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

3

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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

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

45 Starch metabolism is an important determinant of plant growth in a diurnal cycle.
46 In Arabidopsis, genetic evidence demonstrating the relevance of starch metabolism in
47 growth has been obtained from the characterization of "near-starchless" *pgm1* and *agp*
48 mutants impaired in PGM1 and AGP, respectively. When cultured under 12h light and
49 12h dark conditions, these mutants exhibit retarded growth that is likely a consequence
50 of nighttime sugar starvation and soluble sugar-mediated down-regulation of growth- and
51 photosynthesis-related genes (Carspar et al. 1985; Sun et al., 2002; Gibon et al., 2004;
52 Ragel et al., 2013; Bahaji et al., 2015). Further evidence showing the relevance of starch
53 metabolism in Arabidopsis growth has been obtained from "high starch" *gwd*, *mex1* and
54 *mex1/pglcT* starch breakdown mutants. These mutants exhibit low growth (Caspar et al.,
55 1991; Cho et al., 2011; Baslam et al., 2017) likely as a consequence of continuous sugar
56 starvation (Baslam et al., 2017). The overall information obtained using starch synthesis
57 and breakdown mutants indicates that it is not the starch content itself, but the ability to
58 sustain a steady supply of soluble sugar that is crucial for plant growth. Thus, although
59 elevated CO_2 exerts a positive effect on growth of WT plants, no such effect occurs in
60 *agp, pgm* and *gwd* plants (Sun et al., 2002; Rasse and Tocquen, 2006). Also, whereas the
61 A_n of elevated CO_2-grown WT plants is higher than in ambient CO_2-grown WT plants,
62 no such differences are observed in *agp* plants (Sun et al., 1999).

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

67 cultured under ambient or elevated CO_2 conditions. Although Arabidopsis is a facultative
68 long day (LD) plant, these studies were conducted using plants cultured under neutral day
69 conditions. Therefore, we lack knowledge on the role of transient starch in the response
70 of Arabidopsis to long-term elevated CO_2 exposure under LD conditions. To address this
71 question, we assessed responses in LD-grown WT plants and mutants impaired in AGP
72 and GWD cultured under elevated CO_2 conditions. Our hypothesis is that under LD
73 conditions, elevated CO_2 will differentially influence the C metabolism and
74 photosynthetic performance of the different Arabidopsis lines, bearing to (i) the impact
75 of altered sink/source balance on photosynthetic activity; either (ii) the reduced capacity
76 of *agp* mutant to store photoassimilates in the form of starch or (iii) the impossibility of
77 *gwd* mutants to use photoassimilates stored in the form of starch.
78

1 **MATERIALS AND METHODS:**

2 **Plant material and growth conditions**

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

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

30

31 **Gas exchange determinations**

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

35 at 400 and 800 μmol mol^{-1} CO_2, depending on the growth conditions. The photosynthetic
36 responsiveness to elevated CO_2 was evaluated by measuring the response of light-
37 saturated photosynthesis to changes in the ambient [CO_2]. For each plant and treatment
38 combination 3-5 A/Ci curves were conducted, under saturated light conditions (1000
39 μmol m^{-2} s^{-1} irradiance), 300 μmol s^{-1} air flow rate, 25°C, 60 % relative humidity, and the
40 corresponding [CO_2] during growth.. The A/Ci curves started at 400 ppm, then reduce to
41 250, and 99, to up to 250, 400, 600, 800, 1000, 1200 ppm. Estimations of *in vivo*
42 maximum Rubisco carboxylation rates (V_{cmax}) and the maximum electron transport rate
43 contributing to RuBP regeneration (J_{max}) were performed according to McMurtrie and
44 Wang. (1993). The Rd was measured during the night period, using a fluorescence
45 chamber (LFC 6400- 40) coupled to the LI-COR 6400 XT system.
46
47 **Biochemical analysis**
48 *Carbohydrate content:* Frozen plant tissue (0.1 g) ground in a mortar using nitrogen liquid
49 was homogenized in 1 ml of 80% ethanol. The homogenate was collected in an Eppendorf
50 tube, sonicated for 25 min at 30ºC using an ultrasonic bath (Selecta, Barcelona, Spain)
51 and centrifuged at 16000 x g. The supernatant thus obtained was collected in a glass tube,
52 and the solid phase was dried at 70ºC. Starch in the solid phase was measured
53 spectrophotometrically using an amyloglucosidase–based test kit (Boehringer,
54 Mannheim, Germany). The supernatant was evaporated using forced air in a turbovap
55 (Zymark, Carmel, USA) and 1.5 ml of distilled water was added. The soluble sugars in
56 the aqueous fraction (sucrose, glucose, and fructose) were determined using a capillary
57 electrophoresis system (Beckman instruments, Fullerton, USA). The equipment used a
58 fused silica capillary of 50 μm internal diameter and a length of 31.4-38.4 cm. The
59 equipment used a buffer that consisted of a solution of 10 mM benzoic acid and 0.5 mM
60 myristyltrimethylammonium bromide (MTAB), pH 12 (adjusted with 1M NaOH). The
61 method of analysis was performed at a voltage of -15 kV, 20°C and the detections were
62 carried out indirectly at a wavelength of 225 nm. Fucose was used as the internal standard
63 at a final concentration of 0.5 mM.
64 *Amino acid contents*: Frozen plant tissue (0.1 g) was ground in liquid nitrogen and
65 homogenized with 1 ml 1M HCl. The extract was centrifuged at 16000 x g and 4ºC for
66 10 min. Then the supernatant was collected in an Eppendorf tube and neutralized with
67 NaOH to a pH of 7. Amino acids were derivatized at room temperature between 12-16 h
68 with fluorescein isothiocyanate dissolved in 20 mM acetone/borate (pH 10). The amino

69 acid contents were determined with high-performance capillary electrophoresis using a
70 Beckman Coulter PA-800 apparatus (Beckman Coulter, California, USA). The method
71 applied a potential of −20 kV. The equipment used a buffer of 80 mM borax and 45 mM
72 α-cyclodextrine, at pH 9.2. The method cannot separate glycine and serine.
73

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

89 *Mineral determinations*: Nitrogen and carbon concentration was determined in the
90 dry material with a CNS 2500 elemental analyzer (CE Instruments, Milan, Italy). The
91 C/N ratio was calculated as a ratio dividing carbon and nitrogen concentration value.
92

93 **Statistical analysis**

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

35 conditions, *aps1* leaves accumulated WT levels of fructose, and 1.5-fold and 4-fold more
36 sucrose and glucose than WT leaves, respectively (**Figure 3**). Soluble sugar (sucrose,
37 glucose and fructose) content in *gwd* leaves was higher than in WT plants under ambient
38 CO_2 (**Figure 3**), which is consistent with Caspar et al. (1991). Leaf fructose and glucose
39 contents in *gwd* plants cultured under ambient CO_2 were comparable to those of plants
40 cultured under elevated CO_2 conditions, while the leaf sucrose content was higher (**Figure**
41 **3**).

42 No differences in leaf total free amino acid content (TFAC) could be found
43 between the three genotypes cultured under ambient CO_2 conditions (**Supplemental**
44 **Figure 3**). Elevated CO_2 did not greatly alter the TFAC in either WT or *aps1* plants. In
45 clear contrast, the leaf TFAC of *gwd* plants cultured under elevated CO_2 was ca. 30%
46 higher than in leaves of ambient CO_2-grown *gwd* plants. The high leaf TFAC in *gwd*
47 plants cultured under elevated CO_2 was largely the consequence of enhanced levels of
48 asparagine and, to a lesser extent, pyruvate-derived alanine, valine and leucine (**Figure**
49 **4, Supplemental Figure 3**).
50

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

34 Previous studies have shown that elevated CO_2 exposure does not enhance the growth
35 and photosynthesis of neutral day grown Arabidopsis plants impaired in starch synthesis
36 and breakdown, indicating that starch metabolism is an important determinant of
37 Arabidopsis responsiveness to elevated CO_2 (Sun et al., 1999; 2002; Rasse and Tocquin,
38 2006, Gibson et al., 2011). Nevertheless, in the current study we have shown that elevated
39 CO_2 enhances growth and photosynthesis of LD-grown *aps1* plants, indicating that under
40 LD conditions starch granule formation is not an important determinant of promotion of
41 growth and photosynthesis by elevated CO_2. Gibon et al. (2004) showed that under 12 h
42 light/12 h dark conditions, expression levels of hundreds of growth- and photosynthesis-
43 related genes in the near-starchless *pgm1* mutant are lower than in WT plants at the end
44 of the night. The same authors showed that when the night is extended 4-6 hours, global
45 gene expression in WT leaves resembles that in *pgm1* at the end of the night. According
46 to these results, a transient period of acute carbohydrate deficiency occurring during the
47 night triggers a wide-ranging inhibition of biosynthesis and growth. It is therefore
48 conceivable that *pgm1* and *aps1* plants cultured under the neutral day conditions
49 employed by Sun et al. (1999; 2002), Rasse and Tocquin (2006) and Gibson et al. (2011)
50 responded poorly to elevated CO_2 because growth and photosynthesis-related genes are
51 down-regulated at the end of the dark period. As the photoperiod conditions employed in
52 the present study involved a short dark period (and thus a lack of acute sugar starvation),
53 it is also conceivable that *aps1* plants were capable of responding to elevated CO_2 because
54 photosynthesis- and growth-related genes were not down-regulated at the end of the night
55 time.

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

68 accumulate levels of soluble sugars that exert an inhibitory effect on the expression of
69 photosynthesis- and growth-related genes during illumination.
70
71 **Long-term exposure to elevated CO_2 does not promote growth of *gwd* plants**
72 A remarkable feature of the high starch *gwd* mutant is that, unlike WT and *aps1* plants,
73 growth and A_n are not enhanced by elevated CO_2. This would indicate that either starch
74 degradation and/or accumulation of large starch granules are major determinants of
75 Arabidopsis responsiveness to elevated CO_2. As to the possible reason(s) for the non-
76 responsiveness of *gwd* to elevated CO_2 it is worth noting that the J_{max} of *gwd* plants was
77 lower than in the WT under both ambient and elevated CO_2 conditions. It has been
78 suggested that excessive accumulation of starch may negatively affect the internal
79 organization of chloroplasts, disturbing the configuration of granal stacks, distorting the
80 thylakoids and thus negatively affecting electron transport (Yelle et al. 1989; Pritchard et
81 al., 1997). Thus, it is conceivable that the reduced size of *gwd* and the non-responsiveness
82 of this mutant to elevated CO_2 is the consequence of reduced electron transport due to
83 thylakoid distortion, which in turn results in reduced A_n and growth under both ambient
84 and elevated CO_2 conditions.
85
86 **Photosynthetic acclimation to elevated CO_2 in *gwd* plants: a case of accelerated**
87 **senescence?**
88 The photosynthetic acclimation to elevated CO_2 has long been ascribed to sugar-mediated
89 reduction of photosynthetic gene expression (Cheng et al., 1998; Moore et al., 1999;
90 Ainsworth et al., 2004; Aranjuelo *et al.*, 2013). However, in this work we could not find
91 a clear link between the soluble sugar contents, Rubisco content and net photosynthesis
92 in LD-grown *aps1* and *gwd* plants cultured under ambient and elevated CO_2 conditions.
93 Obtained data would indicate that, under LD conditions, sugar-mediated regulation of
94 photosynthetic gene expression does not play an important role in acclimation of
95 Arabidopsis plants to elevated CO_2, at least in *aps1* and *gwd* plants. The case of *gwd*
96 plants was particularly enlightening: although levels of soluble sugars in leaves of
97 ambient CO_2-grown *gwd* plants were comparable to those of plants cultured under
98 elevated CO_2 conditions, Rubisco content and V_{cmax} decreased under elevated CO_2
99 conditions.
100 The N status reduction is a usual response under elevated CO_2 (Stitt & Krapp,
101 1999; (Bloom et al., 2010; Aranjuelo et al., 2011; 2013; Markelz et al., 2013; Jauregui,

102 2016, 2017). In our study, N content significantly decreased in WT and *gwd* plants
103 exposed to elevated 800 ppm Sun and coworkers (2002). The progressive degradation of
104 leaf protein content under elevated CO_2 has been previously associated with an
105 acceleration in leaf protein degradation processes linked with the advanced phenologic
106 status of plants (Miller et al. 1997; Ludewig and Sonnewald 2000). Within this context,
107 the progressive depletion of Rubisco under elevated [CO_2] conditions detected in under
108 elevated CO_2 could be linked with a situation of advanced lead senescence of those plants.
109 As phenology gets closer to the senescence period, N assimilation pathways are altered
110 and the expression of proteases increases (Masclaux-Daubresse et al. 2008). As a
111 consequence of the protease activity and the consequent protein hydrolysis, the resulting
112 N compounds (mostly amino acids) in leaves are released. Within this context, one
113 remarkable feature of this mutant is that elevated CO_2 promotes the accumulation of high
114 levels of asparagine (up to 25% of the total amino acid content). Elevated CO_2 also
115 promoted the accumulation of alanine, leucine and valine. Because *gwd* plants have a
116 poor capacity to accumulate and degrade starch in a diurnal cycle (Caspar et al. 1991),
117 amino acid accumulation could be interpreted as an alternate mechanism for storing
118 photosynthate in a metabolizable form. Alanine is a well-known stress-responsive amino
119 acid (Wallace et al. 1984, Rocha et al. 2010). Furthermore, asparagine, leucine and valine
120 are known to accumulate during senescence (Lea et al. 2007; Watanabe et al. 2013; Avila-
121 Ospina et al. 2015). It is thus likely that photosynthetic acclimation of *gwd* to elevated
122 CO_2 is caused by accelerated leaf senescence rather than sugar accumulation. Further, the
123 fact that Rubisco content was significantly lower in *gwd* than in the WT, together with
124 the large accumulation of high levels of TFAC in *gwd* leaves suggests that Rubisco
125 protein catabolism was associated with amino acid increase and leaf senescence
126 (Huffaker, 1990) in *gwd* plants. Moreover, because excessive accumulation of starch may
127 negatively affect the internal organization of chloroplasts (see above), it is conceivable
128 that *gwd* acclimates to elevated CO_2 to prevent the formation of critically large starch
129 granules that otherwise would compromise chloroplast functionality and the viability of
130 the plant.
131
132 **Conclusion and perspectives**
133 The present work revealed the profound impact of elevated CO_2 on starch metabolism
134 that conditioned plant performance. While in wild type and *aps1* plants exposure to 800
135 ppm increased plant growth, in *gwd* doubling CO_2 availability was not reflected in a larger

136 biomass. Moreover, in plants with the highest starch content, such as wild type grown at
137 elevated CO_2 and *gwd* (at both CO_2 conditions), Rubisco maximum carboxylation activity
138 and photosynthetic apparatus were impaired. Such impairment was explained by the
139 accelerated senescence and hindrance of CO_2 diffusion that was associated with the
140 accumulation of large starch granules rather than sugar accumulation. In summary, our
141 study showed that excessive accumulation of starch negatively affect chloroplast
142 organization and, therefore photosynthesis and growth, in *gwd*.
143 Studies carried out during the last decades with crops such as wheat, alfalfa, rice,
144 soybean, tobacco, etc. exposed to elevated CO_2 condition have shown that, in many cases,
145 plants that suffer photosynthetic acclimation also have high leaf starch content values.
146 Within this context, our results remark the fact that the overflow of starch photosynthate
147 storage negatively affects photosynthetic machinery of Arabidopsis plants. Leaf
148 carbohydrate accumulation probed to be a target factor conditioning plant performance
149 under elevated CO_2 conditions. In agreement with previous studies, our data show that
150 plants with a small sink size will acclimate to high CO_2 by decreasing photosynthetic
151 capacity. Therefore, plants with a large sink size (i.e. large ears in the case of cereals) will
152 benefit more from CO_2 enrichment than those with a small sink size like the plants limited
153 storage organs or the ones that do not have it. The use of near starch-less (*aps1*) and the
154 starch-excess (*gwd*) mutants in this study provided more information on the processes
155 that explain the down regulation of photosynthetic machinery under elevated CO_2
156 conditions. However, we recognize that additional research is needed to discern if it is the
157 accelerated senescence and/or the carbon starvation enhanced under elevated CO_2 of
158 plants. Furthermore, while the use of Arabidopsis as a model organism has enabled
159 advances in understanding plant growth and development, those studies shall be extended
160 to other plants and crops so to better understand how plants will perform under near future
161 environments.
162
163

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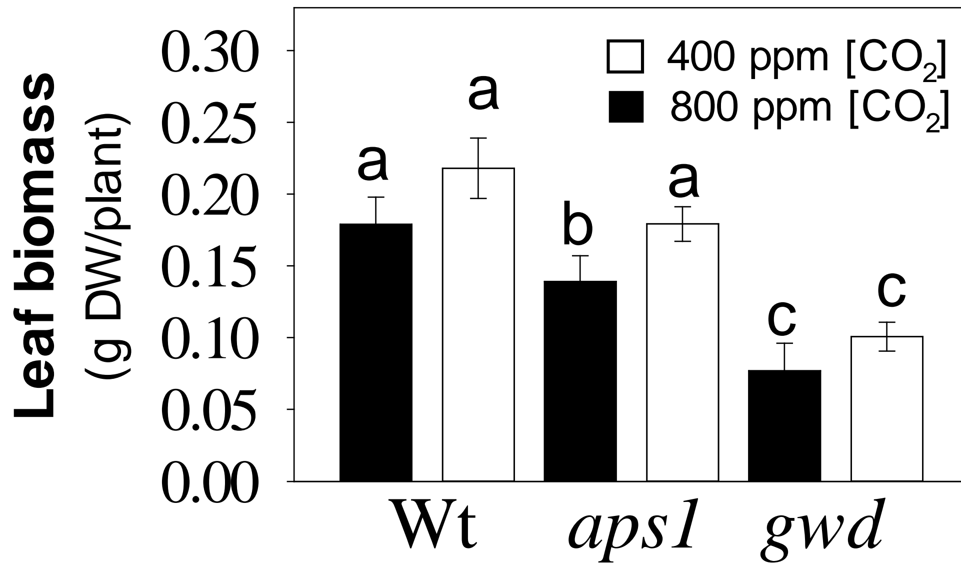

168
169 **Figure 1.** Effect of elevated [CO_2] (800 *versus* 400 ppm) in *Arabidopsis thaliana* (wild
170 type WT, starchless *aps1*, and starchexcess *gwd*) on leaf biomass (dry weight biomass per
171 plant). Bars are means ± SD of 10 replicates, with different letters indicating significant ($P < 0.05$) differences according to Tukey's test.
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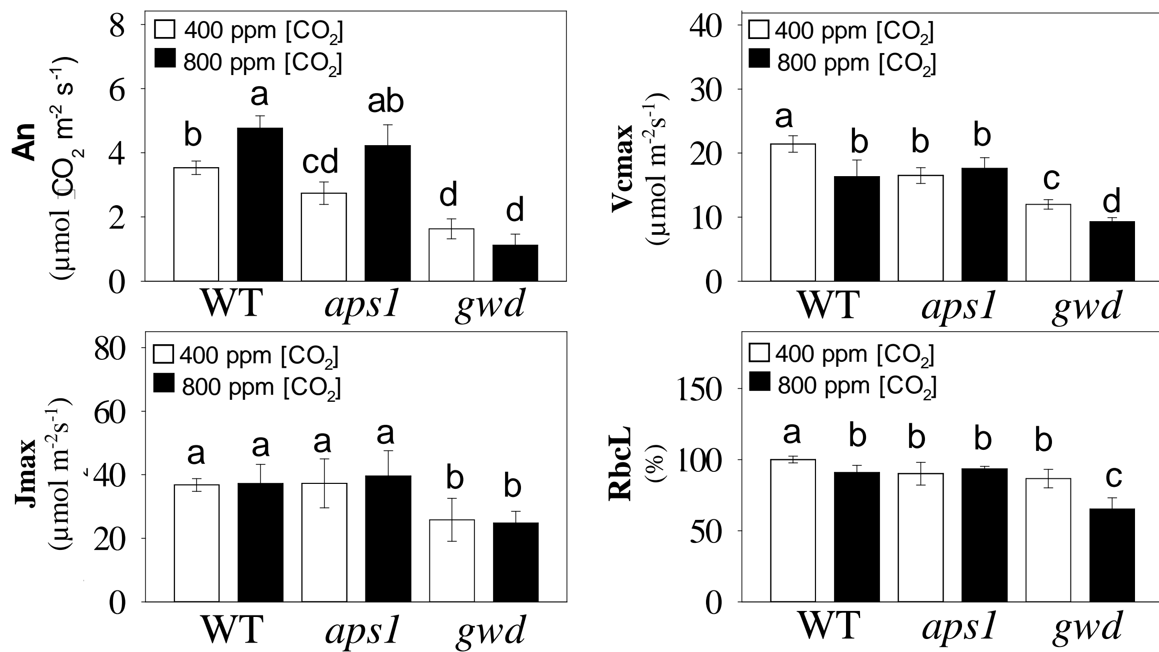

174
175 **Figure 2.** Effect of elevated [CO_2] (800 *versus* 400 ppm) in *Arabidopsis thaliana* (wild
176 type WT, starchless *aps1*, and starchexcess *gwd*) on net photosynthetic rates (An), maximum
177 carboxylation rate (Vc_{max}), maximum electron transport rate contributing to RuBP regeneration
178 (J_{max}) and Rubisco Large Subunit (RbcL). Bars are means ± SD of 5 replicates, with different
179 letters indicating significant ($P< 0.05$) differences according to Tukey's test.
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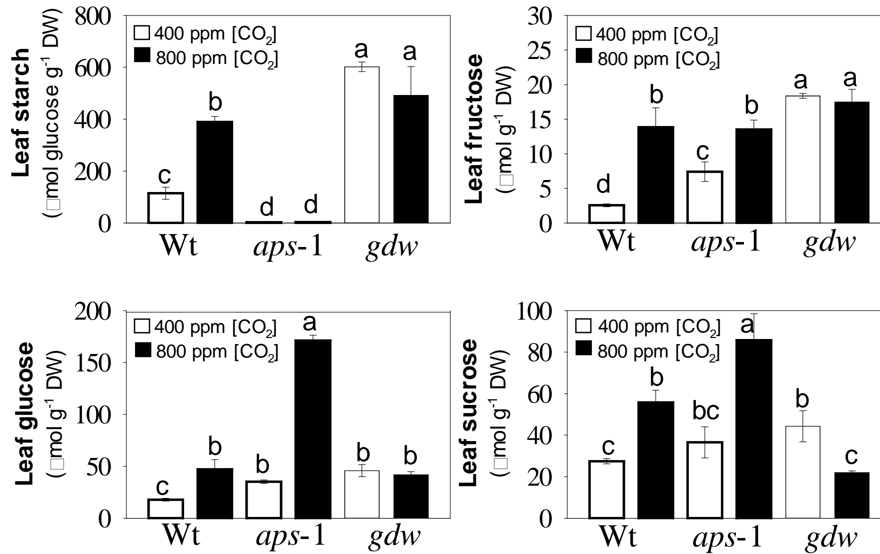

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182 **Figure 3.** Effect of elevated [CO_2] (800 *versus* 400 ppm) in *Arabidopsis thaliana* (wild
183 type wt, starchless *aps*-1, and starchexcess *gdw*) on starch content (µmol glucose g^{-1} DW) and
184 sugars (fructose, glucose, sucrose; µmol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

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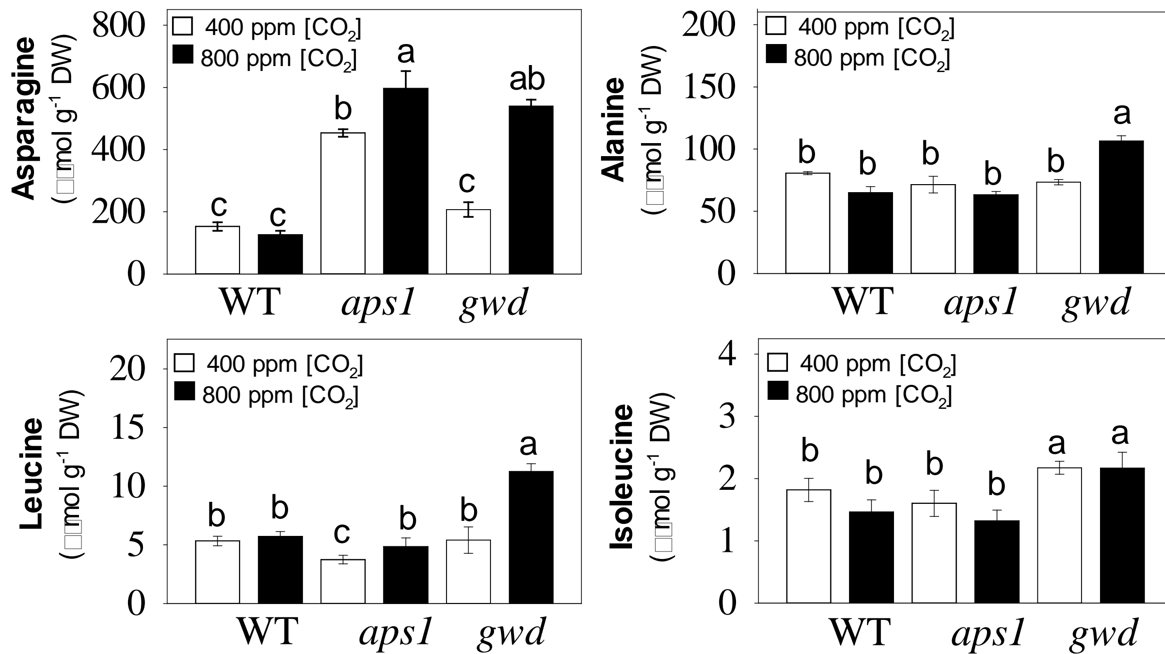

188
189 **Figure 4.** Effect of elevated [CO_2] (800 *versus* 400 ppm) in *Arabidopsis thaliana* (wild
190 type WT, starchless *aps1,* and starchexcess *gwd*) on selected individual amino acid contents in
191 leaves. Bars are means ± SD of 4 replicates, with different letters indicating significant (P<
192 0.05) differences according to Tukey's test.
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194