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Effects of Nitrate and Ammonium on Gene Expression of Phospho*enol*pyruvate Carboxylase and Nitrogen Metabolism in Maize Leaf Tissue during Recovery from Nitrogen Stress[1]

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ABSTRACT

We previously showed that the selective accumulation of phospho*enol*pyruvate carboxylase (PEPC) in photosynthetically maturing maize (*Zea mays* L.) leaf cells induced by nitrate supply to nitrogen-starved plants was primarily a consequence of the level of its mRNA (B Sugiharto, K Miyata, H Nakamoto, H Sasakawa, T Sugiyama [1990] Plant Physiol 92: 963–969). To determine the specificity of inorganic nitrogen sources for the regulation of PEPC gene expression, nitrate (16 millimolar) or ammonium (6 millimolar) was supplied to plants grown previously in low nitrate (0.8 millimolar), and changes in the level of PEPC and its mRNA were measured in the basal region of the youngest, fully developed leaves of plants during recovery from nitrogen stress. The exogenous supply of nitrogen selectively increased the levels of protein and mRNA for PEPC. This increase was more pronounced in plants supplemented with ammonium than with nitrate. The accumulation of PEPC during nitrogen recovery increased in parallel with the increase in the activity of glutamine synthetase and/or ferredoxin-dependent glutamate synthase. Among the major amino acids, glutamine was the most influenced during recovery, and its level increased in parallel with the steady-state level of PEPC mRNA for 7 hours after nitrogen supply. The administration of glutamine (12 millimolar) to nitrogen-starved plants increased the steady-state level of PEPC mRNA 7 hours after administration, whereas 12 millimolar glutamate decreased the level of PEPC mRNA. The results indicate that glutamine and/or its metabolite(s) can be a positive control on the nitrogen-dependent regulation of PEPC gene expression in maize leaf cells.

PEPC[2] is a major soluble leaf protein in maize (*Zea mays* L.) and the initial carboxylation enzyme in the C_4 pathway of photosynthesis. The enzyme is believed to play a variety of physiological roles in plants including: (a) the anaplerotic fixation of CO_2 for the synthesis of dicarboxylic acids used as respiratory substrates (8, 15, 19), (b) provision of carbon skeletons for ammonium assimilation (3, 15), and (c) synthesis of organic acids to maintain charge balance (7, 19). It is therefore not surprising that the enzyme plays a central metabolic role in carbon and nitrogen interaction in plants.

Nitrogen is an important regulator of not only carbon flow but also the gene expression of some proteins in higher plants and algae through mechanisms affecting transcription and/or mRNA stability. These proteins include NR and nitrite reductase (1), Chl *a/b* light-harvesting complex apoproteins (13), vegetative storage proteins (16), and PEPC (17, 23). When maize plants are grown under conditions of nitrogen starvation, the levels of PEPC protein are selectively reduced most conspicuously in the photosynthetic maturing leaf cells primarily as a consequence of the level of PEPC mRNA (17). Despite the major effects of nitrogen on these proteins, information concerning nitrogen availability in the regulation of their gene expression is meager.

In the present study, we measured the total pool sizes of the potentially important cellular metabolites involved in nitrogen/carbon assimilations correlating with the nitrogen-dependent induction of PEPC mRNA in the photosynthetically maturing leaf cells of maize plants during recovery from nitrogen starvation. The objectives were to determine: (a) the specificity of nitrogen sources for the regulation of PEPC gene expression, and (b) possible metabolic signal(s) that control the gene expression in maize.

MATERIALS AND METHODS

Plant Growth

Maize (*Zea mays* L. cv Golden Cross Bantam T51) plants were hydroponically grown for about 2 weeks in low nitrogen (0.8 mM KNO_3) as described previously (17, 18) in a growth chamber in the Experimental System for Gene Analysis and Manipulation, 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% RH. The temperature regimen was 28°C (day)/20°C (night), and the photoperiod was 14 h. To induce transient changes in the concentration of nitrogen, the plants were supplied with either 16 mM KNO_3 or 6 mM NH_4Cl in the daytime (10 AM). A nitrification inhibitor (N-Serve) was included at 1 ppm in the culture solution. NH_4Cl was used as the nitrogen source in this solution, and nitrate was not detected. pH in the culture

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[2] Abbreviations: PEPC, phospho*enol*pyruvate carboxylase; NR, nitrate reductase; GS, glutamine synthetase; GOGAT, glutamate synthase; OG, 2-oxoglutarate; OPA, *o*-phtalalaldehyde.

medium was maintained at 6.5 to 7.0 with KOH during cultivation. The youngest, fully developed leaves, *i.e.* third leaves, were periodically harvested in the daytime under light conditions after nitrogen supply and transversely cut one-third of the way from the base. The basal segments were weighed and plunged into liquid N_2.

Measurement of Metabolite Levels

Ammonia and Nitrate

Frozen leaf material (3 g) was ground in a mortar with a mixture of methanol, chloroform, and water (12:5:3, v/v). The extract was quantitatively transferred into a tube and centrifuged. The pellet was rinsed with the mixture, and the supernatant fractions from five successive washes were combined and concentrated to dryness at 40°C. The residue was dissolved in a small amount of water, and after undissolved materials were removed by centrifugation, the supernatant fluid was stored at −20°C. Ammonia content was determined by a microdiffusion technique in Conway dishes (4), and nitrate content was determined by the method of Cataldo *et al.* (2). Ammonia showed 96% recovery and nitrate 76%.

Amino Acids and OG

Frozen leaf material (3 g) was pulverized in liquid N_2 and ground in a mortar with 10% (v/v) $HClO_4$. The mortar was rinsed three times with 10% $HClO_4$, and the extracts were combined and centrifuged. The combined supernatant fractions were adjusted to pH 6.5 with 5 M K_2CO_3. After the precipitated $KClO_4$ was removed by centrifugation, the supernatant fluid was decolorized by adding activated charcoal. Gln and Glu were immediately determined by HPLC as their OPA derivatives. The derivatization solution consisted of 13.5 mg OPA, 0.25 mL of methanol, 2.25 mL of 0.4 M borate buffer (pH 9.5), and 0.05 mL of 2-mercaptoethanol. A sample aliquot (20–25 μL) was reacted with 0.1 mL of the derivatization solution by mixing for 1 min using a Vortex mixer. A model Σ 871 IRICA liquid chromatograph was used to determine OPA-amino acids in 20 to 30 μL of the mixture. Separation was carried out on a reversed-phase column of Wakosil 5C18 with 40% (v/v) methanol: 60% 0.05 M sodium acetate buffer (pH 6.6). Eluates of OPA-Glu and -Gln were detected at A_{340}. OG was spectrophotometrically measured in a reaction mixture containing 50 mM Tris-HCl (pH 7.0), 2 mM aspartate, 0.16 mM NADH, 1.3 units glutamate-oxaloacetate aminotransferase, and 1.4 units malate dehydrogenase (9). Gln showed 105% recovery, Glu 104%, and OG 77%.

For the determination of free amino acids, 5 g of frozen leaf material was ground in hot 80% (v/v) ethanol. Amino acid content in the extract was determined by HPLC as described before (20) except for the minor modification that a stepwise elution gradient was used (methanol-tetrahydrofuran-0.05 M sodium acetate buffer [pH 6.6] [28:1:71, v/v]) as the starting solvent and 80% (v/v) methanol was used as the final solvent.

Extraction and Analysis of Enzymes

Frozen leaf material (5 g) was ground in a mortar with 0.5 g of insoluble PVP and 3 volumes of an extraction buffer containing 0.1 M Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, and 0.5 mM PMSF. The homogenate was centrifuged at 15,000*g* for 10 min, and the supernatant fluid was applied to a Sephadex G-25 column preequilibrated with the same buffer. NR and GS activities were assayed with the protein eluent (22). For Fd-GOGAT activity, extraction and gel filtration were conducted similarly using 0.1 M sodium phosphate buffer (pH 7.5), 1 mM EDTA, 1 mM PMSF, and 5 mM DTT. One unit of activity corresponds to 1 μmol of product formed per min at 25°C. PEPC protein was determined by single radial immunodiffusion (18).

Other Methods

PEPC mRNA was determined as described previously by dot blot (17) or Northern hybridizations. Protein content was determined according to the method of Lowry *et al.* (10).

RESULTS

Effects of Administration of Nitrate and Ammonium to Nitrogen-Starved Plants on the Accumulation of mRNA and Protein for PEPC

We previously reported that the selective accumulation of PEPC in the photosynthetically maturing cells of nitrogen-recovering maize leaf tissue was primarily a consequence of the level of its mRNA (17). To determine the specificity of an inorganic N source for the accumulation of PEPC, nitrate (16 mM) or ammonia (6 mM) was supplied to plants grown for about 2 weeks in low nitrate (0.8 mM), and the changes in levels of PEPC (Fig. 1A) and its mRNA (Fig. 1B) were measured in the basal region of the youngest, fully developed leaves of plants during recovery from nitrogen starvation. In nitrate-supplemented plants, the PEPC level increased during 72 h of recovery, with a concomitant increase in the level of its mRNA that was measured 24 h after the addition of nitrogen. This result is consistent with our previous observations (17, 23). In ammonium-supplemented plants, there was a more marked increase in the levels of both protein and mRNA for PEPC during recovery; the magnitude was approximately twofold greater than that in nitrate-supplemented plants. The accumulation of PEPC in both sets of plants increased selectively as judged by an increase in the fraction of PEPC in leaf soluble protein (Fig. 1A, inset). The concentrations of N sources used in this study were determined as saturating for the accumulation of PEPC during this recovery process (data not shown). Collectively, the results indicate that the expression of the PEPC gene can be induced by either nitrate or ammonium as the N source. Moreover, it can be concluded that the response to ammonium is faster than that to nitrate.

Levels of Metabolites and Nitrogen Assimilation Enzyme Activities during Nitrogen Recovery

To determine the relationship of nitrogen metabolism to the nitrogen-dependent gene expression of PEPC, we examined the levels of nitrate, ammonium, OG, Gln, and Glu in the photosynthetically maturing leaf cells of nitrogen-starved plants at intervals of 6, 24, 48, and 72 h after the addition of

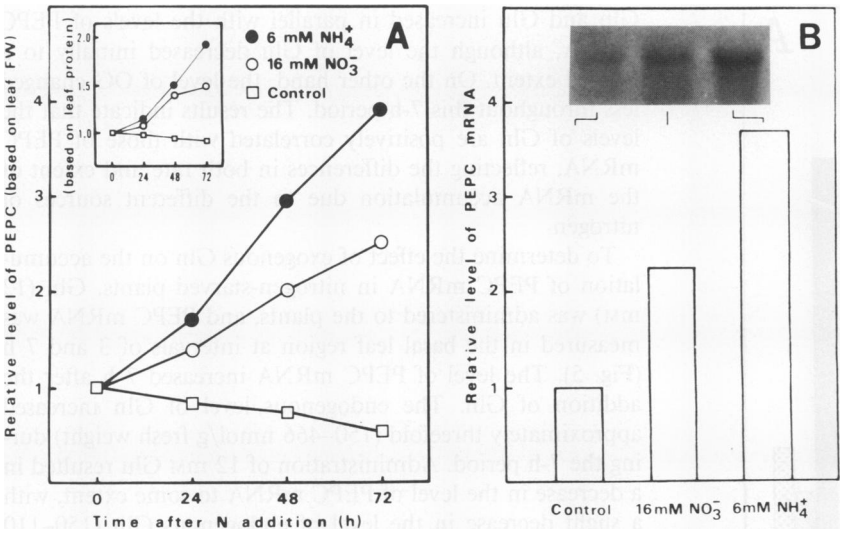

Figure 1. Comparison of nitrate and ammonium as the N source for the accumulation of PEPC protein (A) and its mRNA (B) in the basal region of maize leaves during recovery from nitrogen starvation. The results are expressed as relative values of the control at 0 h. In A, PEPC content was 0.18 mg of PEPC per g fresh weight (FW) at 0 h. In A, inset, relative values of PEPC protein content per unit weight of leaf soluble protein are shown. The value at 0 h was 0.13 mg per mg of leaf soluble protein. B, For the Northern hybridization with 24-h samples, 2 μg of total RNA was applied to each lane.

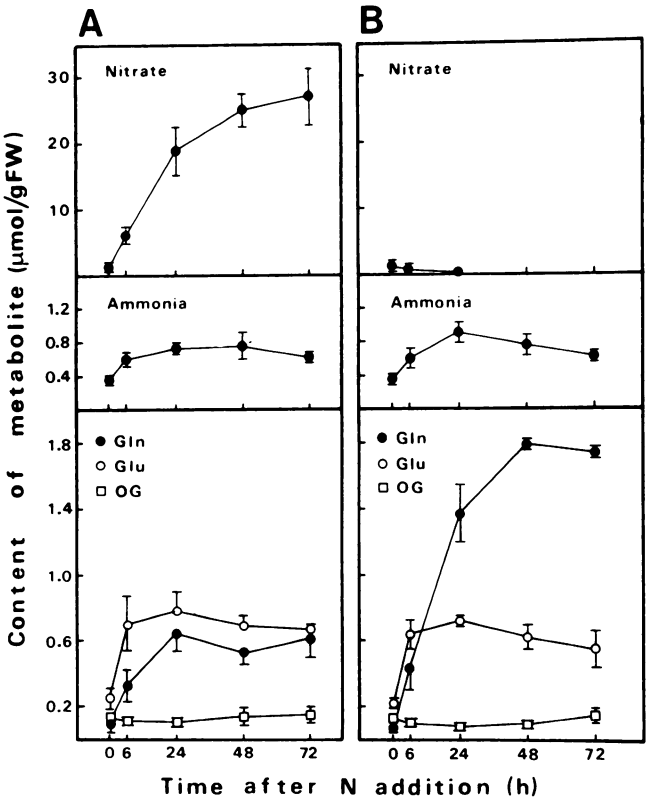

Figure 2. Changes in the levels of nitrate, ammonium, Gln, Glu, and OG in the basal region of nitrate-supplemented (A) and ammonium-supplemented (B) maize leaves during recovery from nitrogen starvation. Vertical bars, SE of data from two independent experiments.

nitrate (Fig. 2A) or ammonium (Fig. 2B). The most prominent differences in the levels of these metabolites between nitrate- and ammonium-supplemented plants were observed for nitrate and Gln. In nitrate-supplemented plants, ammonium, Glu, and Gln reached their saturation levels 24 h after nitrate addition, whereas nitrate continued to accumulate throughout the 72-h period. In ammonium-supplemented plants, Gln accumulated markedly after ammonium addition, reaching a level more than twofold higher than that of nitrate-supplemented plants, whereas ammonium and Glu increased in a similar manner to nitrate-supplemented plants. Preexisting nitrate actually disappeared completely in the early stage of recovery. The levels of OG changed much less, regardless of the nitrogen source. The results indicate that, among the metabolites examined, the level of Gln correlates most closely with the levels of PEPC accumulated during recovery from nitrogen starvation (*cf.* Fig. 1A).

The levels of NR, GS, and Fd-GOGAT activity were monitored during the 72-h recovery process (Fig. 3). In nitrate-supplemented plants (Fig. 3A), a marked increase was observed for NR, as expected. Fd-GOGAT also increased significantly, whereas GS changed much less (Fig. 3A). The increase in NR activity was presumably due to marked nitrate accumulation in leaf cells. It is noteworthy that NR increased before the increase in Fd-GOGAT and in parallel to the increases in ammonium, Glu, and Gln (*cf.* Fig. 2A). In ammonium-supplemented plants (Fig. 3B), the levels of GS and Fd-GOGAT increased in parallel. Despite the disappearance of detectable nitrate in the leaf tissue, there was also a slight increase in NR, although its activity was very low (*cf.* Figs. 2B and 3B). This low activity of NR is consistent with the data reported for hydroponically grown maize plants when NH_4Cl was the only added form of nitrogen (12). Collectively, the results indicate that the accumulation of PEPC during nitrogen recovery is most closely correlated with the increase in the activity of Fd-GOGAT and/or GS.

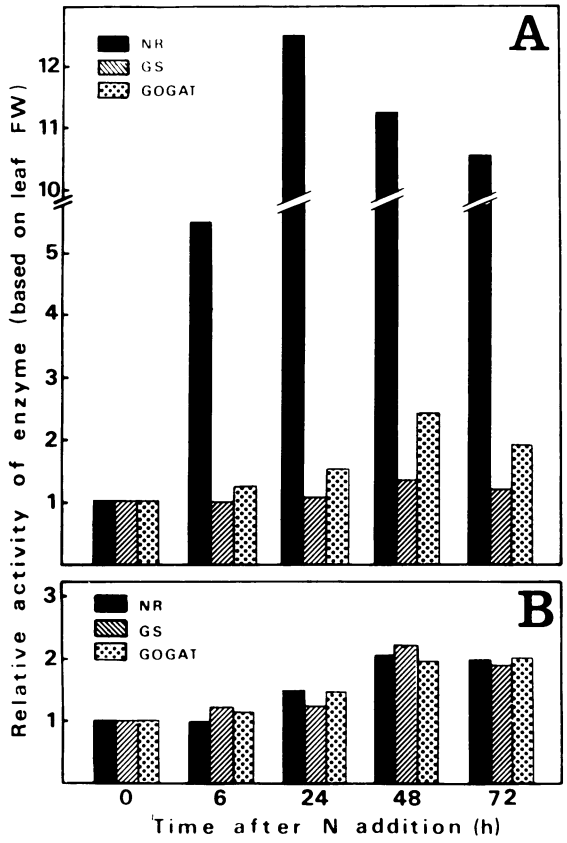

Figure 3. Changes in the activities of nitrogen-assimilating enzymes in the basal region of nitrate-supplemented (A) and ammonium-supplemented (B) maize leaves during recovery from nitrogen starvation. Same shaded patterns for A and B. The value at zero time in each series of treatments was taken as one relative unit. The means in activity of NR, GS, and Fd-GOGAT at zero time for A and B were 0.015, 6.6, and 0.07 units per g fresh weight (FW), respectively.

Short-Term Changes in the Levels of Metabolites and PEPC mRNA and Effect of Gln Administration on the Accumulation of PEPC mRNA during Nitrogen Recovery

We first determined the content of the major amino acids in the basal region of maize leaves during nitrogen recovery (Table I). The sum of these amino acids increased 3 and 7 h after nitrogen administration, regardless of the source of inorganic nitrogen. The most remarkable and selective increase was found for Gln, and this was much more intense in ammonium-supplemented plants than in nitrate-supplemented plants. Glu also showed a notable increase, but there was no difference between the two groups of plants. Other amino acids, such as Ser and Gly, increased to some extent. We then measured the levels of Gln, Glu, and OG, *e.g.* substrates for GS and GOGAT, and compared them with the levels of PEPC mRNA (Fig. 4). The steady-state levels of PEPC mRNA, which were induced in a nitrogen-dependent manner, began to accumulate after 3 h of nitrogen administration, regardless of the N source, but increased thereafter more intensely in ammonium-supplemented plants (Fig. 4B) than in nitrate-supplemented plants (Fig. 4A). The levels of Gln and Glu increased in parallel with the levels of PEPC mRNA, although the level of Glu decreased initially to a limited extent. On the other hand, the level of OG changed less throughout this 7-h period. The results indicate that the levels of Gln are positively correlated with those of PEPC mRNA, reflecting the differences in both rate and extent of the mRNA accumulation due to the different sources of nitrogen.

To determine the effect of exogenous Gln on the accumulation of PEPC mRNA in nitrogen-starved plants, Gln (12 mM) was administered to the plants, and PEPC mRNA was measured in the basal leaf region at intervals of 3 and 7 h (Fig. 5). The level of PEPC mRNA increased 7 h after the addition of Gln. The endogenous level of Gln increased approximately threefold (150–466 nmol/g fresh weight) during the 7-h period. Administration of 12 mM Glu resulted in a decrease in the level of PEPC mRNA to some extent, with a slight decrease in the level of endogenous Gln (150–110 nmol/g fresh weight). Thus, the results suggest that Gln and/or its metabolite(s) may exert a positive control on PEPC expression, which may be involved in the nitrogen-dependent regulation of its mRNA level.

DISCUSSION

The results obtained in the present study show that the expression of the gene for PEPC in photosynthetically maturing leaf cells of nitrogen-starved maize plants can be regulated by either nitrate or ammonium salt as a nitrogen source. The

Table I. *Changes in the Contents of Major Amino Acids in the Basal Region of Maize Leaves during Recovery from Nitrogen Starvation*

The contents of amino acids are expressed in percentages of total major amino acids. All values are the means of duplicate analyses of each sample. ND, Not detected.

Amino Acid	N Starved	Time after Nitrate Supply (h)		Time after Ammonium Supply (h)	
		3	7	3	7
		% of total			
Asp	11.9	16.3	12.9	12.8	9.92
Glu	26.2	27.7	33.2	33.9	28.9
Asn	1.98	0.92	1.26	0.89	1.30
Ser	6.75	7.48	9.16	7.61	8.04
Gln	1.89	4.02	5.22	5.64	11.1
Arg	3.64	3.39	1.53	1.64	1.92
Gly	4.81	4.24	5.72	4.23	7.75
Thr	3.88	2.38	1.35	2.42	1.74
Ala	26.1	25.6	25.2	23.6	23.2
Val	2.83	1.51	1.25	1.65	1.64
Phe	2.36	1.54	1.09	1.19	1.21
Ile	2.88	2.15	0.79	1.47	1.17
Leu	3.28	2.02	0.91	2.29	1.48
Lys	1.59	0.72	0.47	0.65	0.70
His	ND	ND	ND	ND	ND
Met	ND	ND	ND	ND	ND
Total (μmol/g fresh wt.)[a]	2.12	2.98	5.47	3.36	5.42

[a] Equated to 100%.

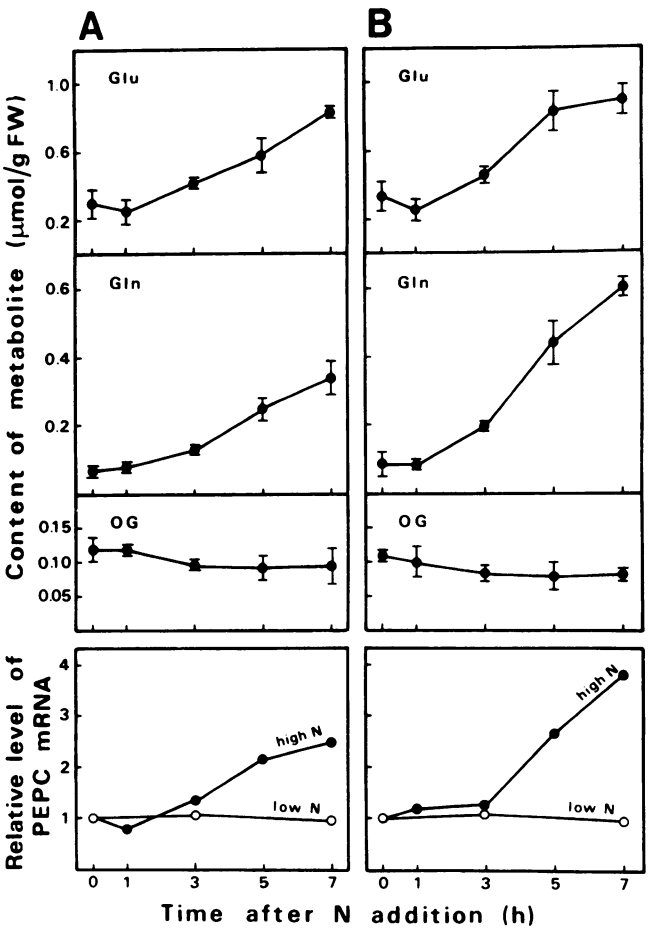

Figure 4. Short-term changes in the levels of Glu, Gln, OG, and mRNA for PEPC in the basal region of nitrate-supplemented (A) and ammonium-supplemented (B) maize leaves during recovery from nitrogen starvation. Vertical bars, SE of data from two independent experiments. mRNA was determined by dot blot hybridization using a fixed amount of total RNA (1–2 μg).

regulation of this nuclear-encoded protein by nitrogen has been suggested to be achieved through mechanisms affecting transcription and/or mRNA stability (17). Recently, we obtained evidence, by conducting *in vitro* transcription assays with isolated nuclei, to indicate that this regulation is most likely a result of control of gene expression at the level of transcription of the PEPC gene and is highly specific for the C_4 form (I. Suzuki, C. Crétin, T. Sugiyama, manuscript in preparation). Lack of the specificity of nitrate and ammonium as the N source for the accumulation of PEPC mRNA excludes the possibility that these inorganic compounds exert positive control as a primary signal for PEPC gene expression. This view is further supported by the evidence that the accumulation of nitrate was insignificant in the basal region of ammonium-supplemented leaf tissue. Ammonium salt was more effective as an inducer of PEPC accumulation, producing a twofold higher rate and extent than nitrate. However, no major difference in accumulation of ammonium was observed between nitrate-supplemented and ammonium-supplemented plants. Consequently, it is unlikely that ammonium, a product of nitrate reduction, is the primary signal for this control. The relatively low accumulation of ammonium in the leaf cells of ammonium-supplemented plants is consistent with the fact that ammonium assimilation in higher plants takes place mainly in the roots (14).

If a metabolite(s) is involved as a signal to modulate the positive control of nitrogen-dependent PEPC gene expression, a likely candidate should be sought among the downstream product(s) of ammonium assimilation. Nitrate in higher plants is reduced to nitrite by NR and then to ammonium by nitrite reductase. Ammonium either produced in this way, taken up from the external medium, or liberated as a result of photorespiration is incorporated into amino acids through the concerted action of GS and Fd-GOGAT. In maize, a C_4 plant, ammonium liberated through photorespiration may be substantially low because of reduced photorespiration. In the present study, we observed an accumulation of Gln, an end product of ammonium assimilation, in parallel with the accumulation of PEPC mRNA in leaf cells during short-term nitrogen recovery. The accumulation of Gln may be a result of the direct assimilation of ammonium via the GS/Fd-GOGAT cycle. On the other hand, in the long-term recovery experiments, there was a difference in the patterns of these enzymes depending upon the nitrogen source. Ammonium supplementation resulted in parallel increases in Fd-GOGAT and GS activity, whereas nitrate supplementation resulted in

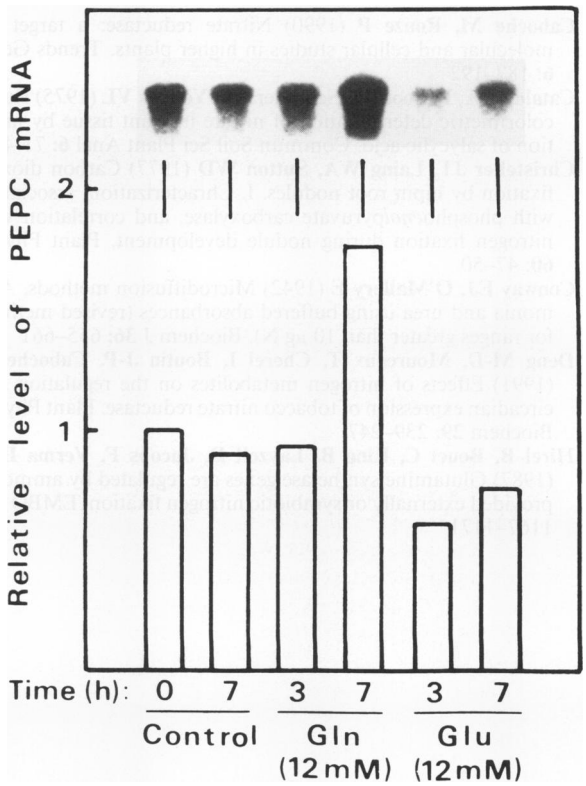

Figure 5. Effects of administration of 12 mM Gln or Glu on the accumulation of mRNA for PEPC in the basal region of nitrogen-starved maize leaves. For the Northern hybridization, 2 μg of total RNA was applied to each lane.

a relative increase in Fd-GOGAT *versus* GS. The increase in GS induced by ammonium is consistent with the data previously reported for both nodules and roots of soybean (6). The parallel increase in Fd-GOGAT and GS activity in ammonium-supplemented plants is consistent with the concept that the GS/Fd-GOGAT cycle can minimize excess levels of endogenous ammonium, as has been proposed (11, 21), and may be the biochemical basis for the accumulation of Gln in the basal region during the recovery of maize leaves with ammonium. However, the possibility that the Gln that accumulated in this region is exported from other cells or leaves as a form of mobile nitrogen cannot be ruled out.

The nature of nitrogen availability, which mediates the response of gene expression in plant cells, is intriguing. In this context, Caboche's group recently reported that Gln and/or other nitrogen metabolites may exert negative control on NR gene expression involved in the diurnal and circadian regulation of NR mRNA in tobacco leaves (5). It is interesting to speculate that Gln may be a common signal that reflects the nitrogen availability in plant cells for the gene expression of PEPC, a nitrogen consumer, and NR, a producer of nitrogen availability, although the effect of this metabolite in the regulation of these nuclear genes is exactly opposite. There is no evidence to show whether or not nitrogen availability is determined by a single metabolite. We are analyzing the expression of PEPC mRNA under inhibitory conditions of GS activity using its specific chemical inhibitor.

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