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RESEARCH PAPER

Does ear C sink strength contribute to overcoming photosynthetic acclimation of wheat plants exposed to elevated CO_2?

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Abstract

Wheat plants (*Triticum durum* Desf., cv. Regallo) were grown in the field to study the effects of contrasting [CO_2] conditions (700 versus 370 μmol mol^{-1}) on growth, photosynthetic performance, and C management during the post-anthesis period. The aim was to test whether a restricted capacity of sink organs to utilize photosynthates drives a loss of photosynthetic capacity in elevated CO_2. The ambient $^{13}C/^{12}C$ isotopic composition ($\delta^{13}C$) of air CO_2 was changed from –10.2‰ in ambient [CO_2] to –23.6‰ under elevated [CO_2] between the 7th and the 14th days after anthesis in order to study C assimilation and partitioning between leaves and ears. Elevated [CO_2] had no significant effect on biomass production and grain filling, and caused an accumulation of C compounds in leaves. This was accompanied by up-regulation of phosphoglycerate mutase and ATP synthase protein content, together with down-regulation of adenosine diphosphate glucose pyrophosphatase protein. Growth in elevated [CO_2] negatively affected Rubisco and Rubisco activase protein content and induced photosynthetic down-regulation. CO_2 enrichment caused a specific decrease in Rubisco content, together with decreases in the amino acid and total N content of leaves. The C labelling revealed that in flag leaves, part of the C fixed during grain filling was stored as starch and structural C compounds whereas the rest of the labelled C (mainly in the form of soluble sugars) was completely respired 48 h after the end of labelling. Although labelled C was not detected in the $\delta^{13}C$ of ear total organic matter and respired CO_2, soluble sugar $\delta^{13}C$ revealed that a small amount of labelled C reached the ear. The $^{12}CO_2$ labelling suggests that during the beginning of post-anthesis the ear did not contribute towards overcoming flag leaf carbohydrate accumulation, and this had a consequent effect on protein expression and photosynthetic acclimation.

Key words: C management, elevated CO_2, photosynthetic acclimation, proteomic characterization, Rubisco, stable isotopes.

Abbreviations: A_{370}, photosynthesis determined at 370 μmol mol$^{-1}CO_2$; A_{700}, photosynthesis determined at 700 μmolmol$^{-1}CO_2$; ADPG, ADPglucose; AGPPase, adenosine diphosphate glucose pyrophosphatase; CA, carbonic anhydrase; Ci_{370}, intercellular [CO_2] determined at 370 μmol mol$^{-1}CO_2$; Ci_{700}, intercellular [CO_2] determined at 700 μmol mol$^{-1}CO_2$; DM, dry matter; g_m, mesophyll conductance; gs_{700}, stomatal conductance determined at 700 μmolmol$^{-1}CO_2$; IRGA, infrared gas analyser; k_{cat}, overall enzyme catalytic rate; PAR, photosynthetically active radiation; PDB, Pee Dee Belemnite; PGAM, Phosphoglycerate mutase; PPFD, photosynthetic photon flux density; RuBP, ribulose bisphosphate; R, dark respiration; T_0, immediately after the end of labelling; T_1, 24 h after the end of labelling; T_2, 48 h after the end of labelling; TOM, total organic matter; TSP, total soluble proteins; TSS, total soluble sugars; $\delta^{13}C$, ^{13}C isotopic composition; δ, $^{13}C_p$plant ^{13}C isotopic composition; Δ, C isotope discrimination; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate.

Introduction

The global atmospheric concentration of carbon dioxide ($[CO_2]$) has increased from ~280 μmol mol^{-1} during the pre-industrial period to 388.5 μmol mol^{-1} in 2010 (Dr Pieter Tans, NOAA/ESRL, www.esrl.noaa.gov/gmd/ccgg/ trends/) and is expected to reach 700 μmol mol^{-1} by the end of this century (Prentice *et al.*, 2001). The primary effects of increased $[CO_2]$ on C_3 plants include (i) increased plant biomass and (ii) leaf net photosynthetic rates, and (iii) decreased stomatal conductance (Long *et al.*, 2004; Nowak *et al.*, 2004; Ainsworth and Long, 2005). The biochemical basis for the leaf CO_2 assimilation response to increased atmospheric $[CO_2]$ is well established (Farquhar *et al.*, 1980). At relatively low $[CO_2]$ concentrations leaf CO_2 assimilation increases because Rubisco carboxylation is enhanced by increased substrate availability and the suppression of competitive Rubisco oxygenation (Ellsworth *et al.*, 2004). Although the initial stimulation of net photosynthesis associated with elevated $[CO_2]$ is sometimes retained (Davey *et al.*, 2006), some species fail to sustain the initial, maximal stimulation (Stitt, 1991; Long *et al.*, 2004; Aranjuelo *et al.*, 2005*b*; Martínez-Carrasco *et al.*, 2005; Ainsworth and Rogers, 2007; Pérez *et al.*, 2007; Alonso *et al.*, 2009; Gutiérrez *et al.*, 2009), a phenomenon called photosynthetic acclimation or down-regulation.

Stomatal limitations reduce photosynthesis due to depletion of intercellular $[CO_2]$ (C_i) as a result of stomatal closure (Naumburg *et al.*, 2004), i.e. a reduced supply of CO_2 to the photosynthetic apparatus within leaves. Limited mesophyll conductance (g_m) to CO_2 diffusion can also significantly constrain photosynthesis, but the extent of this limitation is still not well known (Evans *et al.*, 2009). Previous studies conducted by Singsaas *et al.* (2003) and Flexas *et al.* (2007) with a range of plants exposed to different $[CO_2]$ showed that g_m was involved in photosynthetic acclimation. Non-stomatal limitations reduce photosynthesis due to reduced photosynthetic electron transport (Aranjuelo *et al.*, 2008) or decreased Rubisco carboxylation of RuBP (Stitt and Krapp, 1999; Long *et al.*, 2004; Aranjuelo *et al.*, 2005*b*). Decreased Rubisco carboxylation occurs through two basic mechanisms: one that involves C source–sink relationships and a second that involves N allocation. Enhanced leaf C content caused by greater photosynthetic rates in plants exposed to elevated $[CO_2]$ induces repression of the expression of genes coding for photosynthetic proteins, leading to a down-regulation of photosynthetic capacity (Moore *et al.*, 1999; Jifon and Wolfe, 2002). At the whole-plant level this occurs when photosynthesis exceeds the capacity of sink organs to utilize photosynthate (Lewis *et al.*, 2002; Aranjuelo *et al.*, 2009*b*). In this sense, a previous study conducted by Ainsworth *et al.* (2004) showed that under elevated $[CO_2]$ conditions, a decrease in carboxylation capacity occurred in a determinate soybean mutant, which was genetically limited in its capacity to add 'sinks' for photosynthate, while no acclimation occurred in the indeterminate isogenic line. Accordingly, when plants exposed to elevated $[CO_2]$ have limitations on increasing C sink strength, they decrease their photosynthetic activity to balance C source activity and sink capacity (Thomas and Strain, 1991). The second basic mechanism leading to down-regulation is reduced Rubisco content is caused by non-selective decreases in leaf N content (Ellsworth *et al.*, 2004; Aranjuelo *et al.*, 2005*b*) or by reallocation of N within the plant (Nakano *et al.*, 1997). In both cases, reduced leaf N decreases Rubisco content.

Leaf carbohydrate accumulation is determined by the C source (photosynthesis) and sink balance (i.e. growth, respiration, and partitioning to other organs) (Aranjuelo *et al.*, 2009*b*). Despite the relevance of C loss through respiration, little attention has been given to this topic in cereals (Araus *et al.*, 1993; Bort *et al.*, 1996). Previous studies conducted in wheat and other cereals by Araus *et al.* (1993) revealed that dark respiration (R) in ears during grain filling ranged from 44% to 63% of the gross photosynthesis (net CO_2 assimilation plus R), 12–20 d after ear emergence. Furthermore, as observed in recent studies (Aranjuelo *et al.*, 2009*b*), the 'ability' to respire recently assimilated C may contribute towards preventing carbohydrate build-up and consequently to the avoidance of photosynthetic acclimation. In cereals like wheat, the ear comprises a very important C sink, especially during grain filling (Schnyder, 1993). In wheat, grain filling is sustained by photoassimilates (i) from the flag leaf (Evans *et al.*, 1975), (ii) from C fixed by the ear itself (Tambussi *et al.*, 2007), and (iii) from C remobilized from the stem internodes that was assimilated before anthesis (Gebbing and Schnyder, 1999).

Stable C isotope tracers are a key tool to study C management and its implications in photosynthetic performance (Körner *et al.*, 2005; von Felten *et al.*, 2007; Aranjuelo *et al.*, 2008, 2009*b*). One of the difficulties in analysing the processes of C metabolism (photosynthesis, respiration, allocation, and partitioning) is measuring the different processes simultaneously in the same experiment (Amthor, 2001). The lack of studies analysing the loss of photoassimilates by respiration during grain filling underscores the importance of examining this further. Labelling with $^{13}C/^{12}C$ enables the characterization of assimilated C and its further partitioning into different organs (Nogués *et al.*, 2004; Aranjuelo *et al.*, 2009*a,b*). C allocation and partitioning can be studied further by analysing the isotopic composition of soluble sugars (especially sucrose, glucose, etc.) (Körner *et al.*, 2005; Kodama *et al.*, 2010).

As has been explained above, ensuring adequate sink strength in crops will be essential for maximizing the response to rising $[CO_2]$ conditions. The aim of this study was to determine the role of the ears as major C sinks during grain filling and its effect on the leaf C content, photosynthetic acclimation, and plant growth of wheat plants exposed to elevated $[CO_2]$ under near field conditions. The significance of C management (photosynthesis, respiration, allocation, and partitioning) for grain filling in wheat under elevated $[CO_2]$ was assessed through $^{12}CO_2$

labelling carried out in greenhouses located in the field. $^{12}CO_2$ labelling was conducted at the plant level to gain a better understanding of C management in the whole plant. Furthermore, a biochemical and proteomic characterization was conducted to extend knowledge of the effects of elevated [CO_2] on the expression profile of proteins other than the most extensively characterized Rubisco.

Materials and methods

Experimental design

The experiment was conducted at Muñovela, the experimental farm of the Institute of Natural Resources and Agrobiology of Salamanca, CSIC (Salamanca, Spain). Durum wheat seeds (*Triticum durum* Desf. cv. Regallo) were sown at a rate of 200 kg ha^{-1} and 0.13 m row spacing on 29 October 2007. Before sowing, 60 kg ha^{-1} each of P and K (as P_2O_5 and K_2O, respectively) were added. An application of nitrogen fertilizer [$Ca(NO_3)_2$] as an aqueous solution was made by hand at 140 kg ha^{-1}, on 15 February 2008. The crop was watered weekly with a drip irrigation system, providing the amount of water required to equal potential evapotranspiration. After seedling emergence, six greenhouses (Aranjuelo *et al.*, 2005*a*; Pérez *et al.*, 2005; Gutiérrez *et al.*, 2009), based on those described by Rawson *et al.* (1995), were erected over the crop. The greenhouses were 9 m long, 2.2 m wide, and 1.7 m high at the ridge. They had rigid polycarbonate walls and a UV-stable polyethylene sheet roof. This material has good transmission of photosynthetically active radiation (PAR) and UV radiation, adequately mimicking outdoor conditions. PAR at mid-morning was 1020±187 μmol m^{-2} s^{-1} outdoors, whereas inside the greenhouses the PAR was 825±113 μmol m^{-2} s^{-1}. Three greenhouses were kept at ambient [CO_2] (370 μmol mol^{-1}), while in the other three atmospheric [CO_2] was increased to 700 μmol mol^{-1} (elevated [CO_2]) by injecting pure CO_2 at the two inlet fans during the light hours. CO_2 was not elevated during the night because little or no effect on *R* has been reported (Davey *et al.*, 2004). The atmospheric CO_2 concentration inside the greenhouses was continuously monitored at the plant level and regulated by PID controllers (Aranjuelo *et al.*, 2005*b*). Temperature and humidity were measured with sensors (HMD50; Vaisala, Helsinki, Finland) attached to a computer through analogue–digital convertors (Microlink 751; Biodata Ltd, Manchester, UK). Supplementary Fig. S1 (available at *JXB* online) shows the temperature and relative humidity inside the greenhouses during the experiment.

To analyse C allocation and partitioning in the plants, during the first week after anthesis and coinciding with the period of largest photoassimilate contribution to grain filling (Schnyder *et al.*, 2003), C labelling was conducted over 1 week via modification of the isotopic composition of the air ^{13}C ($\delta^{13}C$). During the C labelling period, the plants exposed to elevated [CO_2] conditions were grown in an environment where the $\delta^{13}C$ of the greenhouses was deliberately modified (–23.6±0.4‰) to distinguish it from the $\delta^{13}C$ of elevated [CO_2] (–20.1±0.4‰) during the previous period. Air $\delta^{13}C$ in the ambient [CO_2] was –10.2±0.4‰. The CO_2 was provided by Air Liquide (Valladolid, Spain). See below for details on air $\delta^{13}C$ collection and measurements. The labelling period lasted for 1 week starting 7 d after anthesis. All the determinations, with the exception of C-labelling-derived parameters, were conducted on the last day of the experiment, 14 d after anthesis. Isotopic characterization data were collected the day before the beginning of labelling (pre-label period), at the end of 7 d labelling (T_0; 2 weeks after anthesis), and 24 h (T_1) and 48 h (T_2) later after labelling (during post-labelling period).

Gas exchange and plant growth

Gas exchange of leaves was recorded in the central segment of flag leaves between 3 h and 8 h after the start of the photoperiod. Measurements were carried out with an air flow rate of 300 ml min^{-1}, 1500 μmol m^{-2} s^{-1} irradiance, and a 1.6±0.23 kPa vapour pressure deficit, using a 1.7-cm^2 window leaf chamber connected to a portable infrared gas analyser (CIRAS-2; PP Systems, Hitchin, Herts, UK) with differential operation in an open system. Temperature was kept at 25 °C with the Peltier system of the analyser. Photosynthesis was recorded at 370 μmol mol^{-1} and 700 μmol mol^{-1} CO_2.

To determine dry matter (DM) accumulation, the number of shoots in 0.5 m of two adjacent rows was counted, five consecutive shoots were harvested from each of the rows, and the dry weight of leaves, stems, and ears was recorded after drying in an oven at 60 °C for 48 h. This allowed the results to be expressed on a ground area basis.

Rubisco protein, amino acids, and Rubisco activity

At mid-morning samples consisting of four leaves were harvested and rapidly plunged *in situ* into liquid nitrogen and then stored at –80 °C until analysed. The fresh weight, leaf area, and chlorophyll content of subsamples of frozen leaves were determined as described (Pérez *et al.*, 2005).This allowed the results to be expressed on a leaf area basis.

Total amino acids were determined spectrophotometrically by the ninhydrin method according to Hare (1977) as described by Morcuende *et al.* (2004). The soluble proteins were extracted and measured spectrophotometrically (Bradford, 1976), and the amount of Rubisco subunits was determined by quantitative electrophoresis followed by densitometry (Pérez *et al.*, 2011).

For Rubisco initial and total activity assays, a NADH-coupled spectrophotometric procedure was followed (Pérez *et al.*, 2005). To estimate the k_{cat}, total Rubisco activity was divided by the number of enzyme active sites, which was obtained by multiplying the number of moles of Rubisco by 8.

Soluble sugar and starch content analyses

For sugar extraction, plant samples were lyophilized and then ground to a fine powder (<10 μm). About 50 mg of the fine powder were suspended in 1 ml of distilled water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany), mixed, and then centrifuged at 12,000×*g* for 5 min at 5 °C. After centrifugation, the supernatant was used for sugar quantification, whereas the pellet was stored at –80 °C for further starch analyses. The supernatant was heated for 3 min at 100 °C and afterward the solution was put on ice for 3 min. The supernatant containing the total soluble sugar (TSS) fraction was centrifuged at 12,000×*g* for 5 min at 5 °C (Nogués *et al.*, 2004). The supernatant was used for quantification of the individual sugars. Soluble sugar samples were purified using a solid phase extraction pre-column (Oasis MCX 3cc; Waters). Sugar content was analysed using a Waters 600 high performance liquid chromatograph (Waters Millipore Corp., Milford, MA, USA). The HPLC refractive index detector (Waters 2414) was set at 37 °C. Samples were eluted from the columns at 85 °C (Aminex HPX-87P and Aminex HPX-87C connected in series, 300×7.8 mm; Bio-Rad) with water at 0.6 ml min^{-1} flow rate and a total run time of 45 min. Sucrose, glucose, and fructans were collected and transferred to tin capsules for isotope analysis. The use of the purification pre-columns, together with the two Aminex columns connected in series enabled the separation of sucrose, glucose, and fructans, avoiding possible contamination problems raised by Richter *et al.* (2009). Furthermore, as an additional precaution, initial and final phases of peaks were discarded when collecting the peaks. As mentioned by Richter *et al.* (2009), there is no method that enables analysis of purified starch $\delta^{13}C$. Following one of the protocols described in the study conducted by Ritcher *et al.* (2009), the $\delta^{13}C$ of the HCl-hydrolysable C (HCl-C), which is composed mainly of starch, was analysed. Therefore, as suggested, the HCl-C was used as a reference for starch C isotopic composition. $\delta^{13}C$ of individual sugars and HCl-C was analysed by isotope ratio mass

spectrometry (Delta C; Finnigan Mat, Bremen, Germany) as described by Nogués *et al.* (2008).

C isotope composition ($\delta^{13}C$) of carbohydrates, total organic matter, together with C and N analyses

Flag leaf and ear samples were collected (T_0, T_1, and T_2) and dried at 60 °C for 48 h and then ground; 1.5 mg samples were used for total organic matter (TOM) analyses, and three biological replicates were analysed for each sample. Determinations of carbohydrates and TOM C, $\delta^{13}C$, and N were conducted at the Serveis Cientifico-Tècnics, University of Barcelona (Barcelona, Spain) using an elemental analyser (EA1108, Series 1; Carbo Erba Instrumentazione, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta C) operating in continuous flow mode.

Closed system for dark respiration sampling

Flag leaves and ears were placed separately in a gas analysis chamber to collect dark-respired CO_2 and to analyse $\delta^{13}C$. The chamber was connected in parallel to the sample air hose of a LI-COR 6400 (LI-COR, Lincoln, NE, USA) (Aranjuelo *et al.*, 2009*b*).

To accumulate CO_2 for the $\delta^{13}C$ analyses, respiration samples of flag leaves and ears were collected separately in the chamber described above. The gas analysis chamber was first flushed with CO_2-free air to ensure that only the CO_2 respired in the chamber was accumulated. The CO_2 concentration inside the chamber was measured by the LI-COR 6400. When the CO_2 inside reached the 300 μmol mol^{-1} concentration value, CO_2 samples were collected and analysed as described (Aranjuelo *et al.*, 2009*b*).

$\delta^{13}C$ measurements corresponding to each greenhouse and plant respiration (flag leaf and ear respiration) of air samples were analysed by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) at the Serveis Cientifico-Tecnics of the University of Barcelona (as previously described by Nogués *et al.*, 2008).

$^{13}C/^{12}C$ ratios of air samples and plant materials were expressed in δ notation:

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{standard}}\right) - 1$$

where R_{sample} refers to plant material and $R_{standard}$ to Pee Dee Belemnite (PDB) calcium carbonate.

C isotope discrimination (Δ) of leaf and ear TOM was calculated as described by Farquhar *et al.* (1989):

$$\Delta = \frac{\delta_a - \delta_p}{\delta_p + 1}$$

where δ_a and δ_p denote air ($\delta^{13}C_a$) and plant ($\delta^{13}C_p$) isotopic composition, respectively.

Proteomic characterization

Four biological replicates of flag leaf samples (200 mg fresh weight) were ground in a mortar using liquid nitrogen and resuspended in 2 ml of cold acetone containing 10% TCA. After centrifugation at 16000×*g* for 3 min at 4 °C, the supernatant was discarded, and the pellet was rinsed with methanol, acetone, and phenol solutions as previously described by Wang *et al.* (2003). The pellet was stored at –20 °C or immediately resuspended in 200 μl of R2D2 rehydration buffer. The total soluble protein (TSP) concentration was determined by the method of Bradford (Bradford, 1976) using BSA as standard. For two-dimensional electrophoresis, the protocol detailed in Aranjuelo *et al.* (2011) was followed.

After staining, the images of the two-dimensional gels were acquired with the ProXPRESS 2D proteomic Imaging System and analysed using Phoretix 2-D Expression Software v2004 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Gels from four independent biological replicates were used and the analysis of gels was performed as previously described by Aranjuelo *et al.* (2011). Molecular mass (M_r) and isoelectric point (pI) were each calculated using Samespots software calibrated with commercial molecular mass standards (precision protein standards prestained; Bio-Rad) run in a separate marker lane on the two-dimensional electrophoresis (2-DE) gel. ANOVA ($P<0.05$) was performed using MiniTAB to compare the relative abundance of the total volume of all detected spots for each gel.

For protein identification by ESI-LC MS/MS, excised spots were washed several times with water and dried for a few minutes. Trypsin digestion was performed overnight with a dedicated automated system (MultiPROBE II; PerkinElmer). The gel fragments were subsequently incubated twice for 15 min in a H_2O/CH_3CN solution to allow extraction of peptides from the gel pieces. Peptide extracts were then dried and dissolved in starting buffer for chromatographic elution, which consisted of 3% CH_3CN and 0.1% HCOOH in water. Peptides were enriched and separated using a lab-on-a-chip technology (Agilent, Massy, France) and fragmented using an on-line XCT mass spectrometer (Agilent). The fragmentation data were interpreted using the Data Analysis program (version 3.4; Bruker Daltonic, Billerica, MA, USA). For protein identification, tandem mass spectrometry peak lists were extracted and compared with the protein database using MASCOT Daemon (version 2.1.3; Matrix Science, London, UK) search engine as previously described by Desclos *et al.* (2009). Once the proteins were identified, their presumed biological function assigned according to Bevan *et al.* (1998).

Statistical analyses

Data were processed by one-factor analysis of variance (ANOVA). Means ±standard errors (SE) were calculated, and when the *F*-ratio was significant, least significant differences were evaluated by the LSD test using the statistical software package SPSS 12.0 (SPSS, Inc., Chicago, IL, USA). The results were accepted as significant at $P<0.05$. All values shown in the figures and tables are means ±SE.

Table 1. Effect of [CO_2] during growth on wheat total, flag leaf, ear, and ear DM/total DM, together with photosynthesis, stomatal conductance (g_s) and intercellular CO_2 (Ci) determined at 370 (A_{370}, gs_{370}, and Ci_{370}, respectively) and 700 (A_{700}, gs_{700}, and Ci_{700}, respectively) μmol mol^{-1} [CO_2] 14 d after anthesis

Each value represents the mean ±SE (n=6). Different letters indicate significant differences ($P<0.05$) between treatments and genotypes as determined by ANOVA test.

Parameter	Ambient CO_2	Elevated CO_2
Total DM (g m^{-2})	2287.1±510.4a	1871.2±171.9a
Flag leaf DM (g m^{-2})	92.3±15.3a	73.3±152a
Ear DM (g m^{-2})	662.0±240.0a	426.7±46.9a (P=0.093)
Ear DM/total DM	0.29±0.09a	0.23±0.01a
A_{370} (μmol m^{-2} s^{-1})	14.59±5.5a	3.77±0.61b
A_{700} (μmol m^{-2} s^{-1})	33.7±6.0a	21.6±4.4b
gs_{370} (mmol m^{-2} s^{-1})	146.9±62.3a	51.8±4.73b
gs_{700} (mmol m^{-2} s^{-1})	184.3±46.4a	124.0±35.7b
Ci_{370} (μmol mol^{-1})	153.6±12.7b	277.0±9.90a
Ci_{700} (μmol mol^{-1})	324.517.6a	345.978.7a

Results

Growth in elevated [CO_2] had no effect on leaf and total biomass; however, ear DM marginally decreased (P=0.093) in these treatments (Table 1). Furthermore, no significant differences were observed in the ear DM/total DM ratio. At the respective CO_2 growth conditions, flag leaf photosynthesis was higher in elevated than ambient CO_2 plants, although the difference was not significant (Table 1). However, when photosynthesis was determined at a common concentration of 370 or 700 μmol m^{-2} s^{-1} (A_{370} and A_{700}, respectively) the results revealed that plants grown under elevated [CO_2] had lower photosynthetic rates. This was associated with higher intercellular [CO_2] (C_i) and lower stomatal conductance values (g_s) than in ambient [CO_2]-grown plants, which suggests that the lower capacity of plants exposed to elevated [CO_2] (Table 1) is attributable to mesophyll reactions.

The leaf carbohydrate determinations (Fig. 1) showed that although glucose and sucrose were not affected by [CO_2], starch (marginally) and fructan concentration increased in plants exposed to 700 μmol mol^{-1}. In ears, no significant differences were detected in any of the analysed carbohydrates. As shown in Fig. 2, N content decreased in leaves exposed to elevated [CO_2], whereas no significant differences were detected in ears. The C/N ratio showed an increase in flag leaves and no significant difference in ears in response to elevated [CO_2]. Leaf N, Rubisco, and amino acid content decreased in elevated [CO_2] (Fig. 3). Although TSP content was not significantly affected by [CO_2], the percentage of Rubisco in TSP decreased in elevated [CO_2]. The authors would like to clarify that apparent discrepancies concerning Rubisco concentration decreases observed by SDS-PAGE and not by the 2-DE were explained by saturation of the silver staining of Rubisco in the 2-DE methodology, due to its abundance. Total Rubisco activity (Fig. 4) was decreased by elevated [CO_2] while initial Rubisco activity was not significantly affected, because Rubisco activation increased. The k_{cat} of Rubisco (Fig. 4) was significantly lower in elevated [CO_2] than in ambient [CO_2].

After 7 d of labelling (during labelling period), the $\delta^{13}C$ in leaf TOM was –39.92‰. This value was constant during the post-labelling period, 24 h and 48 h (–40.22‰ and –40.08‰, respectively) after the end of labelling (Fig. 5). Interestingly, the analyses of leaf respired CO_2 also revealed that in elevated [CO_2], the (T_0) $\delta^{13}C$ was lower in labelled than non-labelled plants (–34.10‰ and –30.72‰, respectively) immediately after the labelling. However, such depletion decreased to –32.36‰ by 24 h and to –31.12‰ by 48 h after the end of labelling (Fig. 5). For ears of labelled and non-labelled plants in elevated [CO_2], the similar $\delta^{13}C$ in TOM (–36.90‰ and –37.75‰, respectively) and in respired CO_2 (–33.51‰ and –33.66‰, respectively) suggests that pre-labelled C was present in ears (Fig. 5). In both flag leaves and ears, the $\delta^{13}C$ of sucrose and fructans were similar in labelled and non-labelled plants exposed to

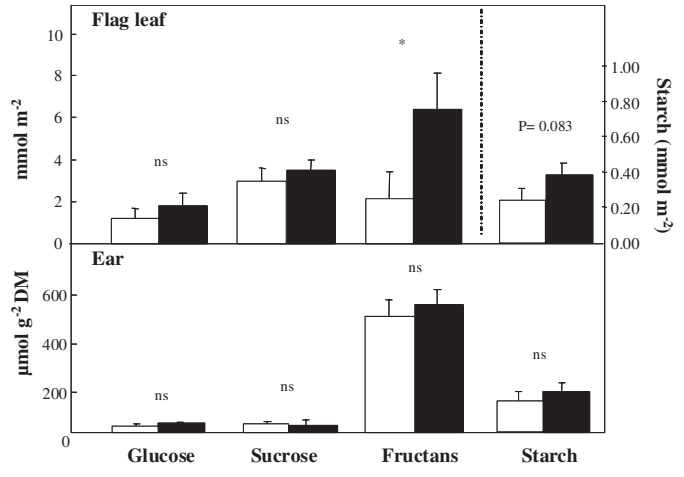

Fig. 1. Elevated [CO_2] effect on wheat flag leaf and ear glucose, sucrose, fructans, and starch content 14 d after anthesis. Open bars correspond to plants grown under ambient CO_2 (~370 μmol mol^{-1}) and closed bars to those grown under elevated CO_2 (~700 μmol mol^{-1}). Each value represents the mean ±SE (n=4). The different symbols indicate non-significant differences (ns), significant differences P<0.05 (*) and P<0.01 (**) between treatments as determined by LSD.

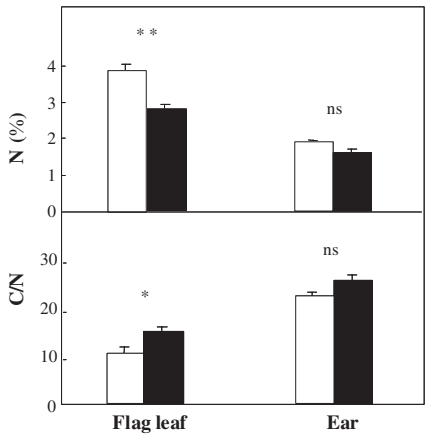

Fig. 2. Elevated [CO_2] effect on wheat flag leaf and ear N content and C/N ratio 14 d after anthesis. Open bars correspond to plants grown under ambient CO_2 (~370 μmol mol^{-1}) and closed bars to those grown under elevated CO_2 (~700 μmol mol^{-1}). Otherwise as in Fig. 1.

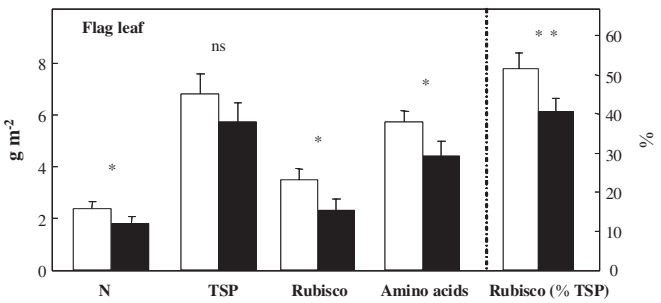

Fig. 3. Elevated [CO_2] effect on wheat flag leaf N, TSP, Rubisco, amino acid content, and Rubisco as a percentage of TSP 14 d after anthesis. Otherwise as in Fig. 1.

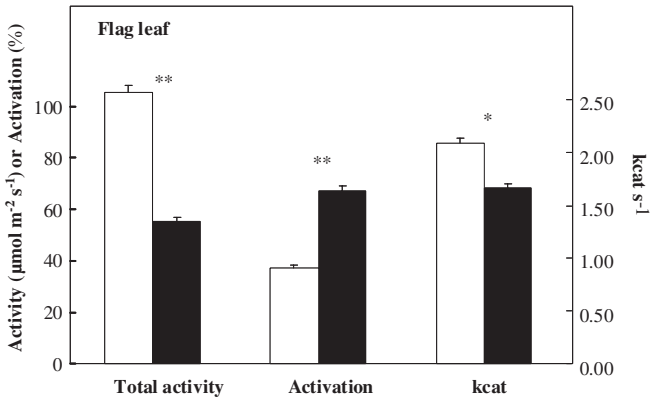

Fig. 4. Elevated [CO_2] effect on wheat flag leaf total Rubisco activity, Rubisco activation, and Rubisco k_{cat} 14 d after anthesis. Open bars correspond to plants grown under ambient CO_2 (~370 μmol mol^{-1}) and closed bars to those grown under elevated CO_2 (~700 μmol mol^{-1}). Otherwise as in Fig. 1.

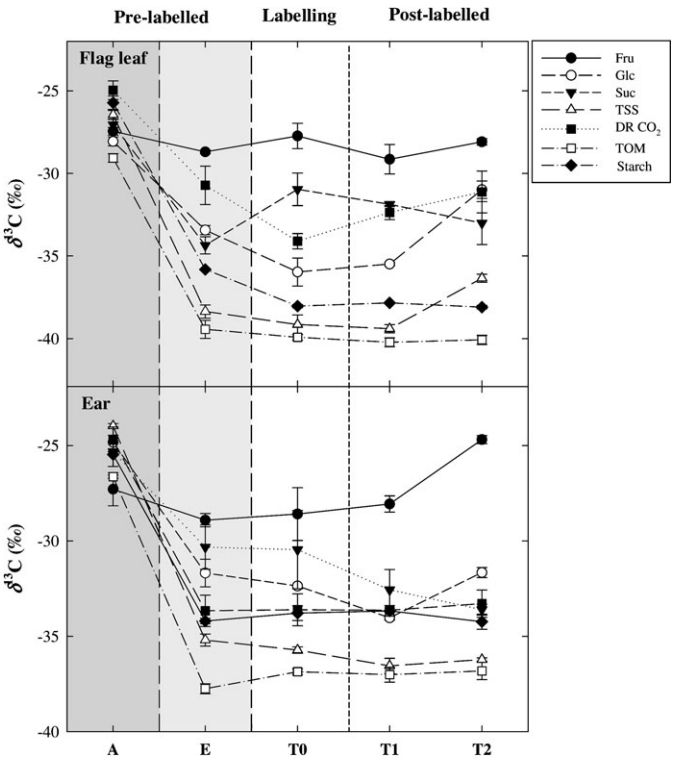

Fig. 5. Elevated [CO_2] effect on wheat flag leaf and ear ^{13}C isotopic composition ($\delta^{13}C$) in TOM, respired CO_2, (DR CO_2), TSS, glucose (Glu), sucrose (Suc), fructans (Fru), and starch (HCl-C). A and E stand for ambient and elevated [CO2], respectively, before labelling (pre-labelling period). T_0 refers to the end of labelling (labelling period; 14 d after anthesis), whereas T_1 and T_2 refer to 24 h and 48 h after the end of labelling (post-labelling period), respectively. Otherwise as in Fig. 1.

700 μmol mol^{-1} (Fig. 5). However, for leaf glucose immediately after the end of labelling (T_0), $\delta^{13}C$ changed from –33.43‰ in unlabelled plants to –35.97‰ in labelled plants. Twenty-four h (T_1) and 48 (T_2) h later, the corresponding $\delta^{13}C$ values were –35.49‰ and –30.98‰ (Fig. 5). Immediately after labelling, the $\delta^{13}C$ of glucose in ears was similar to that of pre-labelled plants in elevated [CO_2] (–32.37‰ and –31.69‰, respectively). As shown in Fig. 5, such values were depleted to –34.03‰ at T_1 and to –31.66‰ at T_2. Figure 5 also shows that $\delta^{13}C$ of leaf starch in elevated [CO_2] conditions was –38.01‰ in labelled plants and –35.81‰ in non-labelled plants. Such values were maintained at T_1 and T_2. However, Fig. 5 also shows that starch in ears of labelled (–34.93‰) and unlabelled (–34.20‰) plants had a similar $\delta^{13}C$.

The effect of elevated CO_2 on the leaf protein pattern in wheat plants was studied using 2-DE (Fig. 6). The protocol used here enabled the identification of 14 proteins that differed in their expression under ambient and elevated CO_2 conditions (Tables 2, 3). Eight of these proteins were up-regulated under elevated CO_2 conditions (Table 2), with the remaining six being down-regulated (Table 3). These proteins were classified in different groups according to their presumed biological function. The up-regulated proteins were classified into six groups: metabolic processes (one protein identified), energy processes (one protein identified), transporters (one protein identified), disease/defence processes (one protein identified), proteins with unclear classification (two proteins identified), and unclassified proteins (two proteins identified). Among the down-regulated proteins, energy processes (two proteins identified), disease/defence (one protein identified), and unclassified proteins (three proteins identified) were detected. The roles of these proteins are discussed in the following section with regard to the changes in physiological traits in response to elevated CO_2 conditions.

Discussion

A review conducted by Amthor (2001), summarizing 156 experiments that analyse wheat yield under elevated [CO_2] conditions, has shown that CO_2 response ranges from no effect or a negative one in some studies to several-fold increases in others. As shown in Table 1, exposure to 700 μmol mol^{-1} CO_2 marginally decreased ear DM during the post-anthesis period (P=0.093) and no effect was observed in total DM and ear DM/total DM ratio. This revealed that elevated [CO_2] did not contribute to increased grain filling, which is in agreement with previous reports (Amthor, 2001; Uddling *et al.*, 2008; Högy *et al.*, 2009). These results were corroborated in the supplementary harvest conducted at the grain maturity stage (see Supplementary Table S1 at *JXB* online). Absence of effects on total DM, together with the lower ear DM suggest that under elevated [CO_2] exposure, the plants invested a larger amount of photoassimilates in the development of vegetative biomass rather than in grain filling. Grain filling may be limited by (i) translocation of photoassimilates from source to sink, (ii) photosynthetic activity, and (iii) ear sink capacity (Uddling *et al.*, 2008). Evans *et al.* (1970) showed that assimilate movement from leaves to ears in wheat was not limited by phloem stem transport. Photosynthesis (measured at the respective

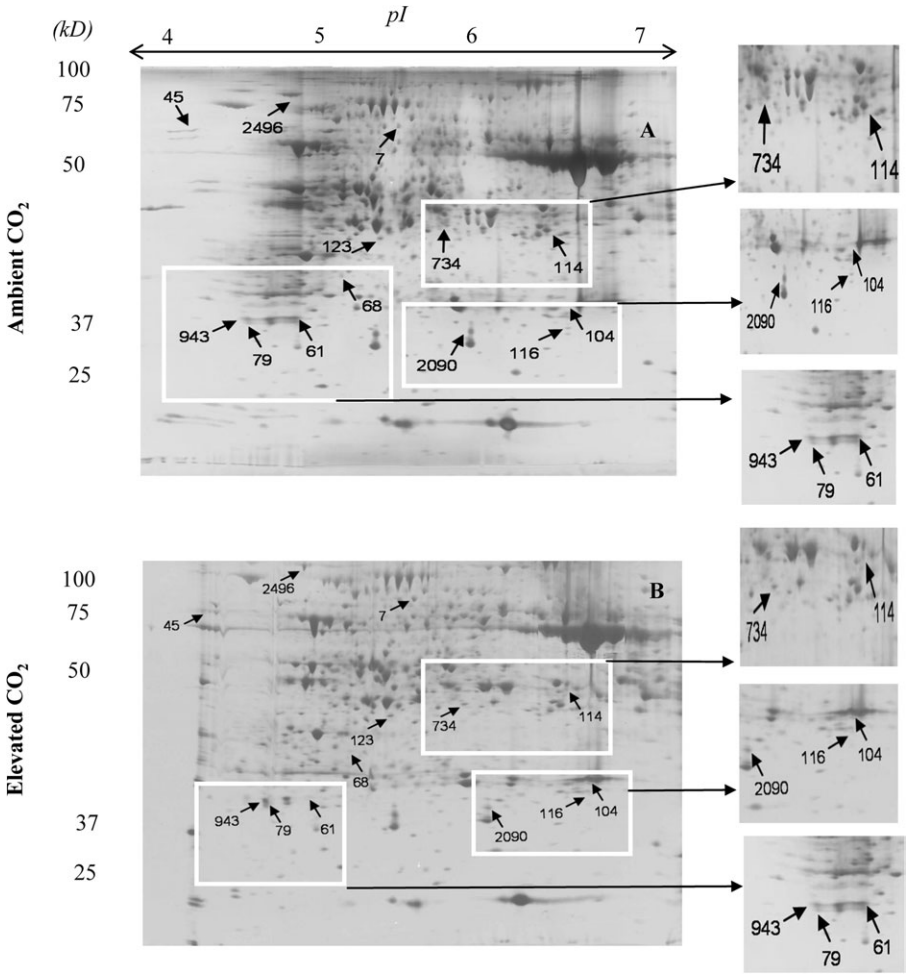

Fig. 6. Silver-stained two-dimensional gel electrophoresis of proteins extracted from wheat leaves grown under ambient and elevated conditions 14 days after anthesis. In the first dimension, 125 mg of total protein was loaded on a 18 cm IEF strip with a linear gradient of pH 4–7. The second dimension was conducted in 12% polyacrylamide (w/v) gels (20 × 20 cm) (for details see ''Materials and Methods''). The gel image analyses was conducted with Progenesis SameSpots software v3.0 and the subsequent mass spectrometry analyses identified up to 14 proteins (marked by arrows) with significantly different expression in elevated [CO_2].

growth conditions) was increased by elevated [CO_2] (Table 1). However, when photosynthetic activity was determined in all plant treatments at 370 μmol mol^{-1} and 700 μmol mol^{-1} [CO_2] (Table 1) it was found that plants grown in elevated [CO_2] had lower photosynthetic capacity than plants grown in ambient [CO_2]. Similar results were described by Zhang *et al.* (2009). Photosynthetic acclimation has been previously described in wheat plants exposed to elevated [CO_2] in greenhouses located in the field (Martínez-Carrasco *et al.*, 2005; Alonso *et al.*, 2009; Gutiérrez *et al.*, 2009). Although exposure to elevated [CO_2] decreased g_s, similar (Ci_{700}) or even higher (Ci_{370}) intercellular CO_2 concentrations (C_i) in elevated [CO_2] than in ambient [CO_2] ruled out stomatal closure as the main cause of the reduction in photosynthetic capacity in elevated [CO_2]. Carbonic anhydrase (CA), a protein that catalyses the reversible conversion of CO_2 to HCO_3^-, has been recognized as an important enzyme that is closely associated with photosynthesis (Jebanathirajah and Coleman, 1998; Sasaki *et al.*, 1998; Evans *et al.*, 2009). CA, together with aquaporins, has been described as a fast-responding biochemical process that regulates mesophyll conductance (Nakhoul *et al.*, 1998; Gillon and Yakir, 2000; Terashima and Ono, 2002; Flexas *et al.*, 2006). A 198% increase was found in this enzyme in elevated [CO_2] relative to control leaves that could partly compensate for the closure of stomata, thus ensuring the supply of CO_2 to the chloroplasts.

The SDS-PAGE densitometric analysis revealed that the photosynthetic down-regulation in elevated [CO_2] was caused by a lower Rubisco protein content (Fig. 3) (Theobald *et al.*, 1998; Aranjuelo *et al.*, 2005*b*). This decrease was not detected by proteomic analysis due to saturation of the silver staining. Moreover, the proteomic characterization showed a decrease in Rubisco activase content in plants exposed to 700 μmol mol^{-1} CO_2 (Table 3). Rubisco activase is essential for the maintenance of Rubisco catalytic activity because it promotes the removal of tightly bound inhibitors from the catalytic sites (Robinson and Portis, 1989; Parry *et al.*, 2008). The lower photosynthetic rates of plants exposed to 700 μmol mol^{-1} [CO_2] (Table 3) may be a consequence of both decreased Rubisco protein and increased binding of inhibitors to Rubisco active sites, which is consistent with the decreased k_{cat} of the enzyme in

Table 2. Annotation of elevated [CO_2] up-regulated spots identified in silver-stained 2-DE gels of leaves collected 14 d after anthesis

A total of 125 μg of total proteins was loaded on an 18-cm gel strip forming an immobilized linear pH gradient of 4–7. Second-dimension electrophoresis (SDS1258-PAGE) was carried out on 12% polyacrylamide (w/v) gels (20×20 cm; for details see Material and methods).

Spot no.	Spot % volume variations	pI/M_r	PM	SC (%)	Score (P<0.05 corresponding to score >51)	Protein name/organism/NCBI accession no.
01. Metabolism						
104	198.03	6.25/32.01	7	28	249	Chloroplastic carbonic anhydrase/gil729003
02. Energy						
7	627.13	5.51/62.91	2	11	55	Phosphoglycerate mutase/gil32400802
07. Transporters						
45	314.21	4.05/54.82	3	6	98	ATP synthase βsubunit/gil3850920
11. Disease/defence						
116	164.06	6.48/24.09	1	6	53	Manganese superoxide dismutase/ gil1621627
12. Unclear classification						
105	188.61	8.67/81.43	2	3	53	Putative bluelight receptor/gil20797092
123	139.12	5.35/42.85	3	3	54	SNF2 superfamilyprotein/gil159466410
13. Unclassified						
79	244.27	4.42/23.86	2	2	63	Predicted protein/gil226460198
943	195.90	4.43/24.77	4	18	122	Hypotheticalprotein/gil1076722

PM, peptides matching; SC, sequence coverage.

Table 3. Annotation of elevated [CO_2] down-regulated spots identified in silver-stained 2-DE gels of leaves collected 14 d after anthesis

A total of 125 μg of total proteins was loaded on an 18-cm gel strip forming an immobilized linear pH gradient of 4–7. Second dimension electrophoresis (SDS1258-PAGE) was carried out on 12% polyacrylamide (w/v) gels (20×20 cm) (for details see Material and methods).

Spot no.	Spot % volume variations	pI/M_r	PM	SC (%)	Score (P<0.05 corresponding to score >51)	Protein name/organism/NCBI accession no.
02. Energy						
114	60.59	6.21/48.39	2	8	112	Ribulose-bisphosphatecarboxylaseactivase/ gil100614
2090	79.12	5.81/23.97	2	5	65	Adenosine diphosphateglucosepyrophosphatase/ gil13160411
11. Disease/defence						
2496	87.75	4.91/85.64	2	3	81	Cytosolic heat shock protein90/ gil32765549
13. Unclassified						
61	34.66	4.95/24.65	2	9	92	Hypotheticalprotein/gil1076722
68	37.85	5.20/38.94	1	91	66	Unknown protein18/gil205830697
734	28.14	5.64/44.58	2	10	103	Hypotheticalprotein/gil125602085

PM, peptides matching; SC, sequence coverage.

elevated [CO_2] found in this (Fig. 4) and previous studies (Pérez *et al.*, 2005, 2007).

Lack of significant differences in TSP content, and the decrease of Rubisco as a fraction of TSP (Fig. 3) revealed that the diminished Rubisco concentration was caused by a specific inhibition of this protein in leaves exposed to elevated [CO_2] (Pérez *et al.*, 2007). According to Zhu *et al.* (2009) and Fangmeier *et al.* (2000), in flag leaves of wheat exposed to elevated [CO_2] there is an increase in protease activity that enables the remobilization of N. In agreement with this finding, the lower amino acid level in flag leaves (Table 2) under elevated [CO_2] suggests that the flag leaf Rubisco-derived N was reallocated to the ear, an organ with high N sink capacity. Furthermore, according to Theobald *et al.* (1998), in elevated [CO_2] there is a greater reduction in Rubisco than in other photosynthetic components (ATP synthase, etc.). Consistent with this, the decrease in Rubisco under elevated [CO_2] was accompanied by the up-regulation of ATP synthase (β subunit), in this experiment. This result suggests a rebalancing away from carboxylation to RuBP-regeneration capacity (Theobald *et al.*, 1998).

The decrease in photosynthetic capacity under elevated [CO_2] has been attributed to end-product inhibition, in which the demand for carbohydrates is insufficient to cope with the enhanced carbohydrate supply (Rogers and Ellsworth, 2002; Ainsworth and Long, 2005; Aranjuelo *et al.*, 2008). The accumulation of fructans and starch in flag leaves in elevated CO_2 (Fig. 1) was associated with decreases in Rubisco (Fig. 2) and Rubisco activase (Table 3), and may be causal in down-regulation of photosynthetic capacity (Moore *et al.*, 1999; Jifon and Wolfe, 2002). As shown in Tables 2 and 3, the proteomic characterization provided relevant information concerning the possible involvement of altered protein levels in carbon metabolism in elevated CO_2. This study revealed that phosphoglycerate mutase (PGAM) content increased by 627.13% in plants grown in elevated [CO_2] (Table 2). PGAM catalyses the intercoversion of 3-phosphoglycerate (3-PGA) to 2-phosphoglycerate (2-PGA) (Batz *et al.*, 1992), and its increase could lead to enhanced glycolysis. Carbohydrate accumulation in leaves, irrespective of whether it is a result of sugar-feeding or an inhibition of phloem transport or growth in elevated [CO_2], has been shown to stimulate organic acid synthesis (Morcuende *et al.*, 1998; Stitt and Krapp, 1999) and respiratory pathways, leading to a decrease in the levels of 3-PGA (Morcuende *et al.*, 1996, 1997) and increased formation of ATP (Stitt and Krapp, 1999). A recent study conducted by Leakey *et al.* (2009) revealed that exposure to elevated [CO_2] increased the abundance of transcripts associated with respiration and carbohydrate metabolism The proteomic characterization conducted in this study also revealed a 79% decrease in adenosine diphosphate glucose pyrophosphatase (AGPPase) in elevated [CO_2] (Table 3). AGPPase catalyses the hydrolytic conversion of ADPglucose (ADPG), the universal glucosyl donor for starch biosynthesis, to AMP and G1P (Rodriguez-López *et al.*, 2000). Although starch and fructan accumulation in leaves in elevated [CO_2] may be accounted for by the observed decrease in leaf nitrogen content, since nitrate is known to repress AGP pyrophosphorylase (Scheible *et al.*, 1997) and at least one enzyme of fructan synthesis (Morcuende *et al.*, 2004), the decrease in AGPPase protein can contribute to the observed starch build-up in elevated [CO_2]. The fact that this protein is inhibited by ATP content (Emes *et al.*, 2003), and that the ATP synthase β subunit increased under elevated [CO_2], points to a tight control of starch build-up in leaves. A previous study conducted by Leakey *et al.* (2009) observed an increase in transcripts associated with starch metabolism in soybean plants exposed to 550 μmol mol^{-1} CO_2. The up-regulation of PGAM and down-regulation of AGPPase show an altered protein pattern that can enhance C utilization for storage and energy in elevated [CO_2].

Carbohydrate build-up in leaves is determined by the plant's ability to develop new sinks (e.g. new vegetative or reproductive structures, enhanced respiratory rates), or to expand the storage capacity or growth rate of existing sinks (Lewis *et al.*, 2002). Although respiration processes require an investment of a large quantity of photoassimilates (Amthor, 2001; Aranjuelo *et al.*, 2009*a*), little attention has been given to this topic (especially in ears) in C balance studies analysing grain filling in cereals. Leaf-respired $\delta^{13}C$ (Fig. 5) was depleted immediately after $^{12}CO_2$ labelling, and 24 h (T_1) and 48 h (T_2) later, showing that these plants were respiring, in part, C assimilated during the labelling period. However, the fact that 48 h later (T_2) the $\delta^{13}C$ was similar to the values obtained before labelling (E) suggests that, 2 d after labelling, the leaves had respired almost all the labelled respiratory substrates. The determination of $\delta^{13}C$ in the various carbohydrates (Fig. 5), suggested that these leaves were respiring the labelled TSS and especially glucose. This point is reinforced by the fact that 48 h after the end of labelling, pre-labelled C was present among glucose C, which is similar to the observation for leaf respiration $\delta^{13}C$. Opposite to the observation for leaf respiration and soluble sugar, the $\delta^{13}C$ of TOM of flag leaves remained constant even 48 h after the end of labelling. Such results could be explained by part of the labelled C being partitioned to structural and storage compounds. While fructan $\delta^{13}C$ did not contribute detectable labelled C in flag leaves, the $\delta^{13}C$ depletion in starch (–35.81‰ in non-labelled plants versus –37.93‰ in labelled plants) revealed that part of the labelled C present in TOM was accounted for by C accumulation in starch. It is very likely that because pre-labelled C was present in soluble sugars 48 h after the labelling, most of the remaining labelled C consisted of structural C compounds. The fact that TOM was more depleted than starch (–39.86‰ and –37.93‰, respectively) confirmed this point.

As mentioned above, leaf carbohydrate in wheat is also determined by ear C sink strength. These data revealed that although exposure to 700 μmol mol^{-1} CO_2 did not modify sucrose and glucose concentrations in ears, fructan and starch concentrations tended to increase. During grain filling, the strong C demand by wheat ears is met by ear photosynthesis and respiration (Tambussi *et al.*, 2007), together with translocation of C from flag leaves and stem internodes (Gebbing and Schnyder, 1999; Aranjuelo *et al.*, 2009*a*). Absence of differences in the $\delta^{13}C$ in ear TOM and respired CO_2 between labelled and non-labelled plants (Fig. 5) confirmed that exposure to elevated [CO_2] did not increase ear filling during the beginning of anthesis, which is in agreement with the data on ear DM/total DM ratios (Table 1). Even if the ear TOM was not labelled, the fact that sucrose and glucose $\delta^{13}C$ was depleted (Fig. 5) highlighted that a small fraction of labelled C reached the ear. Apparent discrepancies in TOM and sugar $\delta^{13}C$ could be explained by the fact that glucose and sucrose concentrations represent a small fraction of ear C, and therefore labelled C was diluted in TOM that was almost totally composed of non-labelled C (see Fig. 2). Although the photosynthetic activity of ears should not be ignored (Tambussi *et al.*, 2007; Zhu *et al.*, 2009), the fact that glucose, TSS, and especially sucrose $\delta^{13}C$ depletion was more marked at 24 h and 48 h after labelling, indicates that this labelled C originated in flag leaves.

In summary this study suggested that the absence of elevated [CO_2] effects on biomass production, and especially ear grain filling, reflected the inability of these wheat plants to increase C sink strength. Absence of elevated [CO_2] effects on biomass production of plants with larger photosynthetic rates caused a leaf carbohydrate build-up. Such an increase induced photosynthetic acclimation, as reflected by the lower carboxylation capacity of plants exposed to 700 μmol mol^{-1}. The $\delta^{13}C$ determinations conducted during the post-anthesis period showed that in flag leaves, under elevated [CO_2], part of the newly assimilated C was allocated to storage compounds and that another part of labelled C (mainly soluble sugars) was totally respired 48 h after the end of labelling. In ears, the differences in the $\delta^{13}C$ data revealed that although no changes were detected in ear TOM, a small amount of C reached the ears in the form of soluble sugars. Proteomic characterization showed that in these plants the changes in protein content enhanced C storage and glycolysis. Furthermore, the protein characterization also revealed that photosynthetic acclimation was caused by a decrease in Rubisco protein content and in the capacity to release Rubisco tight-binding inhibitors. The decreases in leaf N, Rubisco, and amino acid content suggest that under elevated [CO_2] there was a reallocation of leaf N to ears during grain filling. The ear DM, together with the ear isotopic and biochemical determinations revealed that 2 weeks after anthesis, ears of wheat plants exposed to elevated [CO_2] did not contribute to an increase in C sink strength. Therefore, such plants were incapable of overcoming leaf photoassimilate accumulation, with a consequent alteration in leaf N and protein content that caused the photosynthetic down-regulation.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Mean daily courses of temperature (A) and humidity (B) in greenhouses set at either ambient or 700 μmol mol^{-1} CO_2.

Supplementary Table S1. Effect of [CO_2] during growth on total, ear, and grain DM, together with the ear DM/total DM ratio and the grain yield, thousand kernel weight, numbers of ears per unit land area, and grains per ear at grain maturity.

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