Cytokinin and Nitrogen-Mediated Gene Regulation for C_4 Photosynthesis

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Nitrogen (N) is an important regulator of the expression of genes involved in carbon and N assimilation pathways in plants by selectively altering the levels of proteins and/or mRNAs. These in C_4 plants include genes for such as phosphoenolpyruvate carboxylase, carbonic anhydrase, and pyruvate-Pi dikinase. The C_4 genes are regulated in mesophyll cells by N availability both transcriptionally and posttranscriptionally through cytokinins and glutamine as signals. The level of both the signals is up-regulated by N availability: cytokinins in roots and glutamine in leaves. The level of glutamine is controlled by the differential expression by N of glutamine synthetase and ferrdoxin-dependent glutamate synthase genes which locate in the mesophyll cells of C_4 plants. The results is discussed as molecular mechanism for the greater N use efficiency of the plants as well as N partitioning is the photosynthetic cells.

Ⅰ. Introduction

Of the inorganic nutrients required for plant growth, the one that is most commonly limiting is N, and thereby maximized efficiency of use of N by plants is of primarily importance for improvement of the productivity and global environment as well. In this context C_4 plants have a greater N use efficiency, which is defined as biomass production per unit of N take up by the plant, than do C_3 plants (Brown, 1978). In studies of effects of N on C_4 enzymes we found that the levels of enzymes such as phosphoneolpyruvate (PEPC) and pyruvate Pidikinase (PPDK), namely enzymes of carbon dioxide trapping apparatus, in N starved maize plants increase selectively by the supply of N most conspicuously in the photosynthetic maturing cells, while the level of Rubisco decreases (Sugiharto et al, 1990). The regulatory nature of N-distribution into carbon assimilation enzymes interested us to understand the molecular basis for partitioning of N in the photosynthetic cells. Elucidation of the mechanism will also lead us to understand the molecular basis for the greater N use efficiency of the plants. In this paper we will discuss our current data and idea on the molecular mechanism for the N-mediated expression of photosynthesis genes involved in carbon and N assimilation processes in C_4 plants, taking emphasis on cytokinin function in their gene expression

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Abbreviations: N, nitrogen; PEPC, phosphoenolpyruvate carboxylase; PPDK, pyruvate orthophosphate dikinase; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; GS, glutamine synthetase; AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; Fd-GOGAT, ferredoxin-dependent glutamate; MSX, methionine sulfoximine; CA, carbonic anhydrase

Ⅱ. Materials and Methods

1. Plant Growth

Maize (*Zea mays* L. cv Golden Cross Bantam T51) and *Panicum miliaceum* plants were grown with 0.8 mM (low N) or 16 mM (high N) KNO_3 in vermiculite in a growth chamber (Sugiharto et al., 1992a), The chamber was controlled as follows: 14 h light and 10 h dark, 28/20°C (day/night);>60%humidity; 500 umol photon $m^{-2}s^{-1}$ for illumination. When plants grown with low N had the fully developed third leaves, the culture solution was changed to high N in the middle of day. The leaves were periodically harvested and the basal halves were frozen with liquid nitrogen for analysis of biochemical parameters and activity. For some experiments the detached leaf system was used (Sugiharto et al., 1992a and 1992b).

2. Other Methods

Isolation of nuclei, in vitro transcription, and mRNA measurement for C_4 genes in maize were conducted according to the methods previously described (Sugiharto et al, 1992a; Suzuki et al, 1994). Assays for AlaAT and AspAT, mRNA measurement and sequence analysis for *Panicum miliaceum* were conducted as described previously (Son et al, 1992; Son and Sugiyama, 1992; Taniguchi et al, 1992, 1994,and 1995) mRNA measurement, enzyme assays, and sequence analysis for GS and FD-GOGAT were conducted as described previously (Sakakibara et al, 1991, 1992a and 1992b).

Ⅲ. Results and Discussion

1. N-mediated regulation of C_4 photosynthesis genes occurs most conspicuously at photosynthetically maturing cells and requires Gln as a positive signal

To gain a better understanding of the way in which N regulates the selective expression of the major proteins in the leaves of maize (a NADP-malic enzyme-type C_4 plant), PEPC, PPDK, and Rubisco, of which levels are potentially limiting with respect to photosynthetic productivity (Avdeeva and Andreeva,1973;
Sugiyama and Hirayama, 1983; Usuda et al.,1984) we have examined (a) the basipetal distribution of these proteins in response to N status and (b) the effects of N on the synthesis of a range of proteins and the steady state levels of their mRNA during recovery from a N deficit. For analysis we have utilized the cellular differentiation gradient of the developed, youngest leaf to examine the level regulation by N of levels of PEPC, PPDK, and Rubisco in maize. The protein whose level regulated most preferentially by N availability was PEPC, followed by PPDK, and the change in level occurred most conspicuously at the photosynthetically maturing cells (Fig. 1.). Pulse and pulse-chase labeling with [^{35}S] Met and hybridization analyses showed that the increased accumulation of these proteins during N-recovery could largely be accounted for a increase level of synthesis of protein with a concomitant increase in level of their mRNAs (Sugiharto et al., 1990). Thus we concluded that the N-mediated selective accumulation of these proteins is primarily a consequence of level of its mRNAs. Such a selective regulation of the expression of genes for C_4 enzymes, PEPC, AlaAT(Son et al. 1992), and

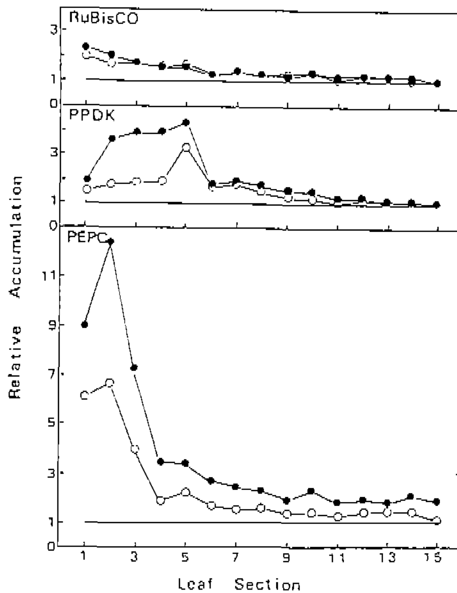

Fig. 1 Relative accumulation of PEPC, PPDK, and Rubisco in developed, youngest leaf of maize plants during recovery from N deficiency
Plants were grown in a growth chamber. The third leaves of low nitrate grown plants were harvested immediately upon supplementing with high nitrate, 2d (open circles) and 3 d (filled circles) thereafter. Leaf segments were prepared from five plants. Relative accumulation was calculated by taking the relative level of each protein in each segment on d 0 as unity.

AspAT (Tangiguchi et al., 1995), have been confirmed to occur also in *Panicum miliaceum* (an NAD-malic enzyme-type C_4 plant).

The specificity of inorganic N sources, nitrate or ammonium, for the regulation of *C4Ppc1*, a gene for C_4-PEPC, was determined by measuring the total pool sizes of the potentially important cellular metabolites involved in N/C assimilations correlating with the N-dependent induction of *C4pc1* mRNA in the photosynthetically maturing cells of maize plants during recovery from N starvation (Sugiharto and Sugiyama, 1992). The increase of *C4pc1* mRNA is more pronounced in plants supplemented with ammonium than with nitrate. The accumulation of mRNA during N recovery increases in parallel with the increase in activity ratio of GS vs Fd-GOGAT and was highly and positively correlated with Gln level among major amino acids. The administration of Gln to N-starved plants efficiently increased the steady-state level of *C4Ppc1* mRNA, indicating that Gln or its metabolite(s) can be a positive signal for the N-dependent regulation of the gene expression. More direct evidence for this notion was obtained by studying the effect of MSX, a specific inhibitor of GS

activity, on the N-dependent gene noteworthy that the gene expression (Sugiharto et al., 1992b) as shown in Table 1. Regarding the data, it is noteworthy that the gene expression of C4ca, a gene for C4-ca, and *C4Ppc1* appears to be coregulated at least in terms of N-response, as was suggested based upon their enzyme activities (Burnell et al., 1990)

Table 1. ***Effects of Administration of N-Compounds on the Levels mRNA for PEPC, CA, and GS1 in MSX-Pretreated Detached Leaves of N-Straved Maize Plants***

The levels of mRNAs were measured by nothern hybridization. The results are expressed as relative values of the controls at 30-min pre-incubation

Treatment	75-min Incubation			150-min Incubation		
	PEPC	CA	GSI	PEPC	CA	GSI
-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

Ⅱ. N-mediated regulation of C_4 photosynthesis genes requires cytokinins in addition to Gln

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-mediated genes expression of maize C_4 enzymes, which mediates the communication between leaf and root tissues, we have chosen detached maize leaves as the experimental material. Two important questions arise un this respect: (a) How

; and (b) What messenger(s) is used to mediate the communication between roots and leaves? We have demonstrated that exogenous cytokinins is absolutely required for the N-mediated accumulation of *C4Ppc1* and *C4Ca* mRNAs in detached maize leaf tissue as shown in Fig. 2 (Sugiharto et al., 1992 a). Our data show that effect of the hormone is dose-dependent and highly specific to cytokinins and that abscisic acid inhibits the gene expression *apparently in a competitive manner against cytokinins.* The fact that both a synthetic and naturally occurring cytokinins give similar results suggests that the effect

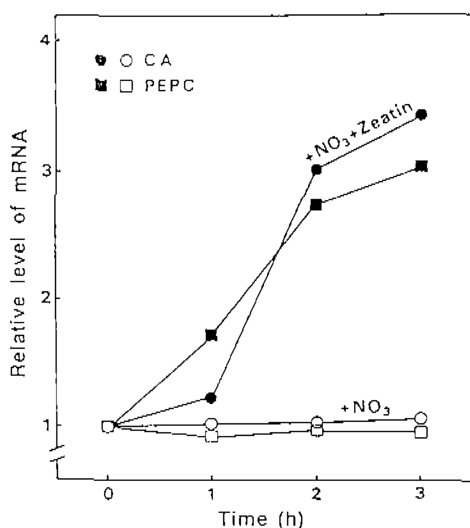

Fig. 2 Effect of coadministration of nitrate and *zeatin on the levels of* PEPC and CA mRNAs in detached leaves of N-starved maize plants. The detached leaves of plants grwon at low nitrate were used for the experiment. The leaves were incubated for up to 3 h with 5 uM zeatin in the presence of 16 mM nitrate or with 16 mM nitrate alone. The results are expressed as relative values of the control at 0h.

of these compounds is due to their function as cytokinins. Our experimental results imply that cytokinins, which are thought to be synthesized in roots (Feldman, 1975), play an essential role in the induction of such C_4 genes in leaves by stimulating transcription and/or by stabilizing the transcripts.

Ⅲ. Cytokinins up-regulate the transcription of C_4 genes whereas Gln up-regulates the level of transcripts

To examine the mode of N-dependent gene expression for C_4 enzymes, we measured *C4Ppc* mRNA levels and the in vitro transcription in leaf nuclei isolated from plants during recovery from N-starvation, taking emphasis in PEPC gene (Fig. 3) (Suzuki et al., 1994). The induction is specific for the C_4-type PEPC gene, and its transcription is N-dependent and increases transiently by supply of an N source, but there is a discrepancy between the steady-state levels of mRNA and the stimulation of *in vitro* transcription, The lack of correlation between these two parameters suggests that the N-meditated expression of *C_4Ppc1* is regulated both transcriptionally and posttranscriptionally by N availability.

To understand the role of cytokinins in the N-mediated accumulation of *C4Ppc1* and *C4Ca* mRNA in detached maize leaves (Sugiharto et al., 1992a), we examined the effects of N sources and zeatin on *in vitro* transcription for *C_4Ppc1*(Table 2) (Suzuki et al., 1994) The *in vitro* transcription rate of *C4Ppc1* is greatly stimulated by incubating detached leaves with zeatin alone, whereas the rate is not affected by incubating with an exogenous N source alone, The result in the table and evidence that both cytokinin and N-source are required for the accumulation of mRNAs, taken together, imply that cytokinins and Gln

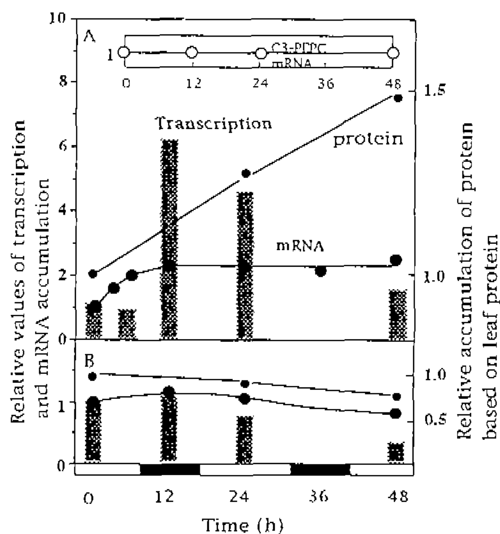

Fig. 3 The gene expression of C4-PEPC is regulated by N both transcriptionally and posttranscriptionally

(A) Plants grown at low nitrate and then supplemented with high nitrate at 0 h..
(B) Plants continueously grwon at low nitrate
Inset represents mRNA pattern for C3-type PEPC. Hatched bas and Open bars represent dark and light period in growth, respectively.

or its metabolite(s) up-regulate the transcription of *C_4Ppc1* and the level of the transcript, respectively.

Table 2. ***Effects of N SOurces and Run-Off Activity for C_4Ppc1***

leaves were detached from plants grown at low nitrate and incubated for 2 h in an N-free medium including an N source and/or zeatin specified

Addition	Relative Run-Off Activity
None	1.0
16 mM nitrate	1.5
12 mM glutamine	1.2
5 uM *zeatin*	6.6
16 mM nitrate + 5 uM zeatin	9.4

The cytokinin-dependent transcription is not general phenomenon during recovery from N starvation; transcription of GS1 (cytosolic GS), GS2 (plastidic GS), Fd-GOGAT, and rRNA genes are not affected by cytokinin. The transcription up-regulated by cytokinin is totally inhibited by cycloheximide, indicating the cytokinin-dependent transcription of *C_4Ppc1* requires the synthesis of protein.

Based upon these results we propose a hypothetical model (Fig. 4) as a mechanism for the N-mediated gene regulation for the N-mediated gene regulation for C4 enzymes in maize plant.

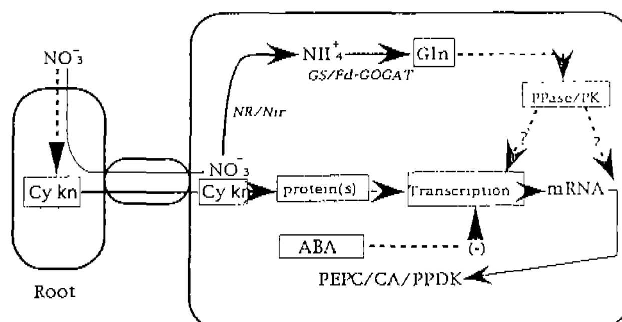

Fig. 4 Proposed scheme for N-mediated regulation of C4-genes
ABA: abscisic acid PPase/PK: protein phosphatase/protein kinase

Ⅳ. Cytokinins accumulate in maize roots in responding to N availability

A most important part of the hypothesis in the model (Fig.4) concerns assignment of a N-mediated accumulation of cytokinins in roots. Although the primary functions in gene expression have not been fully elucidated at the molecular level, it is known that cytokinin levels of roots, shoots, and xylem sap decrease at low N supply with some plant species including sunflower, *Plantago, Betula, and Acer,* and *Lemna*(Salama and Waering, 1979;Horgan and Waering,1980;Kuiper et al., 1989;Thronsteinsson and Eliasson, 1990). More recently, it has been reported with barley that zeatin riboside level of the roots of N-limited plants is non-responsive to nitrate dose in the long term, but transiently responding positively to nitrate supply(Samuelson, Larsson, 1993). To test the possibility we assayed cytokinins in maize roots during recovery from N starvation (Fig.5). The roots of N starved plants in responding to nitrate supply. supports our hypothesis depicted in Fig.4. We are looking for step(s) in cytokinin metabolism, which is responsible to nitrate supply.

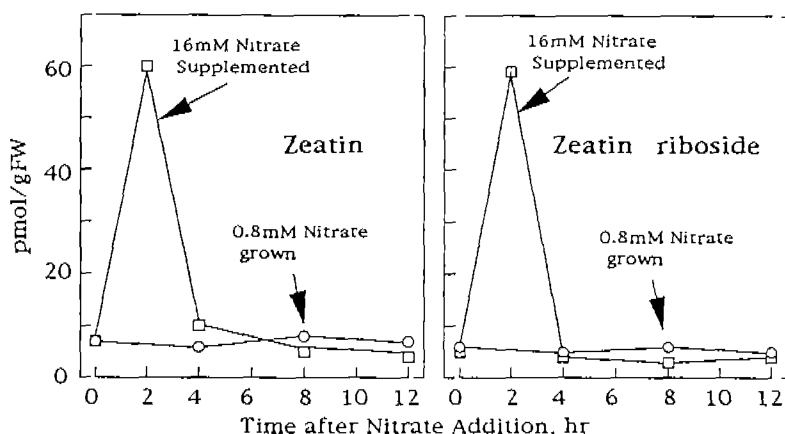

Fig. 5 N-dependent accumulation of cytokinins in maize roots
Plants were grown at low nitrate and then supplied with high nitrate.

V. Gln accumulates in mesophyll cells due to the differential expression of GSs and Fd-GOGAT in responding to N availability

In the leaf of C_4 plants, the enzyme for the reductive assimilation of nitrate is distributed most abundantly in mesophyll cells and most of nitrate taken up by the plant
is assimilated in leaves rather than in roots. In the pathway, GS and Fd-GOGAT play a central role un the assimilation of ammonia. It has been demonstrated that the level of plastidic GS (GS2) increases preferentially in responding to N availability in maize mesophyll cells, whereas Fd-GOGAT, that exists as a gene (Sakakibara et al., 1991), in the cells is non-responsive to N (Sakakibara et al., 1992b). This differential gene expression may be a metabolic basis for a preferential accumulation of Gln in the leaf in response to N availability, which is a positive signal of the N-mediated regulation of expression of C_4 genes.

Primary assimilation of nitrate also occurs in maize roots. In maize, a small multigene family encodes cytosolic isoforms of GS (GS1) and five cDNAs, designated pGS1a, pGS1b, pGS1c, pGS1d, and pGS1e, have been cloned (Sakakibara et al., 1992a; Li M et al., 1993). We identified and characterized the cytosolic isoforms of GS in roots, namely GS1 and GSr. The isoforms were analyzed together with recombinant enzymes that had been overexpressed in *E. coli* by capillary liquid chromatography/electrospray ionization mass spectrometry. The results indicated that GS1 and GSr were the products of the *GS1a/GS1b* and *GS1c/GS1d,* respectively. The expression of *GS1c/GS1d* exhibited an interesting regulatory nature in terms of N response. The significant accumulation of GSr occurred in response to the addition of ammonia to the culture medium and a preferential increase in GS synthetase

activity, as compared to GS transferase activity, was found in the root extract. Assay with the purified recombinant enzymes confirmed that the specific synthetase activity of GSr was 202-fold higher than that of to be advantageous for the primary assimilation of external ammonia by roots.

Ⅵ. Concluding remarks

Collectively, the data described in this paper indicate that gene expression of C_4 genes involved in carbon dioxide trapping apparatus is regulated by two signals, cytokinins and Gln, of which levels are up-regulated in the plant in responding to N availability. These signals are transducers in the gene expression of the C4 apparatus by linking two metabolic pathways, carbon and nitrogen assimilatory processes. Among the signals cytokinin is particularly important and interest since the plant hormone appears to be a transducer for photosynthetic genes not only for carbon dioxide trapping apparatus but also for Rubisco-small subunit and light harvesting chlotophyll binding protein. Elucidation of sensing mechanism and entity of signal for the N-mediated accumulation of cytokinin in maize roots and signaling to expression for the photosynthesis genes will lead us to understand N partitioning mechanism in photosynthetic cells, which will also lead us to understand the molecular basis for the nature of the high responsibility to N in the photosynthesis of C_4 plants in the whole plant level.

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