C and N metabolism in barley leaves and peduncles modulates responsiveness to changing CO₂

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Abstract

Balancing of leaf carbohydrates is a key process for maximising crop performance in elevated CO₂ environments. With the aim of testing the role of the carbon sink–source relationship under different CO₂ conditions, we performed two experiments with two barley genotypes (Harrington and RCSL-89) exposed to changing CO₂. In Experiment 1, the genotypes were exposed to 400 and 700 ppm CO₂. Elevated CO₂ induced photosynthetic acclimation in Harrington that was linked with the depletion of Rubisco protein. In contrast, a higher peduncle carbohydrate-storage capacity in RSCL-89 was associated with a better balance of leaf carbohydrates that could help to maximize the photosynthetic capacity under elevated CO₂. In Experiment 2, plants that were grown at 400 ppm or 700 ppm CO₂ for 5 weeks were switched to 700 ppm or 400 ppm CO₂, respectively. Raising CO₂ to 700 ppm increased photosynthetic rates with a reduction in leaf carbohydrate content and an improvement in N assimilation. The increase in nitrate content was associated with up-regulation of genes of protein transcripts of photosynthesis and N assimilation that favoured plant performance under elevated CO₂. Finally, decreasing the CO₂ from 700 ppm to 400 ppm revealed that both stomatal closure and inhibited expression of light-harvesting proteins negatively affected photosynthetic performance and plant growth.

Key words: Barley, carbohydrates, elevated CO₂, gene expression, N assimilation, peduncle, photosynthetic down-regulation, sink–source.

Introduction

Atmospheric carbon dioxide (CO₂) has increased from around 280 ppm recorded at the beginning of the Industrial Revolution (1780) to approximately 400 ppm at present, and it is expected to increase to over 900 ppm by the end of the 21st century, depending on the climate-change emission scenario (IPCC, 2014). While it would be logical to assume enhanced photosynthetic assimilation in C₃ plants due to the increase in CO₂, several studies have shown that a build-up of leaf carbohydrate linked to higher CO₂ availability might induce a reduction in carboxylation efficiency (Ainsworth et al., 2007; Bloom et al., 2010; Aranjuelo et al., 2015). Prolonged exposure to elevated CO₂ often induces stomatal closure with a consequent impact on CO₂ diffusion into the chloroplast, which would partly explain the decline in photosynthetic carboxylation capacity (Xu et al., 2016). Non-stomatal limitations can also lead to a down-regulation of photosynthesis due to a decrease in the
amount and activity of Rubisco, and this is accompanied by the accumulation of carbohydrates. Several factors have been proposed to explain the phenomenon of CO₂ acclimation, including insufficient plant sink strength (Moore et al., 1999; Ainsworth and Rogers, 2007), nitrogen (N) dilution by accumulation of carbohydrates in leaves (Stütt and Krapp, 1999), and/or inhibition of nitrate assimilation (Bloom et al., 2010). Enhanced leaf C content caused by greater photosynthetic rates in plants exposed to elevated CO₂ could lead to repression of photosynthesis-related genes and to a down-regulation of photosynthetic capacity (Ainsworth et al., 2004; Aranjuelo et al., 2009, 2011, Vicente et al., 2015, 2016). The build-up of leaf carbohydrate has been associated with the capacity to develop strong C sinks, such as developing organs (Lewis et al., 2002; Aranjuelo et al., 2013). Thus, the presence of higher C sink strengths could contribute to preventing photosynthetic down-regulation via a better redistribution and allocation of carbohydrates to the developing sinks under elevated CO₂ conditions (Ainsworth et al., 2004; Aranjuelo et al., 2013). Plants with a greater capacity to remobilize the ‘extra’ photoassimilates to organs with a higher C demand could have a similar advantage.

Nitrogen assimilation has also been identified as a key process that influences photosynthetic performance under elevated CO₂. Photosynthesis provides C skeletons for assimilating N into amino acids to form proteins and other nitrogenous compounds. The imbalance between C fixation and N assimilation has been suggested as the main factor responsible for photosynthetic down-regulation under elevated CO₂ (Ainsworth and Long, 2005; Bloom et al., 2010). In addition, limitations in N assimilation observed in plants grown under elevated CO₂ have been associated with a reduction in energy availability, which would have effects on C and N metabolism (Rachmilevitch et al., 2004; Bloom et al., 2010; Aranjuelo et al., 2013). Such limitations in energy availability would modify the C/N ratio by increasing the carbohydrate content and decreasing the N pool due to competition for reductant (Rachmilevitch et al., 2004; Bloom et al., 2010).

Assimilation and remobilization of C compounds is important during grain filling. To sustain grain filling in C₃ cereals, photoassimilates are mainly provided from photosynthesis in the upper leaves, predominantly the flag leaf and penultimate leaves in barley and the flag leaf in wheat (Evans, 1983; Hosseini et al., 2012; Liu et al., 2015), from the remobilization of C stored in leaves and peduncles that was assimilated before anthesis (Gebbing and Schnyder, 1999), and from photosynthesis in the ear (Tambussi et al., 2007; Zhou et al., 2016). Sucrose, fructans, and starch are the most important carbohydrates that affect crop performance during the grain-filling period in barley. Sucrose is the major transported form of carbohydrate and it provides most of the energy and C necessary for the growth and development of non-photosynthetic organs. Together with starch, fructans have been described as the major C storage compounds in different cereal organs such as the grains, leaves, stems, and roots (Morcuende et al., 2004). In addition to their role as reserve carbohydrates, fructans also provide C and energy to non-photosynthetic tissues when the C demand is high (Xue et al., 2011; Van den Ende, 2013). In addition, carbohydrates can also act as signal molecules that regulate the expression of a wide variety of genes involved in different metabolic pathways and cellular functions (Osuna et al., 2007; Van den Ende, 2013; Valluru, 2015). Fructan synthesis is activated when sucrose content exceeds a threshold concentration (Pollock and Cairns, 1991; Koroleva et al., 1998). The increase in sucrose content increases fructosyltransferase gene expression, whereas high nitrate content inhibits its expression (Morcuende et al., 2004). A close correlation between increases in carbohydrate content and the down-regulation of genes involved in photosynthesis and N metabolism has been recently reported (Vicente et al., 2018).

In the search for more productive varieties, conventional plant-breeding programs have reduced the genetic diversity of crops by the use of ‘elite’ varieties that have lost alleles relevant to specific environmental conditions (Ellis et al., 2000; Dawson et al., 2015). The intensive plant-breeding programs conducted over recent decades have mainly been focused on the selection of genotypes with high harvest indices. However, such selection has not contributed to overcoming sink–source limitations (Maydup et al., 2012; Schmid et al., 2016). To recover some of the favourable alleles lost during plant-breeding programs, Matus et al. (2003) developed a recombinant chromosome substitution line (RCSL) population of 140 lines using the wild ancestor of barley, Hordeum vulgare subsp. spontaneum, as a source of donor alleles for H. vulgare subsp. vulgare cv ‘Harrington’, which is commonly used as a quality standard in North America. The recovered genes in the RCSL-89 line showed higher tolerance to abiotic stress by accumulating more carbohydrates under drought (Méndez et al., 2011).

Adaptation of crops to future atmospheric conditions will require a better understanding of how plants respond to increasing C content. In this study, we took two approaches to investigate the importance of the C sink–source balance in the responsiveness of plants to different CO₂ conditions. The first goal was to determine the relevance of the balance in response to elevated CO₂. For this, barley genotypes with high (RCLS-89) and low (Harrington) capacities to store C/N compounds in the peduncles were exposed to elevated CO₂. The second goal was to characterize and identify mechanisms developed by plants to regulate their C sink–source balance under changing CO₂. For this, two sets of 64 plants were grown. The first set was initially exposed to ambient CO₂ (400 ppm) followed by elevated CO₂ (700 ppm), and the second set was initially exposed to elevated CO₂ (700 ppm) followed by ambient CO₂ (400 ppm). The comparisons made between the responses of elite cultivars and newly developed genotypes have implications for future breeding programs that seek adaptive alleles to maintain the C sink–source balance under changing CO₂.

**Materials and methods**

**Plant material and experimental design**

Seeds of the two barley genotypes, Hordeum vulgare subsp. vulgare cv Harrington and RCSL-89 (Matus et al., 2003), were kept at 4 °C for 10 d to synchronize germination. Once germinated, 64 plants were grown in 32 pots (6.7 l) filled with a mixture of perlite:vermiculite (1:2, v/v) in two controlled environment chambers (Phytotron Service, SGKer, UPV/ EHU). The conditions inside the chambers were 550 µmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD) and a 14/10 h light/dark regime at...
25/17 °C and 50/60% relative humidity. The plants were watered twice a week with Hoagland’s solution (Arnon and Hoagland, 1939) and once a week with deionized water to avoid salt accumulation. The experimental set-up was designed as two experiments in parallel. For Experiment 1, 32 plants were grown at different CO2 concentrations, ambient (400 ppm) or elevated (700 ppm), for 11 weeks (Supplementary Fig. S1 at JXB online). For Experiment 2, a set of 16 plants that had been grown for 5 weeks at ambient CO2 (i.e. up to flag-leaf emergence) were then exposed for the following 6 weeks to elevated CO2 (400–700 treatment). The reverse conditions were applied to another set of 16 plants that had been grown at elevated CO2 for 5 weeks and which were then exposed to ambient CO2 for 6 weeks (700–400 treatment) (Supplementary Fig. S1).

Biomass and gas-exchange measurements

At the end of both experiments (week 11), gas-exchange measurements were conducted, beginning 2 h after the start of the photoperiod. The measurements were made on the flag leaf of plants at the medium milk stage (Zadoks stage 75). The net photosynthetic rate (AN) was measured at 500 μmol m−2 s−1 PPFD with the stomatal conductance (g) and intercellular CO2 (C_i) using a 6400-XT portable gas-exchange system (LI-COR Inc.). Curves of net CO2 assimilation rate versus intercellular CO2 concentration (A/C) were obtained under saturated light (1000 μmol m−2 s−1 PPFD) with the following steps: 400, 300, 200, 100, 400, 500, 600, 800, 1000, 1200, 400 ppm CO2, with typically 2–3 min between each step. For the estimation of the maximum carboxylation velocity of Rubisco (V_{cmax}) we used the equation developed by Sharkey et al. (2007).

After measuring photosynthesis, four plants from each treatment were harvested for biochemical and molecular analyses. The flag leaves and peduncles were immediately plunged into liquid N and stored at −80 °C until further analyses. For determination of biomass, the main stems and tillers of four other plants were dried in an oven at 80 °C for 72 h. The contribution of the ear biomass at the end of the experiment was calculated as (Ear Biomass/Total biomass) × 100 (%).

Carbon and nitrogen contents

Flag leaves and peduncles dried at 80 °C for 72 h were ground and 1 mg of material per sample was loaded into small tin capsules analysed using a Flash 1112 Elemental Analyzer (Carbo Erba, Milan).

Determination of metabolites

Frozen flag leaf and peduncle material was used for ethanol/water extraction for carbohydrate determination according to Morcuende et al. (2004). Sucrose, starch, and fructan contents were subsequently determined spectrophotometrically following the protocol described by Morcuende et al. (2004). In the flag leaf, total amino acids were determined using the ninhydrin method (Hare and Cress, 1997), ammonium quantification was carried out using the Berthelot method (Patton and Crouch, 1977) based on the phenol hypochlorite assay, and nitrate quantification was carried out according to the manufacturer’s instructions. RNA integrity was checked on a 1.5% (v/v) agarose gel and the absence of genomic DNA contamination was confirmed by PCR using a primer pair for the gene encoding glyceraldehyde-3-phosphate dehydrogenase (G3PDH). NADP-hydrogenase synthase (GOGAT) were determined by changes in the NADH concentration at 340 nm in a reaction consisting of 20 μl protein extract and 280 μl of reaction buffer for NADH-dependent GOGAT (100 mM Tris–HCl, pH 8.5, 1 mM CaCl2, 13 mM 2-oxoglutarate, 50 mM (NH4)2SO4, and 0.25 mM NADH) or for NAD-dependent GOGAT (100 mM Tris–HCl, pH 8.6, 10 mM DTT, 1 mM 2-oxoglutarate, and 0.2 mM NADH).

RNA extraction and synthesis of cDNA

RNA was isolated from pulverized frozen flag leaves using the phenol-chloroform method described by Morcuende et al. (1998). Then, 10 μg of RNA for each sample were treated with DNase Turbo (Ambion) according to the manufacturer's instructions. RNA integrity was checked on a 1.5% (v/v) agarose gel. After the quantification of the relative gene expression using the comparative C_{T} method (Schnittenner and Livak, 2008), and using actin as a reference for normalizing the gene expression results (Córdoba et al., 2016).

Quantitative real-time PCR

Gene expression was measured as described by Vicente et al. (2015). Quantitative PCR was performed in an optical 384-well plate with a PRISM 7900 HT Sequence Detection System (Applied Biosystems) in a 10-μl reaction volume using the SYBR Green Maxter Mix reagent (Applied Biosystems), 1 μl of diluted cDNA (1:40), and 200 nM of each gene-specific primer. The PCR thermal profile was as follows: polymerase activation (50 °C for 2 min, 95 °C for 10 min) amplification and quantification cycles repeated for 40 cycles (95 °C for 15 s and 60 °C for 1 min), and a final step of 95 °C for 15 s and 60 °C for 15 s to obtain the dissociation curve. Three biological replicates were used for quantification analysis, with two technical replicates for each biological sample. Transcript levels for genes associated with photosynthesis, carbohydrate metabolism, and N-assimilation in flag leaves were determined using the primers described in Méndez (2014) and Córdoba et al. (2016) (Supplementary Table S1). The data are presented as the log2-fold change (Kolmogorov–Smirnov and Levene tests. Differences in the effects of CO2 on the two barley genotypes were determined by one-way ANOVA with a Duncan post hoc test. Differences between the two genotypes in the same CO2 conditions
were analysed using Student’s t-test. For both analyses, differences were considered significant at $P<0.05$.

**Results**

**Experiment 1: evaluation of the relevance of the plant C sink–source balance in responses to elevated CO$_2$**

Exposure to elevated CO$_2$ did not alter the total biomass but did increased the ear biomass in both barley genotypes, as shown by the higher ear biomass contribution relative to 400 ppm CO$_2$ (Table 1). In the Harrington genotype, the photosynthetic rate ($A_N$) at 700 ppm CO$_2$ was not increased relative to 400 ppm (Fig. 1A) but the maximum carboxylation velocity of Rubisco ($V_{cmax}$) was decreased (Fig. 1B), whilst the stomatal conductance ($g_s$) was similar under both CO$_2$ conditions (Fig. 1C). In RCSL-89 plants exposed to elevated CO$_2$, $A_N$ and $V_{cmax}$ were increased relative to ambient CO$_2$ (Fig. 1A, B). Comparing the Harrington and RCSL-89 plants, the results suggested a possible photosynthetic down-regulation in Harrington, because although there was no difference in the internal CO$_2$ concentration ($C_i$) of Harrington flag leaves relative to RCSL-89, the value of $V_{cmax}$ was decreased at 700 ppm (Fig. 1B, D).

In order to determine the level of photoassimilates and their mobilization to sink organs, the sucrose, starch, and fructan contents were determined in flag leaves and the peduncles of the two barley genotypes (Fig. 2). Flag leaves of Harrington grown under elevated CO$_2$ had lower sucrose levels than under ambient CO$_2$ (Fig. 2A, B). However, the fructan content of the peduncles did not significantly differ between the two CO$_2$ conditions, but the fructan content of the flag leaves was significantly increased under elevated CO$_2$. The sucrose content of the flag leaves was much lower in RCSL-89 than in Harrington under elevated CO$_2$, while there was no significant difference in the sucrose content of the peduncles of both genotypes. The sucrose content of the flag leaves of Harrington grown at 700 ppm CO$_2$ was lower than at 400 ppm CO$_2$ but no significant differences were observed in RCSL-89. Prolonged exposure to elevated CO$_2$ decreased the sucrose content in both genotypes, with the latter maintaining similar activities to plants grown at 400 ppm CO$_2$.

**Experiment 2: testing the plant mechanisms of response to changing CO$_2$**

**Increasing CO$_2$ from 400 ppm to 700 ppm**

Increasing the CO$_2$ concentration from 400 ppm to 700 ppm for a further 6 weeks (400–700) did not significantly alter the ear biomass and the ear biomass contribution (%) of the Harrington and RCSL-89 genotypes compared to those grown continuously at 700 ppm (Table 1). In addition, the ear biomass and the ear biomass contribution (%) in Harrington were similar to plants grown continuously at 400 ppm. On the other hand, while the total biomass in RCSL-89 was not altered when CO$_2$ was increased (Table 1), the ear biomass contribution of these plants was increased, although not as much as in plants that were grown at 700 ppm CO$_2$.

**Table 1.** Total biomass, ear biomass, and the ear biomass contribution (%) of the Harrington and RCSL-89 genotypes at the medium milk stage (Zadoks stage 75)

| CO$_2$ conditions | Total biomass (g) | Ear biomass (g) | Ear biomass contribution (%) |
|-------------------|------------------|----------------|-------------------------------|
| Harrington        |                  |                |                               |
| 400               | 12.88 ± 1.38 a   | 2.18 ± 0.40 b  | 0.17 ± 0.02 b                 |
| 700               | 13.15 ± 1.76 a   | 3.77 ± 0.36 a  | 0.33 ± 0.03 a                 |
| 400–700           | 9.68 ± 0.81 ab   | 2.09 ± 0.27 b  | 0.22 ± 0.02 b                 |
| 700–400           | 8.52 ± 0.53 b    | 1.98 ± 0.32 b  | 0.23 ± 0.04 b                 |
| RCSL-89           |                  |                |                               |
| 400               | 11.40 ± 1.07 a   | 1.05 ± 0.13 a  | 0.10 ± 0.01 a                 |
| 700               | 14.48 ± 1.39 a   | 4.59 ± 0.19 a  | 0.30 ± 0.02 a                 |
| 400–700           | 12.72 ± 1.24 a   | 2.73 ± 0.21 b  | 0.22 ± 0.02 b                 |
| 700–400           | 13.62 ± 1.23 a   | 3.81 ± 0.36 b  | 0.25 ± 0.03 AB                |

CO$_2$ conditions were 400 ppm, 700 ppm, increase from 400 ppm to 700 ppm (400–700), and decrease from 700 ppm to 400 ppm (700–400). Significant differences ($P<0.05$) between each CO$_2$ condition are indicated with different letters: lowercase letters indicate differences for Harrington and capital letters for RCSL-89. * Indicates significant genotype differences. Values are means (±SEM) of 4 biological replicates.
Peduncle metabolism prevents photosynthetic down-regulation continuously in 700 ppm. The photosynthetic parameters in Harrington exposed to the increase in CO2 were similar to those grown continuously at 700 ppm, but $V_{\text{cmax}}$ was lower in for the equivalent plants of RCSL-89 (Fig. 1A, B).

Increasing the CO2 reduced the sucrose and starch contents in Harrington flag leaves compared to plants that were grown continuously at 700 ppm CO2 (Fig. 2A, C, E). On the other hand, the flag leaves of RCSL-89 plants had lower sucrose content than those grown continuously at 700 ppm CO2, but showed similar starch and fructan contents. In the peduncles, the only change observed was that Harrington had a lower fructan content than plants grown continuously at 700 ppm CO2 (Fig. 2F). RCSL-89 maintained higher fructan contents than Harrington in the flag leaves and peduncles when the CO2 was increased (Fig. 2E, F).

Increasing the CO2 concentration reduced the C content of leaves of both genotypes relative to plants grown continuously at 700 ppm, but a similar effect did not occur in the peduncles (Table 2). The increase in CO2 did not result in any substantial changes in N content in Harrington flag leaves and peduncles (Table 2). The increase in CO2 resulted in increases in the ammonium and amino acid contents in Harrington flag leaves (Fig. 3B, C), whilst in RCSL-89 the nitrate and ammonium contents increased (Fig. 3A, B), but the amino acid content did not vary (Fig. 3C) and the protein content decreased relative to plants grown continuously at 700 ppm CO2 (Fig. 3D). Both genotypes showed higher NR activity when the CO2 was increased (Fig. 4A), but the activities of GS and GOGAT were unaffected relative to continuous growth at 700 ppm (Fig. 4B, C). GDH had different patterns of activity, increasing in flag leaves of Harrington but remaining unaltered in those of RCSL-89 (Fig. 4D).

Flag leaves showed differences in transcriptional responses to the increase in CO2 as several photosynthetic genes were induced in both genotypes Fig. 5). Specifically, transcripts of PSII light-harvesting proteins, the Rubisco large subunit, and protochlorophyllide oxidoreductase were induced in Harrington plants compared to those grown continuously at 700 ppm CO2. The gene encoding NR was also up-regulated in both genotypes, whilst the gene encoding fructan 1-exohydrolase was repressed in RCSL-89.

Decreasing CO2 from 700 ppm to 400 ppm

Decreasing the CO2 concentration after 5 weeks of growth at 700 ppm to 400 ppm for a further 6 weeks (700–400) resulted in Harrington having lower biomass compared to plants grown continuously at 400 ppm. However, both the ear biomass and the ear biomass contribution (%) were similar for 700-400 and 400 ppm plants (Table 1). Decreasing the CO2 did not significantly affect the total biomass of RCSL-89, but it did result in higher ear biomass contribution relative to plants grown continuously at 400 ppm (Table 1). In both genotypes, the reduction in CO2 decreased $A_N$ and this was coupled with strong...
stomatal closure compared to plants grown at 400 ppm CO$_2$ (Fig. 1A, C). $V_{\text{max}}$ was significantly decreased in Harrington but not in RCSL-89 (Fig. 1B).

Decreasing the CO$_2$ reduced the sucrose content in the flag leaves of both genotypes compared to those from plants grown continuously at 400 ppm (Fig. 2A), but it did not significantly change the sucrose content in the peduncles (Fig. 2B). The starch content in Harrington flag leaves and peduncles was reduced, whilst in RCSL-89 the starch content in the flag leaves did not vary but it increased in the peduncles in comparison to plants grown at 400 ppm CO$_2$ (Fig. 2C, D). Neither the flag leaves nor peduncles of either Harrington or RCSL-89 plants showed significant differences in fructan content relative to plants grown continuously at 400 ppm (Fig. 2E, F). Corresponding with the decline in $A_N$, decreasing the CO$_2$ reduced the C content in flag leaves of both genotypes compared with plants grown continuously at 400 ppm (Table 2).

Decreasing the CO$_2$ increased the nitrate and ammonium contents but reduced the protein content in Harrington (Fig. 3A, B, D). In RCSL-89, the content of nitrate increased, amino acids decreased, and proteins were unaltered relative to plants grown continuously at 400 ppm CO$_2$ (Fig. 3A, C, D). The decrease in CO$_2$ led to an increase in NR activity in both genotypes (Fig. 4A) but did not significantly affect the activities of the other enzyme studied (Fig. 4B–D).
Table 2. N and C contents in the flag leaves and peduncles of the Harrington and RCLS-89 genotypes grown under different CO₂ conditions

| CO₂ conditions | Leaf C (%) | Leaf N (%) | Leaf C/N | Peduncle C (%) | Peduncle N (%) | Peduncle C/N |
|----------------|------------|------------|----------|----------------|----------------|--------------|
| **Harrington** |            |            |          |                |                |              |
| 400            | 45.19 ± 0.16  a | 3.47 ± 0.26  a | 13.18 ± 0.99  a | 42.36 ± 0.23  a | 1.37 ± 0.99  a | 30.88 ± 0.50  b |
| 700            | 44.57 ± 0.24  a | 3.00 ± 0.14  a | 14.91 ± 0.67  a | 42.39 ± 0.35  a | 0.95 ± 0.67  a | 45.32 ± 3.88  ab |
| 400–700        | 43.47 ± 0.36  b | 3.16 ± 0.49  a | 14.53 ± 2.49  a | 44.60 ± 2.03  a | 0.90 ± 2.49  b | 51.21 ± 7.97  a |
| 700–400        | 44.46 ± 0.36  a | 3.14 ± 0.25  a | 14.36 ± 1.33  a | 41.53 ± 1.04  a | 1.17 ± 1.33  a | 36.93 ± 7.08  ab |
| **RCLS-89**    |            |            |          |                |                |              |
| 400            | 45.22 ± 0.71  A | 4.08 ± 0.47  A | 11.31 ± 1.01  A | 43.34 ± 0.05  A* | 1.34 ± 0.22  A | 34.12 ± 5.41  A |
| 700            | 44.93 ± 0.29  A | 3.25 ± 0.12  AB | 13.84 ± 0.47  A | 43.52 ± 0.07  A* | 1.01 ± 0.11  A | 44.24 ± 5.18  A |
| 400–700        | 43.53 ± 0.16  B | 4.08 ± 0.09  A | 10.67 ± 0.20  A | 42.95 ± 0.31  A* | 1.36 ± 0.20  A | 33.10 ± 5.43  A |
| 700–400        | 43.23 ± 0.31  A | 3.00 ± 0.20  B | 14.51 ± 0.94  B | 42.79 ± 0.51  A* | 1.46 ± 0.30  A | 31.78 ± 6.06  A |

CO₂ conditions were 400 ppm, 700 ppm, increase from 400 ppm to 700 ppm (400–700), and decrease from 700 ppm to 400 ppm (700–400). Significant differences (P<0.05) between each CO₂ condition are indicated with different letters: lowercase letters indicate differences for Harrington and capital letters for RCLS-89. * Indicates significant genotype differences. Values are means (±SEM) of 4 biological replicates.

Fig. 3. Effects of CO₂ on N forms (nitrate and ammonium), amino acids, and soluble proteins in flag leaves of the barley genotypes Harrington and RCLS-89. (A) Nitrate, (B) ammonium, (C) amino acids, (D) soluble protein, and (E) relative Rubisco content. CO₂ growth conditions were 400 ppm, 700 ppm, increase from 400 ppm to 700 ppm (400–700), and decrease from 700 ppm to 400 ppm (700–400). Significant differences (P<0.05) between each CO₂ condition are indicated with different letters: lowercase letters indicate significant differences for Harrington and capital letters for RCLS-89. * Indicates significant genotype differences (P<0.05). Values are means (±SEM) of four biological replicates.
Decreasing the CO₂ repressed several genes that encode photosynthetic proteins (light-harvesting and the Calvin–Benson cycle) as well as genes involved in fructan metabolism (1-FFT, 1-FEH, and 6-FEH) and cell wall synthesis in the flag leaves of both Harrington and RCSL-89 compared with plants that were grown continuously at 400 ppm CO₂ (Fig. 5). Decreasing the CO₂ induced the gene encoding the NR enzyme.

Discussion

The sink–source balance has been postulated as being key to conditioning the responsiveness of photosynthetic capacity to increasing CO₂ (Ainsworth and Long, 2005; Aranjuelo et al., 2011, 2013). In the present study two approaches were used to test the relevance of the capacity of the peduncle to accumulate carbohydrates, and the ‘plasticity’ of leaf C/N metabolism following modifications in the CO₂ conditions.

Experiment 1: a higher peduncle C-storage capacity contributes to overcoming photosynthetic down-regulation under elevated CO₂

Carbon sink–source imbalance has been suggested as being responsible for the photosynthetic down-regulation frequently observed when plants are exposed to elevated CO₂ (Aranjuelo et al., 2011, 2013; White et al., 2016). Indeed, an insufficient demand for carbohydrates from developing C-sinks has been observed to induce leaf C imbalances (White et al., 2016). The peduncle has a special importance in the C-storage capacity for maintaining leaf C balance during the vegetative stage in cereals (Tambussi et al., 2007). Later, during the grain-filling period, the C stored in the peduncle is remobilized towards the grain. In our study it was notable that in both barley genotypes, higher fructan contents were found in peduncles than in flag leaves (Fig. 2), showing the importance of these organs for the subsequent grain filling-stage.

Inadequate C-sink strength can lead to a decrease in photosynthetic activity so that C-source activity and sink capacity are balanced (White et al., 2016). Exposure to elevated CO₂ decreased \( V_{\text{max}} \) and the relative Rubisco content in Harrington (which has a low capacity to store C/N compounds in the peduncles), while an increase in \( V_{\text{max}} \) was found in RCSL-89 (high capacity to store C/N compounds in peduncles) (Fig. 1). The depletion observed in Harrington was consistent with the photosynthetic down-regulation response widely observed under elevated CO₂ (Pérez et al., 2005; Aranjuelo et al., 2011, 2013; Vicente et al., 2015). In addition, the depletion in Rubisco content in these plants, together with the decreases in amino acids and soluble proteins (Fig. 3), reduced the levels of leaf organic-N compounds, as has been
observed in previous studies (Bloom et al., 2002; Pérez et al., 2005; Aranjuelo et al., 2011; Vicente et al., 2015). In contrast to Harrington, elevated CO₂ led to an increase in the soluble protein content in RCSL-89 (Fig. 3). This suggested an improvement in leaf organic-N compounds that could have helped to maximize photosynthetic capacity, which is consistent with the higher \( V_{\text{max}} \) observed under elevated CO₂ in RCSL-89 (Fig. 1). In addition, the drastic increase in ear biomass under elevated CO₂ in RCSL-89 (Table 1) indicated that the strong sink capacity of this organ was especially important in the photosynthetic performance of this genotype under elevated CO₂. The distribution of photoassimilates from flag leaves to the peduncles may have contributed to the avoidance of carbohydrate build-up under elevated CO₂. Our results suggested that the improved leaf C balance in RCSL-89 may have helped to maintain N status, and consequently plants avoided photosynthetic down-regulation under elevated CO₂. Similar to effects observed previously in wheat (Vicente et al., 2015), in Harrington the down-regulation of genes encoding the Rubisco large subunit, together with decreased transcripts for proteins involved in light-harvesting (Fig. 5) and the lower Rubisco content under elevated CO₂ (Fig. 3), may have
contributed to the photosynthetic acclimation that we found in this genotype. The fact that at 700 ppm CO2 Harrington had a higher starch content than RCSL-89 (Fig. 2) may indicate that the flag leaves of Harrington were subjected to C sink–source imbalance. It should be noted that starch has been proposed as a way to store excess C in plants, while leaf sucrose content is suggested to represent the main form of C translocated towards developing sinks (Stitt et al., 2010). This highlights the fact that impaired N assimilation, and consequently reduced Rubisco protein availability, could be linked to leaf C sink–source imbalances (Ainsworth et al., 2004; Aranjuelo et al., 2011, 2013; White et al., 2016).

In our study, NR activity was not significantly affected by elevated CO2 in the flag leaves of either Harrington or RCSL-89 plants (Fig. 4). This suggested that CO2 enrichment did not restrict leaf nitrate reduction, which is in contrast with decreases reported in other species (Bloom et al., 2002, 2010; Vicente et al., 2015). The higher sucrose content in RCSL-89 could have contributed to the maintenance of NR expression and activity (Morcuende et al., 1998) and to sustaining the activity of GS (Robredo et al., 2011), with a consequent impact on amino acid and protein availabilities under elevated CO2 (Fig. 3). However, GS has been described as a target enzyme involved in N and C metabolism (Vega-Mas et al., 2015). The decline in GS and GDH activities decreased the nitrate-assimilation pathway in Harrington flag leaves, which in turn altered the contents of amino acids and other organic-N compounds under elevated CO2. In contrast, the maintenance of the activities of these enzymes observed in RCSL-89 flag leaves would guarantee assimilation of inorganic nitrogen into amino acids. Indeed, total soluble protein levels increased in RCSL-89 flag leaves under exposure to elevated CO2 (Fig. 3). These findings suggest that a limitation in N assimilation could be involved in the decline in organic-N compounds and the down-regulation of photosynthetic capacity found in Harrington plants under elevated CO2. The improved photosynthetic acclimation responses to elevated CO2 in the RCSL-89 genotype were associated with enhanced flag-leaf N assimilation and a consequent increase in organic N compounds (Fig. 3). Moreover, the higher sink capacity of the peduncle and the ears would have facilitated the correct leaf C/N balance and overcome the photosynthetic down-regulation due to elevated CO2, confirming the importance of C-sink strength for increased crop yields under elevated CO2.

**Experiment 2: a balance in C and N metabolism modulates adaptability to changing CO2**

As observed in Experiment 1, photosynthesis in plants grown under elevated CO2 is limited by the ability to adjust photosynthetic activity according to leaf C demand (Ziska et al., 2004). To evaluate the adaptation capacity of the two barley genotypes to changing environmental CO2, plants were grown under ambient CO2 (400 ppm) or elevated CO2 (700 ppm) and then exchanged between treatments.

Increasing the CO2 concentration caused similar responses in Amax, gs, Ci and relative Rubisco content in both genotypes relative to plants grown continuously at 700 ppm (Figs 1, 3). Harrington maintained its photosynthetic capacity compared to plants grown continuously at 700 ppm CO2. However, the increase in Vcmax observed in RCSL-89 grown continuously at 700 ppm was not detected when it was initially grown at 400 ppm (Fig. 1). The ability to overcome photosynthetic acclimation may be linked to the up-regulation of genes encoding proteins involved in light-harvesting and the maintenance of Rubisco gene expression and protein content (Vicente et al., 2015). Hence, our findings suggested that Harrington plants did not suffer photosynthetic down-regulation or, at least, that they showed a better photosynthetic capacity than RCSL-89 under increasing CO2.

In agreement with previous findings by Stitt et al. (2010), the higher starch content observed in Harrington compared to RCSL-89 grown under elevated CO2 (Fig. 2) could be considered a symptom of C overflow due to the rate of photosynthesis exceeding the rate of leaf C demand. This imbalance may have been associated with the down-regulation of amino acid storage, in agreement with previous studies (Yamakawa and Hakata, 2010; Midorikawa et al., 2014). Interestingly, the starch content did not differ in the peduncles of either genotype after increasing the CO2 concentration, but RCSL-89 showed higher storage capacity for fructans in the flag leaves and peduncles than Harrington.

Increasing the CO2 increased the activity of NR in flag leaves of both Harrington and RCSL-89 relative to plants grown continuously at 700 ppm (Fig. 4). This indicated that the reduction of leaf nitrate was unaffected by CO2 enrichment, which is in contrast to the reduction in the N pool reported in other species grown under elevated CO2 (Bloom et al., 2002, 2010; Vicente et al., 2015). More notably than in the first experiment, CO2 enrichment induced the expression of NR genes and increased the nitrate content, as has been observed previously (Stitt and Krapp, 1999; Vicente et al., 2016), while increasing the amino acid content and reducing the sucrose and starch contents relative to plants grown at 700 ppm CO2. The competition for reductants in the chloroplast stroma has been described as a factor that conditions C and N assimilation (Rachmilevitch et al., 2004; Vicente et al., 2016). For this reason, the leaf light-harvesting complexes and proteins involved in electron transport may be particularly important in maintaining the energy necessary for balancing both N and C metabolism. In agreement with Vicente et al. (2017), we observed that exposure to elevated CO2 induced the expression of PSI light-harvesting complexes (Fig. 5). More than 50% of the N that is assimilated by roots is allocated to flag leaves and comprises Rubisco, light-harvesting complexes, and other proteins involved in electron transport (Kitaoka and Koike, 2004). Our results suggested that increasing CO2 from 400 ppm to 700 ppm caused concomitant increases in Amax and nitrate content (Fig. 1, Table 2) and reductions in carbohydrate content (Table 2) by increasing energy availability for co-ordinating C and N assimilation under elevated CO2. These findings suggest that this stimulation of N assimilation could be involved in the increase in the amino acid content and the capacity to overcome the initial photosynthetic down-regulation found in Harrington under elevated CO2.
Decreasing the CO₂ concentration from 700 ppm to 400 ppm after flag-leaf emergence caused significant stomatal closure and reduced photosynthetic rates (Fig. 1), which were associated with lower biomass in Harrington plants (Table 1). Stomatal limitations are one of the mechanisms responsible for photosynthetic down-regulation under elevated CO₂ (Xu et al., 2016). Bloom et al. (2002, 2010) reported that a reduction in A₅ increases nitrate assimilation because NR has access to a larger amount of NADH for reducing nitrate to nitrite. Our results suggested that plants exposed to decreasing CO₂ suffered energy limitations due to a lower expression of light-harvesting complexes and reaction centres when compared to plants grown continuously at 400 ppm CO₂ (Fig. 5). This photosynthetic limitation was reflected by a decrease in the leaf carbohydrate contents (Fig. 2). However, the peduncles of RCSL-89 plants showed a greater accumulation of starch, which is associated with long-term carbohydrate storage. In accordance with the photosynthetic limitations, genes related to photosynthesis, such as light-harvesting, Rubisco, and chlorophyll synthesis, were down-regulated, or at least showed similar expression to plants grown at 700 ppm (Fig. 5). Comparing Harrington and RCSL-89, the higher fructan content in the peduncles of RCSL-89 could have been linked to the repression of fructosyltransferases (particularly sucrose:sucrose 1-fructosyltransferase), which are involved in fructan synthesis. The lower sucrose and starch-storage capacity in flag leaves, together with the accumulation of fructans in the peduncles, revealed that the lower photosynthetic capacity acted to modify the C/N balance. In this regard, the lower biomass, especially in terms of ear weight, together with the lower levels of amino acids and proteins as well as the down-regulation of photosynthesis-related genes suggested that the plants attempted to adapt to the new environment.

Conclusions and future perspectives

Our study has highlighted the importance of the C/N balance as influenced by photosynthesis and N assimilation in two barley genotypes exposed to elevated CO₂, and the relevance of the peduncle sink capacity to this balance. Our study showed that in genotype Harrington, which has a low capacity to store C/N compounds in the peduncles, CO₂ enrichment decreased photosynthetic capacity whereas genotype RCSL-89, which has a higher capacity, could overcome photosynthetic down-regulation under elevated CO₂. The larger C-sink capacity of RCSL-89 enabled the avoidance of a build-up of leaf carbohydrates and enabled the maintenance of Rubisco protein in the flag leaves. On the other hand, the leaf C sink–source imbalance of Harrington plants grown under elevated CO₂ was linked to a depletion of Rubisco content and a consequent decrease in Vₘₙ₉. The increased expression of transcripts associated with light-harvesting complexes and changes to CO₂ diffusion were shown to be significant in influencing plant growth and C and N metabolism when the CO₂ conditions were modified. Increasing CO₂ from 400 ppm to 700 ppm reduced leaf carbohydrate contents and improved N assimilation. On the other hand, decreasing the CO₂ from 700 ppm to 400 ppm led to both stomatal closure and repression of transcripts of light-harvesting proteins, showing them to be the main factors involved in the inhibition of photosynthetic machinery and plant growth.

Within the context of current environmental conditions and those projected for the coming decades, it is crucial to increase crop yields through the development of cultivars that are resilient to environmental changes. While the work now underway in plant breeding programs is of great importance, there is a risk that alleles crucial to adaptations to this unpredictable future might be lost when selecting modern elite crop cultivars. Moreover, as evidenced here and in earlier studies, the introgression of genes from wild genotypes into modern varieties could overcome some sink–source limitations. Together, our results show that the RCSL-89 barley line has a greater responsiveness to elevated CO₂ than the elite Harrington cultivar, highlighting the potential contribution that adaptive alleles from wild genotypes have to breeding programs.

Supplementary data

Supplementary data are available at JXB online.

Table S1. List of primers used for real-time qPCR.

Fig. S1. Schematic diagram of the experimental regimes showing treatments and phenological stages.

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References

Ainsworth EA, Long SP. 2005. What have we learned from 15 years of free-air CO₂ enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO₂. New Phytologist 165, 351–371.

Ainsworth EA, Rogers A. 2004. Testing the ‘source–sink’ hypothesis of down-regulation of photosynthesis in elevated [CO₂] in the field with single gene substitutions in soybean leaves? Journal of Experimental Botany 58, 579–591.

Ainsworth EA, Rogers A, Nelson R, Long SP. 2004. Testing the ‘source–sink’ hypothesis of down-regulation of photosynthesis in elevated [CO₂] in the field with single gene substitutions in Glycine max. Agricultural and Forest Meteorology 122, 85–94.

Aranjuelo I, Cabrera-Bosquet L, Morcuende R, Avíce JC, Nogué S, Araus JL, Martínez-Carrasco R, Pérez P. 2011. Does ear C sink strength contribute to overcoming photosynthetic acclimation of wheat plants exposed to elevated CO₂? Journal of Experimental Botany 62, 3967–3969.
Aranjuelo I, Erice G, Sanz-Sáez A, et al. 2015. Differential CO₂ effect on primary carbon metabolism of flag leaves in durum wheat (Triticum durum Desf.). Plant, Cell & Environment 38, 2780–2794.

Aranjuelo I, Pardo A, Bilc C, Savé R, Azcón-Bieto J, Nogués S. 2009. Leaf carbon management in slow-growing plants exposed to elevated CO₂. Global Change Biology 15, 97–109.

Aranjuelo I, Sanz-Sáez Á, Jauregui I, Irigoyen JJ, Araus JL, Sánchez-Díaz M, Erice G. 2013. Harvest index, a parameter conditioning responsiveness of wheat plants to elevated CO₂. Journal of Experimental Botany 64, 1879–1892.

Arnon DI, Haogland DR. 1939. A comparison of a water culture and soil as media for crop production. Science 89, 512–514.

Baki GKA, Siefritz F, Man H, Weiner H, Kaldenhoff R, Kaiser WM. 2000. Nitrate reductase in Zea mays L. under salinity. Plant, Cell & Environment 23, 515–521.

Bloom AJ, Burger M, Rubio Asensio JS, Cousins AB. 2010. Carbon dioxide enrichment inhibits nitrate assimilation in wheat and Arabidopsis. Science 328, 899–903.

Bloom AJ, Smart DR, Nguyen DT, Searles PS. 2002. Nitrogen assimilation and growth of wheat under elevated carbon dioxide. Proceedings of the National Academy of Sciences, USA 99, 1730–1735.

Cataldo DA, Schrader LE, Youngs VL. 1974. Analysis by digestion and colorimetric assay of total nitrogen in plant tissues high in nitrate. Crop Science 14, 854–856.

Córdoba J, Molina-Cano JL, Martínez-Carrasco R, Morcuende R, Pérez P. 2016. Functional and transcriptional characterization of a barley mutant with impaired photosynthesis. Plant Science 244, 19–30.

Dawson IK, Russell J, Powell W, Steffenson B, Thomas WT, Waugh R. 2015. barley: a translational model for adaptation to climate change. New Phytologist 206, 913–931.

Ellis RP, Forster BP, Robinson D, Handley LL, Gordon DC, Russell JR, Powell W. 2000. Wild barley: a source of genes for crop improvement in the 21st century? Journal of Experimental Botany 51, 9–17.

Evans JR. 1983. Nitrogen and photosynthesis in the flag leaf of wheat (Triticum aestivum L.). Plant Physiology 72, 297–302.

Gebbing T, Schnyder H. 1999. Pre-anthesis reserve utilization for protein and carbohydrate synthesis in grains of wheat. Plant Physiology 121, 871–878.

Gibon Y, Blaesing OE, Hannemann J, Carillo P, Höhne H, Hendriks K, Hülskamp M, Kühn S, Leimgruber A, Lorz J, Morschheuser V, Pollock CJ, Siefritz F, Siefritz M, Schmittgen TD, Livak KJ. 2016. Effects of CO₂ enrichment and drought on photosynthesis, growth and yield of an old and a modern barley cultivar. Plant, Cell & Environment 49, 463–491.

Patton CJ, Crouch SR. 1977. Spectrophotometric and kinetics investigation of the Berthelot reaction for the determination of ammonia. Analytical Chemistry 49, 464–469.

Pérez P, Morcuende R, Molino I, Martínez-Carrasco R. 2005. Diurnal changes of Rubisco in response to elevated CO₂ temperature and nitrogen in wheat grown under temperature gradient tunnels. Environmental and Experimental Botany 53, 13–27.

Pollock CJ, Cairns AJ. 1991. Fructan metabolism in grasses and cereals. Annual Review of Plant Physiology and Plant Molecular Biology 42, 77–101.

Rachmilevitch S, Cousins AB, Bloom AJ. 2004. Nitrate assimilation in plant shoots depends on photosynthesis. Proceedings of the National Academy of Sciences, USA 101, 11506–11510.

Robredo A, Pérez-López U, Miranda-Apodaca J, Lacuesta M, Mena-Petite A, Muñoz-Rueda A. 2011. Elevated CO₂ reduces the drought effect on carbon metabolism in barley plants during drought and subsequent recovery. Environmental and Experimental Botany 71, 399–408.

Schmid I, Franzaring J, Müller M, Brohun N, Calvo OC, Högy P, Fangmeier A. 2016. Effects of CO₂ enrichment and drought on photosynthesis, growth and yield of an old and a modern barley cultivar. Journal of Agronomy and Crop Science 202, 81–95.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative Ct method. Nature Protocols 3, 1101–1108.

Sharkey TD, Bernacchi CJ, Faquhar GD, Singsaas EL. 2007. Fitting photosynthetic carbon dioxide response curves for C₃ leaves. Plant, Cell & Environment 30, 1035–1040.

Stitt M, Krapp A. 1999. The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. Plant, Cell & Environment 22, 583–621.

Stitt M, Lunn J, Usadel B. 2010. Arabidopsis and primary photosynthetic metabolism – more than the icing on the cake. The Plant Journal 61, 1067–1091.

Tambussi EA, Bort Pie J, Guinta MJ, Jorda MM, Jrih G, Arnaud JL. 2007. The photosynthetic role of ears in C₃ cereals: metabolism, water use efficiency and contribution to grain yield. Critical Reviews in Plant Sciences 26, 1–16.

Valluru R. 2015. Fructan and hormone contribution. Frontiers in Plant Science 6, 180.

Van den Ende W. 2013. Multifunctional fructans and raffinose family oligosaccharides. Frontiers in Plant Science 4, 247.
Vega-Mas I, Marino D, Sánchez-Zabala J, González-Murua C, Estavillo JM, González-Moro MB. 2015. CO₂ enrichment modulates ammonium nutrition in tomato adjusting carbon and nitrogen metabolism to stomatal conductance. Plant Science 241, 32–44.

Vicente R, Martínez-Carrasco R, Pérez P, Morcuende R. 2018. New insights into the impacts of elevated CO₂, nitrogen, and temperature levels on the regulation of C and N metabolism in durum wheat using network analysis. New Biotechnology 40, 192–199.

Vicente R, Pérez P, Martínez-Carrasco R, Feil R, Lunn JE, Watanabe M, Arrivault S, Stitt M, Hoefgen R, Morcuende R. 2016. Metabolic and transcriptional analysis of durum wheat responses to elevated CO₂ at low and high nitrate supply. Plant & Cell Physiology 57, 2133–2146.

Vicente R, Pérez P, Martínez-Carrasco R, Morcuende R. 2017. Improved responses to elevated CO₂ in durum wheat at a low nitrate supply associated with the upregulation of photosynthetic genes and the activation of nitrate assimilation. Plant Science 260, 119–128.

Vicente R, Pérez P, Martínez-Carrasco R, Usadel B, Kostadinova S, Morcuende R. 2015. Quantitative RT-PCR platform to measure transcript levels of C and N metabolism-related genes in durum wheat: transcript profiles in elevated [CO₂] and high temperature at different levels of N supply. Plant & Cell Physiology 56, 1556–1573.

White AC, Rogers A, Rees M, Osborne CP. 2016. How can we make plants grow faster? A source–sink perspective on growth rate. Journal of Experimental Botany 67, 31–46.

Xu Z, Jiang Y, Jia B, Zhou G. 2016. Elevated-CO₂ response of stomata and its dependence on environmental factors. Frontiers in Plant Science 7, 1–15.

Xue GP, Kooiker M, Drenth J, McIntyre CL. 2011. TaMYB13 is a transcriptional activator of fructosyltransferase genes involved in β-2,6-linked fructan synthesis in wheat. Plant Journal 68, 857–870.

Yamakawa H, Hakata M. 2010. Atlas of rice grain filling-related metabolism under high temperature: joint analysis of metabolome and transcriptome demonstrated inhibition of starch accumulation and induction of amino acid accumulation. Plant & Cell Physiology 51, 795–809.

Zhou B, Serret MD, Elazab A, Bort Pie J, Araus JL, Aranjuelo I, Sanz-Sáez Á. 2016. Wheat ear carbon assimilation and nitrogen remobilization contribute significantly to grain yield. Journal of Integrative Plant Biology 58, 914–926.

Ziska LH, Morris CF, Goins EW. 2004. Quantitative and qualitative evaluation of selected wheat varieties released since 1903 to increasing atmospheric carbon dioxide: can yield sensitivity to carbon dioxide be a factor in wheat performance? Global Change Biology 10, 1810–1819.