Nitrate assimilation in plant shoots depends on photorespiration

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Communicated by Emanuel Epstein, University of California, Davis, CA, June 18, 2004 (received for review March 1, 2004)

Photorespiration, a process that diminishes net photosynthesis by ≈25% in most plants, has been viewed as the unfavorable consequence of plants having evolved when the atmosphere contained much higher levels of carbon dioxide than it does today. Here we used two independent methods to show that exposure of *Arabidopsis* and wheat shoots to conditions that inhibited photorespiration also strongly inhibited nitrate assimilation. Thus, nitrate assimilation in both dicotyledonous and monocotyledonous species depends on photorespiration. This previously undescribed role for photorespiration (*i*) explains several responses of plants to rising carbon dioxide concentrations, including the inability of many plants to sustain rapid growth under elevated levels of carbon dioxide; and (*ii*) raises concerns about genetic manipulations to diminish photorespiration in crops.

global climate change | CO_2 acclimation | *Arabidopsis* | wheat

Rubisco, the most prevalent protein in plants, indeed in the biosphere, catalyzes the reaction of ribulose-1,5-bisphosphate with either CO_2 or O_2 and thereby initiates, respectively, the CO_2 assimilatory (C_3 reductive) or photorespiratory (C_2 oxidative) pathways. The balance between the two reactions depends on the relative concentrations of CO_2 and O_2 at the site of catalysis. At current atmospheric levels of CO_2 (≈360 μmol·mol^{-1}) and O_2 (≈209,700 μmol·mol^{-1}), photorespiration in C_3 plants dissipates >25% of the carbon fixed during CO_2 assimilation (1). Thus, photorespiration has been viewed as a wasteful process, a vestige of the high CO_2 atmospheres under which plants evolved (2). At best, according to current thought, photorespiration may mitigate photoinhibition under high light and drought stress (2, 3) or may generate amino acids such as glycine for other metabolic pathways (4). Genetic modification of Rubisco to minimize photorespiration in crop plants has been the goal of many investigations (5).

Atmospheric CO_2 concentrations will rise to somewhere between 600 and 1,000 μmol·mol^{-1} by the end of the 21st century (6). Transferring C_3 plants from ambient (≈360 μmol·mol^{-1}) to elevated (≈720 μmol·mol^{-1}) CO_2 concentrations decreases photorespiration and initially stimulates net CO_2 assimilation and growth by ≈30% (7). With longer exposures to elevated CO_2 concentrations (days to weeks), however, net CO_2 assimilation and plant growth slow down until they stabilize at rates that average 12% (8) and 8% (9), respectively, above those of plants kept at ambient CO_2 concentrations. This phenomenon, known as CO_2 acclimation, is often associated with diminished activities of Rubisco and other enzymes in the C_3 reductive photosynthetic carbon cycle (10, 11), but the influence of elevated CO_2 may not be specific to these enzymes (12). Rather, CO_2 acclimation follows a 14% decline in overall shoot nitrogen concentrations (13), a change nearly double what would be expected if a given amount of nitrogen were diluted by the additional biomass that accumulates under elevated CO_2 concentrations (9, 12).

We proposed a relatively simple explanation for these responses: elevated CO_2 concentrations inhibit the assimilation of nitrate (NO_3^-) in shoots of C_3 plants (14–16). Because NO_3^- is the prominent form of inorganic nitrogen available to plants from temperate well aerated soils (17), diminished NO_3^- assimilation dramatically alters the nitrogen balance in C_3 plants (15). Much of our evidence was based on estimates of shoot NO_3^- assimilation derived from calculations of the difference in the assimilatory quotient (ΔAQ, ratio of net CO_2 consumption to net O_2 evolution) between plants that received NO_3^- as their sole nitrogen source and those that received ammonium (NH_4^+) as their sole source. Here, we establish ΔAQ as a measure of NO_3^- assimilation using genotypes of *Arabidopsis* in which NO_3^- reductase activities are enhanced or deficient. We then use both ΔAQ and an independent measure to demonstrate that NO_3^- assimilation depends on photorespiration in a dicotyledon (*Arabidopsis*) and a monocotyledon (wheat). These results offer a different perspective on the importance of photorespiration and on attempts to minimize it.

Materials and Methods

Materials and Growth Conditions. We used three genotypes of *Arabidopsis thaliana* cv. Columbia: (*i*) the wild type, (*ii*) a transgenic line harboring the chimeric gene *Lhch1*3::Nia1*2* that overexpresses one form of NO_3^- reductase (18), and (*iii*) a genotype with mutations in both structural genes for NO_3^- reductase, *nia1 nia2* (19). Seeds were germinated on plates filled with a dilute Murashige–Skoog medium (2.3 g·liter^{-1}) in 0.75% Phytagar (GIBCO/BRL). The plates were placed in controlled environment chambers (Conviron, Winnipeg, MB, Canada) at ambient CO_2 levels and received 9 h of 350 μmol·m^{-2}·s^{-1} photosynthetically active radiation and 24°C. After 10 d, seedlings were transferred one at a time to 5 × 40-mm pieces of rock wool (Grodania, Hovedgaden, Denmark). Twenty seedlings were transplanted to an opaque 4-liter polyethylene container, the end of the rock wool opposite the seedling being immersed in an aerated nutrient solution containing 200 μM NH_4Cl and 200 μM KNO_3 as nitrogen sources (20). This solution was changed every 3 d. The container was placed in the same controlled environment chamber as the plates.

We surface-sterilized wheat (*Triticum aestivum* cv. Veery 10) seeds for 1 min in 2.6% NaClO, washed them thoroughly with water, and germinated them for several days on thick paper toweling saturated with 10 mM $CaSO_4$. Twenty seedlings were transplanted to a 19-liter opaque polyethylene tub filled with an aerated nutrient solution containing 200 μM NH_4NO_3 (21). The solution was replenished every 3 d. The tubs were placed in a controlled environment chamber (Conviron), providing a photosynthetic photon flux density (PFD) of 650 μmol of quanta m^{-2}·s^{-1} at plant height and a 16 h/25°C day and 8 h/15°C night. After ≈14 d, we transferred a seedling that had three true leaves into a gas-exchange measurement system.

Nitrate Reductase Activity. To assess NO_3^- reductase activity in *Arabidopsis*, 1 g of leaf material was ground with fine glass beads in a cold mortar that contained 4 ml of 0.1 M K-phosphate (pH 7.5), 1 mM EDTA, 3 mM cysteine, and 3%

Abbreviations: PFD, photon flux density; ΔAQ, the difference in the assimilatory quotient.

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www.pnas.org/cgi/doi/10.1073/pnas.0404388101

(wt/vol) casein (22). The homogenate was centrifuged at 30,000 × *g* for 10 min and the supernatant assayed for *in vivo* and fully activated NO_3^- reductase activity according to the procedure of Kaiser *et al.* (23).

Gas-Exchange Measurements. A plant was sealed by a rubber stopper around its stem into a shoot and root cuvette (24, 25). Leaves in the shoot cuvette were at their normal orientation; thus the angle of incidence was between 0° and 45° for *Arabidopsis* and 70° and 80° for wheat. Net gas fluxes from the shoot were monitored with the instrumentation described previously (15, 24). In brief, an infrared gas analyzer (Horiba VIA-500R, Kyoto) measured CO_2 fluxes, a custom O_2 analyzer based on heated zirconium oxide ceramic cells measured O_2 fluxes, and relative humidity sensors (Vaisala, Helsinki) measured water vapor fluxes. Mass flow controllers (Tylan, Torrance, CA) prepared the various gas mixtures, and a pressure transducer (Validyne, North Ridge, CA) monitored the gas flows through the shoot cuvette. We also placed wheat leaves in a leaf cuvette (LI-6400–40, Li-Cor, Lincoln, NE) and estimated the gross O_2 exchange from chlorophyll fluorescence, but this measure did not respond to nitrogen source or CO_2 level (26).

Nitrate Absorption and Accumulation. Wild-type *Arabidopsis* and wheat were grown as described above, except that 3 d before measurement for *Arabidopsis* and 2 d for wheat, the plants were shifted from a medium containing 200 μM NH_4Cl and 200 μM KNO_3 to one devoid of nitrogen. This protocol induced NO_3^- absorption and NO_3^- reductase but then depleted the plant tissue of free NO_3^-. The night before measurements, five to eight plants were transferred to a multiplant measurement system (27). The next morning, *Arabidopsis* or wheat plants received, respectively, 500 or 1,000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation at plant height. The plants were exposed to an atmosphere of (*i*) 360 $\mu mol \cdot mol^{-1}$ CO_2 and 21% O_2, (*ii*) 720 $\mu mol \cdot mol^{-1}$ CO_2 and 21% O_2, or (*iii*) 360 $\mu mol \cdot mol^{-1}$ CO_2 and 2% O_2. Then during a measurement period of 1 h for the *Arabidopsis* and 2 h for wheat, the plants were shifted to an aerated medium containing 0 or 5.5 μmol NO_3^-. Absorption was assessed by the amount of NO_3^- remaining in the medium after the measurement period. After the measurement period, the plants were divided into shoots and roots, oven-dried, and ground to a powder in a ball mill. Water extracts of the powder were analyzed for NO_3^- via HPLC (28), and NO_3^- accumulation in the shoots and roots were calculated from the difference in NO_3^- content between the plants that had received NO_3^- during the measurement period and those that had not. Nitrate assimilation was calculated as the difference in the rates of NO_3^- absorption and plant NO_3^- accumulation. The rate of shoot NO_3^- accumulation was the amount of NO_3^- accumulated in the shoots during the measurement period divided by the time.

Statistics. A repeated-measures analysis of variance was performed by using the mixed procedure in SAS (PROC MIXED, SAS Institute, Cary, NC). The PFD was considered to be a repeated factor, because each canopy was measured at all five levels of PFD. Effects of the treatments and their interactions were considered significant when $P < 0.05$.

Results

Nitrate Reductase Activities. In *Arabidopsis*, NO_3^- reductase in the shoot was nearly fully activated (Fig. 1). In 36-d-old wild-type plants, the fully activated rates of reduction in μmol of NO_3^- per g of fresh mass per min (mean ± SE, $n = 10$) were 0.13 ± 0.02 in the shoots (Fig. 1) and 0.030 ± 0.001 in the roots at ambient CO_2 concentrations. The short-day regime under which the *Arabidopsis* plants were grown prevented them from flowering,

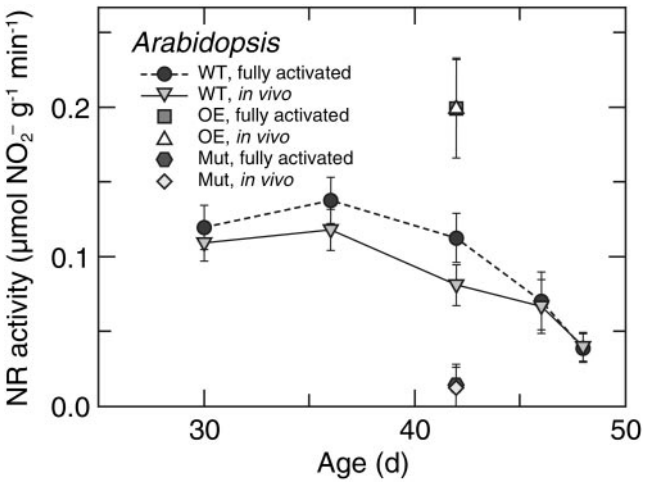

Fig. 1. NO_3^- reductase activity (μmol of NO_2^- generated per g of fresh mass per min) as a function of plant age (d) in leaves of a wild-type *A. thaliana* cv. Columbia (WT), a transgenic line harboring the chimeric gene *Lhch1*3*::*Nia1*2* (OE), and a genotype (*nia1 nia2*) with mutations in both structural genes for NO_3^- reductase (Mut). Because NO_3^- reductase is regulated through phosphorylation, leaf tissue was assayed under conditions that either dephosphorylated the enzyme (fully activated) or did not change its phosphorylation (*in vivo*). Shown are the mean ± SE (n = 5–8 plants).

but as the wild-type plants aged from 36 to 48 d, NO_3^- reductase activity in the shoots diminished markedly (Fig. 1). A transgenic line that harbored the chimeric gene *Lhch1*3*::*Nia1*2* (29) had twice the NO_3^- reductase activity of the wild type, whereas a genotype with mutations in both structural genes for NO_3^- reductase, *nia1 nia2* (19), had no significant activity (Fig. 1). In wheat, the fully activated rates of NO_3^- reductase activity in μmol of NO_3^- per g of fresh mass per min (mean ± SE, $n = 6$) were 0.58 ± 0.03 and 0.021 ± 0.003 in the shoots and roots, respectively, at ambient CO_2 concentrations and 0.46 ± 0.06 and 0.023 ± 0.002 in the shoots and roots, respectively, at elevated CO_2 concentrations (15).

Shoot Gas Fluxes. We simultaneously monitored net CO_2 and O_2 fluxes from shoots of intact *Arabidopsis* and wheat plants as a function of light level. There were six treatments: plants received either NH_4^+ or NO_3^- as a nitrogen source and an atmospheric gas composition of either (*i*) 360 $\mu mol \cdot mol^{-1}$ CO_2 and 21% O_2 (ambient CO_2 and O_2), (*ii*) 700 or 720 $\mu mol \cdot mol^{-1}$ CO_2 and 21% O_2 (elevated CO_2), or (*iii*) 360 $\mu mol \cdot mol^{-1}$ CO_2 and 2% O_2 (low O_2). Net CO_2 consumption was stimulated under elevated CO_2 or low O_2 concentrations but was similar for both nitrogen treatments (Figs. 5 and 6, which are published as supporting information on the PNAS web site), a response typical for C_3 plants that have received ample amounts of nitrogen (30). Net O_2 evolution differed most between NH_4^+ and NO_3^- nutrition under ambient CO_2 and O_2 atmospheres (Figs. 5 and 6).

The ΔAQ, the change in the AQ (the ratio of net CO_2 consumption to net O_2 evolution) with a shift from NO_3^- to NH_4^+ nutrition, highlights these differences (Figs. 2 and 3). Under ambient CO_2 and O_2 atmospheres, ΔAQ was positive in plants having significant NO_3^- activities (36-d-old wild-type *Arabidopsis*, Fig. 2*A*; transgenic *Arabidopsis* overexpressing NO_3^- reductase, Fig. 2*D*; and wheat, Fig. 3), but did not deviate from zero in plants with diminished NO_3^- reductase activities (48-d-old wild-type *Arabidopsis*, Fig. 2*B*; and the *Arabidopsis* knockout mutants, Fig. 2*C*). In *Arabidopsis* and wheat plants having significant NO_3^- activities, ΔAQ decreased at low O_2

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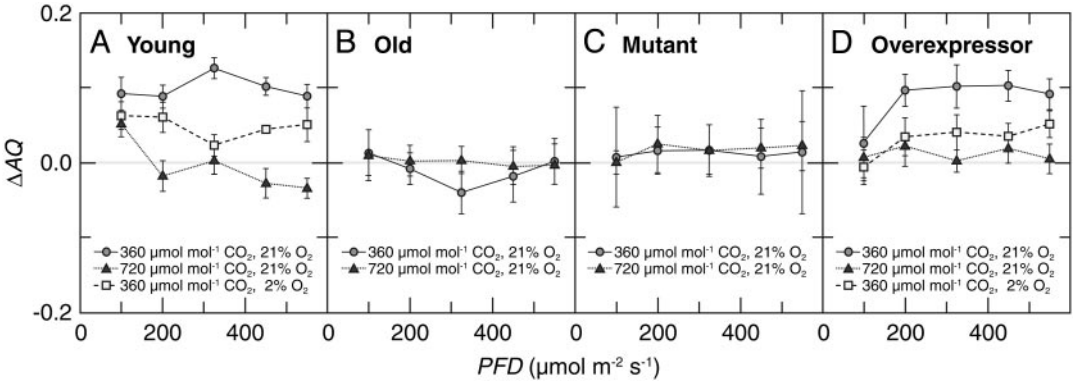

Fig. 2. Changes in assimilatory quotient with the shift from NO_3^- to NH_4^+ (ΔAQ) as a function of photosynthetic PFD in shoots of *A. thaliana* cv. Columbia. Thirty-six-day-old wild-type plants (*A*), 48-d-old wild-type plants (*B*), a genotype with mutations in the two structural genes for NO_3^- reductase (*nia1 nia2*) (*C*), and a transgenic line harboring the chimeric gene *Lhch1*3::Nia1*2* (*D*). The plants were grown under ambient CO_2 (360 μmol·mol^{-1}) and measured under ambient CO_2 and O_2 (360 μmol·mol^{-1} CO_2 and 21% O_2; circles), elevated CO_2 (720 μmol·mol^{-1} CO_2 and 21% O_2; triangles), or low O_2 (360 μmol·mol^{-1} CO_2 and 2% O_2; squares). Shown are the mean ± SE, n = 5–8 plants.

concentrations and became negligible at elevated CO_2 concentrations (Figs. 2 *A* and *D* and 3).

Nitrate Accumulation. Another measure of NO_3^- assimilation is the difference between the amount of NO_3^- that a plant absorbs and that it accumulates in its tissues. According to this measure, both elevated CO_2 and low O_2 concentrations inhibited plant NO_3^- assimilation in *Arabidopsis* and wheat (Fig. 4), although the influence of low O_2 concentrations was significant only at $P <$ 0.2 in *Arabidopsis*. Absorption of NO_3^- also declined at elevated CO_2 and low O_2 concentrations but to a lesser extent than NO_3^- assimilation (Fig. 4). Moreover, the rates at which NO_3^- accumulated in the shoots of either species did not differ significantly among treatments (data not shown).

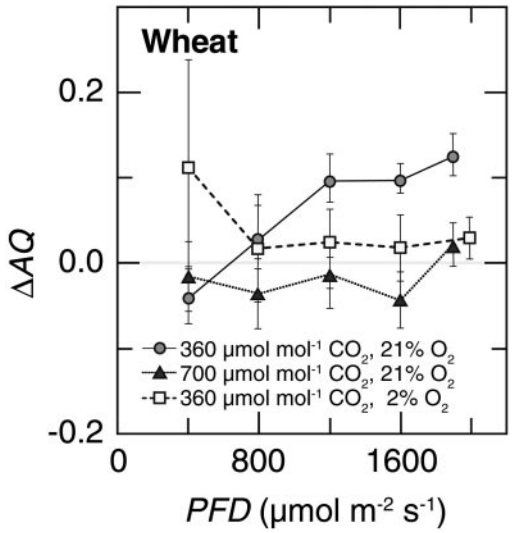

Fig. 3. Changes in assimilatory quotient with the shift from NO_3^- to NH_4^+ (ΔAQ) as a function of photosynthetic PFD in shoots of wheat (*T. aestivum* cv. Veery 10). The plants were grown under ambient CO_2 (360 μmol·mol^{-1}) and measured under ambient CO_2 and O_2 (360 μmol·mol^{-1} CO_2 and 21% O_2; circles), elevated CO_2 (700 μmol·mol^{-1} CO_2 and 21% O_2; triangles), or low O_2 (360 μmol·mol^{-1} CO_2 and 2% O_2; squares). Shown are the mean ± SE, n = 5–8 plants. The data for ambient CO_2 and O_2 and elevated CO_2 and ambient O_2 have been published (15).

Discussion

Two independent methods indicated that NO_3^- assimilation in *Arabidopsis* and wheat decreased under both elevated CO_2 and low O_2 atmospheres.

The first method was a real-time continuous measure involving AQ, the ratio of net CO_2 consumption to net O_2 evolution. The AQ decreases as NO_3^- assimilation increases: additional electrons generated from the light-dependent reactions of photosynthesis are transferred to NO_3^- and hence to NO_2^-, stimulating net O_2 evolution while having little effect on CO_2 consumption (15, 24, 31, 32). We present ΔAQ, the change in AQ under NO_3^- versus NH_4^+ nutrition rather than AQ, because several other biochemical processes such as lipid metabolism can influence AQ, but these processes do not change rapidly with nitrogen source, so ΔAQ should predominantly reflect NO_3^- assimilation (32). The ΔAQ also has appropriate scaling, because it should be zero when NO_3^- assimilation is negligible and should increase as nitrate assim-

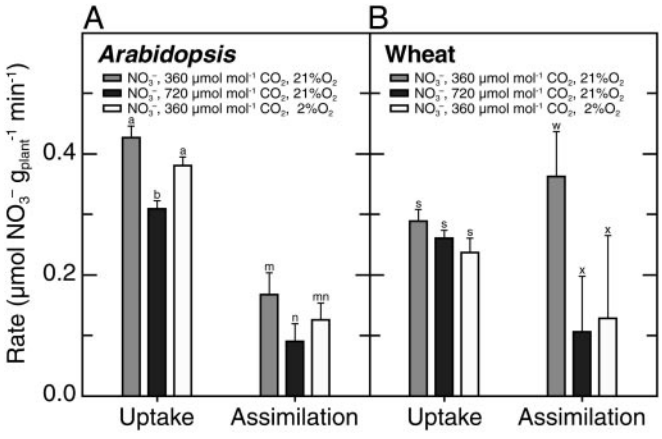

Fig. 4. In wild-type *Arabidopsis* and wheat, NO_3^- uptake as the amount of NO_3^- depleted from a medium and NO_3^- assimilation as the difference between the rates of net NO_3^- uptake and net accumulation of free NO_3^- in plant tissues. Thirty-six-d-old *Arabidopsis* plants (*A*) or 10-d-old wheat (*B*) were exposed to either 360 μmol·mol^{-1} CO_2 and 21% O_2 (gray), 720 μmol·mol^{-1} CO_2 and 21% O_2 (black), or 360 μmol·mol^{-1} CO_2 and 2% O_2 (white). Shown are the mean ± SE (n = 13–16). Treatments labeled with different letters differ significantly ($P \leq 0.05$). The light levels were 500 and 1,000 μmol·m^{-2}·s^{-1} PAR for *Arabidopsis* and wheat, respectively.

ilation increases. Here (Figs. 2 and 3), ΔAQ differed from zero only in plants with relatively high NO_3^- reductase activities, affirming its relationship with NO_3^- assimilation.

The second method for assessing NO_3^- assimilation was a traditional one based on the difference between the total amount of NO_3^- absorbed and that which accumulated in plant tissues (e.g., refs. 33–38). This method has several difficulties.

(*i*) It estimates NO_3^- assimilation in the whole plant, not just in the shoots. Nonetheless, the observed changes in total NO_3^- assimilation with CO_2 levels (Fig. 4) probably reflected mostly the responses of the shoots, because NO_3^- assimilation in the roots usually comprises only a minor percentage of the total during the day (39) and is relatively insensitive to CO_2 levels (15). For example, NO_3^- reductase activity was 27 times greater in wheat shoots than roots and 4.3 times greater in 36-d-old wild-type *Arabidopsis* shoots than roots.

(*ii*) This method requires destructive tissue analysis after the uptake measurement and thus cannot be conducted in real time.

(*iii*) Although the plants were deprived of nitrogen for 3 d, free NO_3^- in the tissues of the controls (those that did not receive NO_3^- during the uptake measurements) spanned a broad range, causing variation in the estimates of NO_3^- accumulation.

(*iv*) Uptake measurements were conducted during the transition from nitrogen deprivation to nitrogen sufficiency. The rates at which NO_3^- accumulated in the shoots, however, were similar in all treatments (data not shown), indicating that NO_3^- availability in the shoots did not limit assimilation at elevated CO_2 concentrations.

Despite these difficulties, the decline in NO_3^- assimilation rates under elevated CO_2 or low O_2 concentrations determined by this method (Fig. 4) paralleled the results based on the ΔAQ (Figs. 2 and 3).

A physiological response common to elevated CO_2 and low O_2 is diminished photorespiration (40). The observed shifts in ΔAQ under elevated CO_2 or low O_2 concentrations did not result directly from photorespiration. Photorespiration releases CO_2 and consumes O_2 in equal amounts (41); therefore, if only the photorespiratory pathway were involved, ΔAQ would shift in the opposite direction to the one we observed. For example, the 36-d-old wild-type *Arabidopsis* under ambient CO_2 and O_2 had an AQ of 0.94 ± 0.01 under NO_3^- and 1.04 ± 0.01 under NH_4^+ (mean ± SE for the five light levels); equal fluxes of CO_2 and O_2 from photorespiration would bring the AQ values for these treatments closer together as photorespiration increases and further apart as it decreases. A straightforward interpretation for the decline in ΔAQ at elevated CO_2 or low O_2 is that NO_3^- assimilation depends on photorespiration. Our results with the second method for assessing NO_3^- assimilation (Fig. 4) affirm this interpretation.

Possible Mechanisms. One part of the photorespiratory pathway is the export of malate from the chloroplast through the cytoplasm and into the peroxisome, where it generates NADH, which reduces hydroxypyruvate. This malate "valve" or "shuttle" increases the NADH/NAD ratio in the cytoplasm (42) and thereby may provide NADH instrumental in the reduction of NO_3^- to NO_2^-. Malate also serves as a counterion that prevents alkalinization when NO_3^-, an anion, becomes incorporated into a neutral amino acid (43). Such processes could explain the observations that NO_3^- assimilation was fastest in *Arabidopsis* and wheat under ambient CO_2 and O_2 concentrations (Figs. 2–4), the treatment under which photorespiration was highest.

The influence of elevated CO_2 concentrations on NO_3^- assimilation was more pronounced than that of low concentrations of O_2 (Figs. 2 *A* and *D*, 3, and 4). Two additional mechanisms contribute to the inhibitory effect of elevated CO_2 concentrations on NO_3^- assimilation. (*i*) Transport of NO_2^- from the cytosol into the chloroplast involves the net diffusion of HNO_2 or cotransport of protons and NO_2^- across the chloroplast membrane. This requires the stroma to be more alkaline than the cytosol (44, 45). Elevated concentrations of CO_2 can dissipate some of this pH gradient, because additional CO_2 movement into the chloroplast acidifies the stroma. As a result, elevated CO_2 concentrations inhibited NO_2^- transport into the chloroplast (15). (*ii*) Several competing processes, the C_3 reductive photosynthetic carbon cycle, the reduction of NO_2^- to NH_4^+, and the incorporation of NH_4^+ into amino acids, occur in the chloroplast stroma (46) and require reduced ferredoxin generated by photosynthetic electron transport (47). Key enzymes in these processes have different affinities for reduced ferredoxin: ferredoxin–NADP reductase has a K_m of 0.1 μM, nitrite reductase has a K_m of 0.6 μM, and glutamate synthase has a K_m of 60 μM (48). As a result, NO_3^- assimilation proceeds only if the availability of reduced ferredoxin exceeds that needed for NADPH formation (49, 50). For wheat (Fig. 3) and tomato (16), this occurred only at high light intensities under ambient CO_2 and O_2 concentrations, conditions under which CO_2 availability limited C_3 photosynthesis.

The responses of CO_2 and O_2 fluxes to the various treatments were similar in the wild-type *Arabidopsis* and the transgenic that overexpresses NO_3^- reductase (Fig. 2 *A* and *D*). This similarity supports the contention that NO_3^- reductase activity by itself limits neither NO_3^- assimilation (23) nor plant performance (51).

Implications. Our finding that CO_2 inhibits NO_3^- assimilation in shoots of *Arabidopsis* and wheat is consistent with previous studies on barley (24), tomato (16), and wheat (14, 15). If CO_2 inhibition of shoot NO_3^- assimilation were common among C_3 species, it could account for several responses of plants to elevated CO_2, including the decline in shoot protein and the diminished activities of photosynthetic enzymes. Nitrogen availability determines plant responses to elevated CO_2 concentrations more than any other environmental factor (52, 53), but ecosystems show a broad range of responses to elevated CO_2 concentrations, possibly as a result of the seasonal and spatial fluctuations in the relative availabilities of NH_4^+ and NO_3^-. For instance, ecosystems in which NH_4^+ is the dominant nitrogen form, such as pine forests (54) or wetlands (55), show a relatively large increase (≈25%) in net primary productivity under CO_2 enrichment, whereas ecosystems in which NO_3^- is dominant, such as grasslands (56) or wheat fields, at standard fertilizer levels (low fertilizer treatment at Maricopa, AZ; ref. 57) show declines in net primary productivity under CO_2 enrichment.

Plants vary in their relative dependence on NH_4^+ and NO_3^- as nitrogen sources and in their balance between shoot and root NO_3^- assimilation (17). Our results suggest that rising atmospheric CO_2 levels will favor taxa that prefer NH_4^+ as a nitrogen source or assimilate NO_3^- primarily in their roots.

Extensive efforts to increase the specificity of Rubisco for CO_2 relative to O_2 and thereby increase the productivity of C_3 crops have proved unsuccessful (5). Our results indicate that such efforts might have hitherto unforeseen consequences: in agricultural systems where NO_3^- is the dominant form of inorganic nitrogen, minimizing photorespiration may be associated with nitrogen deprivation.

We thank Y. M. Heimer (Albert Katz Center for Desert Agrobiology, J. Blaustein Institute for Desert Research) for providing seed of the transgenic *Arabidopsis* that overexpresses NO_3^- reductase and Y. M. Heimer, Aaron Kaplan, and Alan Stemler for comments on the manuscript. Alan Tan and Chang Tun-Hsiang provided technical assistance. This research was funded in part by National Science Foundation Grants IBN-99-74927 and IBN-03-43127 and by U.S. Department of Agriculture National Research Initiative Competitive Grants Program Grant 2000-00647 (to A.J.B.) and an Israel Binational Agricultural Research and Development Fund Fellowship (to S.R.).

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