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Distinctive Responses of Ribulose-1,5-Bisphosphate Carboxylase and Carbonic Anhydrase in Wheat Leaves to Nitrogen Nutrition and their Possible Relationships to CO_2-Transfer Resistance[1]

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ABSTRACT

The amounts of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), total chlorophyll (Chl), and total leaf nitrogen were measured in fully expanded, young leaves of wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), spinach (*Spinacia oleracea* L.), bean (*Phaseolus vulgaris* L.), and pea (*Pisum sativum* L.). In addition, the activities of whole-chain electron transport and carbonic anhydrase were measured. All plants were grown hydroponically at different nitrogen concentrations. Although a greater than proportional increase in Rubisco content relative to leaf nitrogen content and Chl was found with increasing nitrogen supply for rice, spinach, bean, and pea, the ratio of Rubisco to total leaf nitrogen or Chl in wheat was essentially independent of nitrogen treatment. In addition, the ratio of Rubisco to electron transport activities remained constant only in wheat. Nevertheless, gas-exchange analysis showed that the in vivo balance between the capacities of Rubisco and electron transport in wheat, rice, and spinach remained almost constant, irrespective of nitrogen treatment. The in vitro carbonic anhydrase activity in wheat was very low and strongly responsive to increasing nitrogen content. Such a response was not found for the other C_3 plants examined, which had 10- to 30-fold higher carbonic anhydrase activity than wheat at any leaf-nitrogen content. These distinctive responses of carbonic anhydrase activity in wheat were discussed in relation to CO_2-transfer resistance and the in vivo balance between the capacities of Rubisco and electron transport.

The highly positive correlation between photosynthetic capacity and nitrogen content in leaves is often found because the amounts of stromal enzymes and thylakoid proteins account for the majority of leaf nitrogen content (see ref. 6 for a review). Evans and Terashima (9), working with spinach, clarified the relation between nitrogen nutrition and nitrogen partitioning into the various photosynthetic components and activities. They found that although nitrogen supply increased the ratio of Rubisco activity to electron transport activity, ATPase, Chl, or total leaf nitrogen, the balance between the in vivo activities of Rubisco and electron transport remained constant. They concluded that this difference was compensated for by the presence of a CO_2-transfer resistance between intercellular air spaces and the carboxylation sites. As a result of this resistance, the in vivo Rubisco specific activity was reduced progressively with increasing amount of enzyme because the partial pressure of CO_2 at the carboxylation sites was reduced and kept in a constant balance with electron transport activity. The increase in the ratio of Rubisco to total leaf nitrogen or Chl with nitrogen supply is frequently found for other C_3 species, such as tobacco (1), cotton (32), *Solanum* (11), bean (26), and pea (18).

However, in spite of the existence of significant CO_2-transfer resistance in wheat (5, 8, 23, 30), the ratio of Rubisco to total leaf nitrogen or Chl in fully expanded young leaves seems to be independent of nitrogen nutrition (5, 17, 18). Although the proportion of nitrogen in Rubisco was found to increase sometimes with leaf nitrogen content (7, 17), analysis of these data may be complicated by the fact that leaves were sampled at different ages; the Rubisco content declines more rapidly in wheat leaves during senescence (16).

The CO_2-transfer resistance is considered to depend on CA[4] activity (22), mesophyll surface area per unit of leaf area (2, 14), chloroplast surface area adjacent to plasma membranes (30), relative ratio of air space inside a leaf (15), solubility resistance of CO_2 to liquid phase, and permeability of CO_2 through the membranes and cell walls (13). Among these factors, the role of CA in C_3 species is strongly implicated to facilitate the diffusion of CO_2 in the chloroplast because the majority of inorganic carbon is bicarbonate at the alkaline pH within the stroma (see ref. 22 for a review). Cowan (3) developed models of the optimal partitioning of protein into CA in relation to Rubisco for maximum photosynthesis. However, the extent to which CA contributes to

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[4] Abbreviations: CA, carbonic anhydrase; RuBP, ribulose 1,5-bisphosphate.

CO_2 diffusion to the carboxylation sites remains uncertain. In addition, the response of CA activity to changing nitrogen content is not known.

In this study, we used fully expanded, young leaves of several C_3 plants, including wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), spinach (*Spinacia oleracea* L.), bean (*Phaseolus vulgaris* L.), and pea (*Pisum sativum* L.), and first investigated differences in the effects of nitrogen nutrition on the ratio of Rubisco content to Chl content, whole-chain electron transport activity, and total leaf nitrogen between wheat and the other C_3 plants. Using a gas-exchange system, we then examined the in vivo balance between Rubisco activity and electron transport activity. In addition, we found a distinctly different response of CA activity to changing leaf nitrogen content in wheat. Although we could not elucidate the quantitative contribution of CA to CO_2 diffusion to the carboxylation sites, we suggest that the responsiveness of CA to nitrogen supply in wheat, and its role in CO_2 transfer, may be crucial to the in vivo balance between Rubisco and electron transport activities in this species.

MATERIALS AND METHODS

Plant Culture

Wheat (*Triticum aestivum* L. cv Asakaze), pea (*Pisum sativum* L. cv Sugar snap), and spinach (*Spinacia oleracea* L. cv Nobel) plants were grown hydroponically in growth chambers. The chamber for wheat and pea was operated with a day/night temperature of 23/18°C, a PPFD of 1000 μmol quanta m^{-2} s^{-1} at plant level, and a 14-h photoperiod. The chamber for spinach was operated with a day/night temperature of 19/14°C, a PPFD of 900 μmol m^{-2} s^{-1}, and a 9-h photoperiod. Rice (*Oryza sativa* L. cv Sasanishiki) and bean (*Phaseolus vulgaris* L. cv Tendergreen) were grown hydroponically in a greenhouse under natural sunlight conditions from April to July 1991. On a sunny day, maximum PPFD was about 2000 μmol m^{-2} s^{-1}. The hydroponic solution used was described by Makino and Osmond (18), and was continuously aerated. All measurements were made on fully expanded, young leaves of 4- to 10-week-old plants. About 10 to 14 d before the measurements, plants were supplied with a nutrient solution containing different nitrogen concentrations (0.2–12 mM) as described previously (18). These nutrient solutions were changed every 5 d.

Determination of Chl, Total Leaf Nitrogen, Rubisco, and Cyt *f*

Fresh leaves were homogenized in 50 mM lithium-phosphate buffer, pH 7.0, containing 120 mM 2-mercaptoethanol, 2.5 mM iodoacetic acid, and 5% (v/v) glycerol at a leaf:buffer ratio of 1:7 (g/mL) in a chilled mortar with pestle. The total Chl and leaf nitrogen contents were measured from part of this crude homogenate (18). After adding 25% (w/v) lithium dodecylsulfate solution to the homogenate (final concentration, 1.0% [w/v]), this preparation was immediately heated at 100°C for 45 s and centrifuged at 10,000*g* for 5 min. The supernatant fluid was analyzed by SDS-PAGE to determine Rubisco and Cyt *f* contents. The Rubisco content was determined spectrophotometrically by formamide extraction of the Coomassie brilliant blue R-250-stained subunit bands from the gel, using calibration curves made with Rubisco purified from wheat or rice leaves (18). The Cyt *f* content was determined by western blotting using monospecific antisera against Cyt *f* as described previously (12).

Assay of Rubisco, Electron Transport, and CA

The Rubisco and whole-chain ($H_2O \rightarrow$ methyl viologen) electron transport activities were measured as described previously (18). CA activity was measured by the method of Ohki (20) with some modifications. Leaves were homogenized in 50 mM Hepes-NaOH, pH 7.5, containing 10 mM DTT, 0.5 mM EDTA, and 10% (v/v) glycerol in a chilled mortar with pestle. The ratio of leaf to buffer was varied from 1:6 to 1:12 (g/mL) depending on the Chl content, which had been estimated with a Minolta Chl meter SPAD-502 (Minolta Camera, Tokyo, Japan). Triton X-100 was added to a portion of the homogenate to a final concentration of 0.1% (v/v), followed by centrifugation at 15,000*g* for 5 min. The supernatant was used for the enzyme assay.

Addition of 0.1% Triton X-100 enhanced CA activity by 10 to 20%, and there was scarcely any activity remaining in the insoluble fraction. This was verified by western blotting using monospecific antisera against CA (data not shown). CA activity was determined by measuring the pH decrease at 0 to 2°C with a pH electrode. The centrifuged crude extract (25 μL for wheat and 5 μL for other plants) and 2 mL of 20 mM Na-barbital (pH 8.3 with H_2SO_4) were stirred at a constant rate in a small cuvette, and the reaction was initiated by addition of 1.0 mL of CO_2-saturated H_2O (76 mM CO_2 at 0°C). Enzyme activity was defined as 1 unit = $10(T_o - T)/T$, in which T and T_o represent the time(s) at 0 to 2°C needed for a pH decrease from 8.25 to 7.45, with and without enzyme, respectively.

Gas Exchange

The rates of CO_2 and H_2O vapor exchange were measured with an open gas-exchange system using a temperature-controlled chamber equipped with two fans. The system was detailed in Makino et al. (17). Differences in the partial pressures of CO_2 and H_2O entering and leaving the chamber were measured with an IRGA (Horiba ASSA-1110, Horiba, Kyoto, Japan) and a dew point hygrometer (EG&G model 911, EG&G, Waltham, MA), respectively. The PPFD at the position of the leaf in the chamber was adjusted to 1800 μmol quanta m^{-2} s^{-1}. Leaf temperature was controlled at 25°C. Gas-exchange parameters were calculated according to the equations in von Caemmerer and Farquhar (31).

RESULTS

The ratio of Rubisco to total leaf nitrogen and its relationship to leaf nitrogen content are shown in Figure 1A. There was a difference in nitrogen partitioning into Rubisco in response to changing total leaf nitrogen content among the C_3 species examined. The ratio of Rubisco to leaf nitrogen in wheat was independent of leaf nitrogen content, but this ratio from the other plants increased with increasing leaf

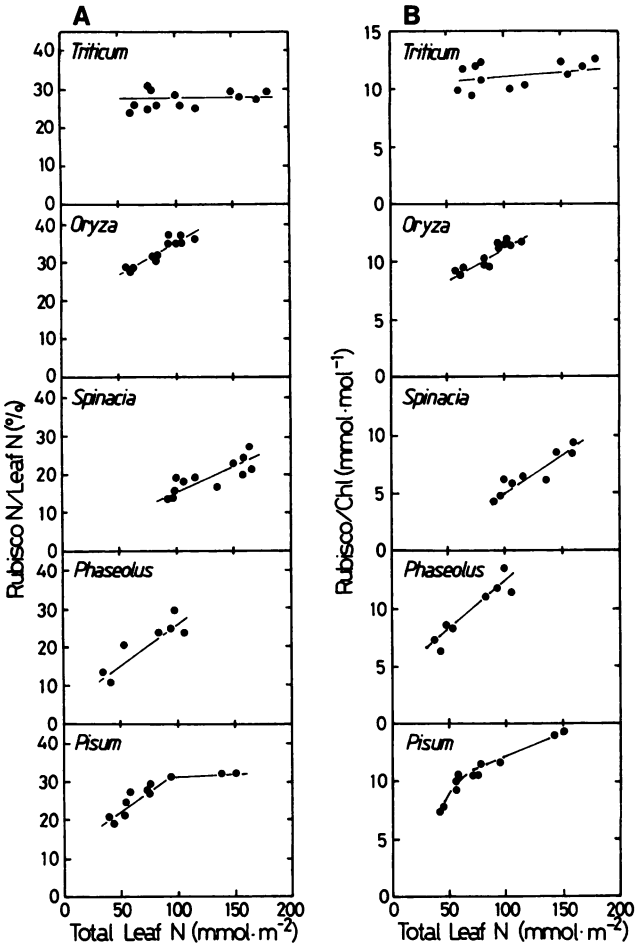

Figure 1. Ratio of Rubisco nitrogen to total leaf nitrogen versus total leaf nitrogen content (A) and the ratio of Rubisco to total Chl contents versus total leaf nitrogen content (B) in fully expanded, young leaves of wheat, rice, spinach, bean, and pea. All plants were grown hydroponically at different nitrogen levels.

nitrogen content. In addition, differences in the absolute ratio were found among species. For example, in wheat and rice, about 28% and 28 to 37% of the leaf nitrogen content was present in Rubisco, respectively, whereas its proportion in spinach was only 13 to 25%. The greater ratio of nitrogen in Rubisco for wheat and rice was also noted by Evans (6). However, the values of Rubisco to leaf nitrogen ratios given in Figure 1 are appreciably higher than those previously reported (7, 17, 25, 26, 29). This may be, in part, because we obtained complete extraction of membrane-bound Rubisco by addition of detergent (19).

The ratio of Rubisco to Chl and its relationship to leaf nitrogen content are shown in Figure 1B. The ratio of Rubisco to Chl in wheat was relatively constant, but that from the other plants increased with increasing leaf nitrogen content. These relationships between Rubisco and Chl are quite similar to those in Figure 1A. This is because the proportion of leaf nitrogen as Chl remained almost constant in all plants, including wheat, irrespective of nitrogen treatment.

We next determined the ratio of Rubisco to whole-chain ($H_2O \rightarrow$ methyl viologen) electron transport activity. Because rice leaves were less suited for assay of electron transport activity, Cyt *f* content was measured as an important indicator of electron transport capacity (12, 29). In wheat leaves, the ratio of Rubisco to electron transport activities was unaffected by nitrogen treatment, but in spinach this ratio increased with increasing nitrogen content (Fig. 2). Similarly, in rice the ratio of Rubisco to Cyt *f* increased. Thus, except for wheat, the greater increase in Rubisco with increasing leaf nitrogen was quite similar to that reported by Evans and Terashima (9) with spinach.

The response of net photosynthetic rate to the intercellular partial pressure of CO_2 was examined using intact leaves of wheat, rice, and spinach. The purpose of this analysis was to ascertain whether there is a species-dependent difference in the in vivo balance between the capacities of Rubisco and electron transport in response to nitrogen nutrition that might account for the distinctive Rubisco responses for wheat shown in Figures 1 and 2. According to the photosynthetic model for C_3 species developed by Farquhar and von Caemmerer (10), the photosynthetic rate at low CO_2 partial pressures is limited by Rubisco capacity, whereas the rate at high CO_2 is limited by electron transport capacity. In addition, photosynthesis under saturating CO_2 conditions can also be limited by the capacity of Pi regeneration during starch and sucrose synthesis (27, 28). Thus, if the in vivo ratio of Rubisco to electron transport capacities is affected by nitrogen supply, we would expect to observe a difference in the ratio of the

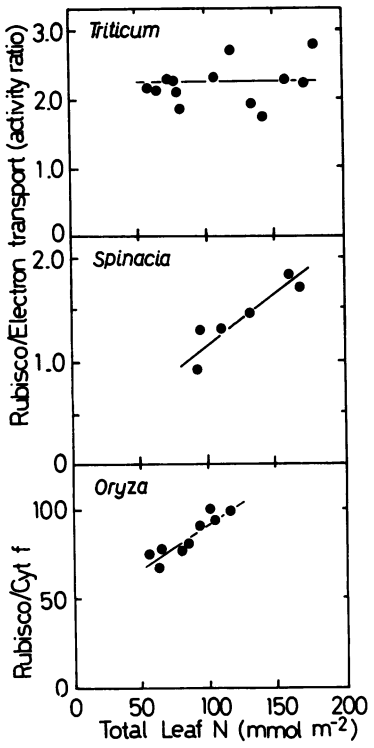

Figure 2. Ratio of Rubisco to whole-chain ($H_2O \rightarrow$ methyl viologen) electron transport activities versus total leaf nitrogen content in leaves of wheat and spinach and the ratio of Rubisco to Cyt *f* content versus total leaf nitrogen content in leaves of rice.

photosynthetic rate at low CO_2 pressures to the rate at high CO_2 among the various nitrogen treatments.

For all plants examined, both the photosynthetic rate at low CO_2 pressures and the rate at high CO_2 increased with increasing nitrogen supply (upper panels in Fig. 3). When the CO_2 response curve for each nitrogen treatment is normalized to the rate at 200 μbar, the respective curves for the three species collapse to nearly identical curves, showing that there is no difference among these species in the relationship between photosynthesis at low and high CO_2 pressures in response to nitrogen nutrition (lower panels in Fig. 3). This finding is surprising, given the absence of nitrogen-dependent changes in the ratios of Rubisco to leaf nitrogen, Chl, and electron transport in wheat (Figs. 1 and 2) compared with the other plants examined. In accord with the view of Evans and Terashima (9), we considered the possibility that the difference in mechanisms for the in vivo balance between Rubisco and electron transport between wheat and the other plants lies in a difference in the response of the CO_2-transfer resistance to changing nitrogen content. Therefore, we examined CA activity as one of the components of the CO_2-transfer resistance.

Figure 4 shows the relationship between the ratio of CA activity to Rubisco content and leaf nitrogen content. Although CA activity on a Rubisco basis in rice, spinach, and pea remained essentially constant irrespective of nitrogen treatment, this enzyme activity from wheat was markedly enhanced with increasing leaf nitrogen content. In addition, the absolute in vitro activity of CA per unit of leaf area in wheat was much lower than that from the other C_3 plants examined over a wide range of leaf nitrogen content, being only 3 to 10% of that from the other plants at any given leaf nitrogen content (Fig. 5). If CA is closely related to the CO_2-transfer resistance, these results suggest that the CO_2-transfer resistance in wheat may be significantly affected by nitrogen treatment, decreasing with increasing leaf nitrogen content. Thus, the change in this resistance may be responsible for the balance between Rubisco and electron transport activities in vivo.

DISCUSSION

With the exception of wheat, the proportion of leaf nitrogen in Rubisco increased with increasing leaf nitrogen content (Fig. 1A). Evans and Terashima (9) concluded that this relative increase in Rubisco content is required to maintain the balance between the in vivo capacities of Rubisco and electron transport because of the presence of a CO_2-transfer resistance. In wheat, however, the proportion of nitrogen in Rubisco was unaffected by nitrogen treatment (Fig. 1A). In addition, the ratio of Rubisco activity to whole-chain electron transport activity in wheat also remained constant over a wide range of total leaf nitrogen content (Fig. 2). Nevertheless, the gas-exchange analysis showed that the in vivo balance between the capacities of Rubisco and electron transport in wheat remains almost constant irrespective of nitrogen treatment (Fig. 3). This finding suggests that wheat has a special mechanism(s) for this in vivo balance. We will first consider the following two possible mechanisms.

One is that the in vivo capacity for RuBP regeneration relative to leaf nitrogen content might have decreased with decreasing in vivo Rubisco specific activity at high leaf nitrogen content. However, the ratio of electron transport activity to Rubisco activity in wheat remained essentially constant

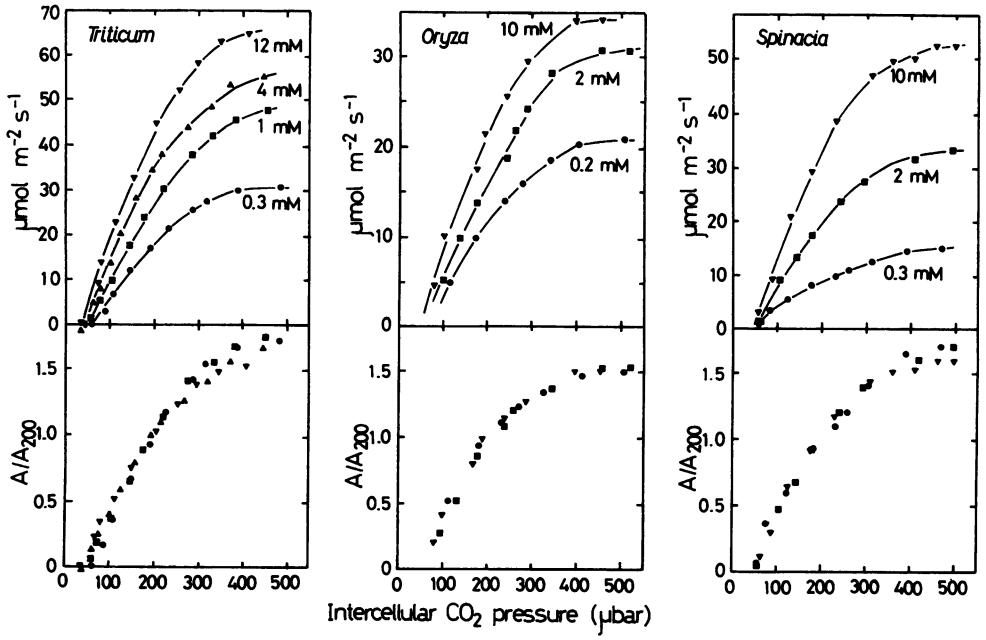

Figure 3. Rate of CO_2 assimilation (A) as a function of the intercellular CO_2 partial pressure in leaves of wheat, rice, and spinach. Upper panels, Response curves expressed in μmol m^{-2} s^{-1}. Lower panels, Response curves that have been normalized relative to the CO_2 assimilation rate at 200 μbar of intercellular CO_2 (A_{200}). Plants were grown at the indicated nitrogen concentrations. Measurements were made at a PPFD of 1800 μmol quanta m^{-2} s^{-1} and a leaf temperature of 25°C.

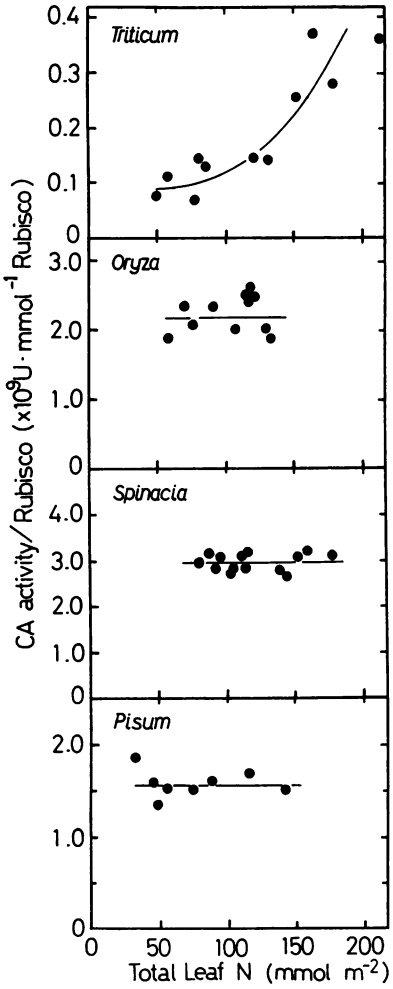

Figure 4. Ratio of CA activity to Rubisco content versus total leaf nitrogen content in leaves of wheat, rice, spinach, and pea.

(Fig. 2), suggesting no decrease in the in vivo RuBP regeneration capacity. RuBP regeneration can also be limited by Pi regeneration during starch and sucrose synthesis (27, 28), and although the results in Figure 3 suggested a relative decrease in Pi regeneration capacity at high nitrogen content, this limitation occurs only at saturating CO_2 pressures (24). Thus, a reduction in the in vivo RuBP regeneration capacity seems unlikely.

Another possible mechanism is that a greater decrease in the CO_2-transfer resistance occurs with increasing leaf nitrogen content in wheat, and this prevented a reduction in the CO_2 partial pressure at the carboxylation sites. The finding that the ratio of CA activity to Rubisco content in wheat was strongly stimulated by nitrogen supply (Fig. 4) lends support to this interpretation. This strong stimulation was not found for the other C_3 plants examined. Although it remains uncertain how much CA contributes to the CO_2 diffusion between leaf intercellular air spaces and the carboxylation sites, it is possible that the greater CA activity in high nitrogen-grown wheat prevented a greater reduction in the CO_2 partial pressure at the carboxylation sites relative to that in the low-nitrogen treatment. In addition, this relative increase in CA activity with increasing Rubisco content is consistent with the optimization model of protein budget between CA and Rubisco developed by Cowan (3). In fact, von Caemmerer and Evans (30), using a combination of conventional gas-exchange measurements with concurrent measurements of carbon isotope discrimination during CO_2 uptake, found an inverse correlation between the CO_2-transfer resistance and leaf nitrogen content in wheat. They also reported that the chloroplast surface area exposed to intercellular air spaces increased with increasing nitrogen content.

At sufficiently high leaf nitrogen content, however, a curvilinear relationship between CO_2-limited photosynthesis and Rubisco content has been reported for wheat (5). This curvilinear relationship was considered to be due to a relatively greater drop in CO_2 pressure at the carboxylation sites in the high-nitrogen leaves, on the assumption that all wheat leaves had a similar CO_2-transfer resistance. However, this interpretation is complicated by the fact that even at an irradiance of 2000 μmol m^{-2} s^{-1}, photosynthesis in the high-nitrogen leaves is not light saturated (29).

Wheat has an unusually low in vitro CA activity, being 10- to 30-fold lower for a given leaf nitrogen content (Fig. 5). If the CA activity in wheat is assumed to be just sufficient to allow CO_2 diffusion for maximum photosynthesis, these results suggest that in the other C_3 plants examined CA is saturating and the CO_2-transfer resistance is primarily de-

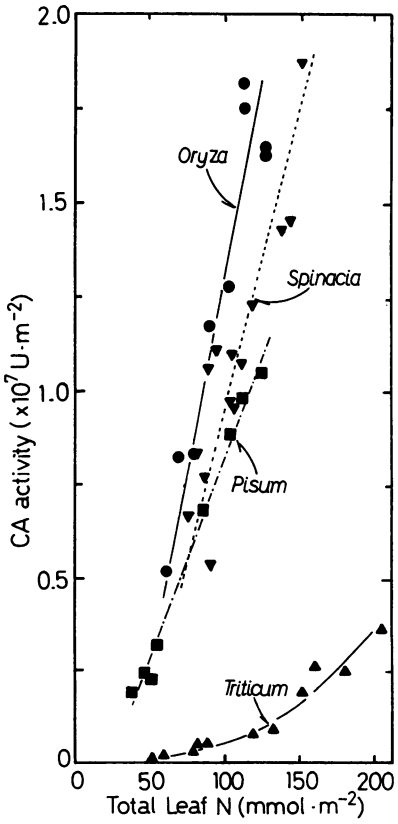

Figure 5. CA activity per unit of leaf area versus total leaf nitrogen content in leaves of wheat (▲), rice (●), spinach (▼), and pea (■).

pendent on leaf anatomical factors such as the mesophyll surface area (2, 14) and chloroplast surface area adjacent to plasma membranes (30). However, our knowledge about these factors in relation to nitrogen nutrition is, at best, limited. In rice leaves, the mesophyll surface area per unit of leaf area increased with increasing nitrogen supply (2). On the other hand, in cotton leaves this surface area was independent of nitrogen nutrition (14). Unfortunately, there are no data available on the mesophyll surface area of wheat grown at different levels of nitrogen, aside from the inferences that can be drawn from von Caemmerer and Evans (30).

There have been reports indicating that CA activity is present in great excess in various plants. Ohki (20) grew cotton at different zinc concentrations and observed that although the CA activity decreased to 15% with decreasing zinc supply, the photosynthetic rate was not affected. Similar results with zinc-deficient plants were obtained for spinach (21) and bean (4). In addition, Jacobson et al. (13), who used intact spinach chloroplasts treated with ethoxyzolamide, an inhibitor of CA, found that the concentration of inhibitor required to inhibit CO_2 fixation was much higher than that required to inhibit CA activity. All these reports suggest that CA is present in great excess over what is required to maximize photosynthesis in C_3 species other than wheat. Thus, it is possible that limitation by CA is species-dependent, at least in C_3 species.

In our study, we found distinctly different responses of Rubisco and CA to changing leaf nitrogen content in wheat compared with several other C_3 species. Although we could not unequivocally elucidate the mechanism(s) for the in vivo balance between Rubisco and electron transport in wheat, we observed consistent changes in Rubisco and CA with increasing leaf nitrogen content in relation to CO_2-transfer resistance and the in vivo balance between the capacities of Rubisco and electron transport. Our results suggest that recent interspecific comparisons using on-line carbon-isotope discrimination analyses by Evans and co-workers (8, 30) might be useful in evaluating the role of CA in the CO_2-transfer resistance of C_3 leaves. These authors found that the CO_2-transfer resistance in rice is smaller than that in wheat at any given leaf nitrogen content (30), which might be due, in part, to the much greater CA activity in rice noted here.

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