Catalase protects against nonenzymatic decarboxylations during photorespiration in Arabidopsis thaliana

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Abstract

Photorespiration recovers carbon that would be otherwise lost following the oxygenation reaction of rubisco and production of glycolate. Photorespiration is essential in plants and recycles glycolate into usable metabolic products through reactions spanning the chloroplast, mitochondrion, and peroxisome. Catalase in peroxisomes plays an important role in this process by disproportionating H2O2 resulting from glycolate oxidation into O2 and water. We hypothesize that catalase in the peroxisome also protects against nonenzymatic decarboxylations between hydrogen peroxide and photorespiratory intermediates (glyoxylate and/or hydroxypyruvate). We test this hypothesis by detailed gas exchange and biochemical analysis of Arabidopsis thaliana mutants lacking peroxisomal catalase. Our results strongly support this hypothesis, with catalase mutants showing gas exchange evidence for an increased stoichiometry of CO2 release from photorespiration, specifically an increase in the CO2 compensation point, a photorespiratory-dependent decrease in the quantum efficiency of CO2 assimilation, increase in the 12CO2 released in a 13CO2 background, and an increase in the postillumination CO2 burst. Further metabolic evidence suggests this excess CO2 release occurred via the nonenzymatic decarboxylation of hydroxypyruvate. Specifically, the catalase mutant showed an accumulation of photorespiratory intermediates during a transient increase in rubisco oxygenation consistent with this hypothesis. Additionally, end products of alternative hypotheses explaining this excess release were similar between wild type and catalase mutants. Furthermore, the calculated rate of hydroxypyruvate decarboxylation in catalase mutant is much higher than that of glyoxylate decarboxylation. This work provides evidence that these nonenzymatic decarboxylation reactions, predominately hydroxypyruvate decarboxylation, can occur in vivo when photorespiratory metabolism is genetically disrupted.

Keywords

metabolism, photorespiration, photosynthesis - carbon reactions/Calvin cycle/C3 photosynthesis, redox homeostasis/redox reactions
**INTRODUCTION**

Photorespiration is the single largest limitation to C3 photosynthesis under current atmospheres, consuming ~30% to 40% of total plant energy in the light and resulting in rates of CO₂ loss approaching 25% the rate of net CO₂ fixation (Sharkey, 1988; Walker, VanLoocke, et al., 2016). Given this major role in determining net rates of energy use and CO₂ exchange, it is vital to understand the biochemical underpinnings of photorespiration to both accurately model plant productivity in response to changing climates and design optimization strategies for improving net photosynthesis. Improvement strategies targeting photorespiration show initial promise both under laboratory conditions (Timm et al., 2012) and more recently under in-field experiments (South et al., 2019); however, future efforts in optimization and improved modeling may require a more mechanistic understanding of the function of native photorespiration.

Photorespiration recycles 2-phosphoglycolate (2-PG) produced following the reaction of ribulose 1,5-bisphosphate (RuBP) with O₂ as catalyzed by the first enzyme of the C3 cycle, RuBP carboxylase/oxygenase (rubisco). This recycling pathway comprises over a dozen enzymatic conversions and transport steps spanning the chloroplast, peroxisome, and mitochondria and results in the partial recycling of 2-PG into the C3-cycle intermediate 3-phosphoglycerate (3-PGA) with the loss of CO₂ and energy (Figure 1; Bauwe et al., 2010; Foyer & Noctor, 2009). The CO₂ loss from photorespiration is assumed to come primarily from glycine decarboxylation in the mitochondria, resulting in a stoichiometric release of 0.5 CO₂ per rubisco oxygenation (Abadie et al., 2016; Somerville, 2001; Somerville & Ogren, 1980). The stoichiometric release of CO₂ per rubisco oxygenation is a cornerstone assumption for biochemical models of leaf photosynthesis, which represent net CO₂ fixation rates in scales ranging from the single cell to the entire globe (Farquhar et al., 1980; Sun et al., 2014; von Caemmerer, 2013; von Caemmerer & Farquhar, 1981).

While there is strong evidence that glycine decarboxylation is the predominate source of CO₂ loss from photorespiration, there are other potential reactions that can result in additional CO₂ loss including the nonenzymatic decarboxylation (NED) of glyoxylate (Grodzinski, 1978; Halliwell & Butt, 1974) and/or hydroxypropyruvate by H₂O₂ (Cousins et al., 2008; Keech et al., 2012). NED reactions would reduce the carbon recycling efficiency of photorespiration by increasing the stoichiometric release of CO₂ per rubisco oxygenation by up to 400%, assuming they processed all of the photorespiratory flux (Cousins et al., 2011). While early in vitro experiments offered support for the importance of NED reactions in explaining in vivo CO₂ loss in wild type (WT) plants (Grodzinski, 1978; Grodzinski & Butt, 1976; Halliwell & Butt, 1974), subsequent genetic and flux labeling experiments demonstrate that glycine decarboxylation explains the majority of CO₂ loss in vivo, at least under ambient (20°C to 25°C) conditions (Abadie et al., 2016; Somerville, 2001; Somerville & Ogren, 1980). These findings indicate that catalase activity, which detoxifies H₂O₂ in the peroxisome, may be present in high enough levels to inhibit NED.
reactions under the conditions measured in WT plants, at least under ambient temperatures.

When photorespiration is disrupted genetically, however, there is evidence that NED reactions drive excess carbon loss. For example, hpr mutants lacking peroxisomal hydroxypyruvate reductase (HPR) have increased photorespiratory CO₂ compensation points (Γ⁺) and release of CO₂ (Cousins et al., 2008; Cousins et al., 2011; Keech et al., 2012; Timm et al., 2008), demonstrating an increase in the stoichiometry of CO₂ released per rubisco oxygenation (see Section 4 for additional theory).

A similar phenotype supporting increased stoichiometry of CO₂ released per rubisco oxygenation is observed in Arabidopsis thaliana mutants lacking the foliar-expressed catalase (CAT) isoform (cat2). Cat2 shows a very subtle photorespiratory phenotype with near-normal rates of growth and development under ambient CO₂ (Queval et al., 2007). These mutants provide a more fundamental approach to studying the effects of elevated peroxisomal H₂O₂ on NED with minimal pleotropic effects. Indeed, cat2 similarly showed increases in the compensation point (Γ) and other gas exchange signatures of CO₂ release from photorespiration (Keech et al., 2012). Notably, these experiments were measured under a constant low CO₂ partial pressure, as required by the membrane-inlet mass spectrometer setup. It is unclear how strong these gas exchange phenotypes are under ambient CO₂ concentrations to understand how prevalent these reactions are under more physiologically relevant conditions. Furthermore, additional evidence is needed to establish which NED reactions (glyoxylate or hydroxypyruvate decarboxylation) explain this in vivo instance of excess carbon loss from photorespiration and that catalase specifically plays a critical role in protecting against this excess loss in WT plants.

Here, we examine the presence of additional decarboxylation reactions in the Arabidopsis mutant cat2 during photorespiration using various gas exchange approaches; specifically, measurements of Γ⁺, Γ, and the quantum efficiency of CO₂ fixation (Φ₀₂) measured under differing photorespiratory conditions. Each of these measurements is effected by the stoichiometry of CO₂ released per rubisco oxygenation from photorespiration in vivo as described in the materials and methods.

In this work, we present gas exchange and metabolic data to demonstrate that catalase mutants show an increase in the stoichiometry of CO₂ release per rubisco oxygenation and this excess CO₂ release from photorespiration most likely comes from decarboxylation of hydroxypyruvate by H₂O₂. These findings suggest that catalase plays a critical role in guarding against additional wasteful loss of CO₂ from photorespiration and provide a set of approaches that could be used to examine the mechanisms governing the efficiency of CO₂ release from photorespiration under elevated temperatures in WT plants.

2 | RESULTS

2.1 | Measurements of Γ⁺ and Γ were higher in cat2

To determine if cat2 had elevated compensation points consistent with an increase in CO₂ release per rubisco oxygenation, Γ⁺ and Γ were measured using the common intersection method (Walker & Ort, 2015; Walker, Skabelund, et al., 2016). Γ⁺ in cat2 is 30% greater than in the WT under 25°C (Figure S1). This increase in Γ⁺ corresponds to an increase in CO₂ release per rubisco oxygenation from 0.5 to 0.64, assuming S_c/o stays constant (Equation 1). Furthermore, Γ was significantly higher for every light intensity used during the common intercept measurement of Γ⁺ except under the lowest light intensity (50 μmol m⁻² s⁻¹), suggesting that the impact of deficient CAT activity is greatest under elevated rates of photorespiration (Table S1).

![Figure 2](https://example.com/figure2.png)

**Figure 2** The response of the quantum efficiency of CO₂ fixation (Φ₀₂) to different rates of rubisco oxygenation (v_o, a) and the ratio of rubisco oxygenation to carboxylation (v_o/v_c, b) in Arabidopsis thaliana wild type (WT) and plants lacking peroxisomal-type catalase expression (cat2). Φ₀₂ was determined from the initial slopes of light-response curves under various CO₂ and O₂ partial pressures measured using the LI-COR 6800 infrared gas analyzer. Shown with n = 5 ± se
2.2 cat2 had a lower efficiency of net carbon assimilation under higher rates of photorespiration

The response of net assimilation to light intensity was used to determine if cat2 had a photorespiratory-dependent decrease in $\Phi_{\text{CO}_2}$ driven by increases in CO$_2$ release per rubisco oxygenation. Consistent with this hypothesis, $\Phi_{\text{CO}_2}$ was much lower in cat2 compared with the WT plants under the highest rates of rubisco oxygenation ($v_o$) but not when $v_o$ was low as measured under high CO$_2$ or low O$_2$ (Figure 2). Furthermore, the decrease in $\Phi_{\text{CO}_2}$ of cat2 compared with the WT plants followed a roughly linear trend with $v_o$. This trend is consistent when $\Phi_{\text{CO}_2}$ is compared with the ratio $v_o/v_c$ with higher ratios in cat2 showing lower efficiencies. Interestingly, under very high rates of photorespiration, cat2 actually loses more CO$_2$ than is fixed as light intensity increases, resulting in a negative $\Phi_{\text{CO}_2}$ (under 5-Pa CO$_2$, Figures 2 and S2).

Fluorescence measurements indicate that decreases in $\Phi_{\text{CO}_2}$ were not due to general damage or inhibition to the core photosynthetic machinery of cat2 but indeed photosynthetic dependent. For example, cat2 and WT have similar dark and light adapted values of the quantum yield of photosystem II ($F_0/F_m$ and $F_c/F_m$) and rates of non-photochemical quenching (NPQ, Table S2).

To determine if other nonphotorespiratory rates of CO$_2$ release changed with photorespiratory conditions to reduce $\Phi_{\text{CO}_2}$, we compared measurements of $R_b$ between WT and cat2 under various CO$_2$ concentrations and O$_2$ concentrations. In all cases, there was either no significant difference or there was a slightly lower $R_b$ in cat2, suggesting that the observed decreases in $\Phi_{\text{CO}_2}$ are unlikely due to changes in nonphotorespiratory CO$_2$ release (Figure S3).

2.3 The stoichiometry of CO$_2$ released per rubisco oxygenation increases in cat2

To confirm more directly that the cat2 plants had an increase in CO$_2$ release per rubisco oxygenation under higher light intensities, we next used membrane-inlet mass spectroscopy to determine relative rates of CO$_2$ release from photorespiration per rubisco oxygenation. This was necessary because $\Gamma^*$ and $\Phi_{\text{CO}_2}$ are both measured under low light intensities due to the nature of the gas exchange approaches and we wanted to confirm this evidence for increased CO$_2$ release under more physiological conditions. Membrane-inlet mass spectroscopy determines rates of rubisco oxygenation and relative rates of CO$_2$ release from photorespiration from net fluxes of O$_2$ and CO$_2$ resolved from photosynthesis using isotopically enriched atmospheres (Canvin et al., 1980; Cousins et al., 2008). Measurements of the total $^{12}\text{CO}_2$ released in the light under a saturating concentration of $^{13}\text{CO}_2$ were higher in cat2, indicating that cat2 had a higher efflux of CO$_2$ compared with WT (Figure 3a). To ensure this release was not due simply to higher rates of rubisco oxygenation, and a true increase in the stoichiometric release of CO$_2$ per oxygenation, $^{12}\text{CO}_2$ release was normalized by rates of rubisco oxygenation (Figure 3b). This normalization also showed that the stoichiometric release of CO$_2$ per rubisco oxygenation was higher in cat2 compared with WT, consistent with additional NED reactions from photorespiration.

2.4 Evidence for higher and alternate sources of CO$_2$ release from the postillumination burst

To determine how prevalent these increases in CO$_2$ release in cat2 are under ambient CO$_2$ concentrations, we next measured the post-illumination burst (PIB). The PIB refers to the burst of CO$_2$ released from leaves immediately after a light to dark transition (Bulley & Tregunna, 1971; Doehlert et al., 1979). Although the PIB is not a strictly quantitative measurement, it can be used to estimate the amount of CO$_2$ release from photorespiration. Our measurements of the PIB revealed that the total CO$_2$ release following a period of illumination was higher in cat2 as compared with WT (Figure 4a). Furthermore, the PIB peak was integrated to determine the magnitude of total CO$_2$ release during PIB. The data show that the CO$_2$ evolution in cat2 was nearly twofold greater than WT with similar rates of A
2.5 | Metabolic transients, formate, and folic acid concentrations suggest hydroxypyruvate decarboxylation releases excess CO₂ in cat2

To resolve the origin of this excess CO₂ release from photorespiration, we next examined the response of photorespiratory metabolites during a transient period of increasing photorespiration induced by measuring a plant switching from a low to high light condition. Metabolite concentrations can be more informative when measured under transient conditions, before a new steady state is established (Abadie et al., 2016). Specifically, we hypothesized that if glyoxylate NED explained this excess release, more carbon would leave photorespiration in the form of formate, decreasing the pool sizes of intermediates downstream of glyoxylate (glycine, serine, hydroxypyruvate, and glycerate) during transient increases in photorespiratory flux in cat2 as compared with WT (Figure 1). Alternatively, hydroxypyruvate NED forms the photorespiratory intermediate glycolate, meaning that this NED reaction should result in relative increases in photorespiratory intermediate pools (for a given change in \( v_o \)) as carbon is maintained in the cycle in cat2 as compared with WT.

Our transient time-course measurements showed higher relative pool sizes of photorespiratory intermediates in cat2, supporting NED of hydroxypyruvate as the source of excess CO₂ release (Figure 5). Specifically, all photorespiratory metabolites increased more with increased photorespiratory rates in cat2, except for glyoxylate (Figure 5). This general trend is consistent with more carbon staying within the photorespiratory cycle in cat2, as expected with the NED of hydroxypyruvate to glycolate, which would maintain more carbon in photorespiration as opposed to converting hydroxypyruvate to glycerate (Figure 1). These general trends, however, could be explained if cat2 had higher relative rates of \( v_o \) during this light transient.

Complementary gas exchange data showed that these differences in metabolite responses are not associated with a higher rate of \( v_o \) in cat2. The rates of \( v_o \) increased immediately upon exposure to high light, as shown by \( v_o \) estimated from gas exchange measurements made over a similar period (Figure S4). Moreover, the two light induction curves were almost identical, indicating similar rates of glycolate influx between WT and cat2. However, WT and cat2 had very different metabolic responses during the photorespiratory transient. For example, the pool size of glycolate in cat2 had a greater proportional increase than that in WT (Figure 5a). Similar trends were also observed for glycine, serine, and hydroxypyruvate (Figure 5c–e), while the opposite trend was seen for glyoxylate (Figure 5b).

The above results show how each individual metabolite responded to the transient, but to understand how much total carbon was present in the photorespiratory intermediates at each time point, we determined the total carbon within photorespiration by summing the carbon present in each individual metabolite. We calculated total carbon concentration based on the five metabolite pools between glycolate and hydroxypyruvate (Figure 5g, glycolate, glyoxylate, glycine, serine, and hydroxypyruvate). These carbon pools should increase in total in the presence of hydroxypyruvate NED during an increase in photorespiration because this NED would increase the carbon that is cycled back into glycolate without additional \( v_o \). Our data showed that, compared with a relatively flat response curve in WT, a larger amount of carbon accumulated in cat2 as compared with WT during the transient period, consistent with this hypothesis (Figure 5g).
Formate is a product of glyoxylate NED. Formate can either be decarboxylated in the mitochondria or enter one carbon metabolism (a cycle involving numerous folate species) following a reaction catalyzed by tetrahydrofolate ligase (Hanson & Roje, 2001). Formate can also be oxidized nonenzymatically by H$_2$O$_2$ directly, resulting in additional CO$_2$ loss. To further test if this NED reaction also contributes to the excess carbon loss, we measured the contents of formate and its downstream folate species. If glyoxylate decarboxylation takes place in vivo, we might expect to see a higher level of formate and/or folates in cat2. However, our data show that there is no significant difference between WT and cat2 in formate or folate contents (Figure S5), suggesting that the glyoxylate decarboxylation is not the predominant NED reaction under physiological conditions, although we cannot rule out a rapid removal of formate by mitochondrial or enzymatic degradation.

### 2.6 Determining H$_2$O$_2$ concentrations

To determine if the metabolite concentrations that we measured were large enough to drive high rates of particular NEDs, we needed to measure levels of H$_2$O$_2$ in WT and cat2 during the transient from low to high photorespiratory rates explored above. There is no difference in H$_2$O$_2$ concentrations measured under the initially low photorespiratory rates (time $t = 0$), implying that cat2 has adapted to the stress of decreased catalase by activating alternate antioxidative systems for H$_2$O$_2$ scavenging to compensate for the shortage in catalase. However, a large divergence was observed after a shift to high light. The H$_2$O$_2$ level was elevated by $\sim$50% in cat2 and was reduced by $\sim$40% in WT by the end of the transient (Figure 6a). We hypothesize that the decrease in H$_2$O$_2$ concentration under elevated light in WT might be due to the light activation of the catalase enzyme, but regardless, cat2 plants had elevated H$_2$O$_2$ content, a key substrate for...
To determine if the content of H$_2$O$_2$ was large enough to drive NED, we next needed some additional parameterizations of the reaction.

### 2.7 Parameterizing rate constants

To determine if the metabolite concentrations that we measured are large enough to drive high rates of NEDs, we characterized the rate constants of the second-order reactions between H$_2$O$_2$ and either glyoxylate or hydroxypyruvate. To characterize the NED reaction order and rate constants, we measured the decay of H$_2$O$_2$ and substrate following reaction with hydroxypyruvate or glyoxylate using UV spectroscopy at various concentrations of each reactant (Figure S6). The response of the reaction rate was linearly related both to [H$_2$O$_2$] and either [glyoxylate] or [hydroxypyruvate], confirming that both reactions are described by a second-order rate equation. The rate constant for decarboxylation of glyoxylate with H$_2$O$_2$ ($7.5$ L mol$^{-1}$ s$^{-1}$) was higher than that describing reaction with hydroxypyruvate and H$_2$O$_2$ ($3.26$ mol$^{-1}$ s$^{-1}$).

### 2.8 Estimating reaction rates of NED

The reaction rates of NED were determined by multiplying the second-order rate constant by the molar concentrations of the reactants (H$_2$O$_2$ and glyoxylate/hydroxypyruvate). Among the four NED reactions (two in WT and two cat2), hydroxypyruvate decarboxylation in cat2 has the highest rate throughout the transient period (Figure 6b). The rate of hydroxypyruvate decarboxylation in cat2 was approximately twofold to fivefold greater than that of the other reactions.

The rates of this CO$_2$ loss estimated from hydroxypyruvate NED was approximately fivefold lower than the excess CO$_2$ loss.
predicted from our gas exchange measurements when expressed on a leaf area basis. Specifically, the metabolite data estimates a loss of 0.03 to 0.05 μmol m⁻² s⁻¹, but the gas exchange measurements suggest an excess rate of CO₂ release of 0.09 to 0.35 μmol m⁻² s⁻¹. We attribute this discrepancy to underestimates of the highly reactive H₂O₂ measured in our leaf tissues, a species that displays large variation in absolute values depending on study and assay technique (Queval et al., 2008). These results provide further evidence supporting the hydroxypyruvate decarboxylation as the predominant NED reaction and source of excess CO₂ release in cat2.

2.9 Decarboxylation of serine

Besides decarboxylation reactions of photorespiration, there is another photorespiratory-linked decarboxylation reaction that could release CO₂. Serine decarboxylase catalyzes the conversion of serine to ethanolamine. Phosphorylated ethanolamine is the precursor for the biosynthesis of polar head groups of two phospholipids classes, phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Because cat2 had a larger pool size of serine (Figure 5d), we wondered whether this could drive a higher rate of serine decarboxylation. To test this hypothesis, we analyzed contents of PC and PE. Our data showed that there were no significant differences in the amount of PC and/or PE between WT and cat2 (Figure S7). Furthermore, fatty acid profiles of PC and PE were also similar (Figure S7). These results further suggest that decarboxylation of hydroxypyruvate is a predominant source of excess release of CO₂ in cat2.

2.10 Total catalase activity

To confirm and quantify the decrease in catalase activity in cat2, total catalase activity was measured via O₂ evolution. Catalase activity decreased in cat2 by almost 80% when expressed both on a leaf area and protein content basis (Table 1). Furthermore, the decrease in catalase activity was not accompanied by a decrease in total protein content.

### Table 1

| Metabolite content, catalase activity, and protein content in Arabidopsis thaliana wild type (WT) and plants lacking peroxisomal-type catalase expression (cat2) |
|---------------------------------|-----------------|-----------------|
| **Catalase activity (mol O₂ m⁻² s⁻¹)** | WT              | cat2            |
| 0.81 ± 0.09a                    | 0.18 ± 0.02a    |
| **Catalase activity (μmol O₂ mg⁻² Prot s⁻¹)** | 1.94 ± 0.18a    | 0.45 ± 0.05a |
| **Protein content (mg m⁻²)**     | 415 ± 34a       | 427 ± 8a       |

Note: Catalase activity was determined from leaf extracts by following the rate of oxygen production following H₂O₂ addition in an O₂ electrode and presented both on a leaf area and mg protein basis. Protein content was determined on the same extract using a Bradford assay of soluble protein. Shown are the averages biological replicates (n = 5 ± ster) determined with technical replicates (n = 3) with significant differences (Student’s t test, α < .05) indicated by different letters.

3 DISCUSSION

In this paper, we demonstrate that catalase protects against excess photorespiratory carbon loss and that this excess loss decreases net photosynthesis under ambient conditions. In cat2 plants, both I° and Γ were greater than in WT, which is explained by an increase in CO₂ release per rubisco oxygenation (Figure S1 and Table S1). Additionally, cat2 ΔCO₂ had a vₐ-dependent decrease, which even became negative under high vₐ indicating an extra loss of CO₂ that negatively impacted net photosynthesis and scaled with rates of photorespiration (Figures 2 and S1). Furthermore, cat2 mutants had elevated 13CO₂ release per rubisco oxygenation and a higher PIB peak area than WT, indicating a higher amount of CO₂ being released under physiological conditions (Figures 3 and 4).

Our metabolite data strongly suggest that the source of this excess CO₂ release from photorespiration arises from the NED reaction between H₂O₂ and hydroxypyruvate. Specifically, a larger carbon accumulation of photorespiratory intermediates formed in cat2 mutant compared with WT during a period of increased photorespiration, suggesting a cyclic route of metabolic flux through photorespiration with NED decarboxylation of hydroxypyruvate to glycolate (Figure 5). Additionally, rates of hydroxypyruvate NED reactions predominated when calculated from measured metabolite pools and reaction rates, providing further evidence for the predominance of the hydroxypyruvate decarboxylation (Figure 6). Furthermore, we did not see evidence for alternative explanations of this loss as metabolite concentrations of downstream products of glyoxylate NED (formate and folates) were similar between WT and cat2, suggesting that the glyoxylate decarboxylation reaction is unlikely to account for the excess CO₂ release in cat2 (Figure S5). We also did not find evidence for elevated downstream products of serine decarboxylation as PC, PE, and fatty acid profiles were similar between cat2 and WT (Figure S7). We note that we cannot exclude the hypothesis that HPR activity is inhibited in cat2 and results in a buildup of downstream metabolites, but the modeling of rates of NED still indicates a certain amount of NED from hydroxypyruvate (Figure 6). This is in line with recent 13C and 14C labeling data in the photorespiratory mutant lacking HPR, although this work also showed support for NED flux to ethanolamine (Timm et al., 2021).

There are other enzymatic decarboxylation reactions that have received attention recently that could help explain this increased CO₂
loss. The import of glucose 6-phosphate (G6P) into the chloroplast could stimulate a G6P shunt that follows the oxidative branch of the pentose phosphate pathway around Calvin-Benson cycle and thus increasing CO₂ release in the light (Sharkey & Weise, 2016). It has been hypothesized that the G6P shunt could cause more CO₂ release and lead to an increase in Rₜ. Our data, showing no significant difference in Rₜ between WT and cat2, do not support that the excess CO₂ release in cat2 is due to additional CO₂ release through the G6P shunt (Figure S3). However, because Rₜ was determined under low CO₂ concentrations and low light intensities, we could not exclude the possibility of excess CO₂ released from G6P shunt under more physiological conditions. Alternatively, recent work highlights the potential for amino acid synthesis to contribute to CO₂ release in a nontargeted metabolic analysis on sunflower showing that CO₂ and O₂ mole fraction changes the flux through several pathways involved in amino acid synthesis (Abadie & Tcherkez, 2021). The CO₂ release associated with these pathways was proposed to have potential impact on the amount of CO₂ release per oxygenation, but it is difficult to evaluate this claim without quantitative flux estimates. Future labeling work combined with formal flux estimates should help resolve this source most conclusively (Xu et al., 2021), but it is outside of the scope of the current work.

This work identifies a probable mechanism for excess CO₂ release measured in mutants with perturbed photorespiratory metabolism, but the presence of NED reactions during photorespiration may not be limited to mutant plants with a disrupted photorespiratory pathway (Cousins et al., 2008, 2011; Keech et al., 2012). Photorespiration is impacted by changes in temperature in ways that could drive NED in WT plants. For example, the activity of glycolate oxidase increases more with temperature than catalase, possibly driving NED reactions (Grodzinski & Butt, 1977). Additionally, posttranslational modifications may modulate catalase activity, which could further modulate NED independently from protein content if activities were kept too low. For example, CAT2 contains a single phosphorylation site spanning residues 79–91 that is phosphorylated in response to nitrogen starvation (Engelsberger & Schulze, 2012; Hodges et al., 2013). An increase in CO₂ release per oxygenation under elevated temperatures would explain discrepancies in measurements of Γ⁺ across many species, resulting in up to a 20% increase in the amount of carbon dioxide a plant loses per rubisco oxygenation reaction (Walker & Cousins, 2013). If present, this increase in CO₂ released from photorespiration could present a potential route for improving the carbon recycling efficiency of photorespiration and subsequent net rates of CO₂ fixation at elevated temperatures.

### 4 MATERIALS AND METHODS

#### 4.1 Plant material and growth

*A. thaliana* cat2 mutants (At4G35090, SALK 076998) were provided by Dr. Graham Noctor (Queval et al., 2007). WT and cat2 plants used for measurements of Γ⁺, Γ⁻, and Φₚₛ₂ were grown under a 12/12 day/night cycle at 90 μmol photons m⁻² s⁻¹ and 23°C/18°C on a standard soil substrate A210 (Stender, Germany). WT and cat2 plants used for PIB, membrane inlet and metabolic analysis were grown under an 11/13 day/night cycle at 100 μmol photons m⁻² s⁻¹ and 23°C/18°C.

#### 4.2 Gas exchange theory

Γ⁺ is a key parameter that links plant biochemistry to rates of net gas exchange by combining rubisco specificity for reaction of CO₂ relative to O₂ $(S/μ)$ with oxygen concentration (O) and the amount of CO₂ lost from photorespiration per rubisco oxygenation $(η)$ according to (Farquhar et al., 1980; von Caemmerer, 2013; von Caemmerer & Farquhar, 1981)

$$Γ⁺ = \frac{ηO}{S/μ}.$$  

Because Γ⁺ is proportional to η and NED reactions increase η, differences in Γ⁺ can indicate changes in the amount of CO₂ released from photorespiration due to increases in NED reactions, especially when measurements are done under the same O and in the same species with identical $S/μ$.

Γ is a more readily measured parameter but is more indirectly related to η because it measures the CO₂ compensation point where a leaf assimilates as much CO₂ as it releases from both photorespiration and nonphotorespiratory CO₂ loss in the light (Rₜ) according to

$$Γ = \frac{Γ⁺ + K_c (1 + \frac{K_o}{c}) R_t / V_{c,max}}{1 - R_o / V_{c,max}},$$

where $K_c$, $K_o$, $R_o$, and $V_{c,max}$ are the Michaelis–Menten enzyme constants of rubisco for CO₂, O₂, rate of mitochondrial respiration in the day, and maximum rate of rubisco carboxylation. Because Γ is also dependent on Γ⁺, it is similarly impacted by changes in η driven by NED reactions.

A third test for the presence of NED reactions during photorespiration is in measurements of Φₚₛ₂. Because Φₚₛ₂ represents net CO₂ fixation per absorbed photon of light, Φₚₛ₂ should decrease when total amounts of CO₂ lost from photorespiration increase and reduce net CO₂ fixation. The total rate of CO₂ lost from photorespiration is equal to η multiplied by rates of rubisco oxygenation $(v_o)$, so $v_o$-dependent decreases in Φₚₛ₂ would provide further evidence for the presence of NED reactions.

#### 4.3 Steady-state gas exchange

Gas exchange was performed on the youngest, fully expanded leaves of 4- to 6-week plants using a LI-6800 with a 3 × 3 cm measuring head (LI-COR Biosciences, Lincoln, Nebraska, USA). After measurements, leaf area enclosed by the cuvette was determined using the ImageJ FIJI distribution (Schindelin et al., 2012). During all gas
exchange measurements, leaf temperature was maintained at 25°C and vapor pressure deficit was controlled at either 1 or 1.5 kPa for 25°C. Measurements were performed in a climate-controlled chamber set to the measurement temperature.

Γ" values were measured using the common intersection method using slope–intercept regression (Laik, 1977; Walker & Ort, 2015; Walker, Skabelund, et al., 2016) and light intensities of 250, 165, 120, 80, and 50 μmol photons m⁻² s⁻¹. No significant Kok effect was seen in light-response curves measured at or above the light intensities used in Γ" measurements. Slope and intercept values were determined from the linear portion of CO₂-response curves measured under each light intensity and CO₂ concentrations between 10- and 3-μmol CO₂ for determination of the common intersection value, which is equal to the intercellular CO₂ concentration (Cᵢ) at Γ" according to the equation Γ" = Cᵢ - Rₑ/γₑ, where Rₑ is determined from the y-axis value of the common intersection point and γₑ was assumed to be 2.23 and 2.01 mol m⁻² s⁻¹ MPa⁻¹ for 25°C according to the temperature response measured previously in A. thaliana (Walker et al., 2013).

Light-response curves for determining Φ₃CO₂ were measured under various CO₂ and O₂ environments controlled either using the native Li-COR 6800 functionality for CO₂ or a synthetic N₂ and O₂ mixing system composed of two mass flow controllers (red-y series, Vögtlin Instruments, Switzerland). Plants were acclimated under 250 μmol photons m⁻² s⁻¹ before being measured under 65, 50, 45, 40, 35, 30, 25, and 20 μmol photons m⁻² s⁻¹. For measurements of Φ₃CO₂, the slope of CO₂ assimilation versus absorbed light intensity was determined from Kok effect-free portions of the initial slope assuming a leaf absorbance of 0.843. For each condition, rates of νₒ and the ratio νₒ/νₑ were determined as described previously from the gas exchange measurements (Walker et al., 2014). Calculations of νₒ and νₒ/νₑ were made using chloroplastic CO₂ concentrations calculated assuming a mesophyll conductance (γₑ) of 2.2 μmol m⁻² s⁻¹ Pa⁻¹ from the intercellular CO₂ concentrations measured at a point midway through the linear section of the light-response curve (usually at 35 μmol photons m⁻² s⁻¹). While each light intensity across this range had slightly different νₒ values, the relationship was linear overall, indicating that Φ₃CO₂ appeared to be constant across the range and justifying a single representative νₒ to be used (Figure 3). Additionally, because the intercellular CO₂ concentration was not greatly impacted across this range due to similar rates of net assimilation, νₒ/νₑ was very constant across the light intensities used to determine Φ₃CO₂. Respiration in the light was estimated according to the method of Kok (Kok, 1948). Light-response curves were measured as subsaturating light intensities. The rate of day respiratory (Rₑ) was determined by the y intercept by extending the part of light-response curve after the compensation point to y axis, removing any potential inflections due to the Kok effect.

4.4 | Membrane-inlet mass spectroscopy

Membrane-inlet mass spectroscopy was measured on leaf disks enclosed in a custom-built, thermostatted cuvette that allowed for inlet sampling and introduction of modified isotopic backgrounds (Cousins et al., 2008; Walker & Cousins, 2013). The cuvette was built with multiple sampling and gas-release ports fitted with sampling septa. The membrane was composed of 0.005° fluorinated ethylene propylene film (CS Hyde Company, Lake Villa, IL, USA). The inlet line passed over a water trap maintained a few centimeters above liquid nitrogen. This was necessary to reach temperatures low enough to trap water at the low inlet pressures but not too low to freeze out carbon dioxide. The inlet line was passed into the ionizing source of a PrismaPlus quadrupole mass spectrometer (Pfeiffer Vacuum), which cycled through relevant masses for detection via an amplified faraday cup. The mass spectrometer, roughing pump, and turbo pump and time-resolved data collection software were provided by Bay Instruments (Port Easton MD, USA).

Measurements were made following a daily oxygen and carbon dioxide calibration and followed previous regimes and calculations (Canvin et al., 1980; Cousins et al., 2008; Walker & Cousins, 2013). In brief, the leaf disk was placed within the chamber and sealed. The chamber was then flushed with nitrogen gas, and ¹⁸O₂ gas was injected to reach the desired atmosphere. Rates of dark CO₂ release, ¹³O₂ uptake, and ¹⁶O₂ release/leaks were monitored for ~10 min before a custom-built LED light source was turned on. Rates of gas exchange were further monitored until a steady state was reached and the chamber was injected with a saturating volume of ¹³CO₂. Following this injection, ¹²CO₂ release from photorespiration was monitored. A CO₂ zero measurement was made before and after each experimental run by momentarily dipping the water trap into the liquid nitrogen. Time-resolved mass spectrometer data were then processed by a pipeline of in-house python scripts to apply the necessary per volt calibrations and calculate the final rates of oxygen exchange, νₒ, νₑ, and ¹²CO₂ release in a ¹³CO₂ background. All processing scripts and chamber designs are available upon request.

4.5 | Postillumination burst

The PIB of CO₂ was measured using a Li-COR-6800 as described previously. During each measurement, the leaf was illuminated at 400 μmol photons m⁻² s⁻¹ for 40 min and then darkened for 20 min. The total amount of CO₂ release during the PIB was estimated as the area of PIB peak determined from the trace of CO₂ release in the dark period after the baseline correction. The baseline was identified from the level of CO₂ release in the last 200 s of dark period.

4.6 | Metabolic response to transient increases in rubisco oxygenation

Plants were treated with a rapid increase of light intensity from 50 to 400 μmol photons m⁻² s⁻¹. Leaf tissues were collected before (time t = 0) and after the shift to high light at the indicated time points. Samples were immediately frozen in liquid nitrogen and weighed before being stored at ~80°C. Five biological replicates were
performed for each genotype. The frozen samples were ground to a fine powder using a bead-beating grinder with a sample holder containing dry ice. Metabolites were extracted with a solution of chloroform:methanol (3:7, v/v), and ribitol was used as an internal standard. After centrifugation, the supernatant was freeze dried using a lyophilizer. Dried metabolites were methoximated (20-mg ml$^{-1}$ methoxyamine in pyridine) and trimethylsilylated (MSTFA:TMS, 99:1) and then analyzed by GC–MS (Agilent 5975, GC/single quadrupole MS). GC–MS data were processed by Agilent MSD ChemStation. Metabolite derivatives were identified by comparison of the retention time with a known standard and comparison of the mass spectra with MS database. The amount of each metabolite was quantified by the total ion current signal of each metabolite peak normalized to the ribitol internal standard and tissue weight.

4.7 Measurements of folates and formate

Formate was extracted by resuspending pulverized leaf tissue (~100 mg) in 0.25 ml of 0.1-M HCl, with 10 μl of 10-mM amino butyric acid (ABA) added as an internal control. After centrifuging at 14,000 rpm for 20 min at 4°C, the supernatant was collected, and the pellet was re-extracted with 0.25 ml of 0.1-M HCl. The supernatants were combined for analysis. Formate was analyzed using a published procedure (Xie et al., 2012) with some modifications. Fifty microliter of each sample was combined with 50 μl of tetrabutylammonium bromide in acetonitrile (20 μmol ml$^{-1}$), 50 μl of triethanolamine, and 1 μl of 9-chloromethyl anthracene as a fluorescence-labeling reagent. The reaction was added up to 500 μl with acetonitrile and then was incubated at 75°C for 50 min. After centrifuging at 14,000 rpm for 10 min at 25°C to precipitate the debris, the samples were separated on the Xterra MS C$_{18}$ column (3.5 μm, 4.6 × 100 mm, Waters, MA) with mobile phase of 64% acetonitrile and 36% water at the 1.0-μl min$^{-1}$ constant rate.

Folates were extracted and analyzed as previously described (Hung et al., 2012). There were five biological replicates performed for each line for formate and folate analysis.

4.8 Measurement of hydrogen peroxide

Hydrogen peroxide concentrations were measured in leaf tissues using a H$_2$O$_2$ Assay Kit from Abcam (ab102500, Cambridge, UK). Leaf tissues were harvested and homogenized as described above for metabolic response study, except that the frozen leaf tissues were immediately used for homogenization without storing at −80°C. Three biological replicates were performed for each genotype. After extraction and centrifugation, samples were deproteinized with 4-M perchloric acid and then neutralized with 2-M KCl until pH between 6.5 and 8.0. All standards and deproteinized samples were incubated with OxiRed probe and horseradish peroxidase for 10 min at room temperature before measurements. Absorbance and fluorescence were measured with a 96-well plate reader (SpectraMax M2) at OD = 570 nm and Ex/Em = 535/587 nm.

4.9 Lipid analysis

Polar glycerolipids were analyzed as described (Wang & Benning, 2011). The Lipids were extracted from fresh leaves tissues. Polar lipids were separated on a silica-gel thin-layer chromatography plate treated with (NH$_4$)$_2$SO$_4$ and a solvent system of acetonit-tenol water (91:30:7, v/v/v). Lipid spots were visualized with brief iodine vapor staining. Individual lipids, PC, and PE were scraped off, and their fatty acid profiles were analyzed using gas–liquid chromatography. Composition is presented as a mole percentage of total fatty methyl esters detected in each lipid. Three biological samples were collected for each line.

4.10 Catalase activity

Catalase enzyme kinetics were determined on raw tissue extracts using an oxygen electrode by following the increase in oxygen production at various [H$_2$O$_2$] in a 50-mM potassium phosphate buffer, pH 8.1 to match the pH of a plant peroxisome (Rørth & Jensen, 1967; Shen et al., 2013; Switala & Loewen, 2002). The oxygen electrode temperature was set to 25°C via a recirculating water bath.

4.11 Determining rate constants and order of nonenzymatic reactions

The reaction between H$_2$O$_2$ and either glyoxylate or hydroxypyruvate was measured using UV spectroscopy at 240 nm in a quartz reaction cuvette at 25°C (Yokota et al., 1985) in a 50-mM potassium phosphate buffer, pH 8.1 to match the pH of a plant peroxisome. Because both glyoxylate and hydroxypyruvate also absorb at 240 nm, the absorbance drop attributed only to H$_2$O$_2$ decay was corrected by also accounting for the extinction coefficients of either glyoxylate (14 L mol$^{-1}$ cm$^{-1}$) or hydroxypyruvate (188.9 L mol$^{-1}$ cm$^{-1}$).

ACKNOWLEDGMENTS

We thank Marion Eisenhut for valuable discussions on photorespiration during the initial periods of planning this research. We additionally thank Ron Cook and Christoph Benning for help with the lipid analysis. We also thank Dr. Graham Noctor for providing the cat2 seeds. B.J.W. was funded under a postdoctoral research fellowship from the Alexander von Humboldt Foundation during the initial experimental work. This material is based upon work supported by the Division of Chemical Sciences, Geosciences and Biosciences, Office of Basic Energy Sciences of the US Department of Energy (DE-FG02-91ER20021) and National Science Foundation (2030337 and 2015843). A.P.M.W. was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany’s Excellence Strategy—EXC-2048/1—Project ID: 390686111, and IRTG 2466. S.R. was funded under Division of Molecular and Cellular Biosciences (2015828).
CONFLICT OF INTEREST
The Authors did not report any conflict of interest.

AUTHOR CONTRIBUTIONS
B.J.W. conceived the original research plans and supervised the research with input from A.P.M.W., H.B., M.M., and S.R. B.J.W. wrote the paper. Further experimental work and analysis was performed by W.R. and S.S.

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