



Root PEPC-based carbon fixation enhances growth and provides limited osmotic stress tolerance in barley: a comparison with Arabidopsis

Liesel Gamarra Reinoso · Imre Majláth · Kamirán Áron Hamow ·
Mihály Dernovics · Csaba Éva

Received: 14 November 2025 / Accepted: 11 June 2026 / Published online: 25 June 2026
© The Author(s) 2026

Abstract

Background and Aims This study aimed to elucidate the mechanisms of root-based inorganic carbon (RIC) uptake and fixation in *Arabidopsis thaliana* and barley (*Hordeum vulgare* ‘Golden Promise’) and compare the physiological response of the two species under osmotic stress.

Methods Barley plants were grown in a growth-promoting inorganic carbon (IC)-containing hydroponic solution. Analyses included fluxomics, physiological measurements, amino acid profiling in leaves, and RNA sequencing. PEG-induced osmotic stress assays were performed for barley and Arabidopsis. Fixation of ^{13}C -labelled carbon into shoot dry mass was studied for *Arabidopsis thaliana* genotypes Col and high suberin-producing mutants *sultr1;2* and *esb1-1*.

Results IC treatment improved barley growth and net photosynthesis, though the effects were less pronounced than in Arabidopsis. In barley, IC fixation occurred mainly in roots via phosphoenolpyruvate carboxylase (PEPC), supported by fluxomic and transcriptome data. The high suberin-producing Arabidopsis mutants took up and fixed less ^{13}C from the hydroponic solution, but no change of suberin-related pathways was observed in the transcriptome of IC-treated barley. Amino acid profiling and transcriptomics in barley leaves revealed an increased use of pyruvate-derived amino acids (leucine, alanine, valine, and isoleucine), supporting protein synthesis and photosynthesis. In the leaves, genes related to cell division and cell expansion, and chloroplast biosynthesis were upregulated. Under PEG-induced osmotic stress, IC-supplementation improved the growth of Arabidopsis and some photosynthetic parameters of barley.

Conclusions The observed growth promotion in barley under root-IC treatment is driven by carbon uptake and fixation pathways that differ significantly from the mechanisms utilized by Arabidopsis.

Responsible Editor: Bruno Rosado.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11104-026-08762-7>.

L. Gamarra Reinoso · I. Majláth · K. Á. Hamow ·
M. Dernovics · C. Éva (✉)
Agricultural Institute, HUN-REN Centre for Agricultural
Research, Brunszvik u. 2, 2462 Martonvásár, Hungary
e-mail: eva.csaba@atk.hun-ren.hu

L. Gamarra Reinoso
PhD School of Biology, Eötvös Loránd University,
Pázmány Péter Sétány 1/A, 1117 Budapest, Hungary

Keywords Root inorganic carbon uptake and fixation · Barley (*Hordeum vulgare*) · Bicarbonate · Phosphoenolpyruvate carboxylase (PEPC) · Osmotic stress tolerance · Arabidopsis

Introduction

Soil CO₂ levels play a crucial role in the global carbon cycle and significantly influence ecosystem health, climate change, and agricultural productivity (Ahmad and Wang 2023). In both natural and agricultural ecosystems, the concentration of CO₂ in soil is often substantially higher than that in the atmosphere (Sotomayor and Rice 1999; Yonemura et al. 2009), primarily due to microbial respiration, root exudation, and decomposition of organic matter, resulting in elevated CO₂ levels in the rhizosphere compared to ambient air (Lin et al. 1999). In land plants, atmospheric CO₂ is fixed via the Calvin cycle (as a first step in C₃ and second step in C₄ plants), serving as the substrate for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). However, in the soil, depending on the pH, dissolved inorganic carbon (IC) can exist as CO₂ in pH levels between 0 and 8, or as bicarbonate (HCO₃[−]) at pH levels between 4 and 11, representing a potential external source of IC accessible to the roots (Shimono et al. 2019). Despite the availability of this root-accessible IC, the mechanisms by which plants take it up and fix it, and the effects on growth and metabolic regulation, remain largely unexplored.

The effects of bicarbonate on plant physiology are concentration-dependent and species-specific. Higher concentrations of bicarbonate, such as 20 mM NaHCO₃, have been associated with growth inhibition and iron deficiency in crops, such as barley, maize, and sorghum (Alhendawi et al. 1997). In *Vicia faba*, treatment with 50 mM NaHCO₃ led to oxidative stress, organic acid accumulation, and nutrient deficiencies, severely inhibiting growth (Sagervanshi et al. 2021). Likewise, in kiwifruit seedlings, 6.5 mM NaHCO₃ induced chlorosis and decreased chlorophyll and carotenoid contents, reflecting nutritional imbalances (Alizadeh et al. 2023). Falade (1972), investigated the effects of HCO₃[−] and pH on cation accumulation. Their findings indicated that bicarbonate enhances cation uptake (e.g., sodium and potassium), whereas elevated pH may inhibit it. Iron uptake, however, was reduced in bicarbonate-supplemented conditions. Notably, cytosolic pH of plant cells is maintained by the activity of bicarbonate, phosphate and protein buffers (Britto and Kronzucker 2005) and Bailey et al. (2023) found greater growth retardation effect of bicarbonate than high pH alone

in *Arabidopsis* which they attributed to the buffering effect of bicarbonate, which would compromise compensatory mechanisms serving to recalibrate both apoplastic and cytoplasmic pH as well as iron distribution and homeostasis.

Conversely to the often-negative effects of high concentration exogenous bicarbonate, low concentrations have demonstrated beneficial effects on several plant systems. For example, in *Coix lacryma-jobi*, treatment with 2 mM NaHCO₃ improved biomass accumulation, water metabolism, nutrient transport, and photosynthetic efficiency (Li et al. 2023). Similarly, in our previous study, the application of 2 mM NaHCO₃ at pH 5.6 resulted in enhanced growth and photosynthetic activity in *Arabidopsis thaliana* (Gamarra Reinoso et al. 2024). Transcriptomic analysis revealed that increased growth was associated with trehalose-6-phosphate (Tre6P) signaling and carbonic anhydrase (CA) activity. Furthermore, sulfate transporter mutants exhibited impaired growth compared to wild-type Columbia (Col-0), and nitrate transporters were upregulated in the transcriptomic data, suggesting a role for these transporters in root-based inorganic carbon uptake or in providing the necessary elements to faster-growing plants under carbon treatment.

In *Orychophragmus violaceus*, the combined application of 10 mM NaHCO₃ and 2 mM NH₄⁺ enhanced biomass in all plant organs without affecting net photosynthesis or intercellular CO₂, although the transpiration rate and stomatal conductance decreased. This treatment also promoted glucose metabolism and nitrogen utilization (Xia et al. 2023). In carob seedlings, supplementation with 10 mM NaHCO₃ and 40 μM Fe increased the leaf number, dry biomass, and root development without exacerbating iron chlorosis, suggesting a positive metabolic response (Gama et al. 2020).

IC taken up by roots may be transported up to the shoot system, in inorganic form for photosynthetic fixation (Shimono et al. 2019; Gamarra Reinoso et al. 2024). Alternatively, bicarbonate could be assimilated through phosphoenolpyruvate carboxylase (PEPC) in the roots and fed directly into the pyruvate, phosphoenolpyruvate and oxaloacetate (PPO) node, which has a central role in integrating IC with broader metabolic networks. PEPC catalyzes the fixation of HCO₃[−] with phosphoenolpyruvate (PEP) to form oxaloacetate (OAA), a central intermediate in the tricarboxylic

acid (TCA) cycle, amino acid biosynthesis, and fatty acid metabolism (Koendjibiharie et al. 2021). When PEP is limited, HCO_3^- can also be carboxylated with pyruvate by pyruvate carboxylase to generate OAA (Jitrapakdee et al. 2008). Moreover, acetyl-CoA can be carboxylated with HCO_3^- via acetyl-CoA carboxylase to produce malonyl-CoA, which initiates fatty acid biosynthesis (Sasaki and Nagano 2004).

As mentioned above, the fixation of HCO_3^- through the PEPC generates organic acids. This process is crucial for maintaining the carbon balance, especially under conditions where traditional photosynthetic pathways are less efficient (O'Leary et al. 2011). Under such stress, PEPC activity increases, leading to elevated malate production, an organic acid that helps to refix CO_2 released during photorespiration and contributes to osmotic regulation and energy supply (Basra and Malik 1985; Abadie and Tcherkez 2019). Organic acids not only support the TCA cycle and energy production but also act as biosynthetic intermediates, essential for cell expansion and growth (Basra and Malik 1985).

Barley (*Hordeum vulgare*) is the fourth most important cereal crop globally after wheat, rice, and maize. It is widely cultivated for its use in animal feed, malting, and human consumption. Barley thrives under diverse environmental conditions, including marginal lands (Verstegen et al. 2014). Previous studies investigating the effects of bicarbonate on barley primarily employed high concentrations, which are often inhibitory, thus limiting insights into growth-promoting responses at lower concentrations. Alhendawi et al. (1997) applied 0, 5, 10, and 20 mM NaHCO_3 to barley, maize, and sorghum and observed reductions in fresh and dry biomass, as well as root length. Maize showed the most severe root inhibition, whereas barley exhibited a moderate response. The iron concentrations declined across all species, although barley was the least affected. Organic acid concentrations in the roots, particularly malate, increased significantly in response to bicarbonate in the three species, peaking by day four of treatment. The accumulation of organic acids correlated with bicarbonate concentration, increasing between two and eleven times compared to the control without bicarbonate. Cram and Laties (1974) examined the exchange kinetics of bicarbonate and malate in root cells of barley. Their results suggest that bicarbonate, as a source of

CO_2 , contributes to malate synthesis and that malate uptake reaches a steady state over time, implying tightly regulated bicarbonate assimilation and CO_2 fixation mechanisms.

Supporting these findings, Vuorinen et al. (1992) demonstrated that malate is among the earliest organic acids labelled with ^{14}C , followed by citrate, pyruvate, and oxaloacetate. These intermediates are eventually converted into phosphorylated sugars, indicating active gluconeogenesis and glycolysis in the light (Walker et al. 2021). PEPC's activity is enhanced in the presence of bicarbonate and magnesium ions, which synergistically improve substrate binding and reduce feedback inhibition by malate (Parvathi et al. 1998; Chinthapalli et al. 2001; Abadie and Tcherkez 2019). Physiologically, preferential fixation of pyruvate over malate may mitigate the inhibitory effects of excessive malate on root elongation and TCA cycle function (Lee and Woolhouse 1969; Jacoby and Laties 1971). Moreover, pyruvate supports energy metabolism and serves as a precursor for acetyl-CoA and amino acids, sustaining vital biosynthetic processes even in the absence of photosynthesis (Splittstoesser 1966; Akram 2014).

A deeper understanding of how plants respond to elevated CO_2 and HCO_3^- levels in the soil is essential for improving nutrient use efficiency, enhancing stress resilience, and predicting plant responses to changing environmental conditions. While the effects of high HCO_3^- concentrations—such as those typically found in alkaline soils—have been well characterized in barley, there remains limited knowledge of the specific metabolic pathways and gene networks activated by growth-promoting bicarbonate treatment. In this study, we aimed to develop a low-concentration, growth-promoting bicarbonate treatment that promotes barley (*Hordeum vulgare* 'Golden Promise') growth, and to investigate its effects on carbon and nitrogen metabolism, key metabolic pathways, and physiological responses. Furthermore, through a comparative experiment using barley and the model plant *Arabidopsis thaliana*, we assessed the potential of bicarbonate supplementation to mitigate osmotic stress, hypothesizing that increased carbon supply at the root level may support plant growth when stomatal CO_2 uptake is limited. This research is particularly relevant in light of the global imperative to enhance crop productivity and resilience under drought conditions, one of the most significant challenges to global

food security and the growing human population (Furbank et al. 2020).

Materials and methods

Plant material and growth conditions

Barley (*Hordeum vulgare* ‘Golden Promise’) was used for most experiments, whereas *Arabidopsis thaliana* ecotype Columbia (Col-0) was included in the osmotic stress treatments. *Arabidopsis thaliana* ecotype Columbia (Col-0) and mutants *sultr1;2* (SALK_122974) and *esb1-1* (N2106042) were subjected to ^{13}C labeling experiments and determination of total shoot carbon content and $\delta^{13}\text{C}$. Plants were grown in a Phytotron chamber (Conviron, Winnipeg, Canada) used in previous publications (Gulyás et al. 2022; Gamarra Reinoso et al. 2024) under a 16-h photoperiod ($100\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$, 21/18 °C Day/night, 75% relative humidity) with illumination provided by L14 LED tubes (NS-12 spectrum; Valoya, Helsinki, Finland).

Bicarbonate and PEG treatments in barley and Arabidopsis

Barley (*Hordeum vulgare* ‘Golden Promise’) seedlings were surface-sterilized and germinated on wet, sterile filter paper in Petri dishes for five days before being transferred to hydroponic culture. A growth-promoting system was first optimized by testing sodium bicarbonate (NaHCO_3 ; Merck-Sigma Group, Darmstadt, Germany) at concentrations of 0 (control), 1, 2, 3, and 4 mM, and at two pH levels (5.6 and 7.2) in half-strength Hoagland medium (composition provided in Supplementary Table 1) for two weeks. The highest growth promotion was observed at 2 mM NaHCO_3 and pH 5.6, hereafter referred to as the “BIC” treatment, with a treatment for three weeks.

Following optimization for growth promotion in barley, seedlings were germinated in Petri dishes as previously described and then transferred to hydroponic systems containing half-strength Hoagland nutrient solution (pH 5.6). After a seven-day acclimation period, plants were exposed to the bicarbonate (BIC) treatment for three weeks, after which physiological and biochemical measurements were conducted.

For osmotic stress evaluation, barley plants were grown as described above. In contrast to the growth-promotion experiment, after the acclimation week, plants were divided into two groups: control and BIC-treated for two weeks. After it, these groups were further subdivided to apply osmotic stress, which was induced using polyethylene glycol (PEG 6000; Duchefa, Haarlem, The Netherlands). This experimental design resulted in four treatments: Control (0 mM NaHCO_3), BIC (2 mM NaHCO_3), PEG (15% PEG), and PEG + BIC (15% PEG + 2 mM NaHCO_3). Osmotic stress evaluation was performed for 10 days, during which the physiological and growth responses of the plants to the different treatments were assessed.

Arabidopsis thaliana seeds were surface-sterilized, cold-stratified, and germinated on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog Basal Salt Mixture, pH 5.7; Merck-Sigma Group) supplemented with 1% (w/v) sucrose (VWR International) in vertical square Petri dishes. After two weeks, seedlings were transferred to a hydroponic system containing quarter-strength MS salts, as described by Gamarra Reinoso et al. (2024). Following the same approach as for barley, plants were divided into two groups, and half were treated with the BIC treatment (2 mM NaHCO_3 , pH 5.6) for 11 days. For osmotic stress evaluation, polyethylene glycol (PEG 6000; 8%) was applied for seven days following the BIC treatment. The same treatment groups used for barley were applied to *Arabidopsis*, namely: Control (0 mM NaHCO_3), BIC (2 mM NaHCO_3), PEG (8% PEG), and PEG + BIC (8% PEG + 2 mM NaHCO_3).

Hydroponic cultures were established in black plastic boxes ($35 \times 25 \times 14$ cm; 9 L; polypropylene), aerated with household aquarium pumps. Each box contained 20 *Arabidopsis* plants or 15 barley plants. The nutrient solutions were changed every week. *Arabidopsis* plants were supported in cut 1.5 mL microcentrifuge tubes, and barley plants were placed in a commercial hydroponic planter kit.

Growth parameters

Root, third leaf, and shoot lengths, and rosette diameter were measured using a ruler. Fresh root weight, third leaf weight, rosette weight, and shoot weight were recorded using a precision scale (Sartorius CP4202S, Germany). These data were collected at the

end of the treatments from the growth standardization, BIC treatments, and osmotic stress treatments.

Gas exchange measurements

Physiological parameters were recorded at the end of growth promotion standardization, BIC, and osmotic stress treatments. Leaf gas exchange measures were performed on the 3rd well-developed leaf at the end of each treatment. The analysis was carried out using a CIRAS-3 portable photosynthesis system (PP Systems, Amesbury, MA, USA) with a narrow leaf chamber (1.75 cm²). The CO₂ fixation rates were investigated at 390 ppm CO₂ in an infrared gas analysis cuvette, with leaf and cuvette temperatures maintained using a Peltier element at 21 °C. The actinic light was provided by a CIRAS 3 LED unit (PPFD=250 µmol m⁻² s⁻¹, RGBW=60:5:25:10). For Arabidopsis, the parameters were followed as mentioned in Gamarra Reinoso et al. (2024).

Collected values for further analysis correspond to net photosynthetic rate (P_n), stomatal conductance (g_s), intracellular CO₂ concentration (C_i), and transpiration rate (E) were measured following a 5 min lag time after the system reached steady-state photosynthesis, and in minute 15th, data was recorded.

Determination of chlorophyll content and chlorophyll-a fluorescence

Chlorophyll contents were determined in 80% (v/v) acetone extracts using an UV–VIS spectrophotometer (Shimadzu) based on the method of (Porra et al. 1989). Chlorophyll-a fluorescence induction and quenching analyses were carried out for the growth standardization and osmotic stress treatments, which were performed using a pulse amplitude modulated fluorometer (PAM) with a blue LED array illumination unit IMAG-MAX/L ($\lambda=450$ nm) (Imaging PAM series, Walz, Effeltrich, Germany). After 30 min of dark adaptation, minimum (F_0), maximum (F_m), and variable fluorescence (F_v) as well as the F_v/F_m ratio were recorded with a 0.8 s saturation pulse (PPFD=3000 µmol m⁻² s⁻¹). Quenching analysis was performed under continuous blue actinic light (PPFD=250 µmol m⁻² s⁻¹) for 15 min, with saturation pulses applied every 30 s at the laboratory temperature (adapted state). Arabidopsis was measured as reported in Gamarra Reinoso et al. (2024).

The quantum yield of PSII [$Y(II)$], quantum yield of regulated non-photochemical quenching [$Y(NPQ)$], electron transport rate (ETR), and non-regulated energy dissipation [$Y(NO)$], non-photochemical quenching (NPQ) were calculated as described by (Klughammer and Schreiber 2008).

Fluxomic analysis

Samples of barley leaf and root were collected from plants that were grown under control (0 mM NaHCO₃) and BIC treatment, and at pH 5.6. The administration of labelled carbon (¹³C NaHCO₃, Merck-Sigma Group) was added to the growth solution of plants of 4-week-old seedlings, one day before sample collection. The treatments were divided as follows: control (not receiving any NaHCO₃), ¹²C-treated (receiving 2 mM ¹²C NaHCO₃, purchased from Merck-Sigma Group, carbon composition according to natural abundance: 1.1% ¹³C), and ¹³C-treated (receiving 2 mM ¹³C NaHCO₃, purchased from Merck-Sigma Group, containing 99% ¹³C).

The samples were ground in liquid nitrogen, and 0.150 g subsamples were weighed into 2 mL safety Eppendorf tubes with the addition of 1.5 mL of 1% (v/v) formic acid (VWR International, Radnor, Pennsylvania, US). After vortex mixing, the tubes were inserted into a pre-cooled (−18 °C) tube holder, and the extraction was carried out in a 1600 MiniG® Automated Tissue Homogenizer and Cell Lyser (SPEX; Rickmansworth, UK) for 3 min at 1500 rpm. The samples were then centrifuged at 15,000×g for 15 min at 4 °C, after which the supernatants were recovered and placed in separate Eppendorf tubes. The residues were re-extracted with 1.5 mL of 1% (v/v) formic acid in a MiniG® instrument (3 min), followed by centrifugation as described above. The matching supernatants were pooled, diluted 1:4 (v/v) with LC–MS grade acetonitrile (VWR), and then centrifuged at 15,000×g for 15 min at 4 °C. The supernatant was filtered through 0.22 µm pore-sized PTFE syringe filters before LC–MS analysis. LC–MS analysis of ¹³C sucrose and ¹³C aspartic acid was carried out as previously described by Gamarra Reinoso et al. (2024) with some modifications, namely for separation an Acquity Premiere BEH AMID (Waters; 150 mm×2.1 mm×1.7 µm) column was used. The applied gradient and the UPLC-ESI-MS/MS

parameters (Supplementary Table 2) are presented. Within the plant concentration of labelled sucrose and aspartic acid have been provided in μM .

Amino acid analysis

Leaf samples from 4-week BIC-treated and untreated (control) barley plants were dried for 24 h at 60 ± 2 °C in an LP-322/1 dryer (Labor-MIM, Esztergom, Hungary). The ground plant material (2 g) was used for subsequent analyses of amino acid composition at the Central Laboratory of Agricultural and Food Products, Faculty of Agriculture, Food Sciences and Environmental Management at the University of Debrecen, Hungary.

Protein concentration was determined following the Kjeldahl method (Lynch and Barbano 1999). After degradation in sulphuric acid and Se-containing catalyzer (VWR International Ltd., Lutterworth, Leicestershire, UK), ammonium was distilled (VEPL UDK 149 Distiller, VELP Scientifica, Usmate Velate, Italy) and titrated with an automatic titrator (Velp Titroline 5000, VELP Scientifica, Usmate Velate, Italy). The protein content was calculated from the nitrogen content using the conversion factor of 6.25 (Supplementary Table 3). The measurements were repeated with a coefficient of variation (CV)% < 10%. For amino acid analysis, 0.5 mg of the samples was hydrolysed with 5 M HCl at 105 ± 2 °C for 5 h (Memmert UN55, Buchenbach, Germany), then filtered through a regenerated cellulose filter (0.2 μm , Whatman Spartan syringe filter, Merck-Millipore) and diluted to an equal protein concentration. An automatic AAA 500 amino acid analyser (INGOS Ltd., Praha, Czech Republic), equipped with low-pressure ion-exchange chromatography and post-column derivatization with ninhydrin, was used to quantitatively determine amino acids. Photometric detection was carried out at wavelengths: 210 nm and 254 nm, respectively. Amino acid standard mixture (INGOS Ltd., Praha, Czech Republic) was used as a reference. The recovery was higher than 95%. The amount of amino acids was expressed as a percentage of the sample weight. Tryptophan content was not determined; and ASN plus ASP, and GLN plus GLU were measured together.

Transcriptome sequencing

Root and leaf samples of control and BIC-treated barley were collected at the end of two-week treatment. Total RNA was extracted from 70 mg samples in three replicates, homogenized in 500 μL of TRI agent (Zymo Research, Irvine, CA, USA). Total RNA was extracted using the Direct-zol RNA MiniPrep System (Zymo Research), according to the manufacturer's protocol, including the on-column DNase digestion. RNA concentrations were measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific), and the A260/A280 ratio was used to estimate the RNA quality.

Total RNA samples were subjected to quality control and custom sequencing. Samples were collected from different treatments as follows: these included L_CTRL (leaf control), L_BC (leaf BIC), R_CTRL (root control), and R_BC (root BIC).

All our samples passed quality control and had an RNA integrity number (RIN) of ≥ 6 . The general parameters of the analysis included the production of 150-bp paired-end reads and 13 Gbp raw data per sample on the Illumina NovaSeq 6000 platform (Novogene, Nanjing, China). The reads were mapped both to the barley reference genome *ensembl_56_hordeum_vulgare_morexv3_pseudomolecules_assembly_toplevel* and to the rice reference genome *ncbi_oryza_sativa_japonica_group_gca_009797565_1_osativa_kitaaake_v2_0*. Only differentially expressed genes (DEGs) with significant ($p < 0.05$) and $\log_2\text{FC} \geq |1|$ were involved in any further investigation, including gene ontology (GO) enrichment (Ashburner et al. 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa and Goto 2000) (org code: osa) analysis. These functional analyses were implemented using the clusterProfiler R package, in which the gene length bias was corrected. The enrichment bubble plot was constructed using SRplot (<https://www.bioinformatics.com.cn/en>). A graphical representation of the pathways activated during the growth promotion system in barley was created using Canva.

qPCR validation of the transcriptome sequencing

Total RNA was extracted as previously described. One microgram of each reaction was then reverse-transcribed using random primers and the RevertAid

First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The obtained cDNA was diluted threefold and used as a template for qPCRs. Expression of six root-based transporter genes (nitrate and phosphate transporters, as well as the S-type anion channel *SLAH3*) was analyzed. The reactions were run in triplicate using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and PCRBIO SyGreen Mix (PCR Biosystems, London, UK). The barley alpha-tubulin 2 gene was used as a normalizer (Schröder et al. 2001; Éva et al. 2018). The gene expression was normalized for these transporter genes and compared between qPCRs and transcriptome sequencing. The transcription levels of the studied genes were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) with an efficiency correction step applied according to (Pfaffl 2004). Primers used for qPCR validation are listed in Supplementary Table 4. The primers used to amplify the HORVU3Hr1G066090/HORVU_MOREX_r3_3HG0283350 gene were obtained from Hajibarat et al. (2023).

¹³C-labelling shoot $\delta^{13}\text{C}$ stable isotope measurement

Plants were cultivated following Gamarra Reinoso et al. (2024); however, once transferred to the hydroponic systems, the plants underwent a three-week control phase (1/4 strength MS-salts, pH 5.6) followed by a one-week treatment with 2 mM ¹³C NaHCO₃. Total shoot samples were then taken, these were lyophilized and 50 mg samples have been sent to Isotopech Zrt. (Debrecen, Hungary) for determining $\delta^{13}\text{C}$ values against the Vienna Pee Dee Belemnite (VPDB) reference. Stable carbon isotope ratios ($\delta^{13}\text{C}$) were measured using a Thermo Scientific™ EA IsoLink™ Elemental Analyzer (Flash EA configuration) coupled to a Thermo Finnigan Delta^{PLUS} XP continuous-flow isotope ratio mass spectrometer (IRMS). The system operates in flash dynamic combustion mode, which produces complete combustion of the sample within a high temperature oxidation reactor at 1020 °C, resulting in the quantitative conversion of all sample carbon to carbon dioxide (CO₂). The resulting CO₂ gas is separated from other gases produced during combustion on a gas chromatographic column and carried by helium into the IRMS for isotopic measurement.

Powdered leaf samples were weighed (0.25–0.35 mg) into aluminum capsules along with small portion of vanadium pentoxide (V₂O₅) to increase their oxidation efficiency and introduced into the autosampler. The carbon isotope composition is reported in conventional delta (δ) notation as:

$$\delta^{13}\text{C}\left(\frac{0}{00}\right) = \left(\frac{R_{\text{sample}}}{R_{\text{reference}}} - 1\right) \cdot 1000 \quad (1)$$

where R is the measured ¹³C/¹²C isotope ratio in the sample or in the reference material.

All $\delta^{13}\text{C}$ values are reported relative to the VPDB scale through normalization using internationally distributed reference materials IVA33802174; IAEA-600; USGS88; USGS41a.

Statistical analysis

All results are expressed as mean ± SD. Fluxomic and amino acid analyses were performed with four biological replicates (n=4) and total chlorophyll content in barley with three biological replicates (n=3). Data were compared using Student's t -test ($p \leq 0.05$).

For the osmotic stress assays performed for barley, plants were grown in two boxes (15 plants each; n=30). Gas exchange was measured in five plants per treatment (n=5), and chlorophyll fluorescence was determined in 30 barley plants (n=30); growth data were taken from the 30 plants per treatment. For growth assays, *Arabidopsis thaliana* plants were cultivated in two independent boxes (20 plants each; n=40). Gas exchange was measured in five plants per treatment (n=5), and chlorophyll fluorescence was determined in 15 *Arabidopsis* (n=15).

Normality and homogeneity of variances were verified with the Shapiro–Wilk and Levene's tests, respectively. When both assumptions were met, data were analyzed by one-way ANOVA, followed by Tukey's HSD test for post hoc comparisons (ETR (barley), intracellular C_i (barley), P_n (barley and Arabidopsis), g_s (barley and Arabidopsis), E (barley and Arabidopsis), root weight (barley), shoot length (barley), Y(NPQ) (Arabidopsis), F_v/F_m (Arabidopsis), Y(II) (Arabidopsis). When homogeneity was not met, the Games–Howell test was applied: root length (barley and Arabidopsis), root weight (Arabidopsis), shoot weight (barley), third leaf weight (barley), Y(NO) (barley), rosette diameter (Arabidopsis),

rosette weight (*Arabidopsis*)). For data that did not meet normality assumptions, the Kruskal–Wallis test followed by Dunn’s test with Bonferroni correction was used (NPQ (barley), Fv/Fm, Y(II) (barley), third leaf length (barley)). Statistical significance was accepted at $p < 0.05$.

For growth-promoting essays for barley, gas exchange ($n=4$), chlorophyll induction ($n=5$), and shoot biomass ($n=8$) were quantified. Two-way ANOVA was performed followed by Tukey post hoc test. The $\delta^{13}\text{C}$ stable isotope data ($n=5$) were evaluated using one-way ANOVA followed by Tukey post hoc test. Statistical significance was established at $p < 0.05$.

The statistical analyses were conducted in RStudio (version 4.3.0).

Results

Optimization of the growth promotion in barley

We first optimized a bicarbonate-based growth-promotion system for barley (*Hordeum vulgare* ‘Golden Promise’). Previous studies have reported that low concentrations of exogenous NaHCO_3 can stimulate plant growth. To evaluate this effect in barley, seedlings were cultivated in a modified half-strength Hoagland solution supplemented with different NaHCO_3 concentrations (0 mM (control), 1, 2, 3, and 4 mM) at two pH levels (5.6 and 7.2) for two weeks. Shoot fresh weight (Fig. S1a) and photosynthetic parameters, including gas exchange and chlorophyll-*a* fluorescence (Figs. S2–S3), were measured. Growth promotion was only observed when 2 mM NaHCO_3 was added, and the pH was 5.6, although the increase was not statistically significant at a 95% confidence level after two weeks of treatment. Net photosynthetic and transpiration rates increased significantly under this treatment, while other parameters did not differ significantly from the control. The experiment was then repeated with a three-week exposure using only 2 mM NaHCO_3 at pH 5.6. Under these conditions, shoot biomass was significantly higher in the bicarbonate-treated plants compared with the control (Fig. S1b). Total chlorophyll content of the leaves was also measured between the control (0 mM NaHCO_3) and the treatment with 2 mM NaHCO_3 (BIC), and it significantly increased as result of the BIC-treatment

($p < 0.01$) (Fig. S1c). Therefore, we decided to follow our study with half-strength Hoagland medium, supplemented with 2 mM NaHCO_3 , and pH 5.6 in a hydroponic system, as our growth promotion treatment (BIC treatment) for barley.

Fluxomics

For fluxomic analysis, leaf and root samples were collected from around four and half-week-old plants that had been treated for three weeks with the BIC treatment. The hydroponic solution was renewed 24 h before sampling, at which point the ^{13}C -labelled group received the labelled solution.

Aspartic acid, produced via the transamination of OAA, itself a product of phosphoenolpyruvate carboxylase (PEPC), was used as a marker of PEPC-mediated carbon fixation, which can operate in both photosynthetic and non-photosynthetic tissues. In contrast, sucrose, the main photoassimilate in land plants, serves as an indirect marker of Rubisco-mediated carbon fixation, which is typically restricted to the photosynthetic tissues.

^{13}C -labelled sucrose was detected only in the leaves, which is consistent with the function of Rubisco. However, its concentration did not vary significantly among the control, non-labelled, and labelled samples (Fig. 1a, Supplementary Table 5), suggesting that the labelled carbon supplied to the roots was not fixed into sucrose during the treatment. In contrast, the ^{13}C -labelled aspartic acid was detected only in roots of the labelled treatment group, at a concentration of 0.06 μM (Fig. 1b, Supplementary Table 5), indicating that PEPC-mediated carbon fixation occurred in root tissues under these conditions and may represent a specific metabolic response to NaHCO_3 supplementation.

Transcriptomic analysis

This transcriptomic analysis aimed to identify pathways, genes, transporters, and channels potentially involved in root-based uptake, translocation and fixation of inorganic carbon, as CO_2 or HCO_3^- , in barley. Although the mechanisms underlying carbon uptake in plants remain largely unclear, this study specifically examined their role in barley. To investigate carbon fixation and its effects, RNA-Seq analysis was performed on the roots and leaves of 4-week-old

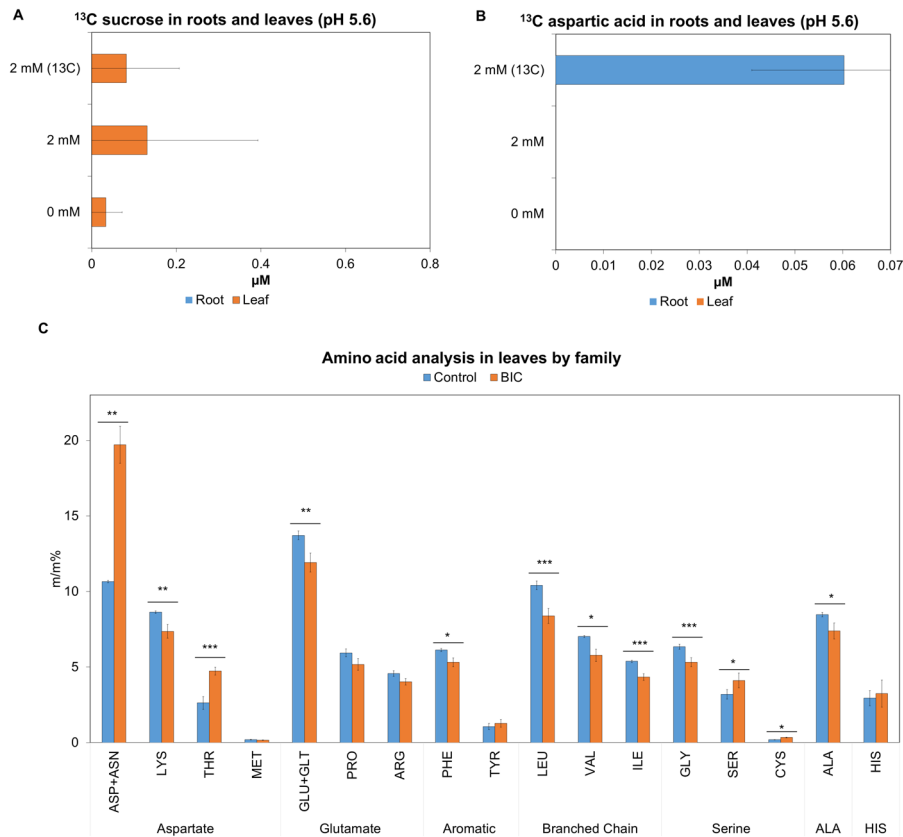


Fig. 1 Fluxomic analysis and amino acid content in barley under NaHCO_3 treatment. Barley (*Hordeum vulgare* 'Golden Promise') plants were subjected to 0 mM NaHCO_3 (Control) or 2 mM NaHCO_3 treatment (BIC). A and B display fluxomic data obtained from leaves and roots under three conditions: Control, BIC (Treated), and BIC supplemented with ^{13}C -labelled 2 mM NaHCO_3 (Treated with labelling). **A.** Shows the relative content of ^{13}C sucrose concentration. **B.** Shows the relative content of ^{13}C aspartic acid concentration

in roots and leaves. These fluxomic data are represented by the mean $\pm$ standard deviation ($n=4$) and were analyzed using one-way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$) different lowercase letters indicate $p \leq 0.05$. **C.** Shows the total amino acid content measured in leaves from Control and BIC-treated plants. This analysis represents the mean $\pm$ standard deviation ($n=4$) and was assessed with a Student's *t*-test. Significance levels for the Student's *t*-test are indicated by asterisks: $p \leq 0.05$ (*), $p \leq 0.005$ (**), and $p \leq 0.0005$ (***)

barley plants grown under control (0 mM NaHCO_3 , pH 5.6) and BIC (2 mM NaHCO_3 , pH 5.6) conditions in triplicate.

Supplementary Table 6 shows that RNA purification and sequencing were of high quality, yielding high RNA integrity numbers ($\text{RIN} \geq 6$), clean read frequencies of 98–99%, and uniquely mapped reads of 90–91%. The error rates were below 0.03% for all samples.

Co-expression analysis using Venn diagrams revealed 17,802 genes that were commonly expressed in the leaves of control and treated plants and 19,835 genes in the roots. In leaves, 698 genes were unique to the control condition and 426 to the treatment

condition (Fig. 2a). In the roots, 772 genes were unique to the control and 405 to the treated roots, with 19,835 genes expressed under both conditions (Fig. 2b).

Differential expression analysis, based on $\log_2\text{FC} \geq 1$ (upregulation), $\log_2\text{FC} \leq -1$ (downregulation), and adjusted p -value ≤ 0.05 , identified 1,376 differentially expressed genes (DEGs) in the leaves, of which 230 were upregulated and 1,146 were downregulated as result of the bicarbonate treatment (Fig. 2c). In the roots, 2,675 DEGs were detected, of which 729 were upregulated and 1,946 were downregulated (Fig. 2d). Notably, leaves exhibited a predominance of downregulated genes

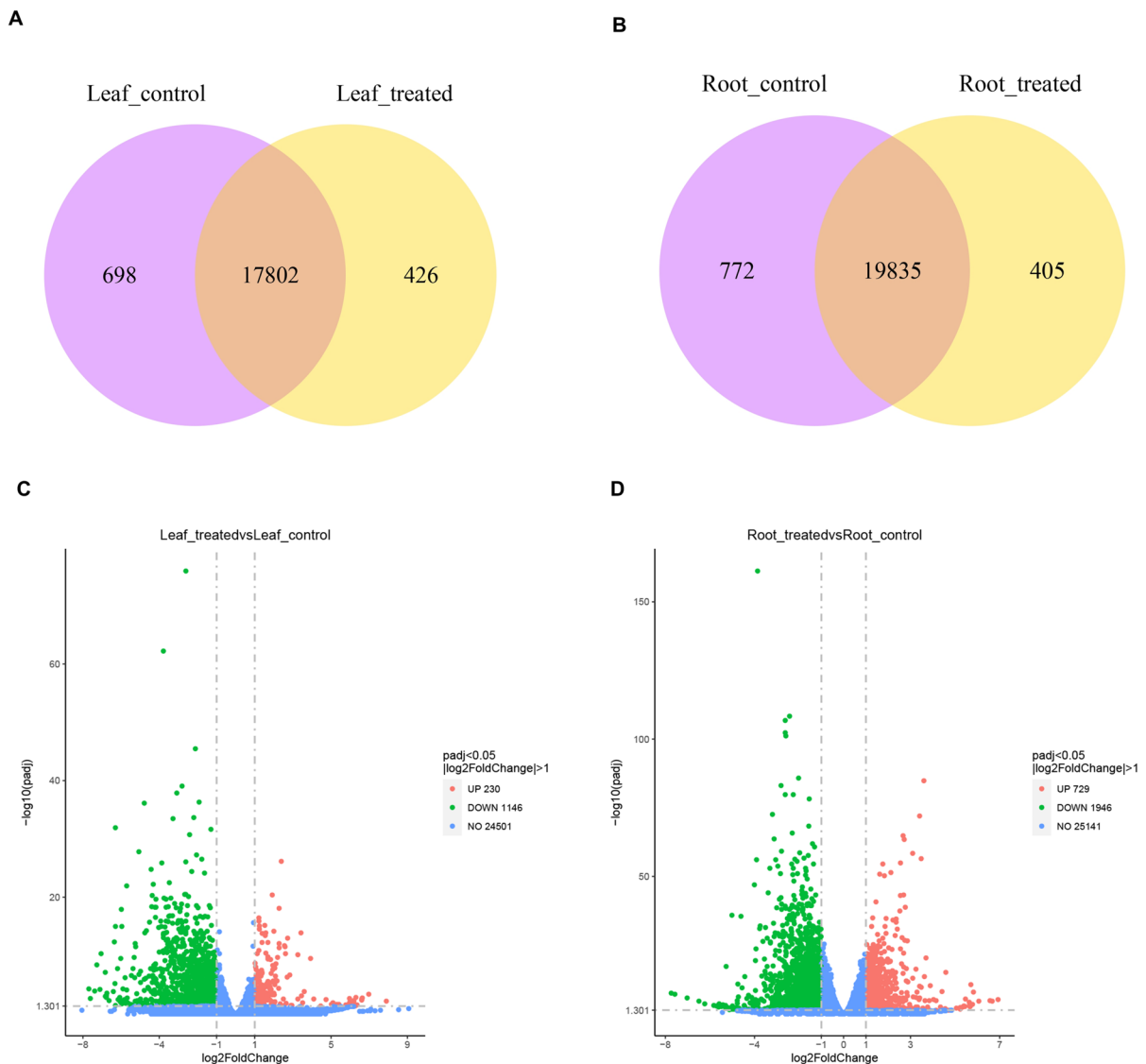


Fig. 2 Differential gene expression analysis in barley (*Hordeum vulgare* ‘Golden Promise’) leaves and roots under control and BIC treatments. Plants were grown under control (0 mM NaHCO_3) or BIC (2 mM NaHCO_3) conditions for 3 weeks. Differentially expressed genes (DEGs) were defined as those

with $|\log_2\text{FC}| \geq 1$ and an adjusted p adjusted value ≤ 0.05 . **A.** Venn diagram showing DEGs identified in leaves. **B.** Venn diagram showing DEGs identified in roots. **C.** Volcano plot illustrating gene expression changes in leaves. **D.** Volcano plot illustrating gene expression changes in roots

under treatment, suggesting that transcriptional repression is a major response to elevated bicarbonate levels in the aerial tissues.

To investigate which pathways or physiological processes were activated under the performed mild bicarbonate treatment, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analyses were

performed for leaves and roots (C, control; T, treated).

In leaves, 19 downregulated GO terms included biological processes (BP), such as response to auxin (GO:0009733), response to organic substances (GO:0010033), response to hormone (GO:0009752), and cell communication (GO:0007154); cellular components (CC), such as cell wall (GO:0005618), cell

periphery (GO: 007144) and apoplast (GO:0048046); and molecular functions (MF), including DNA-binding transcription factor activity (GO:0003700), calcium ion binding (GO:0005509), ubiquitin-protein transferase activity (GO:0004842), and hexosyl transferase activity (GO:0016758) (Fig. 3a, Supplementary Table 7). In leaves, there were 18 upregulated GO terms, from which we can highlight the following: for BP, metal ion transport (GO:003001), and cation transport (GO:0006812); CC included nucleosome (GO:0000786), DNA packaging complex (GO:0044815), non-membrane-bounded organelles (GO:0043228), chromatin (GO:0000785) and chromosome (GO: 0005694); and for MF, metal ion transmembrane transporter activity (GO:0046873), cation transmembrane transporter activity (GO:0008324), and inorganic molecular entity transmembrane

transporter activity (GO:0015318) (Fig. 3b, Supplementary Table 8). These results indicated that metal and cation transport-related genes, including zinc and potassium transporters, were upregulated, whereas hormone signaling, particularly auxin responses, was repressed. Interestingly, leaves upregulated nucleosome, chromatin and DNA packaging closely related to cell division processes taking place in the leaf. These regulators changes were accompanied with downregulation of cell wall and ubiquitin transferase activity. In the leaves, seven KEGG pathways showed decreased activity under treatment, including plant-pathogen interaction (osa04626), plant hormone signal transduction (osa04075), phenylpropanoid biosynthesis (osa00940), glutathione metabolism (osa00480), MAPK signaling (osa04016), phenylalanine metabolism (osa00360), and carotenoid

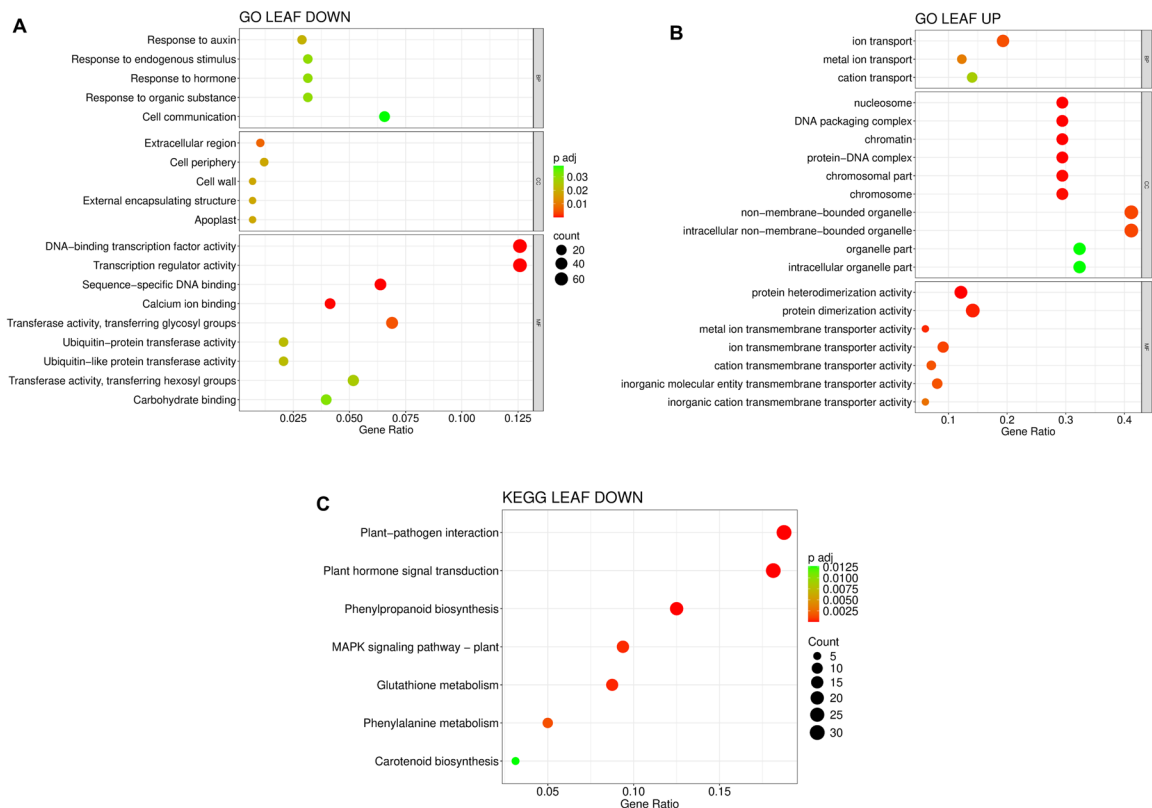


Fig. 3 Functional enrichment analysis of differentially expressed genes (DEGs) in barley leaves. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed for DEGs identified in leaves of barley (*Hordeum vulgare* ‘Golden Promise’) subjected to control (0 mM NaHCO₃) and BIC (2 mM NaHCO₃)

treatment. Differentially expressed genes were defined as those with $|\log_2\text{FC}| \geq 1$ and an adjusted p value ≤ 0.05 . **A.** Significantly enriched GO terms among downregulated genes in leaves. **B.** Significantly enriched GO terms among upregulated genes in leaves. **C.** Significantly enriched KEGG pathways associated with downregulated genes in leaves

biosynthesis (osa00906), (Fig. 3c, Supplementary Table 9) with no significantly upregulated pathways ($p < 0.05$) detected.

In the roots, 39 downregulated GO terms encompassed BP, including protein ubiquitination (GO:0016567), defense response (GO:0006952), aminoglycan metabolic and catabolic processes (GO:0006022, GO:0006026), cell wall macromolecule metabolic (GO:0044036), and catabolic (GO:0016998) processes. For CC, included

oxidoreductase complex (GO:1,990,204) and extrinsic component of membrane (GO:0019898), and MF included DNA-binding transcription factor activity (GO:0003700), transcription regulator activity (GO:0140110), electron transfer activity (GO:0009055), polysaccharide binding (GO:0030247) and calcium and iron ion binding (GO:0005509, GO:0005506) (Fig. 4a, Supplementary Table 10). Sixty GO-terms were upregulated including some important BP terms such

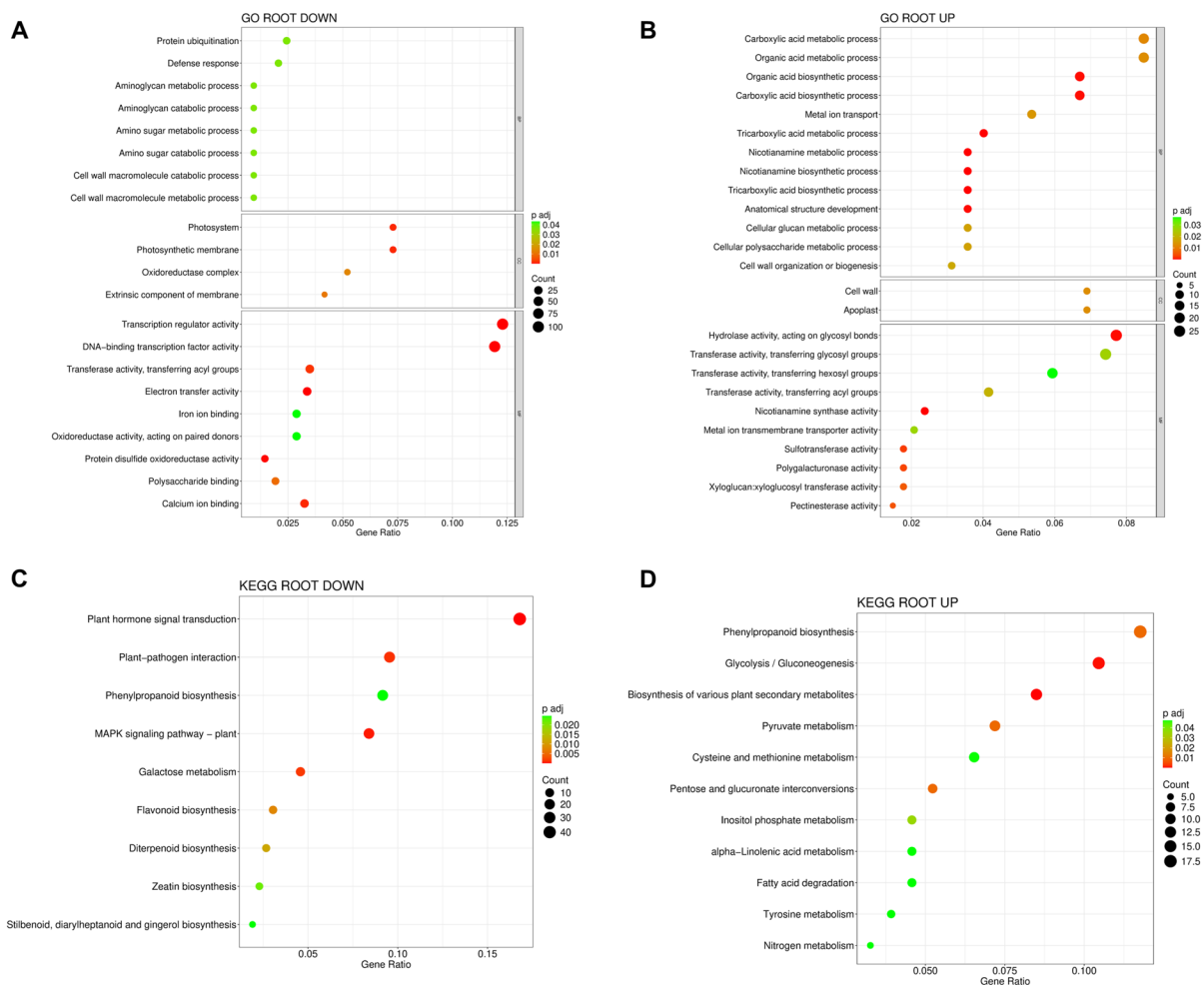


Fig. 4 Functional enrichment analysis of differentially expressed genes (DEGs) in roots of barley (*Hordeum vulgare* ‘Golden Promise’) under control and BIC treatments. Plants were grown under control (0 mM NaHCO_3) or BIC (2 mM NaHCO_3) conditions for 3 weeks. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed to identify significantly affected biological processes and pathways. DEGs

were defined as genes with $|\log_2\text{FC}| \geq 1$ and an adjusted p value ≤ 0.05 . **A.** Enriched GO terms associated with downregulated genes in roots. **B.** Enriched GO terms associated with upregulated genes in roots. **C.** Significantly enriched KEGG pathways associated with downregulated genes. **D.** Significantly enriched KEGG pathways associated with upregulated genes

as nicotianamine metabolism and biosynthesis (GO:0030417, GO:0030418), tricarboxylic acid biosynthetic and metabolic processes (GO:0072350, GO:0072351), amine biosynthetic process (GO:0009308), anatomical structure development (GO:0048856), organic acid biosynthetic process (GO:0016053), metal ion transport (GO:0030001), DNA replication (GO:0006260), response to auxin (GO:0009733), cell wall organization or biogenesis (GO:0071554) and polysaccharide metabolic process (GO:0005976); CC included apoplast (GO:0048046) and cell wall (GO:0005618); MF included hydrolase activity on glycosyl bonds (GO:0016798), sulfotransferase activity (GO:0008146), polygalacturonase and pectinesterase activity (GO:0004650, GO:0030599), xyloglucan:xyloglucosyl transferase activity (GO:0016762), heme binding (GO:0020037) and metal ion transmembrane transporter activity (GO:0046527) (Fig. 4b, Supplementary Table 11). These findings suggest that roots respond to bicarbonate treatment by modifying the cell wall composition, enhancing energy metabolism, and activating transport processes.

In the roots, nine KEGG pathways were down-regulated, including plant hormone signal transduction (osa04075), MAPK signaling (osa04016), galactose metabolism (osa00052), flavonoid biosynthesis (osa00941), zeatin biosynthesis (osa00908), and phenylpropanoid biosynthesis (osa00940) (Fig. 4c, Supplementary Table 12). Eleven upregulated root pathways included the biosynthesis of various secondary metabolites (osa00999), glycolysis/gluconeogenesis (osa00010), pentose and glucuronate interconversions (osa00040), pyruvate metabolism (osa00620), inositol phosphate metabolism (osa00562), cysteine and methionine metabolism (osa00270), nitrogen metabolism (osa00910), tyrosine metabolism (osa00350), and fatty acid degradation (osa00071) (Fig. 4d, Supplementary Table 12).

An overview of the physiological effects of the BIC treatment in barley is presented in Fig. 5a, with the proposed metabolic pathways for root-level inorganic carbon fixation detailed in Fig. 5b. Key genes associated with the PEP-OAA-Pyruvate (PPO) node and the subsequent leaf-level responses are summarized in Tables 1 and 2, respectively.

Integration of GO and KEGG functional analyses with fluxomic data concluded that barley roots primarily assimilate exogenous inorganic carbon

through the PPO node. Within this framework, pyruvate emerges as the central metabolic hub sustaining plant metabolism under BIC conditions. Our model (Fig. 5a) identifies potential bicarbonate and CO₂ transporters, likely members of the MFS superfamily, such as *NRT2;1*, alongside specific aquaporin genes, which regulate the influx and exert significant control over inorganic carbon assimilation. While we did not observe the upregulation of β carbonic anhydrases (β CA) in our transcriptomic data, we identified the presence of α CA7 and γ CA1. These enzymes likely modulate the interconversion of CO₂ and HCO₃⁻, adjusting to environmental pH and cellular demands to facilitate carbon availability. Once assimilated, inorganic carbon enters the PPO node, branching into two primary metabolic streams: organic acids (specifically aspartic acid, but also possible malate and citrate) and amino acids (predominantly alanine).

The influx of inorganic carbon was accompanied by a synergistic increase in the uptake of N, Fe, K⁺, and Zn²⁺. Metal cations were chelated with nicotianamine for efficient xylem transport to the shoot. Upon reaching the leaves, these root-derived elements provide the carbon and nitrogen skeletons, required to drive cell division, chloroplast biogenesis, and cell wall expansion. These elements may support these processes through direct metabolic incorporation or via indirect signaling pathways.

Both the TCA cycle and glycolysis/gluconeogenesis play critical roles in maintaining the energy metabolism and the supply of PEP during inorganic carbon supplementation in the roots. Downstream of glycolysis, myo-inositol metabolism toward inositol pyrophosphates may act as post-translational effectors. Genes upregulated in this pathway participate in cellular morphogenesis, DNA synthesis, cell cycle regulation, signal transduction, secretion, and vesicular trafficking. Phosphatidylinositol-4-phosphate 5-kinases also play important roles in ion channel regulation, membrane dynamics, and actin cytoskeleton organization (Sharma et al. 2020).

The upregulation of nicotianamine biosynthesis is important for maintaining iron and zinc transport, which is essential for preserving barley homeostasis. Amines contribute to plant growth and stress adaptation. Sulfotransferases are important for hormone modification and detoxification as well as for regulating root structure and stress signaling. Enzymes involved in cell wall remodeling, including

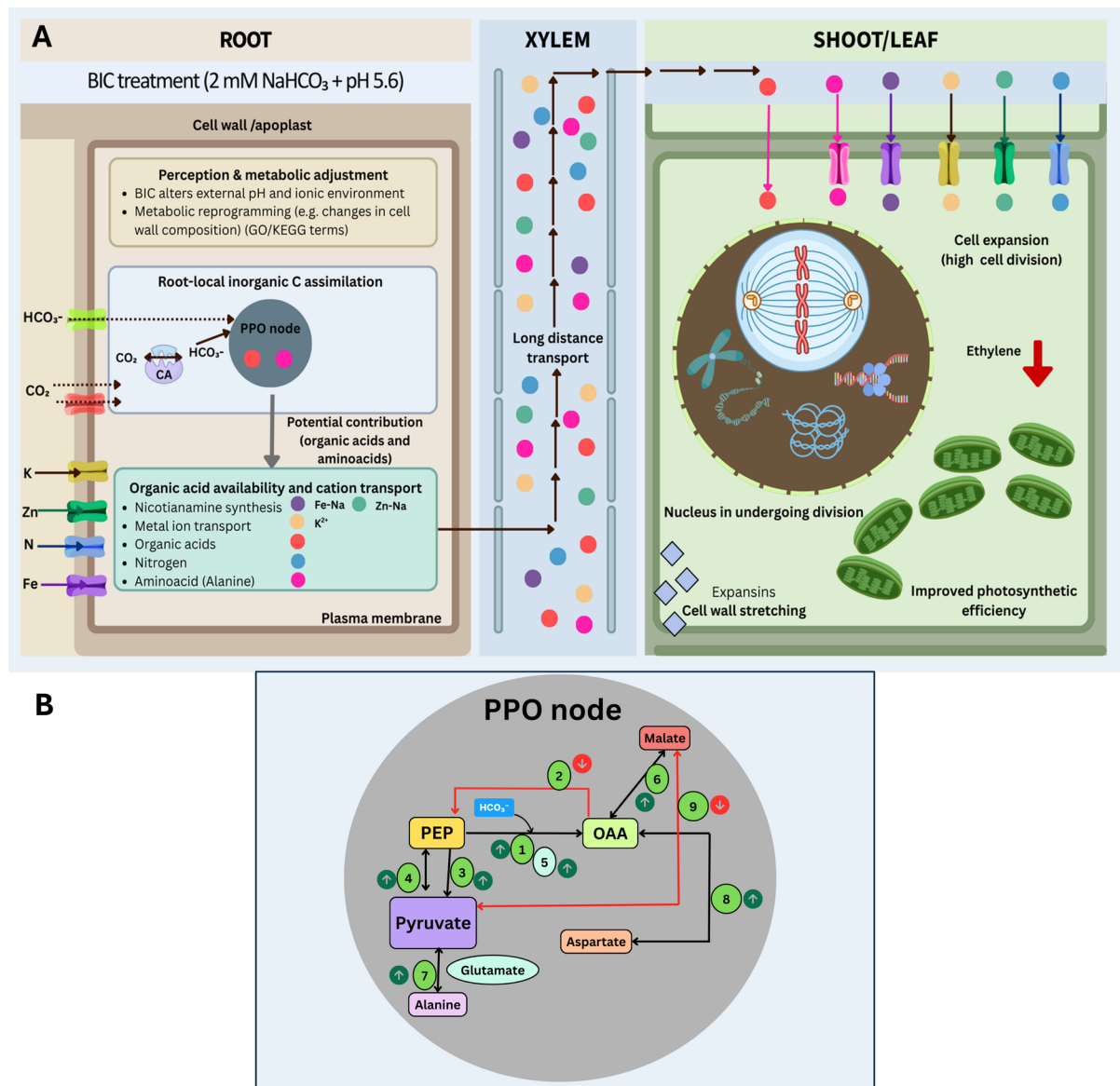


Fig. 5 Proposed model of inorganic carbon (IC) uptake, fixation and metabolic responses in hydroponically grown barley plants under bicarbonate (BIC) treatment (2 mM NaHCO_3 , pH 5.6, half-strength Hoagland solution) based on transcriptomic and fluxomic analysis. **(A)** Proposed pathway of IC assimilation and transport. The left panel illustrates root-level responses: BIC alters pH and ionic balance, driving metabolic reprogramming. Potential IC uptake mechanisms include direct transport (e.g., via NRT or MFS superfamily transporters), transport as CO_2 via aquaporins, or diffusion into the cortex followed by conversion of CO_2 to HCO_3^- via αCA7 or γCA1 . Once converted, HCO_3^- is fixed at the PPO (phosphoenolpyruvate-pyruvate-oxaloacetate) node into organic acids (e.g., aspartate, malate) or supports amino acid synthesis (e.g., alanine) for subsequent transport to the shoots. Upregulation of K,

Zn, N, and Fe transporters facilitates the movement of nitrogen and metal ions, including nicotianamine-metal complexes. The middle panel depicts the xylem, which facilitates the long-distance transport of these organic acids, amino acids, and ions. The right panel summarizes shoot/leaf responses: root-derived metabolic products may act as signals to promote nuclear division and chloroplast synthesis while decreasing ethylene production. This, alongside the strong upregulation of expansins, likely drives the observed growth promotion under BIC treatment. **(B)** Detailed representation of IC fixation in the roots via the PPO node. Numbers correspond to specific enzymes detailed in Table 1. Upward dark green arrows indicate enzyme upregulation, and downward red arrows indicate downregulation

Table 1 Genes present in the pyruvate, phosphoenolpyruvate and oxaloacetate (PPO) node in the barley roots

	Gene ID	Name of the gene	Short name	GE Control (Roots)	GE treated (Roots)	Log ₂ FC
1	HORVU_MOREX_r3_3HG0290090	Phosphoenolpyruvate carboxylase	<i>PEPC</i>	48.91	104.36	1.09
2	HORVU_MOREX_r3_4HG0391040	Phosphoenolpyruvate carboxykinase	<i>PEPCK</i>	2807.05	1111.82	−1.34
3	HORVU_MOREX_r3_2HG0209910	Pyruvate kinase	<i>PK</i>	4118.19	8701.24	1.08
4	HORVU_MOREX_r3_1HG0062940	Pyruvate, phosphate dikinase	<i>PPDK</i>	1539.74	5281.69	1.78
5	HORVU_MOREX_r3_6HG0627600	Phosphoenolpyruvate carboxylase kinase	<i>PEPCK</i>	87.56	227.71	1.38
6	HORVU_MOREX_r3_1HG0043190	Malate dehydrogenase (cytoplasm)	<i>MDH</i>	36,559.75	52,754.86	0.53
7	HORVU_MOREX_r3_1HG0019260	Alanine aminotransferase	<i>ALT</i>	6420.88	14,501.33	1.17
8	HORVU_MOREX_r3_3HG0290290	Aspartate aminotransferase (cytosol)	<i>AST</i>	16,899.29	32,831.16	0.96
9	HORVU_MOREX_r3_3HG0285860	Malic enzyme (cytosol)	<i>ME</i>	4069.29	2971.16	−0.45

pectinesterases, polygalacturonases, and xyloglucans, were also upregulated in the roots.

In addition to the above functional analyses, individual DEGs were monitored, which may be relevant for root-based carbon uptake, fixation or metabolic changes. The full DEG tables are provided as Supplementary Tables 14,15,16, and 17. In leaves, upregulated transporters included zinc transporters (*ZIP3*, *ZIP9*, *ZIP5*), potassium transporters (*HAK5*, *HAK1*), and channels (*SLAC1*, *NIP3;1*) (Supplementary Table 18). In the roots, upregulated transporters included zinc transporters (*ZIP9*, *ZIP3*, *ZIP9*, *ZIP5*, *ZIP8*), nitrogen-related transporters (*NRT2.3*, *NRT2.1*, *NPF6.3*, *NPF8.3*, *NPF6.1*, *NPF4.6*, *BAT1*, *AVT1*, *CAAT5*), phosphate transporters (*PHT1;2*), ABC transporters (*ABCC8*, *ABCG5*, *ABCC10*), sugar transporters (*ERDL4*, *GONST2*, *PLT1*), and channels such as aquaporins (*NIP2;2*, *TIP4;3*, *TIP5;1*) (Supplementary Table 19).

Downregulated transporters in leaves were related to sugar transport (*MST6*, *URGT2*, *HEX6*, *GONS3*, *GPT2*, *PLT5*, *PLT4*, *PLT1*, *SWEET14*), calcium transport (*ECA4*, *ACA5*, *ACA7*), ABC transporters (*ABCG37*, *ABCG44*, *ABCG45*), and channels (*SLAH3*, *PIP2;6*) (Supplementary Table 20). In roots, the downregulated genes were primarily members of ABC transporters (*ABCG44*, *ABCG24*, *ABCA4*, *ABCG1*, *ABCG48*, *ABCG5*) and nitrogen-related

transporters (*AAP4*, *AAP3*, *AMT23*, *ANT1*, *CAAT5*, *NPF6.3*, *NPF5.7*, *NPF8.3*, *NPF7.3*, *NPF5.2*, *NPF4.6*, *NPF6.2*). In the case of channels, we obtained *SLAH1*, *SLAH2*, *CLCe*, *KOR2*, *GLR2.7* and *GLR2.8* (Supplementary Table 21).

Distribution of DEGs that were related to categories including carbon metabolism, cell wall composition, amino acid metabolism, cell division, chloroplast and biosynthesis, transporters, homeostasis, suberin, and cuticle are summarized in Fig. 6a (roots) and 6b (leaves), and more in detail information about the categories can be found in the Supplementary Tables 22 (roots) and 23 (leaves). In the roots, the highest number of DEGs occurred in the homeostasis and transporter categories, both showing a balanced distribution of up- and downregulated genes. In contrast, genes related to cell division, cell wall, and carbon metabolism were predominantly upregulated. A different trend was observed in the leaves, where most categories were largely downregulated. Cell division was the only category showing a primary trend of upregulation, while homeostasis and cell wall were the most significantly downregulated groups.

Proteins involved in the carbon/nitrogen balance, such as *ATL31* (C/N-insensitive 1), were downregulated, suggesting a shift in metabolic prioritization under inorganic carbon treatment (Maekawa et al. 2012).

Table 2 Key upregulated and downregulated genes in leaves involved in cell division, amino acid transport and metabolism, and cell expansion. This table highlights genes with significant relevance to the enhanced growth response observed in barley plants grown under bicarbonate (BIC) treatment

Gene ID	Name of the gene	Short name	GE Control (Leaf)	GE Treated (Leaf)	Log2FC
HORVU_MOREX_r3_7HG0740730	Glutamine dumper 3	<i>GDU3</i>	49.36	179.01	1.85
HORVU_MOREX_r3_4HG0396660	Replication protein A 1B	<i>RPA1B</i>	0	13.47	6.22
HORVU_MOREX_r3_4HG0384560	Pyruvate decarboxylase 2	<i>PDC2</i>	439.19	717.50	0.70
HORVU_MOREX_r3_2HG0121000	Pyruvate dehydrogenase kinase	<i>PDK</i>	3780.81	5437.39	0.52
HORVU_MOREX_r3_1HG0055830	Branched-chain aminotransferase 5	<i>BCAT5</i>	1506.23	2178.17	0.53
HORVU_MOREX_r3_3HG0256620	Expansin A11	<i>EXA11</i>	0.32	16.14	5.52
HORVU_MOREX_r3_6HG0601160	Expansin B11	<i>EXB11</i>	0.61	15.26	4.57
HORVU_MOREX_r3_4HG0409710, HORVU_MOREX_r3_4HG0409760	Expansin like A1	<i>EXLA1</i>	579	1150.74	0.99
HORVU_MOREX_r3_2HG0201400	Serine hydroxymethyltransferase 4	<i>SHM4</i>	2097.68	3515.89	0.74
HORVU_MOREX_r3_2HG0185190	Metal nicotianamine transporter YSL9	<i>YSL9</i>	1083.55	1742.53	0.68
HORVU_MOREX_r3_1HG0072580, HORVU_MOREX_r3_1HG0072590	Nicotianamine transferase A	<i>NAAT</i>	1727.91	3684.22	1.09
HORVU_MOREX_r3_4HG0333550	Mediator of ABA-regulated dormancy 1	<i>MARD1</i>	768.89	1454.18	0.91
HORVU_MOREX_r3_7HG0746970	Cyclin D6-1	<i>CYCD6;1</i>	204.96	647.58	1.66
HORVU_MOREX_r3_1HG0069480	Cyclin B1-5	<i>CYCB1;5</i>	353.72	635.38	0.84
HORVU_MOREX_r3_2HG0198940	Cyclin P1-1	<i>CYCP1;1</i>	3389.83	1959.62	0.79
HORVU_MOREX_r3_4HG0353330	Cyclin-dependent kinase B1-1	<i>CDKB1;1</i>	164.44	276.32	0.74
HORVU_MOREX_r3_6HG0599820	Cyclin P4 –1	<i>CYCP4;1</i>	1653.73	2159.70	0.38
HORVU_MOREX_r3_2HG0182090, HORVU_MOREX_r3_2HG0182080	Phenylalanine ammonia-lyase	<i>PAL</i>	114.55	18.52	–1.66
HORVU_MOREX_r3_2HG0132820, HORVU_MOREX_r3_2HG0132830	Phenylalanine ammonia-lyase	<i>PAL</i>	369.06	66.44	–2.47
HORVU_MOREX_r3_2HG0132660, HORVU_MOREX_r3_2HG0132700, HORVU_MOREX_r3_2HG0132730	Phenylalanine ammonia-lyase		75.77	14.58	–2.37
HORVU_MOREX_r3_6HG0596580	Phenylalanine ammonia-lyase	<i>PAL</i>	346.90	113.48	–1.61

Table 2 (continued)

Gene ID	Name of the gene	Short name	GE Control (Leaf)	GE Treated (Leaf)	Log2FC
HORVU_MOREX_r3_2HG0182020	Phenylalanine ammonia-lyase	<i>PAL</i>	5590.35	3182.85	−0.81
HORVU_MOREX_r3_2HG0182090, HORVU_MOREX_r3_2HG0182080	Phenylalanine ammonia-lyase	<i>PAL</i>	114.55	18.52	−2.62
HORVU_MOREX_r3_7HG0651560	Gamma carbonic anhydrase 1	<i>YCAI</i>	4.31	24.90	2.53
HORVU_MOREX_r3_1HG0021970	1-aminocyclopropane-1-carboxylate oxidase	<i>ACC oxidase</i>	3078.80	857.48	−1.84
HORVU_MOREX_r3_1HG0021960	1-aminocyclopropane-1-carboxylate oxidase	<i>ACC oxidase</i>	25,671.81	10,585.67	−1.27
HORVU_MOREX_r3_2HG0186480	1-aminocyclopropane-1-carboxylate synthase	<i>ACC synthase</i>	125.54	7.29	−4.09
HORVU_MOREX_r3_4HG0339160	1-aminocyclopropane-1-carboxylate synthase	<i>ACC synthase</i>	497.35	65.48	−2.92

As mentioned above, the α -Carbonic anhydrase 7 (*ACA7*) was upregulated in the roots. In Arabidopsis, *ACA7* also associated with reduced Fe and Zn contents in seeds under high CO₂ conditions (Sun et al. 2022). Additionally, *gamma-carbonic anhydrases 1* (*YCAI*), which is part of mitochondrial complex I, were also upregulated, and it is important for plant development and mitochondrial function.

qPCR validation

To validate the transcriptome data, the expression of six root-based transporter genes (nitrate and phosphate transporters, as well as the S-type anion channel *SLAH3*) was followed, and the fold change was compared between qPCRs and the transcriptome analysis (see the primers and results in Supplementary Table 3 and Fig. S4). The fold change (FC) value of gene expression of these genes generated by the two methods was very close to each other (e.g. FC of *HvNRT2.5* was 11.8 in transcriptome sequencing vs. it was 11.3 was in qPCR, FC of *SLAH3* was 1.7 using any of the two methods), though in some cases the transcriptome analysis showed somewhat higher FC for the same gene (FC of the high affinity nitrate transporter HORVU6Hr1G005930 was 3.7 in transcriptome analysis, 2.4 in qPCR) and in other cases the qPCR showed higher FC (FC of the *HvPT2* inorganic phosphate transporter gene was 2.1 in transcriptome sequencing vs. it was 2.6 in qPCR). Gene expression changes shown by qPCR were significant

for all six studied genes at 95% confidence levels (see Fig. S4a and Supplementary Table 24). Overall, the two methods generated similar values, indicating the reliability of our transcriptome data.

Additionally, the results of the qRT-PCR shown in the Supplementary Table 3 and Fig. S4b were largely consistent with the transcriptome data. The linear regression analysis was conducted to assess the correlation between the qRT-PCR and RNA-seq, yielding R² value of 0.8705 for the roots, indicating a strong positive correlation between the two.

Total amino acid analysis in the leaves

To investigate the impact of bicarbonate fixation on the nitrogen-carbon (N/C) balance, we profiled total amino acids in the leaves of four-and-a-half-week-old barley plants. Plants were grown for three weeks under either control conditions (0 mM NaHCO₃) or bicarbonate treatment (BIC; 2 mM NaHCO₃) under pH 5.6.

The BIC treatment induced a distinct divergence in amino acid profiles compared to the control. Total amino acid analysis, encompassing both free and protein-bound fractions, revealed significant increases in the aspartate family members ASN + ASP (59.6%) and THR (57.4%), and a decrease in LYS (15.9%), with no change in MET. Depletion in the glutamate family was observed in the GLU + GLN (14%), while ARG and PRO did not change. In the case of aromatic amino acids, PHE decreased a 14.4%, and TYR

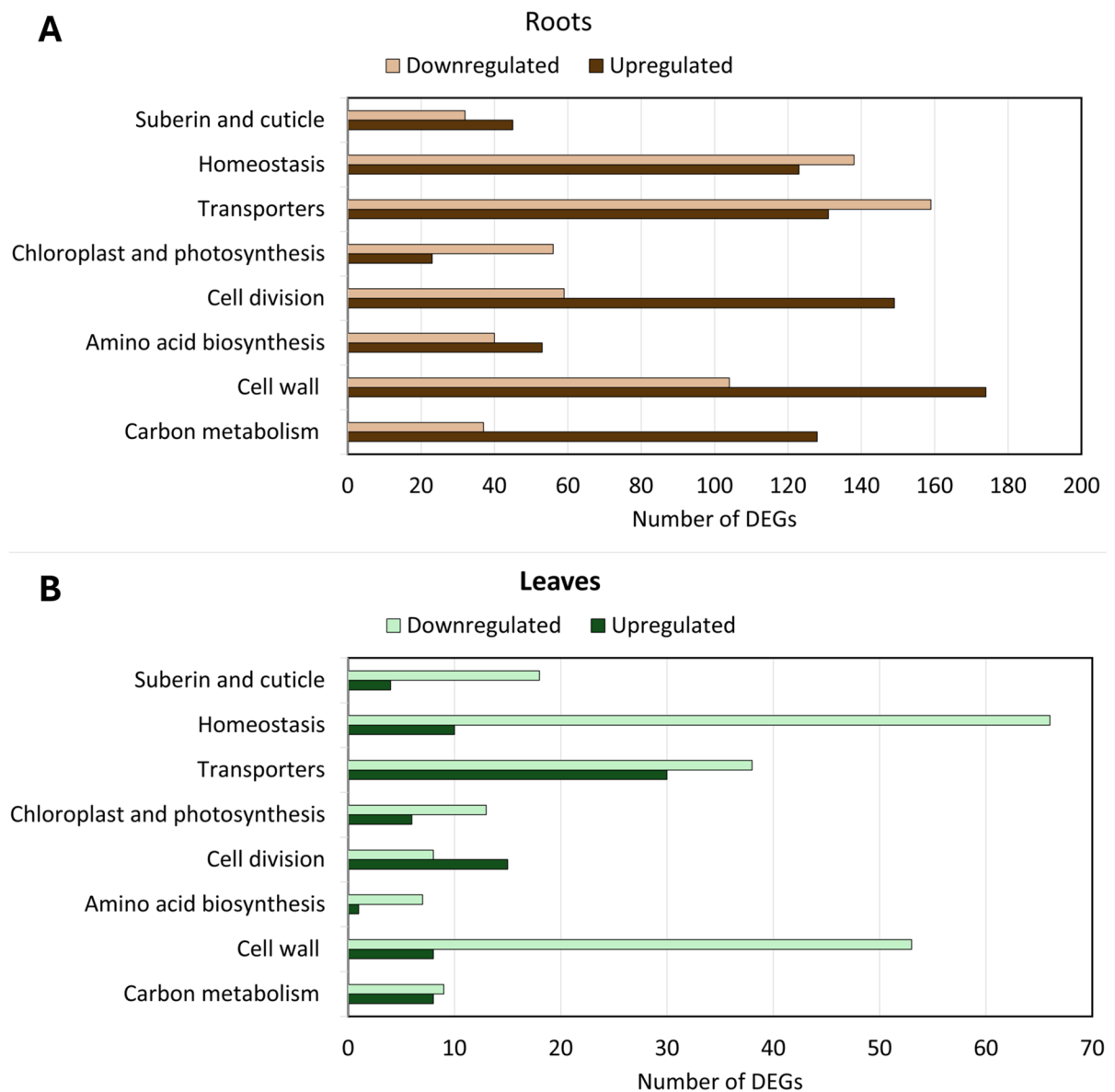


Fig. 6 Functional categorization of differentially expressed genes (DEGs) in roots and leaves. Barley plants were grown in hydroponic cultures supplemented with a BIC treatment (2 mM NaHCO_3 , pH 5.6, half-strength Hoagland medium). DEGs were defined based on a threshold $|\log_2\text{FC}| \geq 0.02$, $p\text{-adj} < 0.05$. Highlighted clusters group genes associated with

suberin and cuticle formation, cellular homeostasis, transport, chloroplast function and photosynthesis, cell division, amino acid biosynthesis, cell wall organization, and carbon metabolism. The complete lists of genes within these groups are provided in Supplementary Tables 22 and 23. (A) it is the representation for the roots. (B) it is the representation for the leaves

increased 18.5%, but it was not statistically significant. All the members of the branched-chain amino acids (BCAAs) decreased VAL (19.5%), ILE (21.4%) and LEU (21.6%). In the serine family, SER (25.2%) and CYS (56.6%) increased, but GLY decreased by 17.6%. The pyruvate-derived amino acid ALA

decreased significantly by 13.7%. Histidine increased by 9.8% and it was significant (Fig. 1c, Supplementary Table 25).

The BIC metabolic signature was characterized by a high ratio of transport-type nitrogen (ASP+ASN) relative to catalytic-type nitrogen (GLU+GLN).

Because the GLU + GLN pool serves as a critical intermediate between nitrogen assimilation and the TCA cycle, this shift suggests a fundamental reallocation of nitrogen and use of this amino acid to support cellular energy demands and rapid growth. Additionally, we could see in the transcriptomic analysis the upregulation of glutamine dumper 3, which might be involved in the regulation of amino acids in the leaves.

Transcriptomic data supported metabolic shifts, showing a high-velocity induction of the translation machinery, including multiple 50S and 30S ribosomal subunits (components of the 70S chloroplast ribosome). A primary nitrogen sink was identified in the nucleus, where histones H3 and H4 were heavily upregulated. This indicates a massive diversion of nitrogen into the structural proteome and DNA/RNA-packaging machinery. Specifically, the decrease in total GLY concentrations correlated with the induction of the DNA replication machinery, including replication protein A (RPA) and Cyclin D6-1, which require glycine-derived purines for nucleotide biosynthesis.

Total pools of BCAAs were significantly depleted. This depletion coincided with the upregulation of leaf-localized pyruvate dehydrogenase kinase (PDHK), pyruvate decarboxylase (PDC), and branched-chain aminotransferase (BCAT), all of which facilitate the oxidative degradation of these amino acids. This pattern suggests that BCAAs turnover is coordinated with the heightened ATP demand of the active cell division phase. Similarly, PHE concentrations were lower in BIC-treated leaves. Pathway analysis revealed a strategic downregulation of lignin-related enzymes, specifically phenylalanine ammonia-lyase (PAL), which deaminates PHE into trans-cinnamic acid and ammonia. This shift effectively suppresses the production of lignin, flavonoids, and salicylic acid in favor of primary protein synthesis. Conversely, genes associated with cell wall loosening and volumetric expansion, such as expansins, endoglucanases, glycosyl hydrolases, and the auxin-responsive SAUR36, were highly induced.

The observed decrease in GLY alongside an increase in SER suggests accelerated glycine-to-serine conversion, likely mediated by serine hydroxymethyltransferase (SHMT). This conversion provides vital bioenergetic byproducts, including CO₂, ammonia, NADH, and ATP, that support growth and prime

the plant for potential drought stress. Furthermore, SER serves as a precursor for phosphatidylserine and phosphatidylethanolamine, essential phospholipids for expanding membrane structures. A significant increase in total CYS further distinguished the metabolic profile. CYS synthesis requires both a carbon skeleton (from SER) and reduced sulfur; its accumulation points to a robust sulfur assimilation capacity in the plastids. Functionally, this CYS pool acts as a metabolic pivot, supplying sulfur for glutathione-based antioxidant defense and the formation of iron-sulfur clusters in respiratory and photosynthetic machinery. This ensures proteomic stability and prevents oxidative damage during high-velocity metabolism. Additionally, CYS is critical for correct protein folding via disulfide bridge formation, maintaining the 3D integrity of the rapidly expanding proteome.

While total leaf ASP + ASN levels were significantly elevated, ¹³C fluxomics failed to detect newly synthesized ¹³C-aspartate within the leaf tissue, despite its clear detection in the roots. This discrepancy suggests that leaf-tissue aspartate is not synthesized *in-situ* but is instead rapidly processed upon arrival from the roots. Root transcriptomic data confirmed a robust induction of aspartate aminotransferase and PEPC, establishing the roots as a high-capacity shipping hub for aspartate-family molecules. In the leaves, the accumulation of THR (functioning as a compatible solute) and ASN (as a high-N storage form) likely serves to stabilize osmotic potential and cellular pH, providing a stable nutrient source during growth.

Evaluation of combined growth promotion and osmotic stress treatments in barley

We investigated whether our growth promotion system (BIC treatment) could promote growth in barley and whether it would be beneficial under osmotic stress (15% PEG). The treatments included control, BIC, PEG, and PEG + BIC. Data can be found in the Supplementary Table 26.

The treatments differentially affected barley growth and biomass. Root length decreased significantly under osmotic stress (43.2% in PEG and 40.6% in PEG + BIC), while the BIC treatment alone increased it by 14.4%, although not significantly. Root weight increased significantly by 28.2% with BIC but declined under PEG and PEG + BIC, following

the same pattern as root length. Aboveground, BIC enhanced shoot and third-leaf weight by 25.1% and 44.4%, and promoted elongation by 8.2% and 20.7%, respectively. PEG inhibited both shoot and root growth, and co-application with BIC substantially attenuated its growth-promoting effects, suggesting

that bicarbonate was unable to offset PEG-induced osmotic stress (Fig. 7a and 7b).

Gas exchange analysis revealed distinct treatment responses. The P_n increased by 29.6% under BIC compared to the control, but declined by 35.5% under PEG. The PEG + BIC combination did not

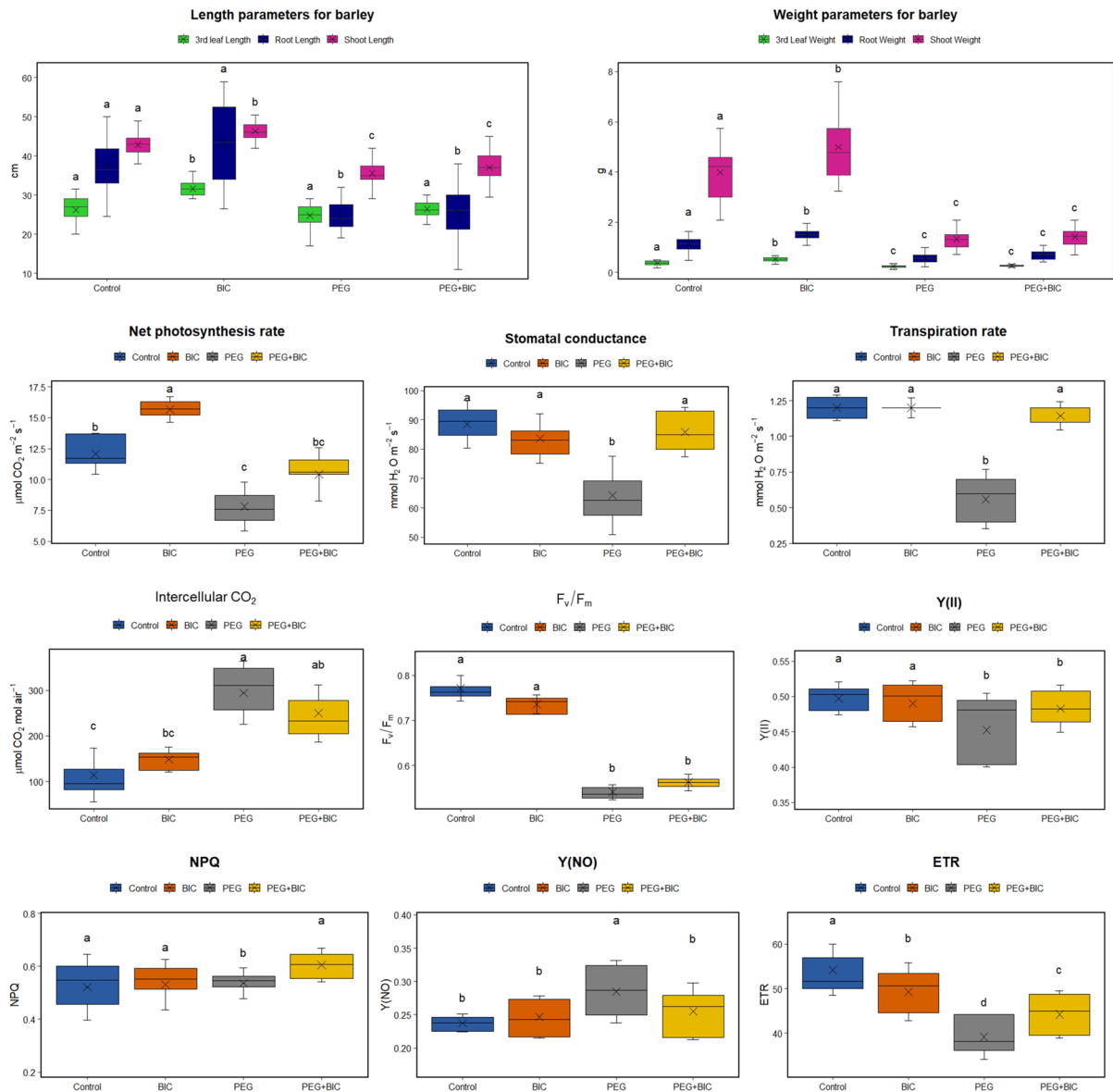


Fig. 7 Growth and photosynthetic responses of barley (*Hordeum vulgare* ‘Golden Promise’) under control (pH 5.6) and stress treatments. Plants were exposed to 0 mM NaHCO_3 (control), 2 mM NaHCO_3 (BIC), 15% PEG (PEG), or 15% PEG + 2 mM NaHCO_3 (PEG + BIC) at pH 5.6. A. Root, shoot, and third leaf length; B. fresh weights of root, shoot, and third

leaf; C. net photosynthesis (P_n); D. stomatal conductance (g_s); E. transpiration rate (E); G. F_v/F_m ; H. Y(II); I. NPQ; J. Y(NO); K. electron transport rate (ETR). Data are means $\pm$ SD. Statistical significance was determined by one-way ANOVA with the corresponding post hoc test; different lowercase letters indicate $p \leq 0.05$

significantly differ from PEG alone (Fig. 7c). The responses of g_s and E followed a similar pattern. Neither g_s nor E differed significantly between the control and BIC treatments, indicating BIC does not impair stomatal function under non-stressed conditions. As expected, PEG significantly decreased both g_s (37.7%) and E (53.3%) relative to the control, reflecting stomatal closure under osmotic stress. However, the inclusion of BIC under stress (PEG + BIC) led to a significant increase in g_s (33.6%) and E (101.8%) compared to PEG alone. This strongly suggests that BIC partially alleviates PEG-induced stomatal closure, allowing for higher water vapor exchange (Fig. 7d and 7e). In contrast, the C_i showed only significant differences between the control and the PEG (Fig. 7f). The significant increase in C_i under PEG stress, combined with the significant decrease in P_n , points towards non-stomatal limitations (e.g., impaired mesophyll capacity or enzyme activity) as the primary cause for the reduced P_n under osmotic stress.

Chlorophyll fluorescence measurements indicated that osmotic stress severely compromised the integrity and function of Photosystem II (PSII). The F_v/F_m , a measure of chronic photoinhibition or damage to the PSII reaction centers, was significantly reduced for the treatments involving PEG. The BIC treatment caused a modest 5.3% decline, suggesting minor downregulation, though it was not significant. However, PEG induced severe photoinhibition, causing the greatest decline (35.1%) and lowering the ratio to approximately 0.54. Although the PEG + BIC combination provided a marginal 3.6% recovery compared to PEG alone, the final low F_v/F_m value confirms that significant PSII damage persists under combined stress (Fig. 7g).

Despite the damage suggested by F_v/F_m , the BIC treatment offered some functional protection under stress. The $Y(II)$ was significantly reduced by 8.5% in the PEG treatment. Critically, $Y(II)$ showed no significant difference among the control, BIC, and PEG + BIC treatments (Fig. 7h). This suggests that while PEG + BIC plants sustained structural damage (F_v/F_m drop), BIC enabled them to maintain high actual photochemical efficiency. This protection is further supported by the response of energy dissipation pathways. The inclusion of BIC in the stress treatment (PEG + BIC) led to a significant 12.4% increase in NPQ compared to PEG alone (Fig. 7i).

This indicates that BIC successfully activated the plant's regulated photoprotective mechanism.

The $Y(NO)$ and ETR patterns confirmed the relative stress levels (Fig. 7j and 7k). The PEG treatment showed the greatest inhibitory effect on ETR (32.3%) and the highest overall increase in $Y(NO)$, confirming the severity of osmotic stress and the lack of regulated defense. Although BIC also reduced ETR (9.6%), the PEG + BIC combination partially restored the electron flow, resulting in an ETR reduction of 20.4% positioning the overall photosynthetic function closer to that of the healthy control.

Evaluation of combined growth promotion and osmotic stress treatments in Arabidopsis

We also investigated whether the growth promotion in Arabidopsis could be beneficial for plants under osmotic stress (8% PEG). The treatments included control, BIC, PEG, and PEG + BIC. Data can be found in the Supplementary Table 27.

In Arabidopsis, belowground responses differed from those in barley. Root length increased significantly by 9% under BIC, indicating a stimulatory effect, while PEG and PEG + BIC caused no significant changes. Root weight rose by 26.1% under BIC but was markedly reduced under osmotic stress, with PEG and PEG + BIC decreasing biomass by 66.7% and 55.3%, respectively. The absence of significant differences between PEG and PEG + BIC treatments suggests that BIC did not mitigate osmotic stress effects on root biomass.

The BIC treatment significantly promoted aboveground growth in Arabidopsis under non-stressed conditions, notably increasing rosette diameter by 15.9% and rosette weight by 30.4% compared to the control. In opposition, osmotic stress inhibited these traits, with the PEG treatment causing the largest reductions in both diameter (59.2%) and biomass (56.4%) compared to the control. However, the addition of BIC to the stress treatment mitigated some of PEG's negative effects; the PEG + BIC combination resulted in a significantly less severe decline in rosette diameter (42.8%) and a substantial 76.7% improvement in rosette dry weight compared to the PEG treatment alone, demonstrating that BIC offered a significant degree of aboveground protection under osmotic stress (Fig. 8a and 8b).

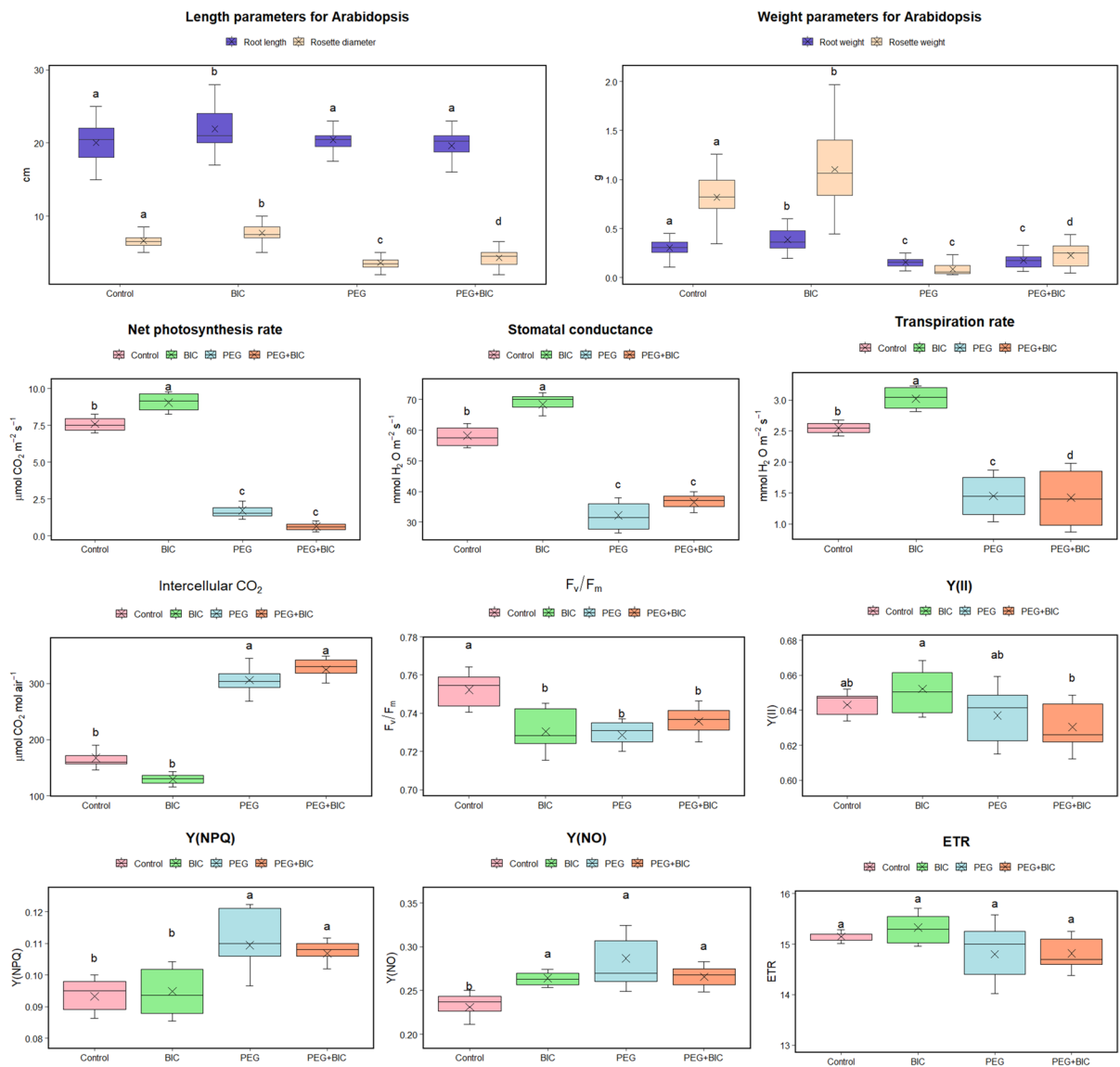


Fig. 8 Growth and photosynthetic responses of *Arabidopsis thaliana* (Col-0) under control and stress treatments. Plants were exposed to 0 mM NaHCO_3 (control), 2 mM NaHCO_3 (BIC), 8% PEG (PEG), or 8% PEG+2 mM NaHCO_3 (PEG+BIC) at pH 5.6. A. Root length and rosette diameter; B. Fresh weights of roots and rosettes; C. Net photosynthesis

(P_n); D. stomatal conductance (g_s); E. transpiration rate (E); F. Intracellular CO_2 (C_i); G. F_v/F_m ; H. Y(NPQ); I. Y(NO); J. Y(II); K. electron transport rate (ETR). Data are means $\pm$ SD. Statistical significance was determined by one-way ANOVA with the corresponding post hoc test; different lowercase letters indicate $p \leq 0.05$

Gas exchange analysis revealed that the P_n significantly increased by 16.9% in the BIC treatment compared to the control, demonstrating a stimulatory effect on carbon fixation. The BIC treatment significantly increased g_s by 16.2% and E by 17% too, suggesting enhanced stomatal opening under non-stressed conditions. Conversely, P_n , g_s and E were

inhibited under osmotic stress conditions. The PEG and PEG+BIC treatments caused reductions in P_n compared to the control by approximately 2.26-fold and 2.69-fold, respectively, with no significant difference observed between the two osmotic stress treatments (Fig. 8c). Similarly, both PEG and PEG+BIC treatments caused a significant decrease in g_s (by

57.5% and 46%) and E (by 55% and 57%), indicating stomatal closure under stress. For these two parameters, there were no significant differences between the PEG and PEG+BIC osmotic stress treatments in the g_s but for the E there were (Fig. 8d and 8e).

In contrast, C_i showed no difference between the control and BIC treatments. However, C_i significantly increased under osmotic stress, rising by 58.2% in PEG and 63.5% in PEG+BIC. The reduction in P_n combined with the significant increase in C_i suggests that the primary limitation to photosynthesis under osmotic stress is non-stomatal (i.e., metabolic impairment) in *Arabidopsis*, as it was visible for barley (Fig. 8f).

Analysis of chlorophyll fluorescence parameters indicated that the PSII reaction centers remained largely intact, as the F_v/F_m stayed close 0.7 across all treatments, despite a small, significant decrease (up to 4.8% in PEG) (Fig. 8g). However, the plants actively responded to the stress; both PEG and PEG+BIC treatments significantly activated $Y(NPQ)$, showing increases of 15.8% and 13.1% respectively, relative to the control, indicating the successful induction of photoprotective mechanisms (Fig. 8h). Conversely, $Y(NO)$ increased significantly across all treatments, with PEG showing the highest rise (21.3%) (Fig. 8i). The $Y(II)$ and ETR showed no significant difference from the control in the stress treatments. Altogether, the gas exchange and chlorophyll fluorescence parameters did not improve significantly in the osmotically stressed *Arabidopsis* plants as result of the additional inclusion of BIC into the medium, but growth parameters improved which can be considered the more important indicators of stress tolerance, therefore the BIC-treatment did offer some protection against the osmotic stress symptoms in *Arabidopsis*.

Study of suberization as a potential mechanism regulating root-based IC uptake

In our previous Gamarra Reinoso et al. (2024) and present work, we aimed to find mechanisms of root-based IC uptake. In the case of *Arabidopsis thaliana*, in our earlier study, we suggested the possible effect of the Casparian strip on the leakage of CO_2 into the xylem through free diffusion. Notably, the KEGG functional group: cutin, suberin and wax biosynthesis (ath00073) was downregulated in the transcriptome of *Arabidopsis* roots as a result of BIC treatment.

It prompted us to study the effect of suberization on root-based inorganic carbon uptake. To this end, *Arabidopsis thaliana* ecotype Columbia (Col-0) and two already characterized, high suberin-producing genotypes, the sulfur transporter mutant *sultr1;2* (Barberon et al. 2016; Jose et al. 2024) and enhanced suberin biosynthesis *esb1-1* (Hosmani et al. 2013) were subjected to a week of ^{13}C $NaHCO_3$ -treatment, and their shoot $\delta^{13}C$ values were then determined. The results (Fig. 9a) show that without ^{13}C labelling, $\delta^{13}C$ values of the three genotypes were similar to each other (no significant difference) and were lower than the VPDB standard, in each case. As a result of the labelling, the $\delta^{13}C$ value of Col became around 55‰ higher than the VPDB, but *sultr1;2* and *esb1-1* took up and fixed much less labelled carbon (around 22 and 9‰ higher than VPDB, 40% and 17% of Col,

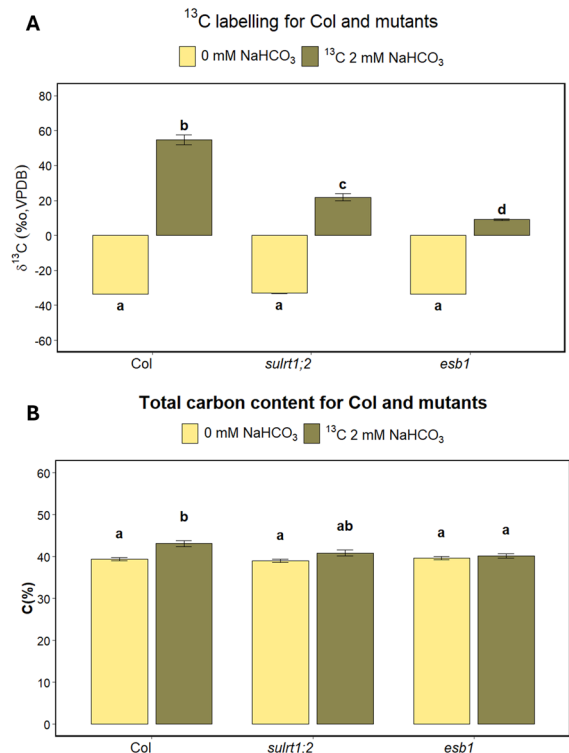


Fig. 9 ^{13}C uptake and fixation from the hydroponic solution in high suberin-producing mutants. *Arabidopsis thaliana* ecotype Columbia (Col-0) and mutants *sultr1;2* (SALK_122974) and *esb1* (N2106042) were subjected to 2 mM ^{13}C $NaHCO_3$ -treatment for a week and then total shoot carbon and $\delta^{13}C$ ratio was determined. Data are means $\pm$ SD. Statistical significance was determined by one-way ANOVA with Tukey post hoc test; different letters indicate $p < 0.05$

respectively) from the hydroponic solution into the dry shoot biomass. Total carbon content was also calculated. In the case of Col, the percentage of carbon increased significantly in the BIC treatment, but in the mutants, there were no significant differences between the control and the BIC treatment in terms of carbon percentage (Fig. 9b).

Discussion

Root-based uptake of IC and its incorporation into central metabolism has been described for different species. Moderate concentrations of bicarbonate supplementation have been applied for *Arabidopsis thaliana* at 2 mM NaHCO_3 (Gamarra Reinoso et al. 2024), *Coix lacryma-jobi* with 2 mM NaHCO_3 (Li et al. 2023), *Broussonetia papyrifera* with 3 mM NaHCO_3 (Yao and Wu 2021), grapevine rootstock with 5 mM NaHCO_3 (Covarrubias and Rombolà 2013), and willow with 3 mM NaHCO_3 (Vuorinen et al. 1992). Therefore, in this study, we evaluated different concentrations of NaHCO_3 and selected an appropriate growth promotion system for barley. We evaluated how this IC is taken up and fixated by barley and its incorporation into carbon central metabolism, and whether IC supplementation confers any benefit under osmotic stress in barley and in *Arabidopsis*.

Supplementation with 2 mM NaHCO_3 at pH 5.6 promoted barley growth and significantly increased P_n , while g_s and E remained unchanged, and C_i trended slightly upward. We believe higher chlorophyll content of the treated plants (see Supplementary Fig. S1c) could have equipped them with higher photosynthesis capability, even having the same stomatal conductance. The growth promotion itself is consistent with previous reports at comparable bicarbonate concentrations in dicot and woody species mentioned above.

As expected, PEG-induced osmotic stress severely inhibited growth in both species (barley and *Arabidopsis*) and induced stomatal closure, similarly to literature examples, e.g. He et al. 2014 and Licaj et al. 2024. Before comparing the two species, one warning must be stated. The barley variety Golden Promise was considerably more PEG-tolerant than the *Arabidopsis* Col ecotype, and in preliminary experiments (data not shown), the PEG concentration had to be raised from 8 to 15% in barley to obtain significant

changes in growth and gas exchange. Absolute stress intensities, therefore, differ between the two species, and the comparison below concerns the qualitative pattern of the BIC response, not its magnitude.

In barley, the marked reduction in P_n under PEG was accompanied by a significant rise in C_i . Decreased P_n with increased C_i indicates that CO_2 is reaching the carboxylation sites but cannot be processed, shifting the primary bottleneck from diffusive supply to the metabolic capacity of the dark reactions and the upstream availability of reducing power and functional electron transport (Flexas and Medrano 2002; Xu et al. 2020). This diagnosis identifies the metabolic machinery of carbon fixation, rather than stomatal gas exchange, as the most vulnerable target under osmotic stress. In barley, PEG additionally caused a significant fall in F_v/F_m and $Y(\text{II})$, indicating sustained PSII photoinhibition, and a drop in NPQ together with a rise in $Y(\text{NO})$. Stressed barley therefore loses both photosynthetic capacity and the ability to safely dispose of absorbed light energy.

Having together the PEG+BIC treatment produced a coherent set of recoveries in barley: g_s , E , and C_i all returned toward control values, ETR rose significantly above the PEG treatment, NPQ recovered to control values, $Y(\text{NO})$ fell back to baseline, and P_n trended upward at the 90% confidence level. However BIC did not reverse the PEG-induced depression of F_v/F_m or $Y(\text{II})$, both of which remained at PEG treatment values. BIC therefore acted protectively rather than reparatively. BIC treatment may be acting intracellularly as a pH buffer to stabilize enzymes, enhancing antioxidant systems or improving osmotic adjustment, thereby reducing cell death and preserving tissue integrity (Ishizawa 2014; Yin et al. 2017).

The downregulation of the GO term 'response to hormone' and the KEGG pathway 'plant hormone signal transduction' in barley suggests a partial relaxation of ABA-mediated stomatal closure (Daszkowska-Golec 2016; Liu et al. 2022). Although this closure is a critical survival response, the osmotic stress imposed by PEG was less physiologically costly than conditions in drying soil. Furthermore, the return of g_s and E to control, rather than supra-control, levels indicates that the response was rebalanced rather than abolished.

In *Arabidopsis*, the pattern was strikingly different. Under PEG, the leaf showed the same diagnostic non-stomatal limitation as barley, but chlorophyll

fluorescence induction and gas exchange parameters did not improve with BIC. Still, aboveground rosette growth under PEG+BIC was significantly higher than under PEG alone. We believe the extra carbon supply, taken up by the roots, could have maintained the growth and kept the photosynthetic dark reactions operating, which could have helped avoiding the oxidative injuries and donor side photoinhibition by replenishing the electron acceptor NADP⁺. The fluxomic analyses also proved that the hydroponically supplied carbon was fixed into sucrose in Arabidopsis, but not in barley, so this avoidance of donor side oxidative injury may have only worked in Arabidopsis. Furthermore, while the BIC-treatment did offer some protection against osmotic stress in barley as it was discussed, the lack of the strong growth regulation of Tre6P signaling may have prevented the advantage from being materialized in increased growth.

As it was mentioned, fluxomic analysis, isotope-labelling experiments were designed to trace the incorporation of bicarbonate-derived carbon into sucrose and aspartic acid, representing Rubisco and PEPC-dependent fixation, respectively. A potential confound must be addressed: at the low pH applied in the hydroponic solution, a significant portion of the bicarbonate could have converted into CO₂, and some of it may have escaped into the air and been absorbed by the plants through their stomata. However, CO₂ is well soluble in water (1.45 g/L at 25 °C, approximately 33 mM) (Devgoswami et al. 2011), and here, only a fraction of it was supplied (2 mM), which could remain in the water solution in any inorganic carbon form. The absence of a significant amount of labelled sucrose in barley leaves also indicates that the outward leakage of carbon and its Rubisco-based fixation in the leaves did not occur at a detectable level. The presence of ¹³C-labelled aspartate in barley roots, even at low concentrations, but not in barley leaf, under BIC treatment is fully consistent with this interpretation, since root PEPC uses HCO₃⁻ directly as substrate and rapidly transaminates the resulting OAA to aspartate (Han et al. 2021). Root PEPC is therefore the dominant entry point for IC into central metabolism in barley.

This pattern stands in sharp contrast to the Arabidopsis result reported in our earlier work (Gamarra Reinoso et al. 2024), where ¹³C-labelled aspartate was detected in both xylem and phloem, and where

¹³C-labelled sucrose was recovered from the phloem sap. In barley, by contrast, the carbon appears to be fixed almost entirely at its point of entry in the roots, through PEPC, into the C₄-acid and amino acids and whatever fraction reaches the shoot does so as organic and amino acids rather than as IC available for shoot Rubisco fixation, or the low CA activity (see below) would prevent its fixation in barley leaves.

Our transcriptomic data situate root PEPC activity within a broader metabolic framework. In heterotrophic plant tissues, central carbon metabolism revolves around the phosphoenolpyruvate–pyruvate–oxaloacetate (PPO) node, the critical intersection of glycolysis and the TCA cycle that partitions carbon flux between energy production and biosynthetic carbon skeleton supply (Koendjibiharie et al. 2021). Under BIC treatment, the barley PPO node was significantly activated: PEPC, PPK, PK, MDH, AlaAT, and AST were all upregulated, with PEPC being the sole exception. Consequently, the root transcriptome is primed for net carbon capture. Beyond its role in incorporating IC into central metabolism via the PEP-to-OAA conversion, PEPC supports the synthesis of root organic and amino acids (Abadie and Tcherkez 2019). Furthermore, by replenishing TCA cycle intermediates, PEPC sustains respiratory activity; its complex regulation, via phosphorylation, monoubiquitination, pH, and allosteric effectors, ensures precise responsiveness to metabolic demands (Hu et al. 2025).

The form of nitrogen supplied to the plant is a critical modulator of PPO node behavior and differs between our two experimental systems; barley received Hoagland medium with nitrate as the sole nitrogen source, whereas the Arabidopsis experiments used MS medium containing both nitrate and ammonium. The form of nitrogen supplied to plants exerts a major influence on carbon metabolism (Pasqualini et al. 2001). Nitrate reduction releases hydroxyl ions and tends to alkalinize the cytosol, increasing the cytosolic pH, which is partially neutralized by the biochemical pH–stat through PEPC-mediated carboxylation (Schubert and Yan 1997). This positions PEPC as an obligatory participant in nitrate-fed metabolism and channels fixed carbon preferentially into organic acids. Additionally, dissolved IC enhances nitrate uptake by influencing the root membrane electrochemical potential (Britto and Kronzucker 2005). On the other hand, ammonium

assimilation, by contrast, releases protons, acidifies the cytosol, and channels carbon directly into amino acid biosynthesis through GS/GOGAT (Schubert and Yan 1997) like it was evaluated in wheat (Sharma and Sirohi 1987), tomato (Alhendawi et al. 1997), and cucumber (Wang et al. 2016), therefore with less involvement of PEPC. The nitrate-only barley environment, therefore, places PEPC under heavy demand, and the additional HCO_3^- supply may be channeled preferentially into organic and amino acid pools.

The downstream consequences of this PPO node and the labelled aspartate result from the fluxomic results are visible in the leaf amino acid pools. BIC-derived carbon assimilated through root PEPC activity was presumably transferred to the shoot and diluted across larger metabolic pools. The detection of labelled aspartate in roots, even at low concentrations, supports this interpretation. In BIC-treated barley leaves, ASP+ASN remained elevated while GLU+GLN decreased significantly. This divergence is the specific metabolic signature expected from a tissue in which OAA supply has been increased by upstream PEPC carboxylation, with ASP and ASN acting as stable, high C: N ratio, nitrogen carriers that support active growth without ammonia toxicity (Lea et al. 2007; Gaufichon et al. 2016; Kambhampati et al. 2017). The concurrent decrease in GLU+GLN more likely reflects redirection toward the GABA shunt, which consumes glutamate to regulate cytosolic pH, protect rapidly dividing cells from oxidative stress, and recapture ammonia released during BCAA and lysine catabolism (Hildebrandt et al. 2015). THR accumulation, downstream of the aspartate pathway, contributes to turgor maintenance during cell expansion as a compatible solute, consistent with parallel expansin upregulation (Joshi et al. 2010). Changes in C_1 metabolism follow the same logic; GLY decreased while SER and CYS increased, consistent with active channeling of GLY through the GDC/SHMT complex to generate the C_1 units required for DNA and histone methylation during cell division (Zrenner et al. 2006; Roell et al. 2025). The increase in CYS has dual significance, supporting glutathione biosynthesis and the ascorbate–glutathione cycle that scavenges ROS and providing sulfhydryl groups for protein folding in rapidly dividing cells (Wirtz and Hell 2006; Romero et al. 2014; Ingrisano et al. 2023). Both arms converge on the same physiological priority, sustaining

the high biosynthetic and replicative demand of tissues undergoing BIC-induced growth.

This convergence on cell division becomes explicit in the transcriptome. PAL was downregulated, reducing flux through the phenylpropanoid pathway (visible as well in the downregulation of the KEGG pathway, phenylpropanoid biosynthesis) and indicating deprioritization of cell wall reinforcement and secondary metabolite synthesis. Ubiquitin-mediated protein degradation and defense response were also deactivated. In parallel, expansins, histones H3 and H4, and components of the DNA replication machinery were upregulated, revealing what those resources were reallocated towards. A coordinated ionic response complements this reorganization; transporters for K^+ , Zn^{2+} , and Fe were synergistically upregulated in both leaves and roots, together with nictianamine synthase genes in the roots. K^+ functions as the preferred counter-ion for balancing the negative charges of root-derived organic acids present in the roots as well as arriving in the leaves while supporting turgor-driven cell expansion (Takahashi and Kinoshita 2017). Beyond osmoregulation, it activates more than 60 enzymes of glycolysis and the Krebs cycle and directly controls Rubisco biosynthesis, maintains stomatal aperture (Sardans and Peñuelas 2021). Zn^{2+} complements this as a structural and catalytic component of zinc-finger transcription factors, RNA polymerases, superoxide dismutase, and CA, regulating carbon metabolism, nucleic acid biosynthesis, and oxidative stress management (Sharma et al. 2013). The picture is one of coherent multi-level reorganization converging on sustained cell division and expansion under BIC supplementation. Collectively, these elemental changes represent an integrated homeostatic strategy in which ionic balance, photosynthetic efficiency, and metabolic flux are jointly optimized to sustain BIC-induced growth.

Three mechanistic differences emerge from our combined datasets to explain the species' asymmetry in carbon fixation and the effect of IC-treatment on osmotic stress tolerance. First, nitrogen form, as discussed above, the nitrate-only barley environment channels PEPC-fixed carbon into pH-stat anaplerosis and organic acid pools, while the mixed-nitrogen Arabidopsis environment leaves more IC available. Second, leaf CA expression, we systematically compared CA expression across the leaf and root transcriptomes of both species (Supplementary Table 28).

The most striking difference was constitutive; the β -carbonic anhydrase 1 (*β CA1*) gene of *Arabidopsis* is expressed at extremely high levels in the leaf transcriptome (TPM $\approx$ 150,000), and no equivalent gene is present in the leaf transcriptome of barley. Many studies have shown the relevance of CAs in root-based IC fixation (Dąbrowska-Bronk et al. 2016; Gamarra Reinoso et al. 2024; Pérez-Martín et al. 2024). *β CA1* in the leaves catalyzes the rapid inter-conversion of CO_2 and HCO_3^- in the chloroplast and cytosol, and its high abundance in *Arabidopsis* leaves may provide the biochemical machinery required to fix dissolved IC delivered to the shoot through the xylem. The near-complete absence of β CAs from the barley leaf transcriptome, only one β CA gene against six in *Arabidopsis*, means that even if barley delivered IC to the shoot through the xylem, the leaf would lack the enzymatic capacity to fix it efficiently, also suggested by the lack of labelled sucrose in the species. Third, Casparian strip suberization. We tested IC uptake in two *Arabidopsis* genotypes with altered suberin biosynthesis, the sulfate transporter mutant *sultr1;2* (which has elevated suberization as a secondary phenotype) (Barberon et al. 2016; Jose et al. 2024) and *esb1-1* (enhanced suberin) (Baxter et al. 2009), and found that both fixed considerably less ^{13}C from the hydroponic solution than wild-type Col. While the CO_2 -diffusion-inhibiting effect of the suberin layer between mesophyll and bundle sheath cells in leaves of C_4 plants was known (Danila et al. 2021) to our knowledge, this is the first report identifying Casparian strip suberization as a regulator of root-based IC uptake. We would also add that while the role of the Casparian strip regulating nutrient uptake can be considered as textbook knowledge, the effect of suberization on the uptake of a given element can be either positive or negative. Interestingly, and contrary to another non-metallic element sulfur, where reduced suberization decreases the uptake (Barberon 2017), our experiment shows that in the case of carbon, the lower suberization increases it. The KEGG pathway 'cutin, suberin and wax biosynthesis' was downregulated in BIC-treated *Arabidopsis* (Gamarra Reinoso et al. 2024) but not in BIC-treated barley, suggesting that *Arabidopsis* additionally enhances IC uptake capacity in response to BIC by reducing root suberization, while barley does not. The interpretation of *sultr1;2* requires a brief logical clarification; If *SULTR1;2* was itself a major IC uptake transporter

(though there is no literature evidence for such activity), loss of *SULTR1;2* in *sultr1;2* should reduce IC uptake additively with the suberization effect, and *sultr1;2* should fix less carbon than *esb1-1*. We observed the opposite, *sultr1;2* fixed more carbon than *esb1-1*, indicating that *SULTR1;2* is not a major IC uptake transporter and that the reduced uptake in *sultr1;2* is largely attributable to its increased suberization.

A final question is whether the growth promotion is best understood as a direct consequence of increased carbon supply or as the result of signaling triggered by it. The evidence points to a combination, with the relative weight differing between species. In *Arabidopsis*, the route from root-supplied IC to growth runs through shoot sucrose and Tre6P signaling. In barley, no labelled sucrose was detected, and Tre6P signaling cannot have been activated through this route; the response is correspondingly more modest and is mediated instead by direct substrate provision through the PPO node and through other signaling routes. Downregulation of ethylene signaling (decreased expression of ACC oxidase, ACC synthase and several ethylene-responsive transcription factors in BIC-treated leaves) could have supported the moderate growth observed in barley. One useful piece of negative evidence constrains the interpretation; cytokinin and zeatin biosynthesis, the canonical eustress signaling response (Nyitrai et al. 2009), was not upregulated in our barley transcriptome under BIC, indicating that the canonical eustress pathway was not engaged and that signaling alone cannot fully explain the growth promotion in either species. The convergent evidence from the *Arabidopsis sultr1;2* mutant supports the same conclusion; it showed less uptake and fixation of carbon and shows correspondingly reduced growth in the BIC treatment. Notably, in *Arabidopsis* hydroponically supplied IC was fixed into sucrose, the main photoassimilate and it partly restored growth during osmotic stress. The carbon diffusion and fixation themselves therefore appear to be the principal driving force of growth in *Arabidopsis*, with Tre6P signaling acting as an amplifier rather than as the sole cause. In case of barley, we have less evidence on the direct effect of IC on the growth and believe that regulatory aspects could have been more important. In general, our results may open new avenues for boosting photosynthesis, growth and osmotic stress tolerance of plants.

Acknowledgements We thank for Prof. Dr. Tünde Pusztahelyi (University of Debrecen, Debrecen, Hungary) for the professional amino acid analysis and also for Dr. Zoltán Balogh (Isotoptech Zrt., Debrecen, Hungary) for the high-quality $\delta^{13}\text{C}$ stable isotope measurement, and also for their help in describing their respective methods. The authors are grateful for the excellent technical assistance of Szilvia Fodor, Szilvia Sipos, and Katalin Gregály. We thank László Sági (Agricultural Institute, HUN-REN Centre for Agricultural Research) for his advice and continuous support. We are grateful to Tibor Janda (Agricultural Institute, HUN-REN Centre for Agricultural Research) for his careful evaluation of the manuscript and his remarks, which improved its quality. The research was financed by the grant NKFI FK 134874.

Author contributions All authors contributed to the study conception and design. Liesel Gamarra Reinoso, Imre Majláth and Csaba Éva carried out the plant physiological experiments and took samples for fluxomic and amino acid analyses, as well as for transcriptome sequencing. Kamirán Áron Hamow and Mihály Dernovics optimised and performed the fluxomic analyses. Liesel Gamarra Reinoso analysed the data and wrote the first version of the manuscript with the help of Csaba Éva. All authors perfected the manuscript and read and approved the final version.

Funding Open access funding provided by HUN-REN Centre for Agricultural Research. The research has been supported by the grant NKFI FK 134874.

Nemzeti Kutatási Fejlesztési és Innovációs Hivatal, NKFI FK 134874, Csaba Éva

Data availability The data supporting the findings of the study are available from the corresponding author upon request. The raw transcriptome data have been deposited to the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) under accession no. of PRJNA1363858, temporary submission ID of SUB15771632 and with release date of 2026–06–30.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Abadie C, Tcherkez G (2019) In vivo phosphoenolpyruvate carboxylase activity is controlled by CO_2 and O_2 mole fractions and represents a major flux at high photorespiration rates. *New Phytol* 221:1843–1852. <https://doi.org/10.1111/nph.15500>
- Ahmad KW, Wang G (2023) Evaluating the crucial relationships between soil health and climate change. *Journal of Environmental Impact and Management Policy*. <https://doi.org/10.55529/jeimp.41.8.21>
- Akram M (2014) Citric acid cycle and role of its intermediates in metabolism. *Cell Biochem Biophys* 68:475–478. <https://doi.org/10.1007/s12013-013-9750-1>
- Alhendawi RA, Römhild V, Kirkby EA, Marschner H (1997) Influence of increasing bicarbonate concentrations on plant growth, organic acid accumulation in roots and iron uptake by barley, sorghum, and maize. *J Plant Nutr* 20:1731–1753. <https://doi.org/10.1080/01904169709365371>
- Alizadeh Z, Ghasemnezhad M, Sangani MF, Atak A (2023) The effects of high concentration of bicarbonate applications on kiwifruit genotypes with different ploidy levels on some growth parameters of leaves. *Turk J Agric for* 47:436–447. <https://doi.org/10.55730/1300-011X.3100>
- Ashburner M, Ball CA, Blake JA et al (2000) Gene ontology: Tool for the unification of biology. *Nat Genet* 25:25–29. <https://doi.org/10.1038/75556>
- Bailey M, Hsieh EJ, Tsai HH, Ravindran A, Schmidt W (2023) Alkalinity modulates a unique suite of genes to recalibrate growth and pH homeostasis. *Frontiers Plant Sci* 14:1100701. <https://doi.org/10.3389/fpls.2023.1100701>
- Barberon M (2017) The endodermis as a checkpoint for nutrients. *New Phytol* 213:1604–1610. <https://doi.org/10.1111/nph.14140>
- Barberon M, Vermeer JEM, De Bellis D et al (2016) Adaptation of Root Function by Nutrient-Induced Plasticity of Endodermal Differentiation. *Cell* 164:447–459. <https://doi.org/10.1016/j.cell.2015.12.021>
- Basra AS, Malik CP (1985) Non-photosynthetic fixation of carbon dioxide and possible biological roles in higher plants. *Biol Rev* 60:357–400. <https://doi.org/10.1111/j.1469-185x.1985.tb00421.x>
- Baxter I, Hosmani PS, Rus A et al (2009) Root suberin forms an extracellular barrier that affects water relations and mineral nutrition in Arabidopsis. *PLoS Genet* 5:e1000492. <https://doi.org/10.1371/journal.pgen.1000492>
- Britto DT, Kronzucker HJ (2005) Nitrogen acquisition, PEP carboxylase, and cellular pH homeostasis: New views on old paradigms. *Plant Cell Environ* 28:1396–1409. <https://doi.org/10.1111/j.1365-3040.2005.01372.x>
- Chinthapalli B, Raghavan C, Bläsing O et al (2001) Phosphoenolpyruvate carboxylase purified from leaves of C3, C4, and C3-C4 intermediate species of *Alternanthera*: properties at limiting and saturating bicarbonate. *Photosynthetica* 38:415–419. <https://doi.org/10.1023/A:1010981807667>
- Covarrubias JI, Rombolà AD (2013) Physiological and biochemical responses of the iron chlorosis tolerant grapevine rootstock 140 Ruggeri to iron deficiency and

- bicarbonate. *Plant Soil* 370:305–315. <https://doi.org/10.1007/s11104-013-1623-2>
- Cram WJ, Laties GG (1974) The kinetics of bicarbonate and malate exchange in carrot and barley root cells. *J Exp Bot* 25:11. <https://doi.org/10.1093/jxb/25.1.11>
- Dąbrowska-Bronk J, Komar DN, Rusaczek A et al (2016) β -carbonic anhydrases and carbonic ions uptake positively influence *Arabidopsis* photosynthesis, oxidative stress tolerance and growth in light dependent manner. *J Plant Physiol* 203:44–54. <https://doi.org/10.1016/j.jplph.2016.05.013>
- Danila FR, Thakur V, Chatterjee J et al (2021) Bundle sheath suberisation is required for C4 photosynthesis in a *Setaria viridis* mutant. *Commun Biol* 4:254. <https://doi.org/10.1038/s42003-021-01772-4>
- Daszkowska-Golec A et al (2016) The role of abscisic acid in drought stress: how ABA helps plants to cope with drought stress. In: Hossain MA, Wani SH, Bhattacharjee S (eds) *Drought stress tolerance in plants*, Vol 2: molecular and genetic perspectives. Springer, Cham, pp 123–151
- Devogswami CR, Kalita MC, Talukdar J et al (2011) Studies on the growth behavior of *Chlorella*, *Haematococcus* and *Scenedesmus* sp. in culture media with different concentrations of sodium bicarbonate and carbon dioxide gas. *Afr J Biotechnol* 10:13128–13138
- Éva C, Téglás F, Zelenyánszki H et al (2018) Cold inducible promoter driven Cre-lox system proved to be highly efficient for marker gene excision in transgenic barley. *J Biotechnol* 265:15–24. <https://doi.org/10.1016/j.jbiotec.2017.10.016>
- Falade JA (1972) Differentiation between bicarbonate effect and pH effect on cation accumulation by barley roots. *Can J Bot* 50:1567–1570. <https://doi.org/10.1139/b72-193>
- Flexas J, Medrano H (2002) Drought-inhibition of photosynthesis in C3 plants: Stomatal and non-stomatal limitations revisited. *Ann Bot* 89:183–189. <https://doi.org/10.1093/aob/mcf027>
- Furbank RT, Sharwood R, Estavillo GM et al (2020) Photons to food: genetic improvement of cereal crop photosynthesis. *J Exp Bot* 71:2226–2238. <https://doi.org/10.1093/jxb/eraa077>
- Gama F, Correia PJ, Saavedra T et al (2020) Can bicarbonate enhance the performance of carob seedlings grown in nutrient solutions with different Fe concentrations? *J Soil Sci Plant Nutr* 20:55–65. <https://doi.org/10.1007/s42729-019-00100-4>
- Gamarra Reinoso L, Majláth I, Dernovics M et al (2024) Root-based inorganic carbon uptake increases the growth of *Arabidopsis thaliana* and changes transporter expression and nitrogen and sulfur metabolism. *Front Plant Sci* 15:1448432. <https://doi.org/10.3389/fpls.2024.1448432>
- Gaufichon L, Rothstein SJ, Suzuki A (2016) Asparagine metabolic pathways in *Arabidopsis*. *Plant Cell Physiol* 57:675. <https://doi.org/10.1093/pcp/pcv184>
- Gulyás Z, Monček B, Hamow KÁ et al (2022) D27-LIKE1 isomerase has a preference towards trans/cis and cis/cis conversions of carotenoids in *Arabidopsis*. *Plant J* 112:1377–1395. <https://doi.org/10.1111/tpj.16017>
- Hajibarat Z, Saidi A, Ghazvini H, Hajibarat Z (2023) Comparative analysis of physiological traits and gene expression patterns in nitrogen deficiency among barley cultivars. *Journal of Genetic Engineering and Biotechnology* 21:110. <https://doi.org/10.1186/s43141-023-00567-w>
- Han M, Zhang C, Suglo P et al (2021) L-aspartate: An essential metabolite for plant growth and stress acclimation. *Molecules* 26:1887. <https://doi.org/10.3390/molecules26071887>
- He Q, Zhao S, Ma Q et al (2014) Endogenous salicylic acid levels and signaling positively regulate *Arabidopsis* response to polyethylene glycol-simulated drought stress. *J Plant Growth Regul* 33:871–880. <https://doi.org/10.1007/s00344-014-9438-9>
- Hildebrandt TM, Nunes Nesi A, Araújo WL, Braun HP (2015) Amino Acid Catabolism in Plants. *Mol Plant* 8:1563–1579. <https://doi.org/10.1016/j.molp.2015.09.005>
- Hosmani PS, Kamiya T, Danku J et al (2013) Dirigent domain-containing protein is part of the machinery required for formation of the lignin-based Casparian strip in the root. *Proc Natl Acad Sci U S A* 110:14498–14503. <https://doi.org/10.1073/pnas.1308412110>
- Hu R, Yu H, Deng J et al (2025) Phosphoenolpyruvate and related metabolic pathways contribute to the regulation of plant growth and development. *Int J Mol Sci*. <https://doi.org/10.3390/ijms26010391>
- Ingrisano R, Tosato E, Trost P et al (2023) Proline, cysteine and branched-chain amino acids in abiotic stress response of land plants and microalgae. *Plants* 12:3410. <https://doi.org/10.3390/plants12193410>
- Ishizawa K (2014) Intracellular pH regulation of plant cells under anaerobic conditions. In: van Dongen JT, Licausi F (eds) *Low-oxygen stress in plants*. Springer, Vienna. Springer, Vienna, pp 59–77
- Jacoby B, Laties GG (1971) Bicarbonate fixation and malate compartmentation in relation to salt-induced stoichiometric synthesis of organic acid. *Plant Physiol* 47:525–531. <https://doi.org/10.1104/pp.47.4.525>
- Jitrapakdee S, St Maurice M, Rayment I et al (2008) Structure, mechanism and regulation of pyruvate carboxylase. *Biochem J* 413:369–387. <https://doi.org/10.1042/BJ20080709>
- Jose J, Bozsó Z, Fábíán A, Éva C, Sági L (2024) The *Arabidopsis* sulfate transporter AtSultr1;2 is a negative regulator of resistance to *Ralstonia solanacearum*. In: L Bóna (ed) XXX. Növénynevelési Tudományos Napok: Összefoglalók p.104–108. ISBN:978-963-8351-50-0 https://real.mtak.hu/205393/2/XXX_NNTN_absztraktotet.pdf
- Joshi V, Joung JG, Fei Z, Jander G (2010) Interdependence of threonine, methionine and isoleucine metabolism in plants: accumulation and transcriptional regulation under abiotic stress. *Amino Acids* 39(4):933. <https://doi.org/10.1007/s00726-010-0505-7>
- Kambhampati S, Ajewole E, Marsolais F (2017) Advances in asparagine metabolism. In: Cánovas F, Lüttge U, Matyssek R (eds) *Progress in botany*. Springer, Cham, pp 49–74
- Kanehisa M, Goto S (2000) KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res* 28:27–30. <https://doi.org/10.1093/nar/28.1.27>
- Klughammer C, Schreiber U (2008) Saturation Pulse method for assessment of energy conversion in PSI. *PAM Application Notes* 1:11–14
- Koendjibiharie JG, Van Kranenburg R, Kengen SWM (2021) The PEP-pyruvate-oxaloacetate node: variation at the

- heart of metabolism. *FEMS Microbiol Rev* 45:1–19. <https://doi.org/10.1093/femsre/fuaa061>
- Lea PJ, Sodek L, Parry MAJ et al (2007) Asparagine in plants. *Annals of Applied Biology* 150:1–26. <https://doi.org/10.1111/j.1744-7348.2006.00104.x>
- Lee JA, Woolhouse HW (1969) Root growth and dark fixation of carbon dioxide in calcicoles and calcifuges. *New Phytol* 68:247–255. <https://doi.org/10.1111/j.1469-8137.1969.tb06437.x>
- Li H, Lv J, Su Y, Wu Y (2023) Appropriate sodium bicarbonate concentration enhances the intracellular water metabolism, nutrient transport and photosynthesis capacities of *Coix lacryma-jobi* L. *Agronomy* 13:1790. <https://doi.org/10.3390/agronomy13071790>
- Licaj I, Fiorillo A, Di Meo MC et al (2024) Effect of polyethylene glycol-simulated drought stress on stomatal opening in “Modern” and “Ancient” wheat varieties. *Plants* 13:1575. <https://doi.org/10.3390/plants13111575>
- Lin G, Ehleringer JR, Rygielwicz PT et al (1999) Elevated CO₂ and temperature impacts on different components of soil CO₂ efflux in Douglas-fir terracosms. *Glob Chang Biol* 5:157–168. <https://doi.org/10.1046/j.1365-2486.1999.00211.x>
- Liu H, Song S, Zhang H et al (2022) Signaling transduction of ABA, ROS, and Ca²⁺ in plant stomatal closure in response to drought. *Int J Mol Sci* 23:14824. <https://doi.org/10.3390/ijms232314824>
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 25:402–408. <https://doi.org/10.1006/meth.2001.1262>
- Lynch JM, Barbano DM (1999) Kjeldahl nitrogen analysis as a reference method for protein determination in dairy products. *J AOAC Int* 82:1389–1398. <https://doi.org/10.1093/jaoac/82.6.1389>
- Maekawa S, Sato T, Asada Y et al (2012) The *Arabidopsis* ubiquitin ligases ATL31 and ATL6 control the defense response as well as the carbon/nitrogen response. *Plant Mol Biol* 79:217–227. <https://doi.org/10.1007/s11103-012-9907-0>
- Nyitrai P, Czövek P, Óvári M, Keresztes Á (2009) Signaling mechanism mediating the anti-senescence effect of low-concentration chemical stressors sprayed onto bean seedlings. *Environ Exp Bot* 66:501–506. <https://doi.org/10.1016/j.envexpbot.2009.04.009>
- O’Leary B, Park J, Plaxton WC (2011) The remarkable diversity of plant PEPC (phosphoenolpyruvate carboxylase): Recent insights into the physiological functions and post-translational controls of non-photosynthetic PEPCs. *Biochemical Journal* 436:15–34. <https://doi.org/10.1042/BJ20110078>
- Parvathi K, Bhagwat AS, Raghavendra AS (1998) Modulation by bicarbonate of catalytic and regulatory properties of C4 phosphoenolpyruvate carboxylase from *Amaranthus hypochondriacus*: Desensitization to malate and glucose 6-phosphate and sensitization to Mg²⁺. *Plant Cell Physiol* 39:1294–1298. <https://doi.org/10.1093/oxfordjournals.pcp.a029333>
- Pasqualini S, Ederli L, Piccioni C et al (2001) Metabolic regulation and gene expression of root phosphoenolpyruvate carboxylase by different nitrogen sources. *Plant Cell Environ* 24:439–447. <https://doi.org/10.1046/j.1365-3040.2001.00692.x>
- Pérez-Martín L, Almira MJ, Estrela-Muriel L et al (2024) A role for root carbonic anhydrase βCA4 in the bicarbonate tolerance of *Arabidopsis thaliana*. *Physiol Plant* 176:e70026. <https://doi.org/10.1111/ppl.70026>
- Pfaffl M (2004) Quantification strategies in real-time PCR. In: Bustin SA (ed) A-Z of quantitative PCR. pp 87–112
- Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *BBA - Bioenergetics* 975:384–394. [https://doi.org/10.1016/S0005-2728\(89\)80347-0](https://doi.org/10.1016/S0005-2728(89)80347-0)
- Roell M-S, Kuhnert F, Westhoff P et al (2025) Mitochondrial formate dehydrogenase links mitochondrial and cytosolic one carbon metabolism via a one-carbon shunt. *Plant Physiol*. <https://doi.org/10.1093/plphys/kiaf623>
- Romero LC, Aroca MÁ, Laureano-Marín AM et al (2014) Cysteine and cysteine-related signaling pathways in *Arabidopsis thaliana*. *Mol Plant* 7:264–276. <https://doi.org/10.1093/mp/sst168>
- Sagervanshi A, Naeem A, Kaiser H et al (2021) Early growth reduction in *Vicia faba* L. under alkali salt stress is mainly caused by excess bicarbonate and related to citrate and malate over accumulation. *Environ Exp Bot* 192:104636. <https://doi.org/10.1016/j.envexpbot.2021.104636>
- Sardans J, Peñuelas J (2021) Potassium control of plant functions: Ecological and agricultural implications. *Plants* 10:419. <https://doi.org/10.3390/plants10020419>
- Sasaki Y, Nagano Y (2004) Plant acetyl-CoA carboxylase: Structure, biosynthesis, regulation, and gene manipulation for plant breeding. *Biosci Biotechnol Biochem* 68:1175–1184. <https://doi.org/10.1271/bbb.68.1175>
- Schröder J, Stenger H, Wernicke W (2001) α-Tubulin genes are differentially expressed during leaf cell development in barley (*Hordeum vulgare* L.). *Plant Mol Biol* 45:723–730. <https://doi.org/10.1023/A:1010648519206>
- Schubert S, Yan F (1997) Nitrate and ammonium nutrition of plants: Effects on acid/base balance and adaptation of root cell plasmalemma H⁺ ATPase. *J Plant Nutr Soil Sci* 160:275–281. <https://doi.org/10.1002/jpln.19971600222>
- Sharma SN, Sirohi GS (1987) The effect of ammonium and nitrate on CO₂ assimilation, RuBP and PEP carboxylase activity and dry matter production in wheat. *Photosynth Res* 12:265–272. <https://doi.org/10.1007/BF00055126>
- Sharma A, Patni B, Shankhdhar D, Shankhdhar SC (2013) Zinc - An Indispensable Micronutrient. *Physiol Mol Biol Plants* 19:11. <https://doi.org/10.1007/s12298-012-0139-1>
- Sharma N, Chaudhary C, Khurana P (2020) Wheat myo-inositol phosphate synthase influences plant growth and stress responses via ethylene mediated signaling. *Sci Rep* 10:10766. <https://doi.org/10.1038/s41598-020-67627-w>
- Shimono H, Kondo M, Evans JR (2019) Internal transport of CO₂ from the root-zone to plant shoot is pH dependent. *Physiol Plant* 165:451–463. <https://doi.org/10.1111/ppl.12767>
- Sotomayor D, Rice CW (1999) Soil air carbon dioxide and nitrous oxide concentrations in profiles under tallgrass

- prairie and cultivation. *J Environ Qual* 28:784–793. <https://doi.org/10.2134/jeq1999.00472425002800030008x>
- Spittstoesser WE (1966) Dark CO₂ fixation and its role in the growth of plant tissue. *Plant Physiol* 41:755–759
- Sun P, Isner JC, Coupel-Ledru A et al (2022) Countering elevated CO₂ induced Fe and Zn reduction in Arabidopsis seeds. *New Phytol* 235:1796–1806. <https://doi.org/10.1111/nph.18290>
- Takahashi K, Kinoshita T (2017) The regulation of plant cell expansion: Auxin-induced turgor-driven cell elongation. In: Rose RJ (ed) *Molecular Cell Biology of the Growth and Differentiation of Plant Cells*. Taylor & Francis, p 156
- Verstegen H, Köneke O, Korzun V, von Broock R (2014) The world importance of barley and challenges to further improvements. In: *Biotechnological Approaches to Barley Improvement*. Biotechnology in Agriculture and Forestry. pp 3–19
- Vuorinen AH, Vapaavuori EM, Raatikainen O, Lapinjoki SP (1992) Metabolism of inorganic carbon taken up by roots in Salix plants. *J Exp Bot* 43:789–795. <https://doi.org/10.1093/jxb/43.6.789>
- Walker RP, Chen ZH, Famiani F (2021) Gluconeogenesis in plants: A key interface between organic acid/amino acid/lipid and sugar metabolism. *Molecules* 26:5129. <https://doi.org/10.3390/molecules26175129>
- Wang M, Sun Y, Gu Z et al (2016) Nitrate protects cucumber plants against fusarium oxysporum by regulating citrate exudation. *Plant Cell Physiol* 57:2001–2012. <https://doi.org/10.1093/pcp/pcw124>
- Wirtz M, Hell R (2006) Functional analysis of the cysteine synthase protein complex from plants: Structural, biochemical and regulatory properties. *J Plant Physiol* 163:273–286. <https://doi.org/10.1016/j.jplph.2005.11.013>
- Xia A, Wu Y, Xiang J et al (2023) Quantification of glucose metabolism and nitrogen utilization in two Brassicaceae species under bicarbonate and variable ammonium soil conditions. *Plants (Basel)* 12:3095. <https://doi.org/10.3390/plants12173095>
- Xu C, He CG, Wang YJ et al (2020) Effect of drought and heat stresses on photosynthesis, pigments, and xanthophyll cycle in Alfalfa (*Medicago sativa* L.). *Photosynthetica* 58:1226–1236. <https://doi.org/10.32615/ps.2020.073>
- Yao K, Wu YY (2021) Rhizospheric bicarbonate improves glucose metabolism and stress tolerance of *Broussonetia papyrifera* L. seedlings under simulated drought stress. *Russ J Plant Physiol* 68:126–135. <https://doi.org/10.1134/S1021443721010209>
- Yin Z, Balmant K, Geng S, et al (2017) Bicarbonate induced redox proteome changes in arabidopsis suspension cells. *Front Plant Sci* 8:. <https://doi.org/10.3389/fpls.2017.00058>
- Yonemura S, Yokozawa M, Shirato Y et al (2009) Soil CO₂ concentrations and their implications in conventional and no-tillage agricultural fields. *J Agric Meteorol* 65:141–149. <https://doi.org/10.2480/agrmet.65.2.2>
- Zrenner R, Stitt M, Sonnewald U, Boldt R (2006) Pyrimidine and purine biosynthesis and degradation in plants. *Annu Rev Plant Biol* 57:805–836. <https://doi.org/10.1146/annurev.arplant.57.032905.105421>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.