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Glutamine Induces the N-Dependent Accumulation of mRNAs Encoding Phospho*enol*pyruvate Carboxylase and Carbonic Anhydrase in Detached Maize Leaf Tissue[1]

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ABSTRACT

We have used detached leaves to study the N-dependent control of expression of phospho*enol*pyruvate carboxylase (PEPC) and carbonic anhydrase (CA) genes in maize (*Zea mays* L. cv Golden Cross Bantam T51). Following supplementation with an N-source and zeatin, PEPC and CA mRNA levels increased in leaves detached from N-deficient maize plants. Addition of methionine sulfoximine (MSX), a specific inhibitor of glutamine synthetase, inhibited the nitrate-dependent increase of PEPC and CA mRNA but did not affect the glutamine-dependent increase of PEPC and CA mRNA levels. Glutamine levels in detached maize leaves treated with various N sources in the presence or absence of MSX correlated with the levels of PEPC and CA mRNA. We conclude that glutamine is the most likely effector for controlling the N-dependent expression of PEPC and CA in maize plants.

Maize leaves selectively accumulate PEPC[2] in response to N availability, presumably at the transcriptional level (14, 15). The expression of CA has also been reported to be regulated by N availability in maize leaf tissue (3). Based on the correlation analysis of the levels of mRNA and intermediates of N assimilation, Gln and/or its metabolite(s) have been implicated in the control of expression of the PEPC gene (15). However, no conclusions can be drawn from this study regarding the cause and effect of the parameters involved.

It has been reported that Gln inhibits the accumulation of NR mRNA in tobacco leaf (4) and squash cotyledons (7). These studies unequivocally demonstrated the importance of Gln in controlling gene expression in higher plants, although its role is the opposite to its effect on the expression of PEPC in maize leaves.

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[2] Abbreviations: PEPC, phospho*enol*pyruvate carboxylase; CA, carbonic anhydrase; NR, nitrate reductase; GS, glutamine synthetase; MSX, methionine sulfoximine.

It is well documented that ammonia assimilation occurs in higher plants through the combined operation of GS and glutamate synthase and that MSX inhibits the production of Gln via GS (for review, see refs. 8 and 9). In maize, this cycle is known to operate in both roots and shoots (11, 16). To simplify the interpretation of experiments designed to determine the role of Gln in the regulation of PEPC and CA gene regulation, we developed a detached leaf system that allows administration of N-containing or related compounds directly to the leaf tissue (13).

The aim of the present work is to study the effect of MSX on the N-dependent accumulation of mRNAs encoding for PEPC and CA, key enzymes of the C_4 pathway of photosynthesis using the detached leaf system of maize (*Zea mays* L.) plants. The results indicate that Gln and/or its downstream metabolite(s) is a positive signal of N availability for the induction of these photosynthetic genes.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Application of Compounds

Maize (*Zea mays* L. cv Golden Cross Bantam T51) plants were grown hydroponically for approximately 2 weeks in low nitrate (0.8 mM KNO_3) in a growth chamber and the youngest, fully developed leaves (third leaves) were used as detached leaves (13). Fifteen of the leaves were placed vertically in a beaker containing 50 mL of 0.5 strength Hoagland and Arnon solution (pH 6.5) containing 5 μM *trans*-zeatin in the presence or absence of various compounds. Incubation of detached leaves was conducted at 28°C under the light, and leaves were harvested and plunged into liquid N_2 as described previously (13).

Extraction and Assay of GS

Leaf proteins were extracted from 3 g of frozen leaf material and the transferase activity of GS was measured as described previously (15).

Measurement of Ammonia, Nitrate, and Amino Acid Content of Maize Leaves

Frozen leaf material (3 g) was ground in a mortar with hot 80% (v/v) ethanol. After centrifugation, the pellet was rinsed

with 80% (v/v) ethanol and the supernatant fractions from successive washes were collected until all green color disappeared. The supernatants were combined and evaporated to dryness at 40°C. The residue was dissolved in a small amount of water and insoluble materials were removed by centrifugation. Ammonia, nitrate, and amino acids were determined in the supernatant as described previously (15).

Measurement of mRNAs

Extraction of RNA and determination of PEPC and CA mRNAs by dot-blot analysis were conducted as described previously using their specific probes (13, 15). α-Tubulin mRNA was determined in the same way using the *Eco*RI and *Hin*dIII DNA fragment of *Arabidopsis thaliana* α3 tubulin gene as a probe, which was kindly provided by Dr. D.P. Snustad (5). GS1 mRNA was measured as described previously (11). PEPC and GS1 mRNAs gave rise to a single band on northern analysis, whereas CA mRNA gave rise to two bands of approximately equal intensity (see refs. 11, 13, 15). Relative accumulation of mRNA was determined after densitometer scans of the resulting autoradiographs.

RESULTS

Effect of Administration of Amino Acids on the Expression of PEPC and CA Genes

When detached leaves of plants grown at low nitrate are incubated with high nitrate and cytokinin, the levels of mRNA encoding PEPC and CA increase relative to total RNA (13). To identify the organic N source effective for the induction of PEPC and CA genes in detached maize leaves, we selected Gln and Glu, which are effective and less effective N source, respectively, for the induction of the PEPC gene in an attached leaf, and Ala, an abundant amino acid in maize leaves (15), and examined their effects on the level of expression of PEPC and CA genes in detached leaves. Amino acids at a concentration of 20 mM in the presence of 5 μM zeatin were administered to detached leaves of N-starved plants and the steady-state levels of mRNAs for PEPC, CA, GS1 (a cytosolic form of GS), and α-tubulin were measured by dot-blot hybridization in the leaves 3 h after administration (Fig. 1). Gln increased the levels of PEPC and CA mRNAs to an extent comparable with nitrate. Glu and Ala were less effective but significantly increased the levels of both mRNAs compared with the control. The effects of these amino acids may be due to an increase in endogenous levels of Gln formed, presumably through their transamination by Glu transaminase and/or Ala-Glu transaminase and GS/glutamate synthase cycle. By contrast, the level of GS1 mRNA remained unchanged regardless of the N source and the level of α-tubulin mRNA did not change by administration of either nitrate, Gln, or Glu. The results suggest that Gln is a primary signal for the induction of N-dependent expression of genes for not only PEPC but also for CA in maize leaves. Furthermore, the results indicate that this induction is gene specific.

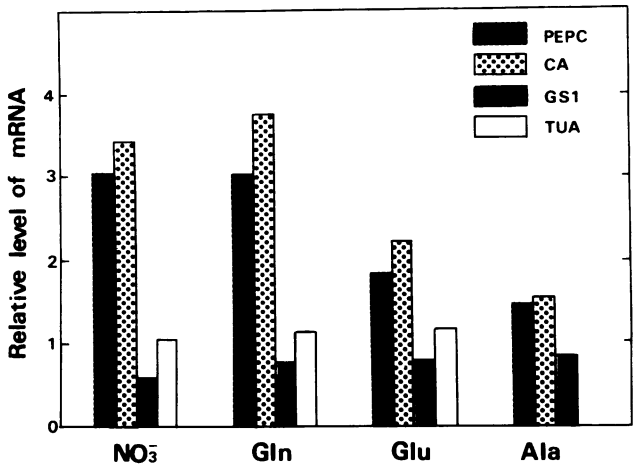

Figure 1. Effects of administration of Gln, Glu, or Ala on the accumulation of mRNAs for PEPC, CA, GS1, and α-tubulin in the detached leaves of N-starved maize plants. The levels of PEPC, CA, GS1, and α-tubulin mRNA were measured by dot-blot hybridization with total RNA 1 to 2, 2 to 4, 5 to 10, and 10 to 20 μg, respectively. The results are expressed as relative values of the control at 0 h. TUA represents α-tubulin.

Effect of MSX on the Expression of PEPC, CA, and GS1 Genes

To confirm the above result, we examined the expression of genes for PEPC and CA, with GS1 as a control in the presence of MSX, a specific inhibitor of GS activity. Initial experiments were conducted to determine the concentration of MSX and incubation time required to inactivate the activity of GS. GS activity was inhibited by 88% 30 min after incubation with 2 mM MSX and inhibited completely after 45 min (Fig. 2). Thus, detached leaves were preincubated for 30 min with 2 mM MSX, then with various N sources for 150 min, and PEPC, CA, and GS1 mRNAs were measured (Table I). The concentrations of nitrate and amino acids used in this study were 16 and 20 mM, respectively, as these concentrations were determined as saturating for the accumulation of PEPC and CA mRNAs under the inhibitory conditions (data not shown). There were two CA mRNA bands on northern blots as reported previously (13). The sum of these two bands are presented as relative values in the Table I because the levels of these mRNAs changed in an identical manner regardless of the treatment. In the leaves pretreated with a minus-MSX solution, administration of nitrate increased the levels of mRNAs for PEPC and CA by 4.2- and 5.5-fold, respectively.

In contrast, in the leaves pretreated with MSX the levels of both mRNAs decreased dramatically in spite of the presence of nitrate. However, administration of Gln to the MSX-pretreated leaves resulted in an increase in the levels of both mRNAs to an extent comparable with that of the minus-MSX control. Administration of either Glu, Gly, or Ser, which are considered to be N compounds on the downstream side of Gln synthesis, did not restore the decreased levels of either PEPC or CA mRNAs. In respect to GS1, the levels of mRNA remained unchanged regardless of the treatment.

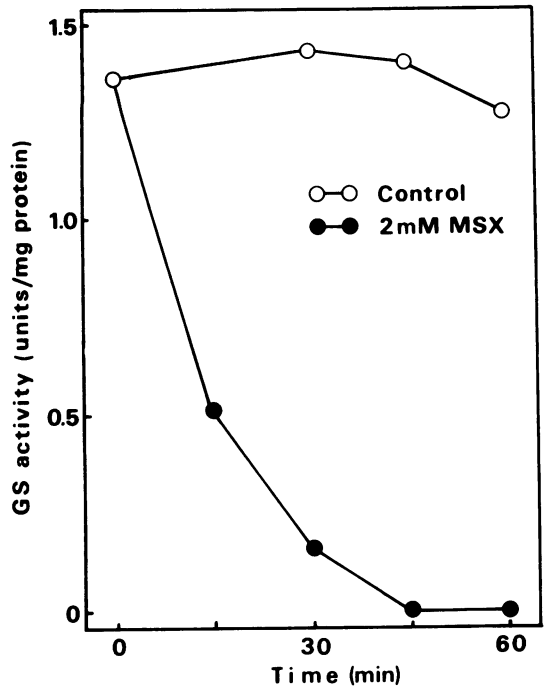

Figure 2. Response of GS activity to MSX treatment in detached leaves of N-starved maize plants. The detached leaves were incubated for 60 min in the medium with or without 2 mM MSX.

Changes in endogenous levels of nitrate, ammonia, Gln, Glu, Ser, and Gly, which increase prominently during recovery from N starvation (cf. table I in ref. 15) were consistent with data obtained from the inhibition experiment (Fig. 3). In the leaves treated with MSX, there was an increase in the level of ammonia between 5.5- and 8.7-fold higher than that in the control and a decrease in the level of Gln to an undetectable level, except where Gln was administered. This change in the levels of ammonia and Gln is a result of the inactivation of GS activity (cf. Fig. 2, see also refs. 2 and 6). Most strikingly, of the N-containing compounds examined, Gln was the only compound in which levels changed with a positive correlation with the changes in levels of PEPC and CA mRNAs.

Collectively, the results imply that Gln and/or its metabolite(s) may be a positive signal for the induction of N-dependent gene expression of PEPC and CA in maize leaves. Furthermore, the failure of Glu, Gly, and Ser to act as an N source for the induction in MSX-pretreated leaves indicates that if there is a positive signal other than Gln, it will probably be a metabolite(s) in the pathway(s) other than in protein synthesis.

DISCUSSION

We have attempted to identify the primary metabolite involved in the induction of the N-dependent accumulation of PEPC and CA mRNAs using detached maize leaves. Based upon correlation analysis of intermediates of N assimilation and gene expression in an attached leaf system, the results of our previous paper indicate that Gln and/or its metabolite(s) may be a primary positive signal for the N-induced accumulation of PEPC mRNA in maize leaves (15). One may argue that correlation of the two parameters cannot refer to their cause and effect relation. However, the results presented in this paper overcome this criticism by showing that inhibition of GS, a Gln-forming enzyme, blocks the expression of PEPC gene in maize leaves. In addition, we have demonstrated that the N-dependent expression of the CA gene is also blocked when GS is inhibited.

Glu, Gly, and Ser, which are considered to be assimilants of nitrate on the downstream side of Gln metabolism, are metabolized in maize leaves through the operation of the integrated photosynthetic carbon oxidation/photorespiratory N cycles and/or transamination reactions (1). With regard to the possible downstream metabolite(s) of Gln acting as the positive signal for induction of PEPC and CA genes, our present data eliminate the possibility of an intermediate in protein synthesis because Glu, Gly, and Ser failed to effectively induce the accumulation of PEPC and CA mRNAs in MSX-treated leaves. This inference is further supported by the finding that Ala, a product of transamination of Glu or other α-amino acids, was less effective as a N source for the induction of these genes than Gln in detached leaves (see Fig. 1).

Gln is also required for the biosynthesis of purine and pyrimidine nucleotides, precursors of RNA (for review see

Table I. *Effects of Administration of N-Compounds on the Levels of mRNAs for PEPC, CA, and GS1 in MSX-Pretreated Detached Leaves of N-Starved Maize Plants*

The levels of PEPC and CA were measured by nothern hybridization (13). The level of GS1 mRNA was measured in the same way, except that the GS1 probe (11) was hybridized with the membrane after removal of the CA probe. The results are expressed as relative values of the controls at 30-min preincubation.

Treatment	75-min Incubation			150-min Incubation		
	PEPC	CA	GS1	PEPC	CA	GS1
−MSX, + nitrate	1.79	1.61	1.37	4.20	5.50	1.20
+MSX, nitrate	0.69	0.95	1.15	0.12	0.50	0.99
+ MSX, + Gln	2.00	1.89	0.83	2.96	4.73	0.90
+ MSX, + Glu	0.39	0.81	0.95	0.15	0.40	1.49
+ MSX, + Gly				0.20	0.23	1.08
+ MSX, + Ser				0.21	0.20	1.20

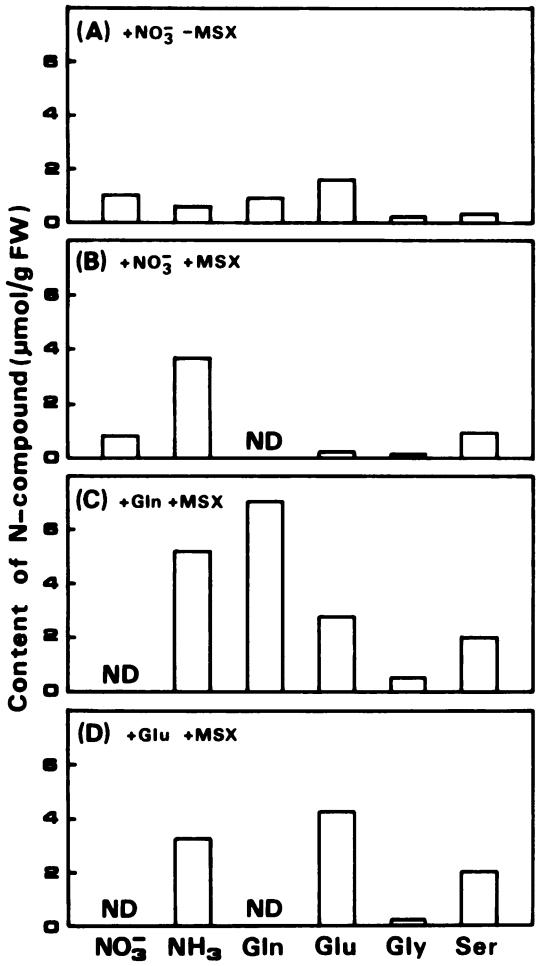

Figure 3. Effects of administration of nitrate, Gln, or Glu on the levels of N compounds in the MSX-treated detached leaves of N-starved maize plants. A, Nitrate-administered leaves without treatment with MSX as a control; B, nitrate-administered leaves after treatment with MSX; C, Gln-administered leaves after treatment with MSX; D, Glu-administered leaves after treatment with MSX. ND, Not detectable.

ref. 17). Gln amidases, which are involved in the biosynthetic pathways, have been shown to be inhibited by azaserine, an analog of Gln (10). In this context, it is noteworthy that administration of 20 mM each of Gln plus Glu failed to induce the accumulation of both mRNAs when detached leaves were pretreated with 2 mM azaserine (B. Sugiharto and T. Sugiyama, unpublished observation). However, it is unlikely that a downstream metabolite(s) in the biosynthetic pathway of nucleotides after the reactions mediated by Gln amidases might act as a positive signal for the N-induced accumulation of PEPC and CA mRNAs in maize leaf. If this were the case, expression of all genes would be blocked because of the lack of nucleotides needed for transcription. Although there is still some doubt about the identity of the effector of N-induction of the PEPC and CA genes, all the evidence so far obtained indicates that Gln is probably the effector for controlling the N-dependent expression of PEPC and CA in maize leaf.

Gln and/or its metabolite(s) has been suggested as a negative signal for NR gene expression in tobacco by Deng et al. (4), who demonstrated that phosphinothricin, an inhibitor of GS, caused a drop in Gln level and abolished the daytime decrease of NR mRNA levels. A similar effect of Gln on the accumulation of NR mRNA has been reported in squash cotyledons (7). In maize leaves, NR levels increase preceding an increase in the accumulation of PEPC when nitrate is supplied to N-starved plants (15). These results predict that the level of NR mRNA would accumulate earlier than a rise in Gln levels, which, in turn, would cause an inhibition of NR gene expression in detached maize leaves after supplying nitrate to N-starved plants.

GS1 mRNA remained unchanged regardless of the treatment. It is relevant to note our observation that the ratios of GS1 protein to leaf protein either in the mesophyll or bundle sheath cells in maize leaves did not respond to N availability (H. Sakakibara and T. Sugiyama, unpublished data). Our work clearly defines a gene specificity in the selective regulation of gene expression by N availability; however, the physiological meaning remains to be answered. In addition to PEPC and CA, the expression of some other C_4 enzyme genes have been known to be regulated by N availability. These include pyruvate Pi dikinase in maize (14) and Ala aminotransferase in *Panicum miliaceum*, an NAD-malic enzyme type C_4 plant (12). We hope that further studies addressing the mode of N-dependent gene regulation of other C_4 enzymes might help to clarify a molecular basis for the high N use efficiency of C_4 plants.

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