Title: Unraveling the role of transient starch in the response of Arabidopsis to elevated CO₂ under long-day conditions

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ABSTRACT:
Previous studies on Arabidopsis under long-term exposure to elevated CO₂ 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 CO₂ (800 ppm) on growth, photosynthesis and content of primary photosynthates in long-day grown wild type plants as well as the near starch-less (apsI) and the starch-excess (gwd) mutants. Notably, elevated CO₂ promoted growth of both wild type and apsI plants but had no effect on gwd plants. Growth promotion by elevated CO₂ was accompanied by an increased net photosynthesis in WT and apsI plants. However, the plants with the highest starch content (wild type at elevated CO₂, gwd at ambient CO₂, and gwd at elevated CO₂) were the ones that suffered decreased in vitro maximum carboxylation rate of Rubisco, and therefore, photosynthetic down-regulation. Further, the photosynthetic rates of wild type at elevated CO₂ and gwd at elevated CO₂ were acclimated to elevated CO₂. Notably, elevated CO₂ 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 CO₂ diffusion to the stroma due to accumulation of large starch granules.
INTRODUCTION:
The concentration of atmospheric CO₂ 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 CO₂ has a profound impact on plant growth. Numerous studies have shown that elevated CO₂ 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 CO₂ fixation ($A_n$) and better plant growth, an “inbalance” between CO₂ fixation and photosynthate utilization under long-term elevated CO₂ 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 CO₂, 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 CO₂. Miller et al. (1997) and Ludewig and Sonnewald (2000) proposed that high CO₂-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 CO₂ is the consequence of hindrance of CO₂ 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-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 CO2 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 CO2-grown WT plants is higher than in ambient CO2-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 CO2 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 CO₂ concentrations: 400 parts per million (ppm) (actual [CO₂]) and 800 ppm (elevated [CO₂]). CO₂ bottles were provided by Praxair (Pamplona, Spain). The air entering in the cabinets was previously filtered (coarse-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 CO₂ analyser (polytron-IRGA, Dragäer, Lübeck, Germany) connected to a microprocessor located inside the cabinet. [CO₂] was analyzed and controlled every second.

All determinations were conducted 4 weeks after initiation of the CO₂ 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 (*Aₙ*) 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 \textit{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 30°C 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 70°C. 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 4°C 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 MgSO4, 1 mM cysteine, 0.5% polyvinylpolypyrrolidone and 1 mM 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 [CO2] 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 *gwd* plants exposed to elevated CO\(_2\).

Under ambient CO\(_2\) the \(V_{\text{cmax}}\) in WT plants was higher than in *aps1* and *gwd* plants (Figure 2). Elevated CO\(_2\) exerted a negative effect on \(V_{\text{cmax}}\) in WT and *gwd* plants, but not in *aps1* plants (Figure 2). Under both ambient and elevated CO\(_2\), the \(J_{\text{max}}\) 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 CO₂

Long-term exposure to elevated CO₂ usually leads to leaf carbohydrate build-up and the consequent decreases in Rubisco content and thereby $V_{\text{cmax}}$, which is thought to represent the acclimation of photosynthesis to elevated CO₂ (Stitt and Krapp, 1999). In this work we have shown that long-term exposure to elevated CO₂ results in reductions in $V_{\text{cmax}}$ 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 CO₂ when these genotypes are grown under LD conditions. In clear contrast, values of $V_{\text{cmax}}$ and Rubisco content in the near-starchless aps1 plants cultured under ambient CO₂ were comparable to those of aps1 plants cultured under elevated CO₂ indicating that this genotype does not exhibit photosynthetic acclimation to elevated CO₂. 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, gwd400 and gwd800), 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 CO₂.

Evidence has been provided that starch over-accumulation hinders CO₂ 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 CO₂ enrichment, accumulation of starch causes a lowering of $V_{\text{cmax}}$ due to hindrance of CO₂ 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 CO₂ conditions would contribute to diminish CO₂ diffusion within the chloroplast. Within this context, the lower stomatal opening values detected in plants grown at 800 ppm CO₂ would support the potential implication of that starch accumulation on CO₂ diffusion and the consequent responsiveness of photosynthetic apparatus to elevated CO₂ condition (Makino and Mae, 1999; Sawada et al., 2001)

Long-term exposure to elevated CO₂ promotes growth and photosynthesis of aps1 plants
Previous studies have shown that elevated CO₂ 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 CO₂ (Sun et al., 1999; 2002; Rasse and Tocquin, 2006, Gibson et al., 2011). Nevertheless, in the current study we have shown that elevated CO₂ 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 CO₂. 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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\textsubscript{2} does not promote growth of gwd plants

A remarkable feature of the high starch gwd mutant is that, unlike WT and aps\textsubscript{1} plants, growth and $A_n$ are not enhanced by elevated CO\textsubscript{2}. This would indicate that either starch degradation and/or accumulation of large starch granules are major determinants of Arabidopsis responsiveness to elevated CO\textsubscript{2}. As to the possible reason(s) for the non-responsiveness of gwd to elevated CO\textsubscript{2} it is worth noting that the $J_{\text{max}}$ of gwd plants was lower than in the WT under both ambient and elevated CO\textsubscript{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\textsubscript{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\textsubscript{2} conditions.

Photosynthetic acclimation to elevated CO\textsubscript{2} in gwd plants: a case of accelerated senescence?

The photosynthetic acclimation to elevated CO\textsubscript{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 aps\textsubscript{1} and gwd plants cultured under ambient and elevated CO\textsubscript{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\textsubscript{2}, at least in aps\textsubscript{1} and gwd plants. The case of gwd plants was particularly enlightening: although levels of soluble sugars in leaves of ambient CO\textsubscript{2}-grown gwd plants were comparable to those of plants cultured under elevated CO\textsubscript{2} conditions, Rubisco content and $V_{\text{c,max}}$ decreased under elevated CO\textsubscript{2} conditions.

The N status reduction is a usual response under elevated CO\textsubscript{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 CO$_2$. 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 CO₂ and gwd (at both CO₂ conditions), Rubisco maximum carboxylation activity and photosynthetic apparatus were impaired. Such impairment was explained by the accelerated senescence and hindrance of CO₂ 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 CO₂ 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 CO₂ conditions. In agreement with previous studies, our data show that plants with a small sink size will acclimate to high CO₂ by decreasing photosynthetic capacity. Therefore, plants with a large sink size (i.e. large ears in the case of cereals) will benefit more from CO₂ 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 CO₂ 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 CO₂ 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.
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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 [CO₂] (800 versus 400 ppm) in Arabidopsis thaliana (wild type WT, starchless *aps1*, and starchexcess *gwd*) on net photosynthetic rates (An), maximum carboxylation rate (Vcₘₐₓ), maximum electron transport rate contributing to RuBP regeneration (Jₘₐₓ) 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 [CO₂] (800 versus 400 ppm) in *Arabidopsis thaliana* (wild type wt, starchless *aps*-1, and starch excess *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 [CO₂] (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.