Plant Physiol. (1992) 100, 153–156
0032-0889/92/100/0153/04/$01.00/0

Received for publication December 26, 1991
Accepted April 5, 1992

Cytokinin Is Required to Induce the Nitrogen-Dependent Accumulation of mRNAs for Phospho*enol*pyruvate Carboxylase and Carbonic Anhydrase in Detached Maize Leaves[1]

Bambang Sugiharto[2], James N. Burnell, and Tatsuo Sugiyama*

Department of Agricultural Chemistry, School of Agriculture, Nagoya University, Nagoya 464–01, Japan (B.S., T.S.); and Centre for Molecular Biotechnology, Queensland University of Technology, PO Box 2434, Brisbane QLD 4001, Australia (J.N.B.)

ABSTRACT

Previous studies with intact maize (*Zea mays* L.) plants indicated that phospho*enol*pyruvate carboxylase (PEPC) levels are controlled by nitrogen (N) availability and that this regulation is presumably at the transcriptional level (B. Sugiharto, K. Miyata, H. Nakamoto, H. Sasakawa, T. Sugiyama [1990] Plant Physiol 92: 963–969; B. Sugiharto, T. Sugiyama [1992] Plant Physiol 98: 1403–1408). In the present study, detached maize leaves were used to investigate further the mechanism of N-dependent regulation of gene expression in C_4 plants. PEPC and carbonic anhydrase (CA) mRNA levels decreased in leaves detached from maize plants. Addition of high nitrate did not prevent this decrease. However, the addition of zeatin to solutions bathing the cut ends of the detached leaves inhibited the decrease of PEPC and CA mRNA levels. Simultaneous addition of high nitrate and zeatin to leaves detached from N-deficient maize plants caused a large and rapid increase in PEPC and CA mRNA levels. Zeatin could be replaced by benzyladenine, but not by indoleacetic acid or abscisic acid. Both CA isozymes were effected and responded in an identical manner. We conclude that detached maize leaves provide an excellent experimental system to study the mechanism(s) of N-mediated regulation of PEPC and CA gene expression. However, zeatin is an essential component of this system.

PEPC[3] catalyzes the carboxylation of phospho*enol*pyruvate to oxaloacetate in the cytosol of mesophyll cells of C_4 plants. The inorganic carbon substrate of this enzyme, HCO_3^-, has recently been recognized as being supplied by CA, which catalyzes the reversible hydration of CO_2 (1, 7). There are recent reports concerning the regulation of expression of C_4 PEPC by environmental factors such as light and nitrogen. With regard to the regulation by nitrogen, the expression of the PEPC gene is influenced by N availability in a maize *Zea mays* L. leaf, presumably at the level of transcription and/or the stability of mRNA (11, 12). In addition, the mechanism controlling the expression of maize leaf PEPC and CA activity, key enzymes in the C_4 pathway, are closely related (2). These studies prompted us to compare the regulation of gene expression of CA and PEPC by N in a maize leaf tissue.

Regulation of gene expression in higher plants by N must require a complex network of intercellular and intracellular communication because N is mobile. To identify a possible messenger(s) in the N-dependent gene expression of maize C_4v enzymes, which mediates the communication between leaf and root tissues, we have chosen detached maize leaves as the experimental material. Two important questions arise in this respect: (a) How is the appearance of N-dependent gene expression in detached leaves regulated; and (b) What messenger(s) is used to mediate the communication between roots and leaves? In this study, we have demonstrated that exogenous cytokinin is necessary for the N-dependent accumulation of PEPC and CA mRNAs in detached maize leaf tissue.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Application of Hormones

Maize (*Zea mays* L. cv Golden Cross Bantam T51) plants were grown for about 2 weeks at low (0.8 mM) or high (16 mM) nitrate as described previously (11, 12). The youngest, fully developed leaves, i.e. the third leaves, were cut at a position 2 to 3 cm below the laminar joint under water with a razor blade during day time (8:00–9:00 AM). The basal ends of 15 randomly selected leaves were placed vertically in a 300-mL beaker containing 50 mL of 0.5 strength Hoagland and Arnon solution (pH 6.5) with low (0.8 mM) or high (16 mM) nitrate in the presence or absence of phytohormones or various compounds. Stock solutions (1 mM) of *trans*-zeatin and BA, and IAA and ABA were prepared by dissolving in 20 mM HCl and 10% (v/v) ethanol, respectively. Incubation of detached leaves was conducted at 28°C under white light

[1] This work was supported in part by grants-in-aid for Scientific Research on Priority Areas (No. 04273103) from the Ministry of Education, Science and Culture of Japan to T.S., by a Fellowship from the Japanese Society for the Promotion of Science to J.N.B., and by a Japanese Government Scholarship (The Ministry of Education, Science and Culture of Japan) to B.S.

[2] Present address: Faculty of Agriculture, Jember University, Kampus Tegalboto, Jember 68121, Indonesia.

[3] Abbreviations: PEPC, phospho*enol*pyruvate carboxylase; CA, carbonic anhydrase.

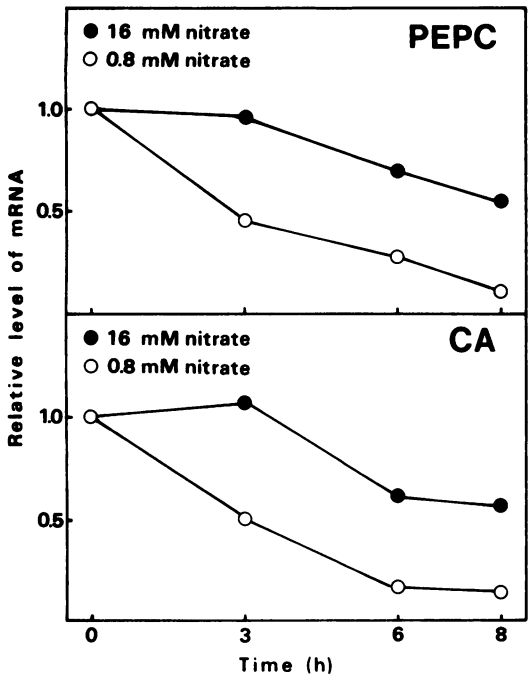

Figure 1. Effect of nitrate administration on the levels of PEPC and CA mRNAs in detached leaves of N-starved plants. The detached leaves of plants grown at 0.8 mM nitrate were used for the experiment. The leaves were incubated with low or high nitrate medium for up to 8 h. The results are expressed as relative values of the control at 0 h (=1.0).

(700 $\mu E \cdot m^{-2} \cdot s^{-1}$ at leaf level) in the chamber used for plant growth. After treatment, the leaves were transversely cut one-third of the way from the base under light. The excised basal segments were weighed and plunged into liquid N_2.

Measurement of mRNA

Extraction of RNA and determination of PEPC mRNA by dot-blot analysis were conducted as described previously (11). CA mRNA was determined in the same way, except a 1.8-kb CA maize insert isolated from a λgt11 expression library and subcloned into pUC19 was used to screen for CA mRNA levels (details of CA clones will be published elsewhere). Probes were labeled with [^{32}P]dCTP using the Multiprime DNA Labelling system (Amersham) and used in dot-blot and northern hybridizations. Two to 4 μg and 15 μg of total RNA were applied for dot-blot and northern analyses of CA mRNA, respectively. Relative accumulation of mRNA was determined after densitometric scanning of the resulting autoradiographs.

RESULTS

Accumulation of mRNAs for PEPC and CA in Detached Leaf Tissue

We previously studied the N-dependent expression of the photosynthetically important PEPC gene using an attached leaf of maize plants (11, 12). To study the mechanism underlying the regulation of PEPC and CA genes, we attempted to examine changes in levels of PEPC and CA mRNAs during recovery from N stress using detached leaves. The youngest, fully developed leaves were detached from plants grown in low nitrate and incubated in either low or high nitrate medium, and PEPC and CA mRNAs were monitored at various intervals (Fig. 1). PEPC and CA mRNAs decreased over the 8-h incubation regardless of the N concentration in the medium, although the rates of decrease in the levels of mRNAs were higher in low-N plants compared to high-N plants. Administration of 5 mM Gln (see ref. 12) or its combination with 2% (w/v) sucrose did not affect the levels of mRNAs for these two enzymes (data not shown).

We next examined the changes in the level of mRNAs for these two enzymes in detached leaves of plants grown continuously in low-N or high-N medium. Detachment of leaves resulted in a decrease in the levels of both mRNAs regardless of the N status of the plants, although the rate of decrease was substantially higher in low-N plants (Fig. 2).

Effects of Hormones on the Levels of mRNAs for PEPC and CA in Detached Leaf Tissue

We examined the effect of zeatin, a naturally occurring cytokinin, on the levels of PEPC and CA mRNAs in detached leaves of N-sufficient and N-starved plants. The detached

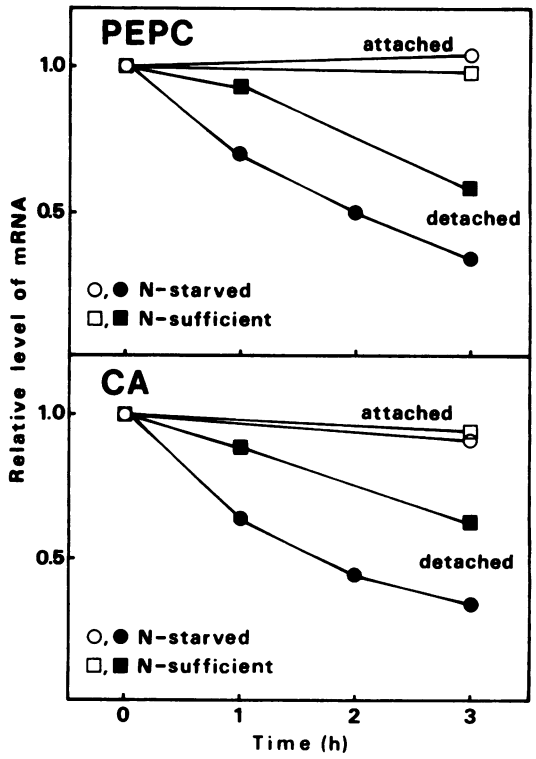

Figure 2. Changes in the levels of PEPC and CA mRNAs in attached and detached leaves of N-sufficient and N-starved plants. The detached leaves of plants grown at 0.8 mM or 16 mM nitrate were incubated for up to 3 h in the corresponding N-medium. The results from both sets of plants are expressed as relative values of the controls at 0 h.

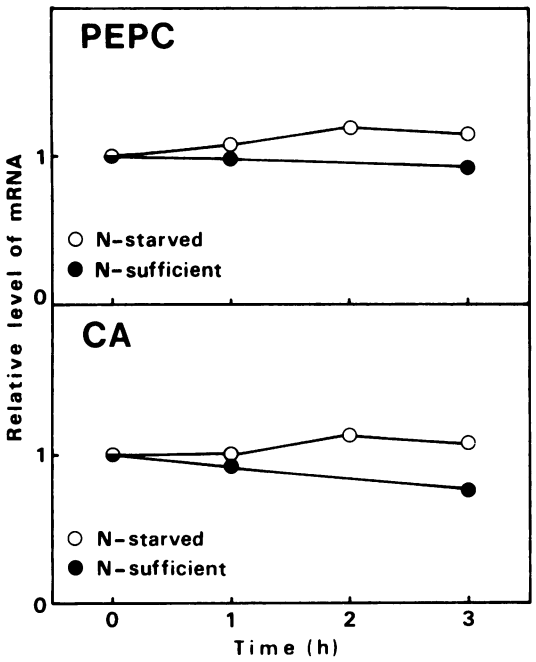

Figure 3. Effect of zeatin administration on the levels of PEPC and CA mRNAs in detached leaves of N-sufficient and N-starved plants. Detached leaves of plants grown at low or high nitrate were used for the experiment. Incubation of detached leaves was carried out as described in the legend to Figure 2, except for the inclusion of 5 μM zeatin. The results are expressed as relative values of the controls at 0 h.

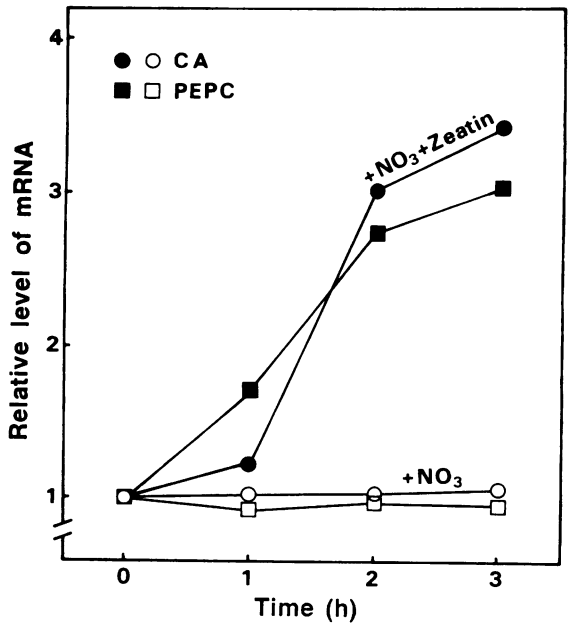

Figure 4. Effect of coadministration of nitrate and zeatin on the levels of PEPC and CA mRNAs in detached leaves of N-starved plants. The detached leaves of plants grown at low nitrate were used for the experiment. The leaves were incubated for up to 3 h with 5 μM zeatin in the presence of 16 mM nitrate or with 16 mM nitrate alone. The results are expressed as relative values of the control at 0 h.

leaves from both sets of plants were incubated in a solution containing 5 μM zeatin and the corresponding level of nitrate, and PEPC and CA mRNA levels were monitored. The decrease in the levels of mRNAs was blocked by administration of zeatin to the detached leaves (Fig. 3), and the changing patterns were comparable to those in attached leaves (cf. Fig. 2). High nitrate (16 mM) plus zeatin (5 μM) was administered to detached leaves of low-N plants, and PEPC and CA mRNAs in the leaves were measured at intervals of 1, 2, and 3 h after incubation (Fig. 4). Both CA and PEPC mRNA levels increased dramatically, with the levels of both mRNAs increasing in parallel over 3 h. This effect of zeatin (plus high nitrate) was dose dependent, and the levels of both mRNAs after 3 h of incubation reached saturation at 1 μM zeatin (Fig. 5). To examine the apparent specificity of this hormonal effect on the expression of PEPC and CA genes, detached leaves of low-N plants were incubated for 3 h with 5 μM zeatin, BA, IAA, or ABA in the presence of 16 mM nitrate, and their mRNAs were measured (Fig. 6). In addition to zeatin, BA, a synthetic cytokinin, was equally effective. However, IAA was ineffective, even when administered together with zeatin. ABA decreased the levels of mRNAs compared to those of the control. To examine the effect of ABA, known as a phytohormone-promoting leaf senescence, zeatin (5 μM) plus ABA (5 μM) was administered. However, the effect of ABA coadministration was not substantial. It should be noted here that there was no change in the levels of mRNA for GS1, a cytosolic form of glutamine synthetase, throughout the experiments (data not shown). We therefore propose that cytokinin regulates the N-dependent expression of PEPC and CA genes in maize leaf tissue. Recent data have shown that there are two CA isozymes in maize and that there are two mRNA bands detected on northern blots probed with the 1.8-kb CA probe from the pUC19 clone (unpublished results).

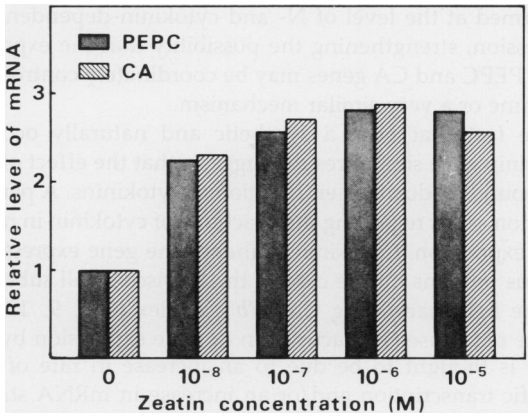

Figure 5. Levels of PEPC and CA mRNAs in detached leaves of N-starved plants as a function of zeatin concentration. The detached leaves of plants grown at low nitrate were used for the experiment. The leaves were incubated for 3 h with the specified concentrations of zeatin in the presence of 16 mM nitrate. The results are expressed as relative values of the control (incubated with 16 mM nitrate alone).

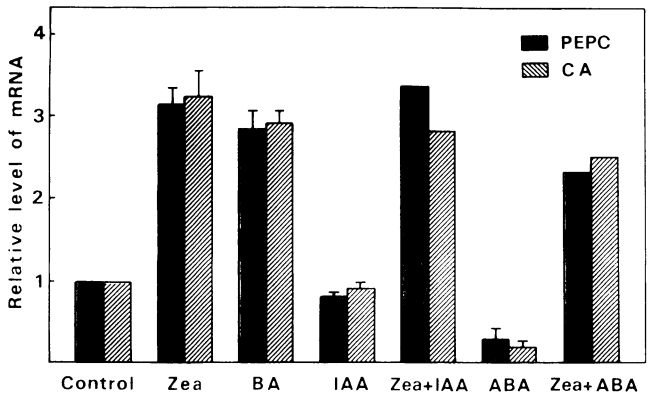

Figure 6. Effect of various phytohormones on the levels of PEPC and CA mRNAs in detached leaves from N-starved plants. Detached leaves of plants grown at low nitrate were used for the experiment. The leaves were incubated for 3 h with 5 μM of each hormone indicated, in the presence of 16 mM nitrate. The results are expressed as relative values of the control incubated with high nitrate alone. The vertical bars represent SE for data from two independent experiments. Zea, Zeatin.

Northern hybridization analysis of total RNA isolated during these studies showed that both CA mRNAs were affected by N and zeatin in an identical manner.

DISCUSSION

Our data show that administration of cytokinin to detached leaves is necessary for an increase in the N-dependent accumulation of PEPC and CA mRNAs. The extent of PEPC mRNA induction by coadministration of cytokinin and high nitrate to detached leaves of low-N plants was comparable to that previously observed with attached leaves of recovering plants (12). However, the kinetics of induction were much faster in detached leaves than in attached leaves. In a previous article (2), we reported that the levels of CA and PEPC activity present in maize leaf tissue changed in parallel in response to light and N nutrition. This correlation was further confirmed at the level of N- and cytokinin-dependent gene expression, strengthening the possibility that the expression of C_4 PEPC and CA genes may be coordinately controlled by the same or a very similar mechanism.

The fact that both a synthetic and naturally occurring cytokinin gave similar results suggests that the effect of these compounds is due to their function as cytokinins. A pertinent question arises regarding the exact role of cytokinin in nuclear gene expression. Cytokinins enhance the gene expression of various proteins. In the case of the Rubisco small subunit (6, 8), the light-harvesting Chl *a/b* complex (4–6, 9, 13), and nitrate reductase (10), activation of gene expression by cytokinin is thought to be due to an increase in rate of gene-specific transcription and/or an increase in mRNA stability. Administration of either cytokinin or nitrate alone to the detached leaves of N-starved maize plants was partially effective in enhancing the levels of PEPC and CA mRNAs. Recently, we proposed that the level of Gln and/or its downstream metabolite(s) could be a metabolic signal for the induction of N-dependent gene expression of maize PEPC (12). Our experimental results in the present study imply that cytokinins, which are thought to be synthesized in roots (3), play an essential role in this induction in leaves by stimulating transcription and/or by stabilizing the transcript.

Although the exact role of cytokinins in gene expression in maize leaves is still unknown, we are using the detached maize leaf system to further study their role in the expression of PEPC and CA genes. In addition, we are using the same system to identify the metabolic signal(s) involved in the N-dependent induction of PEPC and CA mRNA synthesis.

LITERATURE CITED

1. **Burnell JN, Hatch MD** (1988) Low bundle sheath carbonic anhydrase is apparently essential for effective C_4 pathway operation. Plant Physiol **86:** 1252–1256
2. **Burnell JN, Suzuki I, Sugiyama T** (1990) Light induction and the effect of nitrogen status upon the activity of carbonic anhydrase in maize leaves. Plant Physiol **94:** 384–387
3. **Feldman LJ** (1975) Cytokinins and quiescent center activity in roots of *Zea*. *In* JG Torrey, DT Clarkson, eds, The Development and Function of Roots. Academic Press, London, pp 55–72
4. **Flores S, Tobin EM** (1986) Benzyladenine modulation of the expression of two genes for nuclear-encoded chloroplast proteins in *Lemna gibba*: apparent post-transcriptional regulation. Planta **168:** 340–349
5. **Flores S, Tobin EM** (1989) Cytokinin modulation of LHCP mRNA levels: the involvement of post-transcriptional regulation. Plant Mol Biol **11:** 409–415
6. **Funckes-Shippy CL, Levine AD** (1985) Cytokinin regulates the expression of nuclear genes required for photosynthesis. *In* KE Steinback, S Bonitz, CJ Arntzen, L Bogorad, eds, Molecular Biology of the Photosynthetic Apparatus. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 407–411
7. **Hatch MD, Burnell JN** (1990) Carbonic anhydrase activity in leaves and its role in the first step of C4 photosynthesis. Plant Physiol **93:** 825–828
8. **Lerbs S, Lerbs W, Klyachko NL,Romanko EG, Kulaeva ON, Wollgiehn R, Parthier B** (1984) Gene expression in cytokinin- and light-mediated plastogenesis of cucurbita cotyledons: ribulose-1,5-bisphosphate arboxylase/oxygenase. Planta **162:** 289–298
9. **Longo GPM, Bracale M, Rossi G, Longo CP** (1990) Benzyladenine induces the appearance of LHCP-mRNA and of the relevant protein in dark-grown excised watermelon cotyledons. Plant Mol Biol **14:** 569–573
10. **Lu J-I, Ertl JR, Chen C-M** (1990) Cytokinin enhancement of the light induction of nitrate reductase transcript levels in etiolated barley leaves. Plant Mol Biol **14:** 585–594
11. **Sugiharto B, Miyata K, Nakamoto H, Sasakawa H, Sugiyama T** (1990) Regulation of expression of carbon assimilating enzymes by nitrogen in maize leaf. Plant Physiol **92:** 963–969
12. **Sugiharto B, Sugiyama T** (1992) 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. Plant Physiol **98:** 1403–1408
13. **Teyssendier de la Serve B, Axelos M, Peaud-Lenoel C** (1985) Cytokinins modulate the expression of genes encoding the protein of the light- harvesting chlorophyll a/b complex. Plant Mol Biol **5:** 155–163