Acclimation to elevated CO₂ is improved by low Rubisco and carbohydrate content, and enhanced Rubisco transcripts in the G132 barley mutant

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Abstract

Variations induced by mutagenesis may identify targets for adapting to rising atmospheric CO₂ concentrations. G132 is a barley mutant with strong decreases in photosynthesis, Rubisco and carbohydrate content, but increased Rubisco transcripts. We tested the hypothesis that G132 will record a greater stimulation of photosynthesis and dry mass gain than the wild-type (WT) in response to CO₂ enrichment. A growth chamber experiment compared the mutant G132 and the WT developed in ambient (~390 μmol mol⁻¹) and elevated (1200 μmol mol⁻¹) CO₂. Photosynthesis, protein and carbohydrate contents, Rubisco and nitrate reductase activities, leaf morphology, plant dry matter and leaf area were determined. The transcriptome responses of fully expanded leaves of G132 and WT to elevated CO₂ were investigated using microarrays and qRT-PCR. Growth in elevated CO₂ stimulated photosynthesis more in G132 than in WT, in association with a lack of Rubisco down-regulation and lower carbohydrate levels in G132. Electron transport-related proteins increased in G132 and decreased in WT. Elevated CO₂ induced more changes in gene expression in WT than G132. Clustering of the corresponding transcripts showed that the expression of genes involved in carbohydrate synthesis and nitrogen assimilation was enhanced, while that for stress-related genes was repressed in both genotypes, and gene expression for jasmonate metabolism was activated in G132. Elevated CO₂ decreased the transcript levels for a greater number of transcription factors in WT than in G132. CO₂ enrichment increased leaf area in G132 but not in WT, and induced greater dry mass increases in the mutant. The low Rubisco and carbohydrate content and enhanced Rubisco transcripts recorded in G132 are traits that may improve adaptation to rising CO₂.

Key words: Elevated CO₂; gene expression; mutagenesis; photosynthesis; primary metabolism; Rubisco.

Abbreviations: 1-SST, sucrose: sucrose 1-fructosyltransferase; 6-FEH, fructan 6-exohydrolase; 6-SFT, sucrose: fructan 6-fructosyltransferase; cyt b6, cytochrome b6; Fq'/Fm', PSII operating quantum efficiency; Fru, fructose; Ftn, fructan; Fv/Fm, PSII maximum quantum efficiency; HT1, high temperature 1 protein kinase; J, potential rate of photosynthetic electron transport; LHCI, light harvesting complex I; LHCII, light harvesting complex II; OPP, oxidative pentose phosphate

1 pathway; PEPCASE, phosphoenolpyruvate carboxylase; PEPCK, phosphoenolpyruvate
2 carboxylase kinase; PGM, phosphoglucomutase; PORA, protochlorophyllide oxidoreductase A;
3 PSI, Photosystem I; PSII, Photosystem II; rbcL, Rubisco large subunit; rbcS, Rubisco small
4 subunit; R_d , mitochondrial respiration rate in the light; Rubisco, ribulose-1, 5-bisphosphate
5 carboxylase oxygenase; RuBP, ribulose-1, 5-bisphosphate; SLAC1, slow anion channel-
6 associated 1; SLAH3, SLAC1 homologue 3 slow anion channel; TCA, tricarboxylic acid; V_{cmax} ,
7 maximum rate of Rubisco-catalysed carboxylation; WT wild-type.

8

1. Introduction

Long-term records of atmospheric CO₂ concentrations at Mauna Loa Observatory evidence increases from around 315 $\mu\text{mol mol}^{-1}$ in the late 1950s to above 400 $\mu\text{mol mol}^{-1}$ in the present day, with a 2.2 $\mu\text{mol mol}^{-1}$ annual mean growth rate in the last 10 years (Dr. Tans and Dr. Keeling, www.esrl.noaa.gov/gmd/ccgg/trends/). In plants with C3 photosynthesis, the carboxylation enzyme ribulose-1, 5-bisphosphate carboxylase oxygenase (Rubisco) fixes CO₂ at a faster rate with the elevated CO₂ concentrations predicted for the future. With high CO₂ levels, or with low light intensities, where carboxylation is limited by ribulose-1, 5-bisphosphate (RuBP) regeneration, and not by Rubisco activity, CO₂ inhibition of the Rubisco-catalysed RuBP oxygenation also enhances photosynthesis because it releases photosynthetic electron transport products and RuBP (Leakey et al., 2009). Moreover, carbon fixation is stimulated by elevated CO₂ in spite of photosynthetic acclimation, consisting of a decrease in Rubisco activity and content after long-term growth under CO₂ enrichment (Pérez et al., 2005; Sage et al., 1989; Vicente et al., 2015). The maximum rate of electron transport leading to RuBP regeneration also decreases at times (Leakey et al., 2009), although growth in elevated CO₂ increased the photochemical efficiency in our earlier studies (Gutiérrez et al., 2009). A limited sink capacity can lead to photosynthetic acclimation, and diminish the stimulation of carbon fixation by elevated CO₂ (Aranjuelo et al., 2011). It has been suggested that the mechanism involved may be an accumulation of carbohydrates in leaves that represses Rubisco gene expression (Van Oosten et al., 1994), although the correspondence between carbohydrate accumulation and levels of Rubisco transcripts was weak (Nie et al., 1995). Photosynthetic acclimation is associated with a lower nitrogen content in leaves and the whole plant (del Pozo et al., 2007), for which several explanations have been given, including transpiration-limited nitrogen supply and inhibition of nitrate assimilation (Bloom et al., 2014; Taub and Wang, 2008).

Although Rubisco loss and stomatal closure are the most evident responses to rising CO₂ (Long et al., 2004), transcriptomic changes possibly induced indirectly by elevated CO₂ have been found in several plant species. These changes include the induction of genes for enzymes of the tricarboxylic acid cycle (TCA), oxidative pentose phosphate pathway (OPP), glycolysis (Li et al., 2008), starch, sucrose and fructan synthesis (Fukayama et al., 2011; Li et al., 2008; Vicente et al., 2015), cell wall synthesis, signalling, transport, and defence (Ainsworth et al.,

2006; Li et al., 2008). The repression of genes for photosynthesis, nitrogen assimilation and photorespiration (Vicente et al., 2015) has also been reported. The proteomic changes described in plants growing in elevated CO₂ involve increases in the content of the ATP synthase, phosphoglycerate mutase (Aranjuelo et al., 2011), oxygen evolving complex 23 kDa protein, myrosinase precursor, luminal-binding protein, nucleoside diphosphate kinase (Bae and Sicher, 2004), phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase (Aranjuelo et al., 2013), among others. In addition to decreases in Rubisco, losses have been reported in contents of Rubisco activase, adenosine diphosphate glucose pyrophosphatase (Aranjuelo et al., 2011) and the chloroplast glyceraldehyde-3-phosphate dehydrogenase precursor (Bokhari et al., 2007). Together with the transcriptomic and proteomic changes and the increase in carbohydrate levels, elevated CO₂ decreases the content in leaves of amino acids, nitrate (Geiger et al., 1999; Vicente et al., 2015), and organic acids (Aranjuelo et al., 2013).

As plant dry matter is predominantly carbohydrate produced by photosynthesis, an increase in atmospheric CO₂ concentration usually translates into biomass gains in cereals (Gutiérrez et al., 2009; Kimball, 2016; Vicente et al., 2015) and other crops (Bishop et al., 2015), which vary under certain environmental conditions (Ingvordsen et al., 2015; Kimball, 2016). The increase in crop dry matter and yield with elevated CO₂ is generally low (Leahey et al., 2009). Improving photosynthesis adaptation to atmospheric CO₂ enrichment would allow increasing the efficiency of converting intercepted radiation into biomass, which is possibly the only remaining trait for increasing yields (Long et al., 2006) and meeting the increasing demand for food (Tilman et al., 2011). Traditional breeding has not considered the response to rising ambient CO₂ as a selection criterion (Ainsworth et al., 2008), even though genetic improvement may provide a direct strategy for increasing yields through photosynthesis enhancement (Ziska et al., 2012). There have been several studies on genetic variability in photosynthesis and on yield responses to CO₂, but no correlation has been found between photosynthesis and yield variations (Bishop et al., 2015; Driever et al., 2014). In turn, mutagenesis has been used to generate and increase variability in crop species, and eventually modify plant traits (Batista et al., 2008). There are numerous mutants with altered chlorophyll content and changes in photosynthetic rate and Rubisco content (Li et al., 2013). Some stay-green mutants record a slower degradation of

chlorophyll, increased antioxidant capacity, and enhanced photosynthetic competence (Luo et al., 2013; Tian et al., 2013). Moreover, a rice mutation affecting a cytochrome P450 involved in brassinosteroid biosynthesis has erect leaves that increase light capture for photosynthesis and grain yields (Sakamoto et al., 2006). We have reported the characterization of the G132 barely mutant (Córdoba et al., 2016), selected in elevated CO₂ conditions, which shows chlorosis in expanding leaves, low PSII quantum efficiency, and a strong and selective decrease in Rubisco protein and activity, accompanied by increased Rubisco degradation. G132 records increased transcript levels for Rubisco and the PSII light harvesting complex, but lower levels of transcripts for protochlorophyllide oxidoreductase A and psbQ. Despite lower photosynthesis rates and higher intercellular CO₂ concentrations, G132 has greater stomatal conductance than the wild-type (Córdoba et al., 2015). G132 shows Rubisco-limited photosynthesis at very high CO₂ concentrations, and lower non-structural carbohydrate contents than WT. Rubisco-limited photosynthesis has a greater response to CO₂ enrichment than RuBP-limited photosynthesis (Leakey et al., 2009). Moreover, low non-structural carbohydrate levels could minimize photosynthetic acclimation.

The aim of this study was to test the hypothesis that the photosynthesis and growth of the G132 mutant have enhanced responses to elevated CO₂ compared to WT. We selected young plants at the stage of 3-4 leaves unfolded because the larger biomass of older plants in elevated CO₂ often reflects a faster rate of growth at an earlier period of development (Geiger et al., 1998). We have measured the photosynthetic quantum efficiency and gas exchange, and analysed the contents of several photosynthetic proteins, including Rubisco, Rubisco activity and chlorophyll content in both genotypes grown in ambient and elevated CO₂. In addition, we determined the content of soluble carbohydrates and starch, which can trigger the acclimation of photosynthesis to high CO₂. To explore the possible nitrogen limitation of photosynthetic responses to elevated CO₂, we assayed nitrate reductase activity and measured the content of proteins, amino acids and nitrate. The green area and dry matter of plants were recorded to assess the differences in growth responses to CO₂ enrichment. We used genome-wide microarrays and quantitative analyses of selected transcripts to identify genes with a significantly altered expression, as well as possible molecular mechanisms causing the changes

- 1 between genotypes in the response to elevated CO₂. The ultimate goal was to assist in the
- 2 identification of targets for adapting to growth under atmospheric CO₂ enrichment.

2. Materials and methods

2.1. Plant material, growing conditions and experimental factors

Barley (*Hordeum vulgare* L.) seeds of the homozygous genotype Graphic as wild-type (WT) and homozygous sodium azide-mutagenized seeds of the G132 line, grown and selected in 2000 $\mu\text{mol mol}^{-1}$ CO_2 , were obtained from Dr J. L. Molina-Cano's mutant collection at the IRTA research institute. The G132 seed stock was backcrossed once to Graphic (BC1F2), and this material was subjected to the reported experiments. The study of the exome capture of G132 compared with WT is currently a work in progress. The seeds were surface sterilized with hypochlorite, and sown in 5 L pots with 1.2 kg of peat: perlite (3:1) substrate, with a density of four plants per pot after emergence (completed in both genotypes 8 days after sowing). Four g of KNO_3 and 4 g of KH_2PO_4 were added to each pot, with the peat providing a sufficient provision of other nutrients. Water was supplied during growth to maintain pot field capacity. The pots were placed at sowing in controlled environment chambers with 20 °C: 15 °C day: night temperatures, 60±10% relative humidity, and 450 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance provided by fluorescent lamps (Phillips TL5 HO 54W/840 and Phillips TL5 HO 54W/965, with emission temperatures of 4000 K and 6800 K, respectively) in a 16 h : 8 h, light : dark photoperiod. These long day conditions are favourable for growth of spring barley and compensate for the moderate irradiance in the growth chambers.

The treatments were all combinations of two genotypes (G132 mutant and WT) and two growth CO_2 concentrations (ambient, ~390 $\mu\text{mol mol}^{-1}$, and elevated, 1200 $\mu\text{mol mol}^{-1}$). This elevated CO_2 concentration was in keeping with that used in the mutant selection process. Other studies on the effects of elevated CO_2 have selected high CO_2 concentrations within the predicted range for the year 2100 (Aranjuelo et al., 2014). To achieve the elevated CO_2 concentration, a PID controller connected to an infrared gas analyser and acting on a solenoid valve injected pure CO_2 close to the chamber fans homogenizing the air. The experiment was repeated in two successive years with generally similar results. We report the results of the second experiment and present those of the first one in Supplementary Table S1. For microarrays, samples of primary leaves were harvested when the second leaves were fully expanded. All the other measurements were carried out on the youngest fully expanded leaf at

the stage of 3-4 leaves unfolded (13-14 growth stage, Zadoks et al., 1974 scale), with WT being more developed (25-27 days after sowing).

2.2. Chlorophyll fluorescence and gas exchange

In the first hours of the light period in five replicate leaves, the chlorophyll (Chl) fluorescence was measured with a modulated fluorometer (PAM-2000, Walz, Effeltrich, Germany) to record the maximum quantum efficiency of Photosystem II (PSII) (F_v/F_m) after 20 min darkness, as well as the PSII operating quantum efficiency (F_q'/F_m'), the fraction of PSII centres in the open state (qL) and the quantum yield of non-photochemical quenching (Φ_{NPQ}) in light adapted leaves ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$) at both ambient and elevated CO_2 , following the procedure described by Pérez et al. (2011). The gas exchange- CO_2 response curves were recorded in the same leaves with an infrared gas analyser (CIRAS-2, PP Systems, Amesbury, MA, USA) at 20°C , $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance and $1.12 \pm 0.02 \text{ kPa}$ vapour pressure deficit by decreasing CO_2 concentration in five steps from 390 to $60 \mu\text{mol mol}^{-1}$, followed by an increase from 390 to $1800 \mu\text{mol mol}^{-1}$ in six steps. These responses were used according to Farquhar et al. (1980) to determine the maximum rate of Rubisco-catalysed carboxylation (V_{max}), the potential rate of photosynthetic electron transport (J), the mitochondrial respiration rate in the light (R_d) and the limitations of photosynthesis by Rubisco, RuBP regeneration or triose phosphate utilization, fitting the model with the LeafWeb utility (Gu et al., 2010). The acclimatory or long-term response and the direct or short-term response of CO_2 assimilation to elevated CO_2 were quantified as ratios of values recorded at elevated and ambient CO_2 according to (Bunce, 2001), and the net effect as the product of the acclimatory and direct effect (net effect = acclimatory effect $\times$ direct effect), as detailed by del Pozo et al. (2007).

2.3. Chlorophyll, protein and metabolite content, and enzyme activities

At mid-morning on the growth stage indicated above, five replicate samples were harvested per genotype- CO_2 combination, with each one consisting of five leaves from different plants; the leaves were individually cut, immediately plunged into liquid nitrogen under illumination, and then stored at -80°C until analysed. Chlorophyll (Chl), protein (including Rubisco), amino acid, nitrate, glucose, fructose, sucrose, fructan and starch contents, and Rubisco and nitrate

reductase activities were analysed exactly as described (Córdoba et al., 2016). For the quantitation of the Photosystem II (PSII) light harvesting complex b1 protein (Lhcb1), the PSII reaction centre D1 protein and the cytochrome b6 (cyt b6) protein, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and Western blotting were carried out as described (Pérez et al., 2011), except that 300 µg of total protein extracts were mixed with PEB (4x) Protein Extraction buffer (AS08 300, Agrisera, Vännäs, Sweden), 50 mM DTT, 0.01% bromophenol blue and cOmplete, Mini, EDTA-free protease inhibitor (Roche Diagnostics, Spain). Aliquots containing 2.25 µg (for Lhcb1 and cyt b6) or 9 µg (for D1) protein were loaded onto a 12.5% SDS–polyacrylamide gel (Mini-Protean 3 Cell, Bio-Rad). Following electrophoresis and blotting for 90 min to PVDF membranes (Bio-Rad, Madrid, Spain), the blocked blots were probed with the corresponding antibodies (Agrisera) at the recommended dilution: AS01 004 for Lhcb1, AS03 034 for cyt b6 and AS05 084 for D1. The blots were subsequently incubated with a peroxidase conjugated secondary antibody (AS09 602 or AS09 603) and developed with ECL Select Western blotting detection reagent (GE Healthcare, Spain). A ChemiDoc MP System (Bio-Rad) was used for luminescence detection, using D1 protein (AS01 016S) as standard. For Lhcb1 and cyt b6, the samples were quantified relative to the WT grown in ambient CO₂.

2.4. Leaf anatomy and plant growth

The stomatal and epidermal cell numbers and stomatal index on the central segment of the adaxial side of five replicate leaves were recorded according to Córdoba et al. (2015). The area, fresh and dry weights, volume, water content and derived thickness and density of the leaves were determined in five replicate samples, each consisting of two leaves, as described by Gutiérrez et al. (2009b). For whole plant growth measurements, the youngest fully expanded leaf on the main shoot, the remaining main shoot, and the rest of the plant were separated to determine the green area with an electronic planimeter (Li-3000 A, Li-Cor, Lincoln, NE, USA), the fresh weight and, after drying in an oven for 48 h at 60 °C, the dry weight. There were five replicates, each consisting of six plants. The weight of carbohydrates, proteins, nitrate and amino acids per unit leaf weight was estimated (Gutiérrez et al., 2009b) in order to assess the changes across treatments in the weight of leaf structural compounds.

2.5. Microarray and qRT-PCR analyses

Samples of the first leaf were harvested when the second was fully expanded, and immediately frozen in liquid nitrogen under illumination, and then stored at -80 °C for transcript analysis with a microarray. There were six replicates, each consisting of three to five leaves. Customised microarrays have been designed by Dr. Nese Sreenivasulu (formerly at IPK) using 50,938 expression sequence tags from the HarvEST barley database (Assembly 35, <http://harvest-web.org/hweb/pickassy.wc>). With the eArray design application (Agilent), probes of 60 bases were synthesized for unique sequences, and custom 56 K (8 x 60 K format) microarrays were constructed by Agilent. RNA extraction and microarray analyses were performed at the Molecular Genetics Department, IPK (Gatersleben, Germany). The samples were ground in a cryogenic homogenizer and RNA was isolated by the TRIzol reagent method (Invitrogen Life Technologies), treated with DNase (RNase-Free DNase Set, Qiagen), and cleaned with RNeasy mini spin columns (Qiagen). RNA concentrations were measured with a NanoDrop spectrophotometer (ND-1000 UV-VIS Spectrophotometer, Thermo Fisher Scientific) and RNA quality was assessed with an Agilent Bioanalyzer 2100 (Agilent Technologies). cDNA was synthesized with Affinity Script (Agilent) using the T7 promoter as primer. The cDNA was transcribed, and the cRNA labelled with T7 RNA Polymerase Blend and Cyanine-3-CTP (Agilent), following the manufacturer's instructions. The labelled cRNA was purified using RNeasy mini spin columns (Qiagen) and quantified using a NanoDrop spectrophotometer, thereby calculating the reaction's specific activity and yield. For hybridization, 2x HI-RPM Hybridization Buffer (Agilent) was added to 600 ng of labelled cRNA (6 pmol cy3/μg cRNA specific activity), and after centrifugation the sample (40 μl) was immediately dispensed on the 8 x 60 K chip. Hybridization was carried out at 65 °C for 17 h. The chips were scanned at high resolution (3 μm) using an Agilent DNA Microarray Scanner G2565CA (Agilent). The resulting images were processed according to the One-Color Microarray-Based Gene Expression Analysis protocol (Agilent). Data were extracted using Feature Extraction Software (version 11.0, Agilent) and subsequently analysed using RobiNA software (Lohse et al., 2012) for normalization and background subtraction. Transcripts recording significant changes were annotated and assigned to functional categories (BINs) using the HarvEST barley database,

1 Mercator and MapMan (Lohse et al., 2014). The data set for this article is available from
2 ArrayExpress (<http://www.ebi.ac.uk/arrayexpress>, accession number E-MTAB-4942).

3 Transcript analysis by qRT-PCR was carried out on five replicates (each with five leaves
4 pooled from different plants) per treatment combination of the youngest fully expanded leaves at
5 the stage of 3-4 leaves unfolded. Based on the results from the microarrays, we determined the
6 abundance of 26 transcripts, as detailed by Córdoba et al. (2016). Relative quantification
7 involved the comparative C_t (threshold cycle) method ($2^{-\Delta\Delta C_t}$) (Schmittgen and Livak, 2008).

8 9 2.6. Statistical analysis

10 Genotype and CO₂ effects and interactions on physiological and metabolic parameters and
11 qRT-PCR results were determined through a two-way analysis of variance with no blocking
12 (GenStat 6.2). The standard errors of differences and least significant differences were obtained
13 to assess differences between means. To test for consistency of the microarray data and
14 assess the general effect of the genotype and the CO₂ concentration, we analysed the
15 normalized transcript abundances by hierarchical cluster analysis and principal component
16 analysis and examined whether replicate samples grouped together. Those transcripts in the
17 microarray that changed significantly between genotypes or CO₂ concentrations were identified
18 with an analysis of variance, with probability < 0.05 and fold change ≥ 2 , using the Benjamini
19 and Hochberg (1995) false discovery rate correction procedure. The genes differentially
20 expressed between treatments were further analysed using K-means clustering. The number of
21 clusters was chosen from visual inspection of a hierarchical clustering dendrogram of the
22 transcripts. Overrepresentation of the functional categories in each cluster was analysed with
23 hypergeometric tests (Excel Hypergeom.Dist function).

3. Results

3.1. Chlorophyll fluorescence and gas exchange

In G132, elevated CO₂ increased the maximum quantum efficiency of PSII photochemistry (Fv/Fm) (27.5%), the fraction of PSII centres which are in the open state (qL) (34.6%) and the operating quantum efficiency (Fq'/Fm') of PSII (84.6%), while it decreased the quantum yield of non-photochemical quenching (Φ_{NPQ}) (Fig. 1A). In contrast, elevated CO₂ had no effect on the chlorophyll fluorescence quenching parameters in WT. CO₂ enrichment increased V_{cmax} in G132 (27.9%), but had no effect in WT (Fig. 1B). It was not possible to determine J from the photosynthesis-CO₂ response curves for G132 because V_{cmax} limited photosynthesis at all CO₂ concentrations, as observed previously (Córdoba et al. 2015). In WT, J tended to decrease, while the rate of mitochondrial respiration in the light did not change in G132 and WT with the increase in CO₂ (Table 1). The photosynthetic rate was higher with elevated CO₂ than with ambient CO₂ in measurements taken for respective growth CO₂ (Fig. 1B). Measured at a common CO₂ concentration, the photosynthesis rate in WT decreased slightly and not significantly with growth in elevated CO₂. This response showed that the down-regulation of photosynthetic capacity caused by high CO₂ was small. Conversely, the rate of photosynthesis in G132 at a common CO₂ concentration showed an increase with growth in elevated CO₂ which was significant at 1200 $\mu\text{mol mol}^{-1}$ CO₂ (24.6%). This photosynthesis enhancement in G132 suggested an increase in photosynthetic capacity caused by growth in elevated CO₂, although G132 was still underperforming compared to WT. Fig. 1C shows the direct, acclimatory and net photosynthesis responses to CO₂ derived from these measurements. The acclimation or down-regulation of photosynthesis in elevated CO₂ was low in this experiment, but more marked in WT than G132, in which there was a positive acclimation. The direct and net responses to CO₂ were more positive in G132 than WT. Growth in elevated CO₂ decreased g_s in measurements at 390 $\mu\text{mol mol}^{-1}$ CO₂ (Table 1), but not at 1200 $\mu\text{mol mol}^{-1}$ CO₂.

3.2. Enzyme activities

The *in vitro* initial Rubisco activity did not change significantly with elevated CO₂ in G132, at variance with the increase in V_{cmax}, and decreased in WT (-14.3%) (Fig. 1D); the latter result contrasted with the lack of CO₂ effect on V_{cmax} in WT. Although there was no close agreement

between the enzymatic and gas exchange measurements of the Rubisco maximum carboxylation rate, the response to elevated CO₂ of G132 followed a positive trend, at variance with WT. No significant change was observed in Rubisco activation state. As a key enzyme in nitrogen assimilation, and ultimately in protein synthesis, nitrate reductase activity was also measured. CO₂ enrichment increased the maximal (not shown) and selective activities and activation state of nitrate reductase in G132 (75.0% and 14.1%, respectively), but decreased them in WT (-25.1% and -5.6%, respectively) (Fig. 1D).

3.3. Contents of protein, nitrogen compounds and chlorophyll

The effects of CO₂ enrichment on V_{cmax} and *in vitro* Rubisco activity were compared to those induced on Rubisco protein content (Fig. 1E). Elevated CO₂ decreased the Rubisco protein content in WT (-18.6%); this effect is consistent with the fall in Rubisco activity, although not with the absence of significant effects on V_{cmax} . At variance with WT, G132 showed an increase in Rubisco content (58.9%) with elevated CO₂; this effect did not reach significance in the pooled analysis of variance due to the big difference in Rubisco content between genotypes. Thus, the mutant showed a certain parallelism in CO₂ effects on the three Rubisco-related parameters. The content of soluble protein excluding Rubisco (Fig. 1E) responded positively to elevated CO₂ in both genotypes. Membrane and total proteins (Fig. 1E) did not change significantly with growth in elevated CO₂, although both protein fractions tended to increase in G132. As a proportion of the Rubisco large subunit, a 33 kDa degradation product increased with elevated CO₂ in G132 (Table 1).

Genotypic differences in contents of D1, Lhcb1 and cyt b6 proteins strongly depended on CO₂ concentration (Fig. 1F). Thus, in ambient CO₂ G132 had a much lower content of D1 and cyt b6 than WT, while it had a similar Lhcb1 content. In contrast, in elevated CO₂ the mutant had not significantly lower D1 and much higher Lhcb1 and cyt b6 contents than WT. This variable genotypic difference was because the three proteins increased in G132 (81.9%, 84.6% and 143.5%, respectively, for D1, Lhcb1 and cyt b6) and decreased - not significantly for D1 - in WT (-33.0% and -61.0%, respectively, for Lhcb1 and cyt b6) with CO₂ enrichment.

In addition to the proteins described, we analysed the contents of nitrate and total amino acids, which did not change significantly with elevated CO₂ (Fig. 1G). Growth in high CO₂

concentration increased total Chl and Chl a (45.7% and 49.3%, respectively) in G132, but not in WT, and had no significant effect on Chl b (Fig. 1H). In addition, it increased the Chl a/Chl b ratio in WT, but the increase was not significant in G132.

3.4. Carbohydrate content

The free glucose content in leaves was not detectable even with the fluorometric method used. In general, elevated CO₂ increased the contents of the carbohydrates analysed (Fig. 1I), although the increase in free fructose and fructans in G132 did not reach significance in the pooled analysis of variance due to the big difference between genotypes. In G132, at variance with WT, the value below 2 for the ratio of fructose to glucose from the *in vitro* hydrolysis of fructans (data not shown) indicates the absence of fructans; other carbohydrates could have been analysed along with fructans. Despite these increases with elevated CO₂, the carbohydrate content was much lower in G132 than WT.

3.5. Leaf morphology and plant growth

The epidermal cell and stomatal numbers, which were higher in G132 than WT, decreased with elevated CO₂ in the former, but not in the latter (Fig. 2A). The stomatal index (stomata/epidermal cell ratio), however, did not change with genotype or CO₂. The leaf dry weight per unit area (Fig. 2B), thickness (volume/area) and density (dry weight/volume) (Fig. 2C) of the youngest fully expanded leaves were lower in G132 than WT. When the weight of the compounds analysed here was subtracted, the dry weight per unit leaf area was still lower in the mutant (Fig. 2B), pointing to a possible decrease in structural material. Elevated CO₂ increased the dry weight per unit leaf area (Fig. 2B), but when the analysed compounds were discounted, the effect disappeared almost completely, which seems to indicate that the majority of that increase was due to the accumulation of these compounds. Leaf density increased with elevated CO₂, but the small increase in leaf thickness was not significant (Fig. 2C).

The green area and fresh and dry weights of the youngest fully expanded leaf, the main shoot, the tillers and the whole plant were lower in G132 than WT (Table 2). In contrast, the mutant had a higher water per cent of fresh weight. Growth in elevated CO₂ increased the green area of the main shoot, the tillers and the whole plant (35.5%) in G132. At variance with this, in

WT elevated CO₂ decreased the green area of the youngest fully expanded leaf, the main shoot and the whole plant (-6.7%), although not the tiller green area. CO₂ enrichment increased the fresh and dry weights more in G132 (66.1% and 64.9%, respectively) than WT (5.9% and 13.9%, respectively), while slightly decreasing the water content in WT and not affecting it in G132.

3.6. Transcriptomic responses to elevated CO₂

Both the cluster dendrogram and the principal component analysis of the microarray normalized transcript abundances (Fig. 3) showed that the six biological replicates for each genotype x CO₂ combination grouped together, confirming the consistency of the data. The cluster dendrogram showed two major branches, each containing one of the genotypes, which in turn comprised separate branches for plants grown in ambient and elevated CO₂. In the principal component analysis, again, the strongest separation was observed for the genotypes, followed by the CO₂ concentrations, which were more separated in WT than in G132. Of the 56,319 genes with a detected expression in the microarray, 30,706 were annotated using several databases. The transcriptomic changes in G132 relative to WT are described in Supplementary Table-text S2 and Tables S3 and S4.

Compared to ambient CO₂, elevated CO₂ induced more changes (log₂ fold-change>1, P<0.05, with Benjamini and Hochberg adjustment) in gene expression in WT (770 genes) than G132 (165 genes), and there were 36 genes whose expression changed with elevated CO₂ in both genotypes, as shown in a Venn diagram (Fig. 4A). Whilst with elevated CO₂ the number of induced genes (595) was greater than the number of repressed genes in WT (175), the opposite occurred in G132, where the difference between numbers of genes positively and negatively regulated (75 and 90, respectively) was smaller than in WT. The functional categories of genes significantly repressed and induced by elevated CO₂ were K-means grouped into five clusters after visual inspection of a hierarchical clustering dendrogram (not shown). Details of the assignment to clusters of 900 genes with significant changes in expression in response to elevated CO₂ are shown in Table S5; the functional categories significantly overrepresented in each cluster are summarized in Table 3; and the full list is given in Table S6. Genes (241 in total) in clusters 2 and 4, respectively, were repressed by elevated

CO₂ in G132 (79 genes) and WT (162 genes). Clusters 3, 0 and 1 grouped overrepresented categories of genes (659 in total) induced by elevated CO₂, either only in G132 (cluster 3, 72 genes), or only in WT (clusters 0 and 1), to a greater extent (cluster 0, 111 genes) or to a lesser one (cluster 1, 476 genes).

Cluster 2 (genes repressed by elevated CO₂ in G132) had an overrepresentation of the functional categories of major carbohydrate degradation (fructan exohydrolase, alpha amylase); abscisic acid and auxin metabolism; and biotic and abiotic stress. In cluster 4 (genes repressed by elevated CO₂ in WT) there was an overrepresentation of the functional categories of photosynthetic light reactions (LHCII CP29, LHCI Lhca2, psbR, NDH dehydrogenase, and chlororespiration), which conversely was not affected by elevated CO₂ in G132 (cluster 2); metal handling; wax metabolism, included in the secondary metabolism category; abiotic stress (as in G132 in cluster 2); oxidases; regulation of transcription (transcription factors such as AP2/EREBP, C2C2(Zn) GATA, C3H zinc finger, MADS box, MYB, etc.); protein synthesis (chloroplast 30S ribosomal subunit proteins, synthesis initiation); and amino acid transport.

Cluster 3 (genes induced by elevated CO₂ in G132) included the functional categories of major carbohydrate synthesis (sucrose: fructan 6-fructosyltransferase, sucrose: sucrose 1-fructosyltransferase); N metabolism (nitrate reductase); amino acid synthesis (glutamate decarboxylase, cysteine synthase, tryptophan synthase); metal handling; jasmonate metabolism (allene oxide synthase, lipoxygenase); nucleotide degradation; and development (senescence-associated protein). Cluster 0 (categories of genes relatively strongly induced by elevated CO₂ in WT) was enriched in the functional categories of cellulose synthesis (cellulose synthase, COBRA-LIKE 7 protein); fatty acid desaturation; abiotic stress (germin-like protein; heat shock proteins); miscellaneous peroxidases; and signalling in sugar and nutrient physiology. In turn, cluster 1 (relatively weaker gene induction caused by elevated CO₂ in WT) included the overrepresentation of the functional categories of cell wall modification (xyloglucan endotransglycosylase); lipid degradation (triacylglycerol lipase, carboxylesterase, glycerophosphodiester phosphodiesterase, and phospholipases); phenylpropanoids (phenylalanine ammonia-lyase, shikimate O-hydroxycinnamoyltransferase, etc.), included in the secondary metabolism category; DNA synthesis/chromatin structure; and signalling, including receptor kinases.

3.7. *qRT-PCR profiling*

The relative expression levels of a series of genes were determined by qRT-PCR (Table 4) in leaf 3-4, after a longer exposure of plants to elevated CO₂. In elevated compared to ambient CO₂, a decrease was found in transcripts for the PSII light-harvesting complex and psbP and the Rubisco small subunit, while the decrease for the large subunit was not statistically significant. Similarly, elevated CO₂ decreased gene expression for xyloglucan endotransglycosylase (only in G132), glycerophosphodiester phosphodiesterase and a cell wall constituent protease inhibitor-lipid transfer protein. CO₂ enrichment also led to a decrease only in G132 of transcripts for the RNA polymerase alpha subunit and for the chloroplast S3 and L6 ribosomal proteins (30S and 50S subunits, respectively). The abundance of transcript for a leucine-rich repeat family protein involved in protein degradation decreased in G132, and tended to increase in WT with growth in elevated CO₂. The level of transcript for an HT1-like threonine-protein kinase involved in the CO₂ signalling pathway in stomatal guard cells decreased in the mutant, but not in WT under elevated CO₂. In contrast with the effects described, elevated CO₂ induced an increase in transcripts of fructan synthesis-related genes (1-SST), and a significantly higher increase in G132 than WT of transcripts for nitrate reductase.

4. Discussion

4.1. *Transcriptional and functional features of the G132 mutant*

The decreases in G132 as compared to WT in contents of the PSII reaction centre D1 protein and cyt b6 may lead to the inhibition of maximum quantum efficiency in the mutant grown in ambient CO₂ concentration. In addition to the negative regulation of carbohydrate and nitrogen metabolism in G132 (Córdoba et al. 2016), this study shows (Table-text S2) a repression of genes involved in TCA metabolism, OPP and glycolysis, whereby the young mutant plants had a general decrease in transcripts for primary and energy metabolism. Compared to WT, G132 had an activated gene expression for some prokaryotic chloroplast ribosomal proteins, which could favour protein synthesis in these organelles; however, under elevated CO₂ some genes in this category were repressed in G132 relative to WT. The repression in G132 of abiotic stress-related genes, peroxidases and jasmonate biosynthesis genes, which are rapidly activated by singlet oxygen (Khandal et al., 2009), suggests a decrease in oxidative stress. The anatomical characteristics of G132 leaves revealed decreased adaxial epidermal cell expansion (more cells per unit area), and a lower thickness and density attributable to low contents of both structural and soluble compounds. The gene repression for xyloglucan endotransglycosylase in G132 could be involved in cell wall reductions (Sampedro et al., 2010). The inhibitions of primary metabolism and cell wall modification were associated with decreased growth, with subsequent slow recovery (Córdoba et al., 2016). The mutation in G132 has therefore pleiotropic effects, as often found in other mutants (Lunn et al., 2014), particularly for mutations in developmental genes or when a mutated gene is part of a linked biosynthesis pathway (Shu et al., 2012).

4.2. *Improved CO₂ photosynthesis response in the G132 mutant*

The enhancement of Fv/Fm in G132 by elevated CO₂ contrasted with the lack of effects on WT. Other studies have found variable effects of elevated CO₂ on photochemical activity (Hymus et al. 1999, Gutiérrez et al. 2009a). Fv/Fm was low in the mutant (Córdoba et al., 2016) even in elevated CO₂, which indicated an impairment of photochemistry. The improvement in Fv/Fm with CO₂ concentration was associated with increases in the content of Chl and D1 (56%) and Lhcb1 proteins (85%), in spite of the decreased transcripts for LHCI recorded with

qRT-PCR. Conversely, the decreases with rising CO₂ in D1 (-14%) and Lhcb1 proteins (-33%), and the transcripts for LHCl and II proteins, psbQ, psbR, NDH dehydrogenase and chlororespiration (microarray), as well as the increase in Chl a/Chl b ratio, did not decrease the close-to-normal Fv/Fm in WT. The increases in cyt b6 (Yamori et al., 2016) and Rubisco in G132 and the decreases in WT may contribute to the enhancement of Fq'/Fm' in G132 and the insensitivity in WT in response to elevated CO₂. Moreover, when photosynthesis is Rubisco-limited, as in G132, the increase of carboxylation with rising CO₂ is greater than the inhibition of oxygenation, and thus Fq'/Fm' is enhanced. However, when photosynthesis is RuBP regeneration-limited, as in WT, the increase in the carboxylation rate equals the inhibition of oxygenation, with no change in Fq'/Fm' (Hymus et al., 1999). Our results clearly demonstrate the stimulation with elevated CO₂ of the photochemical activity of photosynthesis in G132 and not in WT.

The Farquhar photosynthesis model (Farquhar et al., 1980) allows predicting a more positive response to a short-term increase in CO₂ concentration (the direct response) when V_{cmax} is low, as in G132. As experimentally confirmed (Leakey et al., 2009), the model also predicts greater responses to higher CO₂ levels when photosynthesis is Rubisco-limited in elevated CO₂, as in G132, than when it is RuBP regeneration-limited, as in WT. The greater direct effect of CO₂ on photosynthesis in G132 than in WT fitted these predictions. As distinct from the predictable direct effects, the acclimatory effects of high CO₂ on photosynthesis in our experiments were only moderate compared to prior observations (Bunce, 2001; del Pozo et al., 2007), possibly because our plants were at an early developmental stage. Even so, the acclimatory loss of photosynthetic capacity was lower in G132 than WT, in association with the increase in the former, and the overall decreasing trend in the latter of V_{cmax} and Rubisco *in vitro* activity and protein (del Pozo et al., 2007; Nie et al., 1995; Pérez et al., 2005; Sicher and Bunce, 1997). Moreover, the changes in Rubisco protein in G132 more than compensated for the enhancement by elevated CO₂ of Rubisco degradation recorded by the 33 kDa protein fragment (Córdoba et al., 2016). The greater direct effect of elevated CO₂ on photosynthesis, as well as the attenuated acclimatory effect in G132 compared to WT, led to markedly higher net photosynthesis responses in the mutant (190%) than WT (84%).

There were no changes with elevated CO₂ in the gene expression for Rubisco in the primary leaves analysed with the microarray. However, in leaves from plants exposed to elevated CO₂ for a longer period, the qRT-PCR analysis recorded a repression of Rubisco genes that was significant for the Rubisco small subunit, as reported in many previous studies (Fukayama et al., 2011; Nie et al., 1995; Van Oosten et al., 1994), although not in all (Ainsworth et al., 2006; Pérez et al., 2005). This repression was low in our experiments, as was photosynthetic acclimation. The loss in Rubisco protein with elevated CO₂ may have been due to the decline in Rubisco transcript levels, and the offsetting of this loss in G132 could be attributed to the greater abundance of Rubisco transcripts compared to WT (Table 4 and Córdoba et al., 2016). In G132, the decline with elevated CO₂ in levels of transcripts for chloroplast ribosomal proteins and RNA polymerase alpha-subunit (Table 4) did not seem to restrict protein synthesis in chloroplasts. In WT, in turn, the 30S chloroplast S3 ribosomal protein gene expression decreased with elevated CO₂ according to the microarray results, although not according to the qRT-PCR analysis of older plants.

4.3. CO₂ responses of nitrogen and carbon metabolism in G132 and WT

The loss with elevated CO₂ of Rubisco protein in WT, but not in G132, was selective, with both genotypes showing increases in soluble protein minus Rubisco protein, and little change (WT) or a rising trend (G132) in total protein. These responses show that elevated CO₂ caused no major decrease in leaf organic nitrogen content, in spite of the decrease in WT, in contrast with the increase in G132 of nitrate reductase activity. Such decrease was not observed in the replicate experiment (Table S1), and was not due to a lower level of transcript for nitrate reductase, which rose with elevated CO₂ in both genotypes. Our results contrast with the increase in nitrate and decrease in organic nitrogen content (Rubio Asensio et al., 2015), and with the decrease observed under CO₂ enrichment in leaf and whole shoot nitrogen content (Aranjuelo et al., 2011; del Pozo et al., 2007; Taub and Wang, 2008). It can be inferred that the acclimation to elevated CO₂ in our experiments was not due to a fall in the availability and assimilation of nitrogen, at variance with other studies (McDonald et al., 2002; Pérez et al., 2005), presumably because our data involved young plants.

The rise in carbon fixation with elevated CO₂ led to increases in non-structural carbohydrate contents, as found in prior studies (Nie et al., 1995; Pérez et al., 2005; Stitt, 1991), and in fructan polymerization (higher fructan fructose/glucose ratio) in WT. Carbohydrate accumulation was associated with increases in transcripts for enzymes of non-structural carbohydrate synthesis in both genotypes, and with decreases in those involved in their degradation in G132 (microarray). Our results are consistent with a repression of the Rubisco gene expression induced by carbohydrate accumulation under elevated CO₂ (Van Oosten et al., 1994). The improved response to CO₂ of Rubisco content in G132 was associated with lower carbohydrate contents and a higher level of Rubisco transcripts than in WT.

4.4. Additional differential responses to elevated CO₂ of G132 gene expression

The elevated CO₂-induced changes in gene expression presented here included the decrease in transcripts of genes involved in auxin metabolism in both genotypes and in abscisic acid metabolism in G132. However, only in the mutant were these transcripts overrepresented in the selected clusters. Another difference with WT was the activation by elevated CO₂ of gene expression for lipoxygenase and allene oxide synthase in G132, which can enhance jasmonic acid synthesis and pathogen response. This result contrasts with the observed repression of genes involved in the jasmonate synthesis pathway and decreased tolerance to pathogens under elevated CO₂ (Zavala et al., 2008). Indeed, CO₂ enrichment decreased the level of transcripts of genes involved in biotic stress in G132 and in abiotic stress in both genotypes, at variance with other studies (Ainsworth et al., 2006). The possible decrease with elevated CO₂ in oxidative and drought stress (Erice et al., 2007) could account for the declining levels of abiotic stress transcripts. Nevertheless, elevated CO₂ increased the transcript level for the germin-like proteins in WT (Ainsworth et al., 2006), involved in the response to several stress conditions (Bernier and Berna, 2001). The repression by high CO₂ of genes encoding several transcription factors in WT (MADS box, MYB domain, NAC domain, bZIP family, etc.) may again reflect lower abiotic stress (Century et al., 2008). In G132, in contrast, CO₂ enrichment decreased the transcript level only for the WRKY70 transcription factor (Table S5; not overrepresented in cluster 2), which is consistent with the increased level of transcripts for jasmonate metabolism (Li et al., 2004).

4.5. Improved growth responses to elevated CO₂ in the G132 mutant

The increase in leaf mass per area with elevated CO₂ presented here is consistent with other studies (Ishizaki et al., 2003; Roderick et al., 1999), although we recorded a decrease in earlier research (Gutiérrez et al., 2009b). The increase was almost entirely due to higher contents of the carbohydrates and nitrogen compounds analysed, in agreement with Poorter et al. (1997). It may therefore be inferred that the content of structural compounds such as cell wall components did not appreciably change with elevated CO₂. This occurred in spite of the increases in WT and the decreases in G132 of transcript levels for xyloglucan endotransglycosylase, involved in cell wall extension (Sampedro et al., 2010), and the increase in WT of transcripts for cellulose and lignin synthesis (phenylpropanoids). The higher leaf mass per area did not mean increased leaf thickness, which could decrease light interception per unit of leaf biomass (Evans and Poorter, 2001), but rather greater density (dry weight per unit volume), and a slight decrease in water content. The decline with elevated CO₂ in adaxial epidermal cell numbers per unit leaf area in G132 and in leaf area in WT partly confirm observations by Tsutsumi et al. (2014) and the conclusion that elevated CO₂ affects leaf anatomy and size (Pritchard et al., 1999). The higher relative increase with CO₂ enrichment of dry matter in G132 than in WT is attributable to increases in total green area in the former, but not in the latter, which can increase photosynthesis and dry matter per plant (Raines and Paul, 2006). Most of this advantage could be due to enhanced tillering in G132. Our results indicate that at whole plant level G132 was more efficient than WT in using additional atmospheric CO₂.

The G132 barley mutant provides no obvious advantage, given its low photosynthesis rate and biomass. However, we have shown that G132 has improved responses to elevated CO₂. The parameters correlated with this enhancement are low Rubisco content and enhanced Rubisco gene expression, low starch and soluble carbohydrate content, and increases with CO₂ enrichment in cytochrome b6, PSII components and the allocation of resources toward larger green areas. Genotypic variation in photosynthesis and growth is required for adapting to rising atmospheric CO₂. The traits associated with enhanced responses to elevated CO₂ in G132 may prove valuable for achieving this goal.

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Appendix A. Supplementary data

The following are Supplementary data to this article:

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Table 1. Potential rate of electron transport (J , $\mu\text{mol m}^{-2} \text{s}^{-1}$), dark respiration rate under light (R_d , $\mu\text{mol m}^{-2} \text{s}^{-1}$), intercellular CO_2 concentration (C_i , $\mu\text{mol mol}^{-1}$) and stomatal conductance (g_s , $\text{mmol m}^{-2} \text{s}^{-1}$) determined with air CO_2 concentrations of 390 and 1200 $\mu\text{mol mol}^{-1}$ (indicated with subscripts), 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance, 20 °C and 1.12 ± 0.02 kPa vapour pressure deficit, and content of a 33 kDa polypeptide as a percentage of Rubisco large subunit protein in the youngest fully expanded leaf of Graphis barley (WT) and its G132 mutant grown in ambient (390 $\mu\text{mol mol}^{-1}$) and elevated CO_2 (1200 $\mu\text{mol mol}^{-1}$).

	Ambient CO_2		Elevated CO_2		Isd		
	G132	WT	G132	WT	CO_2	Geno.	$\text{CO}_2 \times \text{Geno.}$
J	-	152	-	144	7.0	-	-
R_d	2.7	4.6	3.2	4.6	0.57	0.57	0.81
C_{i390}	362	233	326	220	14.2	14.2	20.0
g_{s390}	235	157	158	132	31.9	31.9	45.1
C_{i1200}	1355	1060	1300	1096	59.7	59.7	84.4
g_{s1200}	211	129	192	139	33.1	33.1	46.8
33 kDa (% RbcL)	38.4	20.4	43.9	23.3	3.59	3.59	5.08

Measurements at the stage of 3-4 leaves unfolded (13-14 growth stage, Zadoks scale), with WT being more developed. Isd, least significant difference. Numbers in bold type represent significant effects ($P < 0.05$).

Table 2. Leaf area, FW, DW and water content of the main shoot, the rest and the total plant of Graphic barley (WT) and its G132 mutant grown in ambient ($390 \mu\text{mol mol}^{-1}$) and elevated CO_2 ($1200 \mu\text{mol mol}^{-1}$).

		Ambient CO_2		Elevated CO_2		Lsd		
		G132	WT	G132	WT	CO_2	Geno.	$\text{CO}_2 \times \text{Geno.}$
Green area $\text{cm}^2 \text{ plant}^{-1}$	Youngest leaf	12.9	20.4	12.6	17.7	0.81	0.81	1.15
	Main shoot	33.7	62.2	40.9	55.2	2.46	2.46	3.47
	Rest plant	7.5	49.3	19.8	50.1	6.66	6.66	9.42
	Total	54.1	131.9	73.3	123.1	8.99	8.99	12.72
FW g plant^{-1}	Youngest leaf	0.33	0.64	0.37	0.62	0.023	0.023	0.032
	Main shoot	0.67	1.63	1.00	1.68	0.082	0.082	0.116
	Rest plant	0.24	1.82	0.69	2.04	0.235	0.235	0.333
	Total	1.24	4.09	2.06	4.33	0.296	0.296	0.419
DW mg plant^{-1}	Youngest leaf	29.5	86.1	35.5	90.9	3.25	3.25	4.60
	Main shoot	59.1	180.1	86.9	204.6	7.79	7.79	11.01
	Rest plant	19.3	183.0	55.6	216.1	23.25	23.25	32.89
	Total	107.9	449.3	177.9	511.6	29.31	29.31	41.45
Water % FW	Youngest leaf	91.0	86.6	90.5	85.3	0.31	0.31	0.43
	Main shoot	91.2	88.9	91.3	87.8	0.32	0.32	0.45
	Rest plant	91.8	89.9	91.9	89.4	0.67	0.67	0.94
	Total	91.3	89.0	91.4	88.2	0.37	0.37	0.53

Measurements at the stage of 3-4 leaves unfolded (13-14 growth stage, Zadoks scale), with WT being more developed. Lsd, least significant difference. Numbers in bold type represent significant effects ($P < 0.05$).

Table 3. Summary of MapMan functional groups (BINs) overrepresented in clusters of genes differentially expressed in elevated growth CO₂ compared with ambient growth CO₂ concentration in G132 and WT. Genes with significantly different expressions (> 2 fold change, $P < 0.05$) were selected for K-means clustering. Down, up, functional groups repressed and induced, respectively, with elevated CO₂ relative to ambient CO₂. Cluster 2, gene repression in G132; cluster 4, gene repression in WT; cluster 3, gene activation in G132; clusters 0 and 1, stronger and weaker activation, respectively, in WT.

BIN	NAME	Cluster				
		2	4	3	0	1
1.1	PS.lightreaction		down			
2.1	major CHO metabolism.synthesis			up		
2.2	major CHO metabolism.degradation	down				
10.2	cell wall.cellulose synthesis				up	
10.7	cell wall.modification					up
11.2	lipid metabolism.FA desaturation				up	
11.9	lipid metabolism.lipid degradation					up
12.1	N-metabolism.nitrate metabolism			up		
13.1	amino acid metabolism.synthesis			up		
15	metal handling		down			
15.2	metal handling.binding, chelation and storage			up		
16.2	secondary metabolism.phenylpropanoids					up
16.7	secondary metabolism.wax		down			
17.1	hormone metabolism.abscisic acid	down				
17.2	hormone metabolism.auxin	down				
17.7	hormone metabolism.jasmonate			up		
20.1	stress.biotic	down				
20.2	stress.abiotic	down	down		up	
23.2	nucleotide metabolism.degradation			up		
26.12	misc.peroxidases				up	
26.7	misc.oxidases - copper, flavone etc		down			
27.3	RNA.regulation of transcription		down			
28.1	DNA.synthesis/chromatin structure.histone					up
29.2	protein.synthesis		down			
30.1	signalling.in sugar and nutrient physiology				up	
30.2	signalling.receptor kinases					up
33.99	development.unspecified			up		
34.3	transport.amino acids		down			

Table 4. qRT-PCR analysis of gene expression in the youngest fully expanded leaf of Graphic barley (WT) and its G132 mutant grown in ambient (A, 390 $\mu\text{mol mol}^{-1}$) and elevated CO_2 (E, 1200 $\mu\text{mol mol}^{-1}$). The numbers indicate the change in expression ($\log_2$ ratios) between ambient and elevated CO_2 (E/A), between genotypes (G132/WT) and in the CO_2 x genotype (CO_2 x G) interaction, with WT in ambient CO_2 (AWT) as reference: G132 in ambient CO_2 (AG132/AWT), WT in ambient CO_2 (AWT/AWT), G132 in elevated CO_2 (EG132/AWT) and WT in elevated CO_2 (EWT/AWT).

BIN CODE	BIN NAME	DESCRIPTION	CO ₂	Genotype		CO ₂ x Genotype			<i>P</i>		
			E/A	G132/WT	AG132/AWT	AWT/AWT	EG132/AWT	EWT/AWT	CO ₂	G	CO ₂ x G
1.1.1.1	PS.lightreaction.photosystem II.LHC-II	LHCII CAB-1	-0.65	-1.09	-0.79	0.00	-2.01	-0.40	0.006	<.001	0.621
1.1.1.1	PS.lightreaction.photosystem II.LHC-II	LHCII CAB-2	-0.54	1.45	1.67	0.00	0.98	-0.16	0.029	<.001	0.061
1.1.1.2	PS.lightreaction.photosystem II.PSII polypeptide subunits	psbR	-0.19	1.58	2.00	0.00	1.60	0.43	0.578	<.001	0.25
1.1.1.2	PS.lightreaction.photosystem II.PSII polypeptide subunits	psbQ	0.25	-1.41	-0.99	0.00	-1.34	0.48	0.308	<.001	0.087
1.1.1.2	PS.lightreaction.photosystem II.PSII polypeptide subunits	psbP	-0.37	0.98	0.74	0.00	0.56	-0.75	<.001	<.001	0.13
1.1.2.2	PS.lightreaction.photosystem I.PSI polypeptide subunits	psaA	0.23	1.91	1.57	0.00	1.94	-0.32	0.477	<.001	0.267
1.1.2.2	PS.lightreaction.photosystem I.PSI polypeptide subunits	psaB	0.26	2.07	1.73	0.00	2.12	-0.29	0.546	0.001	0.393
1.3.1	PS.calvin cycle.rubisco large subunit	rbcL	-0.17	0.46	0.31	0.00	0.28	-0.36	0.624	0.199	0.704
1.3.2	PS.calvin cycle.rubisco small subunit	rbcS	-0.25	0.36	0.44	0.00	0.11	-0.15	0.052	0.01	0.321
2.2.3.150	major CHO metabolism	6-FEH	0.44	-0.42	-0.51	0.00	0.00	0.37	0.377	0.389	0.991
2.1.3.153	major CHO metabolism	6-SFT	-0.06	-0.13	-0.17	0.00	-0.18	-0.10	0.839	0.672	0.87
2.1.3.1009	major CHO metabolism	1-SST	2.26	-4.73	-3.54	0.00	-2.81	2.33	0.008	<.001	0.01
9.4	mitochondrial electron transport	alternative oxidase 1A	1.83	-2.79	-0.79	0.00	-1.65	2.39	0.063	0.018	0.039
10.7	cell wall.modification	xyloglucan endotransglycosylase	-0.68	-1.31	-0.65	0.00	-2.96	-0.16	0.005	<.001	0.044

BIN CODE	BIN NAME	DESCRIPTION	CO ₂	Genotype		CO ₂ x Genotype			<i>P</i>		
			E/A	G132/WT	AG132/AWT	AWT/AWT	EG132/AWT	EWT/AWT	CO ₂	G	CO ₂ x G
11.9.3.3	lipid degradation.lysophospholipases	glycerophosphodiester phosphodiesterase	-0.57	-1.52	-1.24	0.00	-2.38	-0.38	<.001	<.001	0.994
12.1.1	N-metabolism.nitrate metabolism.NR'	nitrate reductase [NADH]	1.77	-6.74	-7.83	0.00	-4.77	1.76	<.001	<.001	0.001
19.14	tetrapyrrole synthesis	protochlorophyllide reductase A	0.12	-0.63	-0.22	0.00	-0.60	0.44	0.523	0.004	0.035
26.21	misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	protease inhibitor/seed storage/lipid transfer protein	-2.79	-0.19	-0.34	0.00	-2.58	-3.44	<.001	0.536	0.186
27.2	RNA.transcription	plastid RNA polymerase alpha subunit	-0.57	2.06	2.46	0.00	1.70	0.15	0.047	<.001	0.031
28.1	DNA.synthesis/chromatin structure	DNA-dependent ATPase	0.23	-1.80	-1.91	0.00	-1.53	0.19	0.478	<.001	0.854
28.1.3	DNA.synthesis/chromatin structure.histone	histone H3	-0.10	-2.67	-2.28	0.00	-3.20	0.02	0.741	<.001	0.664
29.2.1.1.1.1.3	protein.synthesis.ribosomal protein.prokaryotic.chloroplast.30S subunit	ribosomal protein S3 (chloroplast)	-0.57	1.91	2.29	0.00	1.53	0.11	0.041	<.001	0.031
29.2.1.1.1.2.14	protein.synthesis.ribosomal protein.prokaryotic.chloroplast.50S subunit	ribosomal protein L16 (chloroplast)	-0.57	2.36	2.68	0.00	1.99	0.01	0.01	<.001	0.01
29.4	protein.postranslational modification	threonine-protein kinase HT1-like	-0.61	0.32	0.60	0.00	-0.33	-0.22	0.002	0.06	0.022
29.5.11.4.3.2	protein.degradation.ubiquitin.E3.SCF.FBO X	leucine-rich repeat family protein	-0.15	0.04	0.46	0.00	-0.13	0.30	0.26	0.766	0.004
34.9	transport.metabolite transporters at the mitochondrial membrane	SLAH3 anion transporter	0.01	-3.11	-2.69	0.00	-3.54	0.11	0.961	<.001	0.477

Measurements at the stage of 3-4 leaves unfolded (13-14 growth stage, Zadoks scale), with WT being more developed. The detailed description of genes is recorded in Supplementary Table S7. White indicates no change, blue up-regulation and red down-regulation, as shown in the color bar for a log2 scale. *P*, probability in the analysis of variance. Numbers in bold type represent significant effects ($P < 0.05$).

Figure legends

Fig. 1 (A) PSII maximum (F_v/F_m) and operating (F_q'/F_m') quantum efficiencies, fraction of PSII centres in the open state (q_L) and quantum yield of non-photochemical quenching (Φ_{NPQ}); (B) Maximum Rubisco rate of carboxylation (V_{cmax} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), photosynthesis (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$) measured with air CO_2 concentrations of 390 (A390) and 1200 $\mu\text{mol mol}^{-1}$ (A1200), 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance, 20 °C and 1.2 ± 0.02 kPa vapour pressure deficit; (C) Direct, acclimatory and net effects of elevated CO_2 on photosynthesis; (D) Rubisco initial activity (Rubisco I, $\mu\text{mol m}^{-2} \text{s}^{-1}$) and activation state (Rbco act, %), nitrate reductase selective activity (NR sel, $\text{mmol m}^{-2} \text{h}^{-1}$) and activation state (NR act, %); (E) Rubisco (Rbco), soluble (Sol.), Sol minus Rbco (Sol.-Rbco), membrane (Membr) and total protein contents (g m^{-2}); (F) Content ($\mu\text{g m}^{-2}$) of the D1 protein and abundance relative to the WT at ambient CO_2 of Lhcb1 and cyt b6; (G) Nitrate and amino acid contents (mmol m^{-2}); (H) Chl a+b, Chl a and Chl b contents (g m^{-2}) and Chl a/Chl b ratio; (I) Fructose (Fru), sucrose (Suc), glucose (Glc)+Fru in fructans (Ftn) and starch contents (mmol m^{-2}); Fru, Ftn and starch are given as hexose equivalents. Measurements in the youngest fully expanded leaf of Graphis barley (WT) and its G132 mutant grown in ambient CO_2 (Amb, 390 $\mu\text{mol mol}^{-1}$) and elevated CO_2 (Ele, 1200 $\mu\text{mol mol}^{-1}$), at the stage of 3-4 leaves unfolded (13-14 growth stage, Zadoks scale, with WT being more developed). For each parameter, the columns with the same letter are not significantly different ($P < 0.05$). Vertical bars represent least significant differences ($P < 0.05$)

Fig. 2 (A) Pavement cell and stomatal numbers (mm^{-2}) and stomatal index (stoma/pavement cell) on the adaxial side of leaves; (B) DW area^{-1} (g m^{-2}) and Rest DW area^{-1} (g m^{-2}) of not analysed compounds (Rest); (C) Thickness (volume/area, mm), density (dry weight/volume, g cm^{-3}). Measurements in the youngest fully expanded leaf of Graphis barley (WT) and its G132 mutant grown in ambient CO_2 (Amb, 390 $\mu\text{mol mol}^{-1}$) and elevated CO_2 (Ele, 1200 $\mu\text{mol mol}^{-1}$), at the stage of 3-4 leaves unfolded (13-14 growth stage, Zadoks scale, with WT being more developed). For each parameter, the columns with the same letter are not significantly different ($P < 0.05$). Vertical bars represent least significant differences ($P < 0.05$)

Fig. 3 (A) Cluster analysis and (B) principal component analysis of the microarray normalized transcript abundances with the six biological replicates of the four combinations of genotype (G132 and WT) and CO₂ concentration (A, ambient CO₂, E, elevated CO₂).

Fig. 4 Venn diagrams showing the intersection of all the genes expressed (A) in elevated CO₂ compared with ambient CO₂, both in G132 and WT; and (B) in G132 compared with WT, both in ambient and elevated CO₂. Up, down, induced and repressed genes, respectively.

Fig. 1

Fig. 2

Fig. 3

Fig. 4