Elevated CO_2 induces age-dependent restoration of growth and metabolism in gibberellin-deficient plants

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Abstract

Main conclusion The effect of elevated [CO_2] on the growth of tomato plants with reduced GA content is influenced by developmental stage.

The increase of carbon dioxide (CO_2) in the atmosphere during the last decades has aroused interest in the function of this gas in the growth and development of plants. Despite the known association between elevated CO_2 concentration ([CO_2]) and plant growth, its effects in association with gibberellin (GA), plant hormone that regulates de major aspects of plant growth, are still poorly understood. Therefore, we evaluated the effect of elevated [CO_2] on growth and primary metabolism in tomato plants with drastic reduction in GA content (*gib-1*) at two different growth stages (21 and 35 days after germination, dag). Disruption on growth, photosynthetic parameters and primary

metabolism were restored when *gib-1* plants were transferred to elevated [CO_2] at 21 dag. Elevated [CO_2] also stimulated growth and photosynthetic parameters in Wild type (WT) plants at 21 dag, however, minor changes were observed in the level of primary metabolites. At 35 dag, elevated [CO_2] did not stimulate growth in WT plants and *gib-1* mutants showed their characteristic stunted growth phenotype.

Keywords *gib-1* . Tomato . Plant hormone . phase transition . cell division

Introduction

Plant growth and development involve many endogenous and environmental signals that interact with the plant's genetic program to determine plant architecture (Wang and Li 2008; Achard et al. 2009). Fundamental to this process are chemical regulators known as plant hormones (Santner et al. 2009). Among them, gibberellins (GAs) regulate major aspects of plant growth and development, including seed germination, stem elongation, leaf expansion, trichome development and flowering induction (Hedden and Thomas 2016). Many of these aspects are controlled by the capacity of GAs to stimulate cell division and elongation (de Lucas et al. 2008; Achard et al. 2009), through degradation of transcriptional repressor DELLA proteins (Dill et al. 2001; Alvey and Harberd 2005).

GAs are synthesized via terpenoid pathway by the action of terpene synthase, cytochrome P450 oxygenase and 2-oxoglutarate-dependent dioxygenases (2-ODDs) in plastids, the endomembrane system and the cytosol, respectively. 2-ODDs are 2-oxoglutarate dependent, the key intermediate of one the most fundamental biochemical pathways in carbon metabolism -the tricarboxylic acid (TCA) cycle- and a point of connection between carbon and nitrogen metabolism (Araújo et al. 2014). In this way, the dependence on 2-oxoglutarate (2-OG) links GA biosynthesis and homeostasis

53 directly with primary metabolism (Lancien et al. 2000), since most GA oxidases (GA_{ox})
54 are 2-OG dehydrogenase (2-ODDs) (GA_{3ox}, GA_{20ox}, GA_{2ox}).

55 Alterations in ambient [CO_2] expected for the next few years (IPCC, 2014) may
56 impact plant growth and development (Kimball 2016). Succinctly, [CO_2] directly
57 influences the rate of CO_2 assimilation by Rubisco, and consequently gas exchange
58 rates, which could influence plant growth and crop productivity (Campbell et al. 1988;
59 Igamberdiev 2015; Galmés et al. 2017). Particularly in C3 plants, CO_2 is a limiting
60 substrate for photosynthesis, and elevated [CO_2] usually leads to an increase in
61 photosynthetic assimilation rates and a decrease in photorespiration, stimulating
62 production of sugars and biomass accumulation (Ainsworth and Rogers 2007;
63 Ainsworth 2008; Högy et al. 2010). Higher sugar availability is a trigger for plant
64 growth at elevated [CO_2] (Taylor et al. 1994; Masle 2000; Ferris et al. 2001). However,
65 it is the sink capacity of the plant to use or store additional photoassimilate that
66 determines photosynthesis stimulation and growth at elevated [CO_2] (Arp 1991). In
67 soybean (*Glycine max*), for instance, growth habit was a determinant factor of
68 photosynthetic acclimation at elevated [CO_2] (Ainsworth et al. 2004). In this case, the
69 inability to form sufficient sinks in determinate-growth plants contributed to feedback
70 photosynthesis acclimation, suggesting the participation of sugar sensing and signaling
71 in the growth responses (Paparelli et al. 2013; Wang and Ruan 2013; Lastdrager et al.
72 2014).

73 Plant hormones act as chemical mediators to control growth and development in
74 response to elevated [CO_2]. Increase in GAs in response to elevated [CO_2] have
75 consistently been reported in several species such as *Ginkgo biloba* L.(Li et al. 2002),
76 *Arabidopsis* (Teng et al. 2006) and *Populus* (Liu et al. 2014). Furthermore, growth
77 reduction of *Arabidopsis* treated with the GA biosynthesis inhibitor paclobutrazol

78 (PAC) was reverted by elevated [CO_2] (Ribeiro et al. 2012). This suggests that plant
79 growth at elevated [CO_2] may be partially coupled with the effects of GA (Ribeiro et al.
80 2012), and that elevated [CO_2] and GA act could in similar pathways related with plant
81 growth. Despite this circumstantial evidence of association between elevated [CO_2] and
82 plant hormones, little is known about how elevated [CO_2] coordinates plant growth
83 together with GA.

84 Tomato (*Solanum lycopersicum* L.) is one of the most important horticultural
85 crops in the world and has been widely used as a model organism in several fields of
86 plant research (Kimura and Sinha 2008). The availability of monogenic mutant
87 collections represents a powerful tool for the study of gene function and
88 ecophysiological interactions (Carvalho et al. 2011). For example, three mutants in GA
89 biosynthesis (*gibberellin deficient 1, 2 and 3, gib-1*, *gib-2* and *gib-3* respectively) were
90 identified in tomato plants (Koornneef et al. 1990). The *gib-1* mutant shows reduction in
91 ent-copalyl diphosphate synthase activity, the first enzyme involved in GA biosynthesis
92 leading to a dwarf phenotype due to the drastic reduction in GA content (Bensen and
93 Zeevaart 1990). Different to *gib-1*, both *gib-2* and *gib-3* show less conspicuous
94 reductions in growth (Koornneef et al. 1990). Thus, the availability of these GA-related
95 mutants makes tomato plants a useful model for the study of combinatorial effects of
96 reduced GA content and elevated [CO_2] on the plant growth.

97 Since elevated [CO_2] influenced growth and metabolism of *Arabidopsis* treated
98 with PAC (Ribeiro et al. 2012), here we investigated growth and metabolic responses in
99 tomato plants with drastic reduction in GA content (*gib-1*) transferred to elevated [CO_2]
100 at two different growth stages (21 and 35 days after germination, dag). Mutant *gib-1*
101 plants cultivated in ambient [CO_2] showed stunted growth and reduced biomass
102 accumulation, alterations in photosynthetic parameters and disruption in primary

103 metabolism. Transfer to elevated [CO_2] stimulated growth and most of primary
104 metabolism of GA-deficient plants at 21 dag, but not 35 dag. We discuss the influence
105 of elevated [CO_2] on the growth of tomato plants with reduced GA content and how
106 growth can be influenced by developmental stage in tomato plants submitted to elevated
107 [CO_2].
108
109 **Material and methods**
110 *Growth conditions and experimental design*
111 Seeds of tomato (*Solanum lycopersicum* L.) cv. Moneymaker and GA deficient
112 *gib-1* mutants (kindly donated by M. Koornneef, Max Planck Institute for Plant
113 breeding Research, Cologne, Germany) were germinated in Petri dish containing two
114 layers of filter paper soaker in distilled water. After germination, the seedlings were
115 transferred to pots (1,7L) containing commercial substrate (Tropstrato HT Hortaliças,
116 Vida Verde), supplemented with NPK 20:5:20 fertilizer and cultivated as previously
117 described in Vicente et al. (2015). Twenty-one and 35 days after germination, tomato
118 plants were transferred to open top chamber under either ambient (400 μmol mol^{-1}) and
119 elevated (750 μmol mol^{-1}) [CO_2]. Plants were maintained inside the open top chambers
120 for 21 days. The experiment was conducted in greenhouse localized at Universidade
121 Federal de Viçosa (20° 45'S, 42° 15'W).
122
123 *Growth analysis*
124 Stem length was measured every two days. At the end of the 21-day period at
125 [CO_2] treatment, plants were harvested and divided into leaves, stem and roots. Leaf
126 area was measured using a planimeter (Li-Cor Model 3100 Area Meter, Lincoln, NE,
127 USA). Shoot and root biomass were measured from the dry weight of leaves, stem and

roots. Relative growth ratio (RGR) and specific leaf area (SLA) were determined as described by Hunt (1982).

Leaf anatomy

Leaf discs were collected from the center of the third leaf and fixed in FAA50 (Formaldehyde, acetic acid and ethanol 50%) for 48 h, and then stored in ethanol 70% according to Johansen (1940). Then the plant material was dehydrated in ethanolic series and included in methacrylate (Historesin-Leica), according to the manufacturer's recommendations. For light microscope observation (AX-70 TRF, Olympus Optical, Tokyo, Japan), cross sections 5 µm thick were obtained with an automatic advanced rotary microtome (model RM2155, Leica microsystems Inc., Deerfield, USA), were stained with toluidine blue then photographed using a digital camera (Zeiss AxioCam HRc, Göttinger, Germany). Anatomical features, as leaf thickness and thickness cell layers, were evaluated using Image J (NIH, Bethesda, Ma).

Gas exchanges and fluorescence measurements

The net rate of carbon assimilation (A), stomatal conductance (g_s), internal CO_2 concentration (C_i) and fluorescence parameters were measured in third or fourth fully-expanded leaf from the botton, using infrared gas analyzer (Li 6400XT, Li-Cor, Lincoln, USA) equipped with integrated fluorescence chamber head (Li-6400-40; Li-Cor Inc.). The measurements were conducted with [CO_2] supply of 400 and 750 µmol CO_2 mol^{-1} air with artificial photosynthetically active radiation level of 500 µmol of photons m^{-2} s^{-1}, matching the greenhouse irradiance value. The rate of mitochondrial respiration in darkness (R_D) was measured on dark-adapted leaves for at least 2 hours after the end of the light period. Using the values of these parameter, mitochondrial

153 respiration in light (R_L) was estimated according to Lloyd et al. (1995) as R_L =[0.5 –
154 0.05ln(PPFD)]×R_D. The photorespiratory rate of Rubisco (R_P) was estimated according
155 Valentini et al. (1995), as R_P=1/12[ETR – 4(A+R_L)]. A/C_i curves were measured at
156 saturating irradiance (1000 µmol photons m^{-2} s^{-1}) as described Barbosa et al. (2018).
157 From these curves, maximum rate of carboxylation (V_{cmax}) and the maximum rate of
158 electron transport (J_{max}) were calculated as proposed by Sharkey et al. (2007).
159
160 *Determination of metabolic levels*
161 Samples of third leaf, midpoint region of stem and root were collected,
162 immediately frozen in liquid nitrogen, and stored at -80°C until further analysis.
163 Glucose, fructose, sucrose and starch levels were determined as described by Fernie et
164 al. (2001). Amino acids levels were determined as described by Gibon et al. (2004).
165 Protein levels were measured as described by Bradford (1976), with modifications
166 (Gibon et al. 2004). The levels of nitrate were measured as described by Fritz et al.
167 (2006). Chlorophylls were extracted using acetone 80% and the content of them was
168 determined as described by Lichtenthaler (1987). The levels of all others metabolites
169 were quantified by gas chromatography mass spectrometry (GC-MS) following the
170 protocol described by Lisec et al. (2006).
171
172 *Statistical analysis*
173 The experiments were designed in a completely randomized distribution.
174 Differences described are systematically statistically grounded based on ANOVA,
175 where $P<0.05$ was considered significant. If ANOVA showed significant effects,
176 Student's t test ($P<0.05$) was used to determine differences between each treatment and

177 control. All statistical analyses were made using Statistical Package for the Social
178 Sciences for Windows statistical software (SPSS).
179
180 **Results**
181 *Elevated [CO_2] stimulates impaired growth of the gib-1 mutant*
182 The *gib-1* tomato mutant shows impaired growth due to reduction in the activity
183 of ent-copalyl diphosphate synthase, a key enzyme in the GA biosynthesis pathway
184 (Bensen and Zeevaart 1990). Under ambient [CO_2], *gib-1* plants showed severe
185 reductions in shoot (-82%) and root (-29%) biomass, as well as leaf area (-80%) and
186 stem length (-77%), compared with WT plants (Fig. 1). In addition, SLA and RGR were
187 lower in *gib-1* mutants, compared with WT plants at ambient [CO_2] (Supplementary
188 Figure 1). Elevated [CO_2] did not affect shoot and root biomass of WT plants but led to
189 increased leaf area (22%) and stem length (41%), compared with WT plants at ambient
190 [CO_2] (Fig. 1). In the *gib-1* mutant, elevated [CO_2] restored shoot and root biomass, as
191 well as leaf area, to similar values as WT plants at ambient [CO_2] (Fig. 1). Elevated
192 [CO_2] doubled the stem length in the *gib-1* mutant, compared to *gib-1* at ambient [CO_2],
193 but still fell short of the stem length of WT plants at ambient [CO_2] (Fig. 1 d). *gib-1*
194 plants in elevated CO_2 also showed SLA and RGR values similar to those of ambient
195 [CO_2] WT plants (Supplementary Figure 1).
196
197 *Elevated [CO_2] restores leaf anatomy in gib-1 plants*
198 Given the stimulatory effects of GAs and elevated [CO_2] on cell expansion and
199 division (Taylor et al. 1994, 2005; Achard et al. 2009) we decided to analyze how
200 elevated [CO_2] influences leaf anatomy of *gib-1* mutant and WT plants. Under ambient
201 [CO_2], leaf cross-sections revealed considerable visual differences between *gib-1*

mutants and WT plants (Fig. 2 a and b). Leaf thickness was increased (35%) in *gib-1* compared to WT due to a 18% and 62% increase in the thickness of palisade and spongy parenchyma, respectively (Fig. 2 e, f and g). The greater thickness of spongy parenchyma resulted in the reduction of the palisade-to-spongy parenchyma ratio in *gib-1* (Fig. 2 h)

Under elevated [CO_2], *gib-1* leaf cross-sectional appearance was very similar to that of WT plants at ambient [CO_2] (Fig. 2 a and d). At elevated [CO_2], total leaf thickness, palisade and spongy parenchyma thickness, and the palisade-to-spongy parenchyma ratio of *gib-1* plants were similar to those of WT plants at ambient [CO_2] (Fig. 2 e, f, g and h). Thickness of either upper or lower epidermis was not altered in *gib-1* between [CO_2] levels (Fig. 2 i and j). As for WT plants, most leaf anatomical parameters were similar between treatments, except for upper epidermis thickness, which was reduced in elevated [CO_2] plants compared to ambient [CO_2] (Fig. 2).

Elevated [CO_2] restore photosynthetic function in the gib-1 mutant

We next investigated the combined effects of reduction in endogenous GA content and elevated [CO_2] on gas exchange in *gib-1* mutant tomato plants. Under ambient [CO_2], the *gib-1* mutant showed a marked reduction (~45%) in *A*, compared with WT plants (Fig. 3 a). In addition, the *gib-1* mutation led to reductions in R_D (-52%), V_{cmax} (-45%), J_{max} (-37%), R_P (-41%) and ETR (-44%) at ambient [CO_2]. Under ambient [CO_2], C_i was unaffected in the *gib-1* mutant, compared with WT plants (Fig. 3)

Under elevated [CO_2], *A*, R_D and C_i values were higher in both WT and *gib-1* compared to ambient [CO_2] WT plants (Fig. 3 a, b and g). Elevated [CO_2] increased J_{max} in *gib-1* but not in WT plants, compared to ambient [CO_2] WT plants (Fig. 3 d). *R*p was

227 considerably reduced in both genotypes at elevated [CO_2] (Fig. 3 e). Interestingly, g_s
228 remained stable in WT and *gib-1* under both ambient and elevated [CO_2] (Fig. 3 h). The
229 *F*v/Fm ratio was close to ideal values for non-stressed leaves (~0.83) in WT plants and
230 *gib-1* mutant in both [CO_2] regimes (Fig. 3 i)
231
232 *Nitrogen metabolism is altered by elevated [CO_2] in gib-1 mutant plants*
233 Elevated [CO_2] typically reduces nitrogen content in the tissues of some plant
234 species (Bloom et al. 2002, 2010, 2014; Taub and Wang 2008). We thus determined the
235 content of some of the main nitrogenous compounds in leaves, stem and roots of WT
236 and *gib-1* plants under ambient and elevated [CO_2]. Nitrate levels remained generally
237 unchanged across treatments, except for an increase in *gib-1* at ambient [CO_2]
238 (Supplementary Fig. 2). Alterations in amino acids levels were found for the *gib-1*
239 mutant at ambient [CO_2]. Whereas amino acids levels were reduced in leaf (35%), an
240 increase was observed in the stem (76%) and roots (41%) (Supplementary Fig. 2). At
241 elevated [CO_2], amino acids levels remained unaltered in WT plants and *gib-1* mutants,
242 compared with WT plants at ambient [CO_2]. At ambient [CO_2], leaf protein content was
243 reduced by 24%, while it increased approximately four-fold in stems of *gib-1* mutants
244 (Supplementary Fig. 2). Leaf protein was lower in *gib-1* mutant and WT plants under
245 elevated [CO_2] (Supplementary Fig. 2). In the roots, protein levels were increased in
246 both *gib-1* and WT plants at elevated [CO_2] (Supplementary Fig. 2). Under ambient
247 [CO_2], chlorophyll analysis of *gib-1* mutants showed increases of 18% and 65% in the
248 leaf and stem, respectively. Elevated [CO_2] did not affect leaf chlorophyll content in
249 WT plants.
250

Changes in carbohydrate content and partitioning associated with GA content and elevated [CO_2]

Photosynthetic assimilation of CO_2 and GA are coupled to carbon metabolism and consequently to carbohydrates content (Ainsworth et al. 2002; Ainsworth and Long 2005; Leakey et al. 2009; Paparelli et al. 2013). Since carbohydrates are essential to the fundamental process required to plant growth (Eveland and Jackson 2012), we investigated the levels of soluble sugars and starch in WT and *gib-1* mutant grown both at ambient and elevated [CO_2]. Under ambient [CO_2], *gib-1* mutants showed reduced levels of leaf and stem glucose (Supplementary Fig. 3) compared with WT plants at ambient [CO_2]. By contrast, glucose level in roots increased in *gib-1* mutants at ambient [CO_2] (Supplementary Fig. 3). Under ambient [CO_2], the reduction of leaf fructose in *gib-1* mutants was accompanied by an increase in stem and roots in these plants, compared with WT plants at ambient [CO_2]. The levels of sucrose showed a similar pattern as those of fructose in *gib-1* plants grown at ambient [CO_2]. Growth of *gib-1* mutant at ambient [CO_2] resulted in reduced leaf starch.

Elevated [CO_2] did not affect glucose levels in WT plants but restored them in leaves of *gib-1* mutants. Although higher than *gib-1* at ambient [CO_2], the level of glucose in stem was reduced in *gib-1* mutants grown at elevated [CO_2]. Moreover, the level of glucose in the root increased in *gib-1* mutant at elevated [CO_2]. Although elevated [CO_2] did not affect fructose content in leaf, it increased it in stem and roots of WT plants, compared with WT plants at ambient [CO_2]. At elevated [CO_2], *gib-1* mutants showed increased fructose content in all plant organs analyzed, compared with WT plants at ambient [CO_2]. Elevated [CO_2] did not affect sucrose content in WT plants but restored it in the *gib-1* mutant. The level of starch increased in WT plants and *gib-1*

mutants under elevated [CO_2], compared with WT plants at ambient [CO_2] (Supplementary Fig. 3).

Elevated [CO_2] modifies part of the metabolic profile in gib-1 mutant

To investigate how the metabolism of each organ was modified by GA and elevated [CO_2], we built a metabolic profile in WT and *gib-1* plants under ambient and elevated [CO_2] (Fig. 4).

Under ambient [CO_2], the levels of 34 (out of 40) leaf metabolites were affected in the *gib-1* mutant, of which 30 were reduced and 4 increased compared with WT plants at ambient [CO_2] (Fig. 4). Among the metabolites whose level increased are 3-PGA, trehalose, oxalic acid and leucine. Elevated [CO_2] affected few leaf metabolites in WT plants and restored the levels of 26 of the 34 metabolites affected in the *gib-1* mutant grown at ambient [CO_2]. The levels of galactinol, glyceric acid, glycerol, mannose, 2-OG and proline were reduced in leaf both WT plants and *gib-1* mutants at elevated [CO_2], while citric acid was increased. Elevated [CO_2] increased the level of leaf GABA only in the *gib-1* mutant. Myo-inositol, lactate, glycine, ornithine, serine and valine remained unchanged in WT plants and *gib-1* mutant under both ambient and elevated [CO_2].

Thirty-three metabolites were detected in the stem by GC-MS. In *gib-1* mutant grown at ambient [CO_2], 13 metabolites were reduced, while 11 were increased, compared with WT plants at ambient [CO_2]. Most metabolites were not affected by elevated [CO_2] in the stem of WT plants, compared with WT plants at ambient [CO_2]. However, the level of galactinol, mannose, malate and serine showed reduced and fructose increased levels in WT plants grown at elevated [CO_2] in relation to WT plants at elevated [CO_2]. Elevated [CO_2] restored 19 of 24 metabolites affected in stems of

300 *gib-1* mutants at ambient [CO_2]. The level of fructose, asparagine, glutamate, glutamine
301 remained increased, while malate and serine were reduced in *gib-1* mutants at elevated
302 [CO_2], compared with WT at ambient [CO_2]. No change was observed in myo-inositol,
303 lactate, pyruvate, GABA, glycine, leucine, valine and β-alanine (Fig. 4).

304 In roots, most amino acids were increased in *gib-1* mutants at ambient [CO_2]. In
305 addition, the level of fructose, glucose, 3-PGA, sucrose and 2-OG also increased in the
306 root of *gib-1* mutants, compared with WT at ambient [CO_2]. The level of fumarate,
307 malate and oxalacetate decreased in *gib-1* mutants grown at ambient [CO_2]. Elevated
308 [CO_2] led to increase in the level of fructose, galactinol, 3-PGA, succinate, glycine,
309 phenylalanine, tyrosine and β-alanine in WT plants and *gib-1* mutant, while reducing
310 the level of malate in these plants compared with WT plants at ambient [CO_2]. At
311 elevated [CO_2], an increase in glucose, citrate, isocitrate, glutamate and serine was
312 observed only in the roots of *gib-1* mutant, compared with WT plants at ambient [CO_2].
313 Lastly, no changes were observed in myo-inositol, aconitate, lactate, pyruvate, GABA,
314 ornithine and proline in the roots (Fig. 4).

315

316 *Elevated [CO_2] stimulates growth in tomato plants 21 dag, but not 35 dag*

317 The effect of elevated [CO_2] on plant growth and development was described as
318 age-dependent (Ainsworth 2008; Franks 2013). Thus, the influence of [CO_2] on growth,
319 gas exchange and primary metabolism was evaluated in WT and *gib-1* mutant submitted
320 at 35 dag at ambient and elevated [CO_2]. Under ambient [CO_2], *gib-1* showed a drastic
321 reduction in growth, biomass (~93%) and leaf area (~95%), compared to WT plants
322 (Fig. 5). Growth of WT and *gib-1* at elevated [CO_2] did not affect the growth
323 parameters evaluated at 35 dag (Fig. 5 b and c).

Under ambient [CO_2], reduction GA content in *gib-1* mutant decreases *A* (~35%), R_D (~19%) and R_P (~40%) at 35 dag, compared with WT (Fig 6 a, d and e). Growth in elevated [CO_2] increase *A* and Ci, while reduced R_p in both, WT and *gib-1* (Fig. 6 a, c and e). No alterations in WT plants was observed in R_D, however, these parameters were decreased in *gib-1* mutant at elevated [CO_2], compared to WT plants under ambient [CO_2]. g_s remained stable in WT and *gib-1* under both ambient and elevated [CO_2] (Fig. 6 b). The *F*v/Fm ratio was close to ideal values for non-stressed leaves (~0.83) (Fig. 6 f).

Sugar evaluation of *gib-1* mutant submitted to ambient [CO_2] at 35 dag shows reduction in leaf glucose (~50%) and fructose (~87%) and increased in sucrose compared to WT plants at ambient [CO_2]. Under elevated [CO_2] WT plants did not differ in leaf sugar content when compared to WT plants at ambient [CO_2]. *gib-1* mutants kept reduction on glucose (~51%), fructose (~88%), and increase in sucrose (~27%) at elevated [CO_2] (Supplementary Fig. 4)

Discussion

Elevated [CO_2] stimulates growth at least in part in a GA-independent manner in *Arabidopsis* treated with GA synthesis inhibitor (PAC) (Ribeiro et al. 2012). Here, we evaluated the effect of [CO_2] in tomato mutants with drastic reduction in GA content. GA regulates major aspects of growth and here we show that elevated [CO_2] can directly stimulate mechanisms that compensate GA deficiency in *gib-1* mutants. However, this effect is strongly dependent on plant age.

Source-sink relationships determines carbohydrate allocation

348 Alterations in endogenous level of GAs change the pattern of growth and
349 biomass allocation (Nagel et al. 2001). The concentration of GAs in the *gib-1* mutant is
350 insufficient to maintain normal leaf and stem growth, however, a less drastic effect is
351 observed on root growth (Fig. 1). The marked reduction in growth and shoot biomass
352 allocation impaired RGR in *gib-1* mutants (Supplementary Fig. 1). RGR has a positive
353 relation with leaf mass ratio and SLA (Gleeson and Tilman 1992; Poorter and van der
354 Werf 1998), parameters that are strongly affected by GA. Elevated [CO_2] reestablished
355 biomass allocation and SLA in *gib-1* mutants, which in turn influenced positively RGR.
356 Leaf anatomy influences photosynthetic capacity, determining the diffusion of
357 CO_2 through the mesophyll (Terashima et al. 2011; Tomás et al. 2013). Reduction in
358 GA content acts as a factor disrupting growth and leaf development, since GA acts in
359 the cell division, cell expansion and mesophyll organization (Jiang et al. 2012).
360 Impaired leaf expansion in GA-deficient plants leads to increased number of cells per
361 unit leaf area (Jiang et al. 2012), giving *gib-1* the appearance of a highly packed
362 mesophyll (Fig. 2). In addition, increased mesophyll thickness influenced the reduction
363 of SLA in *gib-1* mutant under ambient [CO_2] (Supplementary Figure 1).
364 The increase in leaf thickness accompanied by the reduction in intercellular
365 spaces in the *gib-1* mutant may have contributed to the reduction in *A*, since they restrict
366 the diffusion of CO_2 through the mesophyll and to the carboxylation site of Rubisco
367 (Evans and Caemmerer 1996; Terashima et al. 2001). Furthermore, reduced lamina size
368 and overlapping leaves may cause self-shading in *gib-1*, which also impairs
369 photosynthetic capacity.
370 Although *gib-1* presented reduced *A* (Fig. 3), CO_2 fixation probably exceeded
371 the demand for growth, leading to the accumulation of carbohydrates in the stem and
372 root (Supplementary Fig. 3). Stem storage of excess photoassimilate during periods of

low sink strength buffers against source-sink changes during the different stages of growth (Slafer 2003). Imbalance between source and sink can lead to downregulation of photosynthesis due to accumulation of non-structural carbohydrates (such as soluble sugars and starch) in leaves (Stitt and Krapp 1999; Ainsworth and Bush 2011; Sugiura et al. 2017). Thus, the allocation of carbohydrates to the stem could be a way of delaying photosynthetic inhibition in *gib-1* mutants. In addition, the larger fraction of carbohydrate allocated to the root supports root respiration, which is less affected by GA deficiency than shoot respiration (Nagel and Lambers 2002).

In general, elevated [CO_2] increases carbon assimilation and the availability of carbohydrates, which contribute to increased plant growth (Ainsworth and Long 2005; Teng et al. 2006; Li et al. 2013). However, it is the ability to grow and produce new sinks that determines photoassimilates consumption, whose accumulation could result in the inhibition of photosynthesis. Dark respiration (R_D) is closely related to carbon balance and therefore the availability of carbohydrates can interfere in R_D. Elevated [CO_2] accelerate the accumulation of carbohydrates, which leads to transcriptional up-regulation of genes associated with respiration pathways and R_D stimulus (Li et al. 2008; Leakey et al. 2009; Markelz et al. 2014; Watanabe et al. 2014). Thus, the increase in R_D observed in WT and *gib-1* mutants can be attributed to the increase in A and consequently to the increase of carbohydrates under elevated [CO_2] (Fig. 3 and Supplementary Fig. 3).

Elevated [CO_2] induces metabolic homeostasis in GA deficient plants

Plant growth is dependent on the interaction between carbon and nitrogen metabolism, which are linked by the tricarboxylic acid (TCA) cycle (Nunes-Nesi et al. 2010). Although the physiological function of genes regulated by GAs has been

addressed (Yamaguchi 2008), studies on the effect of GAs on energy metabolism are scarce. We did not observe any drastic differences in metabolites levels between ambient and elevated CO_2 treatments for WT plants (Fig. 4). Enhanced plant growth under elevated [CO_2] does not induce a massive remodeling of metabolism. Optimization of carbon and nitrogen acquisition under such conditions appears to be dependent mostly on fine-tuning of specific points of the metabolic network. GA-deficient plants, on the other hand, show a general reduction in levels of sugars and amino acids in the leaf and an increase in roots under ambient [CO_2] (Fig 4 and Supplementary Fig. 2, 3). This is probably a consequence of the alteration in carbon allocation in *gib-1*, whereby the mutation leads to increased root-to-shoot biomass ratio.

Elevated [CO_2] restored the levels of most metabolites in *gib-1* to the level of WT (Fig. 4). Alterations in the TCA cycle have been shown to influence GA levels (Margaretha et al. 2009; Araújo et al. 2012), as multiple enzymes in the GA biosynthetic pathway are dependent on a TCA cycle intermediate, 2-oxoglutarate. On the other hand, it is not clear if the reverse is true, *i.e.* how does altered GA impact primary metabolism?

GABA levels increased only in the leaves of *gib-1* under elevated [CO_2] (Fig. 4). Increases in GABA concentration occur in response to extreme conditions, like temperature, dehydration, salinity, oxygen stress (Kinnersley and Turano 2000; Bouché and Fromm 2004). GABA provides an alternative pathway for the conversion of alpha ketoglutarate to succinate in the TCA cycle, and compromising the enzymes of the TCA cycle involved in the steps up to the production of succinate alters GABA shunt activity (Lemaitre et al. 2007; Fait et al. 2008). Exogenous GABA application improved growth of *Zea mays*, *Stellaria longipes* and *Lemna*, possibly by inducing cell elongation and division or/and by maintaining metabolic balance within plant tissues (Kathiresan et al.

1998; Kinnersley and Lin 2000; Li et al. 2016). Thus, elevated [CO_2] in plants with reduction in GA can influence GABA content and consequently carbon flux, ameliorating *gib-1* growth at elevated [CO_2].

The effect of elevated [CO_2] on gib-1 is age-dependent

Both elevated [CO_2] and GA influence the expression of genes related to loosening and rearrangement of the cell wall, besides controlling the rate of cellular proliferation (Vogler et al. 2003; Yang et al. 2004; Achard et al. 2009; Ribeiro et al. 2012). We showed that GA, an essential hormone for the normal growth and development of plants, is dispensable for growth in tomato plants under elevated [CO_2]. Interestingly, we observed that the effects of elevated [CO_2] on tomato growth is age-dependent, regardless of GA content (Fig. 5). These results indicate the existence of a "sensitive phase" in which elevated [CO_2] is able to influence growth in plants. In addition to CO_2, the action of other environmental factors is also restricted to the "sensitive phase" in tomato plants. Calvert (1957) showed that the influence of temperature on tomato flowering occurs in the first two or three weeks from the seedling emergence. This stage corresponds to our treatment of plants submitted to elevated [CO_2] with 21dag.

In Arabidopsis the most pronounced growth under elevated [CO_2] was observed during the vegetative stage (Watanabe et al. 2014). As observed in Arabidopsis, the sensitivity of tomato plants to elevated [CO_2] may be linked to the juvenile phase. The efficiency of enzymes that promote cell expansion and, consequently, plant growth depends on factors controlled by the stage of development, which limits growth to specific periods (Sloan et al. 2009). Addition of new extracellular polymers and remodeling of existing components in the primary cell walls marks the exponential

448 phase of cell expansion. This is followed by cell wall thickening and rigidification to
449 create secondary cell walls that enhance structural integrity, but reduce cell wall
450 extension (Hall and Ellis 2013). Furthermore, the transition from the juvenile to the
451 adult phase can determine plant architecture and growth pattern (Huijser and Schmid
452 2011; Poethig 2013). Phase transition is preceded by a change in the competence of the
453 shoot to respond to stimuli that induce reproductive development (Poethig 2013). The
454 changes in meristem identity during phase transition are accompanied by genetic
455 reprogramming that may trigger changes in leaf and stem morphology, as well as
456 alteration in growth rate (Poethig 2010, 2013). With the development of floral organs
457 most of the photoassimilates are destined for the production of flowers and fruits and
458 for the maintenance of respiration of reproductive structures (Obeso 2002).

459 In conclusion, elevated [CO_2] favors photosynthesis and carbohydrate
460 production, regardless of plant age. However, we showed here that plant age can
461 indirectly influence carbon partitioning via changes in source-sink relationships. These
462 changes are mostly driven by the growth phase, either juvenile or adult, whereby
463 vegetative or reproductive structures will be favored as strong sinks. Plant hormones
464 can act as integrators of growth and development, and GAs, in particular, control cell
465 division and expansion. We have shown here that increased growth under elevated
466 [CO_2] in tomato does not require a functional gibberellin biosynthetic pathway. In the
467 juvenile phase, gibberellin-deficient mutants can grow to the same extent as wild-type
468 plants. In the adult phase, however, elevated [CO_2] does not stimulate growth and
469 gibberellin mutants show their characteristic stunted growth phenotype. This suggests
470 that growth stimulation by [CO_2] is highly dependent on plant developmental stage,
471 possibly linked to the juvenile-to-adult phase transition (Fig. 7). Further work should

explore the potential role of other hormones mediating growth stimulation by elevated [CO_2].

Author contribution statement

KG conducted experiments and wrote the manuscript. LCC, performed the experiments and statistical analysis. FALB, FBC and TMP performed the experiments. WLA contributed reagents, materials, and analysis tools. DMR designed the experiments. AZ finalized manuscript writing. All authors reviewed the final version of the manuscript and approved it.

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Figure legends

Fig. 1 Effects of elevated [CO_2] on growth of wild type (WT) and *gib-1* mutant 21 dag. a, phenotypes of WT and *gib-1* mutant grown at ambient and elevated [CO_2]. b, shoot and root biomass. c, leaf area. d, stem length. Measurements were done in tomato plants after 21 days of growing at 400 or 750 µmol CO_2 mol^{-1}. Asterisks indicate values determined by Student's t test to be significantly different from WT plants in ambient [CO_2] ($P<0.05$). Values are means ± standard error of 10 replicates.

Fig. 2 Effects of GA and elevated [CO_2] at leaf anatomy of wild type (WT) and *gib-1* mutant at 400 or 750 µmol CO_2 mol^{-1}. a, b, c and d cross section of the third leaf of the wild type and *gib-1* plants at 400 or 750 µmol CO_2 mol^{-1} (Scale bar: 100µm). e, total leaf thickness. f, palisade parenchyma thickness. g, spongy parenchyma thickness. h, palisade: spongy parenchyma ratio. i, upper epidermis thickness. j, lower epidermis thickness. Asterisks indicate values determined by Student's t test to be significantly different from WT plants at ambient [CO_2] ($P<0.05$). Values are means ± standard error of 6 replicates. UE, Upper epidermis; LE, lower epidermis; PP, palisade parenchyma; SP, spongy parenchyma.

Fig. 3 Changes in gas exchange and fluorescence parameters in wild type (WT) and *gib-1* mutant at 400 or 750 µmol CO_2 mol^{-1}. a, net rate of carbon assimilation (A). b, rate of mitochondrial respiration in darkness (R_D). c, maximum rate of carboxylation (V_{cmax}). d,

779 maximum rate of electron transport (J_{max}). e, photorespiratory rate of Rubisco (R_P). f,
780 electron transport rate (ETR). g, internal CO_2 concentration (C_i). h, stomatal
781 conductance (g_s). i, variable to maximum fluorescence ratio (F_v/F_m). Asterisks indicate
782 values determined by Student's t test to be significantly different from WT plants in
783 ambient [CO_2] ($P<0.05$). Values are means ± standard error of 10 replicates.

784 **Fig. 4** Relative metabolite level of leaf, stem and root from wild type (WT) and *gib-1*
785 mutant at 400 or 750 µmol CO_2 mol^{-1}. Samples of leaf, stem and root were collected at
786 the end of the light period from plants growing for 21 days at ambient or elevated
787 [CO_2]. Asterisks indicate values determined by Student's t test to be significantly
788 different from wild type (WT) plants in ambient [CO_2] ($P<0.05$). Values are means ±
789 standard error of 6 replicates. Data are normalized with respect to mean response
790 calculated for the wild type (WT) plants in ambient [CO_2]. nd, not detected. The full
791 dataset from the metabolite profiling study is available as Supplementary Table S1.

792 **Fig. 5** Effects of elevated [CO_2] on growth of wild type (WT) and *gib-1* mutant 35 dag.
793 a, phenotypes of WT and *gib-1* mutant grown at ambient and elevated [CO_2]. b, total
794 biomass. c, leaf area. Measurements were done in tomato plants after 21 days of
795 growing at 400 or 750 µmol CO_2 mol^{-1}. Asterisks indicate values determined by
796 Student's t test to be significantly different from WT plants in ambient [CO_2] ($P<0.05$).
797 Values are means ± standard error of 10 replicates.

798 **Fig. 6** Changes in gas exchange in wild type (WT) and *gib-1* mutant at 400 or 750 µmol
799 CO_2 mol^{-1}, 35 dag. a, net rate of carbon assimilation (A). b, stomatal conductance (g_s). c,
800 internal CO_2 concentration (C_i). d, rate of mitochondrial respiration in darkness (R_D). e,
801 photorespiratory rate of Rubisco (R_P). f, variable to maximum fluorescence ratio
802 (F_v/F_m). Asterisks indicate values determined by Student's t test to be significantly

different from WT plants in ambient [CO_2] ($P<0.05$). Values are means ± standard error of 10 replicates.

Fig. 7 Proposed model of growth regulation by elevated [CO_2] in tomato plants. Plants at 21 dag (a) and 35 dag (b) grown under ambient and elevated [CO_2] and their respective meristem development stage. Elevated [CO_2] favors photosynthesis and carbohydrate production independently of the plant age. In the juvenile phase (21 dag), gibberellin-deficient mutants can grow to the same extent as wild-type plants as a response of the cell division and expansion capacity. In the adult phase (35 dag), however, elevated [CO_2] does not stimulate growth and gibberellin mutants show their characteristic stunted growth phenotype. This suggests that growth stimulation by [CO_2] is highly dependent on plant developmental stage, possibly linked to the juvenile-to-adult phase transition. However, which hormones are involved in the control of these events remains an open question.

Supplementary material

Fig. S1 Effects of elevated [CO_2] on specific leaf area (SLA) and relative growth rate (RGR) of wild type (WT) and *gib-1* mutant.

Fig. S2 Levels of nitrate, amino acids, protein and chlorophyll in wild type (WT) and *gib-1* mutant at 400 or 750 µmol CO_2 mol^{-1}.

Fig. S3 Levels of carbohydrate in wild type (WT) and *gib-1* mutant at 400 or 750 µmol CO_2 mol^{-1}.

Fig. S4 Levels of leaf soluble sugars in wild type (WT) and *gib-1* mutant at 400 or 750 µmol CO_2 mol^{-1}, 35 dag.

825 **Table S1** Relative metabolite level of leaf, stem and root from wild type (WT) and *gib-*

826 *1* mutant at 400 or 750 μmol CO_2 mol^{-1}.

827

828

829

A

Ambient [CO_2]

Elevated [CO_2]

WT

gib-1

WT

gib-1

B

Biomass (g)

15

10

5

0

Leaves

Stem

Roots

*

*

WT

gib-1

WT

gib-1

Ambient [CO_2]

Elevated [CO_2]

C

Leaf area (cm^2)

3000

2500

2000

1500

1000

500

0

*

*

WT

gib-1

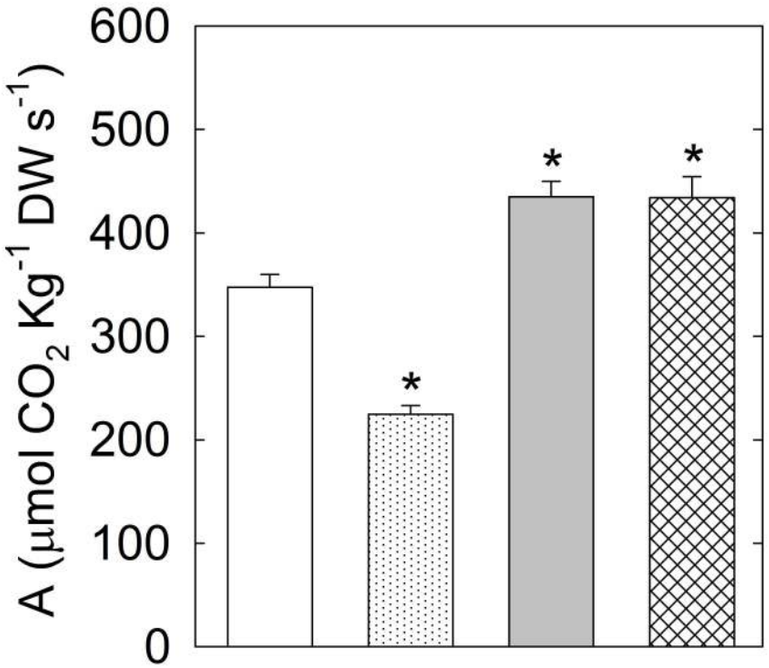
WT

gib-1

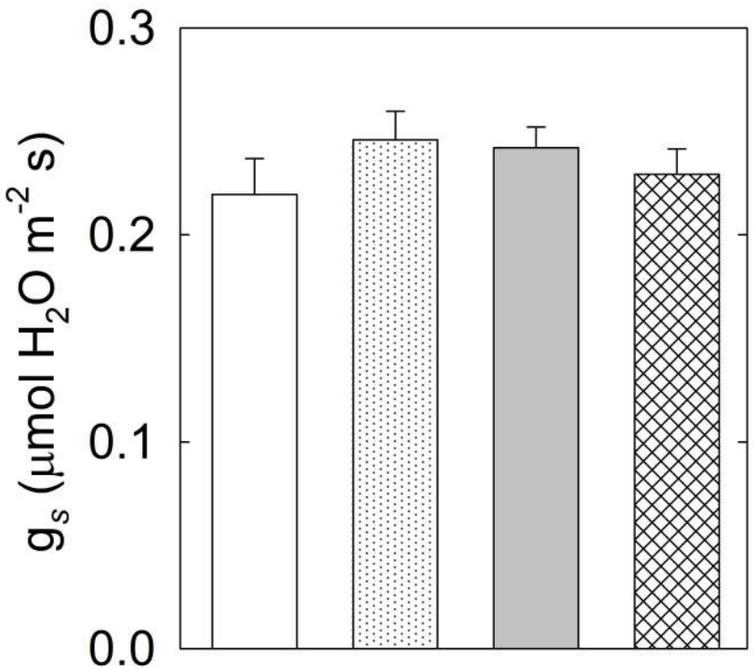
Ambient [CO_2]

Elevated [CO_2]

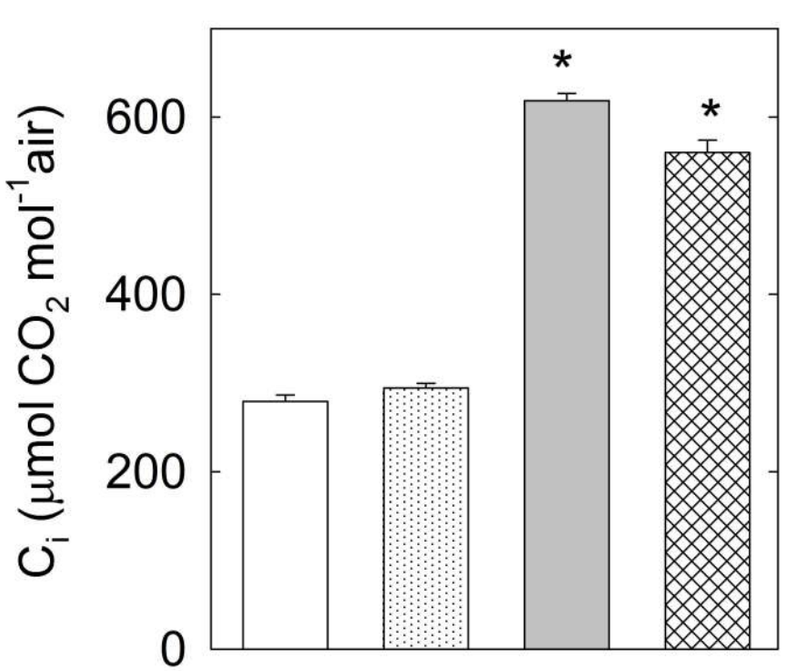
A


600
500
400
300
200
100
0
A (μmol CO2 Kg-1 DW s-1)
*
*

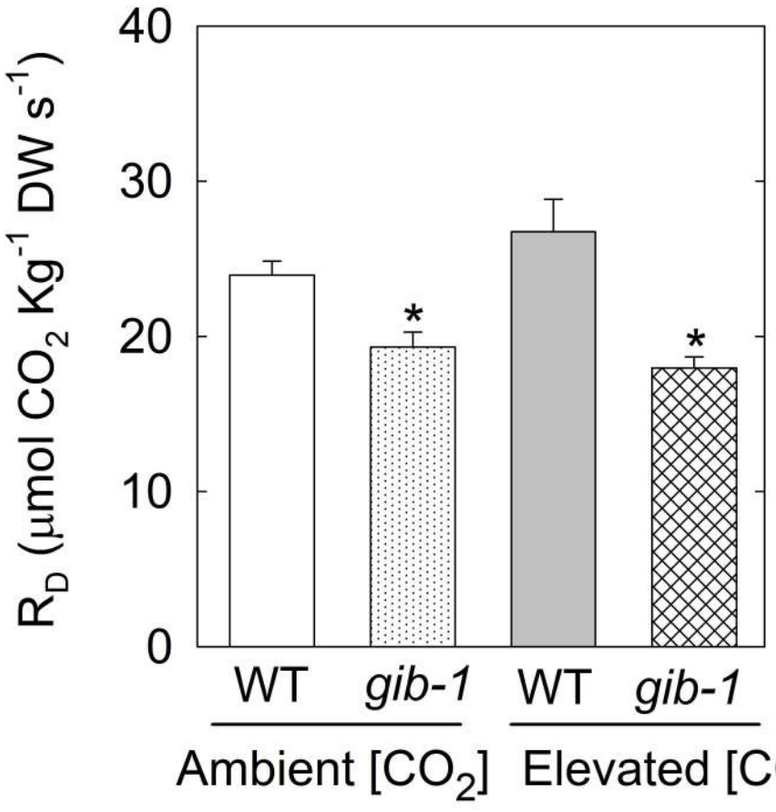
B


0.3
0.2
0.1
0.0
gs (μmol H2O m-2 s)

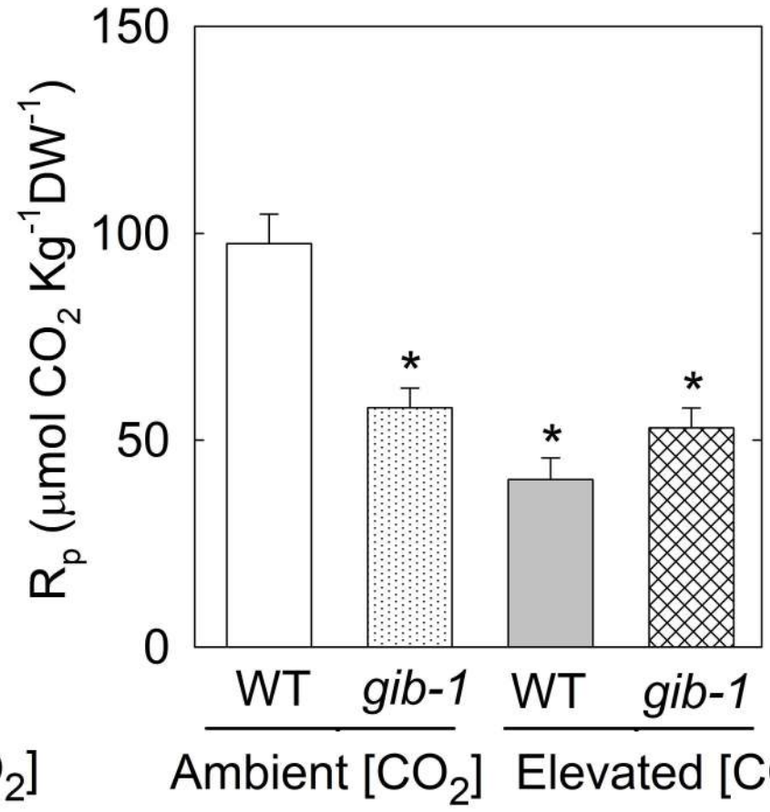
C


600
400
200
0
Ci (μmol CO2 mol-1air)
*
*

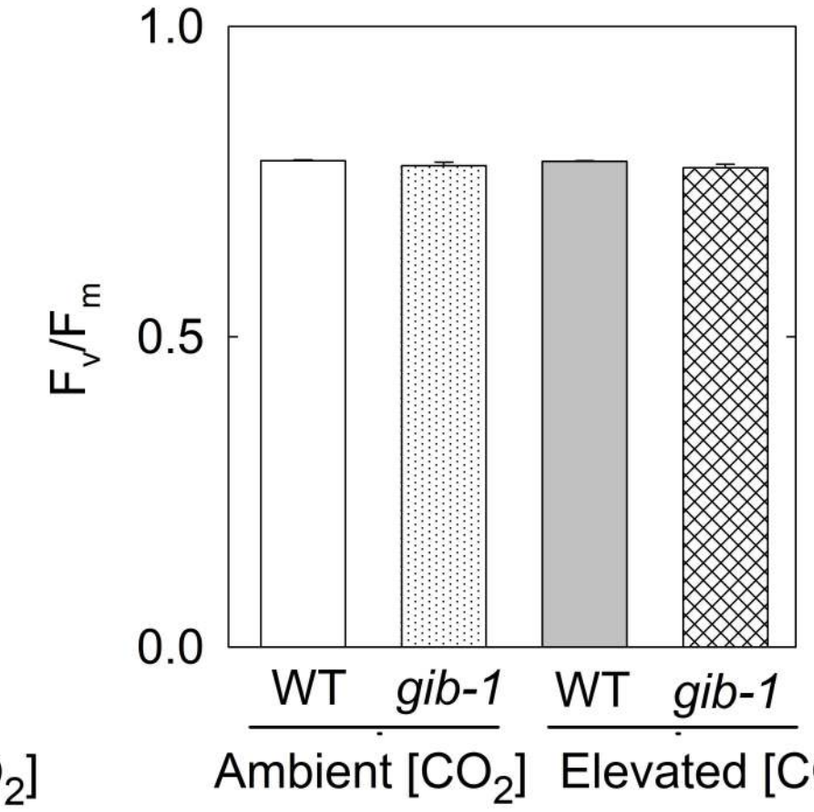
D

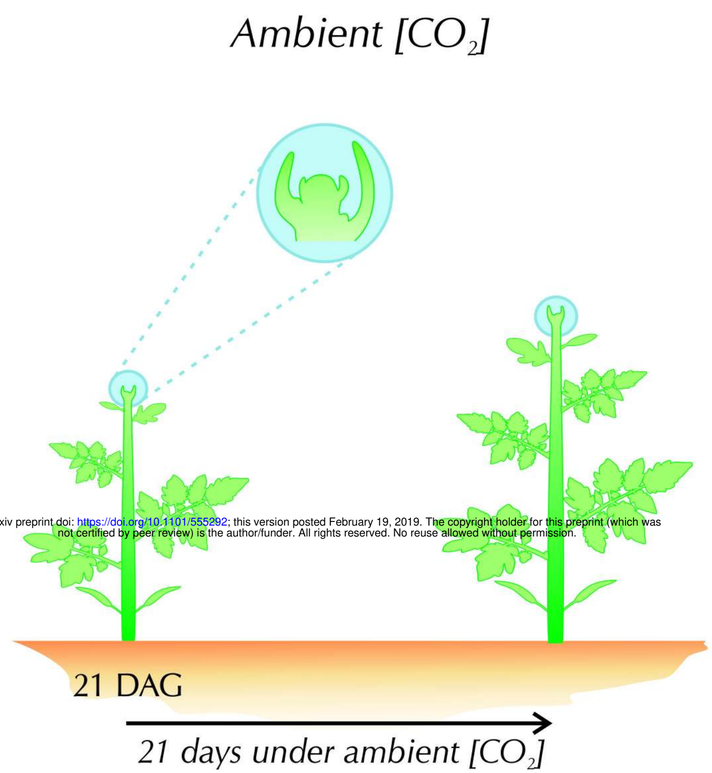

40
30
20
10
0
RD (μmol CO2 Kg-1 DW s-1)
*
*
WT
gib-1
WT
gib-1
Ambient [CO2]
Elevated [CO2]

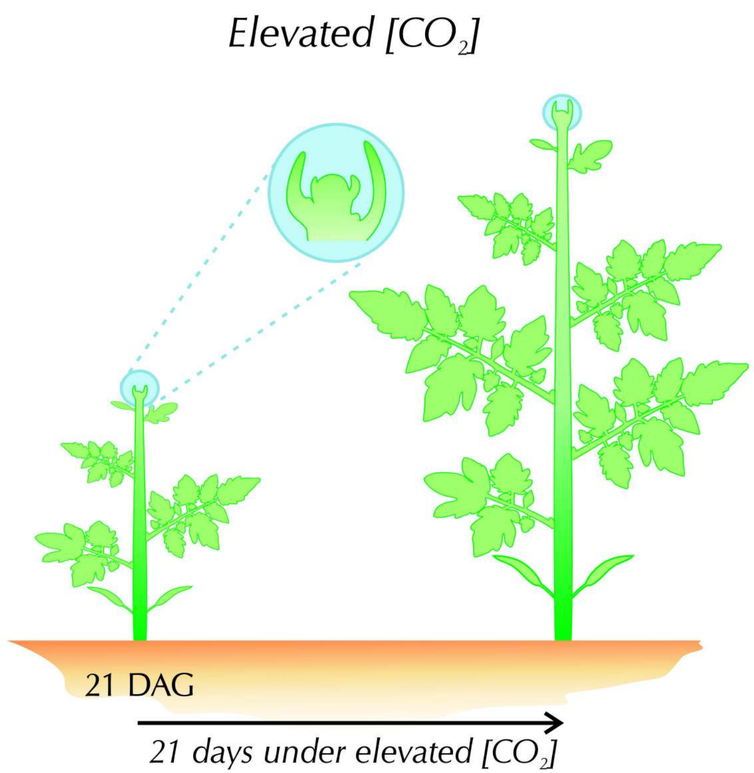
E

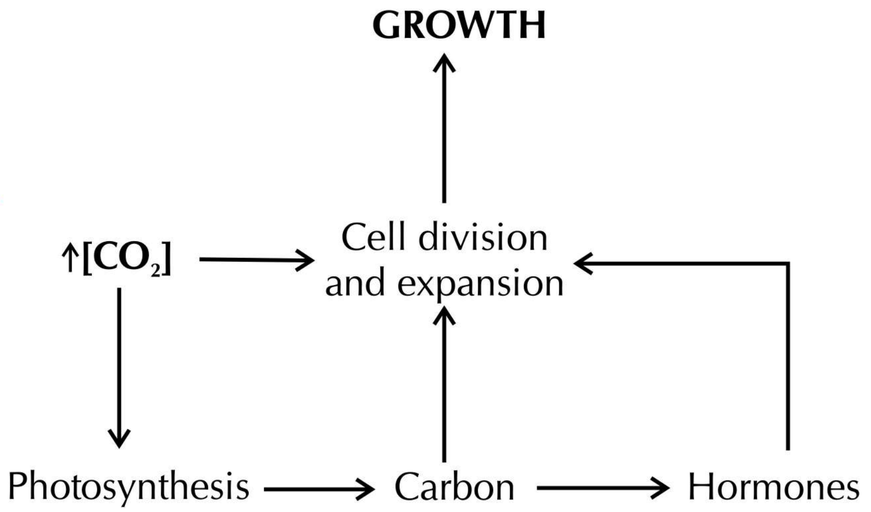

150
100
50
0
Rp (μmol CO2 Kg-1DW-1)
*
*
*
WT
gib-1
WT
gib-1
Ambient [CO2]
Elevated [CO2]

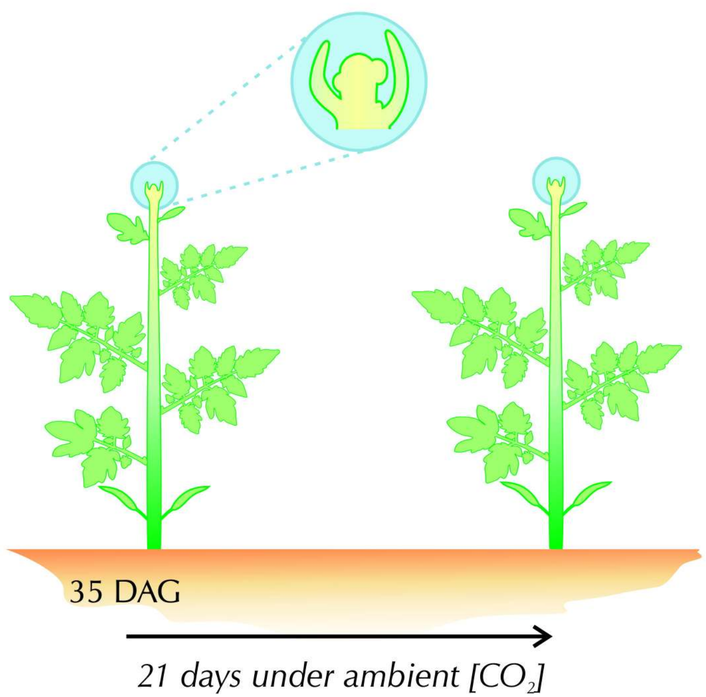
F

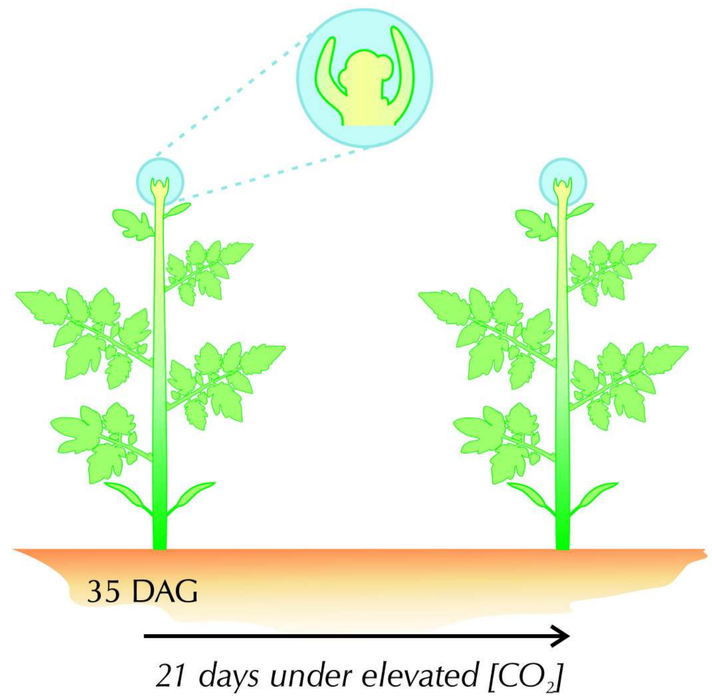

1.0
0.5
0.0
Fv/Fm
WT
gib-1
WT
gib-1
Ambient [CO2]
Elevated [CO2]

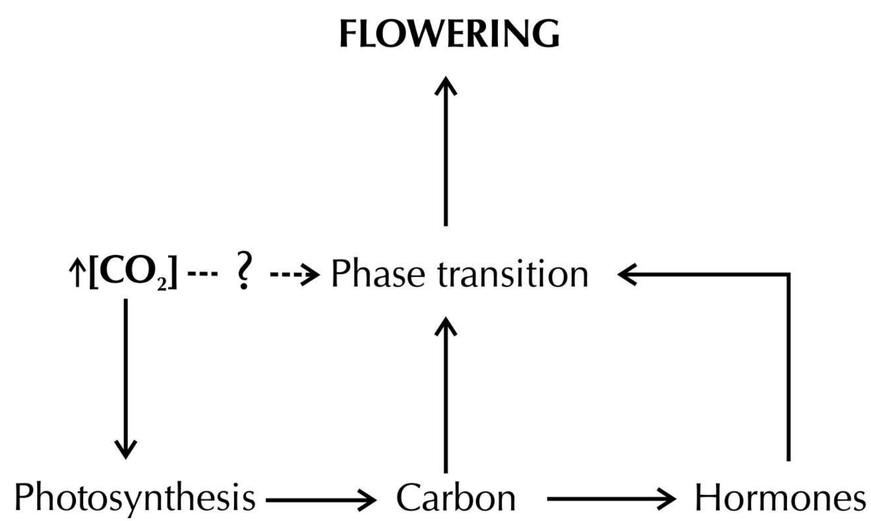


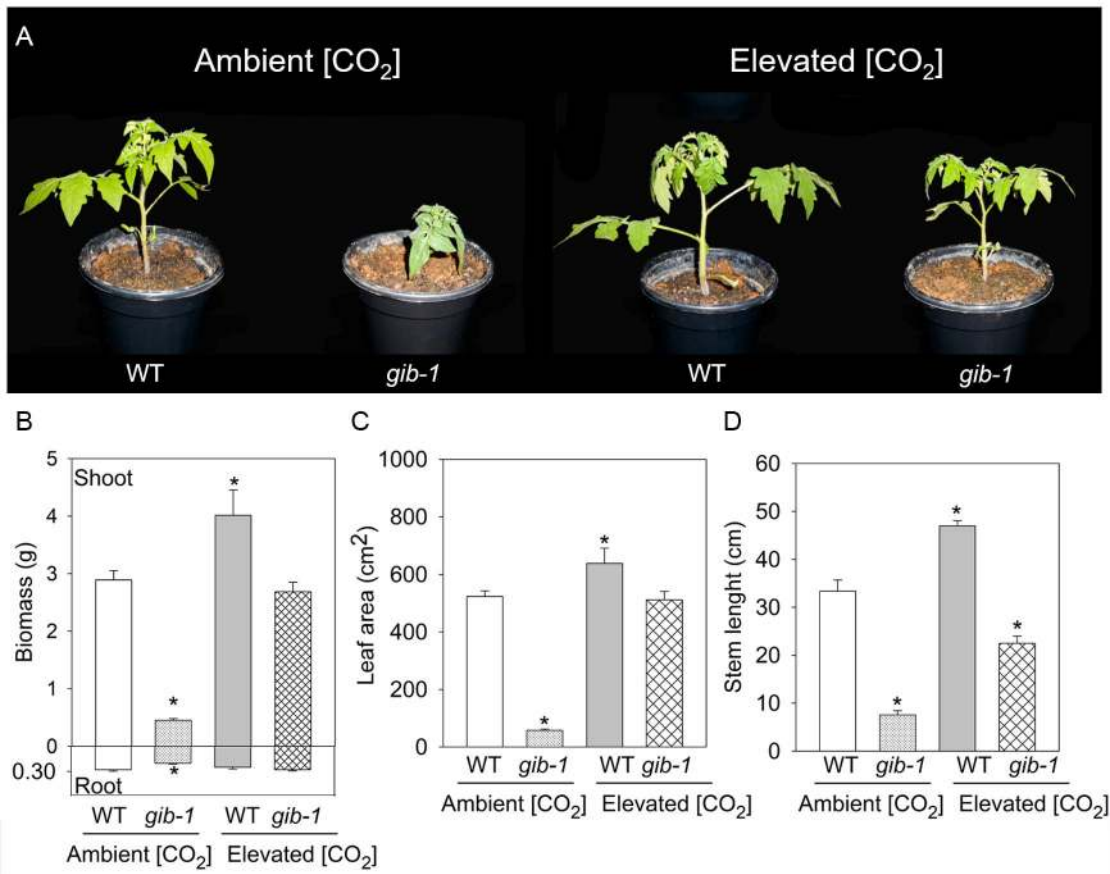


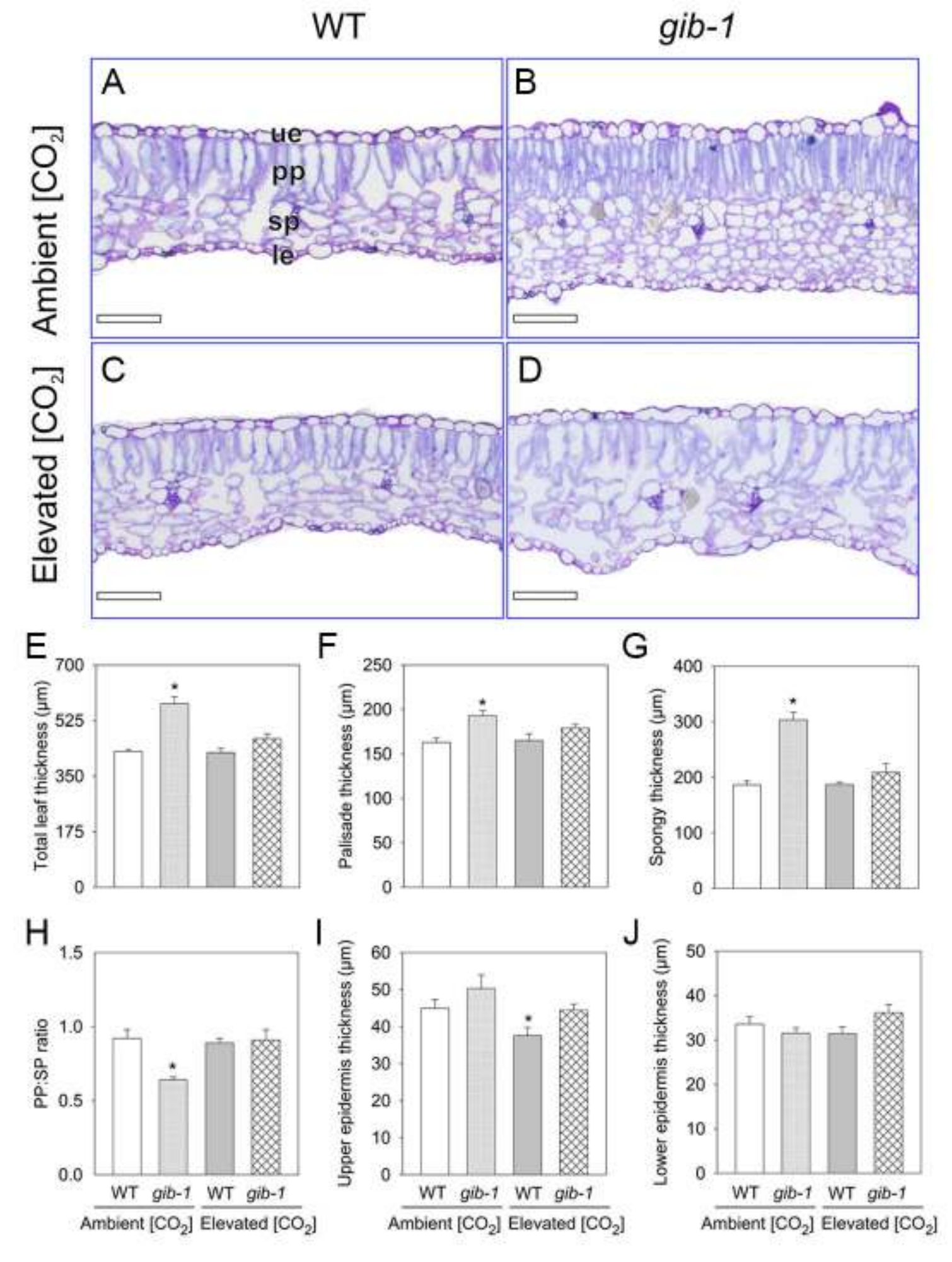









A
Ambient [CO2]
Elevated [CO2]
WT
gib-1
WT
gib-1
B
Shoot
Root
Biomass (g)
5
4
3
2
1
0
0.30
WT
gib-1
WT
gib-1
Ambient [CO2]
Elevated [CO2]
C
Leaf area (cm2)
1000
800
600
400
200
0
WT
gib-1
WT
gib-1
Ambient [CO2]
Elevated [CO2]
D
Stem lenght (cm)
60
50
40
30
20
10
0
WT
gib-1
WT
gib-1
Ambient [CO2]
Elevated [CO2]


WT
gib-1
A
B
C
D
ue
pp
sp
le
Ambient [CO2]
Elevated [CO2]
E
Total leaf thickness (µm)
700
525
350
175
0
F
Palisade thickness (µm)
250
200
150
100
50
0
G
Spongy thickness (µm)
400
300
200
100
0
H
PP:SP ratio
1.5
1.0
0.5
0.0
I
Upper epidermis thickness (µm)
60
50
40
30
20
10
0
J
Lower epidermis thickness (µm)
50
40
30
20
10
0
WT
gib-1
WT
gib-1
Ambient [CO2]
Elevated [CO2]