

## Acclimation of *Arabidopsis thaliana* to long-term CO<sub>2</sub> enrichment and nitrogen supply is basically a matter of growth rate adjustment

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### Abstract:

The long-term response of *Arabidopsis thaliana* to increasing CO<sub>2</sub> was evaluated in plants grown in 800 µl l<sup>-1</sup> CO<sub>2</sub> from sowing and maintained, in hydroponics, on three nitrogen supplies: "low," "medium" and "high." The global response to high CO<sub>2</sub> and N-supply was evaluated by measuring growth parameters in parallel with photosynthetic activity, leaf carbohydrates, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) messenger RNA and protein, stomatal conductance (g<sub>s</sub>) and density. CO<sub>2</sub> enrichment was found to stimulate biomass production, whatever the N-supply. This stimulation was transient on low N-supply and persisted throughout the whole vegetative growth only in high N-supply. Acclimation on low N-high CO<sub>2</sub> was not associated with carbohydrate accumulation or with a strong reduction in Rubisco amount or activity. At high N-supply, growth stimulation by high CO<sub>2</sub> was mainly because of the acceleration of leaf production and expansion while other parameters such as specific leaf area, root/shoot ratio and g<sub>s</sub> appeared to be correlated with total leaf area. Our results thus suggest that, in strictly controlled and stable growing conditions, acclimation of *A. thaliana* to long-term CO<sub>2</sub> enrichment is mostly controlled by growth rate adjustment.

**Abbreviations** -  $A_{sat}$ , assimilation rate at saturating irradiance;  $C_a$ , external CO<sub>2</sub> concentration;  $C_i$ , intercellular CO<sub>2</sub> concentration; FACE, free-air CO<sub>2</sub> enrichment; FW, fresh weight; g<sub>s</sub>, stomatal conductance; IRGA, infrared gas analyzer; mRNA, messenger RNA; NADPH, nicotinamide adenine dinucleotide phosphate; PGI, phosphoglucose isomerase; RBCL, Rubisco large subunit; RBCS, Rubisco small subunit; RH, relative humidity; R/S, root-to-shoot ratio; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SD, stomatal density; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SLA, specific leaf area; TLA, total leaf area;  $V_{c,max}$ , maximum rate of carboxylation of Rubisco.

### INTRODUCTION

Enrichment in atmospheric CO<sub>2</sub> is one of the main causes of the global climate change. Photosynthesis is a major target of the increasing CO<sub>2</sub>, well upstream of other plant responses. Indeed, at present CO<sub>2</sub> concentration (approximately 390 µl l<sup>-1</sup>), C<sub>3</sub> photosynthesis is still limited by the competition between CO<sub>2</sub> and O<sub>2</sub> at the active site of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which is involved in both photosynthesis and photorespiration. Any CO<sub>2</sub> increase should thus stimulate photosynthesis, improve assimilate production and plant growth. If this situation does indeed occur after short-term CO<sub>2</sub> enrichment (from seconds to days), the question of whether it is sustainable over the long term (days to months) or not remains open to discussion (Woodward 2002). Acclimation of photosynthesis is indeed frequently observed, which limits the potential benefit of the CO<sub>2</sub> increase (Moore et al. 1999). Several factors have been proposed to explain this phenomenon: repression of photosynthetic genes by carbohydrates (Krapp et al. 1993, Rolland et al. 2002), limitation by N-availability (Pottersson and McDonald 1994, Stitt and Krapp 1999), insufficient sink capacity (Paul and Foyer 2001), and accelerated leaf senescence (Ludewig and Sonnewald 2000). Accumulation of leaf carbohydrates was observed in a wide variety of plants grown in high CO<sub>2</sub> and has long been proposed to downregulate photosynthetic genes expression—particularly those encoding Rubisco (Van Oosten et al. 1994)—through a sugar-signaling pathway (Jang et al. 1997, Moore et al. 1999). However, this negative feedback might account for short-term response to high CO<sub>2</sub> only (Cheng et al. 1998), and has not been found in all cases (Geiger et al. 1999). Most interestingly, Sims et al. (1998) have reported that, in soybean (*Glycine max*), the photosynthetic capacity of a single leaf does not correlate with local leaf carbohydrate accumulation but with the CO<sub>2</sub> environment sensed by the whole organism. In the longer term, photosynthesis is controlled by a complex network of metabolic and hormonal signals (Paul and Foyer 2001). This allows the plant to balance photosynthesis according to endogenous parameters, such as the source to sink ratio, and not just to primary environmental parameters such as light and CO<sub>2</sub> (Paul and Foyer 2001, Stitt and Krapp 1999).

How plants respond to long-term CO<sub>2</sub> enrichment remains, however, unclear because photosynthesis is not the only process involved. High CO<sub>2</sub> was reported to cause a reduction in stomatal density in different plant species

(Woodward and Kelly 1995) or accessions of *Arabidopsis thaliana* (Woodward et al. 2002). CO<sub>2</sub> enrichment also accelerates leaf senescence and so causes a developmental shift which correlates with reduced photosynthesis (Kauder et al. 2000, Ludewig and Sonnewald 2000, Miller et al. 1997).

During the last decade, *A. thaliana* received increasing interest in the field of global change evaluation. However, getting a comprehensive overview of its response to high CO<sub>2</sub> remains difficult because studies focused either on biochemical and molecular aspects of the photosynthetic response (Bae and Sicher 2004, Cheng et al. 1998, Signora et al. 1998) or on plant growth and development (Van der Kooij et al. 1999) but hardly integrated both aspects (Sun et al. 2002). At the biochemical and molecular level, prolonged exposure to elevated CO<sub>2</sub> was found to cause an increase in carbohydrate content, which consequently represses photosynthetic genes (Cheng et al. 1998, Sun et al. 2002). The capacity to use soluble carbohydrates, and so prevent their accumulation, was further proved to be essential for growth stimulation by high CO<sub>2</sub> because strong downregulation of photosynthesis was observed in mutants unable to store starch (Sun et al. 2002). At the whole-plant level, experiments with *A. thaliana* showed that shoot biomass is generally increased by rising CO<sub>2</sub>. It is clear from this short survey that understanding plant response to high CO<sub>2</sub> will not be achieved unless molecular and organismic aspects are integrated and studied beyond photosynthesis. Furthermore, the fact that other environmental and nutrient factors interfere with plant response to elevated CO<sub>2</sub> imposes special care in experimental design and control of growing conditions. It is also crucial to control the developmental fate of the plants to ensure that growth responses are significantly recorded as direct effects of environmental treatments. Therefore, we took advantage of tools that we had previously designed for *Arabidopsis*: we used an hydroponic system to control the N-supply and sustain vegetative growth for a long time (Tocquin et al. 2003) and a gas exchange measurement device (Tocquin and Périlleux 2004) to integrate the different scales of the plant response to CO<sub>2</sub>, from molecules to organism, from short term to long term.

## MATERIALS AND METHODS

### Plant culture

Plants of *A. thaliana* ecotype Columbia were grown in hydroponics, as previously described (Tocquin et al. 2003). Briefly, seeds were stratified for 3 days on wet filter paper at 2°C, then were sown individually on 0.65% agar-containing plastic "seed-holders" that had been specifically designed and machined (Tocquin et al. 2003). Eight seed-holders were inserted in the cover of a 1-l black plastic container filled with a nutrient solution containing 3.5 mM NO<sub>3</sub><sup>-</sup>. This concentration was determined as optimal for plant growth in ambient CO<sub>2</sub> conditions (Tocquin et al. 2003) and will be hereafter referred to as "1 N-supply." In the experiments reported here, plants even grew better on 1N than was previously reported, showing the significant improvement obtained with the machined seed-holders, that allow easier and faster root growth at the beginning of the culture (Tocquin et al. 2003). Two other N-supplies were tested: a 16-fold lower N-supply (220 μMNO<sub>3</sub><sup>-</sup>, "1/16N"), and a six-fold higher N-supply (21 mMNO<sub>3</sub><sup>-</sup>, "6N"). Based on previous experiments performed in similar growing conditions (Tocquin et al. 2003), we chose to renew the nutrient solution weekly from the third week of growth. This was shown to minimize the impact of nutrient uptake on the pH and the conductivity of the solution. We did not observe any sign of depletion nor toxicity during the whole experiments; plants looked green and healthy in all conditions.

Plants were grown in phytotron Conviron cabinets, in 8-h photoperiod. The photon flux density was 120 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetically active radiations (Very High Output fluorescent tubes, Sylvania, Zaventem, Belgium); temperature was 20°C (day/night) and relative humidity (RH) was 70%. These conditions were selected because they are favorable to vegetative growth of the plants, hence allow to follow-up plants of *Arabidopsis* for a long time without developmental changes (Tocquin et al. 2003).

CO<sub>2</sub> level in the cabinets was recorded and controlled by an infrared gas analyzer (IRGA, WMA-3, PPSystems, Hitchin, UK) equipped with a set point controller (VISIREG+, MCC, Issoudun, France). The atmospheric CO<sub>2</sub> concentration was either ambient (±390 μl l<sup>-1</sup>) or supplied at 800 μl l<sup>-1</sup> (elevated CO<sub>2</sub> conditions).

### Growth measurements

Growth was evaluated by the regular sampling of six plants at least, randomly chosen in the growth cabinet, and the recording of leaf number, total leaf area (TLA) and shoot and root fresh weight (FW). Leaf number was the total number of leaves whose blade was longer than 3 mm. The projected leaf area was measured on a digital picture of intact rosettes, using an image analysis software (SigmaScan Pro4, SPSS Inc., Chicago, IL); a scale was included in each picture for calibration. In the text, this measurement will be referred to as the TLA.

Shoot FW was recorded as the whole rosette biomass measured immediately after harvest. Root FW was measured after mopping up the whole root system with a filter paper. The root-to-shoot ratio (R/S) was calculated on individual plants. TLA and shoot FW were used to calculate, for individual plants, the specific leaf area (SLA), expressed in  $\text{cm}^{-1} \text{g}^{-1}$ .

### Starch, sucrose, fructose and glucose determination

For carbohydrate analyses, a minimum of six whole rosettes, randomly chosen in the growth cabinet, were harvested at the end of the daylight 8-h period. Each sample was extracted in duplicate. About 100 mg of leaf material ground in liquid  $\text{N}_2$  was extracted three times in 500  $\mu\text{l}$  80% (v/v) ethanol at 70°C. The ethanol soluble fractions—containing "soluble carbohydrates"—were pooled, dried under vacuum and dissolved in 250  $\mu\text{l}$  of distilled water. The ethanol insoluble residue—starch—was briefly dried under vacuum and homogenized in 800  $\mu\text{l}$  of 0.1 M citrate buffer, pH 4.6. Starch was then solubilized by 1 h autoclaving. Starch, sucrose, fructose and glucose were assayed enzymatically in separate aliquots. Fructose, sucrose and starch were first converted to glucose by treatment with, respectively, phospho-glucose isomerase (PGI),  $\beta$ -fructosidase/PGI and amylo-glucosidase (all enzymes from Roche Diagnostics, Vilvoorde, Belgium). Glucose was then quantified based on the equimolar release of nicotinamide adenine dinucleotide phosphate (NADPH) by the reaction of glucose with hexokinase/glucose-6-phosphate dehydrogenase, in a reaction mixture containing 0.44 M trietha-nolamine pH 7.6, 5.8 mM  $\text{MgSO}_4$ , 0.3 mM NADP and 0.3 mM ATP. NADPH was subsequently detected by its fluorescence at 445 nm using a fluorimeter (Jones 1979).

### Gas exchanges measurements

The gas exchange measurements were performed as described in Tocquin and Périlleux (2004) using a custom made cuvette enclosing the whole rosette. The cuvette was connected to a differential  $\text{CO}_2/\text{H}_2\text{O}$  IRGA (Ciras-1, PPSsystems, Hitchin, UK) equipped with an automatic air supplier to control flow rate as well as  $\text{CO}_2$  and  $\text{H}_2\text{O}$  partial pressures of the input air. All measurements were performed with plants grown in hydroponics in a phytotron under controlled air temperature (20°C) and humidity (70% RH). In these conditions, the leaf to air water vapor deficit in the cuvette was calculated to be 0.9-1.3 kPa (Tocquin and Périlleux 2004).

Saturating irradiance was estimated by recording  $\text{CO}_2$  uptake under increasing irradiance, as previously described (Tocquin and Périlleux 2004). Because 1000  $\mu\text{mol m}^{-2}\text{s}^{-1}$  was found to saturate  $\text{CO}_2$  assimilation ( $A_{\text{sat}}$ ) in all experimental conditions used, it was selected to measure  $\text{CO}_2$  uptake in response to variation in external  $\text{CO}_2$  concentration ( $C_a$ ).  $\text{CO}_2$  assimilation by unit leaf area was then calculated using TLA as a reliable estimation of the photosynthetic area. It was indeed observed that leaf shading was <5% of total rosette area (Donahue et al. 1997, Sun et al. 1999, Tocquin and Périlleux 2004). Data shown are the mean of five measurements performed on three to five different plants, randomly sampled in the growth cabinet. Data collected by IRGA were used to calculate  $A_{\text{sat}}$ , intercellular  $\text{CO}_2$  ( $C_i$ ) and stomatal conductance ( $g_s$ ) according to von Caemmerer and Farquhar (1981).  $A_{\text{sat}}$  was plotted in response to increasing  $C_i$ , and maximum rate of carbox-ylation of Rubisco ( $V_{\text{C,max}}$ ) was then calculated by fitting these data to model equations from von Caemmerer and Farquhar (1981) by the least squares method using Photosyn Assistant (Dundee Scientific, Dundee, UK).

### Rubisco messenger RNA and protein analyses

For both messenger RNA (mRNA) and protein analyses, a minimum of six whole rosettes were randomly harvested at the onset of the light period and frozen in liquid  $\text{N}_2$ . RNA was extracted as described in Cremer et al. (1991). For Northern blot preparation, 20  $\mu\text{g}$  of total RNA was subjected to electrophoresis on 1.2% formaldehyde-agarose gels and transferred by capillarity onto a Nylon membrane (HybondN 0.45  $\mu\text{m}$ , GE Healthcare Europe GmbH, Diegem, Belgium) as described in Sambrook et al. (1989). RNA was bound to the membrane by cross-linking with ultraviolet (120 000  $\mu\text{l cm}^{-2}$ , Fisher Bioblock Scientific, Tournai, Belgium). Probe labeling was performed with a RadPrime DNA Labeling System (Invitrogen, Belgium). Protocols for hybridization and washes of the Northern blots were as described by Amersham. Probes were synthesized by PCR on *A. thaliana* genomic DNA by using the following primers for the small Rubisco subunit (*RBCS*) 5'-CCTCTATGCTCTCTCCGCTA-3' and 5'-ACAAATCCGTGCTCCAACCTC-3', and for the large sub-unit (*RBCL*) 5'-TTCGGTGGAGGAACCTTAGG-3' and 5' -AAGAAGCGGGG ATACTTGGT-3'. PCR products were cloned and checked by sequencing.

Protein extraction was performed by homogenizing about 50 mg of leaf material in 750  $\mu\text{l}$  extraction buffer (0.1 M Tris-HCl pH 8.3, 0.5 M NaCl, 5 mM dithiothrei-tol, 5 mM ethylenediaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride). Samples were incubated 1 h at 4°C with constant agitation. After centrifugation,

supernatant was collected and pellets were extracted once more. Supernatants were pooled and protein concentrations were determined using the Bradford protein assay (Bradford 1976). One hundred micrograms of proteins was precipitated in 10% trichloroacetic acid (w/v) and dissolved in 30  $\mu$ l sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Twenty-five micrograms of proteins was loaded on SDS-PAGE and submitted to electrophoresis [1 h at 180 V in 0.75-mm running gel containing 15% (w/v) acrylamide]. Gels were then blotted onto a polyvinylidene difluoride membrane and the RBCS was detected with a rabbit anti-chicken IgY antibody from Agrisera (Vannas, Sweden) by using the BM chemiluminescence Western blotting kit from Roche Applied Science.

### Stomatal imprints and stomatal density measurement

Leaves # 5 and 10 (numbered from oldest to youngest) were used for measurement. Leaves were taken off the plant and their abaxial face was covered with transparent nail varnish. After the imprint had air-dried, it was peeled off, laid on a glass slide and examined under a light microscope at 10x magnification. Stomata were counted in the field of the microscope. For each leaf, three microscope fields were examined in the larger part of the blade and mean values were calculated. Three plants were used to determine the mean number of stomata per unit leaf area ( $\text{mm}^2$ ). The leaf blade area was measured on individual leaves by using the same procedure described for the measurement of the TLA (see before).

## RESULTS

### Growth

Plants of *A. thaliana* were grown in ambient  $\text{CO}_2$  ( $\pm 390 \mu\text{l l}^{-1}$ ) or high  $\text{CO}_2$  ( $800 \mu\text{l l}^{-1}$ ), on hydroponic solutions providing low (1/16N), medium (1 N) or high (6N) N-supplies. Other growing conditions were chosen to promote vegetative growth and so elongate the investigated period (see Materials and methods).

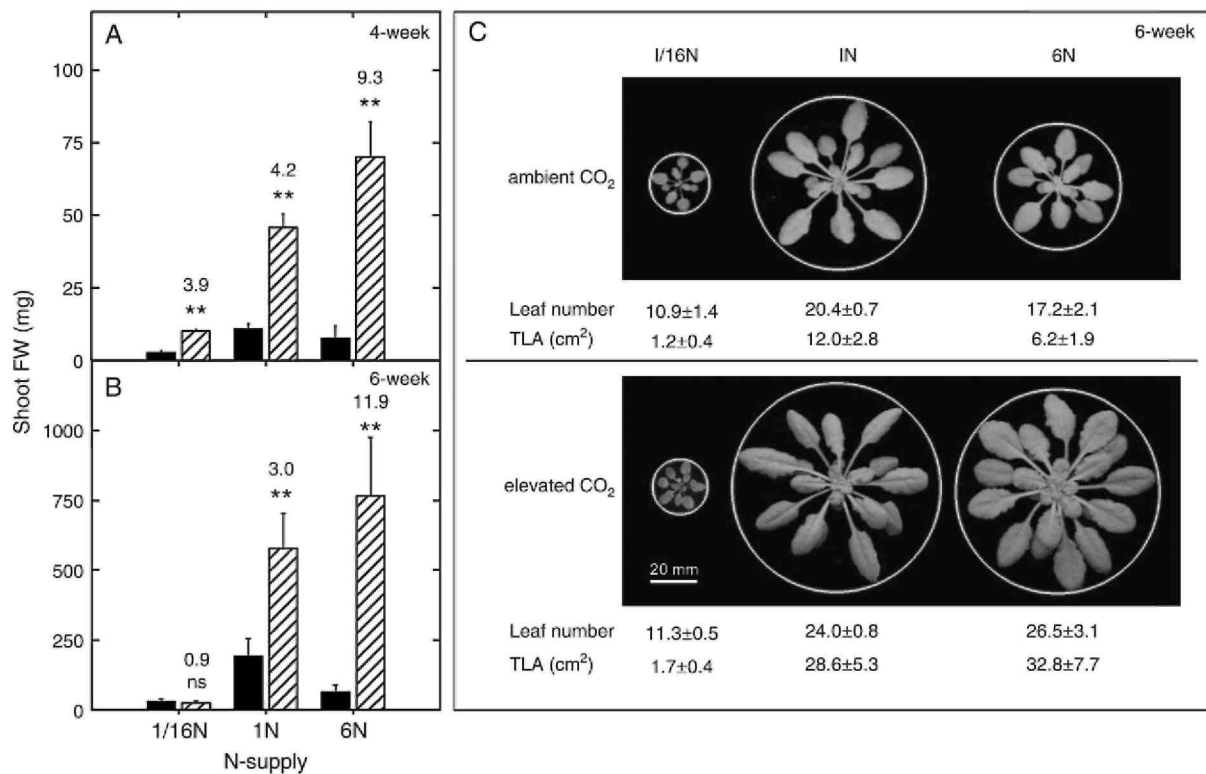
In a preliminary experiment, growth kinetic was recorded by frequent measurements (up to three times a week) of TLA. The exponential phase of rosette growth was found to start around the fourth week in all conditions (data not shown), hence we decided to further characterize 4- and 6-week-old plants, i.e. plants harvested at the beginning and during the exponential growth phase, respectively.

We observed on 4-week-old plants that high  $\text{CO}_2$  led to a remarkable increase in shoot biomass (Fig. 1 A), whose magnitude increased with N-supply. However, after 6 weeks of growth, the stimulation was still detectable on high N only, while plants grown on low N no longer showed a positive effect of high  $\text{CO}_2$  (Fig. 1 B). On the medium level of 1 N, the stimulation also decreased with time: the gain in biomass because of high  $\text{CO}_2$  decreased from a 4.2-fold ratio in 4-week-old plants to a three-fold ratio in 6-week-old plants (Fig. 1 A, B).

These results suggested that the optimal N-supply was different in ambient or high  $\text{CO}_2$ : Fig. 1 indeed shows that in ambient  $\text{CO}_2$  (black bars), the maximum shoot biomass accumulation was observed with the medium 1 N-supply while in high  $\text{CO}_2$  (hatched bars), the optimum was higher.

The increase in shoot biomass observed in high  $\text{CO}_2$  could be because of an increase in leaf number, TLA and/or SLA. We therefore recorded these parameters on 6-week-old plants. Consistent with our observation that the positive effect of high  $\text{CO}_2$  was not visible any more on 1/16N, we observed no difference in leaf number or TLA as compared with control plants grown in ambient  $\text{CO}_2$  (Fig. 1C). In contrast, plants grown on 1 N or 6N still had more leaves and a higher TLA in high  $\text{CO}_2$ . As for the biomass production, the maximum effect of  $\text{CO}_2$  enrichment was observed for 6N plants: TLA was fivefold higher and plants produced 50% more leaves in  $800 \mu\text{l l}^{-1}$  than in ambient  $\text{CO}_2$ . Moreover, under this high N-supply,  $\text{CO}_2$  enrichment led to a modification of leaf morphology: their petiole was shorter and their blade was larger than in control plants. As compared with 1 N plants grown in  $800 \mu\text{l l}^{-1}$   $\text{CO}_2$ , this particular trait of 6N plants resulted in an increase of TLA without significant increase in rosette diameter (Fig. 1C).

**Fig. 1.** Shoot biomass of (A) 4- and (B) 6-week-old *Arabidopsis* plants grown in ambient (black bars) or elevated ( $800 \mu\text{l l}^{-1}$ )  $\text{CO}_2$  (hatched bars). Plants were cultivated in hydroponics on low (1/16N), medium (1N) or high (6N) nitrogen supply. Numbers represent the relative increase of biomass in elevated vs ambient  $\text{CO}_2$ . Data are means  $\pm$  standard deviation, ns, not statistically different.



Concerning SLA ( $\text{cm}^2 \text{g}^{-1} \text{FW}$ ), a significant decrease was observed with increasing  $\text{CO}_2$ , N-supply and plant age (Fig. 2 A). However, these differences were actually caused by TLA because a strong correlation was found between SLA and TLA, whatever the growing conditions (Fig. 2B): SLA decreased to a bottom value of  $48 \text{ cm}^2 \text{g}^{-1} \text{FW}$  when TLA increased up to approximately  $5 \text{ cm}^2$  and beyond.

Root biomass was even more increased than shoot growth by high  $\text{CO}_2$ : we observed that the R/S was higher in  $800 \mu\text{l l}^{-1} \text{CO}_2$  than in ambient  $\text{CO}_2$  (Fig. 2C, D). This effect was moderate in 1 N plants but more pronounced on 1/16N and 6N supplies. Although it was difficult from Fig. 2C, D to find any correlation between R/S and N-supply or plant age, a clear correlation again appeared when R/S was plotted against TLA: irrespective of the  $\text{CO}_2$  concentration, R/S increased to a maximum value when TLA increased up to approximately  $5 \text{ cm}^2$  and beyond (Fig. 2E). This maximum R/S value was different for each N-supply, being the lowest in 1 N plants and the highest in 1/16N plants (but not reached in our experimental conditions).

### Starch and soluble carbohydrates

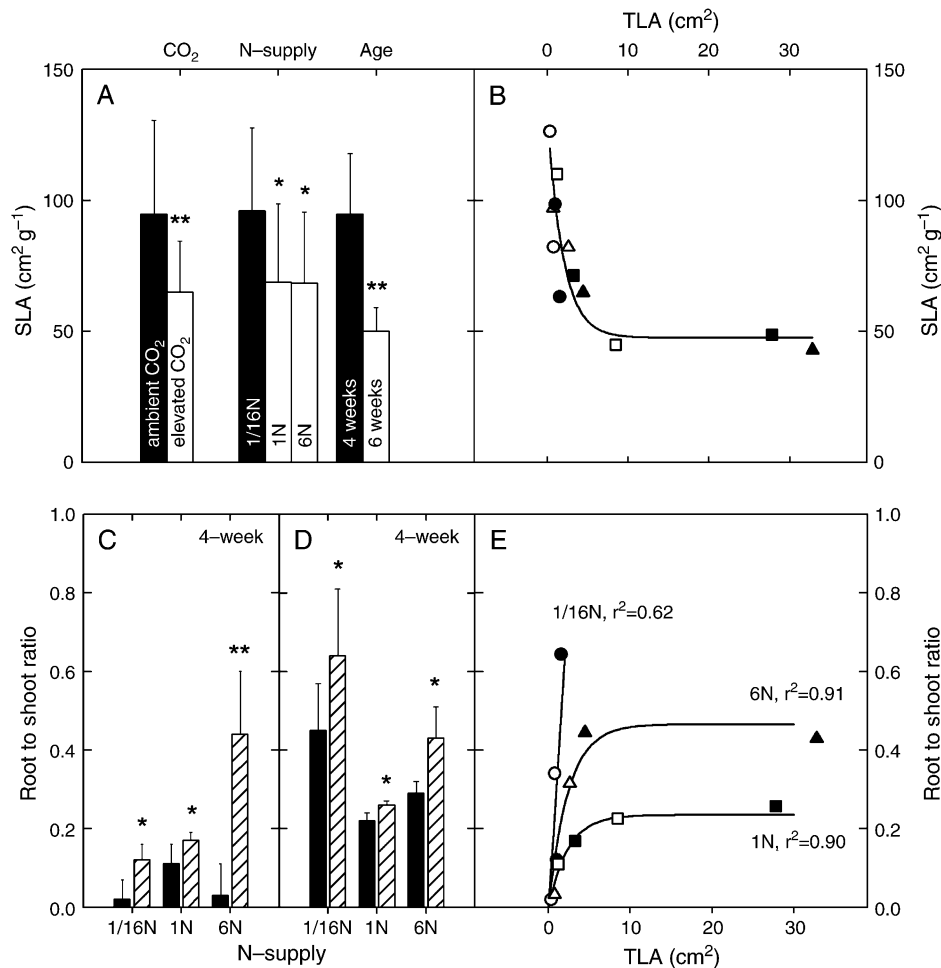
Starch and soluble carbohydrates (glucose, fructose and sucrose) were analyzed in 4- and 6-week-old plants, in all  $\text{CO}_2$ - and N-supplies conditions (Fig. 3); material was harvested at the end of the 8-h light period.

Quite unexpectedly,  $\text{CO}_2$  enrichment had very little effect on starch or soluble carbohydrates content of the rosettes. The only significant difference was observed on low N, where  $\text{CO}_2$  enrichment actually caused a reduction in starch and soluble carbohydrate (Fig. 3). It is also noteworthy that, in ambient  $\text{CO}_2$ , these plants grown on 1/16N contained more sugars than those grown on higher N-supplies, especially after 6 weeks of growth, which suggest that their photosynthesis was in excess as compared with their growth (see below).

### Photosynthesis

The net  $\text{CO}_2$  assimilation rate measured at the saturating irradiance of  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  ( $A_{\text{sat}}$ ) was recorded in all  $\text{CO}_2$ - and N-supplies conditions in response to variation in  $C_a$ , from 0 to  $1400 \mu\text{l l}^{-1}$ . From these assimilation curves,  $V_{\text{C,max}}$  of Rubisco was calculated as explained in Materials and methods.

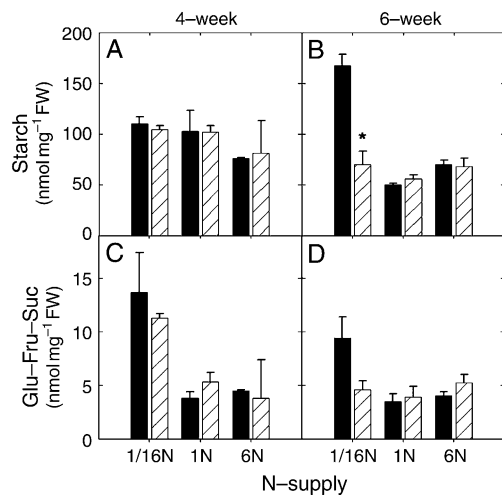
**Fig. 2.** (A and B) Variation of the specific leaf area (SLA) of *Arabidopsis* plants with (A) CO<sub>2</sub> level, N-supply and plant age and (B) total leaf area (TLA). Data shown in this figure are from 12 experimental batches (4- and 6-week-old plants grown in ambient or 800 μl l<sup>-1</sup> CO<sub>2</sub> and in 1/16N, 1N or 6N) of six plants each. These are the 12 experimental points in panel B (mean values; error bars were omitted for clarity). In panel A, data were clustered to show the effect of a single parameter on SLA. (C-E) Root-to-shoot ratio (R/S) of (C) 4- and (D) 6-week-old *Arabidopsis* plants grown in ambient (black bars) or elevated (800 μl l<sup>-1</sup>) CO<sub>2</sub> (hatched bars). (E) Correlation between R/S and TLA for each N-supply. The 12 experimental points are identical to those of panels C and D (mean values; error bars were omitted for clarity). Plants were cultivated in hydroponics on low (1/16N), medium (1N) or high (6N) N-supply. In panels A, C and D, data are means ± standard deviation, ns, not statistically different; \*statistically different at  $P \leq 0.05$ ; \*\*statistically different at  $P \leq 0.001$ . In panels B and E, circles, squares and triangles are, respectively, representing 1/16N, 1N and 6N plants. Open symbols are for ambient CO<sub>2</sub> and filled symbols are for high CO<sub>2</sub> conditions. The regression curves were calculated using SigmaPlot2001 (SPSS, Inc.).



To assess whether acclimation of photosynthesis to high CO<sub>2</sub> occurred in our conditions, we compared A<sub>sat</sub> measured on plants grown in ambient CO<sub>2</sub> at a C<sub>a</sub> of 400 μl l<sup>-1</sup> (Fig. 4A, B, "amb/amb") to (1) A<sub>sat</sub> measured on the same plants at a C<sub>a</sub> of 800 μl l<sup>-1</sup> (Fig. 4A, B, "amb/ elev")—which represents the short-term response to an instant doubling in CO<sub>2</sub> (Fig. 4C, D, open bars)—and to (2) A<sub>sat</sub> measured on plants grown in high CO<sub>2</sub> at a C<sub>a</sub> of 800 μl l<sup>-1</sup> (Fig. 4A, B, "elev/elev"), which accounts for the long-term effect of CO<sub>2</sub> enrichment on photosynthetic activity (Fig. 4C, D, hatched bars).

The short-term response of photosynthesis to CO<sub>2</sub> enrichment (A<sub>sat</sub> amb/elev vs A<sub>sat</sub> amb/amb) was clearly a stimulation, in all N-supplies (Fig. 4A, B). The range of this stimulation (Fig. 4C, D, open bars) was about 50% for 4-week-old plants, whatever the N-supply, and slightly increased with N-supply for 6-week-old plants. These variations were found to correlate with the estimated V<sub>c,max</sub> of ambient CO<sub>2</sub> plants (Fig. 4E, F, black bars).

**Fig. 3.** (A, B) Starch and (C, D) soluble carbohydrates (glucose, fructose and sucrose) content of 4- and 6-week-old plants of *Arabidopsis* plants grown in ambient (black bars) or elevated ( $800 \mu\text{l l}^{-1}$ )  $\text{CO}_2$  (hatched bars). Plants were cultivated in hydroponics on low (1/16N), medium (1N) or high (6N) N-supply. Whole rosettes were harvested at the end of the 8-h light period. Data are means  $\pm$  standard deviation for two independent measurements of two replicates. \*Statistically different at  $P \leq 0.05$ .



The long-term response of photosynthesis to elevated  $\text{CO}_2$  ( $A_{\text{sat}}$  elev/elev vs  $A_{\text{sat}}$  amb/amb) also was a stimulation but only for plants grown on 1 N or 6N (Fig. 4A, B). As compared with plants kept in ambient conditions, the stimulation ranged from 50 to 100% (Fig. 4C, D). On the contrary, when grown on 1/16N, plants in high  $\text{CO}_2$  did not show a higher photosynthetic activity than plants grown in ambient  $\text{CO}_2$ , hence photosynthesis had acclimated. When we compared long-term and short-term responses of photosynthesis to  $\text{CO}_2$  (elev/elev vs amb/elev), we indeed observed that plants grown continuously in 1/16N and  $800 \mu\text{l l}^{-1}$   $\text{CO}_2$  had completely lost the benefit of high  $\text{CO}_2$ . On the contrary, plants grown on 1 N or 6N showed about the same photosynthetic activity in  $800 \mu\text{l l}^{-1}$   $\text{CO}_2$ , whether they had been grown in high or ambient  $\text{CO}_2$ . One exception was found for 4-week-old plants grown in 1N medium, which showed a stronger long-term stimulation than the short-term response (Fig. 4C, hatched vs open bars).

All together, the long-term response of photosynthesis to high  $\text{CO}_2$  could not be explained by effects on the  $V_{\text{C,max}}$  of Rubisco (hatched bars in Fig. 4C, D vs Fig. 4E, F). We therefore further analyzed the Rubisco content of the plants.

### Rubisco

The contribution of Rubisco to the photosynthetic response of plants to  $\text{CO}_2$  and N-supplies was investigated by measuring the steady-state mRNA level of both Rubisco subunits (*RBCL* and *RBCS*) and the amount of the *RBCS* in 6-week-old plants.

Only weak differences in the steady-state amount of mRNA were found between experimental batches: we observed a slight increase in *RBCL* mRNA with  $\text{CO}_2$  level, and a slight decrease in *RBCS* mRNA on the lowest N-supply (Fig. 5). The amount of *RBCS* protein correlated well with the mRNA level: no clear difference was found between  $\text{CO}_2$  treatments but the amount of protein was lower in 1/16N plants as compared with other N-supplies.

### Stomatal conductance and density

$g_s$  was calculated from gas exchange measurements as explained in Materials and methods (Fig. 6). We observed on 4-week-old plants that individuals grown in high  $\text{CO}_2$  had a lower  $g_s$  than plants grown in ambient  $\text{CO}_2$  (Fig. 6A). However, this  $\text{CO}_2$  effect persisted on 1/16N only in 6-week-old plants (Fig. 6B).

Stomata were counted on the abaxial face of leaves # 5 and 10 of 6-week-old plants and stomatal density (SD) was found to be remarkably stable, around  $100 \text{ mm}^{-2}$ , except on 1/16N where values were up to three times

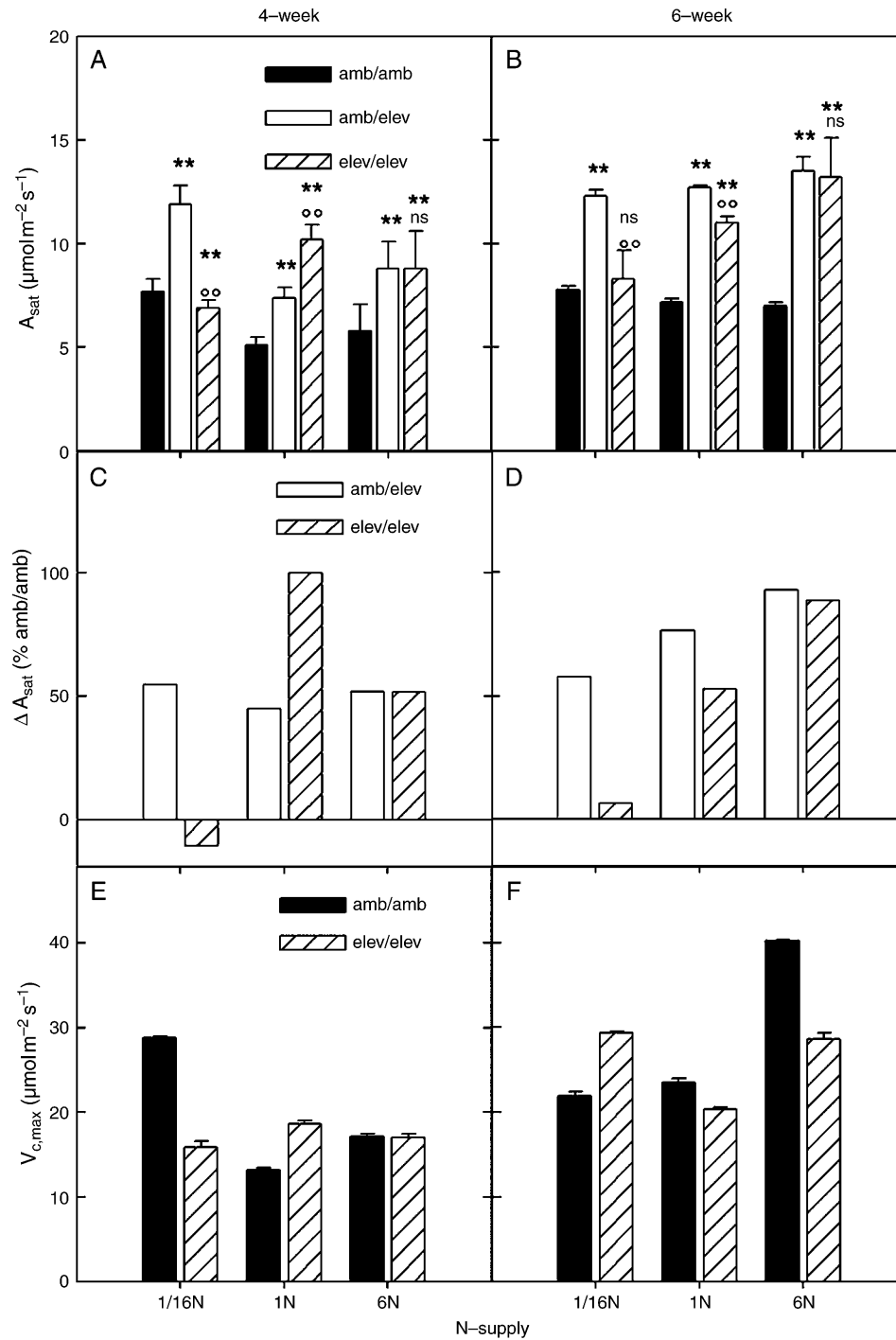
higher (Fig. 6C, D). This difference reflects the impact of CO<sub>2</sub>- and N-supplies on the growth rate of the plants because we found that—when leaves are fully expanded—a strong correlation exists between stomata number and leaf blade area, hence SD is a constant (Fig. 6E). Thus, the higher SD of 1/16N plants could be explained by their slow growing rates: at 6 weeks, leaf blades had not reached their full size, hence SD was not stabilized yet.

## DISCUSSION

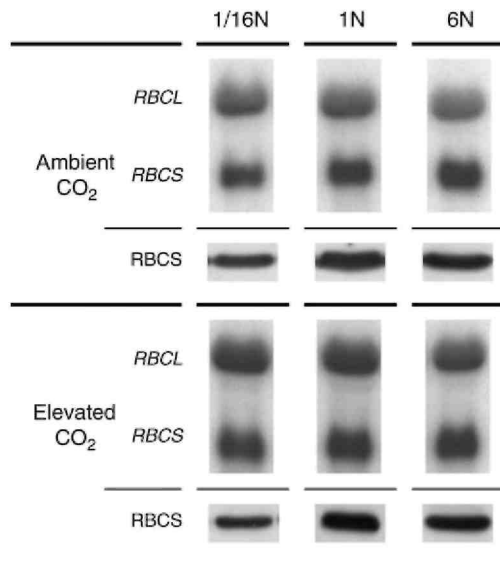
A commonly documented effect of CO<sub>2</sub> enrichment is the acclimation of photosynthesis, generally explained by a negative feedback through accumulating carbohydrates (Moore et al. 1999). This hypothesis is very attractive and it has been demonstrated that carbohydrates do indeed repress photosynthetic genes (Koch 1996, Krapp et al. 1993). However, this single process hardly explains all plant responses to high CO<sub>2</sub> whose understanding requires that whole plant growth and development, as well as interference with other environmental factors, are considered (see Introduction). The goal of our study was to obtain a comprehensive overview of long-term CO<sub>2</sub> enrichment by studying as many responses as possible in the same experiments, on the same material. We used the model plant *Arabidopsis*, but because this fast growing species has a short cycle, long-term experiments must be performed in strictly controlled conditions of light and temperature. Plants were cultivated in phytotronic cabinets, in hydroponics from sowing to adult vegetative size. CO<sub>2</sub> was either "ambient" (390 µl l<sup>-1</sup>) or "elevated" (800 µl l<sup>-1</sup>) and three different N-supplies were tested: "low" (1/16N), "medium" (1 N), and "high" (6N).



**Fig. 4.** Photosynthetic activity and maximum rate of carboxylation of Rubisco of (A, C, E) 4- and (B, D, F) 6-week-old plants of *Arabidopsis* grown in ambient (black bars) or elevated ( $800 \mu\text{l l}^{-1}$ )  $\text{CO}_2$  (hatched bars). Plants were grown in hydroponics on low (1/16N), medium (1N) or high (6N) nitrogen supply.  $\text{CO}_2$  assimilation at saturating irradiance was measured at the  $\text{CO}_2$  concentration of growth for ambient ("amb/amb," black bars) and elevated  $\text{CO}_2$  plants ("elev/elev," hatched bars), and after an instant doubling in  $\text{CO}_2$  level for plants grown in ambient conditions ("amb/elev," open bars). Data are means  $\pm$  standard deviation for three to five plants. The difference was evaluated using the t-test: statistically different at  $P \leq 0.001$  with the corresponding black bar (\*\*) or white bar (°) and not statistically different (ns).



**Fig. 5.** Rubisco messenger RNA and protein analysis in 6-week-old plants of *Arabidopsis* grown in ambient or elevated ( $800 \mu\text{l l}^{-1}$ )  $\text{CO}_2$ . Plants were grown in hydroponics on low (1/16N), medium (1N) or high (6N) N-supply. Transcripts of both subunits were analyzed on Northern blots (RBCL, RBCS) while only the small subunit was analyzed at the protein level.



### **CO<sub>2</sub> enrichment leads to long-term stimulation of growth if N-supply is not limiting**

Biomass accumulation is the most obvious and easy parameter used to evaluate environmental effects on plant growth. As reported by others, we observed that growth is stimulated by high  $\text{CO}_2$ , especially when plants are grown under high N-supply, i.e. when N is not limiting (Fig. 1). In that respect, we have observed that the optimal N-supply is higher in high  $\text{CO}_2$  than in ambient  $\text{CO}_2$  and the most significant gain in biomass was observed for plants grown in 6N. This observation is in good agreement with the well-documented interaction between elevated  $\text{CO}_2$  and nitrogen nutrition (Stitt and Krapp 1999). When N-supply was lower than 6N, we observed that growth stimulation by high  $\text{CO}_2$  decreased in amplitude with time (on 1N), or disappeared (on 1/16N). This was not caused by medium depletion because the hydro-ponic solution was renewed weekly. Thus, the growth stimulation of *Arabidopsis* plants by elevated  $\text{CO}_2$  is a time-dependent process closely linked to N-supply. As a consequence, plant response to high  $\text{CO}_2$  can not be estimated by single biomass comparison, especially in long-term experiments.

### **The growth stimulation at elevated CO<sub>2</sub> results from an acceleration of leaf initiation and expansion**

The increase in biomass under elevated atmospheric  $\text{CO}_2$  has been reported to correlate to a decrease in SLA in many species (Long et al. 2004, Poorter and Pérez-Soba 2002, Woodward 2002), including *Arabidopsis* (Van der Kooij and DeKok 1996). This effect was often shown to result from an increase in non-structural carbohydrates and leaf thickness (Poorter and Navas 2003). N-limitation was also shown to reduce SLA through accumulation of carbohydrates that are not used for size increase (de Groot et al. 2002). In our study, we observed that SLA decreased with increasing  $\text{CO}_2$  but also with increasing N-supply, and plant age (Fig. 2A). However, we found that this effect may be indirect because a strong correlation exists between SLA and TLA, whatever  $\text{CO}_2$  and N conditions (Fig. 2B). This suggests that, in our growing conditions, the primary effect of  $\text{CO}_2$  was to increase TLA and that the decrease in SLA secondarily correlated with this change. This conclusion is supported by the study of Gibeaut et al. (2001) who observed that growing *Arabidopsis* in elevated  $\text{CO}_2$  led to a concomitant increase in relative growth rate and TLA while the SLA was only slightly reduced. They also showed that the activity of UDP-glucose dehydrogenase, a key enzyme in the cell-wall biosynthesis—and thus involved in cell and leaf elongation—was increased in elevated  $\text{CO}_2$ . Moreover, Taylor et al. (1994) showed that leaf expansion is stimulated by high  $\text{CO}_2$  in several species, from trees to herbs.

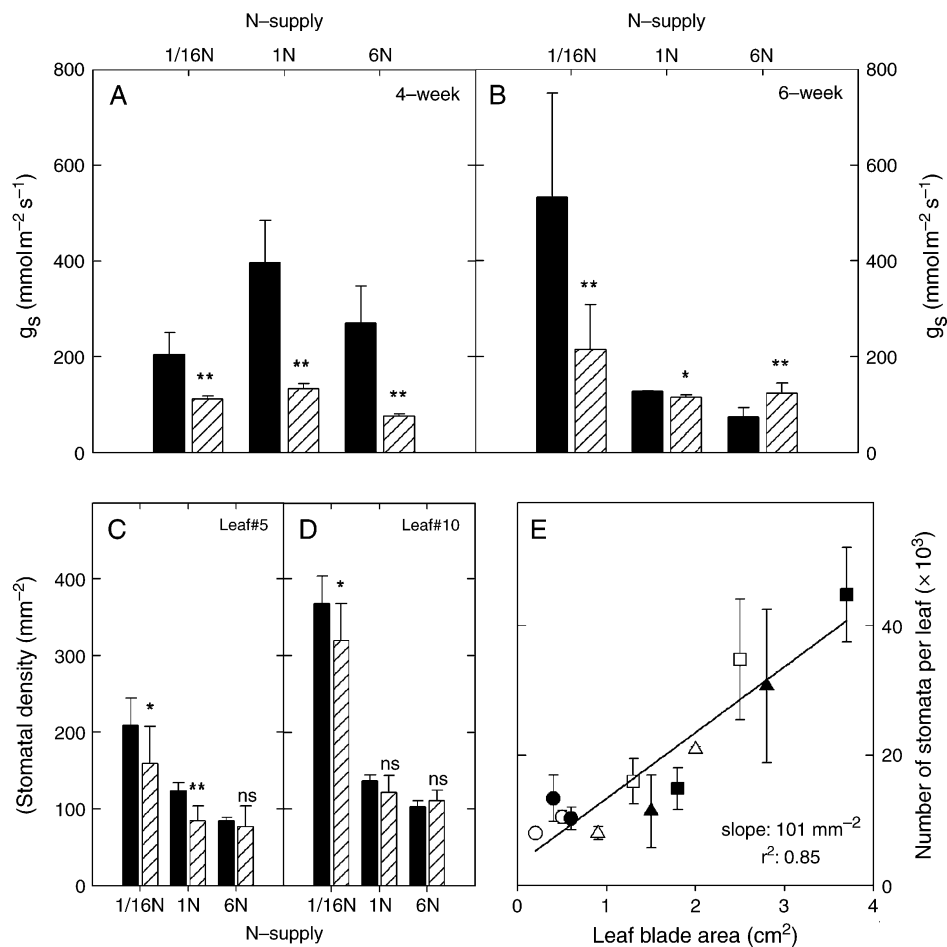
Leaf expansion was not the only growth process stimulated by high  $\text{CO}_2$ : we observed an even more pronounced increase in root biomass (Fig. 2C, D). However, this again appeared as an indirect effect of  $\text{CO}_2$  because the R/S ratio was found to follow TLA, irrespective of  $\text{CO}_2$  conditions (Fig. 2E). Thus, at this stage, we may conclude that the main effect of  $\text{CO}_2$  was to accelerate TLA increase, causing an ontogenic "shift" which correlates with

changes in SLA and R/S ratio.

### Acclimation of *A. thaliana* to long-term elevated CO<sub>2</sub> involves growth rate adjustments rather than a carbohydrate mediated repression of photosynthesis

In our study, acclimation of photosynthesis in high CO<sub>2</sub> was observed under the most limiting N-supply only (1/16N) (Fig. 4A, B). The contribution of nitrogen deficiency in photosynthesis acclimation to elevated CO<sub>2</sub> has been widely reported in many plant species (Stitt and Krapp 1999), including *Arabidopsis* (Sun et al. 2002). In most cases, these non-optimal nutrient conditions led to an increase in non-structural carbohydrates, particularly starch in *Arabidopsis* (Cheng et al. 1998, Sun et al. 2002, Van der Kooij and DeKok 1996, Van der Kooij et al. 1999), and a concomitant decrease in Rubisco content. In our experimental conditions, N-limitation did correlate with an increase in starch and soluble carbohydrates, but CO<sub>2</sub> enrichment did not (Fig. 3). When grown on 1/16N, plants accumulated starch and sugars at ambient CO<sub>2</sub>, but this did not cause a reduction in the photosynthetic activity of the plants as compared with plants grown on higher N-supplies (Fig. 4A, B). Plants grown on 1/16N contained slightly less Rubisco (Fig. 5) but this was compensated by a higher g<sub>s</sub> (Fig. 6). We conclude from this situation that carbohydrate accumulates in low N plants grown at ambient CO<sub>2</sub> because of insufficient sinks.

**Fig. 6.** (A, B) Stomatal conductance of (A) 4- and (B) 6-week-old plants of *Arabidopsis* grown in ambient (black bars) or elevated (800 μl l<sup>-1</sup>) CO<sub>2</sub> (hatched bars). Data are means ± standard deviation for three to five plants. (C, D) Stomatal density (SD) on the abaxial face of leaves # 5 (C) and 10 (D) of 6-week-old plants grown in ambient (black bars) or elevated (800 μl l<sup>-1</sup>) CO<sub>2</sub> (hatched bars). Data are means ± standard deviation for a total of 30 measurements performed on three different leaves. Plants were grown in hydroponics on low (1/16N), medium (1N) or high (6N) N-supply. ns, not statistically different; \*statistically different at P ≤ 0.05; \*\*statistically different at P ≤ 0.001. (E) Correlation between SD and final blade area of expanded leaves. Circles, squares and triangles are, respectively, representing 1/16N, 1N and 6N plants. Open symbols are for ambient CO<sub>2</sub> conditions and filled symbols are for high CO<sub>2</sub> conditions. The regression curve was calculated using SigmaPlot2001 (SPSS, Inc.).



By contrast, enrichment in CO<sub>2</sub> did not cause any accumulation of carbohydrates, whatever the N-supply, and

did not change the levels of Rubisco transcripts, protein (Fig. 5) or capacity ( $V_{c,max}$ , Fig. 4) either. Photosynthesis control by a carbohydrate feedback could thus be involved in short-term response to high  $CO_2$  only. Supporting this hypothesis, it was reported that, when *A. thaliana* plants are transferred to high  $CO_2$  and/or different N-supply conditions (Cheng et al. 1998, Sun et al. 2002, Van der Kooij and DeKok 1996, Van der Kooij et al. 1999), short-term growth, Rubisco content and carbohydrate accumulation are affected to a greater extent than in long-term experiments (Cheng et al. 1998, Gibeau et al. 2001, Ward and Strain 1997). On the other hand, when plants are exposed to high  $CO_2$  throughout their life cycle, other controls are apparently set up at the whole plant level, which affects leaf growth and adjust the photosynthetic capacity to the source to sink balance (Griffin and Seemann 1996, Makino and Mae 1999, Paul and Foyer 2001). Interestingly, similar conclusions were drawn from a metaanalysis of free-air  $CO_2$  enrichment (FACE) experiments: "*loss of Rubisco in FACE is more appropriately described as an acclimatory change benefiting N use efficiency rather than as downregulation*" (Long et al. 2004). Although FACE experiments were designed to avoid some of the constraints of chamber studies, such as root containment, nutrient control and light quality, long-term culture in controlled chambers remains a method of choice for in-depth analysis of the mechanisms involved in the response to elevated  $CO_2$ . Such studies must be performed in stable environments, otherwise "short-term responses" maybe misleadingly generalized. In conclusion, the results presented here support the idea that plants of *Arabidopsis* respond to long-term  $CO_2$  enrichment mainly by adjusting their growth rate. We observed that shoot biomass was higher under high  $CO_2$  when growth was not limited by N-supply. We also showed that changes in SLA, R/S or SD—which are sometimes attributed to  $CO_2$  concentration—actually reflect developmental variations because they are closely related to TLA, irrespective of  $CO_2$  conditions. Only with limiting N was photosynthesis acclimation observed but independently of carbohydrate accumulation and Rubisco decrease. The repression of Rubisco through a carbohydrate-signaling pathway does not seem to control plant response to  $CO_2$  in our experimental conditions.

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