



Sudden elevation of carbon dioxide concentration causes perturbation of the electron transport chain and triggers defense responses in *Arabidopsis thaliana*

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Received: 7 November 2025 / Accepted: 16 February 2026 / Published online: 16 March 2026
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Abstract

Main conclusion *Arabidopsis* wildtype plants suffer symptoms of stress at a sudden increase in CO₂ concentration, resulting from perturbation of photosynthetic electron transport. Defense-related gene induction includes increased methionine cycle and glucosinolates metabolism.

Abstract Elevated CO₂ (eCO₂) increases photosynthetic performance of plants, but also leads to decreased nitrogen-to-carbon ratio and a long-term decline in photosynthetic activity, known as photosynthetic acclimation. It is unclear whether initially increased CO₂ assimilation or perturbation of the physiological homeostasis triggers acclimation. Here, we used a combination of omics analysis to investigate immediate (1 day) and delayed (7 days) responses of plants to rising atmospheric CO₂, thus allowing us to discriminate regulatory from metabolic effects. Responses of wildtype *Arabidopsis* plants, Columbia-0, were compared to those of the *hpr1-1* mutant of peroxisomal hydroxy-pyruvate reductase that has reduced photorespiratory turnover at ambient CO₂. Comparisons enabled separating the impact of eCO₂ (1000 ppm) on increased carbon assimilation from that of reduced photorespiration. While both genotypes had elevated sugar levels at eCO₂, the wildtype displayed symptoms of stress that were accompanied by perturbation of the photosynthetic electron transport chain. These were consistent with physiological parameters, including non-photochemical quenching and chlorophyll fluorescence. The induction of defense-related mechanisms was tightly associated with increased sulfate assimilation, methionine cycle activity and glucosinolates metabolism, all being early responses of the wildtype to eCO₂. Transcriptome data pointed to hexokinase1 as a central regulatory hub in orchestrating these responses. In contrast, eCO₂ enabled the *hpr1-1* mutant to metabolically align with the wildtype. Results offer new interpretations of how the impairment of carbon and nitrogen recycling is compensated in the *hpr1-1* mutant.

Keywords *Arabidopsis thaliana* · Elevated carbon dioxide · FvCB model · Omics analysis · Photorespiration · Photosynthesis · Subcellular metabolism

Abbreviations

a/eCO₂ Ambient/elevated carbon dioxide
 C_i Intercellular carbon dioxide

FvCB Farquhar-von Caemmerer-Berry
 HPR Hydroxypyruvate reductase
J Linear electron transport rate
*J*_{max} Maximum electron transport rate

Communicated by Dorothea Bartels.

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NPQ	Non-photochemical quenching
RuBP	Ribulose-1,5-bisphosphate
TP0 (1, 7)	Harvest at day 0 (1, 7) of eCO ₂ exposure
V_{cmax}	Maximum rate of carboxylation
Φ_{PSII}	Photochemical yield of PSII during light adaptation

Introduction

Industrialization has led to an increasing atmospheric CO₂ concentration that is predicted to reach 800 to 1000 ppm by the end of the century (IPCC 2021). It is known for a long that long-term exposure of plants to eCO₂ causes a decline in the maximum rate of carboxylation (V_{cmax}) of ribulose-bisphosphate carboxylase/oxygenase (Rubisco) and thus photosynthesis (Ainsworth and Long 2005; Ainsworth and Rogers 2007). However, the extent to which this depends on physiological (e.g. stomatal conductance), morphological (canopy traits) or biochemical (e.g. enzyme activity) parameters is still under debate (Krämer et al. 2022b). One obvious effect is an increased carbon-to-nitrogen ratio that may result from (i) high CO₂ levels stimulating carbohydrate accumulation, thus causing a dilution of N-containing compounds (Chen et al. 2005; Krämer et al. 2022a) and (ii) elevated internal CO₂ concentrations that lead to decreased stomatal conductance, causing low transpiration rates that limit mineral uptake and transport (Ainsworth and Rogers 2007; Shi et al. 2021). An additional factor could lie in the reduction of photorespiration at eCO₂ (Krämer et al. 2022b). The photorespiratory pathway starts from oxygenation of ribulose-1,5-bisphosphate (RuBP) and is, therefore, diminished at eCO₂.

Oxygenation of RuBP produces phosphoglycolate, which inhibits enzymes of the Calvin-Benson-Bassham Cycle (CBBC) and must be detoxified. After dephosphorylation, glycolate is exported from plastids and converted to the amino acid glycine in peroxisomes. In the mitochondria, two molecules of glycine are converted to serine which is transported back to the peroxisomes, where it is deaminated and reduced to glycerate. Following transfer to plastids and phosphorylation, phosphoglycerate re-enters the CBBC. The photorespiratory pathway is an important source for serine and glycine in leaves of C₃-plants and the reduction of its turnover may thus affect the whole amino acid metabolism (Friedrichs et al. 2024).

While impacts on N-metabolism are obvious, it has also been reported that photorespiration affects sulfate assimilation. Abadie and Tcherkez (2019) reported a significant enrichment of ³³S labeling of cysteine under photorespiratory conditions and a significant increase in ³³S-methionine and ³³S-cysteine content at high oxygen levels in sunflowers. It is unclear whether this effect depends on the accumulation

of serine, which is the substrate for cysteine synthesis. Li et al. (2008) reported that at eCO₂ serine levels dropped in both *Arabidopsis* accessions, Col-0 and Cvi-0, while cysteine did not change and methionine showed accession-specific effects. In C₄ plants (where photorespiration is suppressed) of the genus *Flaveria*, sulfur assimilation is preferentially localized to roots, because serine production occurs via the phosphorylated pathway mainly in the roots.

The complexity of interactions of C, N and S metabolism is further increased by the fact that plant metabolism is highly compartmentalized, which might result in metabolite levels that are not assessable in whole-cell extracts. In addition to that, isoforms of many enzymes exist in the different compartments, resulting in a redundancy of various metabolic pathways. For example, glycolysis and the oxidative pentose phosphate pathway exist in plastids and cytosol, and synthesis of various amino acids is located in the cytosol, plastids, as well as mitochondria. To disentangle overlapping reactions, mutants lacking specific isoforms of enzymes are frequently utilized that disrupt metabolite conversion in a specific compartment. While this strategy allowed insights into the complex interactions of metabolic pathways, knowledge is still limited due to the high intracellular mobility of metabolic intermediates, and also because of the so-called pleiotropic effects of mutations, which are unexpected based on current knowledge of the functions of the mutated genes (Serrano-Mislata et al. 2017).

Disruption of photorespiration by mutation of essential genes is lethal in C₃ plants at ambient CO₂ (Bauwe 2023) and only very few mutants exist that are severely disturbed in the photorespiratory pathway but can still grow at ambient CO₂. One of these is the *hpr1-1* mutant (Timm et al. 2008), which is deficient in peroxisomal hydroxypyruvate reductase, the second last step in photorespiration before phosphoglycerate re-enters the Calvin-Benson-Bassham Cycle (CBBC). Two other isoforms exist, located to the cytosol and plastids, respectively, and, although mutation of all three isoforms has additive effects, a triple mutant is still viable (Timm et al. 2011). This raises the question whether other pathways exist to detoxify the phosphoglycolate produced upon oxygenation of RuBP.

In the present study, we aimed at disentangling the effects of eCO₂, on the one hand linked to increased CO₂ fixation, and on the other hand caused by reduced photorespiration. To gain insight into early events of regulation, we exposed plants grown at ambient CO₂ (450 ppm) to suddenly elevated (1000 ppm) CO₂ and recorded responses of transcriptome, proteome and subcellularly resolved metabolome over a time course of seven days. This revealed that eCO₂, which provides elevated substrate concentration for photosynthesis, caused symptoms of stress in wild-type plants by perturbing the balance of photosynthetic electron transport. In contrast, it led to a relaxation of stress symptoms in a mutant with

reduced photorespiratory capacity. Comparison of the two responses yielded new insights into how plants cope with different atmospheric CO₂ levels.

Materials and methods

Plant material and growth condition

Arabidopsis thaliana (L.) Heynh. Col-0 as wildtype, and the *hpr1-1* mutant (SALK067724) were used in this study. All plants were grown in fully controlled growth chambers at Stuttgart University for six weeks in soil (mixture of black peat, white peat and coconut macaroon, Art.-Nr 455,433, Klasmann-Deilmann GmbH, Geeste, Germany) under ambient carbon dioxide (aCO₂, 450 ± 20 ppm) and short day (8-h light/16-h dark, 100 μmol m⁻² s⁻¹, 60% relative humidity, temperature 22/16 °C) in order to prevent floral induction. Light was provided by a composition of six LED creating the spectrum shown in Fig. S1. Plants were fertilized with each watering (4% N, 4% P, 3% K fertilizer WUXAL, Hauert MANNA Düngerwerke GmbH, Nürnberg, Germany). After 6 weeks, three biological replicates per genotype, consisting of pools of three plants each (9 plants in total per genotype), were harvested (day 0, aCO₂, “TP0”), and the double number of plants exposed to elevated CO₂ (1000 ± 20 ppm over the full diurnal cycle) at otherwise identical environmental settings. Consecutive sampling was done at days 1 (“TP1”) and 7 (“TP7”), each time 4 h into the light phase at the middle of a short day. Thus, for each genotype, three biological replicates were harvested, each consisting of a pool of three full rosettes (3 replicates per genotype and time point). Aliquots of fresh plant material were prepared for Proteomics and Transcriptomics analysis, and the rest subjected to lyophilization (Heto PowerDry LL3000; Thermo Electron C., Schwerte, Germany) for subcellular fractionation.

RNA extraction and RNA sequencing

100 mg of homogenized plant material of three replicates per genotypes (Col-0, *hpr1-1*) and condition (aCO₂, eCO₂ 1 d and 7 d) was used for RNA extraction. Total RNA was isolated using a single-step Trizol-based method (Zuther et al. 2019). DNase treatment was carried out on 4 μg of RNA (RapidOut DNA-removal Kit, Thermo Fisher Scientific). DNA removal was validated by qPCR using intron-specific primers (AT5G65080) (Zuther et al. 2012). RNA integrity and quality were validated with an Agilent 2100 Bioanalyzer (Agilent Technologies). Library preparation and RNA sequencing (RNA-Seq) were carried out at Genewiz-Azenta Life Sciences (Leipzig, Germany; <https://www.genewiz.com/>). Library preparation and sequencing were performed according to the Genewiz-Azenta workflow.

RNA-seq analysis

Paired-end RNA sequencing data for all 60 samples (35–164 million reads per sample) were checked for quality using FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>, version 0.11.8), and trimmed using the CLC Genomics Workbench (<https://www.qiagen.com/>, version 22.0.2). Using the CLC Genomics Workbench, reads were mapped to the reference transcriptome, Col-0, as obtained from TAIR10 (https://www.arabidopsis.org/download/list?dir=Genes%2FTAIR10_genome_release/) and processed to yield quantitative expression values. On average, 97.18% of the reads mapped uniquely to transcripts, yielding quantitative information for 31,838 transcripts associated with 24,231 genes.

Proteome analysis by mass spectrometry

Protein extraction and trypsin digestion were carried out following the protocol of Marino et al. (2019). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was conducted as previously described, with peptides separated over a 90-min linear gradient of 5–80% (by vol.) acetonitrile (Espinoza-Corral et al. 2023). Raw data files were processed using MaxQuant software version 2.4.14.0 (Cox et al. 2014). Peak lists were searched against the *Arabidopsis* reference proteome (Uniprot, <https://www.uniprot.org/>) using default settings, with the ‘match-between-runs’ feature enabled. Protein quantification was performed using the label-free quantification (LFQ) algorithm (Cox et al. 2014). Subsequent analysis was executed using Perseus version 2.0.11 (Tyanova et al. 2016). Potential contaminants, proteins identified only by site modification, and reverse hits were excluded from further analysis. LFQ intensities were log₂-transformed, and missing values were imputed from a normal distribution using Perseus with standard settings.

Non-aqueous fractionation (NAF)

Subcellular fractionation of lyophilized plant samples by NAF procedures was performed as earlier described (Fürtauer et al. 2019; Hoermiller et al. 2022). Briefly, approximately 80–100 mg of lyophilized leaf homogenate were suspended in 10 ml of ice-cold mixture of tetrachloroethylene-heptane (solvent A, ρ = 1.36 g cm⁻³) and sonified on ice bath for intervals of 5 s with 15 s pauses over a total time course of 12 min (Branson Sonifier 250; Branson, Danbury, Connecticut, USA; output control 3). Under constant cooling, sonified homogenate was sieved through nylon gauze, pore size 30 μm, and the filtrate was centrifuged for 10 min at 2350 g, 4 °C. The supernatant was discarded and the pellet re-suspended in 1.5 ml of fresh cold solvent A, subsequently loaded onto the ice-cold non-aqueous linear gradient

combination of organic solvents initiated by solvent A, and ending up to pure tetrachloroethylene ($\rho = 1.6 \text{ g cm}^{-3}$). Gradients were subjected to ultracentrifugation (Optima™ L-90 K; Beckman Coulter, Krefeld, Germany) for 3 h at 121,000 g, 4 °C. Fractionation of centrifuged gradient was performed into nine 1 ml fractions and each fraction aliquoted to 5 equal sub-fractions and immediately dried under vacuum condition for subsequent metabolite and marker enzyme analysis. Alkaline pyrophosphatase served as plastidial marker, UGPase as cytosolic marker, succinyl-CoA-synthetase as mitochondrial marker, and acid phosphatase as vacuolar marker enzyme, as described earlier (Knaupp et al. 2011; Fürtauer et al. 2019; Hoermiller et al. 2022).

Metabolite profiling

For metabolite analysis, one aliquot of NAF and a respective whole cell sample was subjected to carbohydrate determination by HPLC (Dionex ICS 6000, Thermo Fisher Scientific), yielding concentrations of glucose, fructose, sucrose and raffinose as described by Friedrichs et al. (2024). Briefly, extraction was performed in 80% ethanol at 80 °C, followed by vacuum drying. Dried extracts were re-suspended in Honeywell chromatographic grade water (Honeywell Specialty Chemicals, Seelze, Germany) and subjected to HPLC analysis on Dionex CarboPac PA1 BioLC column (4×250 mm, Thermo Fisher Scientific). Carboxylic acids, including pyruvate, malate, fumarate and citrate, as well as minerals (nitrate, phosphate, sulfate), were quantified by anion-exchange chromatography using another aliquot of samples as described by Friedrichs et al. (2024). Briefly, extraction was done in 1 ml of 55 °C Honeywell water and incubation at 95 °C, followed by separation on Dionex IonPac AS11-HC RFIC column (4×250 mm, Thermo Fisher Scientific).

Amino acid measurements were performed by quantitative GC-MS/MS as described (Molnar-Perl and Katona 2000; Sobolevsky et al. 2003; Guo et al. 2015) with a few modifications using a third sample aliquot. Basically, a modified solid phase extraction of amino acids was performed by suspending NAF samples in 1 ml of 10 mM HCl and 10 min shaking at RT, followed by 2 min centrifugation at 14,000 g, RT. Hundred microliters of supernatant in addition to 10 nmol of norvaline (Acros Organics, Geel, Belgium) as internal standard were subjected to amino acid purification, using homogenized suspension of 100 mg ml⁻¹ of ion-exchange resin (AmberChrom™, Dowex 50WX4, 200–400 mesh, Merck) in 10 mM HCl, incubated for 15 min at RT in Mobicol spin classic tubes equipped with 10 µm pore-sized filters (MoBiTec GmbH, Göttingen, Germany), followed by centrifugation 5 min at 1000 g, RT. Resin was washed twice by 80% methanol and 1 min centrifugation each time at 1000 g, RT, to remove non-amino acid metabolites. Subsequently, resin containing purified amino acids

was suspended in 150 µl of 1:1 mixture of methanol and 8 M ammonia and centrifuged for 2 min at 5000 g, RT. Eluent, containing purified amino acids, was dried in a speed vacuum concentrator (ScanSpeed 32, Lynge, Denmark). Upon drying, derivatization was done by adding 50 µl of both MTBSTFA (Sigma Aldrich) and acetonitrile, 1 h incubation at 95 °C followed by 2 h, at RT and subsequent analysis by GC-MS/MS (TQ8040, Shimadzu). One microliter of the derivatized samples was injected to device, applying helium as carrier gas at a flow of 1.12 ml min⁻¹. Stationary phase was a 30 m Optima 5MS-0.25 µm fused silica capillary column. Ion source, column oven and injection temperature were set up at 250 °C, 100 °C and 250 °C, respectively. A split 10 gradient program was applied with an initial column temperature of 100 °C for 1 min, followed by a 15 °C increment per minute till 290 °C, holding for 3 min, again followed by the same increment rate to reach the final temperature of 330 °C and holding for 10 min. Subsequent to 5 min solvent delay, the spectra of MS device were recorded in Q3 scanning mode.

All other metabolites including carbohydrates, sugar alcohols, carboxylic acids and polyamines were measured using the last remaining sample aliquots by GC-MS/MS as described earlier (Krämer et al. 2022b). Briefly, extraction on ice into methanol-chloroform-water mixture, 2.5:1:0.5 (by vol.) was followed by centrifugation. Polar phase was separated and along with norvaline as internal standard, dried in a speed vacuum concentrator equipped with a cold trap (ScanVac, Lillerød, Denmark). After drying, methoximation was performed using 20 µl of methoxamine hydrochloride (Sigma Aldrich) dissolved in pyridine (40 mg ml⁻¹) by 90 min incubation at 30 °C followed by silylation, using 80 µl of MSTFA (Sigma Aldrich) and 30 min incubation at 50 °C. Detection was done by the same GC-MS/MS device as amino acids and a different split 10 gradient program as 70 °C for initial temperature and one min holding, followed by 15 °C increment per minute till reaching to final 330 °C, and holding for 10 min. Ion source, column oven and injection temperature were set up at 250 °C, 70 °C and 230 °C, respectively, and after 4.7 min solvent delay, spectra were recorded in Q3 scanning mode.

Gas exchange measurements

Diurnal photosynthesis measurements were conducted as described previously by Krämer et al. (2022b). Briefly, an infrared gas analyzer (Uras 3G; Hartmann and Braun AG, Frankfurt am Main, Germany) was utilized. The system operated at a flow rate of 40 l h⁻¹, and each channel was measured sequentially for 6 min across a complete diurnal cycle at a frequency of 0.1 Hz. Spline interpolation was applied to the recorded time points to reconstruct a continuous profile of photosynthetic activity. All measurements

were performed under the same growth light and CO₂ conditions. Dark respiration (R_d) was obtained as an average over the entire dark phase, subtracted for the first five minutes of dark phase. The CO₂ compensation point in the absence of mitochondrial respiration (I^*) was determined following Krämer et al. (2022b).

Instantaneous stomatal conductance for CO₂ (G_{CO_2}), assimilation rate (A) and intercellular CO₂ (C_i) were measured simultaneously by GFS-3000 portable gas exchange fluorescence system (Heinz Walz GmbH, Effeltrich, Germany). Briefly, a standard measuring head, equipped with Arabidopsis chamber 3010-A, was mounted, and all environmental parameters were adjusted as growth condition, including light intensity, relative humidity, temperature and CO₂ concentration. To avoid turbulences of real-time measurements a flow rate of 700 $\mu\text{mol min}^{-1}$ and an impeller speed 6 were selected. The same leaf area of individual replicates and measuring intervals of 5 s over 1 min time courses were considered. Device calibration was performed before starting measurements and at least 30 min of acclimation period were considered for the chamber and sensors to the new adjusted parameters. Collecting data started right after stabilizing parameters. Intercellular CO₂ according to Von Caemmerer and Farquhar (1981) was calculated by the device according to Eq. 1:

$$C_i = \frac{\left(G_{CO_2} - \frac{E}{2}\right) \times C_a - A}{G_{CO_2} + \frac{E}{2}} \quad (1)$$

whereby C_i is intercellular CO₂ mole fraction (ppm), G_{CO_2} is stomatal conductance for CO₂ ($\text{mmol m}^{-2} \text{s}^{-1}$), E is transpiration rate ($\text{mmol m}^{-2} \text{s}^{-1}$), C_a is CO₂ mole fraction in the chamber (ppm) and A is assimilation rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$).

Chlorophyll fluorescence

Chlorophyll fluorescence imaging was performed as described by Hoermiller et al. (2022) using a FluorCam FC 1000-H system (Photon Systems Instruments, Brno, Czech Republic) operated in pulse-amplitude-modulated mode (PAM). Plants were dark-adapted for at least 10 min under growth conditions prior to measurements. Right after dark adaptation, within the minimal fluorescence (F_0) measurement mode, weak far-red illumination was applied by the device automatically to fully oxidize the plastoquinone pool, allowing reliable determination of F_0 .

Fluorescence was excited using the system's internal low-intensity modulated red measuring light (measuring beam). Kautsky induction was initiated by continuous actinic illumination corresponding to 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, as independently verified using a quantum sensor and matching the growth irradiance. Fluorescence induction was recorded

for 120 s. During actinic light exposure, five saturating light pulses for 800 ms duration were applied to determine maximal fluorescence in the light-adapted state (F_m') and to ensure attainment of steady-state photosynthesis. Saturating pulses were empirically verified to fully close PSII reaction centers, as indicated by stable maximal fluorescence signals at steady state level. The measuring beam consisted of low-intensity, modulated red light integrated within the imaging system, fixed by the manufacturer and did not induce detectable actinic effects. After Kautsky induction, a relaxation phase of 20 s was recorded. According to the global PAM mode of FluorCam, 10 μs of shutter frequency and 30% sensitivity was set. Fluorescence parameters were averaged over the whole rosette area using at least eight different regions per leaf. Five biological replicates were analyzed per genotype and CO₂ treatment.

Accordingly, non-photochemical quenching (NPQ) and the maximum quantum efficiency of PSII (F_v/F_m) were determined from chlorophyll fluorescence measurements to assess photoinhibition. According to Eq. 2, the photochemical yield of PSII during light adaptation (Φ_{PSII}) was calculated based on fluorescent measurements and subsequently used to estimate the linear electron transport rate (J), according to Eq. 3:

$$\Phi_{PSII} = (F_m' - F_s')/F_m' \quad (2)$$

$$J = \alpha \times \beta \times PPFD \times \Phi_{PSII} \quad (3)$$

where $\alpha = 0.84$ and $\beta = 0.5$, are representing the leaf absorbance and the fraction of the absorbed irradiance that reaches PSII and PPFD represents the incident photosynthetic photon flux density corresponding to the growth condition, following Pons et al. (2009).

Combining gas-exchange measurements with chlorophyll fluorescence-based estimates of linear electron transport rate, chloroplastic CO₂ concentration (C_c) was estimated according to Eq. 4 following Harley et al. (1992). The resulting C_c values were then used to estimate mesophyll conductance (g_m) based on Fick's law (Eq. 5), as described by Kromdijk et al. (2020):

$$C_c = \frac{\Gamma^* \cdot [J + 8(A + R_d)]}{[J - 4(A + R_d)]} \quad (4)$$

$$g_m = A/(C_i - C_c) \quad (5)$$

Biochemical modeling

The CO₂ dependence curve of photosynthesis ($A-C_i$) was fitted using the Farquhar-von Caemmerer-Berry (FvCB) model as implemented in the *plantecophys* package in R according to Farquhar et al. (1980) and Duursma (2015), assuming net assimilation rate was given according to Eq. 6:

$$A = V_c \cdot (1 - 0.5\phi) - R_d \quad (6)$$

where ϕ is representing the ratio of oxygenation (V_o) to carboxylation (V_c) of Rubisco and defined according to Eq. 7:

$$\phi = 2\Gamma^*/C_c \quad (7)$$

Rearrangement of Eq. 6, following Von Caemmerer (2000), allowed identification of V_c and V_o , and subsequently NADPH and ATP consumption rates were estimated according to Eq. 8 and 9, respectively:

$$\text{NADPH consumption rate} = (2 + 2\phi) \cdot V_c \quad (8)$$

$$\text{ATP consumption rate} = (3 + 3.5\phi) \cdot V_c \quad (9)$$

Average of assimilation rates obtained from seventeen biological replicates at ten given C_i set points were used as fixed input for fitting the FvCB model, which was further constrained by estimated mesophyll conductance and the Γ^* values of the corresponding genotype. The Michaelis–Menten constants of Rubisco for CO_2 (K_c) and O_2 (K_o), at 25 °C were set to 404.9 $\mu\text{mol mol}^{-1}$ and 278.4 mmol mol^{-1} , respectively, and were further temperature-corrected (f) for given leaf temperature (T_{leaf}) using Arrhenius functions according to Eq. 10:

$$f = f(25^\circ\text{C}) \cdot \exp[E_a (T_{\text{leaf}} - 298)/(298RT_{\text{leaf}})] \quad (10)$$

whereby the activation energies (E_a) for K_c and K_o were 79.43 and 36.38 kJ mol^{-1} , respectively. A universal gas constant (R) of 8.314 $\text{J mol}^{-1} \text{K}^{-1}$ and a constant intercellular O_2 concentration (O) of 210 mmol mol^{-1} were assumed (Farquhar et al. 1980; Ishikawa et al. 2007).

Following Von Caemmerer (2000), to define whether net photosynthetic CO_2 assimilation was limited by Rubisco (A_c) or by electron transport (RuBP regeneration, A_j) according to Eq. 11, the V_{cmax} and maximum electron transport rate (J_{max}) were estimated by fitting the FvCB model and subsequent calculation of A_c and A_j according to Eqs. 12 and 13:

$$A_n = \min\{A_c, A_j\} \quad (11)$$

$$A_c = \frac{V_{\text{cmax}}(C_c - \Gamma^*)}{C_c + (K_c(1 + O/K_o))} - R_d \quad (12)$$

$$A_j = \frac{J(C_c - \Gamma^*)}{4C_c + 8\Gamma^*} - R_d \quad (13)$$

Triose phosphate utilization (TPU) limitation of the assimilation rate (A_p) was not estimated, because TPU limitation was not detected within the measured C_i range according to the model fit; therefore, A_p was not considered further as a limiting factor in this study.

Eventually, RuBP saturated carboxylation rate (W_c) under competitive inhibition by O_2 was calculated according to Eq. 14 and compared to electron transport limited rate of RuBP regeneration (W_j) given by Eq. 15 (Farquhar et al. 1980). This comparison was used to identify the dominant biochemical limitation of photosynthesis at given C_i levels and to evaluate its accordance with physiological and omics-based data.

$$W_c = \frac{V_{\text{cmax}} C_c}{C_c + (K_c(1 + O/K_o))} \quad (14)$$

$$W_j = \frac{J_{\text{max}} C_c}{4C_c + 8\Gamma^*} \quad (15)$$

Phloem exudation

In order to quantify loading of carbohydrates into the phloem sap, an exudation experiment was performed according to Tetyuk et al. (2013). Five biological replicates per genotype, consisting of pools of 3 leaves from different plants, were exuded. Leaves were first treated for 1 h with 20 mM $\text{K}_2\text{-EDTA}$ to suppress callose formation, and then gently washed with ddH_2O to remove remaining EDTA. Leaves were left in 1.8 ml water for exudation under the same light intensity, photoperiod and CO_2 conditions. For eCO_2 condition, plants which were incubated for 1 d and 7 d at eCO_2 were used. Exudates were collected for each CO_2 condition 4 h into the light phase at the middle of a short day. Carbohydrate quantification in phloem exudates was performed as described in the metabolite profiling section above, and by HPLC.

Data evaluation, statistics and figure illustration

Gene Ontology Term Enrichment Analysis (GO term analysis) was performed with a significance threshold of $P < 0.01$ using PlantRegMap (<https://plantregmap.gao-lab.org/>) following Tian et al. (2020). Protein–protein interactions were evaluated using the String database for functional protein association networks (<https://string-db.org/>); all active interaction sources were allowed, setting the minimum required interaction score to highest confidence, 0.9). Statistics and plots generation were performed in python (Version 3.11.5), Matlab (<https://de.mathworks.com/>) and R (version 4.5.1). Regarding the Col-0 metabolite profile, Tukey's HSD was applied following one-way ANOVA, as this PostHoc test is well-suited for controlling the family-wise error rate when evaluating pairwise comparisons in a single-factor design. However, for *hpr1-1* vs Col-0 metabolite profiles, Bonferroni correction was employed following two-way ANOVA,

because specific, planned genotype-wise comparisons at each time point were performed, and Bonferroni correction provides a more conservative adjustment appropriate for a limited number of targeted comparisons within a multifactorial framework. In order to calculate the significance of $\log_2FC$ (fold-change) values, a one-tailed z -test was performed after calculating the geometric mean and the propagation of error as suggested by Quackenbush (2002). In addition, the P -value was adjusted for multiple testing using Bonferroni correction. All statistical tests were conducted with $\alpha = 0.05$. Figures 2a, 5a and 6a were illustrated using BioRender (<https://www.biorender.com/>).

Results

Early responses to eCO_2 at the transcriptome and proteome level

Upon shift to eCO_2 after six weeks of growth at aCO_2 , Col-0 and *hpr1-1* responded immediately, but partially contrary, at the level of transcripts, proteins and metabolites (Fig. 1). Loadings of a Principal Component Analysis (PCA) for transcripts (Fig. 1a) revealed a conserved effect after 7 days at eCO_2 along PC2 but no convergence. A GO term enrichment of the top 1000 genes along this trajectory showed that transcripts were predominantly related to defense and stress-response mechanisms (Table S1). In contrast, the immediate response at day 1 of eCO_2 showed a clear separation of *hpr1-1* from Col-0 along PC1, which comprised enrichment of transcripts for cellular, developmental and metabolic processes (Table S1). Interestingly, the distance between the two genotypes decreased after 7 days at eCO_2 in all omics data (Fig. 1a–c). Full lists of loadings of PCA for transcripts, proteome and metabolites and also their GO term enrichments are available as Table S1, respectively.

It is known for a long that eCO_2 causes accumulation of soluble sugars, which in turn trigger down-regulation of photosynthetic genes through sugar sensing by hexokinase 1 (*HXK1*) (Cheng et al. 1998). In agreement with that, upon a shift to eCO_2 and significant accumulation of soluble sugars, we observed induction of the sugar sensor *HXK1*, and down-regulation of carbonic anhydrase (*CA2*) and light harvesting complex2 (*LHCB2.4* and *LHCA4*) at both, transcript and proteome, level in Col-0 (Fig. 2a,b; C1vsC0_T/P). Interestingly, we also found the electron transport chain (ETC) to be affected with down-regulation of both genes encoding plastocyanin (*PC*) (*DRT112* and *PETE*) and the Rieske iron-sulfur protein of *Cytb6f* (*PETC*) (Fig. 2a,b) that transfers electrons from plastoquinol to *Cytb6f* (Knight et al. 2002). While a trimeric complex composed of *HXK1*, *VHA-B1* and *RPT5B* in response to elevated sugars is responsible for suppressing *LHCB2*, the details of the regulatory mechanism

on *PC* and *Cytb6f* genes are not yet fully understood. This prompted us to examine primary reactions of photosynthesis and components of the redox balance. Figure 2c shows analysis of chlorophyll fluorescence, demonstrating that NPQ, i.e. dissipation of excess energy as heat, increased immediately after transfer to eCO_2 , in line with induction of the violaxanthin de-epoxidase *VDE1* that converts the antenna pigment violaxanthin into protective zeaxanthin (Fig. 2a,b). Along with a significant increase in F_0 and decreased F_v/F_m , photochemical quenching (qP) and fluorescence decline ratio (R_{fd}) were reduced (Fig. 2c). A significant decrease in Φ_{PSII} was accompanied by a reduction of the estimated linear electron transport rate and also the NADPH and ATP consumption rate upon shift to eCO_2 (Fig. 2c). Further indications of damage to PSII were obtained, such as evidence for plastoquinone pool over reduction, photoinhibition and oxidative stress. For example, plastidial superoxide dismutase (*SOD3* and *FSD1/2*) and ascorbate peroxidases (*SAPX* and *APX4*) were all induced at the transcript level, clearly pointing to a disturbed redox balance as early response to eCO_2 in Col-0 (Fig. 2b). In accordance with that, the gene for NADP-dependent malic enzyme that decarboxylates malate to pyruvate, producing NADPH, (*NADP-ME4*) was induced as well as several genes of the oxidative pentose-phosphate cycle (*G6PD1*, *G6PD3* and *PGD1/2/3*), and isocitrate-dehydrogenase (*ICDH* and *CICDH*), which are additional sources of reducing equivalents (Fig. 2b).

One of the pathways immediately up-regulated at the transcript level upon shift to eCO_2 in Col-0 was sulfur assimilation, with genes for ATP-sulfurylase 1 and 4 (*ATPS1/4*), APS reductase 1 (*APR1*) and sulfite reductase (*SIR*) being significantly induced in Col-0 at day 1 (Fig. 2b).

Evidence from transcript data pointed to a shift of serine biosynthesis from the photorespiratory to the plastidial phosphorylated pathway (PPSB), supported by induction of the genes for plastidial glycine dehydrogenase (*PGDH1/2/3*), phosphoserine aminotransferase (*PSAT1*) and serine hydroxymethyl transferase 3 (*SHM3*). The PPSB is tightly coupled to sulfur assimilation, and accordingly, genes for plastidial cysteine synthesis, i.e. serine acetyltransferase (*SAT1*), cysteine synthase (*OASB*), cystathionine- γ -synthase (*CGS1*) and cystathionine- β -lyase (*CBL*) as well as methionine synthase 3 (*MS3*) were all induced at eCO_2 . In addition to that, genes involved in aliphatic glucosinolate metabolism (*BCAT4*, *MAM1/3*, *CYP79F1*, *CYP83A1*, *SUR1* and *SOT16/18*) were also induced (Fig. 2b).

Genes involved in the Shikimate pathway (*DHS1/2*, *EPSP*, *EMB1144/3004* and *DHQS*), as well as in subsequent aromatic amino acid metabolism (mainly tryptophan; *ASA1/2*, *PAT1* and *TSA/B1*), were also up-regulated and play important roles in providing substrates for indolic glucosinolate metabolism, of which additional transcripts

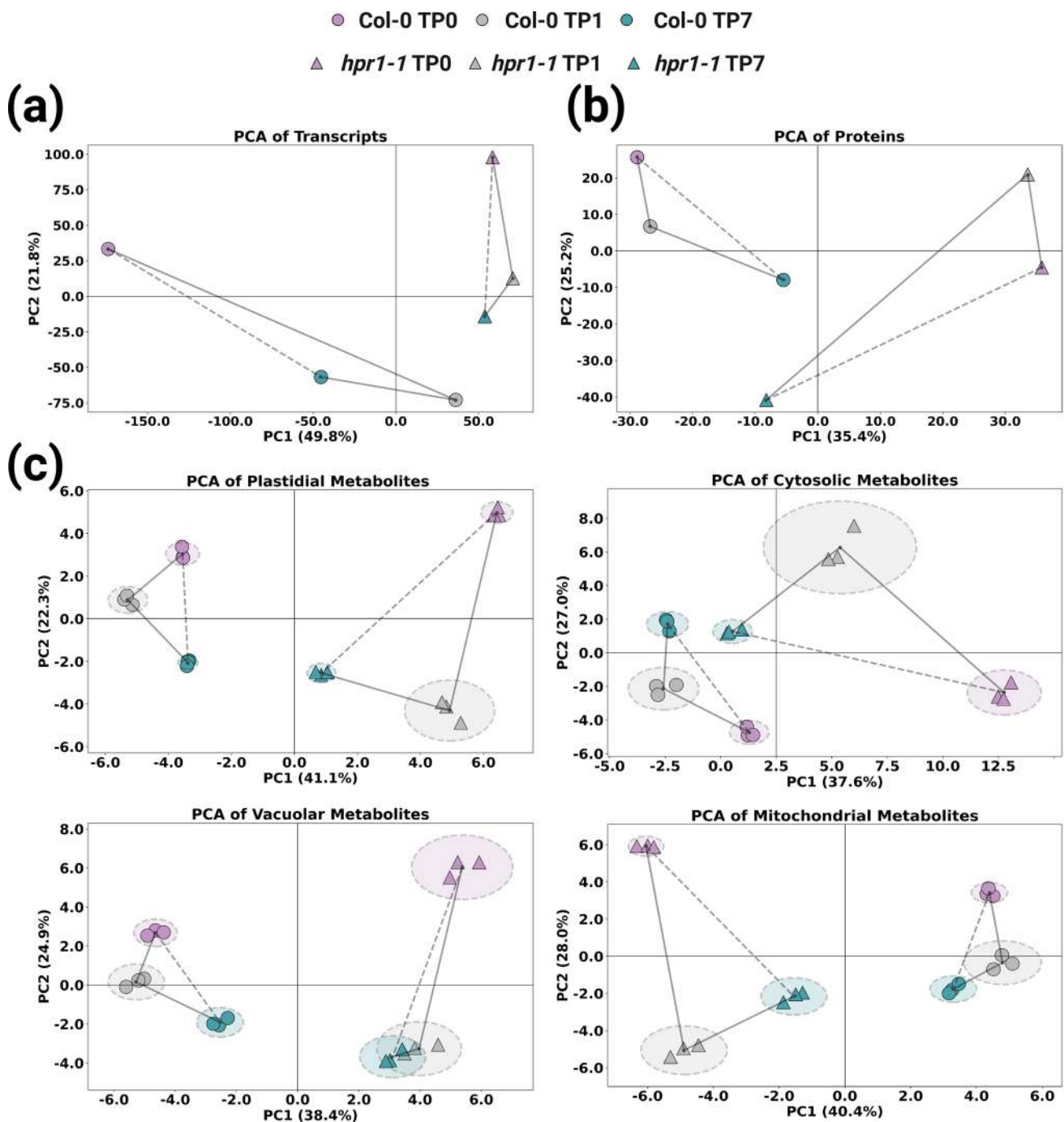


Fig. 1 Dynamics of transcriptomes, proteomes and subcellular metabolomes under elevated CO₂. **a** Principal component analysis (PCA) of mean transcript levels ($n=3$). **b** PCA of mean protein levels ($n=3$). **c** PCA of subcellular metabolites with individual replicates ($n=3$). Colors indicate duration of eCO₂ exposure (purple: 0; gray: 1; green: 7 days) and shapes indicate genotypes (filled circles: Col-0;

filled triangles: *hpr1-1*). Trajectories of full-time series are indicated by solid lines, trajectories from 0 to 7 d eCO₂ are indicated by dashed lines. Semi-transparent dashed ellipses in **c** represent variation of metabolite levels (± 1 SD), scaled for visibility. Full data sets of loadings of every PC and GO enrichment are provided in Table S1

were up-regulated (*CYP83B1* and *CYP79B2*). Besides that, genes involved in salicylic acid synthesis (*ICS2* and *GDG1*) and jasmonic acid synthesis (*LOX2/3/4/6*, *4CLL5*, *ACX1*, *AIM1* and *KAT2*) were induced, all eventually involved in

the induction of defense mechanisms that dominated PC2 (Fig. 1a).

Elevated glucose levels, sensed by *HXK1*, are known to induce aliphatic as well as indolic glucosinolate synthesis

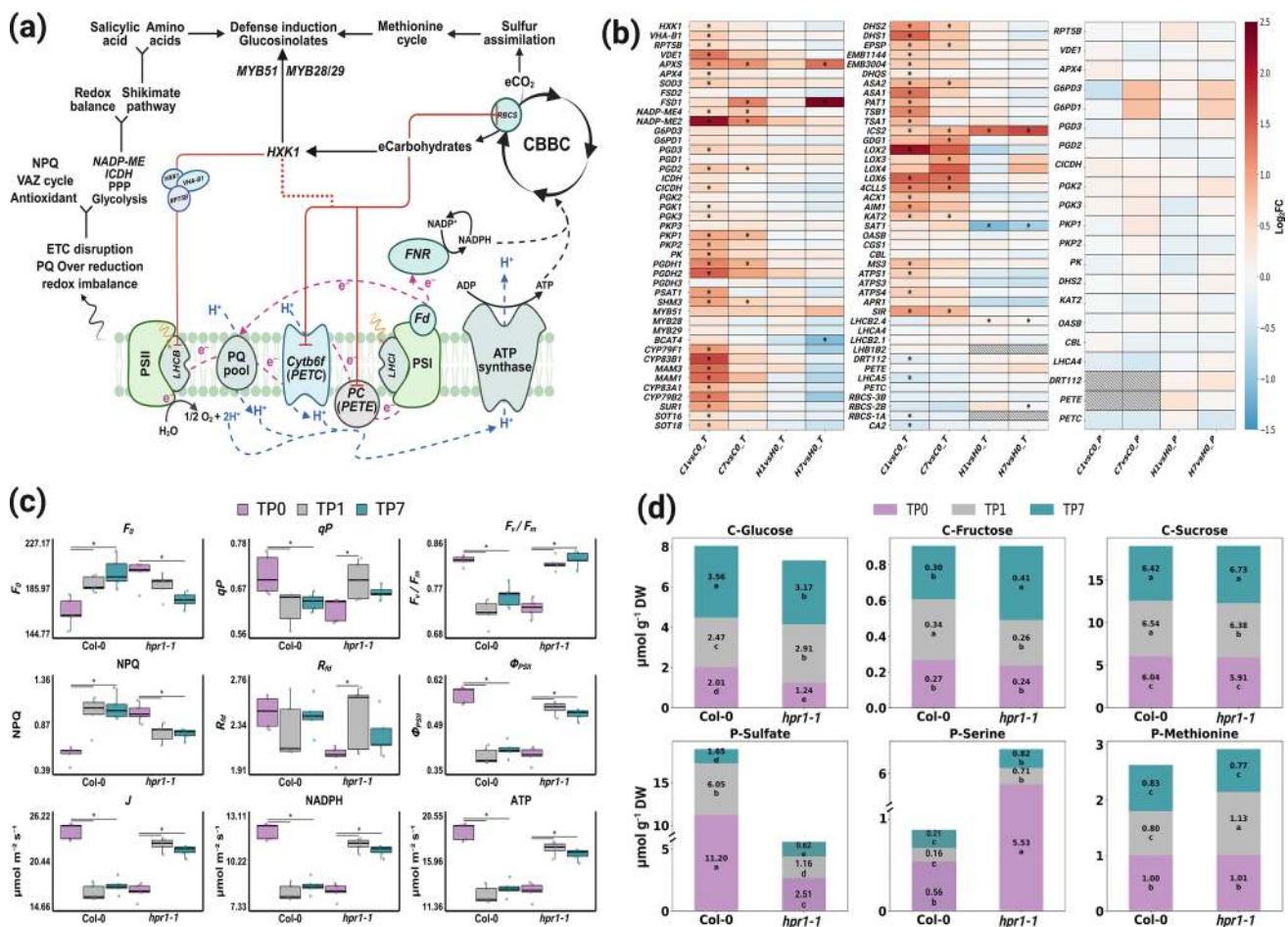


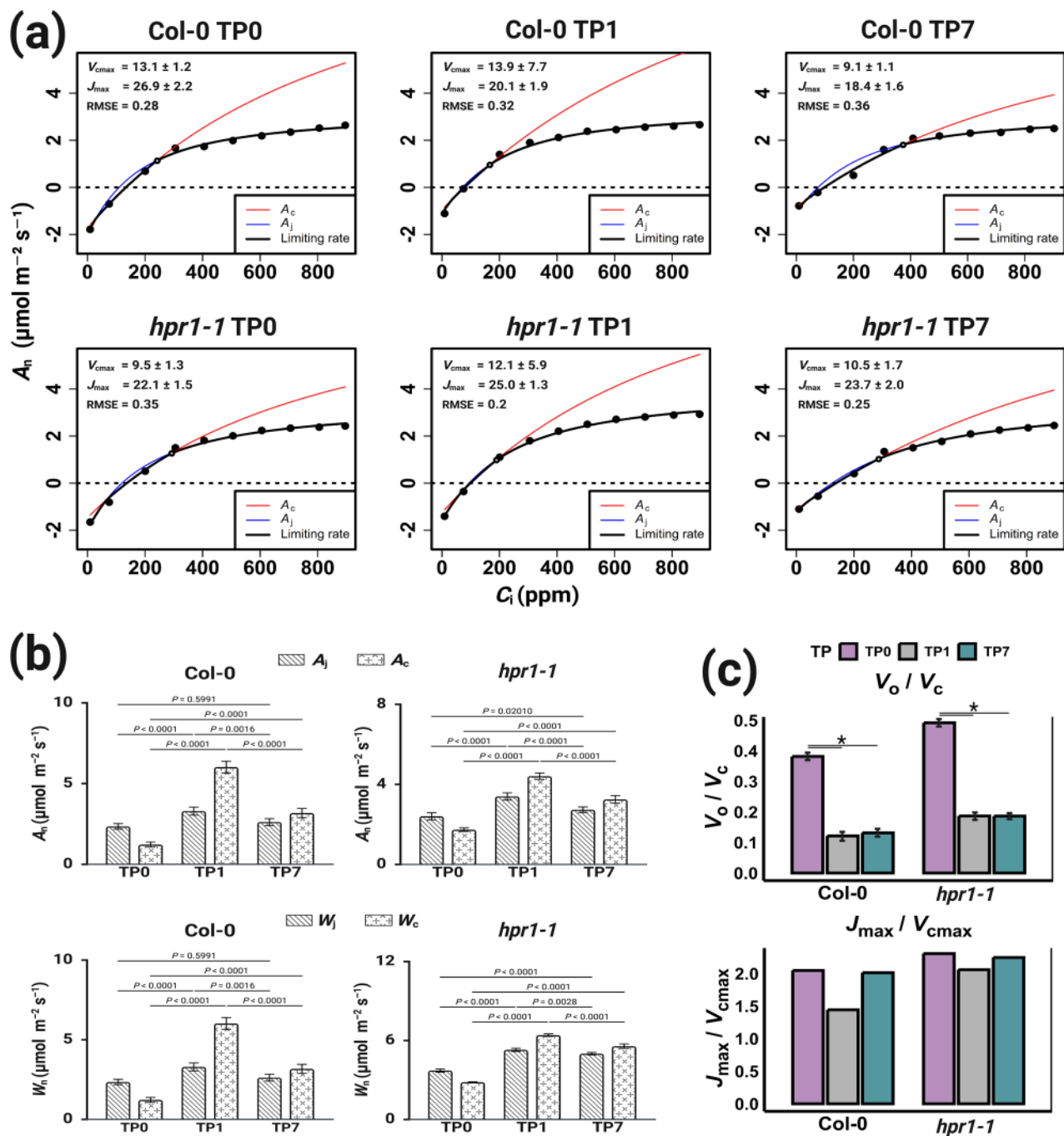
Fig. 2 Immediate responses of plants to eCO₂. Colors are identical for all graphs, indicating eCO₂ duration as purple: 0; gray: 1; green: 7 days. **a** Schematic representation of processes and pathways affected as immediate responses to elevated CO₂ in wildtype. (In)direct conversions and/or induction of pathways are indicated by solid (forked) arrows and suppression is indicated by red solid lines. Electron transport chain, cyclic electron flow and proton transfer are represented by dashed arrows. The possible role of *HXK1* in the regulation of *Cytb6/f* and *PC* is indicated by a red dotted line. **b** Integrated heatmap of log₂FC-transformed differentially expressed transcripts (T) and proteins (P) relative to ambient conditions that relate to metabolic and signaling pathways depicted in (a). C1vsC0 and C7vsC0 compare Col-0, TP1 vs TP0 and TP7 vs TP0, respectively. H1vsH0 and H7vsH0 accordingly compare *hpr1-1* (* adj. *P*-val < 0.05; *n* = 3; one-tailed *z*-test; Bonferroni correction). **c** Chlorophyll

fluorescence measurements under ambient/elevated carbon dioxide (a/eCO₂). Asterisks (*) indicate significant difference (*P* < 0.05) between time points based on two-way ANOVA for genotype and treatment (Bonferroni multiple comparisons test, *n* = 5); boxes represent the interquartile range (IQR), medians and 95% confidence intervals. **d** Subcellular metabolite concentration (means, *n* = 3) as stacked bar plots. Cytosolic and plastidial metabolites are indicated by 'C-' and 'P-' prefix, respectively. Letters indicate significant differences between time points (Bonferroni multiple comparisons test following two-way ANOVA, *n* = 3). *eCarbohydrates* elevated carbohydrates, *Fd* ferredoxin, *FNR* ferredoxin-NADP⁺ reductase, *ICDH* isocitrate dehydrogenase, *LHCB* light-harvesting complex, *NADP-ME* NADP-malic enzyme, *PC* plastocyanin, *PPP* pentose phosphate pathway, *PQ* plastoquinone, *PSI/II* photosystem I/II

via transcription factors MYB28/29 and MYB 34/51/122, respectively (Eom et al. 2024). We found MYB28, 29 and 51 induced as an early response of Col-0 to eCO₂, thus emphasizing the orchestrating role of *HXK1* in early responses to eCO₂ (Fig. 2a,b). The induction of both pathways largely relies on sufficient sulfur supply, which was reflected in a significant depletion of the free sulfate pool upon the shift to eCO₂ (Fig. 2d).

To assess the limitation of net photosynthesis under different CO₂ regimes, A-C_i response curves were fitted using

the FvCB model. Figure 3a shows that the shift to eCO₂ caused a marked decrease in *J*_{max}, while *V*_{cmax} remained largely unchanged. As shown in Fig. 3c, although photorespiration was strongly suppressed under eCO₂, as indicated by a reduced *V*_o/*V*_c ratio, the primary limitation of net assimilation shifted from Rubisco-limited assimilation (*A*_c) under aCO₂ to electron transport-limited assimilation (*A*_j) under eCO₂, which was evidenced by a pronounced decrease in the parameter *A*_j (Fig. 3b). A similar shift was also observed from the RuBP-saturated carboxylation rate (*W*_c) to the



electron transport-limited rate of RuBP regeneration (W_j), as shown in Fig. 3b.

The *hpr1-1* mutant responded differently to eCO_2 , both, at immediate and long-term exposure (H1vsH0_T and H7vsH0_T). We found no evidence for redox imbalance in *hpr1-1* plants shifted to eCO_2 , and this was in line with assessment of chlorophyll fluorescence data for NPQ, F_0 , F_v/F_m and qP and also with estimated Φ_{PSII} , J , NADPH and ATP consumption rates (Fig. 2c). Neither glucosinolate metabolism nor salicylic acid or jasmonate pathways were

induced in *hpr1-1* at eCO_2 , the latter showing even down-regulation at day 1. However, concerted induction of genes related to pathogen responses and plant immunity underlay the shared response of *hpr1-1* and Col-0 along PC2 (Fig. 1a). In addition to that, induction of serine synthesis through the PPSB pathway at eCO_2 was a common feature of both genotypes (Fig. 2b), highlighting the impact of photorespiration on amino acid metabolism.

The situation was different at the protein level. Here, the two genotypes strongly differed initially with respect to PC1,

Fig. 3 Biochemical limitation of net photosynthesis under different CO₂ regimes. **a** Net CO₂ assimilation rate (A_n) as a function of intercellular CO₂ concentration (C_i), fitted according to the FvCB model for Col-0 and *hpr1-1* at different time points (TP0, TP1 and TP7). Filled black circles represent measured data points (means of $n=17$ biological replicates per C_i). The fitted model of the Rubisco-limited assimilation rate (A_c) is shown in red, and the electron transport-limited assimilation rate (A_j) in blue, and the resulting limiting assimilation rate (A_n according to Eq. 11) in black. The C_i transition point between A_c and A_j limitation is indicated by an open black circle. Triose phosphate utilization-limited assimilation rate (A_p) was not detected and therefore not included. Estimated values of V_{cmax} , J_{max} , and the root mean square error (RMSE) of the fitted model are shown within each panel. **b** Derived Rubisco-limited and electron transport-limited assimilation rates (A_c and A_j) and the corresponding RuBP-saturated carboxylation (W_c) and electron transport-limited (W_j) rates in Col-0 and *hpr1-1* across time points, according to Eqs. 12–15. The A_j and W_j are shown as bar plots with diagonal black hatching, while A_c and W_c are shown as black cross-hatched bars. **c** Ratios of Rubisco oxygenation to carboxylation (V_o/V_c) and ratio of maximum electron transport to maximum carboxylation rates (J_{max}/V_{cmax}) in Col-0 and *hpr1-1* across time points with identical colors, illustrating changes in photorespiratory flux and the coordination between electron transport and Rubisco activity under eCO₂. Values represent means $\pm$ SD ($n=5$). significant deviation ($P < 0.05$) is indicated by asterisks (*) and based on one-way ANOVA followed by Bonferroni multiple comparisons test across different time points (ambient/elevated carbon dioxide, a/eCO₂) within wildtype and mutant, respectively (**b** and **c**)

but due to opposite responses showed some convergence after 7 days at eCO₂ (Fig. 1b). For PC2, however, they also responded contrary and were more separated at day 7 as compared to the starting condition.

Biochemical FvCB modeling revealed a marked increase in both V_{cmax} and J_{max} in *hpr1-1* compared with Col-0 (Fig. 3a). The ratio of Rubisco oxygenation to carboxylation (V_o/V_c) was approximately 36% higher in mutant than wildtype under aCO₂. Despite a significant decline at eCO₂, this ratio appeared higher in the mutant (Fig. 3c). Consistent with the alleviation of photorespiration under eCO₂, A_j and W_j became the limiting factors of net photosynthesis and carboxylation rate in *hpr1-1* (Fig. 3b).

Hierarchical clustering of Euclidean distances of protein levels of Col-0 and *hpr1-1* showed that proteomes of both genotypes built different clusters at day 1 at eCO₂, while at day 7 proteomes of both genotypes were least distant (Fig. 4). This was explained by two major clusters of proteins (Fig. 4) which were either depleted in Col-0 and enriched in *hpr1-1* (Fig. 4a) or vice versa (Fig. 4b). Cluster A comprised 1257 out of 1915 proteins (66%) while cluster B comprised 658 out of 1915 proteins (34%; Table S2). A GO term enrichment analysis revealed a highly significant impact on ‘metabolic processes’ in both clusters (Table S2). In cluster A, 951 out of 1257 proteins belonged to this term, in cluster B it comprised 508 out of 658 proteins. To evaluate whether enriched proteins in the *metabolic process* term build (potential) functional or regulatory units of metabolism, putative

protein–protein interactions among them were tested using the String[®] database (<https://string-db.org/>). Filtering on the highest confidence of protein association (for settings see Materials and methods), 25 disconnected graphs were found for cluster A, and 21 for cluster B. A *k-means* clustering comprising the 25 clusters in cluster A revealed that metabolism of cellular amino acids and purine-containing compound metabolic process were most enriched, according to the ‘ $-\log_{10}$ FDR’ filter, in *hpr1-1* at day 1 of eCO₂ (Fig. 4c). In cluster B, most enriched processes revealed by the *k-means* clustering were photosynthesis, electron transport chain, and nucleoside phosphate metabolic process (Fig. 4d). The ‘ $-\log_{10}$ FDR’ filter was chosen because it represents expected gene occurrences in context of its false discovery rate (Szklarczyk et al. 2025). In summary, these observations suggested a significant impact of *HPRI*-deficiency on the supply and metabolism of amino acids and ribonucleotides. The discrepancy between *hpr1-1* and Col-0 in these metabolic processes was mitigated under eCO₂. Second, and maybe as a consequence, proteins involved in nucleoside phosphate biosynthesis processes and photosynthesis were depleted in *hpr1-1*, and only after 7 days at eCO₂ the proteome of these processes became wildtype-like.

Immediate metabolic changes at eCO₂

Whether the responses found at the transcriptome and/or proteome level did affect metabolic processes was analyzed by non-aqueous fractionation of subcellular compartments. This ensured that substrate concentrations were assessed at the site of reactions taking place. Besides the expected rise in sugars, especially hexoses, in cytosol and vacuole (Fig. S2), accumulation of leucine as well as aspartate, asparagine and aromatic amino acids was an immediate response to eCO₂ in wildtype plants (Fig. S2). While glutamine accumulated transiently in the cytosol of Col-0, glutamate accumulated until day 7, and both were also enriched in plastids, indicating stimulated nitrogen assimilation. On the other hand, levels of branched-chain amino acids valine, leucine, and isoleucine sharply dropped in mitochondria on day 1 of the eCO₂ treatment and did not reach initial levels at day 7 (Fig. S2). Carboxylic acids malate and fumarate were not affected, neither in vacuole nor mitochondria, while pyruvate accumulated in mitochondria and citrate in both mitochondria and cytosol, again pointing to enhanced amino acid formation (Fig. S2).

The increase in sugars at eCO₂ was either similar or even stronger in the cytosol and vacuole of *hpr1-1* mutant plants, indicating similar effects on primary carbon fixation (Fig. S2). However, substantial differences were observed for amino acids. While valine dropped at day 1 in plastids of Col-0, it accumulated in plastids of *hpr1-1*. Leucine was generally low in mitochondria of *hpr1-1*, but was higher

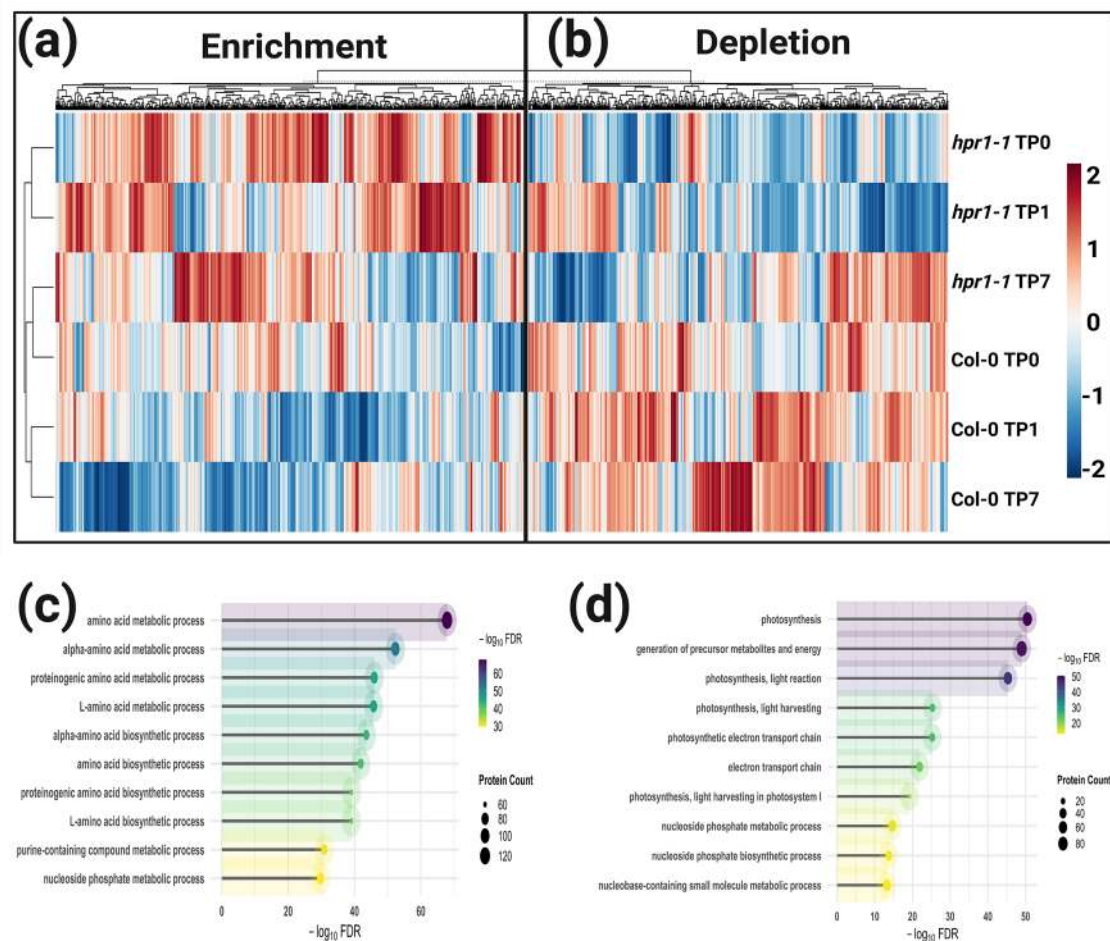


Fig. 4 Cluster analysis of proteome dynamics due to *HPR1*-deficiency. **a** and **b** Hierarchical clustering of mean protein levels in Col-0 and *hpr1-1* based on Euclidean distances ($n=3$). **a** Enrichment cluster of *hpr1-1*. **b** Depletion cluster of *hpr1-1*. **c** Enriched protein functions in cluster a revealed by *k-means* clustering of contained protein association networks (top 10 signals). **d** Enriched protein

functions in cluster b revealed by *k-means* clustering of contained protein association networks (top 10 signals). FDR, false discovery rate. Size of filled circles in **c** and **d** represents the number of gene candidates that were contained in each functional category indicated on the left side. Details of gene identifiers and GO enrichments are available in Table S2

in the cytosol, while isoleucine was very high in *hpr1-1* at aCO_2 in all compartments. Aspartate was generally low in *hpr1-1* at aCO_2 , while asparagine accumulated in plastids at eCO_2 . Glutamate and glutamine transiently increased in mitochondria of *hpr1-1*, and in the case of glutamine this was observed also in the other compartments. Ornithine was very high in *hpr1-1* at aCO_2 and significantly declined at eCO_2 , and the same was observed for arginine in cytosol and plastids. Considering the extremely high levels of glycine and serine in *hpr1-1* at aCO_2 , high levels of cysteine were expected. However, they did not decline in eCO_2 in any of the compartments (Fig. S2).

Long-term effects of eCO_2

To gain a better understanding of the mechanisms underlying metabolic adjustment under prolonged eCO_2 exposure,

plant responses were examined also after one week. While long term responses at day 7 showed a mild alleviation in almost all of the above-mentioned pathways that were induced at transcript levels in the wildtype, transcript levels did not align with initial levels (C7vsC0_T) (Fig. 2b). In addition, chlorophyll fluorescence data for NPQ, F_0 , F_v/F_m and qP at day 7 were still in support of a stress condition in wildtype plants (Fig. 2c). Accordingly, the estimated Φ_{PSII} , J , and rates of NADPH and ATP consumption remained comparable to those observed on the first day of eCO_2 exposure, consistent with a *HXK1*-dependent inhibition of electron transport (Fig. 2a,c). In accordance with that, the NADPH oxidase *RBOHD* that is involved in plant immunity was induced at day 7, and considering that the hydrogen peroxide sensor *HPCA1* and the plasma membrane receptor kinase *GHR1* were also up-regulated, this may underlie reduced stomatal conductance, which was observed in

wildtype plants at day 7 (Fig. 5a) A non-significant induction of these genes was also observed in *hpr1-1* (Fig. 5a).

Despite atmospheric CO₂ enrichment, stomatal closure can impact C_i and carbon assimilation rate (A). As shown in Fig. 5c, wildtype plants exhibited a significant but transient increase of stomatal conductance for CO₂ (G_{CO2}) at day 1, which was lost again at day 7. Consistent with this pattern, the carbon assimilation rate was highest at day 1 and declined until day 7, when C_i was also significantly reduced. Conversely, C_i continuously increased until day 7 in *hpr1-1*. This elevation was not necessarily due to high CO₂ conductance, but may instead have resulted from inefficient assimilation and a limited photosynthetic capacity, as reflected by the decrease in assimilation rate on day 7 (Fig. 5c). The CO₂ compensation point in the absence of dark respiration (I^{*}) was determined to be 50.6 ± 11.1 ppm in the wild type and 73.4 ± 7.44 ppm in *hpr1-1*. Chloroplastic CO₂ concentration

(C_c) estimated using the Harley equation (Eq. 4), reached 256.6 ± 13.8, 770.6 ± 68.3, and 739.4 ± 67.3 ppm at TP0, TP1, and TP7, respectively, in the wildtype, and 299.5 ± 5.3, 782.6 ± 25.4, and 796.2 ± 49.9 ppm in *hpr1-1*. Consequently, mesophyll conductance (g_m, mol m⁻² s⁻¹ bar⁻¹), estimated according to Fick's law (Eq. 5), declined following the shift to eCO₂ in both wildtype (TP0: 0.06; TP1: 0.03; TP7: 0.03) and mutant (TP0: 0.08; TP1: 0.06; TP7: 0.02).

Differential gene expression analysis in wildtype and mutant plants exposed to eCO₂ for 7 days (C7vsCO_T and H7vsH0_T) revealed significant down-regulation of sugar transporters, *SWEET11* and *12*, which are involved in apoplastic sucrose phloem loading (Fig. 5a). Thus, sugar exudation from leaves was analyzed in these plants. As shown in Fig. 5d, sucrose exudation from leaves during an 8 h light phase was reduced in wildtype plants exposed to eCO₂ for 7 days, while the reduction was less pronounced in *hpr1-1*.

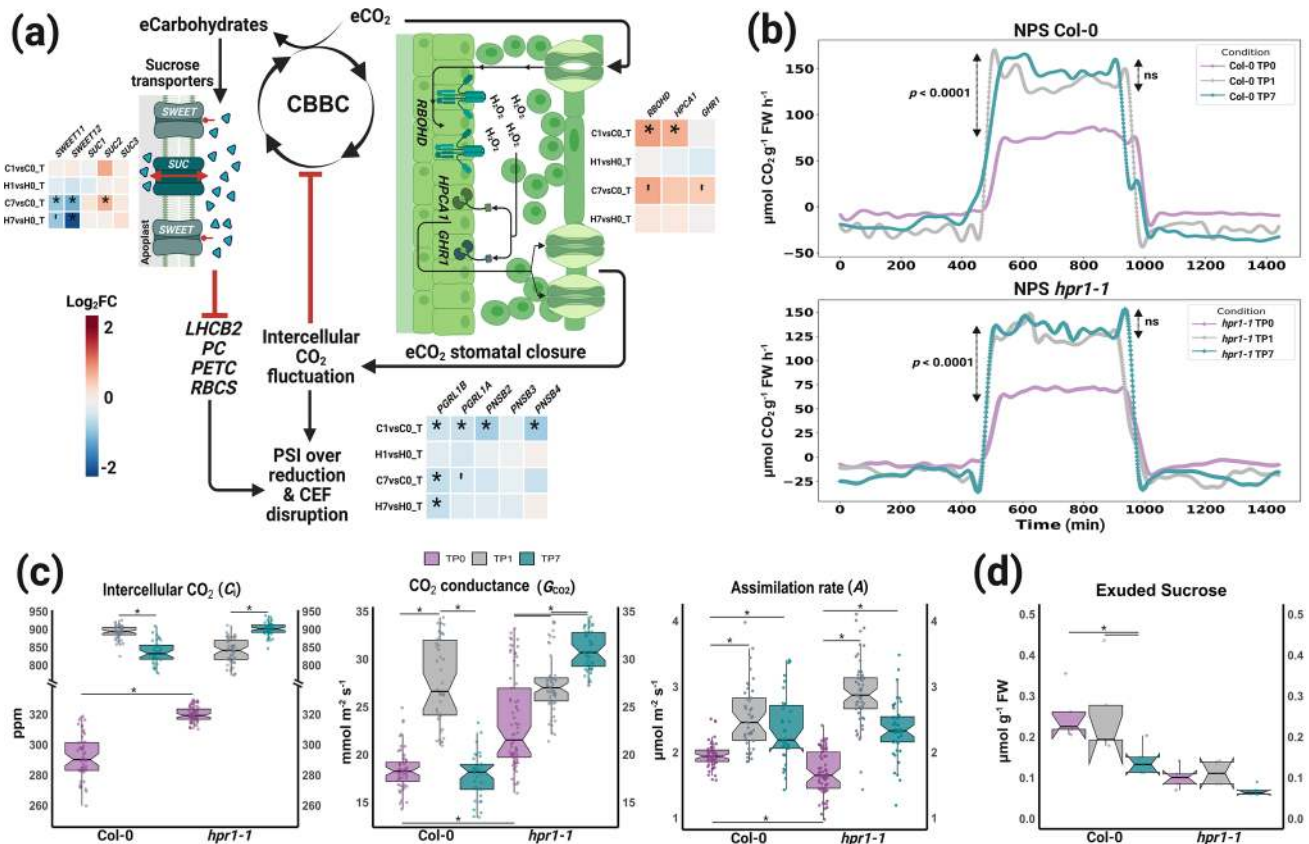


Fig. 5 Gas exchange and assimilate transport in responses to eCO₂. **a** Scheme of processes affected by long-term eCO₂ treatment. Differential transcript abundance at eCO₂ relative to ambient is illustrated as heatmaps reflecting log₂FC (* adj. *P*-val < 0.05 and ' adj. *P*-val < 0.07, *n* = 3; one-tailed *z*-test; Bonferroni correction). **b** Diurnal net CO₂ gas exchange at different time points of eCO₂ exposure. Abscissae reflect time per day (min) and ordinates reflect net photosynthesis (NPS) in μmol CO₂ g⁻¹ FW h⁻¹; (*n* = 3). **c** Calculated internal CO₂ concentration (C_i), stomatal conductance of CO₂ (G_{CO2}), and real-time assimilation rate (A) across different time points of ambient/

elevated carbon dioxide (a/eCO₂) conditions in wildtype and mutant plants. **d** Phloem exudation of sucrose. Ordinate reflects exuded sucrose concentration in phloem sap in μmol g⁻¹ FW. Colors in all sections indicate time at eCO₂ (purple: 0; gray: 1; green: 7 days). Significant deviation (*P* < 0.05) is indicated by asterisks (*) and based on two-way ANOVA followed by Bonferroni multiple comparisons test across different time points (a/eCO₂) within wildtype and mutant (**b** and **d**) or same timepoints among genotypes (**c**). CEF cyclic electron flow, eCarbohydrates elevated carbohydrates

Reduced sucrose loading into the phloem may have contributed to excessive accumulation in mesophyll cells, which was observed especially for cytosol and vacuole in Col-0 at day 7 (Fig. 2d and Fig. S2).

As shown in Fig. 5b, diurnal assessment of photosynthesis revealed a significant difference in net photosynthetic rates between aCO₂ and eCO₂ conditions in both genotypes, while no significant difference was observed between day 1 and day 7 under eCO₂. However, the expression of genes involved in cyclic electron flow (CEF), including *PGRL1A/B*, *PNSB2/3/4* showed a continuous down-regulation from day 1 to day 7 of eCO₂, pointing to the establishment of a new photosynthetic equilibrium in both genotypes (Fig. 5a).

The metabolic phenotype of *hpr1-1* mutants comprises strong effects on cytosolic glycerate and pyruvate metabolism

To gain a detailed understanding of how a bottleneck in the conversion of hydroxypyruvate to glycerate in peroxisomes of the *hpr1-1* mutant impacts each of the steps of the photorespiratory pathway at the different omics levels, *hpr1-1* was compared to Col-0 at aCO₂ and eCO₂ conditions (H0vsC0_T/P, H1vsC1_T/P and H7vs C7_T/P; Fig. 6a–c). Elevated glycerate levels, especially in cytosol and plastids, differentiated *hpr1-1* from Col-0 (Fig. 6c). In stark contrast, the glycolate amount was low in plastids at aCO₂, but accumulated in the cytosol of *hpr1-1* plants (Fig. S2). This pointed to an exchange of both metabolites either via the bile acid/sodium symporter *BASS6* (AT4G22840) or the plastidial glycolate/glycerate antiporter *PLGG1* (AT1G32080) (Fig. S3a,b). Indeed, *PLGG1* had a slightly elevated transcript level in *hpr1-1*, which immediately declined at eCO₂ (Fig. S3b). Although glyoxylate levels were not directly measured, the accumulation of cytosolic glycolate was consistent with the induction of cytosolic glyoxylate reductase *GLYR1* (AT3G25530; log₂FC 0.33, adj. *P*-val 0.008), suggesting enhanced conversion of glyoxylate to glycolate. This reaction would replenish the cytosolic glycolate pool and mitigate the accumulation of the toxic glyoxylate, as previously reported by Jiang et al. (2025). In search of a cytosolic source for glycerate fueling this exchange, the pathways depicted in Fig. 6a were scrutinized at the various omics levels.

As reported by Timm et al. (2011) and Jiang et al. (2025), hydroxypyruvate can be reduced by either peroxisomal (*HPR1*) or cytosolic isoform of *HPR* (*HPR2*), the latter contributing to the cytosolic glycerate pool. Here, we propose an alternative pathway branching from serine (Fig. 6a,b). This route to glycerate would involve malate, pyruvate and cysteine, thus explaining the high cysteine levels in cytosol, plastids and mitochondria of the mutant (Fig. 6c). Key

enzymes in this bypass route are O-acetylserine (thiol) lyase (*OASA1*, *OASB/C*), serine acetyltransferase (*SAT1/2/3*) and also L-cysteine desulfhydrase 1 (*DES1*), which catalyzes the conversion of cysteine to pyruvate thereby releasing ammonia and sulfide. All were up-regulated at the transcript level (*OASA1*, *OASB/C*, *SAT1/2/3*, *DES1* in Fig. 6b). Acetyl-CoA, required for acetylation of serine, could originate from either citrate via the activity of ATP-citrate lyase (*ACL*) or acetate, depending on the compartment of cysteine synthesis. Cytosolic citrate was found to accumulate in *hpr1-1* (Fig. 6c), and transcripts for enzymes related to ATP citrate-lyase and cytosolic acetyl-CoA synthesis, (*ACLA-1*, *ACLB-1/2*, *ACS* and *AEE7*) were found to be up-regulated, too (Fig. 6b).

In accordance with the proposed pathway, the flux of cytosolic pyruvate into mitochondria should be reduced. Indeed, mitochondrial pyruvate carriers 1, 3 and 4 (*MPC1/3/4*; Fig. 6b) displayed lower transcript levels in *hpr1-1* compared to Col-0. In line with this, the transcript of pyruvate dehydrogenase kinase (*PDK*), an inhibitor of the pyruvate dehydrogenase complex, was significantly up-regulated (Fig. S3c). As outlined in Fig. 6a, malate could serve to replenish glycerate via gluconeogenesis. Depending on the malic enzymes, the reaction could proceed via pyruvate and phosphoenolpyruvate (PEP), employing *NADP-ME2/4* and pyruvate kinases (*PK* and *PK9*), or via oxaloacetate and its conversion to PEP, involving malate dehydrogenase (*MDH1*) and phosphoenolpyruvate carboxykinase (*PCK1*). Both pathways are consistent with the observed accumulation of cytosolic malate in *hpr1-1* (Fig. 6a–c). The gluconeogenic route is supported by the significant induction of genes involved in the conversion of PEP to 2-phosphoglycerate (2-PGA; *ENO1/2*), followed by its transformation to 3-phosphoglycerate (3-PGA; *IPGM1/2*), and ultimately to glycerate via glycerate kinase (*GLYK*) (Fig. 6a,b).

Comparative analysis of differentially expressed genes (DEGs) between the mutant and wildtype under aCO₂ (H0vsC0_T) revealed significant induction of genes involved in asparagine metabolism. These genes are known to be regulated in response to cellular sucrose levels via sugar starvation markers such as *SNRK1.1* and *SNRK1.2* (Curtis et al. 2018). In *hpr1-1* under aCO₂, sucrose starvation could be sensed by these markers, which would then lead to the observed induction of genes associated with asparagine synthesis (*ASN1/2/3*). Asparagine biosynthesis through this pathway relies on aspartic acid, which must be provided via transamination of oxaloacetate. Indeed, the genes for aspartate aminotransferase (*ASP1/2/3/5*) were found to be induced in the mutant (Fig. 6b).

In addition, induction of genes involved in the methionine cycle (*MS1/2/3* and *SAMS1/2/3*) and ethylene biosynthesis (*ACO1/2/4*) was observed under aCO₂ in *hpr1-1*. Ethylene biosynthesis is associated with the production of the toxic by-product hydrogen cyanide, which is predominantly

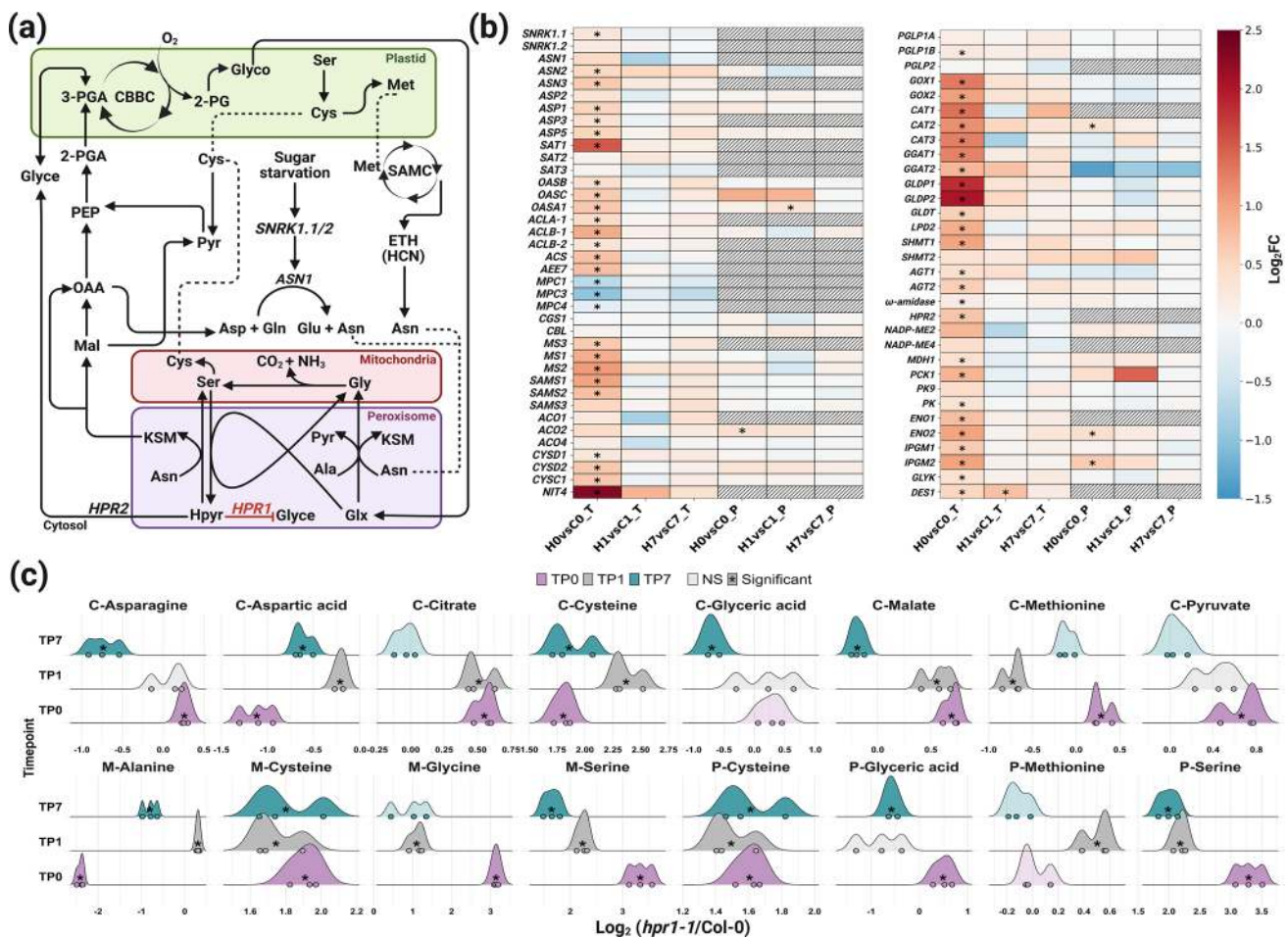


Fig. 6 Differential responses of the *hpr1-1* relative to wildtype across different time points at eCO_2 . **a** Schematic representation of gluconeogenesis-dependent and *HPR2*-mediated bypasses of peroxisomal *HPR1* deficiency in the *hpr1-1* mutant at aCO_2 . (In)direct conversions and/or inductions of pathways are indicated by solid arrows, mutant deficiency is indicated by a red solid line, transfer of metabolites across cellular compartments is indicated by dashed lines. **b** Integrated heatmap of differentially expressed transcripts and proteins associated with hypothesized pathways in (a). Values represent $\log_2FC$ of transcriptome ($_T$) and proteome ($_P$) in mutant relative to wildtype, thus, H0vsC0, H1vsC1, and H7vsC7 indicate mutant-versus-wildtype comparisons at TP0, TP1, and TP7, respectively. (* adj. P -val < 0.05; $n=3$; one-tailed z -test; Bonferroni correction). **c** Subcellular metabolite profiles represented as ridge plots comparing mutant relative to wildtype. Colors indicate duration at eCO_2 (purple:

0; gray: 1; green: 7 days). Abscissae reflect $\log_2$ -transformed ratio of mean metabolite concentrations (*hpr1-1*/Col-0), cytosolic, plastidial and mitochondrial metabolites are indicated by 'C-', 'P-' and 'M-' prefix, respectively, and ordinate indicates time points. Asterisks (*) along with dark colors denote statistically significant differences between time points within each genotype, based on two-way ANOVA followed by Bonferroni multiple comparisons test ($n=3$). Faded color ridges indicate non-significant (NS) changes. 2-PG 2-phosphoglycolate, 2-PGA 2-phosphoglycerate, 3-PGA 3-phosphoglycerate, Ala alanine, Asn asparagine, Asp aspartic acid, Cys cysteine, *ETH* ethylene, Gln glutamine, Glu glutamic acid, Gly glycine, Glyce glycerate, Glyco glycolate, Glx glyoxylate, HCN hydrogen cyanide, Hpyr hydroxypyruvate, KSM α -ketosuccinamate, Mal malate, Met methionine, OAA oxaloacetate, PEP phosphoenolpyruvate, Pyr pyruvate, SAMC s-adenosyl methionine cycle, Ser serine

detoxified in a two-step reaction starting from cysteine. The reaction is catalyzed by β -cyanoalanine synthase (*CYSD1/2* and *CYSC1*) and results in the formation of the less toxic β -cyanoalanine. This compound is subsequently converted to asparagine or aspartate by nitrile hydratase (*NIT4*), providing an alternative source of asparagine. All genes involved in this pathway, particularly *NIT4*, were up-regulated at aCO_2 in *hpr1-1* (Fig. 6a,b).

Discussion

Response of wildtype Col-0 plants to a sudden rise in CO_2 concentration: symptoms of stress

Besides immediate stimulation of primary carbon fixation, an increase in atmospheric carbon dioxide levels has profound consequences for nitrogen and sulfur assimilation, resulting in deflections in amino acid metabolism (Chen

et al. 2005; Abadie and Tcherkez 2019). It has been reported that a decline in photorespiratory activity at $e\text{CO}_2$ could be responsible for reduced N- and S-acquisition (Abadie and Tcherkez 2019; Krämer et al. 2022b), and thus, in the present study, we aimed at disentangling the effects of elevated C-assimilation and reduced photorespiratory activity at $e\text{CO}_2$ by shifting wildtype plants and the *hpr1-1* mutant, which is impaired in recycling carbon and nitrogen released in photorespiration, from ambient to elevated CO_2 levels. It should be emphasized that a CO_2 concentration of 1000 ppm does not completely abolish photorespiration, as stated earlier by Krämer et al. (2022b), but substantially reduces its turnover.

In both genotypes, sugars accumulated immediately at $e\text{CO}_2$, while total amino acids rose in wildtype and declined in the mutant. In fact, responses were not only different among genotypes but also among individual amino acids, especially when their subcellular localization is considered. While an increase in plastidial glutamate and glutamine in both genotypes pointed to a transient stimulation of N-assimilation, possibly linked to the high sugar levels that activate nitrate reductase, methionine levels went down in plastids, but increased in the cytosol of Col-0 and remained unchanged in the cytosol of *hpr1-1*. Thus, methionine accumulation in the cytosol of Col-0 appears to be related to the stress symptoms, displayed by the wildtype but not by the *hpr1-1* mutant.

It has earlier been reported that $e\text{CO}_2$ causes increased transcript levels of disease resistance proteins, heat shock proteins and dehydration-responsive proteins (Li et al. 2008), but the underlying regulatory mechanism is unclear. High sugar levels sensed by the bifunctional enzyme/sugar sensor hexokinase 1, which is involved in various biotic and abiotic stress responses (Shen et al. 2021; Yu et al. 2022) could have triggered this response. The *HXK1* gene is itself responsive to elevated glucose levels (Pourtau et al. 2006) and was up-regulated in Col-0 upon $e\text{CO}_2$. The observed repression of the genes *LHCB2* and *CA2*, both involved in photosynthesis, is in accordance with this view. It has been shown by Cho et al. (2006) that a trimeric complex of *HXK1*, *RPT5B* and *VHA-B1* in response to elevated glucose binds to the promoter region of chlorophyll a-b binding proteins of PSII (*LHCB*) to inhibit transcription and the observed down-regulation of the *RBCS* gene is also in agreement with the reported regulation by *HXK1* (Xu et al. 2015). Considering that sugar levels increased in both genotypes, but stress indicators were up-regulated only in the wildtype, this indicates that sugar accumulation alone is insufficient to trigger the stress response.

What discriminates the two genotypes, was the strong evidence for a disturbed linear electron transport in Col-0 upon the shift to $e\text{CO}_2$. We observed repression of the *PETC* gene, encoding the Rieske-type FeS protein that transfers

electrons from plastoquinol to *Cytb6f*, as well as both genes encoding plastocyanin. *PETC* was shown to be repressed by elevated sucrose levels (Knight et al. 2002) and was significantly down-regulated at day 1 of the $e\text{CO}_2$ treatment. The same is true for the plastocyanin gene, *PETE* (Dijkwel et al. 1996). The observed stimulation of the violaxanthin de-epoxidase gene *VDE1*, involved in the violaxanthin-antheraxanthin-zeaxanthin (VAZ) cycle (Czarnocka and Karpiński 2018) and induction of genes involved in the Foyer-Halliwel-Asada cycle of reactive oxygen defense, including *SOD* and *APX* (Noctor and Foyer 1998), as well as the increase in non-photochemical quenching and F_0 as an indication of oxidative damages to PSII (Baker 2008), shown in Fig. 2c, support this view. A block in photosynthetic electron flux could also have triggered repression of *LHCB2* and *CA2* gene expression (Oswald et al. 2001).

The contrasting light-dependent photosynthetic responses of Col-0 and *hpr1-1* to $e\text{CO}_2$ reveal fundamentally different strategies at the photochemical and biochemical level. In Col-0, the sustained decline in F_v/F_m following transfer to $e\text{CO}_2$ that persisted even after seven days, together with increased F_0 and NPQ, indicates chronic photoinhibition and PSII damage rather than a transient down-regulation (Baker 2008). This interpretation is reinforced by the strong and lasting reductions in Φ_{PSII} and J . Similar reductions in J and Φ_{PSII} under $e\text{CO}_2$ have been reported for soybean, wheat, and woody species, where photosynthetic acclimation was involved in the repression of electron transport capacity and altered excitation energy dissipation (Zheng et al. 2019; Schweiger et al. 2022; Tang et al. 2024). Our omics data provide a mechanistic framework for this response, where repression of *PETC*, downregulation of *LHCB2* and *CA2*, and induction of *VDE1* and antioxidant pathways collectively point to restricted linear electron flow and enhanced photoprotective demand. Such repression is also consistent with carbohydrate-mediated signaling via *HXK1*. In contrast, *hpr1-1* displayed increased F_v/F_m , Φ_{PSII} , and J under $e\text{CO}_2$, suggesting improved photochemical efficiency and protection of PSII. The induction of *VDE1* and antioxidant enzymes in *hpr1-1* supports enhanced photoprotective capacity via the xanthophyll cycle and reactive oxygen species detoxification, consistent with improved redox balance under carbon-replete conditions (Noctor and Foyer 1998; Czarnocka and Karpiński 2018).

The FvCB modeling revealed the mechanistic basis of this divergence. In Col-0, the immediate and progressive decline in $J_{\max}$ without a corresponding change in $V_{\max}$ following $e\text{CO}_2$ exposure indicated a shift towards electron transport limitation, with A_j and W_j becoming strongly limiting relative to A_c and W_c . Such a down-regulation has been widely observed across C_3 species grown under elevated CO_2 for long periods, where both $V_{\max}$ and $J_{\max}$ typically decline relative to ambient conditions, reflecting reduced investment

in photosynthetic machinery under high carbon availability (Zheng et al. 2019; Ancín et al. 2024). This decoupling of photochemistry from carbon metabolism is consistent with reduced NADPH and ATP consumption rates and points to sink limitation and feedback inhibition of photosynthesis.

In contrast, *hpr1-1* exhibited coordinated increase in both V_{cmax} and J_{max} upon the shift to eCO_2 , maintaining close coupling between A_j and A_c , despite its inherently higher photorespiratory burden under aCO_2 (Fig. 3c). This suggests that enhanced photorespiration in *hpr1-1* under aCO_2 may prime electron sinks and redox buffering capacity, allowing more effective utilization of excess reducing power when CO_2 becomes non-limiting. The stability of V_{cmax} and J_{max} after prolonged eCO_2 exposure, together with sustained NADPH and ATP consumption, indicates superior prearrangement of *hpr1-1* to the eCO_2 condition. This exceptional genotype response to eCO_2 contrasts with theoretical and empirical predictions that plants under eCO_2 tend to underinvest in electron transport relative to carboxylation (Smith and Keenan 2020). Although mesophyll conductance declined in both genotypes following eCO_2 exposure, this limitation did not translate into photochemical or biochemical down-regulation in the mutant, highlighting that the failure of the wildtype is not based on diffusional constraints, but is regulatory. Together, these findings support a model in which *HXK1*-mediated sugar signaling, coupled with impaired electron transport and insufficient photoprotection, drives photoinhibition in Col-0, whereas *hpr1-1* maintains photosynthetic balance through reinforced electron sinks, antioxidant capacity, and metabolic flexibility.

Elevated CO_2 is frequently reported to alleviate photoinhibition by enhancing carbon assimilation and increasing the availability of metabolic electron sinks; however, this response is not universal and a growing body of evidence indicates that responses to eCO_2 are highly conditional and frequently accompanied by photosynthetic downregulation. Hymus et al. (2001) demonstrated that growth under eCO_2 can either increase or decrease photochemical efficiency and susceptibility to photoinhibition, depending on nitrogen availability and possible sink limitation. In agreement with this, our data indicate that in wildtype plants, eCO_2 induced a sustained block in electron transport and chronic photoinhibition, resulting in decreased F_v/F_m , reduced Φ_{PSII} and J , and enhanced photoprotective dissipation. By contrast, *hpr1-1* maintained coordinated electron transport and carbon assimilation under eCO_2 , supporting the conclusion that genotype-specific regulation of sink capacity and feedback signaling, rather than CO_2 concentration alone, determine the balance between electron utilization and photoprotection. Multiple studies have shown that exposure to eCO_2 can reduce V_{cmax} and/or J_{max} in diverse species, including forest trees, crops, and legumes, largely due to nitrogen dilution, sink limitation, and feedback inhibition by accumulated

carbohydrates (Medlyn et al. 1999; Arora et al. 2009; Zheng et al. 2019; Sugiura et al. 2024). Consistent with these reports, our FvCB analysis revealed that in wildtype, exposure to eCO_2 caused a rapid and sustained decline in J_{max} without a concomitant increase in V_{cmax} , resulting in a shift towards electron transport limitation ($A_j < A_c$; $W_j < W_c$). This supports the concept that eCO_2 can exacerbate imbalances between photochemical energy provision and downstream carbon metabolism when sink capacity or regulatory coordination is insufficient.

If, indeed, a redox imbalance would have caused the induction of defense-related genes in the wildtype, it would be difficult to separate this effect from a decline in photorespiratory activity, which would, by eliminating an important consumer of reduction equivalents, also result in a disturbance of the redox balance (Voss et al. 2013; Sunil et al. 2019). In this context, the observed stimulation of amino acid production in Col-0 immediately upon the shift to eCO_2 likely serves as an alternative sink for excess reducing equivalents that are no longer utilized by photorespiration. While shikimic acid levels were reduced, aromatic amino acids accumulated in the cytosol of wild-type plants, and the rise in tryptophan could be related to indolic glucosinolate metabolism, being part of an immune response that was significantly up-regulated at the transcript level. In this context, positive feedback driven by elevated glucose on the induction of glucosinolate biosynthetic genes via a *HXK1*-dependent association with MYB transcription factors has been demonstrated by Eom et al. (2024). Induction of genes involved in glucosinolates metabolism under eCO_2 was shown in detail by Landosky and Karowe (2014) and Wiesner-Reinhold et al. (2021). Interestingly, while methionine accumulated in the cytosol of wildtype, cysteine did not change in any of the compartments. Thus, although we observed the previously reported reduction in sulfate content at eCO_2 (Loladze 2014), we did not find a negative impact of reduced photorespiration on sulfur-containing amino acids as reported for sunflower (Abadie and Tcherkez 2019). Note, however, that we measured steady state levels of amino acids, but not *de novo* synthesis, as reported by Abadie and Tcherkez (2019). The rise in methionine is consistent with the reported induction of aliphatic glucosinolate metabolism at eCO_2 (Almuhayawi et al. 2020; AbdElgawad et al. 2023). Methionine, as the precursor for S-adenosylmethionine, is also required for ethylene biosynthesis which is up-regulated at the level of ACC-oxidase 1 (*ACO1*) in *Arabidopsis* at eCO_2 (Smet et al. 2020; Azoulay-Shemer et al. 2023). During ethylene formation, cyanide is released by ACC-oxidase, which is then detoxified in a two-step reaction yielding either asparagine or aspartate (Machingura et al. 2016). The second step is catalyzed by a bifunctional cytosolic nitrilase/nitrile hydratase (*NIT4*), and both products, asparagine and

aspartate, were increased at day 1 of the eCO₂ treatment in wild-type plants.

The sustained down-regulation of genes involved in photosynthesis and the electron transport chain under long-term eCO₂ exposure may result either from carbohydrate accumulation and reduced sucrose loading into the phloem, or from eCO₂-dependent closure of stomata and altered internal CO₂, which has been reviewed in detail by Foyer et al. (2025). Stomatal closure exacerbates the over-reduction of the plastoquinone pool, ultimately disrupting the electron transport chain, as demonstrated by Wang et al. (2016). In addition, sustained stomatal closure has been shown to impair cyclic electron flow and intensify the over-reduction of PSI, as reported by Li et al. (2021). Taken together, the results obtained for wildtype plants gave clear evidence for a stress response, thus extending earlier work on gene expression under eCO₂ (Smet et al. 2020).

The *hpr1-1* mutant shows superior prearrangement to eCO₂

When comparing responses to sudden eCO₂ of wildtype and *hpr1-1* mutant plants, considering the diverse initial condition of both genotypes is vital. The *hpr1-1* mutant is significantly retarded in growth, has very high levels of serine and glycine because of the shortage in photorespiratory turnover, but is also high in threonine, isoleucine, cysteine and arginine, and low in alanine and aspartate levels [this study and Timm et al. (2021)]. Nevertheless, sugar profiles responded similarly to eCO₂ in both genotypes, and carboxylates showed *hpr1-1* to approximate wildtype levels over the time course of 7 days. For example, malate levels that deviated strongly at aCO₂ for cytosol and mitochondria were equal at day 7, as well as mitochondrial fumarate. However, malate in plastids of *hpr1-1* was always depleted. Given its role as a redox shuttle, this indicates a strongly deviating redox situation in the mutant and, as discussed above, may be the reason for the lack of stress symptoms in *hpr1-1*.

While accumulation of glycerate in the cytosol of *hpr1-1* could stem from the non-canonical photorespiratory route catalyzed by cytosolic *HPR2* (Jiang et al. 2025), we propose an alternative pathway depending on pyruvate. This pathway would profit from high cysteine, but also malate levels in *hpr1-1*. Thus, the combined omics analysis applied here points to an alternative route of glycerate regeneration under photorespiratory conditions, which could make a substantial contribution in the *hpr1-1* mutant. The shift to eCO₂ caused a rapid decline in cytosolic pyruvate, malate, glycolate and glycerate, but also in threonine and isoleucine, while aspartic acid increased and reached levels similar to wildtype. It can thus be concluded that eCO₂ caused substantial re-arrangement in the

aspartate family of amino acids, which appears indirectly affected by high glycine and serine levels in the mutant at aCO₂. The high threonine and isoleucine levels found in *hpr1-1* at aCO₂ could originate from a reduced flux from threonine to glycine via threonine aldolase (Lu et al. 2008), as well as from high serine levels (Timm et al. 2021).

We observed induction of asparagine synthesis genes in *hpr1-1* in accordance with an *SNRK*-dependent response to sugar starvation at aCO₂ (Baena-González et al. 2007; Curtis et al. 2018). However, asparagine could also be involved in a reaction by-passing *HPRI*. Although it is not possible, solely based on transcript data, to distinguish the various substrate preference of serine-glyoxylate aminotransferase (*AGT1*), which, besides asparagine, also includes serine and alanine, the significant up-regulation of an upstream *ω*-amidase gene suggests that asparagine could act as a key metabolite in the turnover of accumulated photorespiratory intermediates in *hpr1-1*. Zhang et al. (2013) showed that catalytic efficiency and $V_{\max}$ of *AGT1* were significantly higher for asparagine compared to the other substrates, and Modde et al. (2017) suggested that asparagine, as an additional amino group donor, is a superior substrate in metabolizing photorespiratory intermediates. A role of asparagine in glyoxylate detoxification has also been discussed by Cooper et al. (2022). It is interesting to note that, in the reaction involving *ω*-amidase, malate and oxaloacetate pools are replenished, which are involved in the hypothesized gluconeogenic pathway of glycerate regeneration.

Taken together, our combined and subcellularly resolved omics approach revealed additional, new information on possible metabolic by-passes to peroxisomal *HPR* (*HPRI*) and assisted to elucidate non-canonical phenotypes of the *hpr1-1* mutant. It clearly shows that metabolome analysis at the whole cell level reaches limits when it comes to pathways, such as photorespiration, which are shared among various cellular compartments.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00425-026-04958-4>.

Acknowledgements Prof. Dr. Hermann Bauwe and Dr. Stefan Timm (University of Rostock, Germany) are acknowledged for a generous gift of *hpr1-1* mutant seeds. We thank Annika Allinger and Laura Kalb for expert plant cultivation. This work was supported by the German Science Foundation (DFG), HE3087/12-01 to AGH and NA1545/4-1 to TN.

Author contributions TN and AGH conceptualized and designed the study, DS performed material preparation, developed software, data collection and performed analysis, JH developed software and performed analysis. Data collection and analysis were performed by GK, SS, DL, JG, JA and EZ. The manuscript was written by DS, AGH and TN. All authors read and approved the final manuscript.

Funding Open Access funding enabled and organized by Projekt DEAL. This study was funded by Deutsche Forschungsgemeinschaft, HE3087/12-01, Arnd G. Heyer, NA1545/4-1, Thomas Nägele.

Data availability Details of differential genes expression analysis between conditions and genotypes as well as original data of all measurements and statistical analysis are available in Figs. S4 and S5a–c as well as Tables S3–7. Full list of Tair locus ID versus genes name used in the manuscript are available in Table S8.

Declarations

Conflict of interest Authors declare no conflict of interests.

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References

- Abadie C, Tcherkez G (2019) Plant sulphur metabolism is stimulated by photorespiration. *Commun Biol* 2:379
- Abdelgawad H, Zinta G, Hornbacher J, Papenbrock J, Markakis MN, Asard H, Beemster GT (2023) Elevated CO₂ mitigates the impact of drought stress by upregulating glucosinolate metabolism in *Arabidopsis thaliana*. *Plant Cell Environ* 46:812–830
- Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO₂ enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO₂. *New Phytol* 165:351–372
- Ainsworth EA, Rogers A (2007) The response of photosynthesis and stomatal conductance to rising [CO₂]: mechanisms and environmental interactions. *Plant Cell Environ* 30:258–270
- Almuhayawi MS, Abdelgawad H, Al Jaouni SK, Selim S, Hassan AH, Khamis G (2020) Elevated CO₂ improves glucosinolate metabolism and stimulates anticancer and anti-inflammatory properties of broccoli sprouts. *Food Chem* 328:127102
- Ancín M, Gámez AL, Jauregui I, Galmes J, Sharwood RE, Erice G, Ainsworth EA, Tissue DT, Sanz-Sáez A, Aranjuelo I (2024) Does the response of Rubisco and photosynthesis to elevated [CO₂] change with unfavourable environmental conditions? *J Exp Bot* 75(22):7351–7364
- Arora VK, Boer GJ, Christian JR, Curry CL, Denman KL, Zahariev K, Flato GM, Scinocca JF, Merryfield WJ, Lee WG (2009) The effect of terrestrial photosynthesis down regulation on the twentieth-century carbon budget simulated with the CCCma earth system model. *J Clim* 22:6066–6088
- Azoulay-Shemer T, Schulze S, Nissan-Roda D, Bosmans K, Shapira O, Weckwerth P, Zamora O, Yarmolinsky D, Trainin T, Kollist H, Huffaker A (2023) A role for ethylene signaling and biosynthesis in regulating and accelerating CO₂-and abscisic acid-mediated stomatal movements in *Arabidopsis*. *New Phytol* 238:2460–2475
- Baena-González E, Rolland F, Thevelein JM, Sheen J (2007) A central integrator of transcription networks in plant stress and energy signalling. *Nature* 448:938–942
- Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. *Annu Rev Plant Biol* 59:89–113
- Bauwe H (2023) Humboldt review: Photorespiration–Rubisco's repair crew. *J Plant Physiol* 280:153899
- Chen GY, Yong ZH, Liao Y, Zhang DY, Chen Y, Zhang HB, Chen J, Zhu JG, Xu DQ (2005) Photosynthetic acclimation in rice leaves to free-air CO₂ enrichment related to both ribulose-1, 5-bisphosphate carboxylation limitation and ribulose-1, 5-bisphosphate regeneration limitation. *Plant Cell Physiol* 46:1036–1045
- Cheng SH, Moore B, Seemann JR (1998) Effects of short-and long-term elevated CO₂ on the expression of ribulose-1, 5-bisphosphate carboxylase/oxygenase genes and carbohydrate accumulation in leaves of *Arabidopsis thaliana* (L.) Heynh. *Plant Physiol* 116:715–723
- Cho YH, Yoo SD, Sheen J (2006) Regulatory functions of nuclear hexokinase1 complex in glucose signaling. *Cell* 127:579–589
- Cooper AJL, Dorai T, Pinto JT, Denton TT (2022) The metabolic importance of the overlooked asparaginase II pathway. *Anal Biochem* 644:114084
- Cox J, Hein MY, Lubner CA, Paron I, Nagaraj N, Mann M (2014) Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol Cell Proteomics* 13:2513–2526
- Curtis TY, Bo V, Tucker A, Halford NG (2018) Construction of a network describing asparagine metabolism in plants and its application to the identification of genes affecting asparagine metabolism in wheat under drought and nutritional stress. *Food Energy Secur* 7:e00126
- Czarnocka W, Karpiński S (2018) Friend or foe? Reactive oxygen species production, scavenging and signaling in plant response to environmental stresses. *Free Radic Biol Med* 122:4–20
- Dijkwel PP, Kock PA, Bezemer R, Weisbeek PJ, Smeekens SC (1996) Sucrose represses the developmentally controlled transient activation of the plastocyanin gene in *Arabidopsis thaliana* seedlings. *Plant Physiol* 110:455–463
- Duursma RA (2015) Plantecophys-an R package for analysing and modelling leaf gas exchange data. *PLoS ONE* 10:e0143346
- Eom SH, Kim E, Hyun TK (2024) HXK, SnRK1, and TOR signaling in plants: unraveling mechanisms of stress response and secondary metabolism. *Sci Prog* 107:00368504241301533
- Espinoza-Corral R, Schwenkert S, Schneider A (2023) Characterization of the preferred cation cofactors of chloroplast protein kinases in *Arabidopsis thaliana*. *FEBS Open Biol* 13:511–518
- Farquhar GD, von Caemmerer SV, Berry JA (1980) A biochemical model of photosynthetic CO₂ assimilation in leaves of C₃ species. *Planta* 149:78–90
- Foyer C, Wang A, Shi K (2025) CO₂ signalling in plants. *Phil Trans R Soc Lond B Biol Sci* 380:20240247
- Friedrichs N, Shokouhi D, Heyer AG (2024) Flux calculation for primary metabolism reveals changes in allocation of nitrogen to different amino acid families when photorespiratory activity changes. *Int J Mol Sci* 25:8394
- Fürtauer L, Küstner L, Weckwerth W, Heyer AG, Nägele T (2019) Resolving subcellular plant metabolism. *Plant J* 100:438–455
- Guo M, Shi T, Duan Y, Zhu J, Li J, Cao Y (2015) Investigation of amino acids in wolfberry fruit (*Lycium barbarum*) by solid-phase extraction and liquid chromatography with precolumn derivatization. *J Food Compos Anal* 42:84–90
- Harley PC, Loreto F, Di Marco G, Sharkey TD (1992) Theoretical considerations when estimating the mesophyll conductance to CO₂ flux by analysis of the response of photosynthesis to CO₂. *Plant Physiol* 98:1429–1436

- Hoermiller II, Funck D, Schönewolf L, May H, Heyer AG (2022) Cytosolic proline is required for basal freezing tolerance in *Arabidopsis*. *Plant Cell Environ* 45:147–155
- Hymus GJ, Baker NR, Long SP (2001) Growth in elevated CO₂ can both increase and decrease photochemistry and photoinhibition of photosynthesis in a predictable manner. *Dactylis glomerata* grown in two levels of nitrogen nutrition. *Plant Physiol* 127:1204–1211
- IPCC (2021) Climate change 2021: the physical science basis. Contribution of working group I to the sixth assessment report of the intergovernmental panel on climate change; technical summary. Cambridge University Press, United Kingdom and New York, NY, USA
- Ishikawa K, Onoda Y, Hikosaka K (2007) Intraspecific variation in temperature dependence of gas exchange characteristics among *Plantago asiatica* ecotypes from different temperature regimes. *New Phytol* 176:356–364
- Jiang X, Koenig AM, Walker BJ, Hu J (2025) A cytosolic glyoxylate shunt complements the canonical photorespiratory pathway in *Arabidopsis*. *Nat Commun* 16:4057
- Knaupp M, Mishra KB, Nedbal L, Heyer AG (2011) Evidence for a role of raffinose in stabilizing photosystem II during freeze–thaw cycles. *Planta* 234:477–486
- Knight JS, Duckett CM, Sullivan JA, Walker AR, Gray JC (2002) Tissue-specific, light-regulated and plastid-regulated expression of the single-copy nuclear gene encoding the chloroplast Rieske FeS protein of *Arabidopsis thaliana*. *Plant Cell Physiol* 43:522–531
- Krämer K, Brock J, Heyer AG (2022a) Interaction of nitrate assimilation and photorespiration at elevated CO₂. *Front Plant Sci* 13:897924
- Krämer K, Kepp G, Brock J, Stutz S, Heyer AG (2022b) Acclimation to elevated CO₂ affects the C/N balance by reducing *de novo* N-assimilation. *Physiol Plant* 174:e13615
- Kromdijk J, Glowacka K, Long SP (2020) Photosynthetic efficiency and mesophyll conductance are unaffected in *Arabidopsis thaliana* aquaporin knock-out lines. *J Exp Bot* 71:318–329
- Landsky JM, Karowe DN (2014) Will chemical defenses become more effective against specialist herbivores under elevated CO₂? *Glob Chang Biol* 20:3159–3176
- Li P, Ainsworth EA, Leakey AD, Ulanov A, Lozovaya V, Ort DR, Bohnert HJ (2008) *Arabidopsis* transcript and metabolite profiles: ecotype-specific responses to open-air elevated [CO₂]. *Plant Cell Environ* 31:1673–1687
- Li TY, Shi Q, Sun H, Yue M, Zhang SB, Huang W (2021) Diurnal response of photosystem I to fluctuating light is affected by stomatal conductance. *Cells* 10:3128
- Loladze I (2014) Hidden shift of the ionome of plants exposed to elevated CO₂ depletes minerals at the base of human nutrition. *Elife* 3:e02245
- Lu Y, Savage LJ, Ajjawi I, Imre KM, Yoder DW, Benning C, DellaPenna D, Ohlrogge JB, Osteryoung KW, Weber AP, Wilkerson CG (2008) New connections across pathways and cellular processes: industrialized mutant screening reveals novel associations between diverse phenotypes in *Arabidopsis*. *Plant Physiol* 146:1482–1500
- Machingura M, Salomon E, Jez JM, Ebbs SD (2016) The β -cyanoalanine synthase pathway: beyond cyanide detoxification. *Plant Cell Environ* 39:2329–2341
- Marino G, Naranjo B, Wang J, Penzler JF, Kleint T, Leister D (2019) Relationship of GUN1 to FUG1 in chloroplast protein homeostasis. *Plant J* 99:521–535
- Medlyn BE, Badeck FW, De Pury DG, Barton CV, Broadmeadow M, Ceulemans R, De Angelis P, Forstreuter M, Jach ME, Kellomäki S, Laitat E (1999) Effects of elevated [CO₂] on photosynthesis in European forest species: a meta-analysis of model parameters. *Plant Cell Environ* 22:1475–1495
- Modde K, Timm S, Florian A, Michl K, Fernie AR, Bauwe H (2017) High serine:glyoxylate aminotransferase activity lowers leaf daytime serine levels, inducing the phosphoserine pathway in *Arabidopsis*. *J Exp Bot* 68:643–656
- Molnar-Perl I, Katona ZF (2000) GC-MS of amino acids as their trimethylsilyl/t-butyl dimethylsilyl derivatives: in model solutions III. *Chromatographia* 51:S228–S236
- Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Biol* 49:249–279
- Oswald O, Martin T, Dominy PJ, Graham IA (2001) Plastid redox state and sugars: interactive regulators of nuclear-encoded photosynthetic gene expression. *Proc Natl Acad Sci USA* 98:2047–2052
- Pons TL, Flexas J, Von Caemmerer S, Evans JR, Genty B, Ribas-Carbo M, Brugnoli E (2009) Estimating mesophyll conductance to CO₂: methodology, potential errors, and recommendations. *J Exp Bot* 60:2217–2234
- Pourtau N, Jennings R, Pelzer E, Pallas J, Wingler A (2006) Effect of sugar-induced senescence on gene expression and implications for the regulation of senescence in *Arabidopsis*. *Planta* 224:556–568
- Quackenbush J (2002) Microarray data normalization and transformation. *Nat Genet* 32:496–501
- Schweiger A, Awdamet FT, Drenckhan W, Berauer BJ (2022) Transgenerational effects of elevated CO₂: downregulation of photosynthetic efficiency and stomatal sensitivity to drought. *Authorea Preprints*. <https://doi.org/10.22541/au.166452123.39404860/v1>
- Serrano-Mislata A, Bencivenga S, Bush M, Schiessl K, Boden S, Sablowski R (2017) *DELLA* genes restrict inflorescence meristem function independently of plant height. *Nat Plants* 3:749–754
- Shen C, Zhang Y, Li Q, Liu S, He F, An Y, Zhou Y, Liu C, Yin W, Xia X (2021) *PdGNC* confers drought tolerance by mediating stomatal closure resulting from NO and H₂O₂ production via the direct regulation of *PdHXK1* expression in *Populus*. *New Phytol* 230:1868–1882
- Shi S, Xu X, Dong X, Xu C, Qiu Y, He X (2021) Photosynthetic acclimation and growth responses to elevated CO₂ associate with leaf nitrogen and phosphorus concentrations in mulberry (*Morus muliticaulis* Perr.). *Forests* 12:660
- Smet D, Depaepe T, Vandenbussche F, Callebert P, Nijs I, Ceulemans R, Van Der Straeten D (2020) The involvement of the phytohormone ethylene in the adaptation of *Arabidopsis* rosettes to enhanced atmospheric carbon dioxide concentrations. *Environ Exp Bot* 177:104128
- Smith NG, Keenan TF (2020) Mechanisms underlying leaf photosynthetic acclimation to warming and elevated CO₂ as inferred from least-cost optimality theory. *Abstr Global Change Biol* 26(9):5202–5216. <https://doi.org/10.1111/gcb.v26.910.1111/gcb.15212>
- Sobolevsky TG, Revelsky AI, Miller B, Oriedo V, Chernetsova ES, Revelsky IA (2003) Comparison of silylation and esterification/acylation procedures in GC-MS analysis of amino acids. *J Sep Sci* 26:1474–1478
- Sugiura D, Wang Y, Kono M, Mizokami Y (2024) Exploring the responses of crop photosynthesis to CO₂ elevation at the molecular, physiological, and morphological levels toward increasing crop production. *Crop Environ* 3:75–83
- Sunil B, Saini D, Bapatla RB, Aswani V, Raghavendra AS (2019) Photorespiration is complemented by cyclic electron flow and the alternative oxidase pathway to optimize photosynthesis and protect against abiotic stress. *Photosynth Res* 139:67–79
- Szklarczyk D, Nastou K, Koutrouli M, Kirsch R, Mehryary F, Hachilif R, Hu D, Peluso ME, Huang Q, Fang T, Doncheva NT (2025) The STRING database in 2025: protein networks with directionality of regulation. *Nucleic Acids Res* 53:D730–D737
- Tang X, Zhao J, Zhou J, Zhu Q, Sheng X, Yue C (2024) Elevated CO₂ shifts photosynthetic constraint from stomatal to biochemical

- limitations during induction in *Populus tomentosa* and *Eucalyptus robusta*. *Plants* 14:47
- Tetyuk O, Benning UF, Hoffmann-Benning S (2013) Collection and analysis of *Arabidopsis* phloem exudates using the EDTA-facilitated method. *J vis Exp* 80:51111
- Tian F, Yang DC, Meng YQ, Jin J, Gao G (2020) PlantRegMap: charting functional regulatory maps in plants. *Nucleic Acids Res* 48:D1104–D1113
- Timm S, Nunes-Nesi A, Parnik T, Morgenthal K, Wienkoop S, Keerber O, Weckwerth W, Kleczkowski LA, Fernie AR, Bauwe H (2008) A cytosolic pathway for the conversion of hydroxypyruvate to glycerate during photorespiration in *Arabidopsis*. *Plant Cell* 20:2848–2859
- Timm S, Florian A, Jahnke K, Nunes-Nesi A, Fernie AR, Bauwe H (2011) The hydroxypyruvate-reducing system in *Arabidopsis*: multiple enzymes for the same end. *Plant Physiol* 155:694–705
- Timm S, Nunes-Nesi A, Florian A, Eisenhut M, Morgenthal K, Wirtz M, Hell R, Weckwerth W, Hagemann M, Fernie AR, Bauwe H (2021) Metabolite profiling in *Arabidopsis thaliana* with moderately impaired photorespiration reveals novel metabolic links and compensatory mechanisms of photorespiration. *Metabolites* 11:391
- Tyanova S, Albrechtsen R, Kronqvist P, Cox J, Mann M, Geiger T (2016) Proteomic maps of breast cancer subtypes. *Nat Commun* 7:10259
- Von Caemmerer S (2000) Biochemical models of leaf photosynthesis. CSIRO Publishing, Victoria
- Von Caemmerer SV, Farquhar GD (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* 153:376–387
- Voss I, Sunil B, Scheibe R, Raghavendra AS (2013) Emerging concept for the role of photorespiration as an important part of abiotic stress response. *Plant Biol* 15:713–722
- Wang W, He EM, Chen J, Guo Y, Chen J, Liu X, Zheng HL (2016) The reduced state of the plastoquinone pool is required for chloroplast-mediated stomatal closure in response to calcium stimulation. *Plant J* 86:132–144
- Wiesner-Reinhold M, Nickel M, Graefe J, Schreiner M, Hanschen FS (2021) CO₂ treatment increases glucosinolate hydrolysis products in two *Arabidopsis thaliana* accessions. *J Appl Bot Food Qual* 1:94
- Xu Z, Jiang Y, Zhou G (2015) Response and adaptation of photosynthesis, respiration, and antioxidant systems to elevated CO₂ with environmental stress in plants. *Front Plant Sci* 6:701
- Yu J, Li XM, Wang WY, Gu KD, Sun CH, You CX, Hu DG (2022) Glucose sensor MdHXK1 activates an immune response to the fungal pathogen *Botryosphaeria dothidea* in apple. *Physiol Plant* 174:e13596
- Zhang Q, Lee J, Pandurangan S, Clarke M, Pajak A, Marsolais F (2013) Characterization of *Arabidopsis* serine:glyoxylate aminotransferase, AGT1, as an asparagine aminotransferase. *Phytochemistry* 85:30–35
- Zheng Y, Li F, Hao L, Yu J, Guo L, Zhou H, Ma C, Zhang X, Xu M (2019) Elevated CO₂ concentration induces photosynthetic down-regulation with changes in leaf structure, non-structural carbohydrates and nitrogen content of soybean. *BMC Plant Biol* 19(1):255
- Zuther E, Schulz E, Childs LH, Hinch DK (2012) Clinal variation in the non-acclimated and cold-acclimated freezing tolerance of *Arabidopsis thaliana* accessions. *Plant Cell Environ* 35:1860–1878
- Zuther E, Schaarschmidt S, Fischer A, Erban A, Pagter M, Mubeen U, Giavalisco P, Kopka J, Sprenger H, Hinch DK (2019) Molecular signatures associated with increased freezing tolerance due to low temperature memory in *Arabidopsis*. *Plant Cell Environ* 42:854–873

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