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Elevated CO_2 improved the growth of a double nitrate reductase defective mutant of Arabidopsis thaliana: The importance of maintaining a high energy status

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ABSTRACT

Impairments in leaf nitrogen (N) assimilation in C_3 plants have been identified as processes conditioning photosynthesis under elevated [CO_2], especially when N is supplied as nitrate. Leaf N status is usually improved under ammonium nutrition and elevated [CO_2]. However, ammonium fertilization is usually accompanied by the appearance of oxidative stress symptoms, which constrains plant development. To understand how the limitations of direct fertilization with ammonium (growth reduction attributed to ammonium toxicity) can be overcome, the effects of elevated [CO_2] (800 ppm) exposure were studied in the *Arabidopsis thaliana* double nitrate reductase defective mutant, *nia1-1/chl3-5* (which preferentially assimilates ammonium as its nitrogen source). Analysis of the physiology, metabolites and gene expression was carried out in roots and shoot organs. Our study clearly showed that elevated [CO_2] improved the inhibited phenotype of the nitrate reductase double mutant. Both the photosynthetic rates and the leaf N content of the NR mutant under elevated CO_2 were similar to wild type plants. The growth of the nitrate reductase mutant was linked to its ability to overcome ammonium-associated photoinhibition processes at 800 ppm [CO_2]. More specifically: (i) the capacity of NR mutants to equilibrate energy availability, as reflected by the electron transport equilibrium reached (photosynthesis, photorespiration and respiration), (ii) as well as by the upregulation of genes involved in stress tolerance were identified as the processes involved in the improved performance of NR mutants.

1. Introduction

Although the response of plants under elevated [CO_2] is highly variable, several studies have highlighted the reduction in leaf N concentration (Loladze, 2014) as a factor that conditions photosynthetic rates. The overall leaf N status under elevated [CO_2] has been associated with decreases in photosynthetic enzymes such as Rubisco (Ainsworth and Long, 2005) and reductions in total protein concentration (Taub et al., 2008), which have generally led to decreases in photosynthetic rates. Bloom et al. (2010, 2002) have proposed that elevated [CO_2] directly affects N status in C_3 plants because of the close relationship between photorespiration and leaf nitrate (NO_3^-) assimilation. Guo et al. (2013) found that ammonium assimilation increased due to the increased N demand in N-fixing plants. It was predicted that N assimilation would also be impaired under elevated [CO_2] because photorespiration is diminished by enhanced [CO_2] and consequently ATP synthesis is decreased (Foyer et al., 2012). Furthermore, during the night period, NO_3^- assimilation is also reduced in plants exposed to elevated [CO_2] (Rubio-Asensio et al., 2015). Such an effect would be especially important in cases where plants are exclusively fertilized with NO_3^-.

Leaf N concentration declines under NO_3^- nutrition (Carlisle et al., 2012; Jauregui et al., 2015a, 2016). Nevertheless, when N is provided to the plants in the form of ammonium nitrate (NH_4NO_3), the overall N assimilation is not affected by elevated [CO_2] (Markelz et al., 2013; Jauregui et al., 2015b). However, when ammonium is supplied as the sole N source, "ammonium toxicity" arises in many plant species (Britto and Kronzucker, 2002). Multiple hypotheses have been proposed to explain ammonium toxicity syndrome: cations and root pH disturbance (Britto and Kronzucker, 2002), intercellular pH disruption (Britto et al., 2001), uncoupling of phosphorylation (Gerendás et al., 1997), the futile transmembrane NH_4^+ cycling hypothesis (Britto et al., 2001), or a lack

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of enough C skeletons for ammonium assimilation (Britto and Kronzucker, 2005). Nevertheless, detecting the primary mechanism of ammonium toxicity is very challenging.

Designing appropriate experiments to analyze the impact of NH_4-based nutrition on plant responsiveness to elevated [CO_2] is a matter of great importance and complexity. As shown in Supplemental Figs. 1–3, in previous studies we attested that *Arabidopsis thaliana* plants grown with ammonium as the sole N source were unable to develop in our growth conditions −inhibited phenotype- despite regular pH adjustment of the solution to 5.8 (corresponding to the pH of Murashige and Skoog (MS) medium), buffered with $CaCO_3$ and replaced regularly. Within this context, to test the impact of NH_4-based nutrition in plant responsiveness to elevated [CO_2] we conducted the current study with a double nitrate reductase defective mutant (*nia1-1/chl3-5*; Wilkinson and Crawford, 1993) exposed to elevated [CO_2] (400 *versus* 800 ppm). This mutant mainly uses ammonium as the N source because it has substantially reduced nitrate reductase activity, down to 1% in the leaves and undetectable in the roots (Wang et al., 2004). Although the inhibition of NR activity of the mutant under elevated CO_2 and nitrate nutrition has been previously described (Du et al., 2016) it is unclear whether elevated CO_2 can alleviate ammonium stress. Our working hypothesis is that an increase in C availability promotes ammonium nutrition due to more efficient management of energy and that the biomass and photosynthetic rates of NR mutant plants will be enhanced under elevated [CO_2] conditions. Physiological parameters such as photosynthetic activity, the metabolite profile (carbohydrate and amino acids), total soluble protein and N concentrations, anion and cation concentrations, and the gene expression using microarrays have been determined in shoot and root tissues.

2. Materials and methods

2.1. Plant material and experimental design

The experiments were conducted in hydroponic culture with *Arabidopsis thaliana* plants, Columbia 0 ecotype (wild type) and the Columbia 0 ecotype double nitrate reductase defective mutant *nia1-1/chl3-5* (NR mutant). Seeds were germinated in an agar matrix using a seed holder system (Araponics, Liège, Belgium). Germination took place in a growth chamber that maintained dark conditions for 48 h at 24 °C, with distilled water providing moisture and saturated humidity. Plants were transferred to a growth chamber for 13 days at 150 μmol m^{-2} s^{-1} PAR with a short day photoperiod (8/16 h day/night), a 22–18 °C (day/night) thermoperiod, under saturated humidity and with atmospheric [CO_2] concentration (400 ppm [CO_2]). Subsequently, Arabidopsis plants of uniform size were selected and transferred to hydroponic containers 15 days after sowing (2 days germination and 13 days in a growth chamber). The capacity of the hydroponic containers was 8 liters, and each container had 12 plants (6 wild type and 6 NR mutant plants). Plants were cultured in two different controlled-environment chambers (Heraeus-Votsch HPS-500, Norrkoping, Sweden) of 500 L capacity at two different [CO_2] levels: 400 ppm (atmospheric [CO_2]) and 800 ppm (elevated [CO_2]) for 4 weeks (28 days). The growth chamber conditions were kept at 22/18 °C (day/night), a short day photoperiod (8/16 h day/night), 80% rH and 200 μmol $m^{-2}s^{-1}$ PAR. In order to confirm the physiological response, the experiment was replicated. The plants were rotated between the chambers in order to avoid chamber effect phenomena. In total, the experiment was conducted with 24 replicates per treatment across two experiments for the growth parameters and 3–6 replicates for other measurements. The gene expression analysis was conducted using plants from only one of these pseudoreplicates, as has been done in previous publications (Leakey et al., 2009). Two containers (12 plants per container, 6 for each ecotype) per [CO_2] treatment were used in each experiment. N-free modified Rigaud and Puppo's solution (Puppo and Rigaud, 1975) was used (1.15 mM K_2HPO_4; 2.68 mM KCl; 0.7 mM $CaSO_4$; 0.07 mM Na_2Fe–EDTA; 0.85 mM $MgSO_4$; 16.5 μM Na_2MoO_4; 3.7 μM $FeCl_3$; 3.4 μM $ZnSO_4$; 16 μM H_3BO_3; 0.5 μM $MnSO_4$; 0.1 μM $CuSO_4$; 0.2 μM $AlCl_3$; 0.1 μM $NiCl_2$; 0.06 μM KI). The pH was adjusted to 5.8 with H_3PO_4 and buffered with $CaCO_3$ (0.5 mM) to avoid acidification of the solution. This pH corresponds to the pH of MS medium. The N source was NH_4NO_3 at 1 mM concentration. The solution was replaced every 3-4 days.

All determinations were conducted after 4 weeks exposure to [CO_2] treatments, before the first flower buds were visible and when the more advanced phenology treatment (elevated [CO_2]) was at the 3.7 growth stage of ontology using the scale of Boyes (2001).

2.2. Plant growth determinations and gas exchange

Plant sampling was always carried out 5 h after the beginning of the illumination period. The shoot tissue was harvested and immediately frozen in liquid nitrogen and stored at −80 °C for further analysis. After that, the root tissue was cleaned with abundant MilliQ water, dried with paper, frozen in liquid nitrogen and stored at −80 °C. For plant growth determinations, samples were dried at 70 °C for 48 h to obtain the dry biomass (DM).

Gas exchange measurements were carried out with a Li-COR 6400×T portable gas exchange system (Li-COR, Lincoln, Nebraska, USA) on fully expanded leaves. Such measurements were conducted between 4 h and 7 h after the onset of the light period. The net photosynthetic rates (A_n) were measured at 200 μmol m^{-2} s^{-1} Photosynthetic Photon Flux Density (PPFD). The light-saturated rate of [CO_2] assimilation was measured under saturated light conditions (1000 μmol m^{-2} s^{-1} PPFD) in order to estimate the maximum carboxylation velocity of Rubisco (Vc_{max}) and the maximum electron transport rate contributing to RuBP regeneration (J_{max}) using the model of Harley et al. (1992). The equipment conditions during the measurements were: air flow rate 400 μmol s^{-1}; block and leaf temperature 25 °C; relative humidity in the sample cell 60%. We selected 9 points (7 [CO_2] levels) for these model curves (400, 250, 100, 250, 400, 600, 800, 1000 and 1200 μmol^{-1} [CO_2]). The dark respiration (R_d) measurement was performed 30 min after the dark period started; measurements were made in automatic mode and therefore leaves were placed in the chamber for at least 10 min. The transpiration rate (E) and intercellular [CO_2] (C_i) and were obtained with a Li-COR 6400×T portable photosynthesis system. The relative quantum efficiency of *Psi*I photochemistry (Φ_{PSII}) and the electron transport rate (ETR) were measured simultaneously with a fluorescence chamber (LFC 6400-40; Li-COR) coupled to the Li-COR 6400×T. The rate of electron transport through *Psi*I [Je(*Psi*I)], the electron flux for photosynthetic carbon reduction [*Je*(PCR)] and the electron flux for photorespiratory carbon oxidation [*Je*(PCO)] were measured as described by Epron et al. (1995).

2.3. Metabolite determinations

2.3.1. Soluble sugar and starch determination

Frozen plant material (0.1 g) was ground using liquid nitrogen and then collected in a plastic vial with 1 ml of 80% ethanol. The sample was sonicated for 25 min at 30 °C using an ultrasonic bath (Selecta, Barcelona, Spain) and centrifuged at 16000*g* to collect the supernatant. The same procedure was repeated 3 times. The supernatant was evaporated through a TurboVap (Carmel, New York, USA). After evaporation, the sample was re-suspended in 1.5 ml of H_2O via a vortex step and in an ultrasonic bath. Finally, the extract was centrifuged and the aqueous fraction selected. The solid phase was dried at 70 °C for 24 h to measure starch content. Soluble sugars (sucrose, glucose, and fructose) were determined from the aqueous fraction and starch content from the dry pellet using capillary electrophoresis (Beckman Instruments, Fullerton, California, USA) as detailed in Jauregui et al. (2016). Fucose was used as the internal standard and was added to the extract to be tested (0.5 mM final concentration of fucose).