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RESEARCH PAPER

Harvest index, a parameter conditioning responsiveness of wheat plants to elevated CO_2

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Abstract

The expansion of the world's population requires the development of high production agriculture. For this purpose, it is essential to identify target points conditioning crop responsiveness to predicted [CO_2]. The aim of this study was to determine the relevance of ear sink strength in leaf protein and metabolomic profiles and its implications in photosynthetic activity and yield of durum wheat plants exposed to elevated [CO_2]. For this purpose, a genotype with high harvest index (HI) (*Triticum durum* var. Sula) and another with low HI (*Triticum durum* var. Blanqueta) were exposed to elevated [CO_2] (700 µmol mol^{-1} versus 400 µmol mol^{-1} CO_2) in CO_2 greenhouses. The obtained data highlighted that elevated [CO_2] only increased plant growth in the genotype with the largest HI; Sula. Gas exchange analyses revealed that although exposure to 700 µmol mol^{-1} depleted Rubisco content, Sula was capable of increasing the light-saturated rate of CO_2 assimilation (A_{sat}) whereas, in Blanqueta, the carbohydrate imbalance induced the down-regulation of A_{sat}. The specific depletion of Rubisco in both genotypes under elevated [CO_2], together with the enhancement of other proteins in the Calvin cycle, revealed that there was a redistribution of N from Rubisco towards RuBP regeneration. Moreover, the down-regulation of N, NO_3^-, amino acid, and organic acid content, together with the depletion of proteins involved in amino acid synthesis that was detected in Blanqueta grown at 700 µmol mol^{-1} CO_2, revealed that inhibition of N assimilation was involved in the carbohydrate imbalance and consequently with the down-regulation of photosynthesis and growth in these plants.

Key words: 2D proteomic • Acclimation • C/N • CO_2 • Harvest index • Wheat

Introduction

Although during the past decades increases in cereal productivity have matched population growth, a further understanding of how predicted environmental conditions will affect crop production in the future is a matter of major concern for worldwide food security programmes (Parry and Hawkesford, 2010, 2012; Foulkes *et al*., 2011; Parry *et al*., 2011; Reynolds *et al*., 2011). This is especially relevant considering the ongoing increase in the world population, together with social changes of a diverse nature, including diets, fuels, etc. In this sense, it has been observed that food production needs to increase 100% by 2050 to meet projected demands (Nakicenovic *et al*., 2000).

Experiments analysing wheat performance under the [CO_2] levels predicted for the end of this century have revealed that the initial stimulation of plant growth and photosynthetic capacity is partially or totally reversed (Pérez *et al*.,

2007, 2011; Gutiérrez *et al*., 2009; Aranjuelo *et al*., 2011). Traditional plant breeding programmes have been generally based on empirically selected practices that maximize yield (Sinclair, 1998; Thompson *et al*., 2007). Although wheat yields have increased, this has not been due to an increase in total biomass but rather due to an increase in harvest index (HI) (i.e. the proportion of the total biomass devoted to grain at harvest) and to improvements in agronomic practice. However, the HI for wheat is thought to be approaching a ceiling, and any further increase in yield will need to involve an increase in total biomass and therefore more photosynthesis (Araus *et al*., 2002; Parry and Hawkesford, 2010; Parry *et al*., 2011).

It has been proposed that plants with a small sink size will acclimate to high [CO_2] by decreasing photosynthetic capacity (Aranjuelo *et al*., 2009). Therefore, plants with a large sink size (i.e. large ears in the case of cereals) will benefit more from [CO_2] enrichment than those with a small sink size (Manderscheid and Weigel, 1997; Aranjuelo *et al*., 2009). As mentioned above, leaf C sink/source and therefore photosynthetic performance is also conditioned by the sink strength of other organs such as grains (Uddling *et al*., 2008; Aranjuelo *et al*., 2011). Grain filling and, by extension, grain yield are mainly sustained by assimilation and management of C and N compounds. In wheat, grain N content is the result of the N taken up after anthesis, together with the amount of remobilized N originating from pre-anthesis uptake (Dupont and Altenbach, 2003). On the other hand, C required for grain filling is mostly provided by flag leaf photosynthesis (Evans *et al*., 1975), translocation of C assimilated before anthesis (mainly stored in the internodes; Gebbing *et al*., 1999), and ear photosynthesis (Tambussi *et al*., 2007; Maydup *et al*., 2012). Contrasting results concerning the origin of the C and N that contribute to grain filling have been reported in the literature (Palta and Fillery, 1995; Ercoli *et al*., 2008), with the nature of these sources varying with the growth conditions, the cereal, and the genotype studied (Austin *et al*., 1980; Dupont and Altenbach, 2003; Aranjuelo *et al*., 2009*b*).

Grain yield may be limited by source activity, translocation from source to sink organs, and the sink activity (Borrás *et al*., 2004; Uddling *et al*., 2008; Foulkes *et al*., 2011). It has been observed that grain filling is mostly conditioned by plant sink activity and that grain sink capacity is largely determined by source activity around anthesis (Foulkes *et al*., 2007; Reynolds *et al*., 2007). In this sense, sink strength could be expected to become more important as source activity increases. Limitations on sink activity may induce the above-mentioned accumulation of leaf carbohydrates and altered C/N balance with the consequent inhibition of photosynthetic activity. Down-regulation of photosynthetic activity might be particularly pronounced for plants with relatively low sink strength (Uddling *et al*., 2008). In recent decades, wheat breeding efforts have been devoted to genotypes with a larger investment of resources destined for grain filling (Reynolds *et al*., 2007). Such programmes have sought new cultivars which, compared with the old genotypes, have favoured ear development over vegetative biomass.

The effect of atmospheric [CO_2] on plant growth, and especially on photosynthetic performance, has been extensively studied during recent decades (Ainsworth *et al*., 2002; Long *et al*., 2004; Ainsworth and Rogers, 2007; Leakey *et al*., 2009; Rogers *et al*., 2009; Aranjuelo *et al*., 2011). Although the fact that the current atmospheric [CO_2] is generally limiting for C_3 photosynthesis, the available information suggests enhancement of photosynthetic rates under the predicted increase in [CO_2] (Farquhar *et al*., 1980; Bowes, 1993; Long *et al*., 2004). However, as mentioned before, the initial stimulation has been described as partially reversed in a process of acclimation (Ainsworth *et al*., 2002; Long *et al*., 2004; Ainsworth and Rogers, 2007; Leakey *et al*., 2009; Rogers *et al*., 2009). The 'capacity' to adjust photosynthetic activity with plant C requirements has been described as a key process conditioning photosynthetic performance under elevated [CO_2] (Ainsworth *et al*., 2004; Aranjuelo *et al*., 2009). From this point of view, the reduction in photosynthetic rates would be conditioned by a plant's ability to develop new sinks, or to expand the storage capacity or growth rate of existing sinks. A recent study (Aranjuelo *et al*., 2011) conducted with durum wheat (*Triticum durum*, Desf., cv Regallo) exposed to elevated [CO_2] revealed that the main C and N sinks during grain filling (ears) did not contribute towards overcoming leaf carbohydrate accumulation. As a consequence, these plants underwent Rubisco depletion and photosynthetic down-regulation. Moore *et al*. (1999) proposed a biochemical model where increased hexose levels caused the inhibition of Rubisco content. According to this model, as a consequence of the larger [CO_2], there is an increase in the photosynthetic rates of those plants and consequently in the availability of a major leaf photoassimilate form such as sucrose. The flux of hexoses through hexose kinase (HK) would signal the source/sink imbalance and consequently the down-regulation of Rubisco content (Moore *et al*., 1999; Long *et al*., 2004). More specifically, the enhanced HK catalytic activity initiates the transduction response that induces the repression of promoter activities of the Rubisco small subunit (Jang and Sheen, 1994; Moore *et al*., 1999).

Although the [CO_2] effect on Rubisco has been widely described in the literature (Leakey *et al*., 2009; Aranjuelo *et al*., 2011; Pérez *et al*., 2011), comparatively little research has been devoted to the characterization of the [CO_2] effect on other proteins (Jang and Sheen, 1994; Rogers *et al*., 1998). As observed in recent studies (Stitt *et al*., 2010; Parry *et al*., 2011; Uematsu *et al*., 2012), there is good experimental evidence that Rubisco activity does not always dominate the rate of photosynthesis and that other rate-limiting steps exist. Previous studies (Henkes *et al*., 2001; Raines., 2003; Lefebvre *et al*., 2005; Tamoi *et al*., 2006; Uematsu, *et al*., 2012) have shown that aldolase, sedoheptulose 1,7-bisphosphatase (SBP_{ase}), and transketolase (TK) have a significantly higher control coefficient on photosynthesis than other Calvin cycle enzymes such as Rubisco. Furthermore, according to Bloom *et al*. (2002, 2010), photosynthetic down-regulation might be conditioned by nitrate reductase activity, the inhibition of NO_3^- assimilation, and the consequent decline in plant N compounds.

There is a clear requirement to develop new strategies and tools for agriculture to ensure that high levels of production will accommodate the needs of the expanding world population by developing a highly productive agriculture. To do so, it is essential to identify, understand, and quantify mechanisms associated with crop responses to elevated [CO_2]. The main goal of this study was to characterize the implication of differences in ear sink strength in leaf C and N balance and their implications for photosynthetic performance and plant growth in wheat plants exposed to elevated [CO_2]. Although a [CO_2] effect in plant growth and physiology has been widely described in previous studies, comparatively little attention has been paid to the relevance of HI to plant performance. Furthermore, the mechanisms conditioning the different responses to elevated [CO_2] of wheat plants with contrasting HI remain unclear. For this purpose, the photosynthetic activity and metabolomic profile of a durum wheat genotype with a high HI (cv. Sula) was compared with those of a genotype with low HI (cv. Blanqueta). A second major goal of this study was to deepen knowledge of the leaf C and N metabolism of plants exposed to elevated [CO_2] through proteomic characterization of flag leaves. Most of the previous studies analysing wheat performance under elevated [CO_2] have focused on Rubisco protein. However, recent studies highlight that the activity of other proteins might condition leaf functioning to a greater degree than expected.

Materials and methods

Plant material and experimental design

The experiment was conducted with two durum wheat (*Triticum durum*, Def.) genotypes (cv. Sula and var. Blanqueta) cultivated in the Mediterranean region. Sula is a cultivar with a high HI that is currently grown, whereas Blanqueta has a low HI and was cultivated some decades ago. After the seedlings were vernalized for 1 month at 4 ºC, the plants were transplanted to 13 litre pots (four plants per pot) containing a substrate filled with 2:2:1 (v/v/v) vermiculite/perlite/peat. The experiment was conducted with eight pots of each combination (with four plants per pot). After sowing, the plants were transferred to four [CO_2] controlled greenhouses located at the Universidad de Navarra campus (42.80 N, 1.66 W; Pamplona, Spain). The design of the greenhouses was similar to that described by Sanz-Sáez *et al.* (2012) and based on Aranjuelo *et al.* (2005). Inside the greenhouses, the pots were placed in holes made in the soil in order to provide for natural temperature fluctuations, thus approximating field conditions (Rawson *et al.*, 1995). Plants were watered with a complete Hoagland (Arnon and Hoagland, 1939) solution twice a week and with water once a week to avoid excessive salt accumulation. Half of the plants were divided between greenhouses where no CO_2 was added and [CO_2] was maintained at ambient conditions (~360 µmol mol^{-1}). The other half were transferred to two greenhouses where [CO_2] was increased to ~700 µmol mol^{-1} by injecting pure CO_2 at the two inlet fans during the light hours. The CO_2 was provided by Air Liquide (Bilbao, Spain). The [CO_2] was continuously monitored using a Guardian Plus gas monitor (Edinburgh Instruments Ltd, Livingston, UK). The monitor's signal was fed into a proportional integrative differential controller that regulated the opening time (within a 10 s cycle) of a solenoid valve that injected CO_2 into both inlet fans. Pots were rotated weekly in each module to avoid edge effects. In order to avoid differences derived from chamber effects, the plants were moved from one greenhouse to the other every month. The gas exchange analyses and harvest for metabolomic and proteomic determinations were carried out 2 weeks after anthesis, defined when at least 50% of the spikes in a pot showed protruding anthers. This period was selected because growth conditions between 10 d pre- and 20 d post-anthesis are considered a critical period for grain yield (Martre *et al.*, 2003). Since exposure to elevated [CO_2] advanced the phenology of both genotypes by 5 d, the gas exchange and sample harvesting were separated by 5 d in order to conduct the analyses at the same phenological stage (89 d and 94 d after sowing for elevated and ambient [CO_2] plants, respectively). No phenological differences were observed between Sula and Blanqueta. Plant sampling was always carried out 4 h after the onset of the photoperiod. The samples were immediately plunged into liquid nitrogen and stored at –80 ºC, awaiting analysis. Plant growth data correspond to samples harvested at the grain maturity stage (120-day-old plants)

Plant growth determinations

Ears, flag leaves, and shoots were collected (eight plants per treatment combination). The leaf area was determined with an electronic planimeter (Li-3000 with LI-3050 conveyer accessory, LI-COR, Lincoln, NE, USA). The samples were dried at 60 ºC for 48 h in an oven and afterwards the dry mass (DM) was determined. The HI was calculated as the ratio between grain DM and total DM. Total DM included ear DM, flag leaf DM, and shoot DM.

Gas exchange determinations

Fully expanded flag leaves were enclosed in a GFS-3000 gas exchange portable system (Walz, Effeltreich, Germany). The gas exchange response to [CO_2] was measured from 0 to 1200 µmol mol^{-1}. The light-saturated rate of CO_2 assimilation (A_{sat}), stomatal conductance (g_s), and intercellular [CO_2] (C_i) were estimated at a photosynthetic photon flux density (PPFD) of 1200 µmol m^{-2} s^{-1} using equations developed by von Caemmerer and Farquhar (1981). Estimations of the maximum carboxylation velocity of Rubisco (Vc_{max}) and the maximum electron transport rate contributing to RuBP regeneration (J_{max}) were made using the method of Harley *et al.* (1992).

Metabolomic compound determinations

Soluble sugar, starch, and organic acid content

Leaf extracts were homogenized in 80% ethanol (v/v) and were sonicated for 25 min at 30 ºC using an Ultrasons-H ultrasonic bath (Selecta, Spain). The hydroalcoholic phase was evaporated through a Turbovap evaporator (Zymark, Carmel, IN, USA) and resuspended with 4 ml of distilled water. The sample was centrifuged at 2300 *g* for 10 min and the supernatant and the pellet were stored separately at –80 ºC.

Sucrose, glucose, and fructose content were determined in the supernatant fraction with a Beckman P/ACE5500 capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) (Cabrerizo *et al.*, 2001). Starch content was determined in the pellet according to Ethier and Livingston (2004).

The organic acid extraction protocol was as described by Cabrerizo *et al.* (2001). The extracts were filtered with Milex filters (Millipore, Billerica, MA, USA) and injected in a DX-500 ion chromatograph equipped with an IonPac AS11 column connected to an ATC-1 protecting column and an AG11 pre-column (all chromatography equipment from Dionex, Salt Lake City, UT, USA).

Amino acid content

Leaf samples were homogenized with 1 M HCl, incubated for 10 min at 4 °C, and then centrifuged (20 000 *g*, 4 °C, 10 min). The supernatant was neutralized with NaOH and adjusted to pH 7–9. Internal standards such as norvaline and homoglutamic acid were added. Amino acids were derivatized at 22–25 ºC for 12–16 h with 1 mM FITC (fluorescein isothiocyanate) dissolved in 20 mM acetone/borate, pH 10. The content of single amino acids was determined by capillary electrophoresis in a Beckman-Coulter PA-800 system. The

protocol did not enable the separate analyses of glycine and serine, so they were quantified together. The applied potential was −20 kV, and the capillary tubing was 50 µM i.d. and 31.4/38.4 cm long. The background buffer was 80 mM borax, 45 mM α-cyclodextrin, pH 9.2.

Rubisco protein content

Rubisco protein content was determined according to Sassi *et al.* (2010). The proteins were extracted from frozen leaf subsamples, ground to a fine powder [in 50 mM tricine buffer (pH 8.0), 1 mM EDTA, 5 mM 6-aminocaproic acid, 2 mM benzamidine, 8 mM β-mercaptoethanol, and 100 mM phenylmethylsulphonyl fluoride (PMSF)] for 20 min on ice. This was followed by centrifugation at 12 000 *g* at 4 ºC for 25 min. The protein concentration was measured in the decanted supernatant with the method developed by Bradford (1976). Afterwards, 5 vols of cold acetone were added to an aliquot containing 300 mg of protein, which was left overnight in the freezer or cooler. The sample was then centrifuged at 12 000 *g* at 4 ºC for 15 min. For SDS–PAGE, 4 µg of soluble proteins were prepared, separation performed using a 150 g l^{-1} acrylamide gel, and the gel was stained with silver nitrate (Blum *et al.*, 1987). Gel images were scanned and analysed using ImageQuant TL software (GE Amersham Biosciences, UK). The concentration of Rubisco large (RubL) and small (RubS) subunits was measured against a Rubisco standard protein (Agrisera AB; Vännäs, Sweden) and was calculated on a per area basis (g m^{-2}).

Two-dimensional difference gel electrophoresis (2D-DIGE)

Extracted proteins were precipitated with a ReadyPrep 2D cleanup kit (Bio-Rad), and solubilized in 2D-DIGE sample buffer: 7 M urea, 2 M thiourea, 4% CHAPS, buffered to pH 8.5. Protein concentration was determined using the Bradford Assay (Bio-Rad). Then 50 µg of each sample were labelled with 400 pmol of CyDye DIGE Fluor minimal dyes (GE Healthcare) according to the manufacturer's instructions (Cy3 and Cy5 for samples, and Cy2 for the internal control consisting of equal parts of all samples). Pairs of samples were reverse-labelled in order to eliminate the possibility of dye labelling bias. Samples were cup-loaded in IPG strips, 24 cm, pH 3–11NL (GE Healthcare), and subjected to isoelectrofocusing in an IPGphor™ IEF System (GE Healthcare) according to the manufacturer's recommendations. Upon completion of the first dimension, strips were incubated in an equilibration buffer (50 mM TRIS-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, a trace of bromophenol blue), containing 0.5% DTT for 15 min and thereafter in the same buffer containing 4.5% iodoacetamide for 15 min. For the second dimension, strips were loaded onto 12.5% polyacrylamide gels and run (1 W per gel) for 12–14 h until the bromophenol blue dye reached the gel end. Subsequently, 2D gels were scanned using a Typhoon™ Trio Imager (GE Healthcare) at 100 µm resolution with $\lambda_{ex}/\lambda_{em}$ of 488/520, 532/580, and 633/670 nm for Cy2, Cy3, and Cy5, respectively. The photomultiplier tube was set to ensure that the maximum pixel intensity was between 90 000 and 99 000 pixels. Image analysis was performed using DeCyder 7.0 software (GE Healthcare) as described in the user manual. Briefly, the differential in-gel analysis (DIA) module was used for spot detection, spot volume quantification, and volume ratio normalization of different samples in the same gel. Preparative gels were run with 350 µg of protein following the same procedure described above. Proteins were visualized by staining with SYPRO Ruby Protein Gel Stain (Bio-Rad) and images were acquired with a Typhoon™ Trio Imager (GE Healthcare). Spots differentially represented were excised manually and gel specimens were processed with a MassPrep station (Waters) as described elsewhere (Santamaría *et al.*, 2003). In-gel tryptic digestion was performed with 12.5 ng μl^{-1} trypsin in 50 mM ammonium bicarbonate for 12 h at 37 ºC. The resulting peptides were extracted with 1 % formic acid and 50 % acetonitrile. Then the biological variation analysis (BVA) module was used to match protein spots among different gels and to identify protein spots that exhibited significant differences. Differentially expressed spots were considered for identification when the *P*-value was <0.05 following testing by analysis of variance (ANOVA) or the *t*-test.

Nitrogen content

Three biological replicates of dried flag leaf samples were ground to powder and the 1.0 mg samples were weighed and stored in tin capsules for total organic matter (TOM) analyses. N content was determined at the Serveis Cientifico-Técnics of the University of Barcelona (Barcelona, Spain) using an elemental analyser (EA1108, Series 1; Carbo Erba Instrumentazione, Milan, Italy).

Nitrate content

Nitrate content was determined in the soluble fraction described above for soluble sugar and organic acid content. The samples were injected into capillary electrophoresis equipment (Beckman P/ACE system 5500, Beckman Instruments, Inc., CA, USA). The determinations were conducted with –25 kV voltage at 25 ºC with a photodiode detector at a 254 nm wavelength. The buffer used was composed of 2.25 mM pyromellitic acid, 6.5 mM NaOH, 0.75 mM hexamethonium hydroxide, and 1.6 triethanolamine, where the pH was adjusted to 7.7.

Statistical analyses

Statistical analysis was performed by two-factor ANOVA (SPSS v.12.0; SPSS Inc., Chicago, IL, USA). [CO_2] was used as the first factor (ambient [CO_2], ~400 µmol mol^{-1} versus elevated [CO_2], 700 µmol mol^{-1}) and plant genotype as the second factor. The results were accepted as significant at $P < 0.05$. When differences between treatment [CO_2], and/or genotypes, and/or interactions were significant according to ANOVA, least significant differences were evaluated using the least significant difference post-hoc test (LSD) ($P < 0.05$).

Results

The study showed that elevated [CO_2] increased the total DM of Sula; however, Blanqueta did not show any enhancement in total DM (F=5.53; P=0.03) (Fig. 1A). In the case of Sula, total DM increased 72%, whereas in Blanqueta, exposure to 700 µmol mol^{-1} [CO_2] had no significant effect. The more specific analyses of organ DM reflected that, in Sula, the increase in total DM was mainly the consequence of their larger grain and shoot DM. In Blanqueta, elevated [CO_2] did not alter plant growth (Fig. 1A). Figure 1B revealed that the thousand kernel weight (TKW) was larger in Sula than in Blanqueta and that, regardless of the genotype analysed, [CO_2] had no significant effect on it. Although elevated [CO_2] increased grain number in Sula, no significant effect was detected in Blanqueta (Fig. 1B).

Gas exchange analyses showed that photosynthetic responsiveness (A_{sat}) to 700 µmol mol^{-1} CO_2 exposure was a function of the genotype analysed (F=69.41; $P \leq 0.001$). In Sula, A_n increased by 100%, whereas in Blaqueta it increased by 25% (Table 1). The absence of significant differences in stomatal conductance indicated that the larger the C_i of plants exposed to elevated [CO_2], the larger the A_n. However, the lower Rubisco carboxylation maximum capacity (Vc_{max}) detected in Blanqueta highlighted that in contrast to Sula (where Vc_{max} was not modified), elevated [CO_2] induced the down-regulation of its activity (F=6.30; P=0.03) (Table 1). The maximum electron transport rate contributing to RuBP regeneration

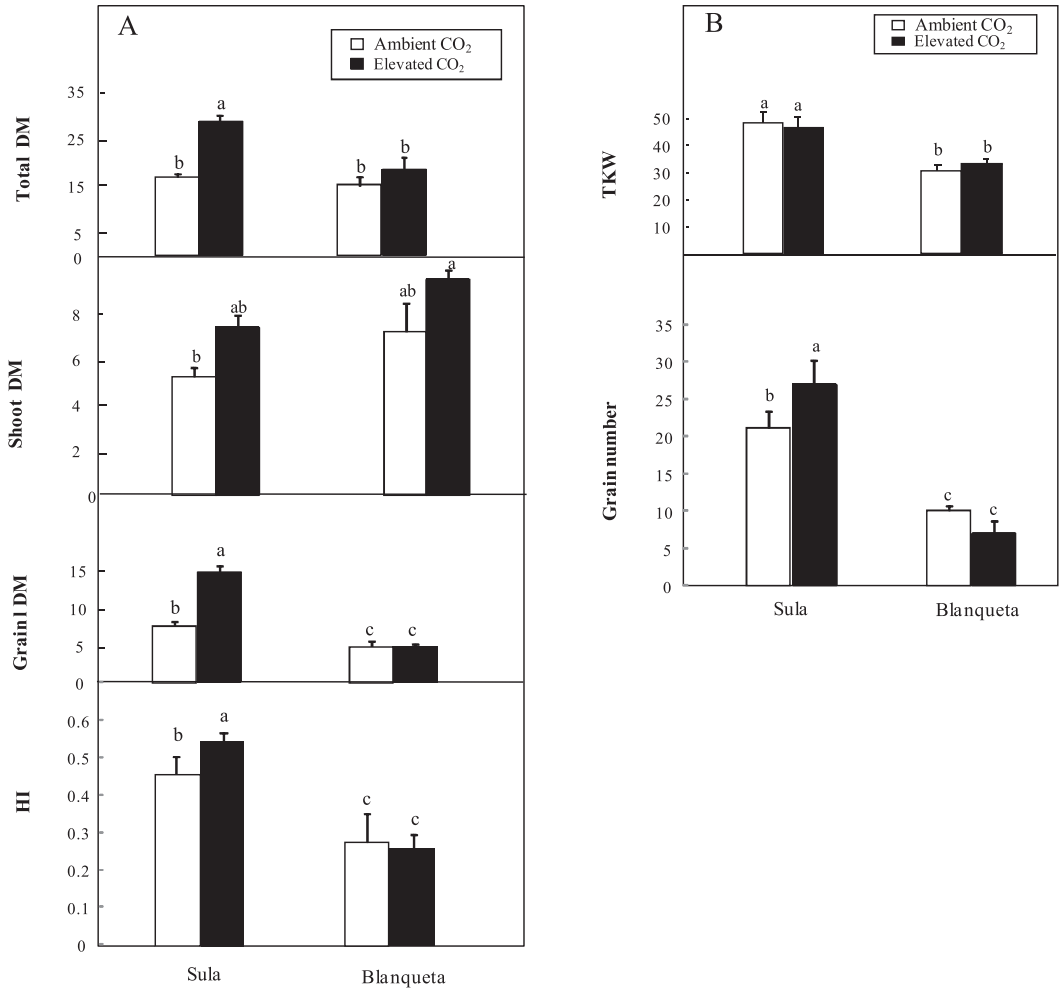

Fig. 1. Effect of elevated [CO2] (700 µmol mol^{-1} versus 360 µmol mol^{-1}) exposure (A) in total dry matter (DM, g plant^{-1}), grain DM (g plant^{-1}), and harvest index (HI, g g^{-1}), and (B) thousand kernel weight (TKW, g plant^{-1}) and number of grains (no. grains plant^{-1}) of durum wheat Sula and Blanqueta genotypes determined during the grain maturity stage. Each value represents the mean of eight replicates ±SE. Two-factor analysis of variance (ANOVA) was used to test significance. When significant differences were detected in ANOVA, LSD analysis was applied. Means that differed significantly ($P > 0.05$) are followed by a different letter according to the LSD test parameters.

(J_{max}) was not altered by [CO_2] (F=0.00; P=0.95), or by studied genotype (F=1.43; P=0.25).

When expressing the A_{sat} on a Rubisco content basis (Table 2), it was observed that its values increased 400% in Sula and 228% in Blanqueta grown at 700 µmol mol^{-1} CO_2 compared with normal [CO_2] (Table 2). Regardless of the genotype, exposure to 700 µmol mol^{-1} CO_2 increased C_i/Rubisco. In both genotypes, [CO_2] deleterious effects on the RubL

Table 1. Effect of elevated [CO_2] (700 µmol mol^{-1} *versus* 360 µmol mol^{-1}) exposure in durum wheat Sula and Blanqueta genotypes on leaf light-saturated rate of CO_2 assimilation (A_{sat}, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mmol CO_2 m^{-2} s^{-1}), intercellular [CO_2] concentration (C_i, µmol CO_2 mol^{-1} air), Rubisco maximum carboxylation rate (Vc_{max}, µmol CO_2 m^{-2} s^{-1}), maximum electron transport rate contributing to RuBP regeneration (J_{max}, µmol CO_2 m^{-2} s^{-1}), and J_{max} /Vc_{max} ratio determined in flag leaves 2 weeks after anthesis. Each value represents the mean of four replicates ±SE. Statistical analysis was made by a two-factors analysis of variance (ANOVA). When significant differences were detected in ANOVA, LSD analysis was applied. Means that differed significantly (P > 0.05) were followed by a different letter according to the LSD test parameters.

	[CO_2]	A_{sat}	g_s	C_i	Vc_{max}	J_{max}	J_{max}/Vc_{max}
Sula	Ambient	10.66±0.49 b	140.50±25.25 a	279.47±8.25 b	52.54±9.31 a,b	93.82±11.87 b	1.88±0.07 c
	Elevated	22.11±0.78 a	173.54±18.00 a	475.25±27.98 a	50.58±6.21 a,b	127.29±11.46 a,b	2.82±0.29 b
Blanqueta	Ambient	16.22±1.11 a,b	240.20±22.11 a	279.57±16.70 b	69.87±3.12 a	144.69±15.54 a	2.06±0.13 c
	Elevated	20.46±2.07a	191.35±26.24 a	512.25±16.73 a	33.31±6.97 b	113.03±18.59 a,b	3.22±0.21 a

Table 2. Elevated [CO_2] (700 µmol mol^{-1} versus 360 µmol mol^{-1}) exposure effect in durum wheat Sula and Blanqueta genotypes on leaf net photosynthesis (A_n/Rubisco, µmol CO_2 $g^{-1}s^{-1}$), Rubisco large (RubL) and small (RubS) subunits (g m^{-2}), nitrate (NO_3^-, g m^{-2}), and N (g m^{-2}) content determined in flag leaves 2 weeks after anthesis. Each value represents the mean of four replicates ±SE. Otherwise as for Table 1.

	[CO_2]	A_n/Rubisco	RubL	RubS	NO_3^-	N
Sula	Ambient	7.45±0.35 b	1.26±0.15 a	0.17±0.02 a,b	5.12±0.47 a,b	1.96±0.15 a,b
	Elevated	29.88±1.06 a	0.67±0.07 b,c	0.08±0.01 c	5.63±0.75 a	2.32±0.21 a
Blanqueta	Ambient	13.63±0.94 b	0.97±0.04 a,b	0.22±0.03 a	3.95±0.37 b	1.60±0.19 b,c
	Elevated	34.10±3.45a	0.51±0.03 c	0.09±0.01 b,c	2.25±0.27 c	1.27±0.18 c

and RubS subunits were similar and the content of these proteins decreased by 52% and 40%, respectively (Table 2). The authors would like to clarify that apparent discrepancies concerning decreases in Rubisco concentration observed with SDS–PAGE (Table 2) but not with 2D electrophoresis (Table 3) can be explained by saturation of the fluorescence staining of Rubisco in the 2D electrophoresis methodology due to its abundance. In addition, N content decreased in Blanqueta under elevated [CO_2] but not in Sula (Table 2). Although [CO_2] had no effect on NO_3^- content in Sula, in the case of Blanqueta, NO_3^- content decreased by 43%. Compared with Sula, NO_3^- levels were lower in Blanqueta, especially under elevated [CO_2] conditions.

The carbohydrate determinations revealed that the [CO_2] effect on sucrose and starch content differed in both genotypes (F=12.81; P=0.01 and F=24.94; P=0.00, respectively) (Fig. 2A). In Sula plants starch was not modified by increased [CO_2], whereas in Blanqueta the starch content increased 60%. Concerning sucrose levels, in the case of Sula there was a 305% increase, whereas in Blanqueta there was no significant difference. Similar responses were observed for fructose content. Regarding glucose, although no [CO_2] effect was observed in Sula, glucose content increased in Blanqueta. Exposure to 700 µmol mol^{-1} CO_2 diminished the organic acid content of both genotypes (Fig. 2B). In Sula plants grown under elevated [CO_2] there was a lower citrate (64%) content, whereas in Blanqueta, in addition to citrate (43%), α-ketoglutarate (31%) and malate (57 %) levels also diminished (Fig. 2B). However, neither the succinate nor the oxaloacetate levels were affected by increased [CO_2] in either cultivar. Glutamate was the most abundant amino acid in both genotypes and, with the exception of cysteine and isoleucine, [CO_2] had no significant effects on Sula amino acid levels, whereas in Blanqueta, the amino acid content decreased by 58% (Fig. 3). More specifically, glutamine, aspartate, threonine, glycine, serine, cysteine, alanine, proline, valine, tyrosine, isoleucine, lysine, leucine, and methionine were decreased in Blanqueta plants exposed to elevated [CO_2].

Finally, the proteomic 2D-DIGE characterization conducted in the flag leaf detected up to 3500 spots from which 59 were shown to differ significantly between genotypes and [CO_2] levels (Table 3). In the case of Sula, 18 proteins were affected by [CO_2], whereas in Blanqueta there were changes to 22 proteins. More specifically, in Sula, with the exception of the RubL subunit and adenosylhomocysteinase (which were down-regulated), the remaining 16 proteins were up-regulated under elevated [CO_2]. In Blanqueta, the levels of adenosylhomocysteinase, homocysteine methyltransferase, and V-ATPase subunit A diminished in response to increasing [CO_2], whereas the remaining 19 proteins were up-regulated. Concerning genotype differences, the study showed that under ambient [CO_2], 13 proteins were affected; 12 of them had lower expression levels in Blanqueta than in Sula and only adenosylhomocysteinase was higher. Under elevated [CO_2], 25 proteins differed between genotypes. Nineteen proteins were diminished in Blanqueta and six increased. Concerning the functioning of detected proteins, the 2D-DIGE analyses detected six proteins involved in metabolism, 31 in energy, nine in disease/defence, one with unclear classification, and four that were not identified.

Discussion

The effects of elevated [CO_2] in wheat plants have been widely characterized (Pérez *et al.*, 2007, 2011; Aranjuelo *et al.*, 2011). The variability of the results reported in the literature highlight the fact that wheat performance under elevated [CO_2] is not unique and depends on exposure time, plant species, etc. The present study showed that the genotype with high HI (Sula) was the only one where elevated [CO_2] increased plant growth. The [CO_2]-derived stimulation of Sula was mainly due to an increase in grain DM. Although sink strength has been described to be a key factor conditioning plant responsiveness to elevated [CO_2] (Ziska *et al.*, 2004; Alonso *et al.*, 2008; Uddling *et al.*, 2008; Högy *et al.*, 2009, 2010; Aranjuelo *et al.*, 2011; Pérez *et al.*, 2011), little attention has been given to HI as a target for research. The fact that Sula had a larger HI under both CO_2 levels reveals that this genotype has been subjected to more intense wheat breeding programmes seeking plants with proportionally larger ear grain DM (Ziska *et al.*, 2004; Uddling *et al.*, 2008; Foulkes *et al.*, 2011; Reynolds *et al.*, 2012).

The capacity of plants to adjust photosynthetic activity with leaf C demand is a major factor conditioning photosynthetic performance under elevated [CO_2] (Ziska *et al.*, 2004). Although analyses of photosynthetic rates at the corresponding growth conditions showed that exposure to 700 µmol mol^{-1} [CO_2] increased photosynthetic activity of both genotypes, the decrease in the Rubisco maximum carboxylation rate (Vc_{max}) of Blanqueta showed that photosynthetic capacity was down-regulated in these plants. These results are in agreement with previous findings (Ainsworth *et al.*, 2004; Ainsworth and Bush, 2011; Aranjuelo *et al.*, 2011). The C_i/Rubisco ratio confirmed that Blanqueta plants exposed to

elevated [CO_2] were capable of increasing A_{sat} with a lower Vc_{max}, thanks to their larger C_i. The photosynthetic down-regulation observed in Blanqueta was caused by depletion of Rubisco and (as explained in more detail below) other component proteins of the Calvin cycle. Although the Rubisco content in Sula also decreased under 700 µmol mol^{-1} CO_2, the increase (400%) in A_n/Rubisco enabled the maintenance of larger photosynthetic rates in wheat plants (Pérez *et al*., 2011). As discussed in more detail below, since plants might have an excess of Rubisco, redistribution of the excess N invested in this protein could be partitioned to other processes and/or organs without impacting leaf C fixation rates, according to Ainsworth and Rogers (2007). In contrast, the increase (228 %) in A_n/Rubisco in Blanqueta was not enough to overcome photosynthetic down-regulation.

Altered source supply, source activity, and sink strength have provided strong evidence for the hypothesis that photosynthesis and sink utilization of carbohydrates are tightly coordinated (Ainsworth and Rogers, 2007). As mentioned above, Rubisco content is regulated by the cytosolic phosphorylation of hexoses by HK availability and/or activity (Kaschuk *et al*., 2010; Ainsworth and Bush, 2011). The enhanced levels of hexoses such as fructose (Sula) and glucose (Blanqueta) suggest that as observed in other plants (Moore *et al*., 1999; Long *et al*., 2004; Ainsworth and Rogers, 2007), HK-mediated depletion of Rubisco content was involved in the Rubisco depletion of plants exposed to elevated [CO_2]. Although exposure to elevated 700 µmol mol^{-1} CO_2 increased carbohydrate content in both genotypes, in Sula the increase was mainly explained by the enhancement of sucrose content

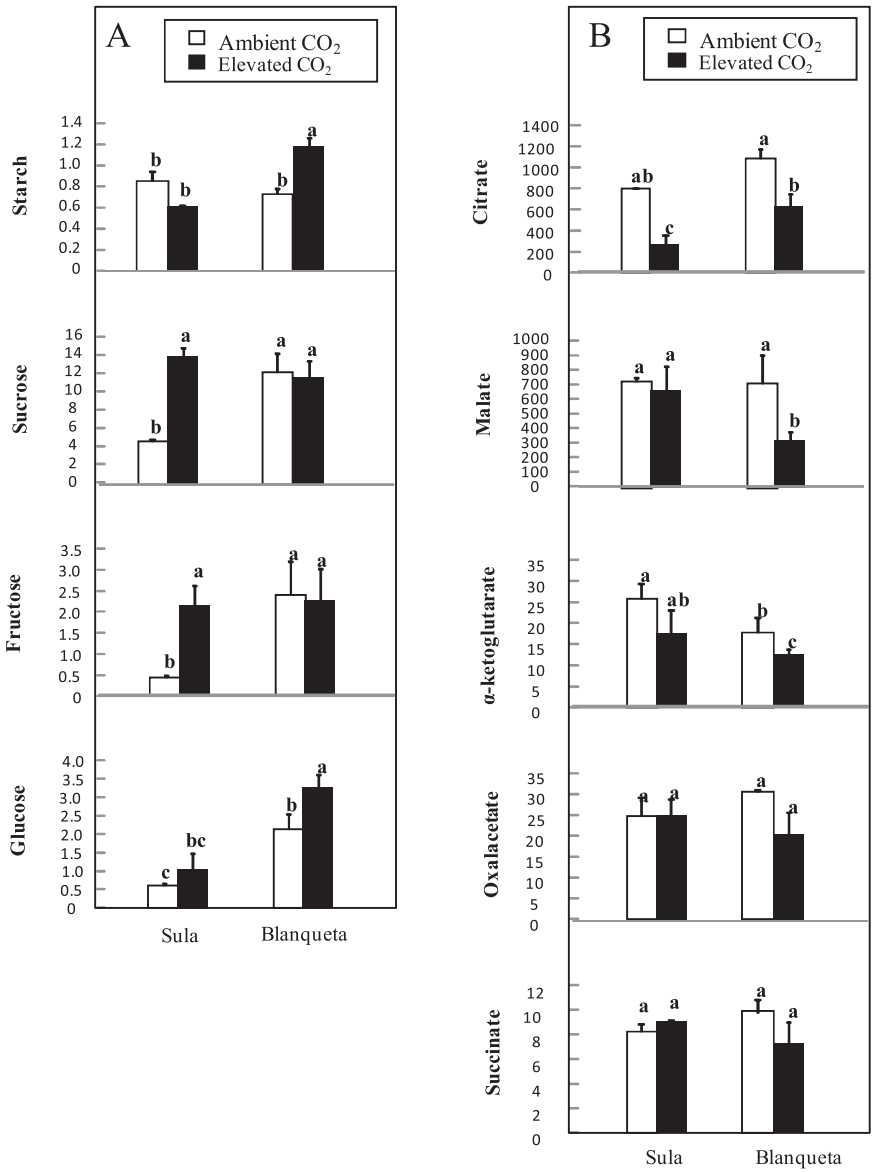

Fig. 2. Effect of elevated [CO_2] (700 µmol mol^{-1} versus 360 µ mol mol^{-1}) exposure (A) on starch (g m^{-2}) and soluble sugars (sucrose, glucose, and fructose; mmol m^{-2}) and (B) organic acid (citrate, malate, oxalacetate, α-ketoglutarate, and succinate; mg m^{-2}) content of durum wheat Sula and Blanqueta genotypes determined in flag leaves 2 weeks after anthesis. Each value represents the mean of four replicates ±SE. Otherwise as for Fig. 1.

Table 3. Identification of differentially expressed proteins in flag leaf of durum wheat Sula (S) and Blanqueta (B) genotypes exposed to elevated (Elev; 700) versus ambient (Amb; 360) [CO_2] (μmol mol^{-1}). Spot number, access sode, protein name, score, mol. wt, pI, peptide number, and average ratio (between the different genotypes and [CO_2] combination) are shown.

	Spot number	Access code	Protein name	Phenyx score	Mol. wt	pI	Peptides	S_{Amb}/S_{Elev}	B_{Amb}/B_{Elev}	S_{Amb}/B_{amb}	S_{Amb}/B_{Elev}	S_{Elev}/B_{Amb}	S_{Elev}/B_{Elev}
0.1 METABOLISM													
	956	P50249	Adenosylhomocysteinase	19.3	53.14	5.89	3	–1.37		1.32		1.8	
	1093	P32112	Adenosylhomocysteinase	89.2	53.44	5.74	12		–1.44		–1.35		
	766	O50008	Homocysteine methyltransferase	9.8	84.85	6.19	1		–1.34			1.19	
	1330	P13564	Glutamine synthetase	79.8	47.09	5.18	12						–1.43
	1332	P13564	Glutamine synthetase	195.2	47.09	5.18	34				–1.43	–1.4	–1.45
	1317	Q56YA5	Serine-glyoxylate aminotransferase	144.6	44.21	8.17	20		1.57			–1.64	
0.2 ENERGY													
	56	A1EA16	RuBisCO large subunit	123	52.81	6.34	16	–1.55				1.71	
	132	A1EA16	RuBisCO large subunit	151.5	52.63	6.34	20	–1.53				1.72	
	539	P28261	RuBisCO large subunit	15.7	52.04	6.34	2	–2.13			–4.64		–2.17
	540	P28261	RuBisCO large subunit	14.7	51.08	6.44	3	–1.72			–3.25		
	1012	P21241	RuBisCO large subunit	34.3	56.59	5.31	4						1.97
	1087	P42862	Glucose-6-phosphate isomerase	41.4	62.52	7	5		1.4		1.69	1.29	1.81
	1188	Q43772	UTP-glucose-1-phosphate uridylyltransferase	153.3	51.64	5.26	19	–1.33				1.77	1.28
	2266	Q6ZBZ2	Germin-like protein	54.8	21.86	6.02	11	2.17	2.66		2.32	–2.48	
	2272	Q6ZBZ2	Germin-like protein	51	21.86	6.37	9	2.13	2.58			–4.48	–1.74
	1339	P12782	Phosphoglycerate kinase. chloroplastic	94.4	49.83	6.98	11	1.72				–2.28	–1.82
	1386	P25857	Glyceraldehyde-3-phosphate dehydrogenase B	40.6	39.3	5.85	5	1.34				–1.53	–1.25
	1404	P13443	Glycerate dehydrogenase	62.5	41.7	6.18	9				–3	–2.39	–3.02
	1433	P12859	Glyceraldehyde-3-phosphate dehydrogenase B	17.9	39.3	6.2	2					–1.52	–1.49
	1434	P26302	Phosphoribulokinase, chloroplastic	53.8	39.18	5.05	7				–1.34		–1.26
	1443	P46285	Sedoheptulose-1.7-bisphosphatase	127.2	42.06	6.26	15					–1.34	–1.22
	1444	P26302	Phosphoribulokinase, chloroplastic	323.8	39.18	5.05	59		1.37	–1.79		–1.99	–1.45
	1455	P26302	Phosphoribulokinase, chloroplastic	111	39.18	5.05	14		1.65	–1.67		–2.11	
	1543	P09315	Glyceraldehyde-3-phosphate dehydrogenase A	82.6	42.86	7.61	11					–1.8	–1.44
	1548	P09315	Glyceraldehyde-3-phosphate dehydrogenase A	118.8	36.09	6.58	18					–2.25	
	1549	P09315	Glyceraldehyde-3-phosphate dehydrogenase A	285	36.09	6.66	56	1.34	1.51	–1.58		–2.11	–1.4
	1552	P09315	Glyceraldehyde-3-phosphate dehydrogenase A	131.8	36.09	6.58	20		1.45	–1.53		–1.96	–1.35
	1554	P09315	Glyceraldehyde-3-phosphate dehydrogenase A	71.9	42.86	7.61	9		1.68			–1.67	
	1560	P09315	Glyceraldehyde-3-phosphate dehydrogenase A	178.1	42.86	7.61	28		1.54	–1.64		–2.04	
	1613	P09315	Glyceraldehyde-3-phosphate dehydrogenase A	89.8	42.86	7.61	12					–1.51	
	1144	P42895	Enolase 2	100.2	48.16	5.84	18	–1.28					
	1997	P46225	Triosephosphate isomerase	63.7	27.28	5.1	7	1.58	1.87	–1.47		–2.33	
	1998	P46225	Triosephosphate isomerase	95	27.28	5.1	12				–1.4		–1.28
	904	P49087	V-type proton ATPase catalytic subunit A	76405,0	61.95	6.03	11		–1.44			1.5	
	1077	A1E9I8	ATP synthase subunit alpha	38.6	55.29	6.59	5			–1.78	–1.56	–1.95	–1.72
	1585	P10933	Ferredoxin-NADP reductase. leaf isozyme	86.4	34.79	7.07	17					–1.37	
	1608	P41343	Ferredoxin-NADP reductase	26.9	41.06	8.68	4			–1.35	–1.29	–1.47	–1.41
	1990	P12330	Chlorophyll *a-b* binding protein 1	115.8	24.83	5.11	14		1.92			–3.12	

Table 3. Continued

	Spot number	Access code	Protein name	Phenyx score	Mol. wt	pI	Peptides	S_{Amb}/S_{Elev}	B_{Amb}/B_{Elev}	S_{Amb}/B_{amb}	S_{Amb}/B_{Elev}	S_{Elev}/B_{Amb}	S_{Elev}/B_{Elev}
	2453	A6MMG6	Photosystem I reaction centre subunit IV	24.1	27.18	8.53	7		1.17	–1.25	–1.07	–1.4	
	1981	A4GG93	NAD(P)H-quinone oxidoreductase subunit K					1.16				–1.48	
11. DISEASE/DEFENCE													
	1789	Q9SGS4	Thioredoxin-like protein CDSP32	15.5	33.68	8.81	3		1.2	–1.43	–1.19	–1.53	
	1981	A4GG93	NAD(P)H-quinone oxidoreductase subunit K	32.6	23.94	6.12	5	1.16				–1.48	
	2120	P80602	2-Cys peroxiredoxin BAS1	24.4	23.29	5.7	3	1.65	1.93			–2.15	
	2171	P80602	2-Cys peroxiredoxin BAS1	45.7	23.29	5.7	6	1.37				–1.66	
	1095	Q43206	Catalase-1		56.8	6.62			1.41		1.59	1.29	1.83
	1085	Q43206	Catalase-1	62	56.5	6.62	7					1.35	1.44
	1086	Q43206	Catalase-1	198	56.5	6.62	28					1.38	1.47
	2560	Q7F8S5	Peroxiredoxin-2E-2	17.7	18.96	5.24	2			–2.56	–2.05	–2.8	–2.24
	2266	Q6ZBZ2	Germin-like protein	54.8	19.37	6.03	11	2.17	2.66		2.32	–2.48	
	2272	Q6ZBZ2	Germin-like protein	51	19.37	6.02	9	2.13	2.58			–4.48	–1.74
12. UNCLEAR CLASSIFICATION													
	1570	P0C1M0	Inceptin [CHAIN 0]	85	36.1	5.95	14		1.09				

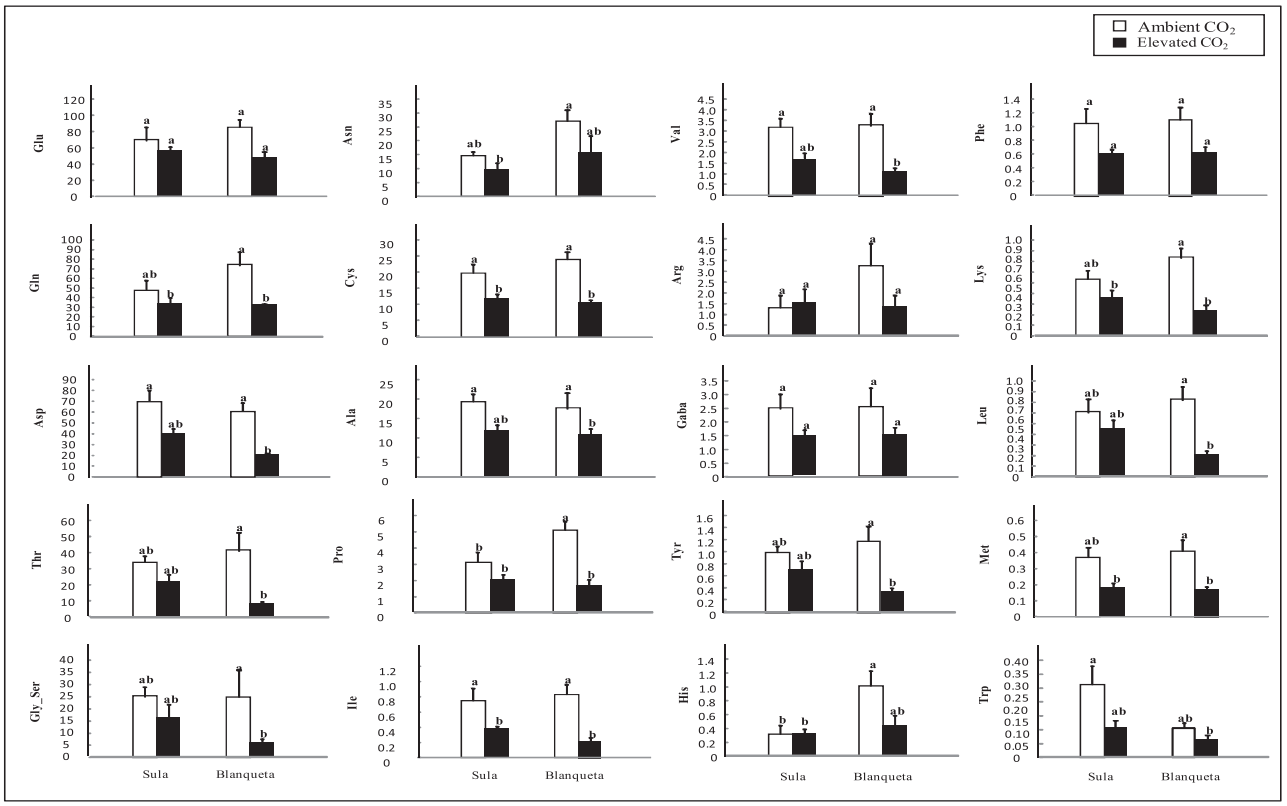

Fig. 3. Effect of elevated [CO_2] (700 µmol mol^{-1} versus 360 µmol mol^{-1}) exposure on amino acid (glutamate, glutamine, aspartate, threonine, glycine, serine, asparagine, cysteine, alanine, proline, valine, arginine, γ-aminobutyric acid, tyrosine, histidine, isoleucine, lysine, phenylalanine, leucine, methionine and tryptophan) content (10^{-5} mol m^{-2}) of durum wheat Sula and Blanqueta genotypes determined in flag leaves 2 weeks after anthesis. Each value represents the mean of four replicates ±SE. Otherwise as for Fig. 1.

and in Blanqueta it was due to an increased starch content. Although the increase in both forms of carbohydrate could be understood as a consequence of C sink limitation, the roles of these carbohydrates in plant performance should be considered. Sucrose is a major form of C translocation to developing organs and/or storage organs (Fallaj *et al.*, 2008). The greater sucrose content of Sula exposed to 700 µmol mol^{-1} CO_2 could be understood as a consequence of the large C requirements of their ears. On the other hand, the increase in starch (a major C storage form; Niittyla *et al.*, 2004) content of Blanqueta grown under elevated [CO_2] would reflect the need to store C due to the absence of a strong C sink. Such results are in agreement with previous findings (Moore *et al.*, 1999; Stitt and Krapp, 1999) that reported that the balance between sucrose synthesis and starch might be involved in photosynthetic down-regulation. The proteomic characterization revealed that the pathway leading to starch synthesis was up-regulated under elevated [CO_2]. More specifically, the study detected the up-regulation of glucose-6-phosphate isomerase (GPI), triose phosphate isomerase (TPI) (Moore *et al.*, 1999), and germin-like proteins (Stitt *et al.*, 2010) in Blanqueta plants exposed to elevated [CO_2], thus highlighting the relevance of starch biosynthesis in those plants. This idea was reinforced by the fact that, regardless of the CO_2 level, Blanqueta leaves showed larger GPI content than Sula.

Although Rubisco is the most abundant leaf protein, recent studies (Rodríguez-López *et al.*, 2001; Dunwell *et al.*, 2008) have shown that the photosynthetic activity, and therefore plant growth, is also conditioned by the availability of other rate-limiting Calvin cycle proteins (Stitt *et al.*, 2010; Parry *et al.*, 2011; Uematsu *et al.*, 2012). The 2D-DIGE characterization highlighted that Sula, where DM increased to a larger extent than in Blanqueta, the Calvin cycle protein content was also larger. More specifically, the content of three isoforms of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), sedoheptulose 1,7-bisphosphatase, and phosphoribulokinase (PRK) was larger in Sula than in the corresponding Blanqueta plants. The larger Calvin cycle protein content of Sula could have contributed towards overcoming photosynthetic acclimation in these plants when exposed to 700 µmol mol^{-1} CO_2. Exposure to elevated [CO_2] in Sula induced the up-regulation of different Calvin cycle enzymes such as chloroplastic phosphoglycerate kinase (PGK) and GAPDH, whereas, in Blanqueta, three chloroplastic GAPDH isoforms and PRK increased. These results highlight the fact that Rubisco was the only Calvin cycle protein that decreased in both genotypes under 700 µmol mol^{-1} CO_2, and were in agreement with previous studies (Henkes *et al.*, 2001; Raines, 2003; Lefebvre *et al.*, 2005; Tamoi *et al.*, 2006; Zhu *et al.*, 2007; Uematsu *et al.*, 2012) where, due to the fixed protein-nitrogen availability, increases in the photosynthetic enzymes have followed the compensatory decrease of others. Among other effects, the increase in Calvin cycle proteins such as

PRK revealed that, in contrast to the observations of Zhu *et al.* (2007), photosynthetic activity was not constrained by limitations in RuBP regeneration under elevated [CO_2]. The absence of significant differences in J_{max} confirmed this point. Furthermore, the increase in J_{max}/Vc_{max} observed in Sula and Blanqueta exposed to 700 µmol mol^{-1} CO_2 corroborated the idea that there was a redistribution of N from Rubisco to RuBP regeneration processes (Zhu *et al.*, 2012).

There is evidence that the carbohydrate-mediated repression of photosynthetic genes is more severe in nitrogen-deficient than in nitrogen-sufficient plants (Geiger *et al.*, 1999; Stitt and Krapp, 1999; Sun *et al.*, 2002; Long *et al.*, 2004; Ainsworth and Long, 2005; Zhu *et al.*, 2010). According to Theobald *et al.* (1998), N assimilation cannot match CO_2 fixation, with a consequent carbohydrate build up and depletion of N content. The data revealed that N and NO_3^- content were negatively affected by elevated [CO_2] in Blanqueta plants, whereas, in Sula, no significant effect was observed. These data highlight that the depletion of NO_3^- assimilation and N content in Blanqueta was related to the inhibition of photosynthetic rates. Bloom *et al.* (2002, 2010) describe two main points where CO_2 fixation and NO_3^- assimilation interfere. The first of these refers to the availability of ferredoxin required for the reduction of NO_2^- to NH_4^+ and the subsequent amino acid and starch synthesis. Ferredoxin-NADP reductase (FNR) has been described (Bloom *et al.*, 2002) as the key protein in the generation of reduced ferredoxin. Although in our case, FNR was not affected by [CO_2] in either of the genotypes, the fact that FNR content in Blanqueta (where starch content increased) was lower than in Sula suggests that the ferredoxin content might have been limiting for N assimilation in Blanqueta plants exposed to elevated [CO_2]. In agreement with Rachmilevitch *et al.* (2004), the enhancement of photosynthetic activity and Calvin cycle proteins (with the exception of Rubisco) of Blanqueta plants exposed to 700 µmol mol^{-1} CO_2 could have decreased the amount of ferredoxin available for NO_2^- reduction. Furthermore, the fact that starch content and proteins involved in its synthesis were up-regulated suggests that starch synthesis was favoured over amino acid assimilation (Ezquer *et al.*, 2010*b*). The second point inferred from Bloom *et al.* (2002, 2010) refers to the NADH required for reduction of NO_3^- to NO_2^- that is produced from malate, and which is shuttled to the chloroplast. The lower V-ATPase detected in Blanqueta exposed to elevated [CO_2], together with the lower levels of ATP synthase in this cultivar, suggest that these plants may have limited ATP and NADH available for CO_2 and NO_3^- assimilation. Such limitations are especially relevant because NO_3^- is the second largest photosynthetic energy sink (Bloom *et al.*, 2002).

It has been described that when there is an accumulation of excess C under limited N availability, the ATP is diverted from protein metabolism toward glycogen biosynthesis (Noctor and Foyer, 1998). According to the model proposed by different authors (Ezquer *et al.*, 2010*b*; Yamakawa and Hakata, 2010; Li *et al.*, 2011), the accumulation of starch includes the down-regulation of proteins involved in internal amino acid provision. This link between C and N metabolism was confirmed by the increases in C storage in Blanqueta exposed to elevated [CO_2] (starch increased by 60%) that were accompanied by decreases in amino acids and proteins involved in amino acid synthesis (adenosylhomocysteinase and homocysteine methyltransferase). Furthermore, limitations to amino acid synthesis were reinforced by the fact that the availability of two isoforms of glutamine synthetase, which is a key protein involved in amino acid assimilation, was lower in Blanqueta under 700 µmol mol^{-1} CO_2 than in the corresponding Sula plants. As a consequence of the protein down-regulation, the availabilities of key amino acids such as glutamine (the major form of N transport; Lea and Azevedo 2007) and essential amino acids such as lysine, methionine, threonine, and isoleucine were lower in Blanqueta plants grown under elevated [CO_2]. The flow of carbon skeletons through the tricarboxylic acid (TCA) cycle has been described as conditioning the assimilation of NO_3^- and NH_4^+ into amino acids (Ezquer *et al.*, 2010*a*). The depletion of a major C skeleton such as α-ketoglutarate for glutamine and proline synthesis also constrained the availability of the amino acid content in Blanqueta plants exposed to elevated [CO_2]. However, in the case of oxalacetate-derived amino acids (aspartate, lysine, methionine, cysteine, threonine, and isoleucine), although the availability of this organic acid was not affected by [CO_2], the decrease in the content of these amino acids (with the exception of asparagine) revealed that the availability of C skeletons was not limiting.

Conclusions

Although the relevance of sink size in photosynthetic acclimation has been known for a long time, this study has revealed the effects of a different HI on the C sink and its implications on the down-regulation of a wide range of leaf proteins and metabolic compounds of photosynthesis. Although exposure to 700 µmol mol^{-1} CO_2 increased Sula growth, in the case of Blanqueta photosynthetic acclimation constrained any significant effect of elevated [CO_2] on plant yield. The sucrose–starch balance was confirmed as a key factor in the responsiveness of photosynthetic machinery to elevated [CO_2]. The larger starch content observed in Blanqueta (compared with Sula) was the consequence of the inability to develop strong C sinks. The present study showed that limitations derived from N assimilation induced the photosynthetic down-regulation. The depletion in N, NO_3^-, amino acid content, and proteins involved in amino acid assimilation, together with the up-regulation of proteins involved in starch synthesis, suggest that the available ferredoxin was destined towards starch synthesis over N assimilation. Furthermore, the depletion in organic acids and proteins involved in energy processes shows that ATP and NADH limitations could also have constrained high energy-demanding processes such as NO_3^- assimilation. The fact that in Sula N assimilation was not diminished by exposure to [CO_2] enabled the photosynthetic acclimation to be overcome,with a consequent increase in plant growth. The data obtained in this study also suggest that the higher Calvin cycle protein content of Sula (compared with Blanqueta) explains the ability of this genotype to overcome photosynthetic down-regulation under elevated [CO_2].

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