Title: Unraveling the role of transient starch in the response of Arabidopsis to elevated CO_2 under long-day conditions

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Running title: The role of starch in the response of LD-grown plants to elevated CO_2

Number of tables: 0

Number of figures: 4

Supplemental data: 4

Keywords: starch, elevated CO_2, photosynthesis, growth, photosynthetic acclimation

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ABSTRACT:

Previous studies on Arabidopsis under long-term exposure to elevated CO_2 have been conducted using starch synthesis and breakdown mutants cultured under short day conditions. These studies showed that starch synthesis can ameliorate the photosynthetic reduction caused by soluble sugar-mediated feedback regulation. In this work we characterized the effect of long-term exposure to elevated CO_2 (800 ppm) on growth, photosynthesis and content of primary photosynthates in long-day grown wild type plants as well as the near starch-less (*aps1*) and the starch-excess (*gwd*) mutants. Notably, elevated CO_2 promoted growth of both wild type and *aps1* plants but had no effect on *gwd* plants. Growth promotion by elevated CO_2 was accompanied by an increased net photosynthesis in WT and *aps1* plants. However, the plants with the highest starch content (wild type at elevated CO_2, *gwd* at ambient CO_2, and *gwd* at elevated CO_2) were the ones that suffered decreased in *in vivo* maximum carboxylation rate of Rubisco, and therefore, photosynthetic down-regulation. Further, the photosynthetic rates of wild type at elevated CO_2 and *gwd* at elevated CO_2 were acclimated to elevated CO_2. Notably, elevated CO_2 promoted the accumulation of stress-responsive and senescence-associated amino acid markers in *gwd* plants. The results presented in this work provide evidence that under long-day conditions, temporary storage of overflow photosynthate as starch negatively affect Rubisco performance. These data are consistent with earlier hypothesis that photosynthetic acclimation can be caused by accelerated senescence and hindrance of CO_2 diffusion to the stroma due to accumulation of large starch granules.

INTRODUCTION:

The concentration of atmospheric CO_2 has risen from pre-industrial revolution levels of ca. 280 ppm to the present level of ca. 400 ppm, and is estimated to reach 500-1200 ppm by 2100 (IPCC 2013)). As the substrate for photosynthesis, the elevated atmospheric CO_2 has a profound impact on plant growth. Numerous studies have shown that elevated CO_2 increases the rates of carboxylation and decreases the rates of oxygenation Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in C3 plants (Ainsworth et al., 2007; Leakey et al., 2009). Although this would in principle result in a higher net rate of CO_2 fixation (A_n) and better plant growth, an "inbalance" between CO_2 fixation and photosynthate utilization under long-term elevated CO_2 conditions has been described as causing a reduction in leaf Rubisco content and consequently a decline in the *in vivo* maximum rate of *in vivo* maximum carboxylation rate of Rubisco (V_{cmax}) (Moore et al., 1999; Ainsworth et al., 2004). This phenomenon, known as photosynthetic acclimation, has been ascribed to sugar-mediated reduction of photosynthetic gene expression through a hexokinase-controlled signaling pathway (Cheng et al., 1998; Moore et al., 1999; Ainsworth et al., 2004; Aranjuelo *et al.*, 2013). To buffer the overload of soluble sugars driving photosynthetic down-regulation in response to elevated CO_2, plants form new tissues, enhance respiration and/or accumulate non-structural carbohydrates such as starch (Long et al., 2004; Aranjuelo et al., 2011; 2013; Markelz et al., 2013). Therefore, many species with strong sinks do not show photosynthetic acclimation (Sage et al., 1989; Yelle et al., 1989; Ainsworth et al., 2007). There are alternative explanations for the decline in photosynthesis in response to elevated CO_2. Miller et al. (1997) and Ludewig and Sonnewald (2000) proposed that high CO_2-mediated down-regulation of photosynthetic gene expression is caused by accelerated leaf senescence rather than sugar accumulation. Also, it has been suggested that acclimation to elevated CO_2 is the consequence of hindrance of CO_2 diffusion from the intracellular space to the stroma in chloroplasts, which is caused by the accumulation of large starch granules (Makino and Mae, 1999; Sawada et al., 2001).

In leaves, up to 50% of the photosynthetically fixed carbon is retained within the chloroplasts during the day in the form of starch (Rao and Terry, 1995). It is widely assumed that this reserve polysaccharide is the end product of a metabolic pathway exclusive to the illuminated chloroplast that involves metabolization of fructose-6-phosphate from the Calvin-Benson cycle (CBC) by the stepwise reactions of plastidic

phosphoglucose isomerase (PGI1), phosphoglucomutase (PGM1), ADP-glucose pyrophosphorylase (AGP) and starch synthase (SS). Recent studies have provided evidence that, in addition to the CBC-PGI1-PGM1-AGP-SS, Arabidopsis plants possess important alternative/additional starch biosynthetic pathways involving the cytosolic and chloroplastic compartments (Bahaji et al., 2014; 2015; Sánchez-López et al., 2016; Baslam et al., 2017). Starch breakdown in leaves requires the coordinated actions of a suite of enzymes including glucan, water dikinase (GWD), phosphoglucan, water dikinase, β-amylases, α-amylases, debranching enzymes and disproportionating enzymes (Streb and Zeeman, 2012; Santelia et al. 2015). These enzymes degrade starch to maltose and glucose, which are transported to the cytosol via the maltose transporter, MEX1 and the glucose transporter pGlcT, respectively (Cho et al., 2011; Baslam et al., 2017).

Starch metabolism is an important determinant of plant growth in a diurnal cycle. In Arabidopsis, genetic evidence demonstrating the relevance of starch metabolism in growth has been obtained from the characterization of "near-starchless" *pgm1* and *agp* mutants impaired in PGM1 and AGP, respectively. When cultured under 12h light and 12h dark conditions, these mutants exhibit retarded growth that is likely a consequence of nighttime sugar starvation and soluble sugar-mediated down-regulation of growth- and photosynthesis-related genes (Carspar et al. 1985; Sun et al., 2002; Gibon et al., 2004; Ragel et al., 2013; Bahaji et al., 2015). Further evidence showing the relevance of starch metabolism in Arabidopsis growth has been obtained from "high starch" *gwd*, *mex1* and *mex1/pglcT* starch breakdown mutants. These mutants exhibit low growth (Caspar et al., 1991; Cho et al., 2011; Baslam et al., 2017) likely as a consequence of continuous sugar starvation (Baslam et al., 2017). The overall information obtained using starch synthesis and breakdown mutants indicates that it is not the starch content itself, but the ability to sustain a steady supply of soluble sugar that is crucial for plant growth. Thus, although elevated CO_2 exerts a positive effect on growth of WT plants, no such effect occurs in *agp, pgm* and *gwd* plants (Sun et al., 2002; Rasse and Tocquen, 2006). Also, whereas the A_n of elevated CO_2-grown WT plants is higher than in ambient CO_2-grown WT plants, no such differences are observed in *agp* plants (Sun et al., 1999).

Previous studies on the role of starch in the response of Arabidopsis to long-term exposure to elevated CO_2 have been mainly focused on growth, Rubisco activity, A_n and soluble sugar content in WT and *agp* plants (Sun et al., 1999; 2002; Gibson et al., 2011). In addition, Rasse and Tocquen (2006) compared the growth of WT, *pgm1* and *gwd* plants

cultured under ambient or elevated CO_2 conditions. Although Arabidopsis is a facultative long day (LD) plant, these studies were conducted using plants cultured under neutral day conditions. Therefore, we lack knowledge on the role of transient starch in the response of Arabidopsis to long-term elevated CO_2 exposure under LD conditions. To address this question, we assessed responses in LD-grown WT plants and mutants impaired in AGP and GWD cultured under elevated CO_2 conditions. Our hypothesis is that under LD conditions, elevated CO_2 will differentially influence the C metabolism and photosynthetic performance of the different Arabidopsis lines, bearing to (i) the impact of altered sink/source balance on photosynthetic activity; either (ii) the reduced capacity of *agp* mutant to store photoassimilates in the form of starch or (iii) the impossibility of *gwd* mutants to use photoassimilates stored in the form of starch.

MATERIALS AND METHODS:

Plant material and growth conditions

The study was carried out using *Arabidopsis thaliana* WT (ecotype Col-*0*), and the *gwd* (SALK_077211) and AGP-lacking *aps1* (SALK_040155) mutants (Ventriglia et al., 2008; Li et al., 2012). The experiment has been repeated in two consecutive years (2014 and 2015). The second year, the assay was performed to confirm the results of the first year. Biomass and N content analyses carried out in both experiments did not significantly differ. Seeds were placed at -80ºC in a freezer for 2 hours to improve the germination rates. Then the seeds were germinated on 0.65% agar using the Araponics (Araponics SA, Liege, Belgium) seed holders system to support the experiment under hydroponic conditions. The seed holders were placed in a germination chamber under continuous darkness for 48 h at 25ºC, with saturated humidity conditions and distilled water. Subsequently the plants were cultured in chambers at 22/18ºC (day/night) with a LD photoperiod of 16 hours of 200 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) and a relative humidity of 70/80 % (day/night). The distilled water was replaced every 3-4 days. Plants were transferred to 8 L containers filled with Rigaud and Puppo solution with modifications as detailed by Jauregui et al. (2016). The solution was replaced every 3-4 days. Plants were cultured in two different environment-controlled chambers (Heraeus-Votsch hps-500, Norrkoping, Sweden) under above described growth conditions and at two different atmospheric CO_2 concentrations: 400 parts per million (ppm) (actual [CO_2]) and 800 ppm (elevated [CO_2]). CO_2 bottles were provided by Praxair (Pamplona, Spain). The air entering in the cabinets was previously filtered (coarse-5 μm and 1 μm Ø particle and 0.01 μm Ø particle physical filters and a charcoal chemical filter) to prevent the entrance of anomalous components to the chambers. The air were taken from outside the building Cabinets were equipped with an infrared CO_2 analyser (polytron-IRGA, Dragäer, Lübeck, Germany) connected to a microprocessor located inside the cabinet. [CO_2] was analyzed and controlled every second.

All determinations were conducted 4 weeks after initiation of the CO_2 treatment, prior to when the first flower buds were visible at the 3.6 growth stage of the ontological scale described by Boyes et al. (2001). The harvesting was carry on 3h after the dawn, in 1 h.

Gas exchange determinations

Gas exchange measurements in the last fully expanded leaf per plant were carried out using a LI-COR 6400 XT portable photosynthesis gas exchange system (Li-COR, Nebraska, USA). Net photosynthesis (A_n) and stomatal conductance (gs) were recorded

at 400 and 800 µmol mol^{-1} CO_2, depending on the growth conditions. The photosynthetic responsiveness to elevated CO_2 was evaluated by measuring the response of light-saturated photosynthesis to changes in the ambient [CO_2]. For each plant and treatment combination 3-5 A/Ci curves were conducted, under saturated light conditions (1000 µmol m^{-2} s^{-1} irradiance), 300 µmol s^{-1} air flow rate, 25°C, 60 % relative humidity, and the corresponding [CO_2] during growth.. The A/Ci curves started at 400 ppm, then reduce to 250, and 99, to up to 250, 400, 600, 800, 1000, 1200 ppm. Estimations of *in vivo* maximum Rubisco carboxylation rates (V_{cmax}) and the maximum electron transport rate contributing to RuBP regeneration (J_{max}) were performed according to McMurtrie and Wang. (1993). The Rd was measured during the night period, using a fluorescence chamber (LFC 6400- 40) coupled to the LI-COR 6400 XT system.

Biochemical analysis

Carbohydrate content: Frozen plant tissue (0.1 g) ground in a mortar using nitrogen liquid was homogenized in 1 ml of 80% ethanol. The homogenate was collected in an Eppendorf tube, sonicated for 25 min at 30ºC using an ultrasonic bath (Selecta, Barcelona, Spain) and centrifuged at 16000 x g. The supernatant thus obtained was collected in a glass tube, and the solid phase was dried at 70ºC. Starch in the solid phase was measured spectrophotometrically using an amyloglucosidase–based test kit (Boehringer, Mannheim, Germany). The supernatant was evaporated using forced air in a turbovap (Zymark, Carmel, USA) and 1.5 ml of distilled water was added. The soluble sugars in the aqueous fraction (sucrose, glucose, and fructose) were determined using a capillary electrophoresis system (Beckman instruments, Fullerton, USA). The equipment used a fused silica capillary of 50 µm internal diameter and a length of 31.4-38.4 cm. The equipment used a buffer that consisted of a solution of 10 mM benzoic acid and 0.5 mM myristyltrimethylammonium bromide (MTAB), pH 12 (adjusted with 1M NaOH). The method of analysis was performed at a voltage of -15 kV, 20°C and the detections were carried out indirectly at a wavelength of 225 nm. Fucose was used as the internal standard at a final concentration of 0.5 mM.

Amino acid contents: Frozen plant tissue (0.1 g) was ground in liquid nitrogen and homogenized with 1 ml 1M HCl. The extract was centrifuged at 16000 x g and 4ºC for 10 min. Then the supernatant was collected in an Eppendorf tube and neutralized with NaOH to a pH of 7. Amino acids were derivatized at room temperature between 12-16 h with fluorescein isothiocyanate dissolved in 20 mM acetone/borate (pH 10). The amino

acid contents were determined with high-performance capillary electrophoresis using a Beckman Coulter PA-800 apparatus (Beckman Coulter, California, USA). The method applied a potential of −20 kV. The equipment used a buffer of 80 mM borax and 45 mM α-cyclodextrine, at pH 9.2. The method cannot separate glycine and serine.

Rubisco content: Frozen plant tissue (0.1 g) was ground with nitrogen liquid and homogenized with 1 ml of 50 mM TRIS-HCl pH 8, 1 mM EDTA, 10 mM 2-mercaptoethanol, 5 mM DTT, 10 mM $MgSO_4$, 1 mM cysteine, 0.5% polyvinylpolypyrrolidone and 1mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 16000 x g and 4 ºC for 10 min. Five µl of soluble protein was mixed and denatured with the following loading buffer: 62 mM TRIS-HCl, pH 6.8, 50% glycerol, 5% 2-mercaptoethanol, 2.3% sodium dodecyl sulfate (SDS) and 0.1% bromophenol blue. Then the extract was boiled at 100ºC for 5 min. Protein samples were loaded onto acrylamide gels (12.5%) and run at 125 V for 1 hour with the following running buffer: 25 mM TRIS, 192 mM glycine, and 0.1 mM SDS. Gels were then stained with GelCode Blue Stain Reagent (Pierce Biotechnology, Rockford, USA) and were scanned and quantified with the "quant 1" software in a Geldoc 2000 (Bio-Rad, Watford, UK) for the determination of abundance of the Rubisco large subunit (RbcL). Gel data were normalized to standards and recorded as a percentage, taking the content obtained in the 400 ppm [CO_2] treatment as a reference.

Mineral determinations: Nitrogen and carbon concentration was determined in the dry material with a CNS 2500 elemental analyzer (CE Instruments, Milan, Italy). The C/N ratio was calculated as a ratio dividing carbon and nitrogen concentration value.

Statistical analysis

Statistical analysis was performed by one factor ANOVA (SPSS v.12.0; SPSS Inc., Chicago, USA). Differences between treatments were determined by using the *Tukey-b* test. The results were accepted as significant at a *P value* ≤ 0.05.

RESULTS

Growth

LD-grown *aps1* and *gwd* plants cultured under ambient CO_2 conditions showed lower biomass values than WT plants (**Figure 1, Supplementary Figure 1**). Long-term exposure to elevated CO_2 promoted growth of WT plants, but not the growth of *gwd* plants (**Figure 1**). Notably, elevated CO_2 exerted a positive effect on the growth of *aps1* plants, with a value of fresh weight (FW) comparable to that of WT plants cultured under elevated CO_2 conditions (**Figure 1**).

Photosynthesis

A_n values in *aps1* and *gwd* plants were lower than in WT plants under ambient CO_2 (**Figure 2**). The A_n in *gwd* plants cultured under elevated CO_2 was comparable to ambient CO_2-grown plants. It is noteworthy that under elevated CO_2 the A_n of WT plants was higher than under ambient CO_2, and that this was also the case in *aps1* plants. Furthermore, the A_n of *aps1* plants was comparable to that of WT plants when cultured under elevated CO_2 conditions (**Figure 2**). Regardless of analyzed genotype, plants grown under 800 ppm showed lower stomatal conductance (g_s; **Supplemental Table 2**). The lowest g_s values were detected in g*wd* plants exposed to elevated CO_2.

Under ambient CO_2 the V_{cmax} in WT plants was higher than in *aps1* and *gwd* plants (**Figure 2**). Elevated CO_2 exerted a negative effect on V_{cmax} in WT and *gwd* plants, but not in *aps1* plants (**Figure 2**). Under both ambient and elevated CO_2, the J_{max} of WT was comparable to that of *aps1* plants, and higher than that of *gwd* plants (**Figure 2**). No growth CO_2 linked significant differences on dark respiration rates (R_d) were detected on the different genotypes (**Supplemental Table 1**).

Exposure to elevated CO_2 promoted a significant reduction in leaf Rubisco large subunit and N content in WT and *gwd* plants, but not in *aps1* plants (**Figure 2** and **Supplemental Figure 1** respectively).

Primary photosynthate content

The starch content in leaves of WT plants cultured under elevated CO_2 conditions was ca. 3-fold higher than under ambient CO_2 conditions (**Figure 3**). No differences in starch content could be found between ambient and elevated CO_2 conditions in *aps1* and *gwd* plants (**Figure 3**). Under ambient CO_2 conditions *aps1* leaves accumulated nearly WT levels of sucrose, and ca. 2-fold more glucose and fructose than WT leaves. Leaves of WT plants cultured under elevated CO_2 conditions accumulated 2-3-fold more glucose, fructose and sucrose than under ambient CO_2 conditions (**Figure 3**). Under the same

conditions, *aps1* leaves accumulated WT levels of fructose, and 1.5-fold and 4-fold more sucrose and glucose than WT leaves, respectively (**Figure 3**). Soluble sugar (sucrose, glucose and fructose) content in *gwd* leaves was higher than in WT plants under ambient CO_2 (**Figure 3**), which is consistent with Caspar et al. (1991). Leaf fructose and glucose contents in *gwd* plants cultured under ambient CO_2 were comparable to those of plants cultured under elevated CO_2 conditions, while the leaf sucrose content was higher (**Figure 3**).

No differences in leaf total free amino acid content (TFAC) could be found between the three genotypes cultured under ambient CO_2 conditions (**Supplemental Figure 3**). Elevated CO_2 did not greatly alter the TFAC in either WT or *aps1* plants. In clear contrast, the leaf TFAC of *gwd* plants cultured under elevated CO_2 was ca. 30% higher than in leaves of ambient CO_2-grown *gwd* plants. The high leaf TFAC in *gwd* plants cultured under elevated CO_2 was largely the consequence of enhanced levels of asparagine and, to a lesser extent, pyruvate-derived alanine, valine and leucine (**Figure 4, Supplemental Figure 3**).

DISCUSSION

Starch granule formation is an important determinant of photosynthetic acclimation to elevated CO_2

Long-term exposure to elevated CO_2 usually leads to leaf carbohydrate build-up and the consequent decreases in Rubisco content and thereby V_{cmax}, which is thought to represent the acclimation of photosynthesis to elevated CO_2 (Stitt and Krapp, 1999). In this work we have shown that long-term exposure to elevated CO_2 results in reductions in V_{cmax} and Rubisco content in WT and *gwd* plants cultured under a 16 h light/8 h dark photoregime. This indicates that the photosynthesis of WT and *gwd* plants acclimates to elevated CO_2 when these genotypes are grown under LD conditions. In clear contrast, values of V_{cmax} and Rubisco content in the near-starchless *aps1* plants cultured under ambient CO_2 were comparable to those of *aps1* plants cultured under elevated CO_2 indicating that this genotype does not exhibit photosynthetic acclimation to elevated CO_2. Starch content has been traditionally associated with leaf C sink/source imbalance causing photosynthetic down-regulation (Long et al., 2004). This study showed that the plants with the highest starch content (WT800, *gwd*400 and *gwd*800), where the ones in which photosynthetic down-regulation was more severe. This would indicate, in principle, that starch granule formation is an important determinant of photosynthetic acclimation to elevated CO_2.

Evidence has been provided that starch over-accumulation hinders CO_2 diffusion in the chloroplast (Nafziger and Koller, 1976; Nakano et al., 2000; Sawada et al., 2001). Thus, it has been suggested that during the acclimation to CO_2 enrichment, accumulation of starch causes a lowering of V_{cmax} due to hindrance of CO_2 diffusion from the intracellular space to the stroma in the chloroplasts (Makino and Mae, 1999; Sawada et al., 2001; Singsaas et al., 2004). According to Kitao and coworkers (2015) leaf cell wall thickness, together with leaf the starch accumulation detected under elevated CO_2 conditions would contribute to diminish CO_2 diffusion within the chloroplast. Within this context, the lower stomatal opening values detected in plants grown at 800 ppm CO_2 would support the potential implication of that starch accumulation on CO_2 diffusion and the consequent responsiveness of photosynthetic apparatus to elevated CO_2 condition (Makino and Mae, 1999; Sawada et al., 2001)

Long-term exposure to elevated CO_2 promotes growth and photosynthesis of *aps1* plants

Previous studies have shown that elevated CO_2 exposure does not enhance the growth and photosynthesis of neutral day grown Arabidopsis plants impaired in starch synthesis and breakdown, indicating that starch metabolism is an important determinant of Arabidopsis responsiveness to elevated CO_2 (Sun et al., 1999; 2002; Rasse and Tocquin, 2006, Gibson et al., 2011). Nevertheless, in the current study we have shown that elevated CO_2 enhances growth and photosynthesis of LD-grown *aps1* plants, indicating that under LD conditions starch granule formation is not an important determinant of promotion of growth and photosynthesis by elevated CO_2. Gibon et al. (2004) showed that under 12 h light/12 h dark conditions, expression levels of hundreds of growth- and photosynthesis-related genes in the near-starchless *pgm1* mutant are lower than in WT plants at the end of the night. The same authors showed that when the night is extended 4-6 hours, global gene expression in WT leaves resembles that in *pgm1* at the end of the night. According to these results, a transient period of acute carbohydrate deficiency occurring during the night triggers a wide-ranging inhibition of biosynthesis and growth. It is therefore conceivable that *pgm1* and *aps1* plants cultured under the neutral day conditions employed by Sun et al. (1999; 2002), Rasse and Tocquin (2006) and Gibson et al. (2011) responded poorly to elevated CO_2 because growth and photosynthesis-related genes are down-regulated at the end of the dark period. As the photoperiod conditions employed in the present study involved a short dark period (and thus a lack of acute sugar starvation), it is also conceivable that *aps1* plants were capable of responding to elevated CO_2 because photosynthesis- and growth-related genes were not down-regulated at the end of the night time.

An increase in leaf carbohydrates has long been associated with an inhibition of photosynthesis, and carbohydrates are known to modulate the expression of many photosynthesis- and growth-related genes (Jang and Sheen, 1994; Moore et al., 2003). In this work we found that, under elevated CO_2 conditions, illuminated leaves of LD-grown *aps1* plants accumulate WT-levels of fructose, and 1.5-fold and 4-fold more sucrose and glucose than WT leaves, respectively. This moderate increase in soluble sugars in *aps1* plants contrasts with the work of Sun et al. (2002) who showed that leaves of plants grown under neutral day impaired in AGP and cultured under CO_2 conditions accumulate ca. 5-fold more glucose, fructose and sucrose than WT leaves during illumination. Therefore, the differences between our results and those reported by Sun et al. (1999; 2002), Rasse and Tocquin (2006) and Gibson et al. (2011) could be due to the fact that under neutral day, but not under the LD conditions (employed in this work), *pgm1* and *agp* plants

accumulate levels of soluble sugars that exert an inhibitory effect on the expression of photosynthesis- and growth-related genes during illumination.

Long-term exposure to elevated CO_2 does not promote growth of *gwd* plants

A remarkable feature of the high starch *gwd* mutant is that, unlike WT and *aps1* plants, growth and A_n are not enhanced by elevated CO_2. This would indicate that either starch degradation and/or accumulation of large starch granules are major determinants of Arabidopsis responsiveness to elevated CO_2. As to the possible reason(s) for the non-responsiveness of *gwd* to elevated CO_2 it is worth noting that the J_{max} of *gwd* plants was lower than in the WT under both ambient and elevated CO_2 conditions. It has been suggested that excessive accumulation of starch may negatively affect the internal organization of chloroplasts, disturbing the configuration of granal stacks, distorting the thylakoids and thus negatively affecting electron transport (Yelle et al. 1989; Pritchard et al., 1997). Thus, it is conceivable that the reduced size of *gwd* and the non-responsiveness of this mutant to elevated CO_2 is the consequence of reduced electron transport due to thylakoid distortion, which in turn results in reduced A_n and growth under both ambient and elevated CO_2 conditions.

Photosynthetic acclimation to elevated CO_2 in *gwd* plants: a case of accelerated senescence?

The photosynthetic acclimation to elevated CO_2 has long been ascribed to sugar-mediated reduction of photosynthetic gene expression (Cheng et al., 1998; Moore et al., 1999; Ainsworth et al., 2004; Aranjuelo *et al.*, 2013). However, in this work we could not find a clear link between the soluble sugar contents, Rubisco content and net photosynthesis in LD-grown *aps1* and *gwd* plants cultured under ambient and elevated CO_2 conditions. Obtained data would indicate that, under LD conditions, sugar-mediated regulation of photosynthetic gene expression does not play an important role in acclimation of Arabidopsis plants to elevated CO_2, at least in *aps1* and *gwd* plants. The case of *gwd* plants was particularly enlightening: although levels of soluble sugars in leaves of ambient CO_2-grown *gwd* plants were comparable to those of plants cultured under elevated CO_2 conditions, Rubisco content and V_{cmax} decreased under elevated CO_2 conditions.

The N status reduction is a usual response under elevated CO_2 (Stitt & Krapp, 1999; (Bloom et al., 2010; Aranjuelo et al., 2011; 2013; Markelz et al., 2013; Jauregui,

2016, 2017). In our study, N content significantly decreased in WT and *gwd* plants exposed to elevated 800 ppm Sun and coworkers (2002). The progressive degradation of leaf protein content under elevated CO_2 has been previously associated with an acceleration in leaf protein degradation processes linked with the advanced phenologic status of plants (Miller et al. 1997; Ludewig and Sonnewald 2000). Within this context, the progressive depletion of Rubisco under elevated [CO_2] conditions detected in under elevated CO_2 could be linked with a situation of advanced lead senescence of those plants. As phenology gets closer to the senescence period, N assimilation pathways are altered and the expression of proteases increases (Masclaux-Daubresse et al. 2008). As a consequence of the protease activity and the consequent protein hydrolysis, the resulting N compounds (mostly amino acids) in leaves are released. Within this context, one remarkable feature of this mutant is that elevated CO_2 promotes the accumulation of high levels of asparagine (up to 25% of the total amino acid content). Elevated CO_2 also promoted the accumulation of alanine, leucine and valine. Because *gwd* plants have a poor capacity to accumulate and degrade starch in a diurnal cycle (Caspar et al. 1991), amino acid accumulation could be interpreted as an alternate mechanism for storing photosynthate in a metabolizable form. Alanine is a well-known stress-responsive amino acid (Wallace et al. 1984, Rocha et al. 2010). Furthermore, asparagine, leucine and valine are known to accumulate during senescence (Lea et al. 2007; Watanabe et al. 2013; Avila-Ospina et al. 2015). It is thus likely that photosynthetic acclimation of *gwd* to elevated CO_2 is caused by accelerated leaf senescence rather than sugar accumulation. Further, the fact that Rubisco content was significantly lower in *gwd* than in the WT, together with the large accumulation of high levels of TFAC in *gwd* leaves suggests that Rubisco protein catabolism was associated with amino acid increase and leaf senescence (Huffaker, 1990) in *gwd* plants. Moreover, because excessive accumulation of starch may negatively affect the internal organization of chloroplasts (see above), it is conceivable that *gwd* acclimates to elevated CO_2 to prevent the formation of critically large starch granules that otherwise would compromise chloroplast functionality and the viability of the plant.

Conclusion and perspectives

The present work revealed the profound impact of elevated CO_2 on starch metabolism that conditioned plant performance. While in wild type and *aps1* plants exposure to 800 ppm increased plant growth, in *gwd* doubling CO_2 availability was not reflected in a larger

biomass. Moreover, in plants with the highest starch content, such as wild type grown at elevated CO_2 and *gwd* (at both CO_2 conditions), Rubisco maximum carboxylation activity and photosynthetic apparatus were impaired. Such impairment was explained by the accelerated senescence and hindrance of CO_2 diffusion that was associated with the accumulation of large starch granules rather than sugar accumulation. In summary, our study showed that excessive accumulation of starch negatively affect chloroplast organization and, therefore photosynthesis and growth, in *gwd*.

Studies carried out during the last decades with crops such as wheat, alfalfa, rice, soybean, tobacco, etc. exposed to elevated CO_2 condition have shown that, in many cases, plants that suffer photosynthetic acclimation also have high leaf starch content values. Within this context, our results remark the fact that the overflow of starch photosynthate storage negatively affects photosynthetic machinery of Arabidopsis plants. Leaf carbohydrate accumulation probed to be a target factor conditioning plant performance under elevated CO_2 conditions. In agreement with previous studies, our data show that plants with a small sink size will acclimate to high CO_2 by decreasing photosynthetic capacity. Therefore, plants with a large sink size (i.e. large ears in the case of cereals) will benefit more from CO_2 enrichment than those with a small sink size like the plants limited storage organs or the ones that do not have it. The use of near starch-less (*aps1*) and the starch-excess (*gwd*) mutants in this study provided more information on the processes that explain the down regulation of photosynthetic machinery under elevated CO_2 conditions. However, we recognize that additional research is needed to discern if it is the accelerated senescence and/or the carbon starvation enhanced under elevated CO_2 of plants. Furthermore, while the use of Arabidopsis as a model organism has enabled advances in understanding plant growth and development, those studies shall be extended to other plants and crops so to better understand how plants will perform under near future environments.

ACKNOWLEDGEMENTS

This work was partially supported by the Spanish National Research and Development Programme (AGL2016-79868-R), by Comisión Interministerial de Ciencia y Tecnología and Fondo Europeo de Desarrollo Regional (Spain) (grant number BIO2016-78747-P) and the Basque Government (IT-932-16). The authors would like to acknowledge the technical support provided by Dr. Philippe D'Hooghe from the UMR INRA/UCN 950 Ecophysiologie Végétale et Agronomy (Université de Caen Normandie).

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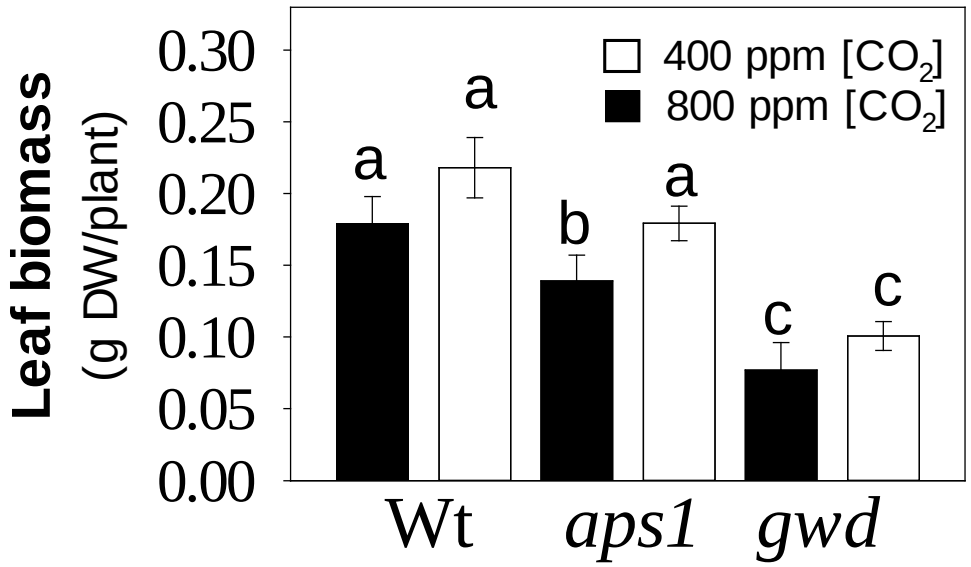

Figure 1. Effect of elevated [CO_2] (800 *versus* 400 ppm) in *Arabidopsis thaliana* (wild type WT, starchless *aps1*, and starchexcess *gwd*) on leaf biomass (dry weight biomass per plant). Bars are means ± SD of 10 replicates, with different letters indicating significant ($P < 0.05$) differences according to Tukey's test.

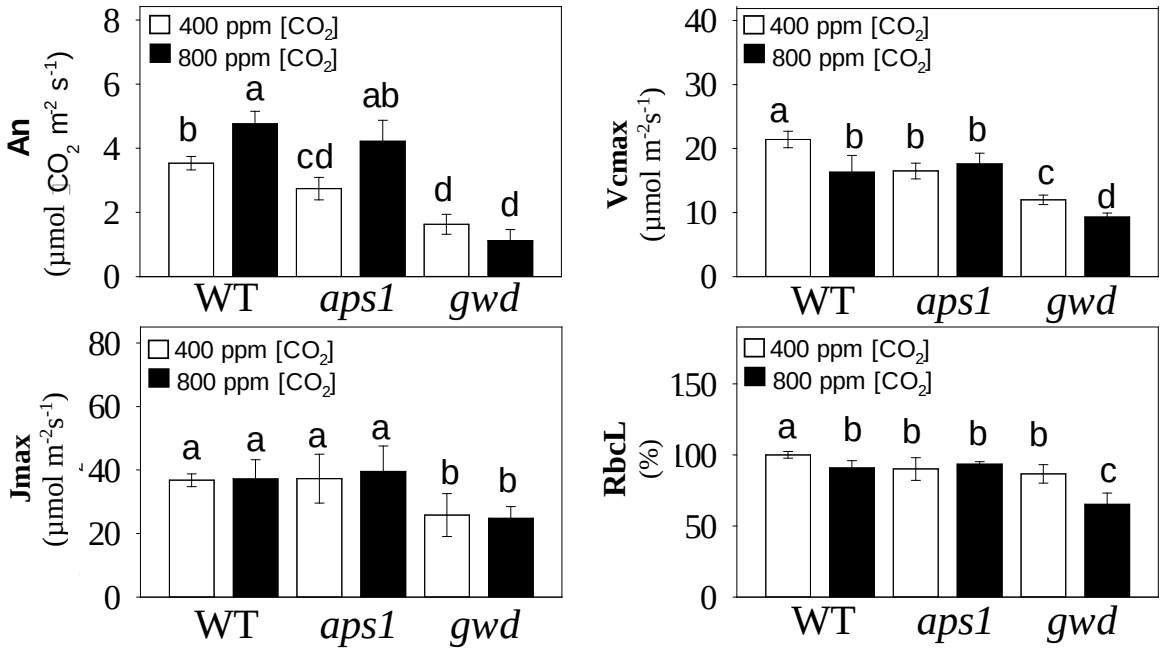

Figure 2. Effect of elevated [CO_2] (800 *versus* 400 ppm) in *Arabidopsis thaliana* (wild type WT, starchless *aps1*, and starchexcess *gwd*) on net photosynthetic rates (An), maximum carboxylation rate (Vc_{max}), maximum electron transport rate contributing to RuBP regeneration (J_{max}) and Rubisco Large Subunit (RbcL). Bars are means ± SD of 5 replicates, with different letters indicating significant ($P < 0.05$) differences according to Tukey's test.

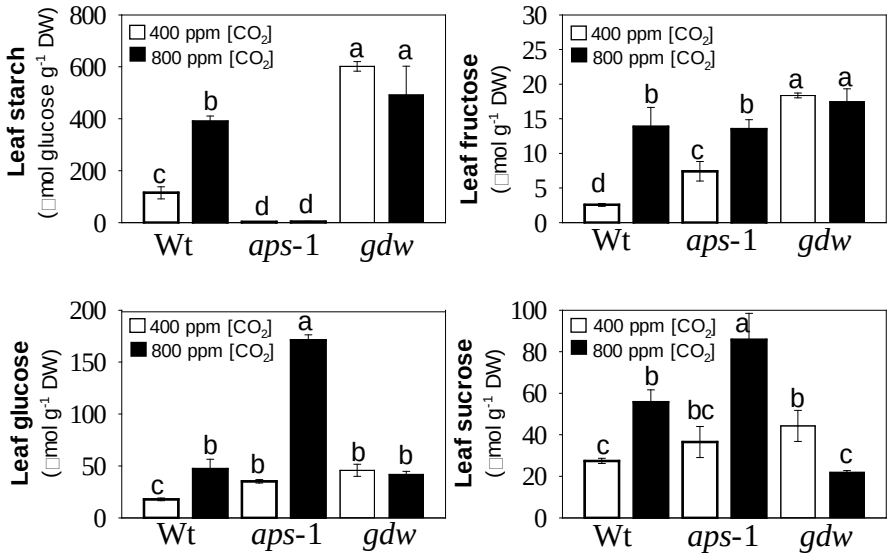

Figure 3. Effect of elevated [CO_2] (800 *versus* 400 ppm) in *Arabidopsis thaliana* (wild type wt, starchless *aps*-1, and starchexcess *gdw*) on starch content (µmol glucose g^{-1} DW) and sugars (fructose, glucose, sucrose; µmol g^{-1} DW) in leaves. Bars are means ± SD of 3 replicates for sugars and 6 for starch, with different letters indicating significant ($P < 0.05$) differences according to a Tukey test.

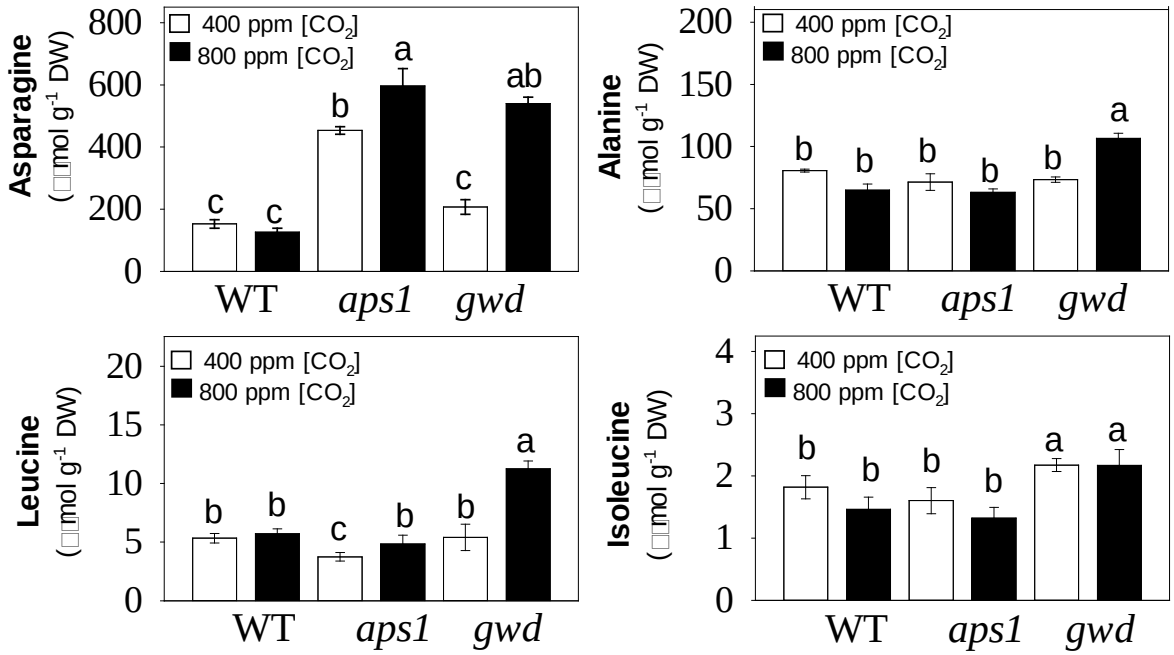

Figure 4. Effect of elevated [CO_2] (800 *versus* 400 ppm) in *Arabidopsis thaliana* (wild type WT, starchless *aps1*, and starchexcess *gwd*) on selected individual amino acid contents in leaves. Bars are means ± SD of 4 replicates, with different letters indicating significant ($P < 0.05$) differences according to Tukey's test.