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Regulation of Expression of Carbon-Assimilating Enzymes by Nitrogen in Maize Leaf[1]

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ABSTRACT

We have utilized the cellular differentiation gradient of the developed, youngest leaf to examine the regulation by nitrogen of levels of phospho*enol*pyruvate carboxylase (PEPCase), pyruvate orthophosphate dikinase (PPDK), and ribulose 1,5-bisphosphate carboxylase in maize (*Zea mays* L.). The protein whose level regulated most preferentially by N availability was PEPCase, followed by PPDK, and the changes in level occurred most conspicuously at the photosynthetically maturing cells. Pulse and pulse-chase experiments to analyze photosynthetic fixation of $[^{14}C]CO_2$ indicate that maize leaf primarily exploited a C_4-mode of photosynthetic fixation of carbon dioxide even under a selective reduction in levels of these proteins. The effects of N on the synthesis of these proteins and the accumulation of corresponding mRNAs during recovery from a deficiency were examined by pulse and pulse-chase labeling with [^{35}S]Met and by hybridization, respectively. The rate of turnover of PPDK was substantially higher than that of the other proteins. Results also showed that the reduced accumulation of PEPCase, as well as PPDK, under N deficiency could largely be accounted for a reduced level of synthesis of protein with a concomitant reduction in level of their mRNAs. This indicates that the N-dependent selective accumulation of these enzymes is primarily a consequence of level of its mRNAs.

PEPCase[3], PPDK, and Rubisco are major carbon-assimilating enzymes in maize leaf, and their levels are potentially limiting with respect to photosynthetic productivity (2, 20, 22–24). These proteins can, therefore, be considered as essential in maize with regard to productivity as well as nitrogen economy. In the hybrid used in our experiments, these three proteins comprise approximately 8, 6, and 35% of the total soluble protein in the leaf, respectively, under near optimal growth conditions, and plants grown with a suboptimal supply of nitrogen have a modified investment in leaf proteins (21). Such plants show a decrease in the proportions of PEPCase and PPDK with a concomitant increase in the proportion of Rubisco.

To gain a better understanding of the way in which nitrogen regulates the selective expression of the major leaf proteins, we have examined (a) the basipetal distribution of these proteins in response to nitrogen status and (b) the effects of nitrogen on the synthesis of a range of proteins and the steady-state levels of their mRNAs during recovery from a nitrogen deficit.

MATERIALS AND METHODS

Plant Growth

Maize (*Zea mays* L. cv Golden Cross Bantam T51) plants were grown for 21 to 24 d with 0.8 mM (low) or 16 mM (high) KNO_3 in vermiculite as described previously (20, 21) under greenhouse conditions or in a growth chamber in the System for Biological Fixation of Solar Energy, Nagoya University. The light intensity of the chamber was approximately 700 $\mu E \cdot m^{-2} \cdot s^{-1}$ at plant level. Room humidity was maintained at 70%. The temperature was maintained at 30°C by day and 25°C at night with a photoperiod of 12 h. To induce transient changes in the concentration of nitrogen, plants were grown with low nitrate for 21 d and then were supplied with high nitrate. The fully developed, youngest leaves were periodically harvested at the same day time (10 AM) before additional nitrogen was supplied, and 2 and 3 d thereafter. The change in length of the leaves during nitrogen supplementation was less than 3%.

Measurements of Levels of Proteins

Proteins were extracted and levels of PEPCase, PPDK, and Rubisco were determined by single radial immunodiffusion, as described previously (21). For each experiment, quoted values are the mean of values from duplicate or more samples.

Photosynthetic Fixation of $[^{14}C]CO_2$ and Analysis of Intermediates

The fully developed youngest leaves of control and nitrogen-deficient plants grown for 21 d were transversely cut at 4, 8, and 12 cm from the base. Immediately before introduction of [^{14}C], the basal (0–4 cm) and middle (8–12 cm) leaf sections were placed in a glass chamber (20 mL), capped with a rubber stopper that contained 0.5 mL water, and preilluminated for at least 20 min with white light (100 w/m^2) at 30°C, with passage of humidified air through the chamber. Two mL of air was replaced by air containing 0.25 μmol of $[^{14}C]CO_2$

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[3] Abbreviations: PEPCase, phospho*enol*pyruvate carboxylase; PPDK, pyruvate orthophosphate dikinase; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; PGA, 3-phosphoglyceric acid; M, mesophyll; BS, bundle sheath.

(12.5 μCi) through the stopper with a glass syringe. After 10 s the stopper was removed and the leaf section was frozen in liquid nitrogen. For pulse-chase experiments, leaf sections, fed as above with [^{14}C]CO_2, were transferred to other glass vials that contained 0.5 mL water, illuminated for additional 60 s, and then frozen. The frozen sections were pulverized in liquid nitrogen and extracted successively with acetic acid/ethanol (10:90, v/v), with 80% ethanol, and then with 50% ethanol. Each extraction was conducted at 60°C for 10 min and the supernatants were obtained by centrifugation. An aliquot of the combined supernatants (9×10^4 dpm) was spotted onto Whatman No. 1 filter paper for separation and identification of individual compounds by two-dimensional paper chromatography (16). Radioactivity in compounds was located by a autoradiogram and measured with a GM-counter. The compounds in spots were identified by comparisons with unlabeled standards. For each experiment, quoted values are means of values from duplicate samples.

Labeling of Proteins with [^{35}S]Met and Isolation of Labeled Proteins

Four of the fully developed youngest leaves were detached from plants and kept at 25 to 30°C under white light (500–600 $\mu E \cdot m^{-2} \cdot s^{-1}$ at leaf level) in 0.3 mL of [^{35}S]Met (125 μCi) in a glass tube for 3 and 6 h. The radioisotope solution was taken up by the leaf blades within 30 min, and water was supplied thereafter. After a given period of time, the leaf blades were cut transversely into three segments of equal length from base to tip. Proteins were extracted as described in the preceding section after homogenates had been centrifuged at 50,000*g* for 20 min, 4°C. Labeled PEPCase, PPDK, and Rubisco were immunochemically isolated essentially according to the procedure described previously (8), using their specific antibodies (21) and Protein A-Sepharose, and the radioactivity of resultant immunocomplex was measured in a scintillation counter. Purity of labeled antigen in the immunocomplex was determined by SDS-PAGE with 10% (for PEPCase and PPDK) and 12.5% (for Rubisco) acrylamide gels, followed by fluorography (19). For each sample, quoted values are means of at least duplicate analyses. For the measurement of radioactivity in TCA-insoluble fractions, an aliquot of leaf extract was soaked on a GF/C Whatman filter disc, washed with 5% (w/v) TCA and ethanol, dried, and radioactivity on the disc was measured in a scintillation counter.

Measurement of mRNAs

Total RNA was isolated in guanidine/CsCl as previously described (25) from the basal sections (1 g) of fully developed, youngest leaves prepared as indicated earlier and varied amounts of glyoxalated RNA (0.1–4 μg) of each sample were dot-blotted on nylon filters (Biodine A, Pall). The 1.1 and 1.0 kb fragments digested with *Hin*dIII and *Eco*RI of maize pM52 cDNA for PEPCase (7) and maize pPPDK1067 cDNA for PPDK (12), respectively, were labeled with [^{32}P]dCTP using Multiprime DNA Labeling System (Amersham) and used as hybridization probes. The filters were prehybridized and then hybridized at 42°C in 50 mM sodium phosphate buffer (pH 7.0), 5 × SSC, 5 × Denhardt's solution, 50% (v/v) formamide, calf thymus DNA (0.125 mg/mL), and 0.1% SDS with the labeled probes (1×10^6 cpm/μg), washed four times at room temperature in 2 × SSC, 0.1% SDS for 10 min each, then two times at 50°C in 0.1 × SSC, 0.1% SDS for 30 min each. Densitometer scans of the resulting autographs were performed over a range of RNA dilutions giving a linear response.

RESULTS

Distribution of PEPCase, PPDK, and Rubisco Proteins as a Function of Position in Leaves under Nitrogen Stress

To compare the positional distribution of carbon-assimilation enzymes in the leaves of nitrogen-deficient and control plants, we determined the levels of proteins for PEPCase, PPDK, and Rubisco in the extracts of leaves of seedlings grown with low (0.8 mM) or high (16 mM) levels of nitrate. At harvest, the fifth and sixth leaves were actively developing in control plants while the primary leaf was chlorotic and the sixth leaf was just starting to emerge in nitrogen-deficient plants. As summarized in Figure 1, each protein in the control plants was at its highest level in fourth leaf and the levels were lower both in the younger and older leaves. The ratio of levels of PEPCase, PPDK, and Rubisco in the fourth leaf was 1.0:0.9:3.4 on a unit fresh-weight basis. By contrast, in nitrogen-deficient plants, the accumulation of each protein was clearly shifted to younger leaves. This shift is consistent with the well-documented concept that available nitrogen moves toward younger leaves in plants under growth-limiting conditions. The most intensive accumulation of these proteins in nitrogen deficient plants was observed in the fourth and fifth leaves, which are, respectively, the fully and almost fully developed youngest leaves; the average ratio of levels of PEPCase, PPDK, and Rubisco in the fourth leaf was 1.0:1.3:6.3 on a unit fresh-weight basis. The changes in levels of accumulation of these three proteins in the two groups of plants indicate that the accumulation of PEPCase and PPDK in maize leaf is selectively reduced under nitrogen stress, a result

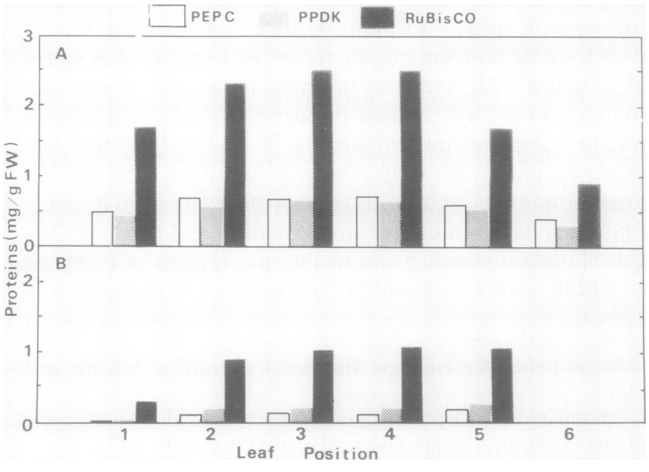

Figure 1. Positional distribution in leaves of PEPCase, PPDK, and Rubisco of control (A) and nitrogen-deficient (B) plants. Plants were grown in a greenhouse for 21 d at 30°C by day and 25°C by night.

that is in agreement with our previously reported data obtained from the analysis of whole plants (21).

Basipetal Distribution in Developed Youngest Leaves of Carbon-Assimilation Enzymes under Nitrogen Stress

To specify the region of the leaf that is most affected by the selective reduction in levels of PEPCase and PPDK under nitrogen stress, we determined the basipetal distribution of the three proteins in the developed youngest leaves of plants grown under control and nitrogen-deficient conditions. The level of each protein in the leaves increased exponentially from base to tip regardless of the nitrogen status and corresponded to the degree of maturity of the cells (Fig. 2A). Under conditions of nitrogen deficiency (Fig. 2B), levels of all of these proteins fell markedly, as was predicted. The most remarkable reduction in the levels of protein occurred at the leaf base where cells were maturing (sections 1–5). Among the three proteins of interest, the reduction in accumulation was much greater in the case of PEPCase than the cases of PPDK and Rubisco, with the values in reduction to controls of approximately 95, 50, and 15%, respectively. At the leaf tip, the level of PEPCase was extremely low, presumably as a result of the selective degradation and/or reduced rate of synthesis induced by nitrogen stress.

Changes in Levels of Proteins in Developed Youngest Leaves of Plants during Recovery from Nitrogen Deficiency

In any comparison of control and nitrogen-deficient plants, one might argue that tissues of the same chronological age may be at different developmental stages. Therefore, an analysis of recovery of proteins was undertaken by monitoring the levels of proteins in the transversely cut sections of the developed youngest leaf upon supply of high levels of nitrate to deficient plants. In control plants, the relative levels of all the proteins, over the course of 2 d of normal development, increased almost evenly by 1.5- to 2.5-fold at the base and decreased to some extent near the leaf tip (Fig. 3). This decrease reflects a reduction in rates of synthesis and/or an acceleration of degradation of proteins due to senescence. In sharp contrast, however, each protein in plants recovering from nitrogen stress accumulated preferentially and predominantly at the leaf base (Fig. 4). The protein whose level increased most markedly was PEPCase, followed by PPDK, with maximal increases in the levels of 12- and 4-fold, respectively, during the 3 d after the addition of the high levels of nitrate. The increase in accumulation of Rubisco during this period was also substantial but smaller (2.5-fold).

Mode of Photosynthetic Fixation of Carbon Dioxide in Nitrogen-Deficient Leaves

With regard to the most prominent reduction in the level of PEPCase observed under nitrogen stress, a pertinent question is whether or not maize exploits a C_4-mode of photosynthetic fixation of CO_2 under these conditions. To answer this question, pulse- and pulse-chase labeling experiments were conducted to investigate photosynthetic fixation of [^{14}C]-CO_2. As summarized in Table I, the most intensive incorporation of label into the alcohol-soluble fraction after a 10-s pulse was seen in the middle sections of the leaves of control plants. The incorporation of label into this fraction during the period was approximately four-fold higher in the basal sections of the leaves of control plants than in those of nitrogen-deficient plants, on a unit-weight basis. This difference may approximate the difference in the rates of photosynthetic fixation of carbon dioxide between the two segments with different nitrogen status, since the result of labeling for 10 s under these conditions should provide an estimate of the initial rate of fixation of CO_2 in the maize leaf (see ref. 5 for an example). Percentages of label that disappeared from the fractions during the subsequent 60-s chase were approximately 30 and 10% in segments from control and deficient leaves, respectively, indicating a reduction in the rate of

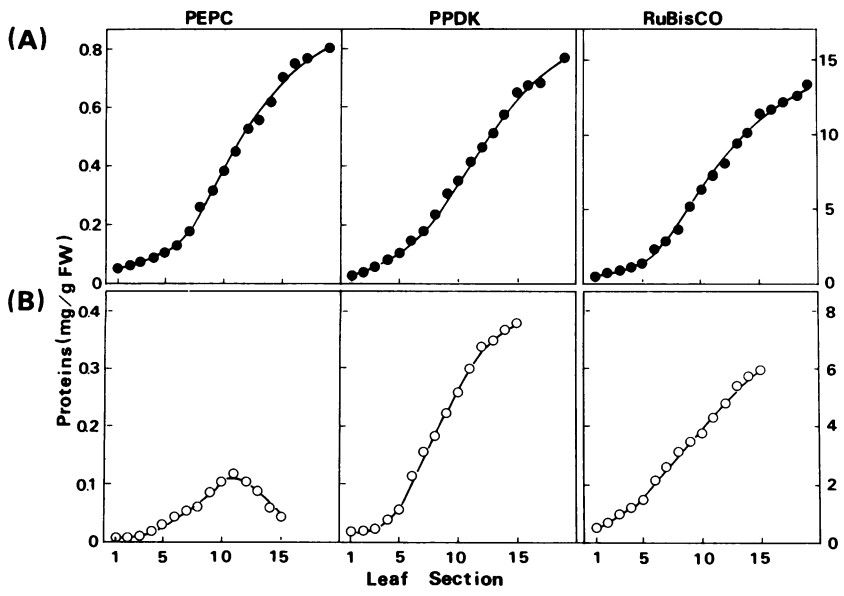

Figure 2. Basipetal distribution of PEPCase, PPDK, and Rubisco in developed youngest leaves of control (A) and nitrogen-deficient (B) plants. Plants were grown in a growth chamber for 3 weeks. When the fourth leaf and third leaf of control and nitrogen-deficient plants, respectively, were fully developed as the youngest leaves, the leaves of at least five plants were cut transversely into 2-cm segments and subjected to immunochemical quantification.

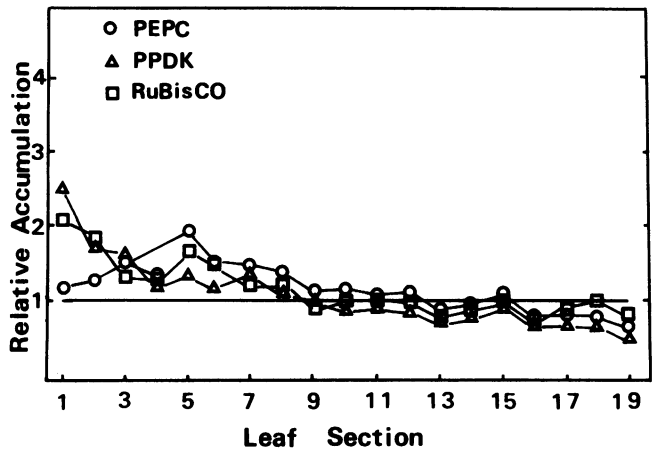

Figure 3. Patterns of the relative accumulation of PEPCase, PPDK, and Rubisco in developed youngest leaves during the course of 2 d of normal development. Growth of control plants and preparations of leaf segments were conducted as described in the legend to Figure 2, using the fourth leaves of five plants. Levels of proteins were determined immunochemically and relative accumulation was calculated by taking the relative level of each protein in each segment on d 0 as unity.

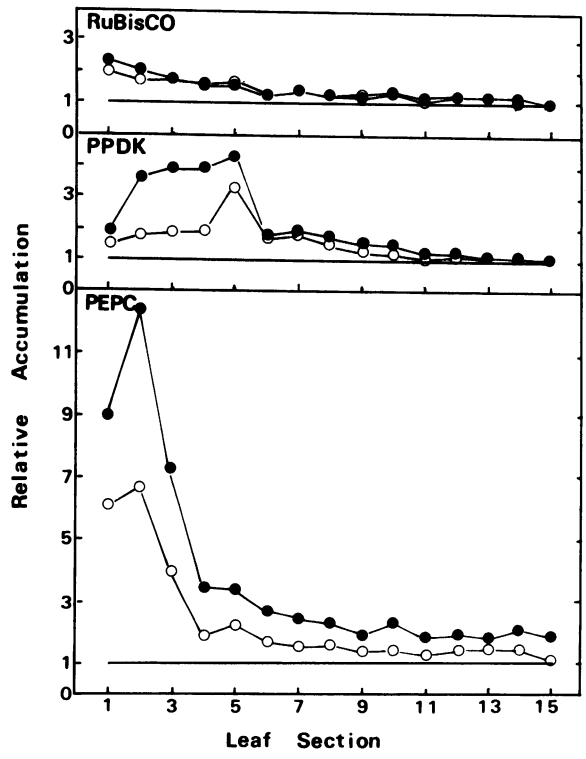

Figure 4. Relative accumulation of PEPCase, PPDK, and Rubisco in developed youngest leaf of plants during recovery from nitrogen deficiency. Plants were grown in a growth chamber. The third leaves of nitrogen-deficient plants were harvested immediately upon supplementing with nitrogen, 2 d (open circles) and 3 d (filled circles) thereafter. Leaf segments were prepared from five plants as described in the legend to Figure 2. Relative accumulation was calculated in the same way as described in the legend to Figure 3.

conversion from alcohol-soluble into alcohol-insoluble compounds under conditions of nitrogen deficiency. There was no significant difference in the pattern of distribution of labeled intermediates after the 10-s pulse in any of the leaf segments. Furthermore, in the 10-s pulse-labeling and subsequent 60-s chase, there was no significant difference in the pattern of distribution of labeled C_4 acids and PGA between the two segments of the different plants. We conclude that maize leaf exploits primarily a C_4-mode of photosynthetic fixation of carbon dioxide, even when photosynthetic activity is reduced by nitrogen deficiency. In segments of deficient leaves, there was a marked decrease in label into sucrose with a concomitant increase in label in sugar-phosphates. This change may be at least partly due to the altered rates of synthesis and/or degradation of sucrose induced by nitrogen stress.

Protein Synthesis in Developed Youngest Leaves of Nitrogen-Deficient and Recovering Plants

Reduced accumulation of proteins in cells could be due either to a reduced rate of synthesis or to an increased rate of breakdown. To distinguish between these alternatives, incorporation of [^{35}S]Met into proteins at leaf base was examined in detached leaf blades from nitrogen-deficient and recovering plants. Developed youngest leaves were harvested at 0 and 2 d after supply of nitrogen to deficient plants and were incubated with a solution of [^{35}S]Met. The radioactive solution was taken up into leaf tissues in less than 30 min so that subsequent incubation with water for 3 and 6 h results in an almost complete pulse-chase experiment.

Considering the experimental conditions and the evidence that the contents, as a percentage of this amino acid residue in PEPCase, PPDK, and Rubisco are similar (7, 12, 13), the proportion of ^{35}S-labeled proteins detected after incubation for 3 compared to 6 h should largely reflect the stability of the newly synthesized proteins. Relatively small differences were observed in the levels of label in PEPCase and Rubisco proteins between the two feeding times, both in the leaves of deficient and recovering plants (Table II). Therefore, we conclude that there was no evidence for a large-scale degradation of these two proteins in leaf blades, regardless of nitrogen status. There were, however, relatively large differences (of approximately 50% or more) in the levels of labeled PPDK between the two feeding periods in the leaves of deficient and recovering plants. This result indicates that substantial breakdown of newly synthesized PPDK occurs in leaves, regardless of nitrogen status. The incorporation of [^{35}S]Met into proteins increased approximately 4.0-, 2.0-, and 1.3-fold for PEPCase, PPDK, and Rubisco, respectively, when nitrogen was supplied to deficient plants. These results, taken together, indicate that the nitrogen-dependent changes in accumulation of PEPCase and PPDK in plants may be mainly due to changes in the rates of protein synthesis, although the possibility of small changes in rates of breakdown of proteins cannot be excluded. It should be stressed that the rate of degradation of newly synthesized PPDK appears to be substantially higher than that of the other two enzymes.

Table I. *Distribution of ^{14}C-Labeled Photosynthetic Products in Leaf Segments of Control and Nitrogen Deficient Plants*

Leaf Segment and Treatment	Total ^{14}C in Soluble Fraction ($\times 10^8$ dpm/g fresh wt)	Total Radioactivity in Compounds			
		Mal + Asp	PGA	Sugar-P	Sucrose
		%			
10-s pulse					
Control					
Base section	4.56	44.8	23.0	9.0	ND[a]
Middle section	14.7	36.7	24.3	9.3	ND
Nitrogen-deficient					
Base section	1.08	42.2	19.1	8.9	ND
10-s pulse/60-s chase					
Control					
Base section	3.16	12.4	11.9	14.4	13.8
Middle section	9.73	8.1	11.8	11.0	24.9
Nitrogen-deficient					
Base section	0.98	14.9	9.2	22.0	6.6

[a] Not determined.

Table II. *Synthesis of PEPC, PPDK, and Rubisco, as Percentages of Synthesis of Total Soluble Protein, in the Leaf Base of Plants during Recovery from Nitrogen Deficiency*

Sample Source	Labeling time	[^{35}S] Proteins (% of total [^{35}S] soluble protein)					
		PEPC		PPDK		Rubisco	
		I[a]	II	I	II	I	II
	h						
Deficient	3	1.34	2.13	0.22	0.19	9.93	6.18
	6	1.18	1.34	0.13	0.08	9.08	5.02
Recovering	3	6.49	7.21	0.43	0.68	9.80	11.0
	6	4.30	6.91	0.26	0.21	8.41	8.78

[a] I and II represent results of two independent experiments.

Table III. *Changes in Accumulation of Hybridizable mRNAs and Proteins for PEPCase and PPDK in Leaf Base of Plants during Recovery from Nitrogen Deficiency*

Randomly selected leaves harvested at 0 and 2 d after supplying a high nitrate to deficient plants were assayed. Quoted values are means of values from duplicate samples.

Protein	Recovering Leaf/Deficient Leaf	
	Hybridizable RNA	Protein
PEPCase	6.3	2.7
PPDK	1.3	1.4

Changes in Levels of mRNAs in Maturing Cells of Developed Youngest Leaves of Plants from Nitrogen Deficiency

The reduced level of synthesis of PEPCase and PPDK in nitrogen-deficient leaves could be due to either reduced levels of their mRNAs, or to less efficient translation of control levels of their mRNAs. Thus, we determined the changes in levels of their mRNAs relative to total RNA by dot blot hybridization. The results are summarized in Table III. The level of hybridizable mRNAs for PEPCase and PPDK in the basal sections of developed youngest leaf of nitrogen-deficient plants increased 6.3- and 1.3-fold, respectively, concomitant with the increase in the levels of respective proteins, during 2 d after the addition of the high levels of nitrate.

DISCUSSION

We have exploited the basipetal gradients of prevalent carbon assimilating enzymes in the developed youngest leaves of maize plants as a system to analyze the selective expression of enzymes in response to nitrogen status. PEPCase, PPDK, and Rubisco were detectable at substantial levels, even in the basal sections of the leaves, regardless of nitrogen status. This result indicates a complete cellular differentiation of M and BS cells, as reported by Langdale *et al.* (9, 10) who demonstrated that the accumulation of products of photosynthesis-related genes in both types of cell is associated with vascular development. Our data appear to differ from those reported by Taylor's group (11, 14) who found no detectable levels of the carboxylase enzymes, despite the presence of significant amounts of mRNA for the subunits of both enzymes in the most immature leaf tissue of maize. The discrepancy between the results of the two sets of experiments may be due to differences in the developmental stages of leaves used for analysis.

An important finding from the present study was the nitrogen-dependent selective change in levels of PEPCase and PPDK which was evident at the leaf base where cells are maturing photosynthetically. The selective change in the CO_2-trapping machinery in C_4 photosynthesis in response to the availability of nitrogen is consistent with the previous results (21) from the analysis of whole maize plants. The present study provides further insight by specifying the region of the leaf in which the predominant regulation by nitrogen of nitrate-partitioning into these photosynthetic proteins occurs. The changes in levels of proteins for these enzymes should reflect the changes in activity of the enzymes, at least in the

case of PEPCase and PPDK, since equivalence between the amount of immunochemically determined protein and the activity of the enzyme has been established for these enzymes in maize leaves (21). Thus, a selective reduction in levels of these enzymic proteins in maturing cells may imply a limited function under nitrogen stress of the CO_2-trapping apparatus. Nitrogen deficiency in the maturing cells of maize leaves resulted in a large reduction of photosynthesis and biomass production. As has been reported previously, the levels of PEPCase and PPDK in terms of both activity and protein are closely correlated with the rate of photosynthesis (22, 23) and the amount of leaf biomass that is produced (20, 21). This correlation is also borne out by the present results for the basal region of the leaf. Pulse and pulse-chase experiments with $[^{14}C]CO_2$ showed that ambient CO_2 could not be directly fixed by Rubisco in BS cells even given a reduced level of CO_2-trapping enzymes in M cells.

Feeding experiments with [^{35}S]Met revealed the patterns of labeling of proteins in maturing cells during recovery from nitrogen deficiency. In addition to PEPCase, the nitrogen-dependent change in levels of PPDK may also be regulated by a selective change in the rate of synthesis, although the range of variation in levels of PPDK is smaller than that of PEPCase. Another important characteristic of PPDK found in this labeling study is reflected in the substantially high turnover rate of the polypeptide compared to PEPCase and Rubisco. The labeled polypeptide of PPDK should be for the most part the completely processed, mature enzyme; since no precursor-sized immunochemically cross-reactive polypeptide, such as has been identified as a product of the *in vitro* translation products of its mRNA (1), was detectable in this study.

Nitrogen deficiency in maize leaves resulted in a reduced rate of synthesis most prominently of PEPCase. This result immediately suggests the possibility of a translational control mechanism exercised through the availability of nitrogen for protein synthesis. However, our present results, obtained at the level of maturing cells, indicate a major regulatory effect of nitrogen status in maize leaves at the steady-state level of RNAs hybridizable to their cDNAs, as had been confirmed in our previous study by measuring translatable mRNAs at the whole-plant level (21). Consequently, the regulatory event by nitrogen is likely to occur in response to changes in the rate of (a) transcription of the genes, (b) processing of the mRNA precursors, (c) degradation of mRNAs, or (d) any combination of these processes. To distinguish between these possibilities we are undertaking *in vitro* transcription with isolated nuclei from stressed and unstressed plants. As for PPDK, the mechanism could be more complex because of its lower stability.

The expression of PEPCase and PPDK in maize leaf is also regulated by light, which causes an increase in the steady-state level of their mRNAs (4, 6, 15, 17, 18), requiring nitrogen as an essential factor (25). Interestingly, this indicates that these two environmental factors, light and nitrogen, interact in the regulation of gene expression for these two enzymes. Understanding of the way in which the factors interact might reveal basic mechanisms underlying the metabolic interaction of carbon and nitrogen in plants.

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