7 to −3.9 μmol m^{-2} s^{-1}, *cia2–1* ranging from −8.8 to −4.5 μmol m^{-2} s^{-1}, and *glk11–1 glk2–1* ranging from −17.2 to −9 μmol m^{-2} s^{-1} (Fig. S2; Table S1). Therefore, the respiration originating from the soil is unique to each genotype and significantly influences respiration rates depending on the growth condition.

2.2. Testing methods to measure CO_2 assimilation rates of various genotypes in different growth conditions

Before adjustment for soil-based respiration, differences between genotypes could be observed; however, the CO_2 assimilation rates were almost always negative (Fig. 2a, d, g; Fig. S3a, d, g). In addition, following subtraction of the soil-based respiration (Fig. 2b, e, h; Fig. S3b,

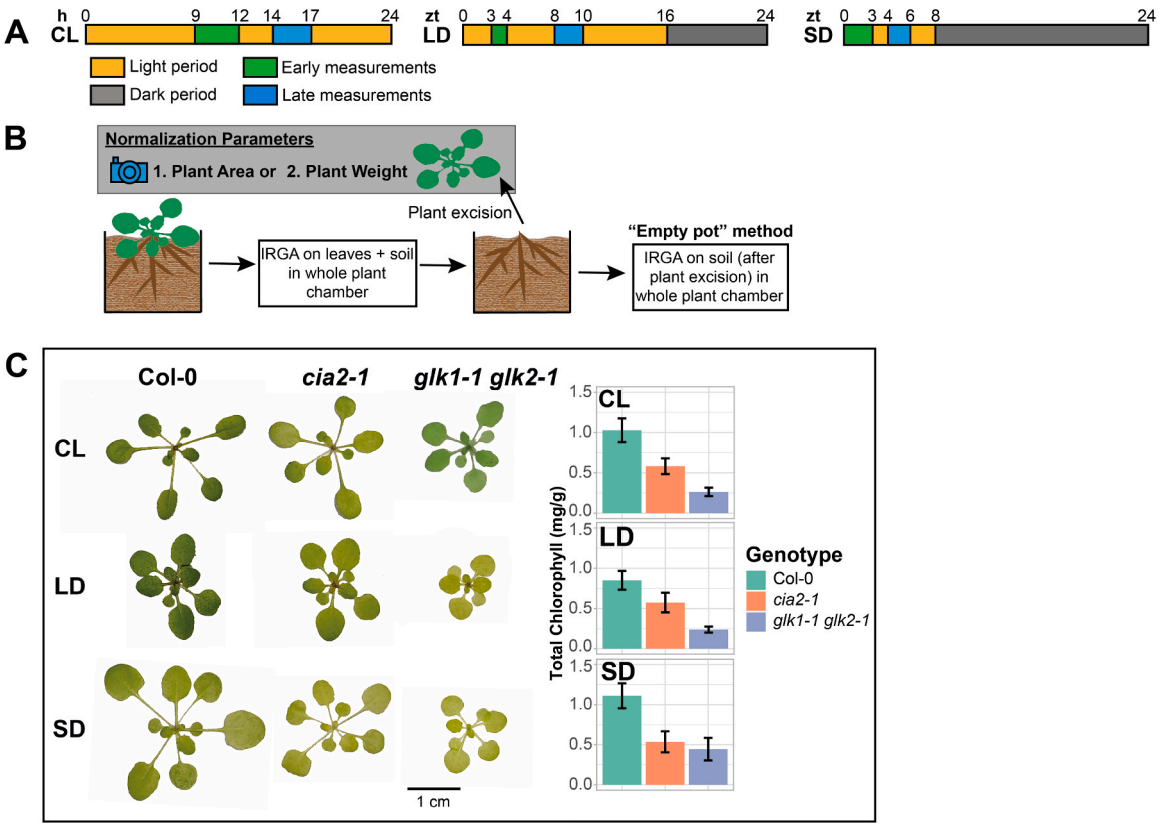

Fig. 1. Strategy for measuring photosynthetic rates by IRGA. (A) *A. thaliana* plants were grown in three growth conditions with different light regimes. The light regimes, "continuous light" (CL), "long day" (LD) and "short day" (SD) are indicated by orange light period and gray dark period. Within each light regime, CO_2 gas exchange measurements were started at "Early" (green) or "Late" (blue) times of day, as indicated in the figure. See Materials and Methods for more details. (B) CO_2 gas exchange was measured by IRGA of whole pots containing an *Arabidopsis* plant and the soil in which it was grown. The aerial section of the plant was then excised and photographed to measure its area and the fresh weight. For the EP method, IRGA was then performed on the pot without the aerial section of the plant. CO_2 gas exchange data was normalized using either plant area or plant fresh weight measurements. The CO_2 gas exchange data from the pot without the aerial section of the plant was subtracted from the data from the pot containing the aerial section to obtain measurements of leaf photosynthesis that removed the contribution of respiration originating from the soil. (C) Representative images and total chlorophyll analysis of Col-0, *cia2–1* and *glk1–1 glk2–1* adult plants grown in CL, LD and SD conditions for 3–3.5 w. Error bars are s.e.m. from 3 to 6 biologically independent replicates.

e, h), the differences between genotypes were not the same as observed before the correction for soil-based respiration (e.g. Fig. 2d-f). Furthermore, the variation associated with each measurement was significantly reduced using the EP method (Fig. 2; Fig. S3; Table S1).

In each growth condition, *glk1–1 glk2–1* adult plants were the smallest in size followed by *cia2–1* (Fig. 1c; Table S2), suggesting a reduction in photosynthetic capacity, in keeping with the pale phenotypes and lower chlorophyll levels of *cia2–1* and *glk1–1 glk2–1*, or an increase in respiration (Fig. 1c; Fig. 2; Table S1; Table S3). Using the EP method combined with an area-based normalization there was a clear difference in CO_2 assimilation rates ($\Delta 1.5 - 2.8$ μmol m^{-2} s^{-1}) between the mutant genotypes and Col-0 under CL condition at all light intensities above 250 μmol m^{-2} s^{-1} at Early times of day ($p.adj < 0.03$; Fig. 2c; Table S2). Under CL at Late times of day, differences between *cia2–1* and Col-0 were observed at the three highest light intensities ($\Delta 1.6 - 2.3$ μmol m^{-2} s^{-1}, $p.adj < 0.07$; Fig. S3c; Table S2). Mean CO_2 assimilation rates of *glk1–1 glk2–1* were lower than Col-0 at all light intensities but statistical support for these differences were only observed at 250 μmol m^{-2} s^{-1} ($\Delta 2.197$ μmol m^{-2} s^{-1}, $p.adj = 0.008$; Fig. S3c; Table S2).

At Early times of day under LD conditions, CO_2 assimilation of *cia2–1* was lower than Col-0 ($\Delta 1.2 - 1.9$ μmol m^{-2} s^{-1}, $p.adj < 0.04$) at light intensities from 500 – 1000 μmol m^{-2} s^{-1} with *glk1–1 glk2–1* CO_2 assimilation only being reproducibly lower than Col-0 ($\Delta 1.2$ μmol m^{-2} s^{-1}, $p.adj = 0.04$) at 250 μmol m^{-2} s^{-1} (Fig. 2f; Table S2). At Late times of day under LD conditions, differences between Col-0 and the mutant genotypes were much more pronounced from 500 – 1000 μmol m^{-2} s^{-1} ($\Delta 2.2 - 3$ μmol m^{-2} s^{-1}, $p.adj < 0.006$; Fig. S3f; Table S2).

The largest magnitude of CO_2 assimilation rate differences was between Col-0 and the two mutant genotypes under SD conditions (Fig. 2g-i; Fig S3g-i; Table S2). Col-0 and *cia2–1* displayed similar differences at Early ($\Delta 1.9 - 2.8$ μmol m^{-2} s^{-1}, $p.adj < 0.05$) and Late ($\Delta 1.5 - 2.5$ μmol m^{-2} s^{-1}, $p.adj = 0.05 - 0.33$) times of day, though variation was higher in the latter measurements. Differences between Col-0 and *glk1–1 glk2–1* were much larger at Late ($\Delta 4 - 6.8$ μmol m^{-2} s^{-1}, $p.adj < 0.0008$) than at Early ($\Delta 2.1 - 3.1$ μmol m^{-2} s^{-1}, $p.adj < 0.03$) times of day. This demonstrates that using our methodology we can discriminate between CO_2 assimilation rates of mutant and wild-type plants.

Though the area based normalization worked well with the EP method, we also tested whether a fresh weight-based measurement would also be suitable (Fig. 1b). In comparison to the area-based assessment of mean CO_2 assimilation rates under CL conditions at Early times of day (Fig. 2c), the differences between Col-0 and *cia2–1* were compressed ($\Delta 0.6 - 1.25$ μmol m^{-2} s^{-1}, $p.adj = 0.06 - 0.51$; Fig. S4a-b; Table S2). In contrast, mean *glk1–1 glk2–1* CO_2 assimilation rates were similar but slightly higher than Col-0 at the three highest light intensities ($\Delta 0.13 - 0.64$ μmol m^{-2} s^{-1}, $p.adj > 0.4$; Fig. S4b; Table S2). The EP method did reduce the variation for each measurement in comparison to when the EP method was not used (Fig. S4a-b). However, we concluded that the area-based normalization was more suitable as fresh-weight measurements introduce variation due to moisture content, which could vary between genotypes. A dry-weight normalization is an

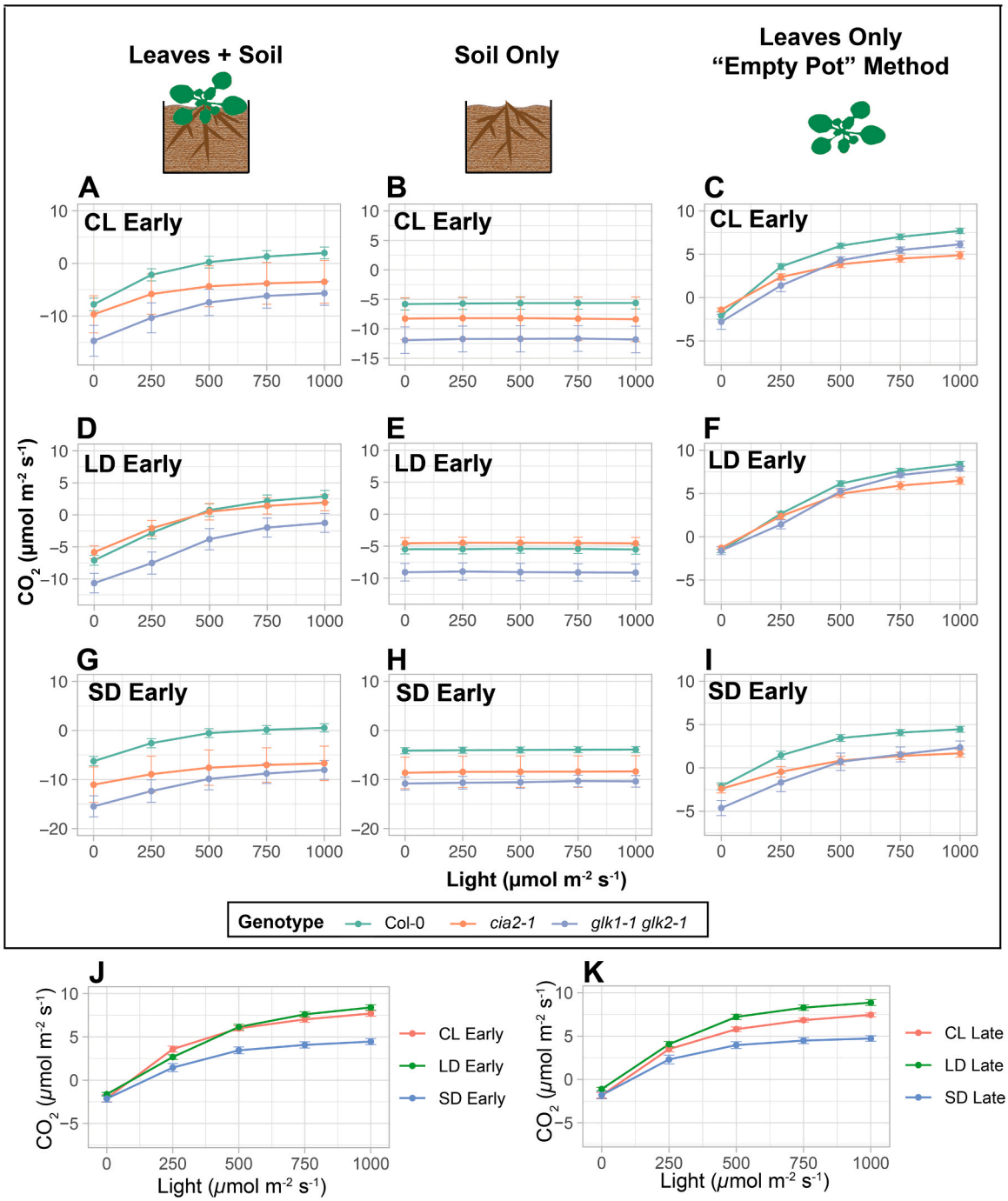

Fig. 2. Gas exchange measurements using IRGA. (A-I) CO_2 gas exchange rates at "Early" time points at varying light intensities are shown for whole adult plants grown in (A-C) continuous light (CL), (D-F) long day (LD) and (G-I) short day (SD) conditions. Measurements were taken of (A, D, G) whole pots containing an *Arabidopsis* plant and the soil in which it was grown and (B, E, H) the pot without the aerial section of the plant, allowing (C, F, I) analysis of the photosynthetic rate of the aerial section of the plant using the EP method. (J-K) CO_2 gas exchange rates at varying light intensities are shown for whole Col-0 plants grown in CL, LD and SD conditions measured at (J) "Early" and (K) "Late" time points using the EP method. Error bars in are s.e.m. of (A-C) 8–16 individual plants from 3 to 7 biologically independent replicates, (D-F) 8–18 individual plants from 4 to 9 biologically independent replicates, (G-I) 6–12 individual plants from 3 to 6 biologically independent replicates and (J-K) 12–18 individual plants from 6 to 9 biologically independent replicates. See Table S2 for statistical analysis of the data.

alternative possibility, however, since the area-based normalization produced robust results, we continued with this approach.

We also tested another recommended method to account for gas exchange from the soil by using pressure differentials. Here, we applied a 50 % leak to create pressure in the small plant chamber and prevent gasses diffusing from the soil into the chamber. With this method, differences in dark respiration of each genotype were larger when compared to the EP method (Fig. S4c). Using the pressure method under CL conditions, mean CO_2 assimilation rates were lower for *glk1–1 glk2–1* compared to Col-0 ($p.adj > 0.1$, Δ4.8 – 5.3 μmol m^{-2} s^{-1}) in all light conditions whereas mean CO_2 assimilation rates for *cia2–1* was higher compared to Col-0 ($p.adj > 0.64$, Δ1.3 – 2.6 μmol m^{-2} s^{-1}) (Fig. S4c; Table S2). Notably, the variation of each measurement was much higher compared to the EP method meaning that the differences between the means of the mutant genotypes relative to Col-0 were not supported by statistical analysis (Table S2). Furthermore, CO_2 assimilation rates of

Col-0 measured with the pressure method were consistently lower than values measured with the EP method ($p.adj < 0.06$, Δ1.2 – 4.3 μmol m^{-2} s^{-1}; Fig. 2c; Fig. S3c; Fig. S4c; Table S2), likely due to a loss of gasses due to the leak that was applied. Taking these data together, we concluded that the EP method with an area-based normalization was the most suitable and reproducible of the methods tested.

2.3. Testing CO_2 assimilation at different times of day and daylengths

As mentioned above, some differences were observed in CO_2 assimilation rates between plants measured at Early and Late times of day (Fig. 2; Fig. S3; Table S2). Under CL and LD conditions, Col-0 plants that were measured at Early timepoints had very similar CO_2 assimilation rates in all light intensities ($p.adj > 0.1$, Δ0.1 – 0.9 μmol m^{-2} s^{-1}; Fig. 2j; Table S2). However, when measuring plants at Late timepoints, CO_2 assimilation of LD grown plants lower than CL grown plants in light intensities from 500 – 1000 μmol m^{-2} s^{-1} ($p.adj < 0.004$, Δ1.4 μmol m^{-2} s^{-1}; Fig. 2k; Table S2). SD grown plants had consistently lower CO_2 assimilation rates compared to both CL and LD grown plants regardless of the time of day the plants were measured ($p.adj < 0.04$, Δ1.2 – 4.1 μmol m^{-2} s^{-1}; Fig. 2j-k; Table S2). Col-0 plants grown under CL conditions displayed almost identical CO_2 assimilation profiles at Early and Late times of day ($p.adj > 0.98$, Δ0.09 – 0.24 μmol m^{-2} s^{-1}; Fig. S4d; Table S2). Col-0 plants grown under LD conditions displayed lower CO_2 assimilation at Early timepoints at 250 μmol m^{-2} s^{-1} ($p.adj = 0.02$, Δ1.4 μmol m^{-2} s^{-1}; Fig. S4e; Table S2) but became more similar at the light intensity increased ($p.adj < 0.1$, Δ0.47 – 1.1 μmol m^{-2} s^{-1}; Fig. S4e; Table S2). Col-0 plants grown under SDs displayed very similar CO_2

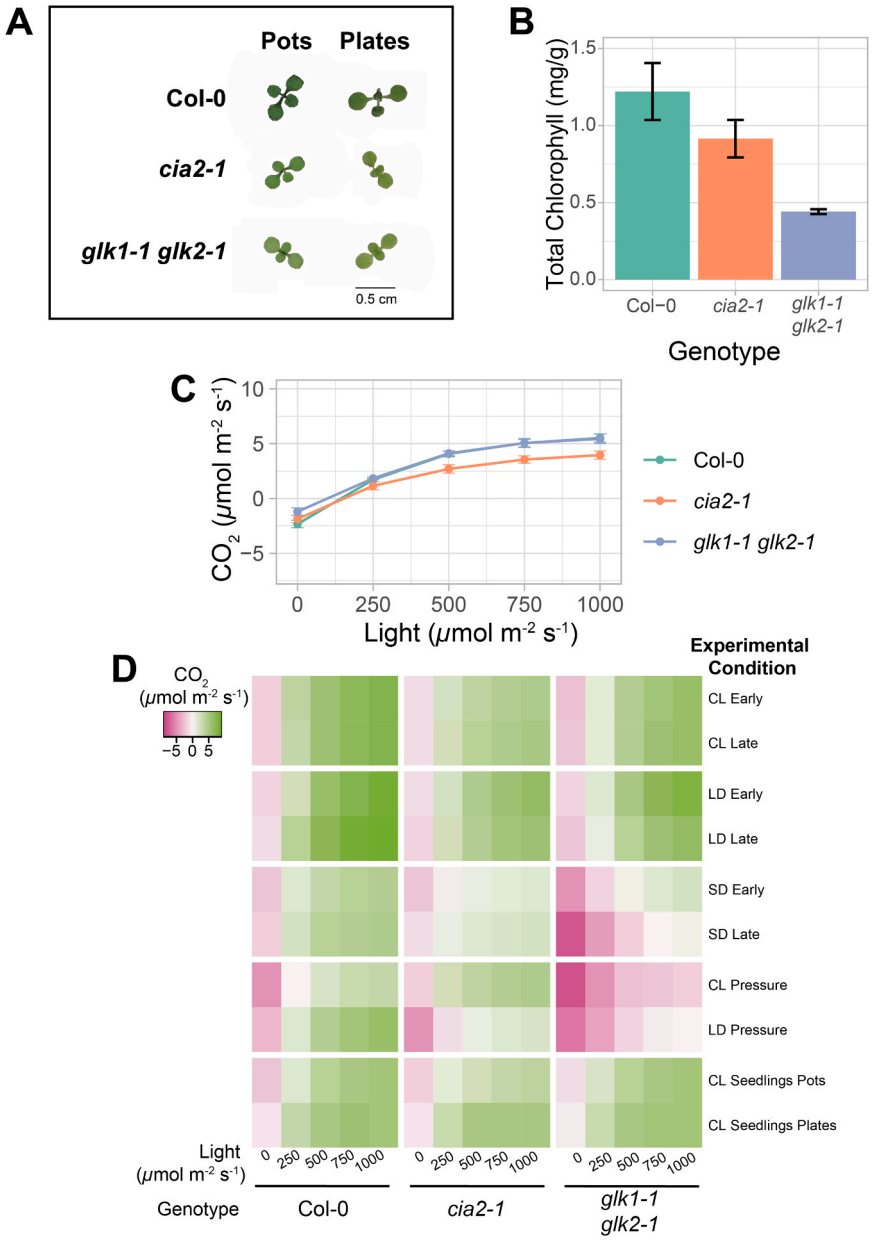

Fig. 3. Comparison of gas exchange measurements across genotypes, plant age, growth conditions and measurement time. (A) Representative photographs of Col-0, *cia2–1* and *glk1–1 glk2–1* adult plants grown in CL conditions for 10 d in pots/plates. (B) Total chlorophyll analysis of Col-0, *cia2–1* and *glk1–1 glk2–1* seedlings grown in CL conditions for 10 d in pots. Error bars are s.e.m. from 5 to 6 biologically independent replicates. (C) CO_2 gas exchange rates at varying light intensities are shown for 10 d seedlings grown in CL conditions in pots. Error bars are s.e.m. of 11–14 individual pots, each containing 5–16 seedlings, from 4 biologically independent replicates. (D) Heatmap summary of average CO_2 gas exchange rates at varying light intensities for Col-0, *cia2–1* and *glk1–1 glk2–1* in all experimental conditions tested in this study. See Table S2 for statistical analysis of the data.

assimilation rates at both times of day across all light conditions ($p.adj >$ 0.5, Δ0.28 – 0.86 μmol m^{-2} s^{-1}; Fig. S4f; Table S2). The differences of CO_2 assimilation rates between growth conditions were further verified in all light intensities using an individual leaf clamp ($p.adj \leq 0.01$, Δ1.2 – 2.6 μmol m^{-2} s^{-1}; Fig. S4g; Table S2).

2.4. Testing methods to detect CO_2 assimilation rates in A. thaliana seedlings

To our knowledge, CO_2 flux experiments have not yet been performed on *A. thaliana* seedlings, which is desirable as 10–14 d old seedlings are widely used in biochemical and genome-wide approaches to study protein activity and GRNs. To test whether differences in CO_2 assimilation rates could be detected in seedlings with defects in chloroplast development, we performed IRGA on 10 d old Col-0, *cia2–1* and *glk1–1 glk2–1* seedlings grown under CL growth conditions on both soil-filled pots and MS-agar plates (see Materials and Methods). As with the adult plants (Fig. 1c), both the *cia2–1* and *glk1–1 glk2–1* seedlings were paler in appearance (Fig. 3a) and had lower levels of chlorophyll (Fig. 3b; Table S3) than Col-0. Remarkably, despite the small size of the 10 d old *A. thaliana* seedlings, we were able to detect differences in CO_2 gas exchange rates in plants grown on soil in pots (Fig. 3c). The rate of CO_2 assimilation in *cia2–1* seedlings was lower than that of Col-0 seedlings in light intensities from 500 – 1000 μmol m^{-2} s^{-1} ($p.adj < 0.0001$, Δ1.4 – 1.6 μmol m^{-2} s^{-1}; Fig. 3c; Table S2). Interestingly, there was less dark respiration observed of *glk1–1 glk2–1* seedlings (-1.2 μmol m^{-2} s^{-1}, respectively) compared to Col-0 (-2.3 μmol m^{-2} s^{-1}, $p.adj < 0.0001$; Fig. 3c; Table S2). We were, however, unable to detect differences in CO_2 gas exchange rates in plants grown on plates (Fig. 3d; Table S2), possibly due to the smaller average size of seedlings grown on plates compared to pots (Fig. S5).

3. Discussion

Here, we present a large-scale dataset in which we use an "empty-pot" method to measure CO_2 assimilation rates in *A. thaliana* wild-type and photosynthesis mutant adult plants and 10 d old seedlings (Fig. 2; Fig. 3c-d; Fig S3). With this method we could detect differences in CO_2 assimilation rates between plants of the same genotype grown in different conditions, including different daylengths and times of day (Fig. 2; Fig. 3d; Fig. S3). We also detected differences between genotypes in adult plants (Fig. 2; Fig. 3d; Fig. S3), which were also influenced by growth conditions, and seedlings (Fig. 3c-d). The simplicity of this method combined with the verifiable measurement of confounding factors (respiration from soil microorganisms and/or root respiration) makes it a very attractive method to implement.

Although leaf clamps can be used to measure CO_2 assimilation rates, the small leaf size and close rosette structure of *A. thaliana* plants make the use of bulky leaf clamps difficult. If measuring attached leaves, it is therefore necessary to wait until the plants have advanced in age and the leaves have expanded sufficiently before the use of a leaf clamp is possible. The consistent selection of leaves at the same developmental stage is critical as leaves at different ages have different photosynthetic rates (Bielczynski et al. 2017). However, this becomes more difficult with older *A. thaliana* plants, particularly when dealing with mutant lines where developmental progression is affected, and leaf size may be reduced (Fig. 1c). It is also important to note that many transcriptomic studies in *A. thaliana* are performed on young plants (10–14 d) (Sun et al. 2009; Ahmad et al. 2019; Susila et al. 2023) and direct comparison of these data with physiological measurements is valuable.

Whole plant chambers have been utilized to overcome the challenges associated with leaf-clamp measurements in *A. thaliana* plants. CO_2 analysis using whole plant chambers detects respiration/photosynthesis from both the plant of interest and any microorganisms present in the soil unless methods are used to block diffusion from the soil. However, the effectiveness of these methods to prevent contamination from soil-based microorganisms or root respiration (Fig. S2) is not well described, while some of the methods used are cumbersome, risk damaging the plant tissue, and controls to verify the absence of soil-based contamination of CO_2 assimilation rates are not easily included. Furthermore, customized solutions to this problem are not commercially available (Kollist et al. 2007), which reduces accessibility.

The method we present here is a simple way to ensure contamination from soil-based microorganisms are removed from CO_2 assimilation analysis (Fig. 1). We found a substantial amount of respiration from the Col-0 "empty pots" up to −6.1 μmol m^{-2} s^{-1} (Fig. S2; Table S2), which contrasts sharply with the maximum CO_2 assimilation rate of 8.9 μmol m^{-2} s^{-1} observed for Col-0 following adjustment for soil-based respiration (Fig. 2; Fig. S3; Table S2). Mutant genotypes were associated with a much larger amount of respiration up to −17.2 μmol m^{-2} s^{-1} (Fig. S2; Table S2), further highlighting the necessity to accurately account for soil-based respiration. The origin of these soil-based respiration values is probably a combination of both respiration from soil-based microorganisms and root respiration, although we are unable to discriminate between the contributions with our experiments. Although we sterilized seeds and autoclaved the soil, the plants were grown in non-sterile pots and conditions, making it likely that a substantial number of microorganisms are present. Notably, the respiration did not respond to light intensity, indicating that there are no photosynthetic microorganisms present in the soil (e.g. algae) (Fig. S2; Table S2). Regardless of the origin of the respiration values, by subtracting values measured from an "empty pot" from values obtained from a pot containing the plant of interest, we have completely removed the contribution of soil-based microorganisms and/or root respiration from our photosynthetic rate analysis. The presented method produced estimates of gross photosynthetic rates similar to those produced with a leaf clamp (Fig. S4g) and to those previously reported (Jenny et al. 2003; Nelson et al. 2007; Msanne et al. 2011; Conn et al. 2013; Ré et al. 2014).

Notably, before adjusting for soil-based respiration, the CO_2 assimilation rates were negative, and the differences observed before adjustment did not translate when compared with those following the adjustment with the EP method (Fig. 2; Fig. S3; Table S2). This method also improves the reproducibility of gas exchange analysis and reduces the rate of error in these measurements associated with contamination from soil-based microorganisms and root respiration (Fig. 2; Fig. S3; Table S2). We also use this method to measure photosynthetic rates in 10 d old seedlings and show that *cia2–1* mutants also display a reduced CO_2 assimilation rate at this young developmental stage (Fig. 3c). The reproducible results obtained with the EP method can help improve detection of defects in photosynthesis at multiple developmental stages. Our results also highlight the importance of consistent conditions, including daylength and time of day, when carrying out these measurements.

4. Materials and methods

4.1. Plant growth and materials

Seeds were sown on autoclaved (60 min at 125°C) potting medium (10:6:4, compost:vermiculite:perlite) in 6 cm pots, or on 3.5 cm plates with 0.5x MS agar. Seeds were stratified for at least two days in the dark at 4°C. Plants were then moved to growth rooms in continuous light (CL), long day (LD) 16:8 h light:dark, short day (SD) 8:16 h light:dark or neutral day (ND) 12:12 h light:dark conditions, as described in the text and Table S1. The specific ages that plants were measured at is described in Table S1. For adult plant measurements, 1 plant was grown per pot while for 10 d seedling measurements 5–17 seedlings were planted per pot or plate. The plant lines used in this study are *Arabidopsis thaliana* ecotype Col-0, *cia2–1* (Sun et al. 2001) (N6522) and *glk1–1 glk2–1* (Fitter et al. 2002) (N9807). These plant lines were acquired from the Nottingham Arabidopsis Stock Centre.

4.2. Infrared gas exchange analysis

Plants were grown to the age indicated in Table S1 and well-watered before their photosynthetic CO_2 assimilation rates were measured using an LI-6800 Portable Photosynthesis System (LI-COR, USA). Although we have not used them, alternative photosynthesis systems such as the GFS-3000 Gas-Exchange System (WALZ, Germany) or CIRAS-4 Portable Photosynthesis System (PP Systems, USA) should be suitable for similar comparisons depending on the plant chambers available.

For plants grown in CL growth conditions, "Early" measurements were initiated between approximately 09:00 and 12:00 while "Late" measurements were initiated between 14:00 and 17:00. For plants grown in LD growth conditions, "Early" measurements were initiated between approximately Zeitgeber Time (ZT) 3 and ZT4, while "Late" measurements were initiated between ZT8 and ZT10. For plants grown in SD growth conditions, "Early" measurements were initiated between approximately ZT0 and ZT3, while "Late" measurements were initiated between ZT4 and ZT6.

CO_2 assimilation measurements were carried out using a small plant chamber with a mounted large light source. Where indicated, a leaf clamp chamber with a 6 cm^2 aperture was used for IRGA. Plants were placed into the sealed small plant chamber with the light source switched off and allowed to acclimate for at least 5 min in the dark, or until the CO_2 sample readings stabilized. A light response curve was performed using the following settings; a flow rate of 600 μmol s^{-1}, sample water vapor set point of 19 mmol mol^{-1}, reference CO_2 of 400 μmol^{-1}, mixing fan speed of 10,000 rpm, heat exchanger temperature setpoint of 22°C, chamber pressure 0.2 kPa, light source composition 90 % red 10 % blue light, light intensity curve set points 0, 250, 500, 750 and 1000 μmol m^{-2} s^{-1} with a 120 – 180 s wait time between set points (no early match allowed). For experiments with the individual leaf clamp chamber, plants were grown to 3–4 weeks old and the same light response curve was applied with the following settings on the two largest most expanded leaves per plant; a flow rate of 400 μmol s^{-1}, relative humidity of air setpoint of 55–60 %, reference CO_2 of 400 μmol^{-1}, mixing fan speed of 10,000 rpm, heat exchanger temperature setpoint of 20°C, chamber pressure 0.2 kPa and light source composition 90 % red 10 % blue light. When the "Pressure" method was used, a leak of approximately 50 % or 300 μmol m^{-1} was applied to release valve beneath the pot. When the "empty pot" (EP) method was used, the aerial part of the plant was excised from the pot without disturbing the soil and without removing the pot from the chamber after the light response curve measurement was completed. The chamber was then sealed again with the "empty pot" within and another light response curve measurement was performed.

CO_2 assimilation measurements of seedlings in plates were also carried out using a small plant chamber with a mounted large light source. A clean, empty 6 cm plastic pot was mounted in the whole plant chamber and a small plastic disk was placed over the pot. A 3.5 cm agar plate was seated on top of the disk such that the seedlings in the plate were approximately the same distance from the light source as is the case when a pot containing soil is used. CO_2 assimilation measurements of seedlings in plates were then performed as described above.

4.3. Measurement of Plant Size

Following the measurement of photosynthetic rate, the plant was imaged and weighed. Plant images were processed in Adobe Photoshop 2022 (Release 21.2.12, Photography Plan). The plant area was measured by using the magic wand and magnetic lasso tool to quickly select the plant tissue (Fig. S1). For the seedling area measurements, average seedling size was calculated by dividing the total area calculated for all the seedlings in an individual pot/plate by the number of seedlings measured. Area measurements can also be calculated from the images collected using open-source software, such as Fiji (Schindelin et al. 2012).

4.4. Chlorophyll analysis

Chlorophyll was extracted through acetone extraction as previously described (Ni et al. 2009). Briefly, leaves from adult plants or approximately 20 10 d-old seedlings were harvested and flash frozen in liquid nitrogen in 1.5 mL centrifuge tubes. Samples were pulverized at −80°C using a TissueLyser II (Qiagen) and 1–2 mL of 80 % v/v acetone was added to the pulverized tissue. The samples were incubated in the dark at room temperature on a rocking shaker for 30 min. Samples were then centrifuged at 850 *g* for 15 min at 4°C. The supernatant was removed and placed into a black centrifuge tube. Samples were placed in 1 mL cuvettes and measured at 663 nm and 645 nm with a UVmini-1240 Spectrophotometer (Shimadzu) and calculated as described (Ni et al. 2009).

4.5. Data analysis

CO_2 assimilation rates were normalized on an area (μmol CO_2 m^{-2} s^{-1}) or fresh-weight (μmol CO_2 mg^{-1} s^{-1}) basis, as described in the text. When the EP method was used, the CO_2 assimilation rate measured for the "empty pot" at each light intensity was subtracted from the value obtained for the pot containing the plant at each corresponding light intensity.

Data were analyzed using Prism 10 for macOS, Version 10.1.0 (264) or Microsoft Excel, graphs were made using the *ggplot2* package from R studio software (R Core Team, 2022) and figures were edited for appearance using Adobe Illustrator 2022 (Release 21.2.12, Photography Plan). For multiple comparisons, data were analyzed using a two-way analysis of variance (ANOVA). Post-hoc tests were employed if a significant result was obtained from the above tests ($p < 0.05$). Following an ANOVA test, the Šídák multiple comparisons test was used when two groups were being compared, while the Tukey's multiple comparisons test was used when three groups were being compared. The heatmap was generated using the Heatmapper web tool (Babicki et al. 2016).

CRediT authorship contribution statement

Ailbhe J. Brazel: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Niranjana S. Manoj:** Writing – review & editing, Investigation, Formal analysis. **Franziska Turck:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Diarmuid S. Ó'Maoiléidigh:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2024.112295.

Data Availability

All data related to this manuscript is shared in the main figures, supplementary figures and supplementary tables.

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