Functional and transcriptional characterization of a barley mutant with impaired photosynthesis

Javier Córdoba^{a,b}, José-Luis Molina-Cano^{b,1}, Rafael Martínez-Carrasco^a, Rosa Morcuende^a, Pilar Pérez^{a*},

^aInstitute of Natural Resources and Agrobiology of Salamanca, IRNASA-CSIC, Cordel de Merinas 40, E-37008 Salamanca, Spain

^bIRTA (Institute for Food and Agricultural Research and Technology), Field Crops, Av. Alcalde Rovira i Roure, 191, E-25198 Llérida, Spain

¹Retired

*Corresponding author:

Pilar Pérez

Phone: +34 923 219606

E-mail address: pilar.perez@imasa.csic.es

Abstract

Chemical mutagenesis induces variations that may assist in the identification of targets for adaptation to growth under atmospheric CO₂ enrichment. The aim of this work was to characterize the limitations causing reduced photosynthetic capacity in G132 mutagenized barley (*Hordeum vulgare* L. cv. Graphic) grown in a glasshouse. Compared to the wild type (WT) G132 showed increased transcript levels for the PSII light harvesting complex, but lower levels of chlorophyll, transcripts for protochlorophyllide oxidoreductase A and psbQ, and PSII quantum efficiency in young leaves. Rubisco limitation had an overriding influence on G132 photosynthesis, and was due to strong and selective decreases in Rubisco protein and activity. These reductions were accompanied by enhanced Rubisco transcripts, but increased levels of a Rubisco degradation product. G132 showed lower levels of carbohydrates, amino acids and corresponding transcripts, and proteins, but not of nitrate. Many of the measured parameters recovered in the mutant as development progressed, or decreased less than in the WT, indicating that senescence was delayed. G132 had a longer growth period than the WT and similar final plant dry matter. The reduced resource investment in Rubisco of G132 may prove useful for studies on barley adaptation to elevated CO₂ and climate change.

Keywords: *Hordeum vulgare*, mutagenesis, gene expression, photosynthesis, Rubisco, primary metabolism.

Abbreviations: A, rate of CO₂ assimilation; A_c, rate of CO₂ assimilation limited by Rubisco; A_j, rate of CO₂ assimilation limited by RuBP regeneration; Chl, chlorophyll; C_i, intercellular CO₂ concentration in leaves; FEH, fructan exohydrolase; Fv/Fm, maximum quantum efficiency of PSII photochemistry; Fq'/Fm', PSII operating efficiency; Φ_{NPQ}, quantum yield of non-photochemical quenching; g_s, stomatal conductance; J, potential rate of photosynthetic electron transport; LHCII, PSII light-harvesting complex; PORA, protochlorophyllide oxidoreductase A; PSI, photosystem I; PSII, photosystem II; qL, fraction of PSII centres which are in the open state; qRT-PCR, quantitative real-time polymerase chain reaction; rbcL Rubisco large subunit; R_d, mitochondrial respiration rate in the light; RuBP, Ribulose-1, 5-bisphosphate; Rubisco, Ribulose-1, 5-bisphosphate carboxylase/oxygenase; SLA, specific leaf area; 1-SST, sucrose: sucrose 1-fructosyltransferase; 6-SFT, sucrose: fructan 6-fructosyltransferase; SBPase, sedoheptulose-1, 7-bisphosphatase; TPU, triose-phosphate use; V_{cmax}, maximum rate of Rubisco-catalyzed carboxylation; WT, wild-type.

1. Introduction

The development of new crop varieties and agronomic practices led to an increase in food production in the 1960s and 1970s, but several recent studies indicate that yields may no longer be increasing in different regions of the world [1]. The demand for crops has similarly been increasing since 1960, and may rise by 100%-110% between 2005 and 2050 [2]. Yields in four major crops -maize, rice, wheat, and soybean- are increasing at 0.9%-1.6% per year, which is less than the annual rate of 2.4% required to double global production by 2050 [1]. Climate trends can account for ~10% of the yield stagnation observed in Europe over the last two decades; thus, temperature and precipitation changes in Europe have reduced average wheat and barley yields by 2.5% and 3.8%, respectively [3]. Consistent with these climate effects on crops a study on 138 spring barley accessions found that grain yield decreased by 29%, with a concurrent rise in temperature (+5 °C) and CO₂ concentration (700 μmol mol⁻¹), in spite of the 16% yield increase caused by elevated CO₂ [4]. In addition to changes in agricultural subsidies and environmental policies [3], proximity to the theoretical peak in biomass allocation to the harvestable part of agricultural crops is a likely explanation for declining yield increases [5, 6]. Extending the cultivated land area is not a sustainable option, and thus the productivity of existing arable land will have to be improved. It is generally accepted that the only way to improve yield potential is through an enhancement of radiation use efficiency [6]. Studies with crops grown under elevated CO₂ conditions [5] and model simulations [7] indicate that increasing photosynthetic performance may raise efficiency in energy conversion and increase crop yields to meet global food demand [8] in the future's, CO₂-rich atmosphere.

As CO₂ increases, photosynthesis in C₃ crops shifts from a Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) limitation to a Ribulose-1,5-bisphosphate (RuBP)-regeneration limitation [9]. Therefore, one way to improve crop adaptation to atmospheric CO₂ enrichment may be to increase the capacity for RuBP-regeneration to match the enhanced rates of carboxylation. Similarly, there is evidence of increases in the RuBP-regeneration capacity of crops under elevated CO₂. Thus, fructose-1, 6-bisphosphatase levels increased in *Lolium perenne* grown at high nitrogen supply [10], and the content of Rubisco decreased in tobacco while that of other Calvin-Benson enzymes increased [11]. The impacts on photosynthesis of changes in the activity of several Calvin-Benson cycle enzymes have been assessed in studies of antisense transformants. Transgenic plants with more than 50% reductions in the amounts of carbon-reduction-cycle enzymes have shown that some of them are present in excess. Included among these enzymes are glyceraldehyde 3-phosphate dehydrogenase [12], fructose-1, 6-bisphosphatase [13], phosphoribulokinase [14],

plastid aldolase [15] and, under moderate light, Rubisco, but not under high light intensity [16]. In contrast, photosynthesis is sensitive to small reductions in transketolase [17] and sedoheptulose-1,7-bisphosphatase (SBPase; [18]). Photosynthesis is inhibited by decreases in contents of cytochrome *b₆f*, which is involved in photosynthetic electron transport, and thus in RuBP-regeneration [19, 20]. Conversely, SBPase overexpression increased carbon fixation and photosynthesis enhancement in plants grown in elevated CO₂ [8]. The use of a numerical simulation to optimize resource allocation among enzymes of carbon metabolism in elevated CO₂ (700 $\mu\text{mol mol}^{-1}$) has revealed that Rubisco and the enzymes of photorespiration should decrease, while the other enzymes of the Calvin-Benson cycle, sucrose synthesis in the cytosol and adenosine diphosphate glucose pyrophosphorylase should increase [7]. These studies present the perspectives of increasing photosynthetic efficiency through manipulation of the enzymes involved in carbon fixation.

Traditional breeding has not considered the response to rising ambient CO₂ as a selection criterion [21], and yet genetic improvement, either through breeding or biotechnology, may provide a direct strategy for increasing yields [22] through enhanced photosynthesis. Mutagenesis, which may induce greater changes in the pattern of gene expression than transgene insertion, has been used to rapidly create and increase variability in crop species and eventually modify plant traits [23]. The mutant lines can be initially evaluated by visual inspection [24], and transcriptomics resources can be used to gather information on the candidate genes potentially related to agronomically important traits [25] or to environmental adaptation. Mutant lines, identified either by forward or reverse screening, will carry many other mutations in addition to the one selected [26]. A substantial part of these undesirable mutations can be removed through backcrossing. There are numerous leaf colour mutants with altered chlorophyll content, either with reduced photosynthetic rate [27], or with a relatively higher quantum yield of PSII together with chloroplastic CO₂ concentration, Rubisco content and photosynthetic rate similar to those of the wild type [28]. Some stay-green mutants exhibit delayed senescence associated with slower degradation of chlorophyll, increased antioxidant capacity and enhanced photosynthetic competence [29-31]. The *tigrina* (*tig*)-*d.12* mutant of barley, with excess protochlorophyllide accumulation in the dark, provokes singlet oxygen production and photooxidative damage, which causes suppression of synthesis of Rubisco small and large subunits, the major PSII light harvesting chlorophyll *a/b*-binding protein, as well as other chlorophyll binding proteins [32]. Furthermore, a rice brassinosteroid-deficient mutant, *osdwarf4-1*, with erect leaves which increase light capture for photosynthesis, has enhanced grain yields; the mutation affects a cytochrome P450 involved in

brassinosteroid biosynthesis [33]. The T-DNA knockout mutant for chloroplastic fructose-1,6-bisphosphatase [34], and the T-DNA insertion null mutant for plastidic phosphoglucose isomerase [35] display lower rates of photosynthesis.

This article reports the characterization of a barley mutant, selected in elevated CO₂ conditions, which shows decreased carbon assimilation rates. The aim of this work was to understand the causes of the mutant's reduced photosynthetic capacity and the limitation of this capacity by Rubisco activity, RuBP-regeneration or triose-phosphate use (TPU). To achieve this goal, we determined photosystem II quantum efficiency and gas exchange, and analysed Rubisco content and activity. These measurements were complemented with nitrate reductase activity assays and analyses of proteins, amino acids and nitrate, to explore possible limitations to the synthesis of photosynthetic components. Furthermore, we determined the contents of soluble carbohydrates, which are synthesized from carbon fixed in photosynthesis and make up the bulk of plant biomass, and the abundance of transcripts involved in all these processes. To assess the possible changes with development in the effects of the mutation, our approach was to consider different leaf and plant growth stages. The consequences for growth of the mutation were appraised by determining plant dry matter at the vegetative stage and at maturity. The longer-term goal would be to assist in the identification of targets for adapting to growth under atmospheric CO₂ enrichment.

2. Material and methods

2.1. Plant material and growing conditions

Barley (*Hordeum vulgare* L.) seeds of the pure pedigree Graphic variety as wild type (WT) and homozygous sodium azide mutagenized seeds of the line G132, grown and selected in 2000 $\mu\text{mol mol}^{-1} \text{CO}_2$, were obtained from Dr J. L. Molina-Cano's mutant collection at the IRTA research institute. Preliminary gas exchange measurements revealed that G132 showed lower photosynthesis and higher stomatal conductance than the WT, and the study of the exome capture of the mutant compared with WT is currently a work in progress. Seeds of G132 and WT were surface sterilized with hypochlorite and sown in 5 L pots with 1.2 kg of peat: perlite (4:1) substrate, with a density of four plants per pot after emergence. 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 in a glasshouse with partial temperature control to achieve c. 20:15 °C day:night temperatures, supplementary illumination to extend the photoperiod to 16 h with a minimum of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance, and 60±10% relative humidity. All measurements were taken on the youngest fully expanded leaf (leaf *a*) and on the penultimate (leaf *b*) and antepenultimate (leaf *c*) ones of a shoot (Supplementary Fig. 1), representing successive leaf developmental phases, at the growth stages of 3-4 leaves or 5-7 leaves (13-14 or 15-17 stages of the Zadoks scale [36], respectively), with WT being more developed than G132.

2.2. Chlorophyll fluorescence and gas exchange

The chlorophyll (Chl) fluorescence quenching analysis followed the procedure described by Pérez et al. [37] 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 (q_L) and the quantum yield of non-photochemical quenching (Φ_{NPQ}) in light adapted leaves (1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

Gas exchange was measured in the central segment of leaves using a 1.7 cm^2 -window leaf chamber connected to an infrared gas analyser (PLC6 [rice] and CIRAS-2, respectively, PP Systems, Amesbury, MA, USA). The air flow rate was 300 ml min^{-1} , leaf temperature was kept at 20 °C using the Peltier system in the leaf chamber, irradiance was set at 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by red LEDs supplemented with white ones, and the vapour pressure deficit was 0.95±0.12 kPa. The irradiance was not above 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to prevent photo-inhibition in leaves adapted to grow in 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The gas exchange- CO_2 response curves were recorded by decreasing

CO₂ 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. Gas exchange parameters were recorded as soon as ambient CO₂ concentration was stable, but not necessarily steady-state [38]. The leaks into or out of the leaf chamber were considered [39]. The photosynthesis-intercellular CO₂ concentration (C_i) responses were used to determine the maximum rate of Rubisco-catalysed carboxylation (V_{cmax}), 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 TPU, according to Farquhar et al. [40], fitting the model with the LeafWeb utility [41].

2.3. Chlorophyll and protein analysis

At mid-morning on the growth stages indicated above, five replicate samples of the three uppermost leaves were harvested per genotype, each consisting of five leaves from different plants; each leaf was cut, immediately plunged into liquid nitrogen under illumination and then stored at -80 °C until analysed. Subsamples (100 mg) of frozen leaf material were ground to a fine powder in a mortar with 1.5 ml of 50 mM N-[tri(hydroxymethyl)methyl] glycine (Tricine) buffer (pH 8.0), 1 mM EDTA, 10 mM NaCl, 5 mM MgCl₂, 75 mM sucrose, 5 mM ϵ -aminocaproic acid, 2 mM benzamidine, 8 mM β -mercaptoethanol, and 15 μl of 100 mM PMSF for 5 min on ice. For Chl analysis, 40 μl of the suspension were diluted to 200 μl with water, stored in the dark for 30 min at 4 °C, made up to 80% acetone concentration and centrifuged at 13000 g for 5 min. Chl was determined according to Arnon [42].

Part of the leaf extract was centrifuged at 13000 g at 4 °C for 30 min and soluble protein was determined in an aliquot of the decanted supernatant [43]. For total protein analysis, 800 μl of a mixture of 20 % trichloroacetic acid and 0.07 % β -mercaptoethanol in acetone were added to 800 μl of the leaf extract, and the mixture was kept at -20 °C for 2 h for protein precipitation. Following centrifugation at 13000 g at 4 °C for 15 min, the supernatant was discarded. The precipitate was washed three times with 1 ml acetone with 0.07 % β -mercaptoethanol, incubated 30 min at -20 °C and centrifuged at 13000 g at 4 °C for 15 min, discarding the supernatants. The precipitate was dried at 40 °C for 30 min. This was followed by the addition of 800 μl of 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer pH 8.0, 100 mM sucrose, 3.5 % sodium dodecyl sulfate (SDS, w/v), 1 mM EDTA, 0.07 % β -mercaptoethanol, and the solution was shaken for 20 min at room temperature, followed by another 20 min at 70 °C. After cooling, the extract was centrifuged at 15000 g for 15 min at room temperature. Total protein was determined in the supernatant [44]. Membrane protein content was estimated as the difference between total and soluble protein contents.

The quantitation of Rubisco protein by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and Western blotting was carried out as described [37], except that the samples loaded onto the gel contained 1.5 µg (WT) or 6 µg (G132) of protein, the antibody for the Rubisco large subunit detection (anti-Rbcl rabbit polyclonal AS03 037, Agrisera) and the secondary antibody (goat anti-rabbit IgG horseradish peroxidase conjugated AS09 602, Agrisera) were diluted at 1:50000, and a ChemiDoc MP System (Bio-Rad) was used for luminescence detection, using purified Rubisco as a standard.

2.4. Nitrate, amino acid and carbohydrate analysis

Additional subsamples (100 mg) of frozen and ground leaves were sequentially extracted with ethanol and water [45]. The extracts were used for the spectrophotometric analysis of nitrate at 210 nm [46], amino acid analysis by the ninhydrin colorimetric method at 570 nm [47], and enzymatic analyses of free glucose and fructose, sucrose, fructans and starch coupled to NADP reduction, recorded either at 460 nm with excitation at 340 nm with a fluorometer (glucose and fructose, [48]), or at 340 nm with a spectrophotometer [45].

2.5. Enzyme activity assays

Frozen leaf aliquots (80 mg) were ground in a buffer and used for *in vitro* coupled spectrophotometric assays of initial and total Rubisco activity [37] by continuously recording the decrease in absorbance at 340 nm due to NADH oxidation, with a stoichiometry of 2:1 with RuBP carboxylation. To assay total Rubisco activity, the extracts were first incubated with NaHCO₃ and MgCl₂ in the absence of RuBP for 10 min. The activation state was estimated as initial activity, as a percentage of total activity. Frozen leaf material (150 mg) was extracted for nitrate reductase assay according to Morcuende et al. [45]. The activity of nitrate reductase was measured in the absence of magnesium (maximal activity) and in the presence of 10 mM magnesium acetate (selective activity, the phosphorylated form of the enzyme) in the assay buffer. The activation state of the enzyme was given as selective activity, as a percentage of maximal activity.

2.6. Analysis of transcripts by qRT-PCR

Based on the results of a microarray analysis of WT and G132 primary leaves, to be reported elsewhere, a series of genes were selected for the qRT-PCR analysis of transcript levels. The genes selected and the corresponding designed primers are listed in Supplementary Table 1. The formation with each primer pair of a single PCR product of the expected size was assessed by agarose electrophoresis and PCR

melting curve data. RNA extraction and purification followed the procedure described by Morcuende et al. [49]. Total RNA was treated with TURBO DNase (Ambion) before proceeding with cDNA synthesis using the SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The qRT-PCR assays were performed in 384-well plates with a sequence detector system (ABI PRISM 7900 HT, Applied Biosystems), using Power SYBR Green PCR Master Mix (Applied Biosystems), cDNA and gene-specific primers in a total volume of 10 μ l. The thermal profile was as follows: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, then 60 °C for 1 min, and a final temperature ramp from 60 °C to 95 °C at 1.9 °C min⁻¹. For normalization, use was made of the genes of ubiquitin (GenBank: M60175, forward CACCCTCGCCGACTACAA and reverse CTTGGGCTTGGTGTACGTCT primers) and actin (GenBank: AY145451, forward GGCACACTGGTGTTCATGG and reverse CTCCATGTCATCCCAGTT primers). Relative quantification involved the comparative C_t (threshold cycle) method ($2^{-\Delta\Delta C_t}$, [50]).

2.7. Statistical analysis

For each growth stage, the experiment consisted of two genotypes and three leaf positions, with five replicates. Factor effects and interactions were determined through analysis of variance with no blocking (GenStat 6.2). The standard errors of differences and least significant differences were obtained to assess differences between means.

3. Results

A feature of the G132 mutant was that emerging leaves showed a marked chlorosis, from which they progressively recovered, whereby the penultimate leaf from the apex was typically greener than the uppermost leaf (Supplementary Fig. 2a). G132 seedlings grew at a slower rate than their WT counterparts (Supplementary Fig. 2b).

3.1. Chlorophyll fluorescence

The maximum quantum efficiency (F_v/F_m , Table 1) and the PSII operating quantum efficiency (F_q'/F_m') were significantly lower in G132 than in WT at the two growth stages examined (3-4 leaves unfolded, 13-14 Zadoks stage, and 5-7 leaves unfolded, 15-17 Zadoks stage, with the WT being more developed), although in some cases the difference in F_v/F_m between genotypes did not reach significance for the older leaves. F_v/F_m in G132 had always values below the typical 0.81 of healthy leaves shown by WT. Compared with the two preceding leaves, in G132 leaf a had lower F_v/F_m at both growth stages, and lower F_q'/F_m' at the 13-14 stage, but not at the 15-17 stage. In WT these parameters showed no differences among leaves. At variance with F_q'/F_m' , the fraction of PSII centres in the open state (q_L) did not change with the genotype at any growth stage or with leaf positions at the 13-14 growth stage, while it decreased with leaf age in both genotypes at the 15-17 stage. The quantum yield of non-photochemical quenching (Φ_{NPQ}) was higher in G132 and showed no significant difference with leaf age, although it tended to decline in older leaves of G132 at the first sampling.

3.2. Gas exchange

No gas exchange was recorded in leaf c at the 13-14 growth stage due to the sample's small size relative to the CIRAS-2 leaf chamber window. The maximum rate of Rubisco-catalysed carboxylation (V_{cmax}) was lower in G132 than in WT at both growth stages (Table 2). V_{cmax} tended to increase from leaf a to leaf b in both genotypes at the 13-14 growth stage, but the difference was not significant (Table 2); at the 15-17 stage, V_{cmax} was similar in successive leaves in the two genotypes. It was not possible to determine the potential rate of photosynthetic electron transport (J) from the photosynthesis- CO_2 response curves in G132 because V_{cmax} limited photosynthesis at all CO_2 concentrations (Fig. 1). The fitting of the A- C_i curves showed no TPU limitation of photosynthesis. In turn, J was similar among WT leaves at both growth stages, with a (non-significant) downward trend with leaf age at the last one. The rate of dark respiration in the light (R_d) tended to be higher in WT than in G132 at the 13-14 stage,

with the difference reaching statistical significance at the 15-17 stage. No difference was recorded among leaves in dark respiration at any stage.

With high light intensity ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$), both with ambient ($390 \mu\text{mol mol}^{-1}$) and elevated ($1300 \mu\text{mol mol}^{-1}$) air CO_2 concentrations, CO_2 assimilation (A) was lower, and intercellular CO_2 concentrations (C_i), as well as stomatal conductances (g_s) and transpiration rates (E) were higher in G132 than in WT at both growth stages (Table 2). Exceptions with no significant differences between genotypes were A measured with ambient CO_2 in leaves *b* and *c* at the 15-17 stage, g_s recorded with elevated CO_2 at the first growth stage, and E measured with elevated CO_2 at both stages. At the first sampling date, A was lower in leaf *a* than in leaf *b* in G132, being significantly so with elevated CO_2 , and showed no significant differences between WT leaves. At the 15-17 growth stage, A decreased from leaf *a* to leaf *c* in both genotypes. No difference was recorded among leaves in C_i , g_s and E at any growth stage, except for the decreases with leaf age in g_s in both genotypes and in E in G132 with measurements in ambient CO_2 at the 15-17 stage.

3.3. Chlorophyll contents

Total Chl and Chl *a* contents were lower in all G132 leaves compared to WT (80% of the latter, on average) at the first growth stage, and lower in leaf *a*, but similar in the two older leaves at the 15-17 stage (Fig. 2). The Chl *b* content was lower in leaf *a* of G132 compared to WT, but similar in leaves *b* and *c* at both growth stages. At the 13-14 stage, the Chl contents increased in both genotypes from the last to the penultimate leaf, and decreased in the antepenultimate one; at the 15-17 stage, the Chl contents showed little change in G132, and decreased in WT, as the leaves aged. At the first growth stage, the Chl *a*/Chl *b* ratio was also lower in G132 than in WT, except in leaf *c*; this difference disappeared at the 15-17 stage. The Chl *a*/Chl *b* ratio increased with leaf age in G132 at the earlier growth stage and was not significantly different among leaves in other instances.

3.4. Nitrogen compound content

Rubisco, soluble, membrane and total protein contents were lower in G132 than in WT at both plant growth stages (31%, 59%, 82% and 69% the WT contents, respectively, at the 13-14 growth stage, and 39%, 72%, 53% and 65% of this content at the 15-17 stage; Table 3). Therefore, Rubisco protein in G132 decreased more than other proteins relative to WT, accounting for most of the soluble protein decrease. Membrane protein content decreased in G132 relatively less than soluble protein at the 13-14 stage, but decreased more than soluble protein at the 15-17 stage. In addition,

genotypic differences in the content of proteins, especially Rubisco, were greater than those found in Chl contents (see above). At the first sampling date, Rubisco protein content in G132 increased from leaf *a* to leaf *b* and declined in leaf *c*, while the youngest leaf had more Rubisco protein than the older ones in WT. In both genotypes, soluble, membrane and total protein contents among leaves followed the same pattern as Rubisco in G132. The content of soluble protein excluding Rubisco was lower in leaf *b* than in the other leaves in G132, whereas it increased from leaf *a* to leaf *b* and decreased in leaf *c* in WT. As development progressed (15-17 stage), all protein fractions decreased as leaves aged in WT, while they showed little differences among leaves in G132. The Western blots showed a 33 kDa polypeptide that reacted with the Rubisco large subunit (RbcL) antibody. As a fraction of total RbcL, the 33 kDa polypeptide was greater in G132 than in WT at both growth stages, particularly at the second one. The oldest leaf had more 33 kDa polypeptide as a fraction of total RbcL than the youngest one at both growth stages in both genotypes.

Like protein content, amino acid content was lower in G132 than in WT at both growth stages (Table 3). The amino acid content showed no significant differences among leaves at the 13-14 stage (Table 3), although it tended to decrease in the antepenultimate leaf relative to the younger ones. At the second sampling date, the amino acid content decreased with leaf age in both genotypes. In contrast with the amino acid content, the overall leaf nitrate content in the mutant was not lower at the 13-14 stage, and surpassed that of WT at the 15-17 stage. Among leaves, leaf *c* in both genotypes had a lower nitrate content than the younger leaves at the 13-14 stage, but this difference disappeared at the 15-17 stage.

3.5. Carbohydrate contents

At the first growth stage, the free hexose content was almost undetectable (under $0.3 \mu\text{mol g Fwt}^{-1}$) even with the fluorometric method used. The contents in the remaining carbohydrates analysed (Table 4) was lower in G132 than in WT at both sampling dates, although the difference between genotypes in fructan content did not reach significance at the second date ($P < 0.08$). The ratio of fructose to glucose from *in vitro* hydrolysis of fructans is indicative of the presence of fructans and their degree of polymerization. The values lower than 2 in G132 leaves (except leaf *b*) at both growth stages indicate the absence of fructans; other carbohydrates, such as those of the raffinose series, may have been analysed along with fructans. The fructan fructose/glucose ratio in WT reached or surpassed that threshold, with values generally higher at the second growth stage.

Except for starch, whose content was lower in the youngest leaf than in the preceding leaves in both genotypes (Table 4), there were no significant differences among leaves in terms of carbohydrate contents at the 13-14 growth stage. Nevertheless, in G132 these compounds showed a trend to increase from the last to the penultimate leaf and to decrease in the antepenultimate one, while in WT the carbohydrate contents increased with leaf age. At the 15-17 stage, there were no significant differences in carbohydrate contents among G132 leaves, while sucrose and starch decreased with leaf age in WT.

3.6. Enzyme activities

The initial and total Rubisco activities were lower and the activation state of the enzyme was higher in G132 than in WT at both growth stages (Table 5). No significant differences were recorded among leaves in terms of Rubisco activity or activation state at the first growth stage (Table 5), although initial and total activities in G132 tended to increase from the youngest to the penultimate leaf, and then decrease in the antepenultimate one. At the 15-17 growth stage, initial and total Rubisco activities showed significant downward trends from the youngest to the oldest leaves, particularly in WT, while there were no differences among leaves in the activation state.

The maximal and selective nitrate reductase activities and the enzyme's activation were lower at the 13-14 growth stage, but higher at the 15-17 stage in G132 than in WT. This shift was due to the sharp decrease in nitrate reductase activity of WT at the second sampling compared to the first. Like Rubisco activity and activation, nitrate reductase maximal and selective activities and activation states increased at the first growth stage from the ultimate to the penultimate leaf and declined in the oldest one in G132, while minimal differences were recorded in WT. At the second sampling, the nitrate reductase activity and activation state were similar among leaves, although in WT the activity tended to increase with leaf age.

3.7. Transcript profiling

Both at the 13-14 and the 15-17 growth stages, the transcript levels for proteins linked to photosynthesis were enhanced in G132 relative to WT: PSII light harvesting complex (LHCII) and psbP, Photosystem I (PSI) psaA and psaB, and the Rubisco large subunit (rbcL); as well as the chloroplast RNA polymerase alpha subunit (Supplementary Tables 2 and 3). In contrast, at both growth stages a decrease was recorded in transcript levels for the PSII extrinsic protein psbQ, the mitochondrial alternative oxidase 1A, DNA-dependent ATPase (except for leaf *a* at the 15-17 stage), histone H3 and (with the exception of leaf *b* at the 13-14 stage) SLAH3, involved in

CO₂ signalling for stomatal aperture. At the first growth stage, but not at the second, the mutant's abundance of transcripts for chloroplast ribosomal proteins S3 and L16 (30S and 50S subunits, respectively) increased strongly, while a decrease in transcripts was recorded for *psbR*, associated to the PSII oxygen evolving complex, protochlorophyllide oxidoreductase A (PORA), involved in Chl (tetrapyrrole) synthesis, sucrose: sucrose 1-fructosyltransferase (1-SST) and sucrose: fructan 6-fructosyltransferase (6-SFT), of the fructan synthesis pathway, as well as for an HT1-like protein kinase involved in the stomatal aperture's response to CO₂. At variance with the first growth stage, the second showed in G132 increases in transcripts for *psbR* and for fructan synthesis (6-SFT) and degradation (fructan exohydrolase, FEH), and decreases in those for ribosomal proteins S3 and L16. At the second growth stage in G132 there was also a significant increase in the transcripts for *rbcS*, xyloglucan endotransglycosylase 3 (in leaf *a*, but not in leaves *b* and *c*), with a function in cell wall modification, and nitrate reductase, as well as decreases in transcripts for protein degradation (an F-box leucine-rich repeat protein involved in ubiquitination).

When the three uppermost leaves on the stem, representing successive leaf developmental stages, were compared at both growth stages sampled, the youngest leaf had, compared to the older ones, a higher level of transcripts for LHCII, *psaA*, PORA, xyloglucan endotransglycosylase, nitrate reductase, and, with some variation, chloroplast RNA polymerase alpha subunit and HT1. In contrast, the youngest leaf had lower levels of transcripts for *psbR*, alternative oxidase 1A and histone H3. At the 13-14 stage, but not at the 15-17 stage, the transcript abundance for *psaB* and FEH was greatest in the youngest leaf, while the transcript levels for *psbP* increased in G132 with leaf age, but decreased in WT. The abundance of transcripts for the Rubisco subunits showed little variation among leaves in G132, while it was greater in the youngest leaf in WT compared to the older ones. The transcript levels for fructan synthesis (1-SST and 6-SFT) were lower in leaves *a* and *c* than in leaf *b* in G132, but were higher in the youngest leaf or showed little change with leaf age in WT. At the 15-17 growth stage, leaf *a* recorded a higher level than the older leaves of transcripts for *rbcS*, the ribosomal proteins S3 and L16 and a leucine-rich repeat protein. In addition, the levels of transcripts for DNA-dependent ATPase, SLAH3, and (except for leaf *b* in WT) a cell-wall constituent lipid transfer protein and FEH were lower in the youngest leaf compared to the older ones.

Summarizing this section, we recorded seven up-regulated genes and five down-regulated ones at both growth stages in G132 relative to WT, plus eight up-regulated and eight down-regulated genes at either the first or second of these stages (Fig. 3). Comparing the youngest leaf with the two preceding ones, there were seven up-

regulated and three down-regulated genes in both genotypes and growth stages; a total of 13 and seven genes were up- and down-regulated, respectively, in one or both genotypes at either the first or the second growth stage.

3.8. Plant growth

At the 15-17 growth stage, plant dry weight in G132 was approximately 25% that of WT (Table 6). Thereafter, the duration of G132 growth through to maturity was longer (16 more days) and was probably faster than in WT, whereby the grain and total dry weights, although somewhat lower in G132, were not significantly different from those in WT. The ratio of grain to total dry weight at maturity (harvest index) was similar in both genotypes.

4. Discussion

The sharp decrease in photosynthesis in the G132 mutant compared to WT was associated with the inhibition of the maximum quantum efficiency of PSII photochemistry (Table 1). This inhibition was accompanied by a lower Chl content (Fig. 2), especially at the 13-14 Zadoks growth stage. The analysis of transcript abundance at this stage revealed a repression of transcripts for PORA (Supplementary Table 2), whose scarcity could limit Chl synthesis either directly [51] or by inhibiting aminolevulinate formation through protochlorophyllide accumulation [52]. The higher transcript levels for LHCII in G132, together with low Chl a/Chl b ratios in younger leaves, could point to the depletion of PSII reaction centres relative to light harvesting complexes [53], although the Chl a/Chl b ratio could also be due to increased PSII:PSI ratios [54]. There are also likely to be decreases in G132 of other transcripts and proteins of the PSII reaction centre that have not been analysed. Comparisons among successive leaves and plant growth stages indicate that G132 deficiency in Chl, and possibly also in PSII reaction centres (Chl a/Chl b ratio), was attenuated in step with development, in spite of which Fv/Fm was still lower than in WT. At the 13-14 growth stage, but not at the 15-17 stage, there was a decrease in gene expression for the psbR involved in the PSII core assembly of the psbP and psbQ O₂ evolving complex proteins [55]. In addition, the abundance of psbQ transcript decreased in G132 at both growth stages. Although psbQ is required in low light conditions [56], it is not essential for the regulation and stabilization of PSII under normal growth conditions [57]. On the other hand, psbQ can play a role in grana stacking [58], so that its deficiency, together with decreased membrane protein content in G132 (Table 3), could indicate the lower abundance and altered organization of thylakoid membranes. These changes may have contributed to the decreased Fv/Fm in the mutant. The decrease in WT Chl content as leaf aged at the 15-17 growth stage, in contrast with G132, can indicate that senescence was delayed in the latter.

In illuminated leaves, the low Fq'/Fm' in G132 and, at the 13-14 growth stage, the increase in this parameter in older G132 leaves, were not due to changes in the fraction of open PSII centres (qL). The increases in G132 and decreases in older G132 leaves in Φ_{NPQ} accounted for differences in Fq'/Fm' [59]. Although repressed mitochondrial alternative oxidase 1A expression in G132 could deprive the mutant of a photoprotective mechanism [60], efficient non-photochemical quenching may have avoided an overreduction in the chloroplast. At the later plant growth stage (15-17), leaf ageing decreased qL while Fq'/Fm' and Φ_{NPQ} remained almost constant, implying an increase in the quantum efficiency of open PSII centres. Overall, the differences in Fq'/Fm' and Φ_{NPQ} between genotypes and consecutive leaves may be attributable to

changes in CO₂ assimilation (Table 2) and the corresponding use of electron transport products.

The high stomatal conductance of G132 (Table 2) was proposed to be related to a higher ratio of electron transport to carboxylation capacities and to lower gene expression for SLAH3 [61]. The elevated stomatal conductance implies that photosynthesis was not restricted by CO₂ diffusion into the intercellular air spaces. Differences in mesophyll conductance, which was not determined in this experiment, can affect V_{cmax} estimations. However, the measurements of Rubisco protein content and *in vitro* activity rule out mesophyll conductance as the sole cause for the sharp photosynthesis decrease in the mutant. G132 had Rubisco-limited photosynthesis up to very high atmospheric CO₂ concentrations (Fig. 1), indicating that carboxylation capacity, rather than photosynthetic electron transport or TPU capacities, strongly inhibited CO₂ assimilation. A similar result was obtained with Rubisco antisense plants with decreased Rubisco activity [9]. Declines in G132 relative to WT of V_{cmax} (Table 2) and *in vitro* Rubisco activity (Table 5) were due to a drop in Rubisco protein content (Table 3), which was selective among soluble proteins. Rubisco synthesis ends soon after the time of maximum leaf expansion, while Rubisco degradation continues during senescence [62]. Either nitrogen availability [63] or transcription regulation [62] may be the major determinant of the rate of Rubisco biosynthesis. The similar or greater nitrate content, but lower amino acid content in G132 than in WT (Table 3) could suggest an inhibition in the mutant of nitrogen assimilation and protein synthesis at the nitrate reduction step. Indeed, G132 had lower *in vitro* nitrate reductase activity than WT (Table 5) at the 13-14 growth stage. However, the opposite occurred at the 15-17 stage, when the level of nitrate reductase transcript also increased sharply in the mutant (Supplementary Table 3). Moreover, limited nitrate reductase activity cannot account for the preferential decrease in Rubisco, which could be due to some other cause. Consistent with our results, nitrate reductase activity decreased in tobacco mutants with a reduced number of functional copies of nitrate reductase genes, but Rubisco activity did not [64]. Notably, the transcript abundance for the Rubisco large subunit and, at the 15-17 growth stage, also for the small subunit, was greater in G132 than in WT (Supplementary Tables 2 and 3), implying that the mutant's low enzyme content is not attributable to reductions in Rubisco gene expression. The altered abundance of Rubisco transcripts was not mirrored by changes in the transcript levels involved in chromatin structure, which were lower in the mutant, or in chloroplast ribosomal proteins, which increased in G132 at the 13-14 growth stage, but decreased at the 15-17 stage. Independently of gene expression, mRNA processing, edition and stability, as well as translation initiation, play a major regulatory role in chloroplast

protein synthesis [65]. Thus, although low Rubisco content in G132 could depend, at least in part, on Rubisco synthesis, it was clearly not related to restrictions in gene expression for this enzyme.

Remarkably, compared to WT, G132 had higher content of a 33 kDa polypeptide that reacted against a Rubisco large subunit antibody (Table 3). Different Rubisco large subunit fragments have been described [66, 67] that are Rubisco degradation products. Oxidising conditions enhance Rubisco degradation, which involves the oxidation of cysteine residues [68], cysteine proteinases [69] and peptidases [70]. The results indicate that an increase in Rubisco degradation largely accounts for the low Rubisco content in G132. This content increased in step with leaf development at the earlier growth stage, but never reached the Rubisco content in WT. The decline in Rubisco protein with leaf age was associated, particularly in WT at the 15-17 stage, with the decreased transcripts for the Rubisco small subunit and increasing content of the 33 kDa polypeptide (Table 3). These changes are consistent with those reported by Suzuki et al. [51] for senescing rice leaves. At the 15-17 growth stage, the contents of Rubisco and other proteins were similar among G132 leaves of different ages, which contrasted with a decreasing content in WT as leaves aged. This genotypic difference suggests a delay of leaf senescence in the mutant.

The low photosynthesis rate in G132 was probably the cause of decreased non-structural carbohydrate contents (Table 4), which was particularly strong for starch. Reduced flux through the Calvin-Benson cycle may lead to low levels of 3-phosphoglycerate that negatively affect ADP-glucose pyrophosphorylase activity [71] and starch synthesis. The preferential inhibition of starch accumulation shows that assimilated carbon is shifted towards sucrose synthesis, which can be used for export and growth [14]. The marked decrease in fructan content in G132 was correlated with the repression of gene expression for fructan synthesis at the 13-14 growth stage (Supplementary Table 2). These drops were mitigated (fructan content) or disappeared (fructan transcripts) at the 15-17 stage (Supplementary Table 3). The differences in fructan content and transcript abundance between genotypes did not correlate with nitrate content (Table 3), which is a negative signal for fructan synthesis [45], while changes between plant growth stages were associated with declining nitrate content. In turn, the lower sucrose content (Table 4), which is a substrate and also an effector for fructosyltransferase activity [72], could limit fructan accumulation in G132, especially at the 13-14 growth stage. Together with the drop in nitrate content, a higher sucrose content at the 15-17 growth stage could favour fructan synthesis. It is therefore likely that starch content in G132 decreased due to the metabolic regulation of enzyme activity, while fructan content decreased due to the metabolic regulation of gene

expression. Like protein content, starch, sucrose and fructan contents at the 15-17 growth stage decreased with leaf age in WT, but not in G132, which again suggests that senescence was delayed in the mutant.

According to observations in Rubisco antisense plants [73], G132 developed slowly with delayed senescence. Reduced photosynthetic rates and starch and sucrose contents are correlated with increases in specific leaf area (SLA) in Calvin-Benson cycle antisense plants [74]. We have observed (Córdoba et al. unpublished) increases in SLA in G132 compared to the WT, together with a decreased expression of genes for cell wall constituents. The trend towards a lower xyloglucan endotransglycosylase transcript level in younger G132 leaves at the 13-14 growth stage is consistent with this finding. A higher SLA may compensate for the decrease in photosynthetic capacity at the whole plant level [74]. Thus, delayed senescence and a higher SLA in G132 could account for the small difference with WT in plant dry matter and grain yield at maturity (Table 6).

In conclusion, G132 may be unique among transformed plants with reduced amounts of Rubisco [9, 75] in showing no inhibition of Rubisco gene expression and enhanced Rubisco degradation. Although with low level of PORA transcript, G132 slowly accumulates Chl, with no inhibition of LHCII genes by protochlorophyllide accumulation [32]. In spite of the reduced PSII quantum efficiency, low Rubisco content and activity have an overriding influence on the photosynthesis of G132. The mutation has far-reaching effects on primary metabolism, development and final yield [74]. Due to its decrease in Rubisco content, which may optimize resource allocation among enzymes of carbon metabolism [7], and to a lesser extent, its lower g_s sensitivity to CO_2 [61], the G132 mutant may prove to be a valuable resource for studies on barley adaptation to rising atmospheric CO_2 and climate change.

Acknowledgements

A.L. Verdejo and M.A. Boyero contributed to plant growing, gas exchange measurements and leaf analyses. This work was funded by the Spanish Research and Development Programme-European Regional Development Fund, ERDF (Project RTA2009-00006-C04-01) and the regional government, the Junta de Castilla y León (Projects CSI148A11-2 and CSI250U13). J. Córdoba was the recipient of a pre-doctoral contract from the National Institute of Agricultural and Food Research-INIA.

References

- [1] D.K. Ray, N.D. Mueller, P.C. West, J.A. Foley, Yield trends are insufficient to double global crop production by 2050, *PLOS One* 8 (2013) e66428.
- [2] D. Tilman, C. Balzer, J. Hill, B.L. Befort, Global food demand and the sustainable intensification of agriculture, *P. Natl. Acad. Sci. USA* 108 (2011) 20260- 20264.
- [3] F.C. Moore, D.B. Lobell, The fingerprint of climate trends on European crop yields, *P. Natl. Acad. Sci. USA* 112 (2015) 2670-2675.
- [4] C.H. Ingvordsen, G. Backes, M.F. Lyngkjær, P. Peltonen-Sainio, J.D. Jensen, M. Jalli, A. Jahoor, M. Rasmussen, T.N. Mikkelsen, A. Stockmarr, R.B. Jørgensen, Significant decrease in yield under future climate conditions: Stability and production of 138 spring barley accessions, *Eur. J. Agron.* 63 (2015) 105-113.
- [5] S.P. Long, X.G. Zhu, S.L. Naidu, D.R. Ort, Can improvement in photosynthesis increase crop yields?, *Plant Cell Environ.* 29 (2006) 315-330.
- [6] X.G. Zhu, S.P. Long, D.R. Ort, What is the maximum efficiency with which photosynthesis can convert solar energy into biomass?, *Curr. Opin. Biotech.* 19 (2008) 153-159.
- [7] X.G. Zhu, E. de Sturler, S.P. Long, Optimizing the distribution of resources between enzymes of carbon metabolism can dramatically increase photosynthetic rate: a numerical simulation using an evolutionary algorithm, *Plant Physiol.* 145 (2007) 513-526.
- [8] D.M. Rosenthal, A.M. Locke, M. Khozaei, C.A. Raines, S.P. Long, D.R. Ort, Over-expressing the C3 photosynthesis cycle enzyme Sedoheptulose-1-7 Biphosphatase improves photosynthetic carbon gain and yield under fully open air CO₂ fumigation (FACE), *BMC Plant Biol.* 11 (2011) 123.
- [9] G.S. Hudson, J.R. Evans, S. von Caemmerer, Y.B.C. Arvidsson, T.J. Andrews, Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase content by antisense RNA reduces photosynthesis in transgenic tobacco plants, *Plant Physiol.* 98 (1992) 294-302.
- [10] A. Rogers, B.U. Fischer, J. Bryant, M. Frehner, H. Blum, C.A. Raines, S.P. Long, Acclimation of photosynthesis to elevated CO₂ under low N nutrition is affected by the capacity for assimilate utilization. Perennial ryegrass under Free-Air CO₂ Enrichment, *Plant Physiol.* 118 (1998) 683-692.
- [11] M. Geiger, V. Haake, F. Ludewig, U. Sonnewald, M. Stitt, The nitrate and ammonium nitrate supply have a major influence on the response of photosynthesis, carbon metabolism, nitrogen metabolism and growth to elevated carbon dioxide in tobacco, *Plant Cell Environ.* 22 (1999) 1177-1199.

- [12] G.D. Price, J.R. Evans, S. von Caemmerer, J.-W. Yu, M.R. Badger, Specific reduction of chloroplast glyceraldehyde-3-phosphate dehydrogenase activity by antisense RNA reduces CO₂ assimilation via a reduction in ribulose biphosphate regeneration in transgenic tobacco plants, *Planta* 195 (1995) 369-378.
- [13] J. Kossmann, U. Sonnewald, L. Willmitzer, Reduction of the chloroplastic fructose-1,6-bisphosphatase in transgenic potato plants impairs photosynthesis and plant growth, *Plant J.* 6 (1994) 637–650.
- [14] M.J. Paul, J.S. Knight, D. Habash, M.A.J. Parry, D.W. Lawlor, S.A. Barnes, A. Loynes, J.C. Gray, Reduction in phosphoribulokinase activity by antisense RNA in transgenic tobacco: effects on CO₂ assimilation and growth in low irradiance, *Plant J.* 7 (1995) 535-542.
- [15] V. Haake, R. Zrenner, U. Sonnewald, M. Stitt, A moderate decrease of plastid aldolase activity inhibits photosynthesis, alters the levels of sugars and starch, and inhibits growth of potato plants, *Plant J.* 14 (1998) 147–157.
- [16] M. Stitt, W.P. Quick, U. Schurr, E.D. Schulze, S.R. Rodermeier, L. Bogorad, Decreased ribulose-1,5-bisphosphate carboxylase/oxygenase in transgenic tobacco transformed with antisense *rbcS*: flux control coefficients for photosynthesis in varying light, CO₂ and air humidity, *Planta* 183 (1991) 555-566.
- [17] S. Henkes, U. Sonnewald, R. Badur, R. Flachmann, M. Stitt, A small decrease of plastid transketolase activity in antisense tobacco transformants has dramatic effects on photosynthesis and phenylpropanoid metabolism, *Plant Cell* 13 (2001) 535–551.
- [18] C.A. Raines, E.P. Harrison, H. Olcer, J.C. Lloyd, Investigating the role of the thiol-regulated enzyme sedoheptulose-1,7-bisphosphatase in the control of photosynthesis, *Physiol. Plant.* 110 (2000) 303-308.
- [19] Price GD, S. von Caemmerer, J.R. Evans, K. Siebke, J.M. Anderson, M.R. Badger, Photosynthesis is strongly reduced by antisense suppression of chloroplastic cytochrome *b₆/f* complex in transgenic tobacco, *Aust. J. Plant Physiol.* 25 (1998) 445-452.
- [20] W. Yamori, S. Takahashi, A. Makino, G.D. Price, M.R. Badger, S. von Caemmerer, The roles of ATP synthase and the cytochrome *b₆/f* complexes in limiting chloroplast electron transport and determining photosynthetic capacity, *Plant Physiol.* 155 (2011) 956-962.
- [21] E.A. Ainsworth, C. Beier, C. Calfapietra et al., Next generation of elevated [CO₂] experiments with crops: a critical investment for feeding the future world, *Plant Cell Environ.* 31 (2008)1317-24.

- [22] L.H. Ziska, J.A. Bunce, H. Shimono, D.R. Gealy, J.T. Baker, P.C.D. Newton, M.P. Reynolds, K.S.V. Jagadish, C. Zhu, M. Howden, L.T. Wilson, Food security and climate change: on the potential to adapt global crop production by active selection to rising atmospheric carbon dioxide. *P. Roy. Soc. B- Biol. Sci.* 279 (2012) 4097-4105.
- [23] R. Batista, N. Saibo, T. Lourenço, M.M. Oliveira, Microarray analyses reveal that plant mutagenesis may induce more transcriptomic changes than transgene insertion, *P. Natl. Acad. Sci. USA* 105 (2008) 3640–3645.
- [24] A. Chawade, P. Sikora, M. Bräutigam, M. Larsson, V. Vivekanand, M. A. Nakash, T. Chen, O. Olsson, Development and characterization of an oat TILLING-population and identification of mutations in lignin and β -glucan biosynthesis genes, *BMC Plant Biol.* 10 (2010) 86.
- [25] M. Chantreau, S. Grec, L. Gutierrez, M. Dalmais, C. Pineau, H. Demailly, C. Paysant-Leroux, R. Tavernier, J.P. Trouvé, M. Chatterjee, X. Guillot, V. Brunaud, B. Chabbert, O. van Wuytswinkel, A. Bendahmane, B. Thomasset, S. Hawkins, PT-Flax (phenotyping and TILLinG of flax): development of a flax (*Linum usitatissimum* L.) mutant population and TILLinG platform for forward and reverse genetics, *BMC Plant Biol.* 13 (2013) 159.
- [26] M.A.J. Parry, P.J. Madgwick, C. Bayon, K. Tearall, A. Hernandez-Lopez, M. Baudo, M. Rakszegi, W. Hamada, A. Al-Yassin, H. Ouabbou, M. Labhilili, A.L. Phillips, Mutation discovery for crop improvement, *J. Exp. Bot.* 60 (2009) 2817-2825.
- [27] N. Li, J. Jia, C. Xia, X. Liu, X. Kong, Characterization and mapping of novel chlorophyll deficient mutant genes in durum wheat, *Breeding Sci.* 63 (2013) 169-175.
- [28] Y. Li, B. Ren, L. Gao, L. Ding, D. Jiang, X. Xu, Q. Shen, S. Guo, Less chlorophyll does not necessarily restrain light capture ability and photosynthesis in a chlorophyll-deficient rice mutant, *J. Agron. Crop Sci.* 199 (2013) 49-56.
- [29] P.G. Luo, K.J. Deng, X.Y. Hu, L.Q. Li, X. Li, J. Chen, H.Y. Zhang, Z.X. Tang, Y. Zhang, Q.X. Sun, F.Q. Tan, Z.L. Ren, Chloroplast ultrastructure regeneration with protection of photosystem II is responsible for the functional stay-green' trait in wheat, *Plant Cell Environ* 36 (2013) 683-696.
- [30] F. Tian, J. Gong, J. Zhang, M. Zhang, G. Wang, A. Li, W. Wang, Enhanced stability of thylakoid membrane proteins and antioxidant competence contribute to drought stress resistance in the *tasg1* wheat stay-green mutant, *J. Exp. Bot* 64 (2013) 1509-1520.
- [31] V. De Simone, M. Soccio, G.M. Borrelli, D. Pastore, D. Trono, Stay-green trait-antioxidant status interrelationship in durum wheat (*Triticum durum*) flag leaf during

- post-flowering, J. Plant Res. 127 (2014) 159-171.[32] D. Khandal, I Samol, F. Buhr, S. Pollmann, H. Schmidt, S. Clemens, S. Reinbothe, C. Reinbothe, Singlet oxygen-dependent translational control in the *tigrina-d.12* mutant of barley, P. Natl. Acad. Sci. USA 106 (2009) 13112-13117.
- [33] T. Sakamoto, Y.Morinaka, T.Ohnishi, H.Sunohara, S.Fujioka, M. Ueguchi-Tanaka, M. Mizutani, K. Sakata, S. Takatsuto, S. Yoshida, H. Tanaka, H. Kitano, M. Matsuoka, Erect leaves caused by brassinosteroid deficiency increase biomass production and grain yield in rice, Nat Biotechnol. 24 (2006) 105-109.
- [34] J.A. Rojas-Gonzalez, M. Soto-Suarez, A. Garcia-Diaz, M.C. Romero-Puertas, L.M. Sandalio, A. Merida, I. Thormaehlen, P. Geigenberger, A.J. Serrato, M. Sahrawy, Disruption of both chloroplastic and cytosolic FBPase genes results in a dwarf phenotype and important starch and metabolite changes in *Arabidopsis thaliana*, J. Exp. Bot. 66 (2015) 2673-2689.
- [35] A. Bahaji, A.M. Sanchez-Lopez, N. De Diego, F.J. Munoz, E. Baroja-Fernandez, J. Li, A. Ricarte-Bermejo, M. Baslam, I. Aranjuelo, G. Almagro, J.F. Humplik, O. Novak, L. Spichal, K. Dolezal, J. Pozueta-Romero, Plastidic phosphoglucose isomerase is an important determinant of starch accumulation in mesophyll cells, growth, photosynthetic capacity, and biosynthesis of plastidic cytokinins in *Arabidopsis*, PLOS One 10 (2015) e0119641.
- [36] J.C. Zadoks, T.T. Chang, C.F. Konzak, A decimal code for the growth stages of cereals, Weed Research 14 (1974) 415-421.
- [37] P. Pérez, G. Rabnecz, Z. Laufer, D. Gutiérrez, Z. Tuba, R. Martínez-Carrasco, Restoration of Photosystem II photochemistry and carbon assimilation and related changes in chlorophyll and protein contents during the rehydration of desiccated *Xerophyta scabrifolia* leaves, J. Exp. Bot. 62 (2011) 895-905.
- [38] S.P. Long, C.J. Bernacchi, Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error, J. Exp. Bot. 392 (2003) 2393-2401.
- [39] A. Alonso, P. Pérez, R. Martínez-Carrasco, Growth in elevated CO₂ enhances temperature response of photosynthesis in wheat, Physiol. Plant. 135 (2009) 109–120.
- [40] G.D. Farquhar, S. von Caemmerer, J.A. Berry, A biochemical model of photosynthetic CO₂ assimilation in leaves of C₃ species, Planta 149 (1980) 78–90.
- [41] L. Gu, S.G. Pallardy, K. Tu, B.E. Law, S.D. Wullschleger, Reliable estimation of biochemical parameters from C₃ leaf photosynthesis-intercellular carbon dioxide response curves, Plant Cell Environ. 33 (2010) 1852-1874.

- [42] D.I. Arnon Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*, Plant Physiol. 24 (1949) 1-15.
- [43] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
- [44] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265-275.
- [45] R. Morcuende, S. Kostadinova, P. Pérez, I.M. Martín del Molino, R. Martínez-Carrasco, Nitrate is a negative signal for fructan synthesis, and the fructosyltransferase-inducing trehalose inhibits nitrogen and carbon assimilation in excised barley leaves, New Phytol. 161 (2004) 749-759.
- [46] P.A. Cawse, The determination of nitrate in soil solutions by ultraviolet spectrophotometry, Analyst 92 (1967) 311-315.
- [47] P.E. Hare, Subnanomole-range amino acid analysis, Method. Enzymol. 47 (1977) 3-18.
- [48] M.G.K. Jones, W.H. Outlaw, O.H. Lowry, Enzymic assay of 10^{-7} to 10^{-14} moles of sucrose in plant tissues, Plant Physiol. 60 (1977) 379-383.
- [49] R. Morcuende, A. Krapp, V. Hurry, M. Stitt, Sucrose-feeding leads to increased rates of nitrate assimilation, increased rates of α -oxoglutarate synthesis, and increased synthesis of a wide spectrum of amino acids in tobacco leaves, Planta 206 (1998) 394-409.
- [50] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C_T method, Nat. Protoc. 3 (2008) 1101-1108.
- [51] J. Papenbrock, B. Grimm, Regulatory network of tetrapyrrole biosynthesis- studies of intracellular signalling involved in metabolic and developmental control of plastids, Planta 213 (2001) 667-681.
- [52] N. La Rocca, N. Rascio, U. Oster, W. Rüdiger, Amitrole treatment of etiolated barley seedlings leads to deregulation of tetrapyrrole synthesis and to reduced expression of *Lhc* and *RbcS* genes, Planta 213 (2001) 101-108.
- [53] D.Z. Habash, J.P. Matthew, M.A.J. Parry, A.J. Keys, D.W. Lawlor, Increased capacity for photosynthesis in wheat grown at elevated CO_2 : the relationship between electron transport and carbon metabolism, Planta 197 (1995) 482–489.
- [54] M. Rott, N.F. Martins, W. Thiele, W. Lein, R. Bock, D.M. Kramer, M.A. Schöttler, ATP synthase repression in tobacco restricts photosynthetic electron transport, CO_2 assimilation, and plant growth by overacidification of the thylakoid lumen, Plant Cell 23 (2011) 304-321.

- [55] M. Suorsa, S. Sirpiö, Y. Allahverdiyeva, V. Paakkarinen, F. Mamedov, S. Styring, E.-M. Aro, PsbR, a missing link in the assembly of the oxygen-evolving complex of plant Photosystem II, *J. Biol. Chem.* 281 (2006) 145-150.
- [56] X. Yi, S.R. Hargett, L.K. Frankel, T.M. Bricker, The effects of simultaneous RNAi suppression of PsbO and PsbP protein expression in photosystem II of *Arabidopsis*, *Photosynth. Res.* 98 (2008) 439-448.
- [57] K. Ifuku, Y. Yamamoto, T. Ono, S. Ishihara, F. Sato, PsbP protein, but not PsbQ protein, is essential for the regulation and stabilization of Photosystem II in higher plants, *Plant Physiol.* 139 (2005) 1175-1184.
- [58] J.P. Dekker, E.J. Boekema, Supramolecular organization of thylakoid membrane proteins in green plants, *Biochim. Biophys. Acta* 1706 (2005) 12-39.
- [59] N.R. Baker, J. Harbinson, D.M. Kramer, Determining the limitations and regulation of photosynthetic energy transduction in leaves, *Plant Cell Environ.* 30 (2007) 1107-1125.
- [60] K. Yoshida, C.K. Watanabe, I. Terashima, K. Noguchi, Physiological impact of mitochondrial alternative oxidase on photosynthesis and growth in *Arabidopsis thaliana*, *Plant Cell Environ.* 34 (2011) 1890-1899.
- [61] J. Córdoba, J.L. Molina-Cano, P. Pérez, R. Morcuende, M. Moralejo, R. Savé, R. Martínez-Carrasco, Photosynthesis-dependent/independent control of stomatal responses to CO₂ in mutant barley with surplus electron transport capacity and reduced SLAH3 anion channel transcript, *Plant Sci.* 239 (2015) 15-25.
- [62] Y. Suzuki, A. Makino, T. Mae, Changes in the turnover of Rubisco and levels of mRNAs of *rbcL* and *rbcS* in rice leaves from emergence to senescence, *Plant Cell Environ.* 24 (2001) 1353-1360.
- [63] K. Imai, Y. Suzuki, A. Makino, T. Mae, Effects of nitrogen nutrition on the relationships between the levels of *rbcS* and *rbcL* mRNAs and the amount of ribulose 1·5-bisphosphate carboxylase/oxygenase synthesized in the eighth leaves of rice from emergence through senescence, *Plant Cell Environ.* 28 (2005) 1589-1600.
- [64] W.-R. Scheible, A. González-Fontes, R. Morcuende, M. Lauerer, M. Geiger, J. Glaab, A. Gojon, E.-D. Schulze, M. Stitt, Tobacco mutants with a decreased number of functional *nia* genes compensate by modifying the diurnal regulation of transcription, post-translational modification and turnover of nitrate reductase, *Planta* 203 (1997) 304-319.
- [65] K. Yamaguchi, A.R. Subramanian, Proteomic identification of all plastid-specific ribosomal proteins in higher plant chloroplast 30S ribosomal subunit, *Eur. J. Biochem.* 270 (2003) 190-205.

- [66] H. Ishida, Y. Nishimori, M. Sugisawa, A. Makino, T. Mae, The large subunit of Ribulose-1,5-bisphosphate carboxylase/oxygenase is fragmented into 37-kDa and 16-kDa polypeptides by active oxygen in the lysates of chloroplasts from primary leaves of wheat, *Plant Cell Physiol.* 38 (1997) 471-479.
- [67] N. Kokubun, H. Ishida, A. Makino, T. Mae, The degradation of the large subunit of Ribulose-1,5-bisphosphate Carboxylase/oxygenase into the 44-kDa fragment in the lysates of chloroplasts incubated in darkness, *Plant Cell Physiol.* 43 (2002) 1390-1395.
- [68] Y. Marcus, H. Altman-Gueta, A. Finkler, M. Gurevitz, Dual role of cysteine 172 in redox regulation of Ribulose 1,5-bisphosphate carboxylase/oxygenase activity and degradation. *J. Bacteriol.* 185 (2003) 1509-1517.
- [69] A. Prins, P.D.R. van Heerden, E. Olmos, K.J. Kunert, C.H. Foyer, Cysteine proteinases regulate chloroplast protein content and composition in tobacco leaves: a model for dynamic interactions with ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) vesicular bodies, *J. Exp. Bot.* 59 (2008) 1935-1950.
- [70] U. Feller, I. Anders, T. Mae, (2008) Rubiscolytics: fate of Rubisco after its enzymatic function in a cell is terminated. *Journal of Experimental Botany* 59: 1615-1624.
- [71] J. Preiss, Biosynthesis of starch and its regulation. In: J. Preiss (Ed.), *The Biochemistry of Plants*, Vol. 14, Academic Press, San Diego, CA, 1988, pp. 181–254.
- [72] W. Wagner, A. Wiemken, P. Matile, Regulation of fructan metabolism in leaves of barley (*Hordeum vulgare* L. cv. Gerbel), *Plant Physiol.* 81 (1986) 444-447.
- [73] A. Miller, C. Schlaghaufer, M. Spalding, S. Rodermeil, Carbohydrate regulation of leaf development: prolongation of leaf senescence in Rubisco antisense mutants of tobacco, *Photosynth. Res.* 63 (2000) 1-8.
- [74] C.A. Raines, M.J. Paul, Products of leaf primary carbon metabolism modulate the developmental programme determining plant morphology, *J. Exp. Bot.* 57 (2006) 1857-1862.
- [75] W.P. Quick, U. Schurr, R. Scheibe, E.D. Schulze, S.R. Rodermeil, L. Bogorad, M. Stitt, Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" *rbcS*. I. Impact on photosynthesis in ambient growth conditions, *Planta* 183 (1991) 542-554.

Legends to figures

Fig. 1. Rate of CO₂ assimilation limited by Rubisco (A_c , thick lines) and by RuBP regeneration (A_j , thin line) in barley (*Hordeum vulgare* L.). Measured and fitted values for the G132 mutant (closed symbols, broken line) and Graphic WT (open symbols, solid lines). There was no RuBP-limited CO₂ assimilation in G132 (see text for details). Curves were fitted with the LeafWeb (leafweb.ornl.gov) utility to five replicate leaves per genotype and the mean values are presented. Measurements in (a, c) the youngest fully expanded leaf (leaf *a*), and (b, d) the penultimate (leaf *b*) and (e) antepenultimate (leaf *c*) leaves of plants with (a, b) 3-4 leaves unfolded (13-14 growth stage, Zadoks scale) and (c-e) 5-7 leaves unfolded (15-17 stage), with the WT being more developed. Vertical bars represent twice the standard error of means. The corresponding V_{cmax} and J values and probability in the analysis of variance are shown in Table 2.

Fig. 2. Chl content (mg g Fwt⁻¹) and Chl *a*/Chl *b* ratios of the youngest fully expanded leaf (leaf *a*, black columns) and the two preceding leaves (leaf *b*, white columns, and leaf *c*, grey columns) of Graphic barley (WT) and its G132 mutant with (a) 3-4 leaves unfolded (13-14 growth stage, Zadoks scale) and (b) 5-7 leaves unfolded (15-17 stage), with the WT being more developed. Within each group, columns with the same letter are not significantly different ($P < 0.05$).

Fig. 3. Up- and down-regulated genes in G132 relative to Graphic barley (WT) and in the youngest fully expanded leaf compared to the two preceding leaves at growth stages 1 (3-4 leaves unfolded, 13-14 stage of Zadoks scale) and 2 (5-7 leaves unfolded, 15-17 stage of Zadoks scale). Data from Supplementary Tables 2 and 3, where the statistical significance of the differences is shown.

Table 1

Chlorophyll fluorescence parameters in the youngest fully expanded leaf (leaf *a*) and the two preceding leaves (leaves *b* and *c*) of Graphic barley (WT) and its G132 mutant with 3-4 leaves unfolded (13-14 growth stage, Zadoks scale) and 5-7 leaves unfolded (15-17 stage), with the WT being more developed. Measurements under ambient CO₂ concentration (390 $\mu\text{mol mol}^{-1}$) and either darkness (Fv/Fm) or 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance. Isd, least significant difference. Numbers in bold type represent significant effects ($P < 0.05$). When an interaction is present, the significance of a main factor effect may depend on the level of the other main factor.

Growth Stage		Genotype						Isd		
		G132			WT			Genotype	Leaf	Gen. x Leaf
		a	b	c	a	b	c			
13-14	Fv/Fm	0.52	0.73	0.72	0.81	0.80	0.81	0.058	0.072	0.101
	qL	0.43	0.42	0.37	0.43	0.39	0.34	0.075	0.092	0.130
	Fq'/Fm'	0.14	0.22	0.24	0.36	0.35	0.35	0.026	0.032	0.045
	Φ_{NPQ}	0.73	0.68	0.63	0.50	0.52	0.49	0.047	0.058	0.082
15-17	Fv/Fm	0.61	0.70	0.74	0.81	0.81	0.79	0.033	0.040	0.057
	qL	0.54	0.49	0.35	0.60	0.43	0.42	0.095	0.116	0.164
	Fq'/Fm'	0.17	0.19	0.18	0.41	0.36	0.34	0.048	0.059	0.084
	Φ_{NPQ}	0.70	0.70	0.71	0.48	0.52	0.51	0.048	0.059	0.083

Table 2

Maximum Rubisco rate of carboxylation (V_{cmax} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), potential rate of electron transport (J , $\mu\text{mol m}^{-2} \text{s}^{-1}$), mitochondrial respiration rate in the light (R_d) rate of CO_2 assimilation (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$), intercellular CO_2 concentration (C_i , $\mu\text{mol mol}^{-1}$), stomatal conductance (g_s , $\text{mmol m}^{-2} \text{s}^{-1}$) and transpiration (E , $\text{mmol m}^{-2} \text{s}^{-1}$) in the youngest fully expanded leaf (leaf *a*) and the two preceding leaves (leaves *b* and *c*) of Graphic barley (WT) and its G132 mutant with 3-4 leaves unfolded (13-14 growth stage, Zadoks scale) and 5-7 leaves unfolded (15-17 stage), with the WT being more developed. Measurements with air CO_2 concentrations of 390 and 1300 $\mu\text{mol mol}^{-1}$, 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance, 20 °C and 0.95 kPa vapour pressure deficit. lsd, least significant difference. Numbers in bold type represent significant effects ($P < 0.05$). When an interaction is present, the significance of a main factor effect may depend on the level of the other main factor.

		Genotype						lsd		
Growth Stage		G132			WT			Genotype	Leaf	Gen. x Leaf
		a	b	c	a	b	c			
13-14	V_{cmax}	18.5	23.2		45.2	51.1		12.07	12.07	17.06
	J	-	-		106.7	102.4			12.62	
	R_d	1.69	1.66		2.87	2.81		1.334	1.334	1.887
	A_{390}	5.6	7.4		13.6	12.5		2.30	2.30	3.26
	C_{i390}	334	327		245	262		9	9	12
	g_{s390}	289	314		205	217		59	59	83
	E_{390}	2.6	3.1		2.1	2.1		0.62	0.62	0.87
	A_{1300}	11.4	15.5		21.9	20.4		2.21	2.21	3.12
	C_{i1300}	1293	1250		1203	1202		24	24	34
	g_{s1300}	334	285		271	254		56	56	79
	E_{1300}	2.8	2.9		2.5	2.3		0.55	0.55	0.77
15-17	V_{cmax}	24.4	24.4	21.6	69.3	64.1	58.6	8.43	10.32	14.6
	J	-	-	-	112.9	107.6	98.9		14.16	
	R_d	2.63	2.6	2.62	6.05	5.42	5.36	0.744	0.911	1.289
	A_{390}	6.6	5.0	3.8	9.3	4.3	5.1	0.99	1.21	1.71
	C_{i390}	318	300	317	214	236	256	20	25	35
	g_{s390}	197	105	107	99	48	65	28	34	48
	E_{390}	2.1	1.4	1.5	1.5	0.9	1.3	0.29	0.36	0.51
	A_{1300}	15.3	14.6	12.5	19.6	18.2	16.5	1.66	2.04	2.88
	C_{i1300}	1214	1246	1238	1027	1035	1088	56	69	97
	g_{s1300}	160	171	156	102	96	109	26	32	45
	E_{1300}	1.8	2.0	2.0	1.6	1.7	2.0	0.31	0.38	0.54

Table 3

Content of proteins, amino acids and nitrate (mg g Fwt^{-1}), and of a 33 kDa polypeptide as a percentage of Rubisco large subunit protein, in the youngest fully expanded leaf (leaf *a*) and the two preceding leaves (leaves *b* and *c*) of Graphic barley (WT) and its G132 mutant with 3-4 leaves unfolded (13-14 growth stage, Zadoks scale) and 5-7 leaves unfolded (15-17 stage), with the WT being more developed. Lsd, least significant difference. Numbers in bold type represent significant effects ($P < 0.05$). When an interaction is present, the significance of a main factor effect may depend on the level of the other main factor.

Growth Stage		Genotype						Lsd		
		G132			WT			Genotype	Leaf	Gen. x Leaf
		a	b	c	a	b	c			
13-14	Rubisco protein	1.4	2.5	0.95	6	4.6	5.1	0.45	0.52	0.77
	Soluble protein	7.1	7.3	5.7	11.0	12.2	10.8	0.52	0.64	0.9
	Membrane protein	6.8	9.0	5.2	8.9	9.5	7.2	1.00	1.23	1.73
	Soluble-Rbco protein	5.8	3.7	5.9	5.3	6.8	6.2	0.86	1.05	1.48
	Total protein	13.8	16.4	10.9	19.9	21.8	18	1.11	1.36	1.92
	33 kDa	24.9	42.8	41.9	19.8	22	39.4	9.38	11.49	16.25
	Amino acids	13.7	14.8	11.4	25.4	24.3	23.1	3.08	3.77	5.33
	Nitrate	161	135	126	147	161	131	16.4	20.0	28.3
15-17	Rubisco protein	1.6	1.6	1.1	5.3	3.3	2.3	0.8	0.98	1.39
	Soluble protein	7.5	6.2	6	12.4	8.7	6.4	1.01	1.24	1.75
	Membrane protein	2.9	3.2	3.1	8.7	5.0	3.6	1.14	1.40	1.98
	Soluble-Rbco protein	5.9	4.9	5.1	7.1	5.4	4.1	1.08	1.33	1.87
	Total protein	10.5	9.4	9.1	21.1	13.7	10.0	1.73	2.12	3.00
	33 kDa	35.1	37.7	46.3	8.6	11.3	36.0	7.95	9.74	13.77
	Amino acids	12.5	8.2	8.2	15.6	11.4	9.1	2.43	2.98	4.21
	Nitrate	96.1	101.1	94.2	55.0	37.5	38.4	13.07	16.00	22.63

Table 4

Contents ($\mu\text{mol g Fwt}^{-1}$) of carbohydrates (glucose, Glc; fructose, Fru; fructans, Ftn; starch) and Fru/Glc ratio of Ftn in the youngest fully expanded leaf (leaf *a*) and the two preceding leaves (leaves *b* and *c*) of Graphic barley (WT) and its G132 mutant with 3-4 leaves unfolded (13-14 growth stage, Zadoks scale) and 5-7 leaves unfolded (15-17 stage), with the WT being more developed. Starch is expressed as Glc equivalents and Ftn as Glc plus Fru equivalents. Isd, least significant difference. Numbers in bold type represent significant effects ($P < 0.05$). When an interaction is present, the significance of a main factor effect may depend on the level of the other main factor.

Growth Stage		Genotype						Isd		
		G132			WT			Genotype	Leaf	Gen. x Leaf
		a	b	c	a	b	c			
13-14	Glc	-	-	-	-	-	-			
	Fru	-	-	-	-	-	-			
	Sac	5.6	7.6	4.9	13.9	15.9	16.7	2.01	2.46	3.48
	Ftn (Glu+Fru)	0.92	1.32	1.05	1.94	2.51	3.1	0.765	0.937	1.325
	Ftn(Fru)/Ftn(Glc)	0.5	2.8	0.9	3.7	2.3	2.0	1.56	1.91	2.70
	Starch	0.75	1.74	0.93	2.71	4.00	4.10	0.577	0.707	1.000
15-17	Glc	3.4	1.7	0.9	6.3	7.3	5.5	1.31	1.60	2.26
	Fru	3.1	1.7	1.1	6.6	10.2	9.5	2.24	2.75	3.88
	Sac (Glu)	9.1	9.2	9.0	20.7	18.1	12.9	1.94	2.38	3.37
	Ftn (Glu+Fru)	13.9	14.1	16.9	26.2	30.7	17.5	10.98	13.44	19.01
	Ftn(Fru)/Ftn(Glc)	1.8	2.3	1.9	3.4	4.0	3.8	1.10	1.34	1.90
	Starch	0.33	0.28	0.49	7.34	3.80	1.18	0.847	1.037	1.467

Table 5

Initial and total Rubisco activities ($\text{nmol s}^{-1} \text{g Fwt}^{-1}$), maximal (max) and selective (sel) nitrate reductase activities (NR, $\mu\text{mol h}^{-1} \text{g Fwt}^{-1}$) and activation state of the enzymes (%) in the youngest fully expanded leaf (leaf *a*) and the two preceding leaves (leaves *b* and *c*) of Graphic barley (WT) and its G132 mutant with 3-4 leaves unfolded (13-14 growth stage, Zadoks scale) and 5-7 leaves unfolded (15-17 stage), with the WT being more developed. Isd, least significant difference. Numbers in bold type represent significant effects ($P < 0.05$). When an interaction is present, the significance of a main factor effect may depend on the level of the other main factor.

Growth Stage		Genotype						Isd		
		G132			WT			Genotype	Leaf	Gen. x Leaf
		a	b	c	a	b	c			
13-14	Initial Rubisco	35.5	52.9	41.9	85.0	71.0	86.7	11.01	13.49	19.08
	Total Rubisco	53.6	69.9	41.5	139.6	123.4	153.4	22.11	27.07	38.29
	Rbco activation	76.9	76.4	103.8	61.8	56.5	59	13.06	15.99	22.61
	NR max	25.7	29.7	23.6	33.8	34.4	33.9	2.13	2.61	3.69
	NR sel	22.7	27.2	19.5	33.0	33.3	31.7	2.45	3	4.243
	NR activation	88.0	91.5	82.8	97.6	96.8	93.5	4.16	5.09	7.20
15-17	Initial Rubisco	59	41.3	31.3	125.5	97.3	48.1	19.72	24.15	34.16
	Total Rubisco	57.6	40.8	32.2	140.9	102.9	48.6	20.46	25.05	35.43
	Rbco activation	102.3	100.9	99.9	89.4	93.4	99.2	6.83	8.37	11.84
	NR max	13.1	12.6	11.8	1.73	2.04	2.80	1.46	1.79	2.53
	NR sel	11.1	11.7	10.3	1.42	1.61	2.31	0.67	0.82	1.16
	NR activation	85.2	92.0	87.2	79.0	75.6	79.6	7.84	9.6	13.58

Table 6

Dry weight (D wt) and yield components in G132 and Graphic barley (WT) at the 15-17 Zadoks' growth stage, with the WT being more developed, and at maturity. lsd, least significant difference. Numbers in bold type represent significant effects ($P < 0.05$).

		G132	WT	lsd
15-17 stage	Total D wt g/plant	1.0	4.2	0.46
Maturity	No. ears/plant	10.8	13.2	2.59
	No. grains/ear	18.5	17.0	2.51
	D wt per grain, mg	45.2	44.6	3.22
	Grain D wt, g/plant	8.9	9.9	1.66
	Glumes and rachis D wt, g/plant	2.1	2.5	0.47
	Ear D wt, g/plant	11.0	12.5	0.90
	Straw D wt, g/plant	12.1	13.4	2.30
	Total D wt, g/plant	23.1	25.9	4.22
	Harvest index	0.39	0.38	0.024
	Days to maturity	224	208

Fig. 1

Fig. 2

Fig. 3

Supplementary information

Functional and transcriptional characterization of a barley mutant with impaired photosynthesis

Javier Córdoba^{a,b}, José-Luis Molina-Cano^{b,1}, Rafael Martínez-Carrasco^a, Rosa Morcuende^a, Pilar Pérez^{a*},

^aInstitute of Natural Resources and Agrobiology of Salamanca, IRNASA-CSIC, Cordel de Merinas 40, E-37008 Salamanca, Spain

^bIRTA (Institute for Food and Agricultural Research and Technology), Field Crops, Av. Alcalde Rovira i Roure, 191, E-25198 Lérida, Spain

¹Retired

*Corresponding author:

Pilar Pérez

Phone: +34 923 219606

E-mail address: pilar.perez@irnasa.csic.es

Supplementary Table 1. List of genes selected for qRT-PCR and their corresponding primer pairs. The GenBank accessions, unigene codes from HarvEST: Barley v.1.83, Assembly 35 database, database descriptions, and forward (F) and reverse (R) primers designed are shown.

GenBank	Unigene	Description	Sequence (5'-3')
AK356022	189	photosystem II light harvesting complex protein 1 (LHCII CAB-1)	F CATCCCCTCACGGCTTTCTT R CGCCGCCATTGTAGAGCTAA
AK361860	28165	photosystem II light harvesting complex protein 2 (LHCII CAB-2)	F CGCCACCAACTTTGTTCTG R ATCGAAGGCGGGCAAATCTT
AK365564	15169	photosystem II subunit R (PsbR)	F GCGGATTATAACCGTCAGGACA R TGTGAGAGAGCTTAGCACTGAA
AK360942	26681	photosystem II subunit Q (PsbQ)	F AAAGGGGACTACGCAGAAGC R AGCTCTTGATCCGGCAAACA
AK252670	6275	photosystem II subunit P (PsbP)	F GACCTAGGCCCTCTGAGAA R ATAGAGCTTGCCATCGTCCG
KC912689	31646	photosystem I apoprotein A1 (PsaA)	F CGCAAGGAAAGCGAAAACCT R ATTTGCTCGGAGTTCCCGTT
AGP50910	34138	photosystem I apoprotein A2 (PsaB)	F CATTGAAAGCGGGGCCATTC R TGCTCATGGCAAGACGACAT
AGP50919	38001	Rubisco large subunit (RbcL)	F ACGTGCTCTACGTTTGAGG R GCGGGCCTTGAAAGTTTTT
U43493		Rubisco small subunit (RbcS)	F ACCAACATGCTCGAGAAAGCA R GTGTGGCGTGCAAAGATGT
AK357958	2595	fructan exohydrolase (FEH)	F GTCAGAGGAGGAACACGCAC R GGCGGTCGAGTCTGTCATAA
AK253058	1173	sucrose: fructan 6-fructosyltransferase (6-SFT)	F GTCCCGGAACCTTGCCAAT R GGTAGCACCGTCATAGAGCC
AK366020	15653	sucrose: sucrose 1-fructosyltransferase (1-SST)	F GGCCAGGAAACAATCTACCCA R GGGATGAGAATGACGCGAGA
AK369112	Contig15936_at	alternative oxidase 1A	F CCGCTCGGGTACCACTAATC R TATGGTCCGTTGAACCAACCG
AK358343	31642	xyloglucan endotransglycosylase 3	F AACACAAGTTTCCATCCAGCA R GTTGCGTGCTCCCAAAAGTA
AK248297	17079	glycerophosphodiester phosphodiesterase	F TTTCTGACTCCAGTGACGC R GTCTGGAAGGCAGATGCCAA
AK358128	17981	nitrate reductase [NADH]	F ACCGATCGATGTTTTGCCCT R ACAAACACACCCTCAATCGGA
X15869	14488	protochlorophyllide oxidoreductase A (PORA)	F CGTGTAAGGAGCTGGAACA R GGATTTGCGGTGGATCATGC
AK359654	1389	cell wall constituent	F CATAGATCGAGCGGTGGCTA R AATCCGGGCCATCATGCTC
AGP50941	13021	plastid RNA polymerase alpha subunit	F TGCTTCTCTAGAGTGCCCA R TGAGAATTGACAGTTTCGTATGGA
AK357412	15515	DNA-dependent ATPase	F GAAGGGCGTGGGATCATCAA R GGTCACCTCGCTCTTGAACA

GenBank	Unigene	Description	Sequence (5'-3')
AK354750	14051	histone H3	F TCACCATCATGCCCAAGGAC R CGCAGCTGACACATCACAGA
AGP50948	8551	ribosomal protein S3 (chloroplast)	F TGTTTGGAGGGGAAGTCTGC R GGGTCGTCTCGGAGGAAAAG
AGP50947	16486	ribosomal protein L16 (chloroplast)	F AGTGTAACCGGTTTGTCTGGG R ATATGCACGTCGTGGTGGAA
AK369833	20431	threonine-protein kinase HT1-like	F CAACGGCAACGCTTACAACA R TCACAGCAGTAGACCTCCCA
AK365098	1989	leucine-rich repeat family protein	F TAATCGTCACGCCCTGGAAT R ACGAGTTGTTGCGCTGAAGA
AK368060	38703	SLAH3 C4-dicarboxylate transporter	F CCAACACAAGCAGCAAGACC R GAAGCCGTCGAGATGGGAAA

Supplementary Table 2. Level of gene expression in the youngest fully expanded leaf (leaf *a*) and the two preceding leaves (leaves *b* and *c*) of Graphic barley (WT) and its G132 mutant with 3-4 leaves unfolded (13-14 growth stage, Zadoks scale), with the WT being more developed. In a heat map, the numbers indicate the change in expression ($\log_2$ ratios) between genotypes (WT as reference), between leaves (leaf *a* as reference) and in the genotype x leaf interaction (WT leaf *a* as reference); white indicates no change, blue up-regulation and red down-regulation, as shown in the color bar for a $\log_2$ scale. The functional categories of genes were annotated with Mercator. *P*, probability in the analysis of variance for genotype, leaf and genotype x leaf (G x L) interaction. Numbers in bold type represent significant effects ($n=5$; $P<0.05$).

		<div><div></div><div>-44</div></div>												
		Genotype		Leaf		G x L						<i>P</i>		
Functional category	Name	G132/WT	leaf <i>b</i>	leaf <i>c</i>	leaf <i>a</i>	leaf <i>b</i>	leaf <i>c</i>	leaf <i>b</i>	leaf <i>c</i>	Genotype	Leaf	G x L		
PS.lightreaction.photosystem II.LHC-II	LHCII CAB-1	2.12	-2.91	-2.27	1.85	-1.02	-0.12	-3.02	-4.44	<.001	<.001	0.025		
PS.lightreaction.photosystem II.LHC-II	LHCII CAB-2	1.40	-1.36	-1.72	0.86	-0.14	-0.45	-2.39	-3.07	<.001	<.001	0.841		
PS.lightreaction.photosystem II.PSII polypeptide	psbR	-1.41	1.26	1.32	-1.98	-1.05	0.37	1.34	0.89	<.001	<.001	0.002		
PS.lightreaction.photosystem II.PSII polypeptide	psbQ	-0.53	0.46	0.35	-0.19	0.17	-0.30	0.55	0.66	0.006	0.098	0.148		
PS.lightreaction.photosystem II.PSII polypeptide	psbP	1.16	0.25	0.11	0.48	0.98	1.03	-0.18	-0.88	<.001	0.108	<.001		
PS.lightreaction.photosystem I.PSI polypeptide	psaA	2.68	-0.12	-0.45	2.24	2.25	1.86	-1.05	-0.86	<.001	0.029	0.108		
PS.lightreaction.photosystem I.PSI polypeptide	psaB	1.88	-0.29	-0.48	1.44	1.37	1.09	-1.07	-0.88	<.001	0.019	0.308		
PS.calvin cycle.rubisco large subunit	rbcL	1.26	-0.43	-0.17	0.63	0.45	0.85	-0.94	-1.14	<.001	0.031	0.006		
PS.calvin cycle.rubisco small subunit	rbcS	0.17	-0.42	-0.56	-0.37	-0.37	-0.45	-0.85	-1.06	0.334	0.028	0.051		
major CHO metabolism	FEH	-0.68	-0.78	-1.76	-1.08	-1.38	-2.27	-1.08	-2.15	0.027	<.001	0.061		
major CHO metabolism	6-SFT	-2.69	0.03	-0.48	-5.55	-2.04	-3.17	-0.32	-0.69	<.001	0.214	0.045		
major CHO metabolism	1-SST	-2.50	0.05	0.12	-3.35	-2.08	-2.47	-0.15	0.01	<.001	0.957	0.731		
mitochondrial electron transport	alternative oxidase 1A	-1.25	1.65	2.31	0.58	1.67	1.05	2.21	3.38	<.001	<.001	<.001		

		G x L								P		
		Genotype	Leaf		G132			WT		Genotype	Leaf	G x L
Functional category	Name	G132/WT	leaf b	leaf c	leaf a	leaf b	leaf c	leaf b	leaf c			
cell wall.modification	xyloglucan endotransglycosylase 3	-0.47	-3.63	-3.43	-0.59	-5.27	-2.95	-3.20	-5.39	0.14	<.001	0.052
lipid degradation.lysophospholipases	glycerophosphodiester phosphodiesterase	-0.01	-0.86	-1.22	0.16	-1.05	-1.03	-0.55	-1.23	0.957	0.001	0.469
N-metabolism.nitrate metabolism.NR	nitrate reductase [NADH]	-0.14	-0.59	-0.26	-0.07	-1.00	-0.21	-0.34	-0.38	0.205	0.001	0.057
tetrapyrrole synthesis	PORA	-4.84	-1.44	-3.54	-5.34	-6.16	-6.45	-1.45	-3.71	<.001	<.001	<.001
misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	cell wall constituent	0.31	0.25	0.34	0.34	0.83	0.37	-0.15	0.66	0.245	0.579	0.164
RNA.transcription	plastid RNA polymerase alpha subunit	2.11	-0.96	-0.21	2.19	1.05	2.05	-0.32	-0.56	<.001	0.021	0.032
DNA.synthesis/chromatin structure	DNA-dependent ATPase	-3.15	-0.86	-0.49	-3.70	-3.27	-3.91	-1.03	-0.51	<.001	0.244	0.193
DNA.synthesis/chromatin structure.histone	histone H3	-2.13	1.20	1.96	-1.19	-1.00	-0.02	1.49	2.20	0.003	0.038	0.136
protein.synthesis.ribosomal	ribosomal protein S3 (chloroplast)	2.42	-0.17	0.56	2.08	1.99	2.73	-0.54	0.12	<.001	<.001	0.005
protein.prokaryotic.chloroplast.30S subunit	ribosomal protein L16 (chloroplast)	2.36	-0.70	-0.09	2.24	1.52	2.22	-0.65	-0.46	<.001	0.041	0.130
protein.prokaryotic.chloroplast.50S subunit	threonine-protein kinase HT1-like	-0.86	-1.00	-1.10	-1.18	-1.87	-1.77	-1.17	-1.40	0.002	<.001	0.040
protein.postranslational modification	leucine-rich repeat family protein	0.04	0.16	0.59	-0.36	-0.01	0.58	-0.01	0.26	0.783	0.003	0.120
transport.metabolite transporters	SLAH3 C4-dicarboxylate transporter	-0.62	0.14	0.60	-0.13	0.12	-0.46	0.02	1.12	0.021	0.109	0.009

Supplementary Table 3. Level of gene expression in the youngest fully expanded leaf (leaf *a*) and the two preceding leaves (leaves *b* and *c*) of Graphic barley (WT) and its G132 mutant with 5-7 leaves unfolded (15-17 growth stage, Zadoks scale), with the WT being more developed. In a heat map, the numbers indicate the change in expression ($\log_2$ ratios) between genotypes (WT as reference), between leaves (leaf *a* as reference) and in the genotype x leaf interaction (WT leaf *a* as reference); white indicates no change, blue up-regulation and red down-regulation, as shown in the color bar for a $\log_2$ scale. The functional categories of genes were annotated with Mercator. *P*, probability in the analysis of variance for genotype, leaf and genotype x leaf (G x L) interaction. Numbers in bold type represent significant effects ($n=5$; $P<0.05$).

Functional category	Name	G x L										
		Genotype	Leaf		G132			WT		<i>P</i>		
		G132/WT	leaf <i>b</i>	leaf <i>c</i>	leaf <i>a</i>	leaf <i>b</i>	leaf <i>c</i>	leaf <i>b</i>	leaf <i>c</i>	Genotype	Leaf	G x L
PS.lightreaction.photosystem II.LHC-II	LHCII CAB-1	2.80	-4.19	-3.67	3.47	-1.32	-1.08	-2.04	-1.04	<.001	<.001	<.001
PS.lightreaction.photosystem II.LHC-II	LHCII CAB-2	3.68	-2.30	-3.05	3.46	1.24	0.46	-3.68	-3.58	<.001	<.001	0.002
PS.lightreaction.photosystem II.PSII polypeptide	psbR	2.80	0.81	1.58	3.73	4.35	5.25	2.19	2.23	<.001	<.001	0.007
PS.lightreaction.photosystem II.PSII polypeptide	psbQ	-1.83	-0.62	1.25	-1.48	-1.90	-0.80	-0.71	1.41	<.001	<.001	<.001
PS.lightreaction.photosystem II.PSII polypeptide	psbP	1.74	-1.14	-0.78	1.09	0.21	0.69	-1.93	-2.29	<.001	0.008	0.603
PS.lightreaction.photosystem I.PSI polypeptide	psaA	2.21	-0.78	-1.44	1.96	1.29	0.59	-1.32	-1.77	<.001	<.001	0.004
PS.lightreaction.photosystem I.PSI polypeptide	psaB	2.60	0.07	-0.22	3.80	3.78	3.26	0.94	1.68	<.001	0.591	0.076
PS.calvin cycle.rubisco large subunit	rbcL	2.23	-0.60	0.09	3.27	2.67	2.98	-0.51	1.78	<.001	0.270	0.307
PS.calvin cycle.rubisco small subunit	rbcS	0.81	-1.38	-1.52	0.31	-0.58	-0.82	-2.38	-2.19	0.004	<.001	0.685
major CHO metabolism	FEH	1.73	0.32	1.28	2.13	2.57	3.18	-0.32	2.02	<.001	0.001	0.552
major CHO metabolism	6-SFT	2.05	-0.55	0.07	3.20	2.62	2.82	-0.35	1.87	<.001	0.296	0.183
major CHO metabolism	1-SST	0.04	0.08	0.06	0.05	0.14	0.09	0.06	0.08	0.723	0.870	0.973
mitochondrial electron transport	alternative oxidase 1A	-4.70	0.81	1.05	-5.33	-4.70	-3.09	0.81	1.00	<.001	0.019	0.045

Functional category	Name	G x L										
		Genotype	Leaf		G132			WT		P		
		G132/WT	leaf b	leaf c	leaf a	leaf b	leaf c	leaf b	leaf c	Genotype	Leaf	G x L
cell wall.modification	xyloglucan endotransglycosylase	0.75	-1.29	-1.94	1.45	-0.51	-1.08	-0.28	-1.02	0.044	<.001	0.010
lipid degradation.lysophospholipases	glycerophosphodiester phosphodiesterase	-0.41	0.30	1.58	1.10	1.55	0.97	-0.10	2.89	0.098	<.001	<.001
N-metabolism.nitrate metabolism.NR	nitrate reductase [NADH]	2.61	-0.68	-1.46	2.64	1.97	1.13	-0.74	-1.22	<.001	<.001	<.001
tetrapyrrole synthesis	PORA	0.06	-1.34	-1.29	0.37	-1.09	-1.51	-1.19	-0.77	0.688	<.001	0.032
misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	cell wall constituent	-0.03	0.85	2.50	4.67	5.55	5.25	-0.58	6.79	0.898	<.001	<.001
RNA.transcription	plastid RNA polymerase alpha subunit	1.72	-1.13	-1.25	1.57	0.44	0.48	-1.12	-1.88	<.001	<.001	0.155
DNA.synthesis/chromatin structure	DNA-dependent ATPase	-2.43	0.62	4.04	-0.09	0.60	1.71	0.51	4.83	<.001	<.001	<.001
DNA.synthesis/chromatin structure.histone	histone H3	-2.30	0.71	5.13	-0.40	0.40	3.18	0.64	5.71	<.001	<.001	<.001
protein.synthesis.ribosomal	ribosomal protein S3 (chloroplast)	-1.90	-1.26	-0.36	-1.97	-2.22	-3.16	-1.71	-0.20	<.001	0.009	0.008
protein.prokaryotic.chloroplast.30S subunit	ribosomal protein L16 (chloroplast)	-4.06	-0.68	-2.21	-4.19	-5.03	-5.38	-0.67	-2.29	<.001	<.001	<.001
protein.prokaryotic.chloroplast.50S subunit	threonine-protein kinase HT1-like	0.33	-1.14	-1.22	0.57	-0.90	-0.77	-0.76	-1.06	0.184	<.001	0.249
protein.postranslational modification	leucine-rich repeat family protein	-3.03	-0.67	-1.73	-3.19	-4.02	-4.02	-0.65	-1.88	<.001	<.001	<.001
transport.metabolite transporters	SLAH3 C4-dicarboxylate transporter	-1.36	0.02	2.66	-0.95	-0.50	1.22	-0.26	2.86	<.001	<.001	<.001

Supplementary Fig. 1. Leaves *a*, *b*, and *c* of barley sampled in this study, at the 13 Zadoks stage.

Supplementary Fig. 2. (a) G132 mutant barley plants showing the chlorosis of young leaves and its correction in older leaves. (b) Comparison of Graphic barley (WT) (left) and G132 (right) plants of the same age.