

REPLENISHMENT OF CARBON SKELETONS FOR AMMONIUM ASSIMILATION IN PLANT ROOTS

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<https://doi.org/10.11501/3142493>

出版情報 : 九州大学, 1998, 博士 (農学), 課程博士
バージョン :
権利関係 :



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1998

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THESIS

SUBMITTED TO GRADUATE SCHOOL, KYUSHU UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY IN AGRICULTURAL SCIENCE

NOBUHISA KOGA

1998

ACKNOWLEDGMENTS

The author wishes to express his thanks to Dr. Motoki Ikeda, Professor of Plant Nutrition, Department of Agricultural Chemistry, Kyushu University for his invaluable instructions throughout the author's course work.

He is grateful to Dr. Kazuhiko Egashira, Professor of Soil Science for his critical review of the dissertation.

He is also indebted to Associate Professor Dr. Takeo Yamakawa of Plant Nutrition for his helpful suggestions.

The author would extend to thank his colleagues in the Laboratory of Plant Nutrition and Soil Fertility, Kyushu University for their encouragements and helps in his study and student life.

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Abbreviations

AS	asparagine synthetase
BSA	bovine serum albumin
CAM	Crassulacean acid metabolism
CBB	Coomassie Brilliant Blue
DEAE	diethylaminoethyl
EDTA	ethylenediaminetetraacetate
FW	fresh weight
GOGAT	glutamate synthase
GS	glutamine synthetase
HEPES	N-[hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid]
HPLC	high performance liquid chromatography
I ₅₀	half-inhibitory dose
K _m	Michaelis constant
LSC	liquid scintillation counter
MDH	malate dehydrogenase
MSX	L-methionine sulfoximine
NAD(H)	nicotineamide adenine dinucleotide
OAA	oxaloacetate
2-OG	2-oxoglutarate
PAGE	polyacrylamide gel electrophoresis
PEP	phosphoenolpyruvate
PEPC	phosphoenolpyruvate carboxylase
PITC	phenyl isothiocyanate
PMSF	phenylmethylsulfonyl fluoride
PPO	2,5-diphenyloxazole
PTC	phenyl thiocarbamoyl
PVPP	polyvinylpolypyrrolidone
RH	relative humidity
rpm	revolutions per minute
SDS	sodium dodecylsulfate

Chapter 1

INTRODUCTION

1.1 Differential Plant Growth between Nitrate and Ammonium Nutrition.

Nitrogen (N) is a major constituent within plants and a rate-limiting element in plant growth. Plants are basically able to absorb and utilize nitrate and ammonium as inorganic forms of N. In agricultural practices, ammonium-N is mainly fertilized because nitrate-N is lost from soils by leaching. Ammonium in soils is oxidized to nitrate by the action of nitrifying bacteria, thereby both nitrate and ammonium are available for plants under upland soil conditions (Sechley et al. 1992).

Generally, the supply of nitrate or ammonium makes a difference to plant growth or biomass production. Numerous researchers have extensively studied the effects of nitrogen form on plant growth. The supply of ammonium generally displayed the smaller biomass production than the supply of nitrate or mixed nitrogen nutrition (Cramer and Lewis 1993)

although it depended, to some extent, on plant species or cultivars. Particularly, it was apparent that ammonium nutrition considerably repressed the root mass production and raised a shoot/root mass ratio (Lewis et al. 1987 ; Cramer and Lewis 1993). In addition, the experiment of split-root system confirmed that one half of the root system fed with ammonium showed a more depressed dry matter production than the other half of the root system fed with nitrate in soybean plants (Chaillou et al. 1994).

Within plant cells, one direct toxicity of ammonium is suggested to be uncoupling of electron transport and oxidative phosphorylation at mitochondria (Lang and Kaiser 1994). However, the causation of the poor growth of plants during ammonium nutrition appears to be very complicated. The repressed mass production in ammonium-fed plants is often characterized by the decreased rate of photosynthesis or root respiration. (Cramer and Lewis 1993 ; Chaillou et al. 1994). Schortemeyer et al. (1997) demonstrated that ammonium-sensitive cultivars accumulated a higher level of ammonium and a lower level of water-soluble carbohydrates in roots than ammonium-insensitive cultivars employing maize cultivars differing in ammonium sensitivity. It was thus hypothesized that the sufficient supply of carbon (C) skeletons in roots during ammonium assimilation was required for the sound growth of plants (Schortemeyer et al. 1997). Magalhães et al. (1992) also showed that the exogenous supply of 2-OG contributed to the decreased concentration of ammonium in

tomato roots and improved the growth.

1.2 Replenishment of Carbon Skeletons for Nitrogen Assimilation in Plants.

Nitrate is able to be stored in vacuoles and exported to shoots without reduction. Subsequently, nitrate is reduced in shoots or roots by nitrate reductase and subsequent nitrite reductase and resultant ammonium is assimilated to amides and amino acids. The main site of nitrate reduction depends not only on plant species but also on temperature, light intensity and plant age (Engels and Marschner 1995 ; Crawford 1995).

In contrast, when plants are situated in a condition that ammonium is dominantly absorbed, in practice, an excess of ammonium-N is fertilized with the limited nitrification, absorbed ammonium is immediately assimilated in roots to detoxify ammonium and roots consequently accumulate glutamine and/or asparagine (Givan 1979). At the onset, ammonium is combined to glutamate to produce glutamine by the action of cytoplasmic GS. Then, glutamine reacts with 2-OG to regenerate two molecules of glutamate by NADH-dependent GOGAT. The absorbed ammonium is mostly assimilated in this GS/GOGAT cycle in roots (Joy 1988 ; Sechley et al. 1992 ; Oaks 1994). Ammonium produced by the reduction of nitrate is also fixed in the GS/GOGAT cycle (Sechley et al. 1992 ; Sivasankar and Oaks 1996). Ammonium released by foliar photorespiration in the process of the conversion of two molecules of glycine to

serine is assimilated in a distinct GS/GOGAT cycle (Hayakawa et al. 1990 ; Peat and Tobin 1996). Asparagine, another dominant nitrogen assimilate for transport and storage in plants, is synthesized by the transfer of amide group of glutamine to carboxyl group of aspartate by the function of glutamine-dependent AS (Givan 1979). Aspartate is formed by a transamination reaction of glutamate and OAA (Lea 1993 ; Oaks 1994).

When ammonium is continuously supplied, the assimilates produced in ammonium assimilation are to be translocated into shoots. As mentioned above, since the oxo acids such as 2-OG and OAA derived from TCA cycle are precursors of the assimilates, these oxo acids as carbon skeletons for ammonium assimilation have to be provided from the TCA cycle and other sources when ammonium continuously enters to roots. The withdrawal of 2-OG and OAA results in the decreased pool of the TCA cycle intermediates. Therefore, C₄-dicarboxylic acids such as malate and OAA are to be replenished into the TCA cycle. The C₄-dicarboxylic acids can be synthesized from a C₃ compound via β -carboxylation. In this case, the carbon fixation is especially referred to as anaplerotic dark carbon fixation.

The carbon fixation in roots is virtually mediated by PEPC (EC 4.1.1.31), which converts PEP and bicarbonate to OAA and orthophosphate. PEP is provided through the glycolytic degradation of hexoses originating from the photosynthates (mainly sucrose) translocated into roots. Bicarbonate,

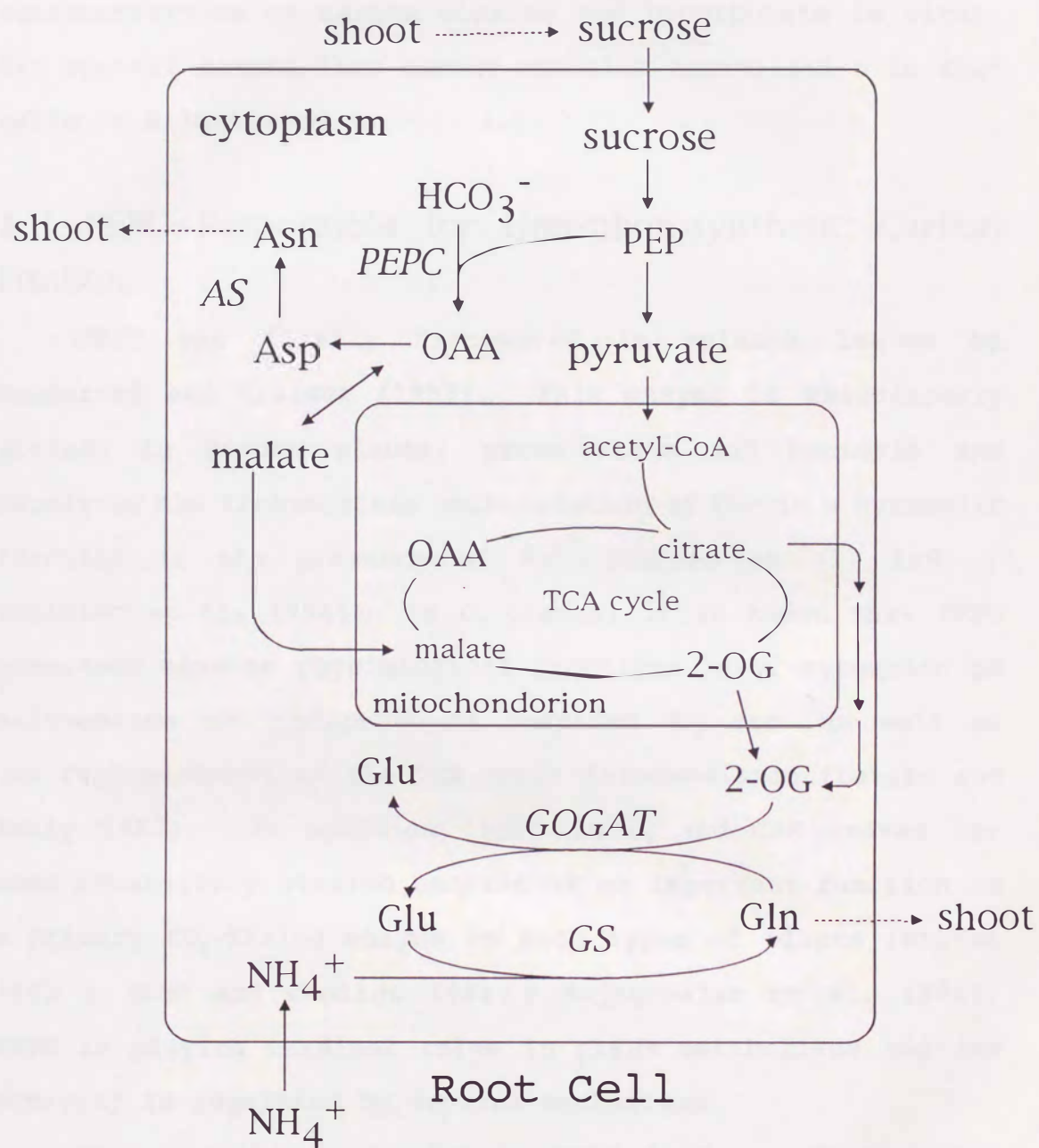


Fig. 1-1. The carbon flow related to ammonium assimilation in roots.

another substrate for the dark carbon fixation, is consecutively provided by carbonic anhydrase, which catalyzes the rapid interconversion of carbon dioxide and bicarbonate in water. The overall carbon flow during ammonium assimilation in root cells is illustrated in Fig. 1-1.

1.3 PEPC Responsible for Non-photosynthetic Carbon Fixation.

PEPC was firstly discovered in spinach leaves by Bandurski and Greiner (1953). This enzyme is ubiquitously present in higher plants, green algae and bacteria and catalyzes the irreversible carboxylation of PEP in a cytosolic fraction in the presence of Mg^{2+} (Andreo et al. 1987 ; Lepiniec et al. 1994). In C_3 plants, it is known that PEPC possesses diverse physiological functions, e.g. cytosolic pH maintenance and recapture of respired CO_2 etc. as well as the replenishment of the TCA cycle intermediates (Latzko and Kelly 1983). In addition, PEPC in C_4 and CAM leaves has been intensively studied because of an important function as a primary CO_2 -fixing enzyme in such types of plants (Winter 1982 ; Jiao and Chollet 1991 ; Rajagopalan et al. 1994). PEPC is playing cardinal roles in plant metabolisms and its activity is regulated by several mechanisms.

The activities of various PEPC isoforms from higher plants, root nodules and green algae are allosterically controlled by positive or negative metabolite effectors (Lepiniec et al. 1994 ; Chollet et al. 1996). Generally,

organic acids including malate represent negative effectors while sugar phosphates positively affect PEPC activities. Which compounds and how strongly those function as effectors appear to depend on the source of PEPC. Therefore, *in vivo* PEPC activities presumably vary with the changes in the concentration of metabolite effectors in cells. In relation to ammonium nutrition in plant roots, it should be noted that a PEPC isoform separated from a green alga was activated by glutamine, the product of ammonium assimilation (Schuller et al. 1990a).

In C_4 and CAM leaves, PEPC activity is known to be regulated by reversible protein phosphorylation (Chollet et al. 1996). PEPC from these materials is covalently modified by protein kinase and phosphatase in a light-dependent manner. Jiao and Chollet (1990) have already identified the phosphorylated serine residue near N-terminus of maize leaf PEPC. The phosphorylation of PEPC elevates the catalytic activity and renders the enzyme activity less sensitive to the inhibition by malate (Brulfert et al. 1986 ; Jiao and Chollet 1988 ; Jiao and Chollet 1989).

In addition to the case of C_4 and CAM leaves, it has been reported that PEPCs also undergo the regulatory phosphorylation in C_3 leaves (Wang and Chollet 1993 ; Duff and Chollet 1995) and root nodules (Schuller and Werner 1993 ; Zhang et al. 1995). From the results of the stem girdling experiment, Zhang et al. (1995) suggested that shoot-derived materials might have a significant influence on the *in vivo*

phosphorylation state of nodule PEPC. It is very noteworthy that PEPC in root nodules, which are not exposed to direct illumination, is consistently phosphorylated.

The metabolite effects and the reversible protein phosphorylation are responsible for the rapid modulation of the PEPC activity. Hence, both are called short-term modulations.

In contrast with metabolite effects and regulatory phosphorylation, *de novo* protein synthesis is referred to as a long-term modulation. Sugiharto and Sugiyama (1992) showed that glutamine had a inducible effect on the accumulation of PEPC protein in maize leaves during recovery from nitrogen deficiency. Manh et al. (1993) also indicated that glutamine was a likely compound for the induction of PEPC in detached wheat leaves.

These informations on the regulations of plant PEPC activity will be helpful when root PEPC activity is discussed in response to the supply of ammonium.

1.4 Aim of This Study.

A continuous supply of ammonium is considered to require the replenishment of carbon skeletons for the assimilation of ammonium to amides and amino acids in plant roots. Since the carbon skeletons are replenished via anaplerotic dark carbon fixation, it is conceivable that *in vivo* PEPC activity and/or the provision of the substrate for the dark carbon fixation in roots may be responsible for the

regulation in the replenishment of carbon skeletons. The root is a specific site for ammonium assimilation in plants. Nevertheless, the carbon metabolism in roots is not sufficiently studied from the viewpoint of ammonium nutrition. The present study is designed to understand the regulations of the replenishment of carbon skeletons in roots during ammonium assimilation.

Chapter 2

STIMULATION OF DARK CARBON FIXATION IN ROOTS DURING AMMONIUM NUTRITION

2.1 Introduction

Dark (non-photosynthetic) carbon fixation is a usual metabolic incident in plants and algae etc. This reaction is responsible for C_4 -dicarboxylic acid synthesis and mostly takes place as a carboxylation of PEP. Stimulation of the dark carbon fixation under various nutritional conditions has been already reported in higher plants (Ikeda et al. 1992 ; Johnson et al. 1994), cultured plant cells (Wright and Givan 1988) and algal cells (Vanlerberghe et al. 1990 ; Müller et al. 1990). The rate of dark carbon fixation is substantially increased when organic acid synthesis is reinforced. For example, phosphorus-stressed white lupin showed a stimulated dark carbon fixation in roots for citrate exudation to rhizosphere to acquire phosphorus (Johnson et al. 1994).

It is reported that the supply of ammonium stimulates

the dark carbon fixation in roots of higher plants (Ikeda et al. 1992 ; Cramer et al. 1993) and green algae (Wright and Givan 1988 ; Müller et al. 1990). Once ammonium enters into roots, the ammonium immediately has to be assimilated to counteract its toxicity although ammonium is a major source of N. This involves an alteration of carbon metabolism in roots because it demands the supply of oxo acids such as OAA and 2-OG in order to accept ammonium. These dicarboxylic acids are synthesized via the dark carbon fixation. Thereby, C fixed in roots is considered to play an important role in carbon metabolism of roots in the course of ammonium assimilation.

This chapter addressed the determination of the rate of dark carbon fixation and the destinations of the fixed C in wheat roots supplied with ammonium and pretreated with or without MSX that is a specific inhibitor for GS.

2.2 Materials and Methods

Plant Growth.

Seeds of wheat plant (*Triticum aestivum* L. var. Saitama) were germinated on moist filter paper for 2 d in the dark. The seedlings were grown on washed sand for several days and thereafter hydroponically cultured in a 7.5 L plastic container (10 seedlings per container) in a glasshouse. One-eighth modified Hoagland solution (pH 6,

Table 2-1) containing 2 mM NaNO_3 was supplied to the plants with continuous aeration. The nutrient solution was basically prepared with deionized water and renewed twice a week.

Nitrogen Treatment and MSX Pretreatment.

Prior to the supply of ammonium, 3-week-old wheat plants were supplied with N-free 1/4 strength modified Hoagland solution (pH 7) for 3 d. For pretreatment with MSX, 100 μM MSX was supplemented in the nutrient solution for 3 h just before the initiation of the supply of ammonium. Subsequently, all plants were supplied with 1/4 strength nutrient solution containing 2 mM $(\text{NH}_4)_2\text{SO}_4$ and withdrawn at 0 h, 3 h, 1 d and 3 d for ^{14}C -bicarbonate feeding experiments. The nutrient solution was renewed every day during the supply of ammonium.

^{14}C -bicarbonate Feeding.

The withdrawn wheat plants were transferred into flasks containing 100 mL of 1/4 strength nutrient solution containing 2 mM $(\text{NH}_4)_2\text{SO}_4$ except for the plants withdrawn at 0 h, which were placed in N-free nutrient solution. All plants were then acclimated to darkness for 1 h with shaking (100 rpm) in a dark growth chamber (20 °C). The plants were supplied with 100 mL of the solution containing 2 mM $(\text{NH}_4)_2\text{SO}_4$ and 1 mM $\text{NaH}^{14}\text{CO}_3$ (3.7 MBq mmol^{-1}) at the initiation of dark carbon fixation and then incubated for 3 h with shaking in the dark chamber.

Table 2-1. Composition of 1/4 strength modified Hoagland solution in which nitrogen was omitted.

Element	Chemical	Concentration (mg L ⁻¹)
P	KH ₂ PO ₄	7.75
K	K ₂ SO ₄ , KH ₂ PO ₄	58.8
Mg	MgSO ₄ · 7H ₂ O	12.4
Ca	CaCl ₂ · 2H ₂ O	51.5
Fe	NaFe · EDTA	5
Mn	MnSO ₄ · H ₂ O	0.127
Zn	ZnSO ₄ · 7H ₂ O	0.0118
Cu	CuSO ₄ · 5H ₂ O	0.005
B	H ₃ BO ₃	0.127
Mo	MoO ₃	0.0025
S		16.6

After the ^{14}C -bicarbonate feeding, the plants were harvested and the roots were rinsed with water and 0.01 N HCl to remove adhering ^{14}C -bicarbonate. The plants were separated into shoot and root parts with a razor. Each part was chopped, followed by measuring the fresh weight. The chopped samples were put into 50 mL flasks and kept frozen at -70°C until extraction.

Aqueous solution of $\text{NaH}^{14}\text{CO}_3$ was purchased from Amersham (England).

Distribution of ^{14}C within the Plant.

The excised roots were extracted three times with 30 mL of 80 % (v/v) ethanol at 80°C . The combined extract was made up to 100 mL. The radioactivity of "root-soluble fraction" was determined with an LSC (LSC-5100 model, Aloka, Japan). The sample cocktail for liquid scintillation counting consisted of 0.5 mL of the extract, 5 mL of methanol-ethanol (1:1 in v/v) and 5 mL of PPO-toluene (4 g PPO per 1 L toluene).

The root residue after the extraction with 80 % (v/v) ethanol was dried overnight at 50°C and designated as "root-insoluble fraction". The radioactivity was determined after immersing the dried residue into PPO-toluene overnight.

The chopped leaves were hydrolyzed with 30 mL of 6 N HCl at 80°C for 6 h. The hydrolysate was referred to as "shoot fraction". Its radioactivity was determined with an LSC.

Ion-exchange Chromatography.

Further fractionation was performed for the root-soluble fraction. The root extract (25 mL) was concentrated by rotary evaporation. A whole aliquot of the concentrate was loaded onto the columns of Dowex 50W (H^+ form) and Dowex 1 $\times$ 8 (CH_3COO^- form) connected in series.

The neutral fraction was eluted with 12 mL of deionized water. After the columns were disconnected, the basic and acidic materials were eluted from Dowex 50W and Dowex 1 $\times$ 8 columns with 7 mL of 2 N NH_4OH and 8 N $HCOOH$, respectively. The eluate was collected to a vial that was previously weighed. The radioactivity was determined with an LSC for each fraction.

HPLC Analysis for Major Organic Acids.

The acidic fraction was evaporated to dryness. The dried samples were dissolved in 100 μ L of 0.1 % H_3PO_4 and syringe-filtered (0.45 μ m). The individual organic acids were separated by HPLC (PV-980 model pump, Jasco, Tokyo) equipped with both of a Shim-Pack SCR-102H column (Shimadzu, Japan) and a guard column recommended by the manufacturer. Ten μ L of the sample was injected and eluted with 0.1 % H_3PO_4 at 40 °C and at a flow rate of 1.0 mL min⁻¹. Column pressure was 29 kg cm⁻². Individual peaks were detected at 210 nm with a UV detector (UVDEC-100-III model, Jasco) and the peak areas were calculated with an integrator (Chromatocoder 12 model, SIC, Japan). Individual acids were collected to a

vial by manual handling and the radioactivity was determined in a sample cocktail with an LSC. For malate, citrate, succinate and fumarate, the retention time and area/nmol value were determined in the preliminary experiments. The solvents used in HPLC analyses were previously degassed and filtered (0.45 μm).

Derivatization of Amino Acids with PITC.

Preparation of PTC-amino acids was according to the method of Yamaya and Matsumoto (1988). The sample of basic fraction (5 mL) was evaporated to dryness. Hydroxyproline (12.5 nmol) was added as an internal standard. After evaporation to dryness, the sample was mixed with 50 μL of ethanol- H_2O -triethylamine (TEA) (2:2:1 [v/v/v]) and allowed to stand for 20 min. After evaporation, 50 μL of freshly prepared derivatization reagent consisting of ethanol- H_2O -TEA-PITC (7:1:1:1 [v/v/v/v]) was added and the sample was incubated for 20 min at room temperature. The sample was evaporated to dryness and stored at - 20 °C until HPLC analysis. According to the same procedure as the sample, amino acids in standard solution (type H) from Wako Pure Chemical Industries, LTD. supplemented with asparagine, glutamine and hydroxyproline were derivatized with PITC.

HPLC Analysis for Amino Acids.

HPLC analysis for amino acids was performed by the method of Yamaya and Matsumoto (1988). PTC-amino acids were

dissolved in 0.2 mL of solvent A (0.1 M ammonium acetate pH 6.5, 2.5 % [v/v] acetonitrile) and syringe-filtered (0.45 μ m). Twenty μ L of the sample was applied to an HPLC system equipped with a Cosmosil 5C18 (4.6 $\times$ 150 mm) column (Nacalai tesque Inc, Kyoto). For Asp, Asn, Glu and Gln, PTC-amino acids were separated by a isocratic elution for 15 min at 45 °C and at a flow rate of 2.0 mL min⁻¹ and detected at 254 nm. Individual peaks were collected to a vial by manual handling and the radioactivity was determined in a sample cocktail with an LSC. The column was washed with solvent B (60 % [v/v] acetonitrile) for 5 min after the separation of amino acids. All the solvents were degassed and filtered prior to HPLC analysis.

Ammonium Quantification.

One mL of root extract (root-soluble fraction) was placed in the outer room of a Warburg flask and 1 mL of 0.5 N H₂SO₄ was placed in the inner room of the flask. Gaseous NH₃ released by the addition of 1 mL of saturated borate buffer (pH 10) to the outer room was collected into the inner room for 2 d at 30 °C.

Ammonium concentration was colormetrically measured by the method of McCullough (1967). Sample (0.2 mL) was mixed with 2.5 mL of solution C (10 g phenol and 100 mg Na nitroprusside L⁻¹) and 2.5 mL of solution D (10 g NaOH, 9.43 g Na₂HPO₄·12H₂O, 31.8 g Na₃PO₄·12H₂O and 10 mL 5.25 % NaClO L⁻¹). The mixture was incubated for more than 35 min at 37

°C, followed by reading the absorbance at 625 nm. Ammonium concentration was determined with the calibration curve prepared with NH_4Cl standard solutions (0, 0.05, 0.1, 0.2 and 0.45 mM).

2.3 Results

The rate of dark carbon fixation was examined in roots of young wheat plants supplied with ammonium and pretreated with or without MSX (Fig. 2-1). ^{14}C -bicarbonate feeding was imposed for 3 h on the plants having different careers of ammonium treatment and ammonium was supplied during the feeding. The rate of dark carbon fixation was increased as a period of the pretreatment with ammonium was prolonged (Fig. 2-1). When the plants were grown on ammonium media for 3 h, the rate of the dark carbon fixation became two times as high as that in -N plants. The plants pretreated with ammonium for 1 and 3 d showed the remarkable stimulation of dark carbon fixation. The rates were 6-fold higher compared to the rate in -N plants. The rates obtained in the present study were much higher than the rates reported for rice and tomato roots by Ikeda et al. (1992). They showed that the rate in ammonium-fed plants was 3-fold increased at the highest compared to the rate in -N plants. A significant difference of the stimulation of the dark carbon fixation in wheat roots pretreated with ammonium

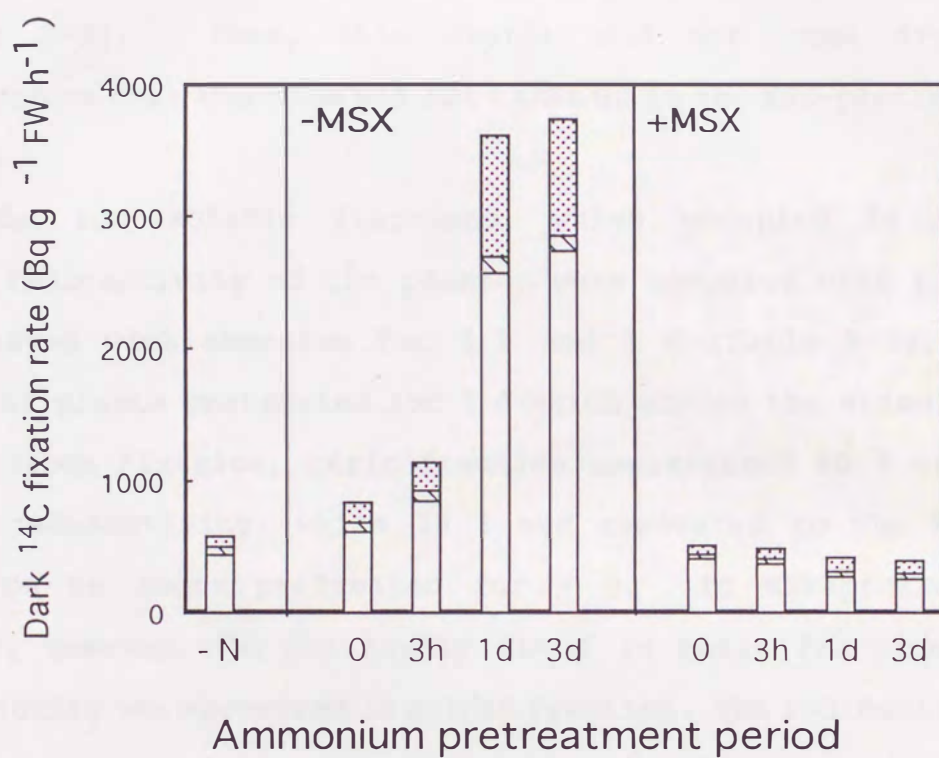


Fig. 2-1. Dark $\text{H}^{14}\text{CO}_3^-$ fixation rate in wheat roots supplied with ammonium.

shoot fr.
 root-insoluble fr.
 root-soluble fr.

between 3 h and 1 d (Fig. 2-1). On the other hand, all the MSX-pretreated plants, failing to assimilate ammonium, did not display the stimulation of the dark carbon fixation despite the supply of ammonium (Fig. 2-1). However, MSX-pretreated plants contained substantial amounts of ammonium (Table 2-2). Thus, this result did not come from a consequence that ammonium was not taken up in the MSX-pretreated plants.

The root-soluble fractions, which occupied 70 % of total radioactivity of the plants, were compared with plants pretreated with ammonium for 3 h and 1 d (Table 2-3). In roots of plants pretreated for 1 d which showed the stimulated dark carbon fixation, basic fraction represented 60 % of the total radioactivity, while 33 % was recovered to the basic fraction in roots pretreated for 3 h. In MSX-pretreated plants, however, ^{14}C was hardly found in basic fraction and the majority was recovered in acidic fraction. The radioactivity was not recovered in neutral fraction in all the treatments when ^{14}C -bicarbonate was fed. Similar results were obtained in maize roots supplied with nitrate or ammonium (Cramer et al. 1993).

The concentration and radioactivity of individual organic acids were measured for citrate, malate, succinate and fumarate (Table 2-4). Other several acids were also detected by HPLC analysis (data not shown). As shown in Table 2-4, malate and citrate were mainly labeled but the incorporation of ^{14}C into succinate and fumarate was

Table 2-2. Concentration of free ammonium in wheat roots.
($\mu\text{mol g}^{-1}\text{FW}$)

Ammonium pretreatment	— MSX	+ MSX
0 h	20.6 ($\pm$ 0.2)*	25.0 ($\pm$ 1.5)
3 h	30.8 ($\pm$ 1.8)	32.6 ($\pm$ 0.9)
1 d	48.2 ($\pm$ 1.3)	36.1 ($\pm$ 1.1)
3 d	51.8 ($\pm$ 2.2)	24.3 ($\pm$ 0.3)

* Standard deviation

Table 2-3. Distribution of radioactivity from ^{14}C -bicarbonate in soluble fractions of wheat roots. (%)

Ammonium pretreatment	Neutral (sugars)	Acidic (organic acids)	Basic (amino acids)
3 h — MSX	0.6	66.0	33.4
1 d — MSX	0.7	40.3	59.0
3 h + MSX	0.1	92.4	7.5
1 d + MSX	0.4	90.4	9.2

^{14}C -bicarbonate was fed for 3 h after the pretreatment with ammonium.

Table 2-4. Concentration, radioactivity and specific radioactivity of major organic acids in acidic fractions in roots of wheat plants.

		Concentration				Radioactivity				Specific radioactivity			
Ammonium pretreatment period		cit	mal	suc	fum	cit	mal	suc	fum	cit	mal	suc	fum
		$\mu\text{mol g}^{-1} \text{FW}$				$\text{Bq g}^{-1} \text{FW}$				$\text{Bq } \mu\text{mol}^{-1}$			
3h	- MSX	3.7	22.2	5.1	0.09	114	941	N.D.	N.D.	30.8	42.3	N.D.	N.D.
1d		1.6	6.2	4.0	0.06	47	214	N.D.	N.D.	29.4	34.7	N.D.	N.D.
3h	+ MSX	3.4	39.2	9.0	0.10	66	737	N.D.	N.D.	19.1	18.8	N.D.	N.D.
1d		3.0	21.9	7.4	0.07	24	392	N.D.	N.D.	7.9	17.9	N.D.	N.D.

cit : citrate mal : malate suc : succinate fum : fumarate N.D. : Not detected

Table 2-5. Concentration, radioactivity and specific radioactivity of major amino acids in basic fractions in roots of wheat plants.

		Concentration				Radioactivity				Specific radioactivity			
Ammonium pretreatment period		Asp	Glu	Asn	Gln	Asp	Glu	Asn	Gln	Asp	Glu	Asn	Gln
		$\mu\text{mol g}^{-1} \text{FW}$				$\text{Bq g}^{-1} \text{FW}$				$\text{Bq } \mu\text{mol}^{-1}$			
3h	- MSX	0.13	0.23	0.58	0.23	28	39	134	56	215	167	231	243
1d		0.20	0.50	2.87	0.54	58	66	476	73	290	132	166	135

undetectable. This may imply that carbon flow is not via succinate and fumarate for the ammonium assimilation. As ammonium was supplied longer, the concentration and radioactivity of malate and citrate were drastically declined but the specific radioactivity was not influenced (Table 2-4). In addition, an unidentified peak detected at 12.7 min of retention time in HPLC analysis showed a high radioactivity only in ammonium-treated plants (data not shown). As for the basic fractions, asparagine content was 5-fold increased from 3 h pretreatment to 1 d pretreatment and asparagine was the most dominant amino acid in quantity (Table 2-5). Cramer et al. (1993) showed that asparagine was the most ^{14}C -labeled and accumulated amino acid in maize roots supplied with ammonium when ^{14}C -bicarbonate was incorporated. On the whole, the specific radioactivity in the amino acids was considerably higher compared to that in the organic acids (Tables 2-4, 2-5). The reason may be due to the larger non-radioactive pool of organic acids compared to amino acids.

2.4 Discussion

It has been already documented that nitrate nutrition did not stimulate the dark carbon fixation in tomato and rice roots (Ikeda et al. 1992). Hence, the present study was focused on ammonium nutrition. Consistent with the

previous work in tomato and rice roots (Ikeda et al. 1992) and in maize roots (Cramer et al. 1993), ammonium nutrition did stimulate the rate of the dark carbon fixation in wheat roots (Fig. 2-1). However, the plants pretreated with ammonium for less than 3 h did not show the marked increase which was observed in plants pretreated for 1 and 3 d (Fig. 2-1). The reaction of dark carbon fixation mostly occurs as the carboxylation of PEP. Therefore, the stimulation of the dark carbon fixation in roots may be linked to *in situ* root PEPC activity, the supply of PEP due to carbohydrate degradation in roots and the translocation of photosynthates from shoots. MSX pretreatment experiment may propose that ammonium assimilation is a requisite for the stimulated dark carbon fixation in roots rather than the entry of ammonium itself (Fig. 2-1, Table 2-2). However, the supply of glutamine, the product of ammonium assimilation, did not stimulate the bicarbonate incorporation although the supply of ammonium resulted in the stimulated bicarbonate incorporation in cultured cells of *Acer pseudoplatanus* (Goodchild and Givan 1991).

There was a marked difference in the distribution of ^{14}C in root-soluble fractions (Table 2-3). The plants which showed the stimulated dark carbon fixation by the supply of ammonium represented higher radioactivity in the basic fraction than in the acidic fraction (Table 2-3). When the rate of the dark carbon fixation for the ammonium assimilation was low, citrate and malate were heavily labeled (Table 2-4).

Therefore, C₄-dicarboxylic acids synthesized by the dark carbon fixation were mainly converted to citrate and malate in this case. On the other hand, when the dark carbon fixation was stimulated by the supply of ammonium, the radioactivity in citrate and malate became low (Table 2-4). These results imply that the carbon flow to amides through organic acids was accelerated when the dark carbon fixation was stimulated for ammonium assimilation.

In the basic fractions, asparagine was strongly labeled (Table 2-5). Similar trends were observed in maize roots by the supply of ammonium rather than by the supply of nitrate (Cramer et al. 1993). These results confirm that C fixed by the dark carbon fixation in roots is of great importance for the replenishment of carbon skeletons to assimilate ammonium and to prevent its toxicity.

Generally, the plants accumulate asparagine and/or glutamine in roots when ammonium is supplied (Oaks and Hirel 1985 ; Joy 1988). Which amide compound is dominantly accumulated in roots appears to depend on plant species. As shown in Table 2-3, MSX-pretreatment consistently repressed the incorporation of ¹⁴C into basic compounds even if ammonium was supplied. Since MSX inhibits the action of GS, this result suggests that glutamine is the primary assimilate of ammonium in wheat roots. However, wheat roots specifically accumulated asparagine and the C fixed by the dark carbon fixation was also utilized for asparagine synthesis (Table 2-5). Since the radioactivity was not detected in succinate

and fumarate among the TCA cycle intermediates examined, it is considered that the carbon skeletons of asparagine are not compounds which come through citrate and 2-OG in the TCA cycle but are OAA which was produced in the dark carbon fixation. In other words, it is likely that OAA produced in the dark carbon fixation was directly converted to aspartate and subsequently to asparagine.

2.5 Summary

The rate of dark carbon fixation in roots of young wheat plants differing in ammonium pretreatments was determined using ^{14}C -bicarbonate since the dark carbon fixation was considered to be responsible for C_4 -dicarboxylic acid synthesis necessary for the imperative assimilation of ammonium. As a period of ammonium pretreatment was prolonged, the rate of dark carbon fixation was accelerated. The rate in plants pretreated with ammonium for 1 or 3 d was about 6-fold higher than the rate in plants grown on N-free media. On the other hand, the pretreatment with MSX did not result in such a stimulation despite the entry of ammonium into roots. These results indicated that the dark carbon fixation was stimulated in a gradual manner in response to the supply of ammonium. It was shown that the stimulation required not only the absorption of ammonium but also the assimilation of ammonium in roots.

As a period of ammonium pretreatment was prolonged, the incorporation of ^{14}C derived from bicarbonate into organic acids such as malate and citrate was decreased and asparagine was strongly labeled. Therefore, C fixed by the dark carbon fixation was exclusively utilized for the synthesis of asparagine, which is one of important nitrogenous compounds in accumulation and export of N. It was made clear that the stimulation of dark carbon fixation in roots was closely associated with the replenishment of carbon skeletons for ammonium assimilation to prevent its toxicity.

Chapter 3

CHANGES IN PHOSPHOENOLPYRUVATE CARBOXYLASE ACTIVITY IN ROOTS DURING AMMONIUM NUTRITION

3.1 Introduction

Ammonium nutrition influenced carbon metabolism in roots and stimulated the dark carbon fixation for amide synthesis (Chapter 2). The dark carbon fixation is mainly mediated by PEPC although some other carboxylating enzymes exist in roots (Izui 1994).

Extensive work has been dedicated to PEPC in leaves of C_4 and CAM plants because of its significant function as a primary CO_2 assimilating enzyme in photosynthesis (Jiao and Chollet 1991 ; Rajagopalan et al. 1994). Recently, the informations have been accumulated on PEPC from other species than C_4 and CAM plants. According to the previous work on plant PEPC, it is well-known that PEPC activity is regulated by two mechanisms (Chollet et al. 1996). One is a short-term

modulation such as metabolite effects and reversible protein phosphorylation, which is effective at a post-translational level. The other is a long-term modulation including *de novo* protein synthesis, that is a regulation at a transcriptional or translational level.

In addition, it is reported that PEPC activity was increased by the supply of ammonium in green algae (Schuller et al. 1990a) and N-stressed maize leaves (Sugiharto and Sugiyama 1992). When ammonium was produced in N_2 fixation and in nitrate reduction, the increase in PEPC activity was noticed in soybean root nodules (Schuller and Werner 1993) and in illuminated wheat leaves supplied with nitrate (Van Quy et al. 1991), respectively. Moreover, Sugiharto et al. (1992) reported that glutamine induced N-dependent accumulation of mRNAs encoding PEPC in maize leaf. Therefore, PEPC activity seems closely related to nitrogen metabolism that involves the ammonium assimilation.

It was documented that NADH-dependent GOGAT was responsive to ammonium in rice roots, depending on *de novo* protein synthesis (Yamaya et al. 1995). To evaluate the role of PEPC in the stimulation of dark carbon fixation, the activity and amounts of PEPC in the roots of plants were determined in relation to ammonium nutrition.

3.2 Materials and Methods

3.2.1 Time-course Changes in PEPC Activity in Roots of Plants Fed with Different Nitrogen Sources.

Plant Growth.

Seeds of wheat (*Triticum aestivum* L. var. Roland), barley (*Hordeum vulgare* L. var. Fuji Nijou) and tomato (*Lycopersicon esculentum* Mill. var. Ohgata Fukuju) plants were germinated on moist filter paper for 2 d (4 d in tomato) at 25 °C in the dark. The seedlings were grown as described in Chapter 2.

Nitrogen Treatment and Rapid Preparation of Root Extracts.

Prior to nitrogen treatment, 21-d-old wheat and barley plants and 47-d-old tomato plants were supplied with the N-free 1/4 strength modified Hoagland solution (pH 6, Table 2-1) for 3 d. Subsequently, the plants were supplied with the 1/4 strength nutrient solution containing 4 mM NaNO₃, 2 mM (NH₄)₂SO₄ or no N. The fresh roots were periodically harvested and immediately subjected to extraction. The nutrient solution was renewed every second day.

One g of fresh roots washed with deionized water was chopped and homogenized in an ice-cold mortar with a pestle with 0.1 g of PVPP, 1 g of washed sea sand, 5 mL of extraction medium containing 50 mM imidazole-HCl (pH 7.1), 20 % (v/v) glycerol, 5 mM MgCl₂, 5 mM NaF, 5 mM 2-mercaptoethanol

and 1 mM fresh PMSF (from 100 mM ethanolic solution). After another addition of 2.5 mL of extraction medium, further homogenization was completed. The homogenate was filtered through one layer of Miracloth (Calbiochem) and the filtrate was centrifuged at 30,000 g for 30 min at 4 °C. The clear supernatant was kept on ice until use.

Standard PEPC Assay.

The PEPC activity was spectrophotometrically measured by monitoring NADH oxidation at 340 nm with an assay mixture for the determination of the carboxylation reaction coupled to exogenous malate dehydrogenase activity at 30 °C (V-530 model, JASCO). The assay mixture contained 50 mM HEPES-KOH (pH 8.0), 0.2 mM NADH, 10 mM MgCl₂, 10 mM NaHCO₃, 2 units of malate dehydrogenase (from pig heart, Boehringer Mannheim) and 2 mM PEP (trisodium salt) in a final volume of 0.8 mL. One unit of PEPC activity corresponds to the oxidation of 1 μmol NADH per minute under standard assay conditions. The data was obtained from two independent plants. The extinction of 6.2 of 1 mM NADH solution at 340 nm was employed.

3.2.2 Changes in Amounts of PEPC Protein in Roots of Tomato Plants Fed with Different Nitrogen Sources.

SDS-PAGE.

SDS-PAGE was according to the method of Laemmli (1970). Sample was mixed with an equal volume of SDS sample buffer

and boiled for 5 min. The sample was resolved in a slab gel (10 % separation and 4 % stacking gel) at 20 mA.

The gel was stained with CBB solution (1 g of CBB R-250, 250 mL of methanol and 50 mL of acetic acid 500 mL⁻¹) and destained with 5 % methanol and 10 % acetic acid solution.

Production of Antiserum to Tomato Root PEPC.

Partially purified PEPC from tomato roots (Chapter 4) was desalted through a Sephadex G-25 column and then concentrated by lyophilization. The lyophilized proteins were dissolved in SDS sample buffer and resolved by SDS-PAGE (10 %). After the gel was stained with CBB, the band corresponding to PEPC subunit was excised from the gel with a razor blade and pulverized in a mortar. The pulverized gel was suspended in 10 mM Na phosphate buffer (pH 7.2) containing 150 mM NaCl.

A 4-week-old female mouse (strain : ddY) was immunized by five intraperitoneal injections with the gel suspension. A total of 30 µg of PEPC protein was injected per mouse over 50 d.

In order to check the antiserum production, a few µL of the blood was collected from the mouse's tail and the serum was obtained by centrifugation. Antiserum production was tested with the serum by ELISA (Fig.3-1).

After the production of the antibodies was confirmed, whole blood was collected from axillary plexus. The blood was allowed to coagulate at 37 °C for 1 h and then to stand

96 well ELISA plate (flat bottom)

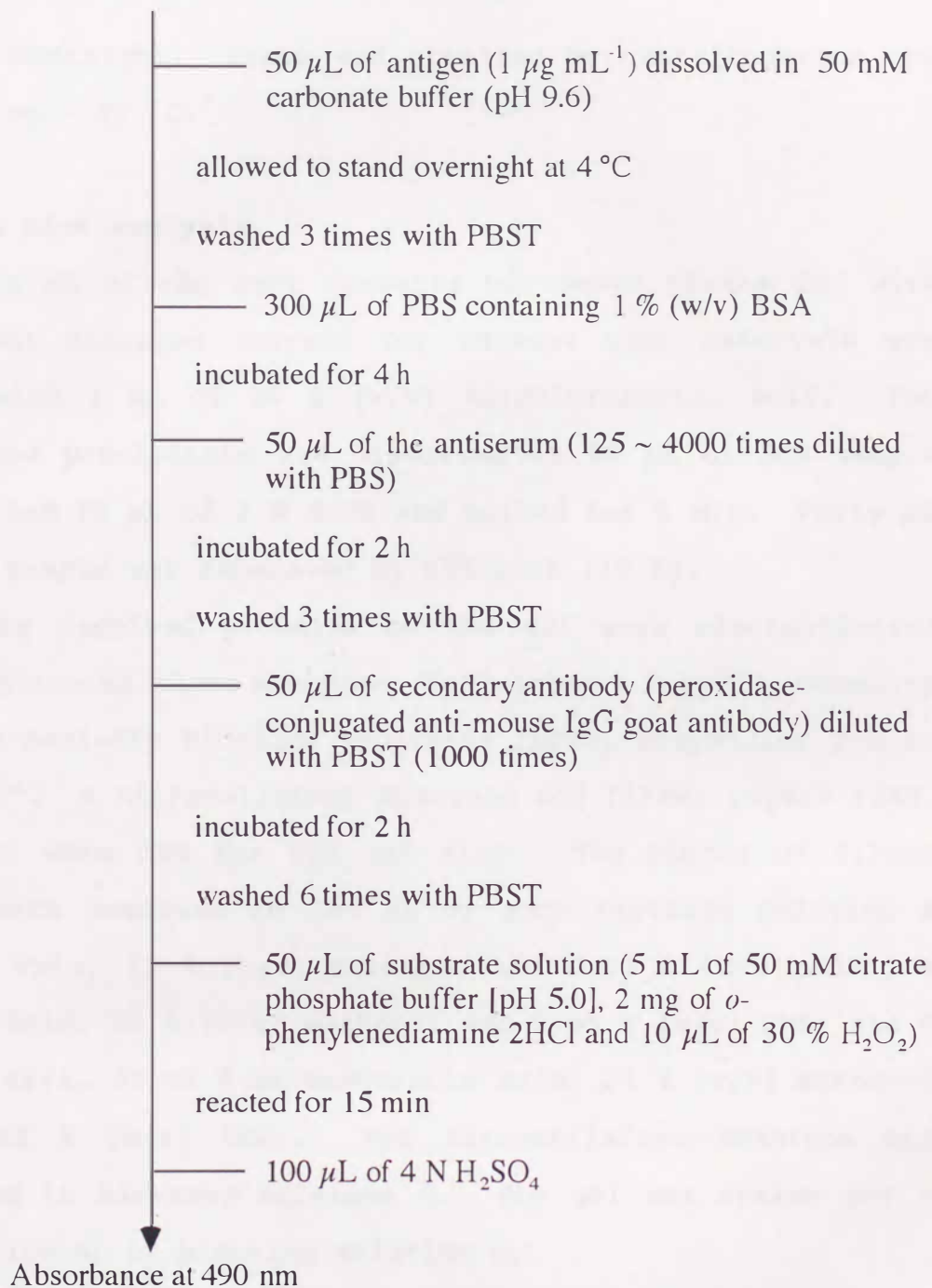


Fig. 3-1. Procedure for ELISA
(Enzyme-linked immunosorbent assay) .

PBS : 10 mM Na phosphate buffer (pH 7.2) plus 0.15 M NaCl

PBST : PBS plus 0.05 % Tween-20

All above steps were carried out at room temperature unless otherwise mentioned.

Secondary antibody was purchased from Organon Teknika Corp.

on ice overnight. Serum was obtained by centrifugation and stored at - 80 °C.

Western blot analysis.

One mL of the root extracts of tomato plants fed with different nitrogen sources for various time intervals was mixed with 1 mL of 30 % (w/v) trichloroacetic acid. The collected precipitate was dissolved in 80 μ L of SDS sample buffer and 20 μ L of 2 N NaOH and boiled for 5 min. Forty μ L of the sample was separated by SDS-PAGE (10 %).

The resolved proteins on the gel were electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Germany) using a semi-dry blotting apparatus (ATTO, Tokyo) for 2 h at 1 mA cm⁻². A nitrocellulose membrane and filter papers (3MM, whatman) were cut for the gel size. Two pieces of filter paper were immersed in 100 mL of each blotting solution A (0.3 M Tris, 20 % [v/v] methanol and 0.05 % [w/v] SDS), B (25 mM Tris, 20 % [v/v] methanol and 0.05 % [w/v] SDS) and C (25 mM Tris, 40 mM 6-aminohexanoic acid, 20 % [v/v] methanol and 0.05 % [w/v] SDS). The nitrocellulose membrane was immersed in blotting solution C. The gel was shaken for 5 min in 100 mL of blotting solution C.

After electroblotting, the immunoreactive bands on the membrane were visualized as shown in Fig. 3-2.

Electroblotted proteins on the nitrocellulose membrane

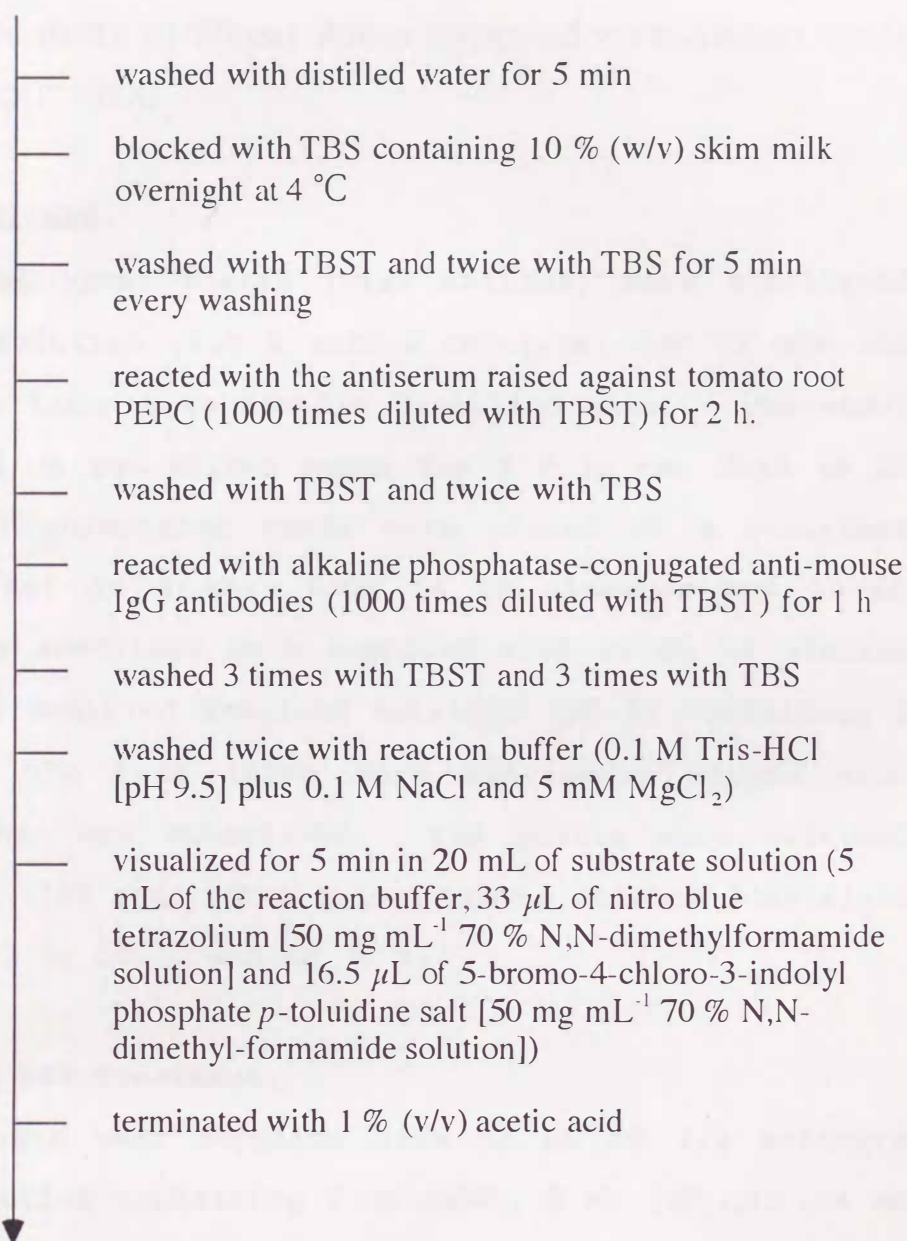


Fig. 3-2. Visualization of immunoreactive proteins on the nitrocellulose membrane.

TBS : 20 mM Tris-HCl buffer (pH 7.5) plus 0.5 M NaCl

TBST : TBS plus 0.05 % Tween-20

All above steps were performed at room temperature unless otherwise stated.

Substrate stock solutions were stored at - 20 °C.

The secondary antibody was purchased from Organon Teknika Corp.

3.2.3 PEPC Activity in Wheat Roots Supplied with Amino Acids with or without MSX.

Germ-free Culture.

Seeds of wheat plants (var. Saitama) were sterilized with NaClO solution (1.5 % active chlorine) for 15 min and washed three times with sterile distilled water. The seeds were imbibed on wet filter paper for 3 d in the dark at 20 °C. The 13 germinated seeds were placed on a stainless steel wire net in a test tube (4 cm diameter and 20 cm depth). The seedlings were supplied with 50 mL of sterile 1/8 strength modified Hoagland solution (pH 6) containing 2 mM NaNO_3 . The test tubes were previously capped with aluminium foil and autoclaved. The plants were cultured with shaking (100 rpm) for 5 d in a growth chamber (day/night cycle 12 h/12 h, 20 °C and RH 70 %).

Nitrogen and MSX Treatment.

The plants were supplied with 50 mL of 1/4 strength nutrient solution containing 4 mM NaNO_3 , 2 mM $(\text{NH}_4)_2\text{SO}_4$, 4 mM Na glutamate, 4 mM glutamine or 4 mM asparagine. In MSX-treatments, 50 μM MSX was supplemented throughout the N supply. To all the treatment solution, 100 μL of antibiotics mixture (5000 units of penicillin, 5 mg of streptomycin and 10 mg of neomycin mL^{-1} , Sigma) was added. The treatment solution was renewed every day. The plants were subject to the treatments for 3 d. Each treatment was duplicated. The

nutrient solutions containing inorganic nitrogen were autoclaved. The amino acids and MSX stock solution were syringe-filtered (0.20 μm cellulose acetate membrane, Toyo Roshi Kaisha, Ltd., Tokyo).

Crude root extracts were rapidly prepared as described previously in this chapter (3.2.1). The activity and amounts of PEPC and GS activity were examined for the extracts.

GS (Transferase) Assay.

GS transferase activity was assayed according to Rhodes et al (1975). The composition of the reaction mixture was 100 μL of 1 M imidazole-HCl (pH 6.4), 250 μL of 0.26 M L-glutamine, 100 μL of 0.18 M $\text{NH}_2\text{OH}\cdot\text{HCl}$, 100 μL of 25 mM MnCl_2 , 100 μL of 2 mM ADP, 100 μL of 0.33 M $\text{Na}_2\text{HAsO}_4\cdot 7\text{H}_2\text{O}$ and 250 μL of enzyme solution. The mixture was incubated for 15 min at 30 °C and then the reaction was terminated with 1 mL of ferric chloride reagent (0.37 M FeCl_3 , 0.67 N HCl and 0.2 M trichloroacetic acid). Absorbance of the mixture was read at 540 nm. For calibration, γ -GHA (L-glutamic acid γ -monohydroxamate ; 0 ~ 3 $\mu\text{mol mL}^{-1}$) standard solution was mixed with the ferric chloride reagent.

3.3 Results

For wheat, barley and tomato plants, time-course

changes in extractable root PEPC activity on a fresh weight basis were examined in response to different forms of N (Fig. 3-3). In all the three species, nitrate-fed plants showed a slightly higher root PEPC activity than -N plants but the root PEPC activities in nitrate-fed and -N plants remained unchanged throughout the nitrogen treatment. On the other hand, the PEPC activity in roots of ammonium-fed plants gradually and consistently increased. Finally, ammonium-fed plants showed 2- to 2.5-fold higher PEPC activities compared to nitrate-fed plants at 7 d after the nitrogen treatment. Consistent with these results, Arnozis et al. (1988) showed that ammonium-fed plants had 2- to 3-fold higher PEPC activities than nitrate-fed plants at 12 d in maize, wheat and tomato roots. The increase in the activity was not observed within 3 h but 24 h after the ammonium treatment in any species (Fig. 3-3). Wheat plants exhibited about 2-fold higher root PEPC activity than barley and tomato plants, regardless of nitrogen sources.

The amounts of PEPC subunit protein in the root extracts were examined in tomato plants treated with the different nitrogen sources for a few days. By immunoblot analyses, tomato root contained PEPC subunit proteins with about 106 kDa of molecular weight (Fig. 3-4). Each lane included the soluble proteins equivalent to 47 mg fresh roots. The immunoblot analyses revealed that the ammonium nutrition showed the most positive effect on the amounts of PEPC subunit protein in tomato roots among the three nitrogen

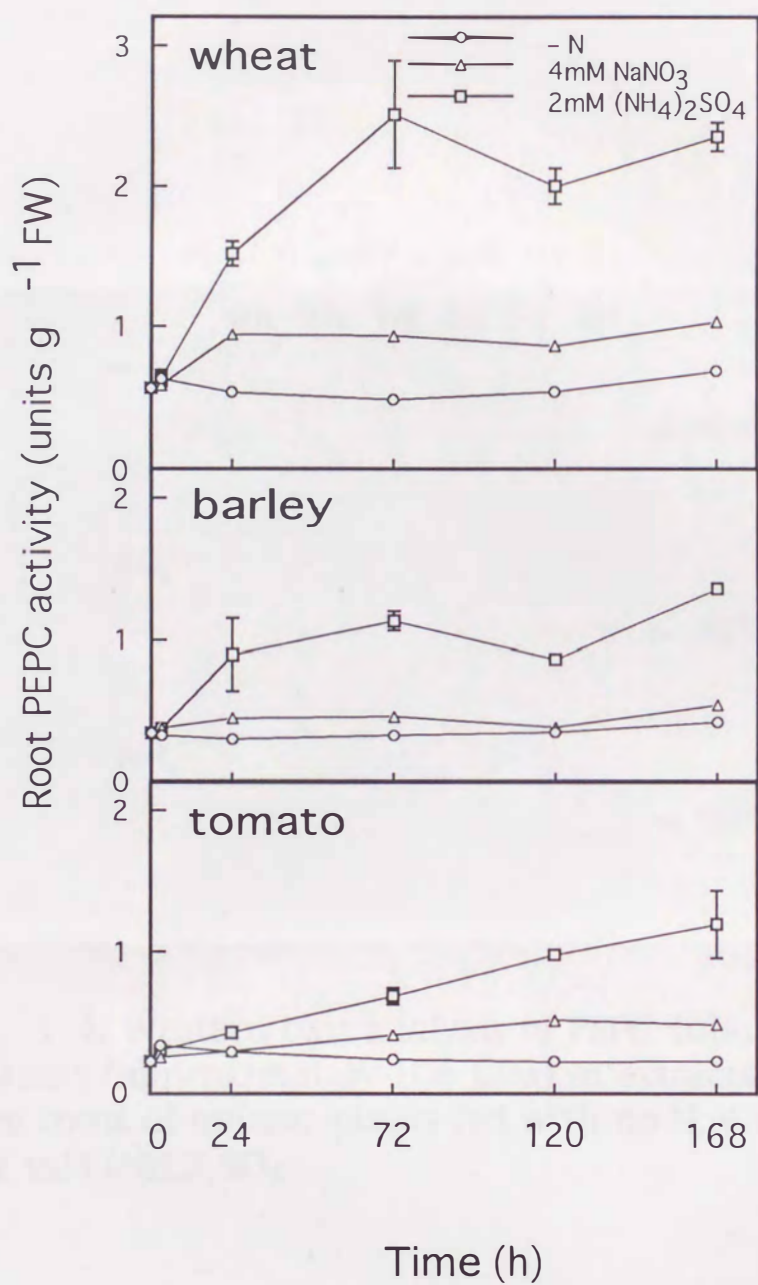


Fig. 3-3. Time-course changes in extractable PEPC activity from roots of wheat, barley and tomato plants fed with no N, nitrate or ammonium.

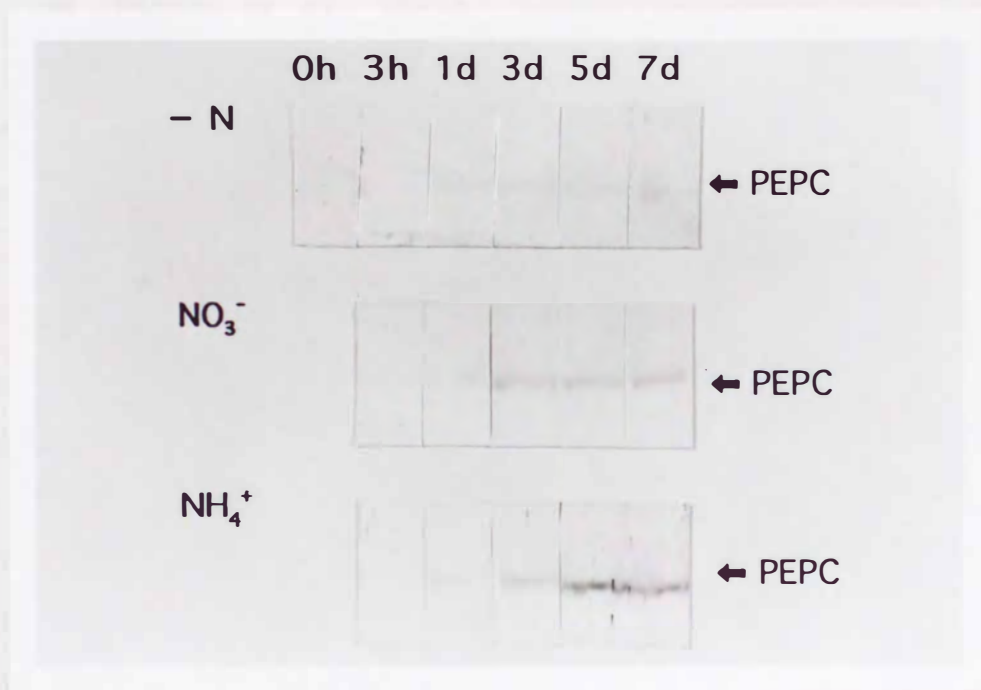


Fig. 3-4. Western blot analysis of PEPC subunit proteins (approximately 106 kDa) in extracts prepared from roots of tomato plants fed with no N, 4 mM NaNO_3 or 2 mM $(\text{NH}_4)_2\text{SO}_4$.

treatments. In addition, the intensity of the immunoreactive bands seemed to coincide with the extractable root PEPC activities (Figs. 3-3, 3-4). As observed in tomato root PEPC, the activity of NADH-dependent GOGAT in rice roots was coincided with amounts of this enzyme protein in response to ammonium (Yamaya et al. 1995).

PEPC activity was measured in wheat roots when the plants were supplied with several amino acids as organic nitrogen sources and treated with or without MSX. The supply of asparagine or glutamine at a concentration of 4 mM resulted in a 1.5-fold higher root PEPC activity than the supply of ammonium (Fig. 3-5). The supply of glutamate and ammonium increased the PEPC activity to a similar extent with each other. In contrast, the PEPC activity was repressed in all the roots where the action of GS was inhibited by MSX treatment although ammonium was supplied. Speculating from the report on N-stressed maize leaves of Sugiharto et al. (1992), it was expected that glutamine would increase the PEPC activity even if the roots were treated with MSX. However, the different result was obtained in wheat roots of the present study. In addition, levels of the PEPC activity in wheat roots with several kinds of nitrogen sources were proportional to the amounts of PEPC subunit protein revealed by Western blot analyses (Fig. 3-6).

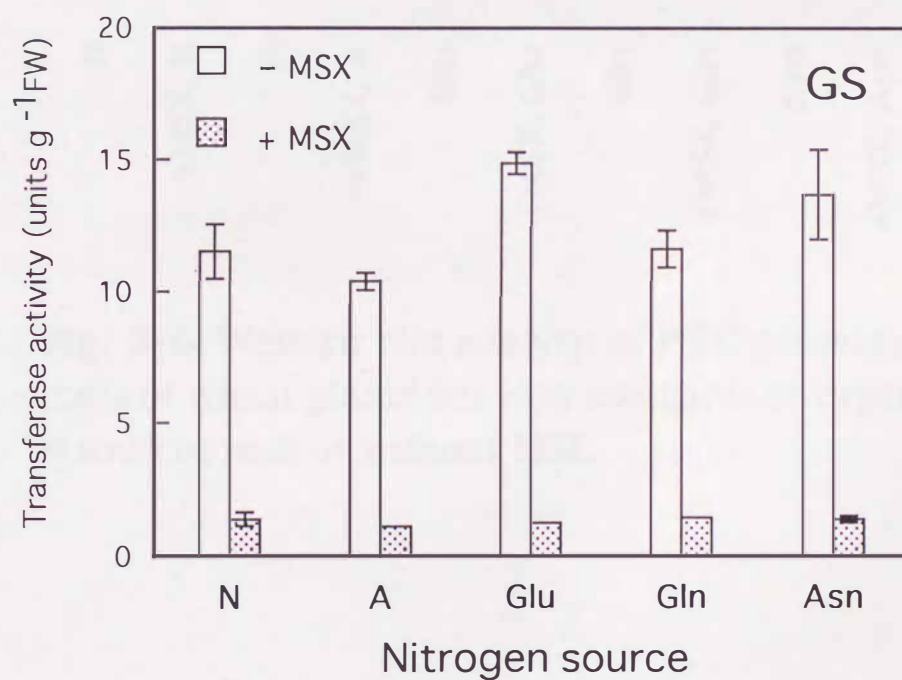
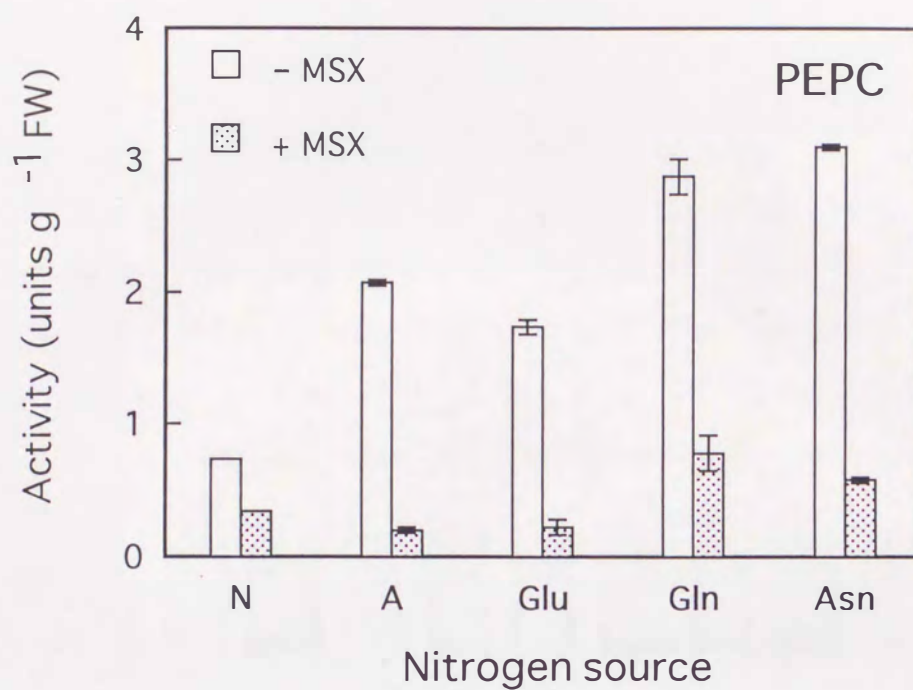


Fig.3-5. PEPC and GS activities in roots of wheat plants supplied with amino acids with or without MSX.

N; nitrate A; ammonium

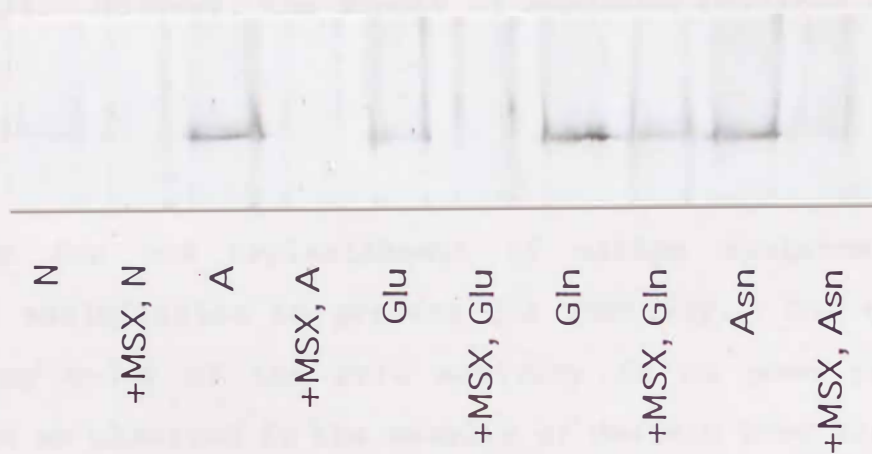


Fig. 3-6. Western blot analysis of PEPC proteins in roots of wheat plants fed with inorganic or organic N sources with or without MSX.

3.4 Discussion

This chapter was focused on the response of PEPC activity to nitrogen sources to make clear the function of the enzyme in the replenishment of carbon skeletons during ammonium assimilation in plant roots. Nitrate-fed plants showed a higher root PEPC activity than -N plants, implying that a part of nitrate was reduced in roots in those species (Fig. 3-3). However, the supply of ammonium resulted in the most conspicuous increase in root PEPC activity. The work of Arnozis et al. (1988) supports the results of the present study. Increased PEPC activity in roots is unequivocally favorable for the replenishment of carbon skeletons for ammonium assimilation to prevent its toxicity. One of the regulating modes of the PEPC activity is *de novo* protein synthesis as observed in the results of Western blot analyses (Fig. 3-4). Increases in both activity and amounts of NADH-dependent GOGAT were also found in response to ammonium in rice roots (Yamaya et al. 1995). Therefore, in roots of plants during ammonium nutrition, *de novo* synthesis of PEPC and GOGAT protein is a cardinal strategy for ammonium assimilation or its detoxification.

When GS was inhibited by the treatment with MSX, the supply of ammonium did not result in the increases in both PEPC activity and PEPC protein (Figs 3-5, 3-6). Hence, the assimilation of ammonium by the action of GS is necessary for the induction of synthesis of PEPC protein in roots. In

other words, glutamine, the product of the GS reaction, or its down-stream compounds seem to be responsible for the increase of PEPC activity, depending on *de novo* synthesis.

Manh et al. (1993) reported that the supply of glutamine or the treatment with azaserine, which causes an accumulation of glutamine by inhibiting the action of GOGAT, resulted in the substantial increase in PEPC activity when wheat leaves were supplied with various nitrogen treatment solutions through cut end. In the present study, however, the supply of asparagine also resulted in the increases in both activity and amounts of PEPC in wheat roots (Figs. 3-5, 3-6). As shown in Table 2-5, wheat roots accumulated asparagine rather than glutamine. In this species, amides and their down-stream compounds were likely to be a trigger of the induction of PEPC activity. On the contrary, Sivasankar and Oaks (1996) showed that there was no effect on a level of PEPC protein when asparagine or glutamine was supplemented to maize roots. Therefore, the effects of these amides on the synthesis of PEPC protein may not yet be conclusively clarified.

3.5 Summary

In Chapter 2, it was revealed that the supply of ammonium consistently stimulated the dark carbon fixation in roots. In order to understand the mechanism of the stimulation,

attentions were paid to root PEPC activity since the dark carbon fixation was predominantly mediated by PEPC.

In wheat, barley and tomato plants, the extractable activity of PEPC in roots of plants treated with no N or nitrate remained unchanged throughout the treatments. In contrast, the PEPC activity was gradually and consistently increased by the supply of ammonium. It was made clear that PEPC activity in roots specifically responded to the supply of ammonium. Western blot analyses revealed that in tomato roots the amounts of PEPC proteins were increased gradually by the supply of ammonium and reflected the changes in extractable activity of PEPC. This result shows that the increase in PEPC activity in roots is due to *de novo* synthesis of PEPC protein.

When wheat roots were treated with MSX during the supply of ammonium, PEPC activity and amounts of PEPC protein in the roots were not increased by the supply of ammonium. The supply of glutamine or asparagine had a remarkable effect on the induction of PEPC activity. From these results, it is concluded that *de novo* synthesis of PEPC protein induced by the assimilates of ammonium contributes to the replenishment of carbon skeletons for ammonium assimilation in roots.

Chapter 4

PURIFICATION OF ROOT PHOSPHOENOLPYRUVATE CARBOXYLASE AND POST-TRANSLATIONAL REGULATION OF ITS ACTIVITY

4.1 Introduction

It is well documented that the activity of plant PEPC responsible for non-photosynthetic carbon fixation is regulated by post-translational modulations. The regulation of PEPC activity was first studied in leaves of C_4 (Doncaster and Leegood 1987 ; Jiao and Chollet 1988) and CAM plants (Winter 1982 ; Brulfert et al. 1986). The PEPC activity from these sources is controlled by so-called metabolite effectors such as malate and glucose-6-phosphate (Winter 1982 ; Doncaster and Leegood 1987). Similar to C_4 and CAM leaves, it has been shown that PEPC activities from C_3 leaves (Gupta et al. 1994), soybean nodules (Schuller et al. 1990b) and green algae (Schuller et al. 1990a) are modulated by metabolite effectors although there are considerable differences in the

intensity of the effects among various effectors.

The supply of ammonium affects the intracellular levels of various compounds in barley roots (Rigano et al. 1996) and green algal cells (Turpin et al. 1990). Hence, it is considered that the changes in metabolite concentration may alter *in vivo* PEPC activity according to its regulatory properties.

PEPC from C₄ and CAM leaves undergoes reversible protein phosphorylation that is mediated by protein kinase and phosphatase (Brulfert et al. 1986 ; Jiao and Chollet 1988). The seryl residue near N-terminus of the PEPC subunit is phosphorylated in a light-dependent manner (Jiao and Chollet 1990). The phosphorylated PEPC exhibits a decreased sensitivity to metabolite effectors and an increased catalytic activity (Jiao and Chollet 1990). In soybean nodules, which are not directly affected by the light/dark conditions, PEPC is potentially able to be phosphorylated (Schuller and Werner 1993 ; Zhang et al. 1995).

This chapter was focused on the post-translational regulation of root PEPC activity. The regulatory properties of root PEPC were determined using the enzyme purified according to a newly developed strategy. The aim was set on understanding of the correlation between *in vivo* PEPC activity and changes in metabolite levels in roots caused by ammonium nutrition.

4.2 Materials and Methods

4.2.1 Purification of PEPC from Tomato Roots.

Plant Growth and Preparation of Crude Extract.

Tomato plants (*Lycopersicon esculentum* Mill. var. Fukuju Nigo) were hydroponically grown and supplied with 1/4 strength modified Hoagland solution (pH 6, Table 2-1) containing 2 mM NaNO_3 for 2 months. Prior to the harvest of the roots, tomato plants were supplied with the nutrient solution containing 2 mM $(\text{NH}_4)_2\text{SO}_4$ for 5 d. The harvested roots were washed with water and stored at -70°C until extraction.

The roots (400 g FW) were pulverized in liquid N_2 and further homogenized in a chilled mortar with 1.6 L of extraction medium containing 100 mM Na phosphate (pH 7.3), 10 mM MgCl_2 , 1 mM EDTA, 15 mM 2-mercaptoethanol and 1 mM fresh PMSF. Washed sea sand and 2 % (w/v) PVPP were also included. The homogenate was filtered through two layers of Miracloth and one layer of gauze, and the clear supernatant was obtained by centrifugation at 15,000 g for 30 min at 4°C .

Fractionation by Ammonium Sulfate Precipitation.

The clear supernatant was 40 % saturated with solid ammonium sulfate and stirred for 30 min. After centrifugation, the precipitate was removed. The supernatant was brought to 70 % saturated with solid ammonium sulfate and allowed to

stand at 4 °C. The collected precipitate (40-70 % saturated fraction) contained PEPC activity.

Separation of PEPC by Column Chromatographies.

The collected precipitate was dissolved in a minimum volume of buffer A (50 mM Na phosphate [pH 7.0], 5 mM MgSO₄ and 5 mM 2-mercaptoethanol) and applied onto a Sepharose CL-6B (Pharmacia Biotech) column (3.5 × 100 cm) equilibrated with buffer A. Active fractions were collected after PEPC assay and reading the absorbance at 280 nm. Subsequently, the combined eluates were loaded onto a DEAE cellulose column (2 × 15 cm) equilibrated with buffer A. PEPC was eluted by a linear 400 mL gradient of 0-500 mM NaCl in buffer A. Collected active fractions were precipitated by the addition of solid ammonium sulfate at 70 % saturation.

The pellet obtained by centrifugation was resuspended in a minimum volume of buffer A and separated by the second gel filtration on a Sepharose CL-6B column under the same conditions as those for the first gel filtration. Combined active fractions were applied onto a Q-Sepharose (Pharmacia Biotech) column (1.2 × 9.5 cm) equilibrated with buffer A. The enzyme was eluted by a linear 100 mL gradient of 0-500 mM NaCl in buffer A. All the above steps were carried out at 4 °C.

The fractions containing a high PEPC activity were pooled and mixed with an equal volume of glycerol. The enzyme preparation obtained at the final step was stored at

- 20 °C.

The molecular weight of PEPC subunit was estimated in SDS-PAGE by the method of Weber and Osborn (1969) as standards of beta-galactosidase (116.0 kDa), phosphorylase b (97.4 kDa), BSA (66.0 kDa), egg albumin (45.0 kDa) and carbonic anhydrase (29.0 kDa). Proteins on the gel were stained by silver staining (Oakley et al. 1980).

Soluble Protein Assay.

Total soluble protein concentration was determined by the method of Bradford (1976) using BSA as a standard. Dye reagent (250 mg of CBB G-250, 265 mL of 85 % phosphoric acid and 117 mL of 99.5 % ethanol 500 mL⁻¹) was five times diluted with deionized water and filtered. Subsequently, 0.1 mL of sample was mixed with 5 mL of the diluted dye solution. The mixture was vortexed and allowed to stand for 10 min. BSA standard solutions (0, 0.2, 0.4, 0.6 and 0.8 mg mL⁻¹) were reacted as above. Absorbance was read at 595 nm and the protein concentration was determined from the calibration curve.

4.2.2 Enzymatic Properties of Tomato Root PEPC.

Metabolite Effects on Tomato Root PEPC.

Metabolite effects on PEPC activity from tomato roots were assessed using the purified enzyme with respect to organic acids, amino acids and hexose phosphates. The

enzyme (stored in 50 % glycerol) was passed through a Sephadex G-25 (Pharmacia Biotech) column equilibrated with 10 mM HEPES-KOH (pH 7) containing 20 % (v/v) glycerol and 1 mM MgSO_4 and kept on ice to keep the enzyme activity stable during measurements.

The PEPC assay was performed at an optimal pH of 8.0 or a suboptimal pH of 7.2 and at final PEP concentrations corresponding to saturation (2 mM) or subsaturation (0.2 mM). Some metabolite solutions were neutralized or adjusted to the desired pH.

Quantification of Major Organic and Amino Acids in Roots.

One g of fresh roots of tomato (var. Ohgata Fukuju) and wheat (*Triticum aestivum* L. var. Saitama) plants was homogenized in a mortar with ice-cold 80 % ethanol. The homogenate was filtered, and the filtrate was then made up to 25 mL with 80 % ethanol.

Malate, citrate and 2-OG were enzymatically quantitated. Malate and citrate were determined by the method of Delhaize et al. (1993). 2-OG was assayed according to Turpin et al. (1990) with some modifications.

Major amino acids were quantitated as PTC-amino acids with HPLC by the method of Yamaya and Matsumoto (1988).

4.2.3 Malate Sensitivity of PEPCs from Roots of Nitrate- and Ammonium-fed Tomato Plants.

Preparation of Enzyme Extracts from Tomato Roots Treated with Different Nitrogen Sources.

Tomato plants (var. Ohgata Fukuju) were supplied for 5 d with 1/4 strength modified Hoagland solution containing either 4 mM NaNO_3 or 2 mM $(\text{NH}_4)_2\text{SO}_4$. Thirty-five g of fresh roots were homogenized in a mortar with 140 mL of extraction medium (50 mM imidazole-HCl [pH 7.1], 5 mM MgCl_2 , 5 mM NaF, 5 mM 2-mercaptoethanol and 1 mM fresh PMSF) and with 3.5 g of PVPP and a small amount of washed sea sand. The clear supernatant was obtained after filtration and centrifugation and it was brought to 70 % saturation with solid ammonium sulfate. The precipitate was stirred for 1 h on ice and allowed to stand at 4 °C. The precipitate collected by centrifugation was resuspended in the extraction medium. The resuspension was desalted through a Sephadex G-25 column (2 × 24.5 cm) equilibrated with the extraction medium.

Determination of I_{50} Value for Malate.

The crude desalted PEPC was provided for the determination of malate sensitivity. PEPC activity was assayed under suboptimal conditions in the presence of malate. The sensitivity of PEPC to malate was evaluated by I_{50} value. I_{50} value was determined according to the method of Job et al. (1978).

4.3 Results

4.3.1 Purification of PEPC from Tomato Roots.

PEPC was purified from roots of tomato plants treated with ammonium as shown in Table 4-1. Purification of PEPC was originally reported in C_4 and CAM leaves containing abundant PEPC proteins. Recently, the work on C_3 plant materials showed that hydroxyapatite and hydrophobic steps were effectively employed for tobacco leaves (Wang and Chollet 1993) and cultured green tobacco cells (Sato et al. 1988). Nevertheless, when these steps were adopted to purification of PEPC from tomato roots, there was a very low recovery of activity due to the strong binding to the column material (data not shown). In place of these steps, a combination of gel filtrations and anion exchange chromatographies was employed. The presence of phosphate was found to be very effective to keep the activity stable during the extraction and the column chromatographies. Therefore, phosphate buffer solution was used in all procedures.

The crude extract from tomato roots contained a lot of root pigments detrimental to enzyme activity but the first gel filtration enabled to separate completely PEPC from the pigments. A single peak of PEPC activity was detected in each step (Figs. 4-1 to 4-4). At the end of Q-Sepharose column chromatography, PEPC was 84-fold purified and the specific activity of the partially purified enzyme was 10.3

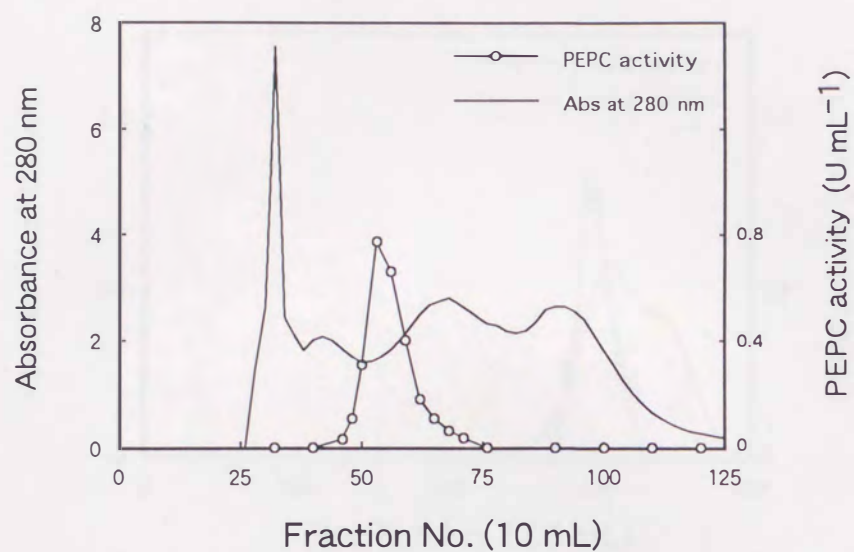


Fig. 4-1. Elution profile of gel filtration on a Sepharose CL-6B column of crude PEPC from tomato roots.

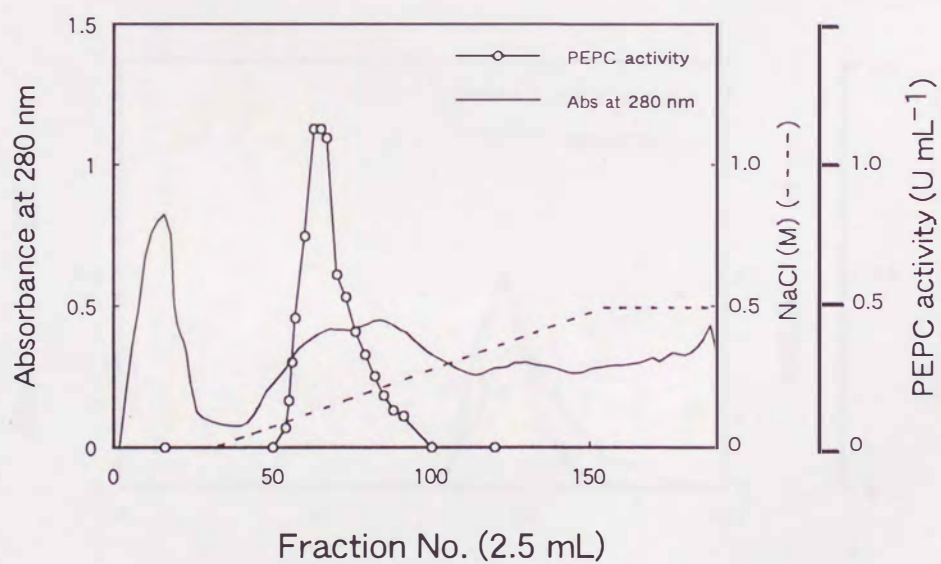


Fig. 4-2. Chromatography on a DEAE cellulose column.

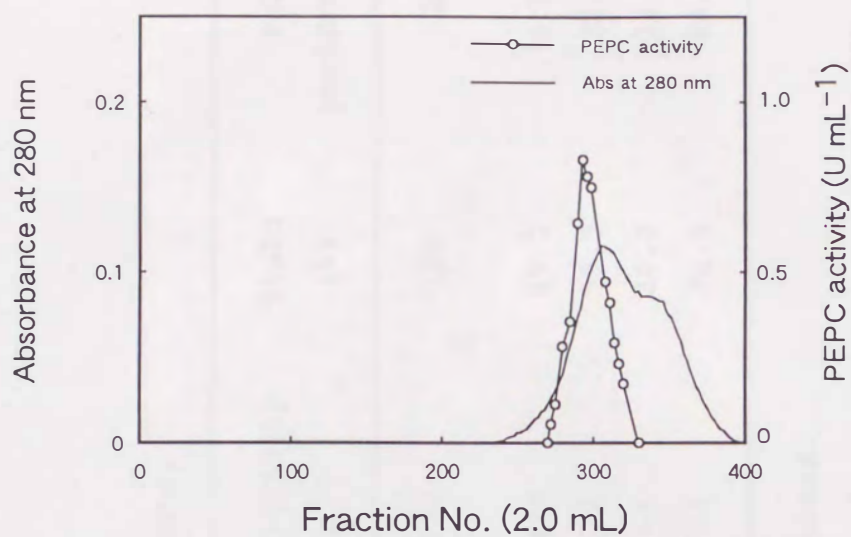


Fig. 4-3. Elution profile of the second gel filtration on a Sepharose CL-6B column.

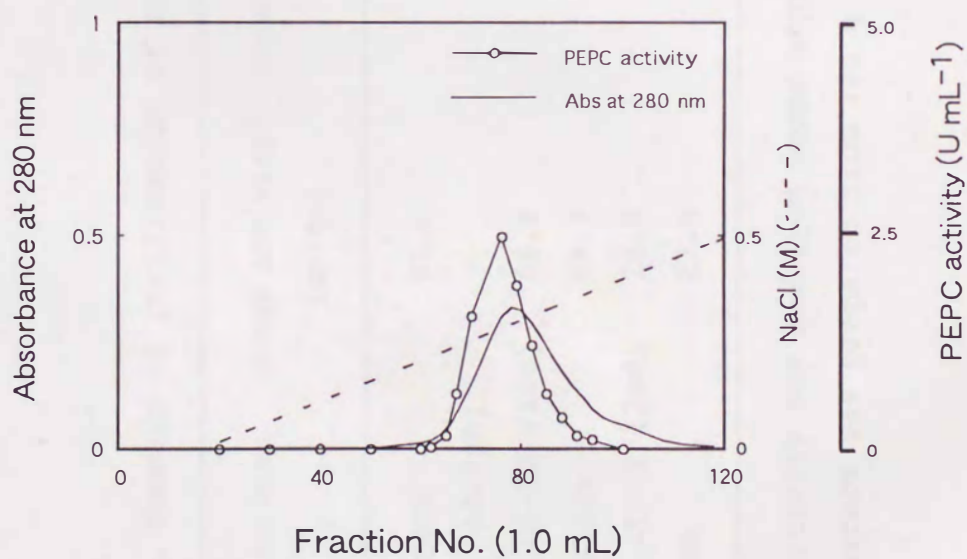


Fig. 4-4. Chromatography on a Q-Sepharose column.

Table 4-1. Summary of purification of PEPC from tomato roots.

Purification step	Total activity ^a (units)	Total protein (mg)	Specific activity (U mg ⁻¹)	Yield (%)	Fold purification
Crude extract ^b (NH ₄) ₂ SO ₄ (40-70%)	85.0	695	0.122	100	1
Sepharose CL-6B (1st)	58.8	121	0.486	69.2	3.98
DEAE-cellulose	49.7	18.3	2.72	58.5	22.3
Sepharose CL-6B (2nd)	28.9	7.36	3.93	34.0	32.2
Q-Sepharose	27.6	2.68	10.3	32.5	84.4

^a Enzyme activity was measured under standard assay conditions.

^b Crude extract was prepared from 400 g fresh roots of plants grown on ammonium medium.

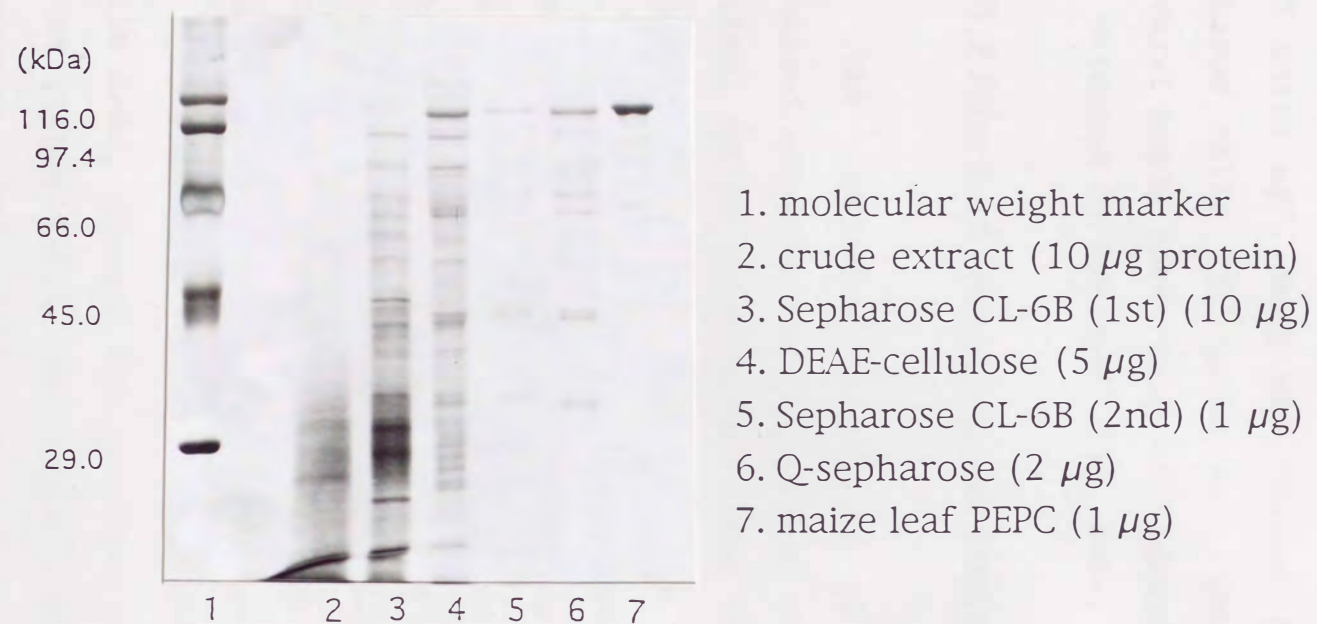


Fig. 4-5. SDS-PAGE profile of PEPC fraction from tomato roots at each purification step.

units mg^{-1} protein (Table 4-1). This value was lower than the values from C_3 plant materials such as tobacco leaves (35 units mg^{-1} , Wang and Chollet 1993) and cultured green tobacco cells (32 units mg^{-1} , Sato et al. 1988) because several bands other than PEPC subunit protein still remained as detected by SDS-PAGE (Fig. 4-5).

4.3.2 Effects of Metabolites on Tomato Root PEPC Activity.

The regulatory properties of tomato root PEPC were examined using partially purified enzyme (Table 4-1). At an optimal pH of 8.0, tomato root PEPC showed a hyperbolic kinetics for PEP in the substrate saturation curve and the K_m value for PEP was approximately 0.2 mM (Koga 1995). Thereafter, the metabolite effects were examined at final PEP concentrations equivalent to saturation (2 mM) or subsaturation (0.2 mM) and at an optimal pH of 8.0 or a suboptimal pH of 7.2 (Tables 4-2, 4-3). However, the effects were not determined with 0.2 mM PEP at pH 7.2 because of an extremely low PEPC activity.

Malate strongly inhibited PEPC activity even at 1 mM while 2-OG, citrate and pyruvate inhibited the activity at 10 mM (Table 4-2). Only acidic amino acids, aspartate and glutamate, were inhibitory. The inhibition by those acids was not observed at a 0.1 mM level. Asparagine and glutamine greatly accumulated in tomato roots in the course of ammonium assimilation (Table 4-5) but these amino acids

Table 4-2. Effects of organic acids and amino acids on activity of partially purified tomato root PEPC under different assay conditions.

Metabolite Concentration (mM)		Relative activity (%)		
		2 mM PEP pH 8.0	2 mM PEP pH 7.2	0.2 mM PEP pH 8.0
none		100	71 (100)	55 (100)
malate	10	73	5 (7)	33 (59)
	1	99	15 (21)	52 (95)
	0.1	100	60 (85)	62 (112)
2-oxoglutarate	10	117	16 (23)	43 (78)
	1	98	66 (94)	51 (93)
citrate	10	88	43 (61)	34 (61)
	1	102	65 (92)	58 (104)
pyruvate	10	49	16 (23)	29 (52)
	1	84	65 (92)	52 (94)
	0.1	97	67 (95)	54 (97)
aspartate	10	102	14 (19)	52 (94)
	1	99	49 (69)	55 (99)
	0.1	104	72 (103)	56 (100)
glutamate	10	110	36 (51)	60 (109)
	1	100	67 (95)	48 (87)
	0.1	100	73 (104)	60 (109)
asparagine	10	113	73 (103)	58 (105)
glutamine	10	112	76 (107)	55 (98)

Table 4-3. Effects of hexose phosphates on activity of partially purified tomato root PEPC under different assay conditions.

Metabolite Concentration (mM)		Relative activity (%)		
		2 mM PEP pH 8.0	2 mM PEP pH 7.2	0.2 mM PEP pH 8.0
none		100	71 (100)	55 (100)
glucose-6-phosphate	10	129	91 (129)	108 (194)
	1	109	80 (114)	96 (173)
	0.1	97	69 (98)	73 (131)
glucose-1-phosphate	10	108	80 (113)	90 (163)
	1	111	79 (112)	84 (152)
	0.1	106	75 (106)	68 (123)
fructose-6-phosphate	10	127	92 (131)	108 (195)
	1	119	84 (120)	93 (167)
	0.1	101	67 (94)	71 (129)
fructose-1,6-bisphosphate	10	100	70 (100)	51 (92)

did not affect the activity (Table 4-2). Serine, alanine, proline, threonine, glycine, arginine and lysine were also not effective (data not shown). The inhibition by organic acids and acidic amino acids was more conspicuous for 2 mM PEP at pH 7.2 than for 0.2 mM PEP at pH 8.0 (Table 4-2). In contrast to them, glucose-6-phosphate, glucose-1-phosphate and fructose-6-phosphate consistently activated the activity of root PEPC at a 1 mM level whereas fructose-1,6-bisphosphate was not effective (Table 4-3). The activation with 0.2 mM PEP at pH 8.0 by hexose phosphates was more conspicuous than the activation with 2 mM PEP at pH 7.2. If these effects were determined under more physiological conditions with 0.2 mM PEP at pH 7.2, the magnitude of the effects would be greater. ATP and ADP (10 mM) strongly inhibited the activity of PEPC (data not shown). This inhibition is probably due to their chelation with Mg^{2+} . Ammonium ions themselves had no direct effects on root PEPC activity (data not shown).

The contents of malate and 2-OG in roots were drastically reduced when ammonium was supplied for 3 d to tomato plants grown on N-free media (Table 4-4). In wheat roots that contained a high level of malate, on the other hand, only malate content was declined by the supply of ammonium (Table 4-6). The declines in contents of malate and 2-OG were not observed in both plants at 3 h after the supply of ammonium (Tables 4-4, 4-6).

When tomato plants were supplied with ammonium for 3 d,

Table 4-4. Major organic acids in roots of tomato plants treated with different sources of N. ($\mu\text{mol g}^{-1}$ FW)

N source	Treatment period	malate	citrate	2-OG
	0 h	2.50 (± 0.17)*	0.96 (± 0.28)	3.24 (± 0.16)
4 mM NaNO_3	3 h	1.76 (± 0.13)	0.34 (± 0.00)	2.20 (± 0.04)
	1 d	1.01 (± 0.14)	0.50 (± 0.19)	0.48 (± 0.18)
	3 d	2.73 (± 0.75)	0.88 (± 0.13)	0.66 (± 0.18)
2 mM $(\text{NH}_4)_2\text{SO}_4$	3 h	3.00 (± 0.00)	1.00 (± 0.02)	3.15 (± 0.07)
	1 d	0.61 (± 0.05)	0.66 (± 0.09)	1.56 (± 0.15)
	3 d	0.31 (± 0.04)	0.66 (± 0.47)	0.58 (± 0.26)

Prior to the supply of nitrate or ammonium, N-free nutrient solution was supplied for 3 d.

* Standard deviation

Table 4-5. Major amino acids in roots of tomato plants treated with different forms of N. ($\mu\text{mol g}^{-1}$ FW)

N source	Treatment period	Asp	Glu	Asn	Gln
	0 h	0.03 (± 0.00)*	0.08 (± 0.01)	0.06 (± 0.01)	0.34 (± 0.05)
4 mM NaNO_3	3 h	0.03 (± 0.01)	0.11 (± 0.01)	0.07 (± 0.02)	0.48 (± 0.00)
	1 d	0.11 (± 0.01)	0.25 (± 0.00)	0.13 (± 0.01)	1.13 (± 0.13)
	3 d	0.12 (± 0.02)	0.31 (± 0.06)	0.21 (± 0.01)	0.77 (± 0.24)
2 mM $(\text{NH}_4)_2\text{SO}_4$	3 h	0.02 (± 0.00)	0.12 (± 0.00)	0.07 (± 0.02)	2.02 (± 0.05)
	1 d	0.07 (± 0.00)	0.17 (± 0.01)	0.42 (± 0.01)	5.88 (± 0.73)
	3 d	0.14 (± 0.00)	0.40 (± 0.06)	3.39 (± 1.60)	13.42 (± 1.94)

* Standard deviation

Table 4-6. Major organic acids in roots of wheat plants treated with different sources of N. ($\mu\text{mol g}^{-1}$ FW)

N source	Treatment period	malate	citrate	2-OG
	0 h	11.88 (± 0.42)*	1.06 (± 0.24)	1.33 (± 0.32)
4 mM NaNO_3	3 h	11.06 (± 0.70)	0.68 (± 0.04)	0.84 (± 0.15)
	1 d	3.61 (± 0.24)	0.89 (± 0.05)	1.35 (± 0.13)
	3 d	3.71 (± 0.36)	0.51 (± 0.14)	0.30 (± 0.10)
2 mM $(\text{NH}_4)_2\text{SO}_4$	3 h	11.58 (± 0.28)	0.89 (± 0.16)	0.96 (± 0.16)
	1 d	5.55 (± 0.34)	0.57 (± 0.38)	0.50 (± 0.38)
	3 d	1.15 (± 0.26)	0.42 (± 0.10)	1.14 (± 0.23)

Prior to the supply of nitrate or ammonium, N-free nutrient solution was supplied for 3 d.

* Standard deviation

Table 4-7. Major amino acids in roots of wheat plants treated with different forms of N. ($\mu\text{mol g}^{-1}$ FW)

N source	Treatment period	Asp	Glu	Asn	Gln
	0 h	0.14 (± 0.00)*	0.82 (± 0.02)	0.36 (± 0.09)	0.79 (± 0.13)
4 mM NaNO_3	3 h	0.14 (± 0.03)	0.82 (± 0.04)	0.22 (± 0.07)	0.94 (± 0.10)
	1 d	0.27 (± 0.05)	1.41 (± 0.07)	0.54 (± 0.12)	1.88 (± 0.24)
	3 d	0.30 (± 0.01)	1.59 (± 0.15)	0.44 (± 0.13)	1.73 (± 0.15)
2 mM $(\text{NH}_4)_2\text{SO}_4$	3 h	0.11 (± 0.01)	0.04 (± 0.01)	0.25 (± 0.01)	3.01 (± 0.18)
	1 d	0.20 (± 0.01)	0.71 (± 0.10)	3.56 (± 0.55)	11.31 (± 2.04)
	3 d	0.47 (± 0.00)	1.87 (± 0.05)	18.99 (± 2.44)	16.41 (± 1.33)

* Standard deviation

the levels of asparagine and glutamine increased 56- and 46-fold compared to the initial levels, respectively and glutamine was the most dominant amino acid in tomato roots (Table 4-5). A small increase in glutamine was already found at 3 h. In wheat roots, glutamine and asparagine were greatly increased by the supply of ammonium (Table 4-7). The increase in the level of asparagine in wheat roots were noteworthy.

4.3.3 Malate Sensitivity between PEPCs Prepared from Roots of Tomato Plants Grown with Nitrate or Ammonium.

It was investigated whether the regulation of root PEPC activity in relation to ammonium nutrition involves the protein phosphorylation or not. The involvement was evaluated by the difference of the sensitivity to malate since it is well accepted that the phosphorylated enzyme shows a lower sensitivity to malate (Jiao and Chollet 1989 ; Schuller and Werner 1993 ; Chollet et al. 1996). Protein phosphatase could be inhibited by the addition of NaF to the buffer solutions. The sensitivity to malate was examined under suboptimal conditions (2 mM PEP, pH 7.2) and evaluated by I_{50} values (Fig. 4-6). The specific activities of PEPC were 0.264 and 0.475 units g^{-1} FW in roots of tomato plants supplied with nitrate and ammonium, respectively. The I_{50} values were 5.86 mM for PEPC from nitrate-fed roots and 6.09 mM for PEPC from ammonium-fed roots (Fig. 4-6). These

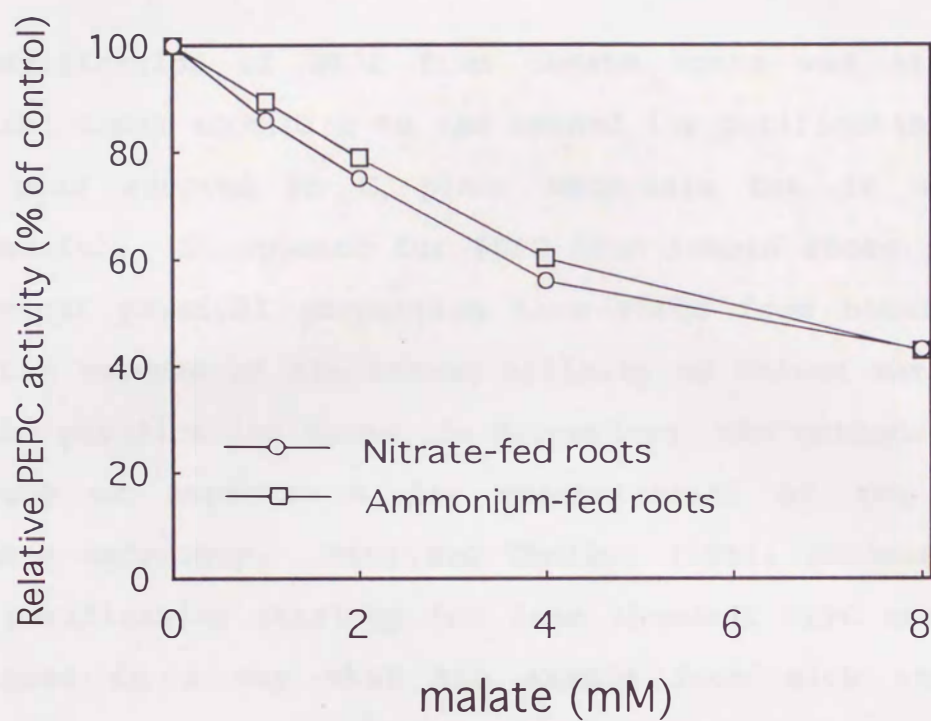


Fig.4-6. Inhibition by malate of PEPC activity from roots of tomato plants fed with nitrate or ammonium.

results indicated that PEPCs from both materials had a similar sensitivity to malate.

4.4 Discussion

Purification of PEPC from tomato roots was attempted several times according to the method for purification which has been adopted to C_3 plant materials but it was not successful. It appears for PEPC from tomato roots to have different physical properties from PEPCs from other plant sources because of the strong affinity to column materials. As the purification steps are proceeding, the methodology to operate or separate a low concentration of the sample becomes necessary. Wang and Chollet (1993) proposed that the purification strategy for less abundant PEPC should be designed in a way that the sample from each step was directly brought to the next.

Tomato root PEPC was obviously inhibited by organic acids and acidic amino acids (Table 4-2). Malate was found to be the most potent inhibitor among those acids tested. In roots of both tomato and wheat plants, malate content was decreased to 10 % of the initial content at 3 d after the supply of ammonium as shown in Tables 4-4, 4-6. The supply of ammonium caused a decrease in the level of malate in algal cells as well (Turpin et al. 1990). It can be considered that the decrease in malate content will alleviate

the malate inhibition of root PEPC and consequently enhance *in vivo* PEPC activity and the rate of dark carbon fixation by the supply of ammonium. At 3 h after the supply of ammonium, however, there was not a decrease in malate content (Tables 4-4, 4-6). At this time, high levels of malate may limit PEPC activity and may not stimulate the dark carbon fixation. Large amounts of asparagine and glutamine accumulated in tomato and wheat roots when ammonium was supplied (Tables 4-5, 4-7). The activity of a PEPC isoform separated from a green alga *Selenastrum minutum* is reported to be activated by glutamine (Schuller et al. 1990a). The activation by glutamine would be convenient for the replenishment of carbon skeletons in roots during ammonium assimilation. Nevertheless, asparagine and glutamine did not increase tomato root PEPC activity (Table 4-2).

When ammonium is supplied, a decrease in 2-OG level and a remarkable increase in glutamine level were noticed in tomato roots, whereas 2-OG level did not decrease in wheat roots with a remarkable increase in asparagine level (Tables 4-4 to 4-7). The different changes in 2-OG level may enable us to consider that ammonium absorbed into roots is mainly accumulated as glutamine in tomato roots and as asparagine in wheat roots. Therefore, the replenishment of 2-OG is necessary in tomato roots and that of OAA is more favorable in wheat roots.

The regulatory protein phosphorylation of plant PEPC was mostly light-dependent (Jiao and Chollet 1988 ; Chollet

et al. 1996). However, it has been reported that the activity of soybean nodule PEPC is also regulated by the protein phosphorylation, which requires signals from shoot component (Schuller and Werner 1993 ; Zhang et al. 1995). Generally, the phosphorylation of PEPC results in the increase in catalytic activity and the decreased sensitivity to malate (Jiao and Chollet 1988 ; Schuller and Werner 1993 ; Chollet et al. 1996). In this study, however, there was no significant difference in sensitivity to malate between PEPCs prepared from tomato roots treated with nitrate and ammonium (Fig. 4-6). In addition, the protein phosphorylation is generally accepted to be effective as a rapid regulation of its activity. Previous work showed that PEPC activity in tomato roots was increased in response to ammonium in a gradual manner (Fig. 3-4). The evidence of the present study strongly supports that the protein phosphorylation is not responsible for the positive response of root PEPC activity to the supply of ammonium. Further work is required to examine whether the protein phosphorylation takes place *in situ* in roots.

4.5 Summary

The objective of this chapter is to understand how *in vivo* activity of PEPC is regulated in relation to ammonium assimilation. PEPC was 84-fold purified from roots of

tomato plants grown on ammonium media. Its specific activity was 10.3 units mg^{-1} protein which was lower than that of PEPC from other sources. The PEPC preparation contained several other bands than the PEPC subunit band as revealed by SDS-PAGE.

The activity of tomato root PEPC was decreased by organic acids and acidic amino acids. Particularly, malate was the most inhibitory metabolic compound. As reported in the materials such as bacteria and higher plants, root PEPC was activated by hexose phosphates. When ammonium was supplied, the level of malate in tomato roots was decreased in a marked degree and the decreased level of malate was considered to increase *in vivo* PEPC activity. Therefore, this increase in root PEPC activity presumably contributes to the replenishment of carbon skeletons for ammonium assimilation. Asparagine and/or glutamine greatly accumulated in tomato and wheat roots when ammonium was supplied. Since PEPC activity in roots is little affected by these amides, these compounds are considered to have no significant influence on *in vivo* PEPC activity in roots.

In general, the sensitivity of PEPC to malate decreases due to the phosphorylation of PEPC protein. In the present experiment, however, there was no clear difference in the sensitivity between PEPCs prepared from tomato roots supplied with nitrate and ammonium. Therefore, it was suggested that the increased PEPC activity by the supply of ammonium did not involve the protein phosphorylation unlike leaf PEPC.

Chapter 5

GLUCOSE METABOLISM IN ROOTS DURING AMMONIUM NUTRITION

5.1 Introduction

As described in Chapter 2, the supply of ammonium stimulated the rate of dark carbon fixation in roots. Since PEP, the substrate for the dark carbon fixation, is provided through the glycolytic pathway, the stimulation may be accompanied by the elevated glycolytic activity. In a green alga, the supply of N accelerated the carbon flow in the glycolytic pathway by the action of phosphofructokinase activated in combination with the rapid decrease in its inhibitors such as PEP and 3-phosphoglycerate (Botha and Turpin 1990 ; Huppe and Turpin 1994). This chapter focused on the flow of the intermediates formed in the degradation of hexoses in roots when plants were grown with different nitrogen sources.

5.2 Materials and Methods

Treatment with Nitrogen and MSX.

Wheat plants (*Triticum aestivum* L. var. Saitama) were hydroponically grown with 1/8 strength modified Hoagland solution (pH 6, Table 2-1) containing 2 mM NaNO_3 in a phytotron (20 °C, 70 % RH) for 3 weeks after sowing. The plants were supplied with N-free 1/4 strength solution for 3 d. Subsequently, these plants were supplied with 4 mM NaNO_3 or 2 mM $(\text{NH}_4)_2\text{SO}_4$ in the nutrient solution for 1 d. Some plants were treated with 100 μM MSX in N-free nutrient solution for 3 h prior to the supply of ammonium nitrogen. The plants grown in N-free nutrient solution (0 h) and the plants grown in nitrate or ammonium media for 1 d were used for following ^{14}C -glucose feeding experiments.

^{14}C -Glucose Feeding.

The wheat plants were acclimated for 1 h in a dark growth chamber (20 °C). These plants were then inserted into a hole of a rubber stopper that was sealed with moist flour wad and set on 250 mL wide-mouth bottle in which 100 mL of glucose solution was poured (Fig. 5-1). The glucose solution contained 0.1 mM glucose (with 148 kBq D-[U- ^{14}C] glucose [10.8 GBq mmol^{-1}]) and either 4 mM NaNO_3 or 2 mM $(\text{NH}_4)_2\text{SO}_4$ in 1/4 strength nutrient solution. The plants were incubated with shaking (100 rpm) for 3 h in the dark chamber. During feeding, $^{14}\text{CO}_2$ released from roots was

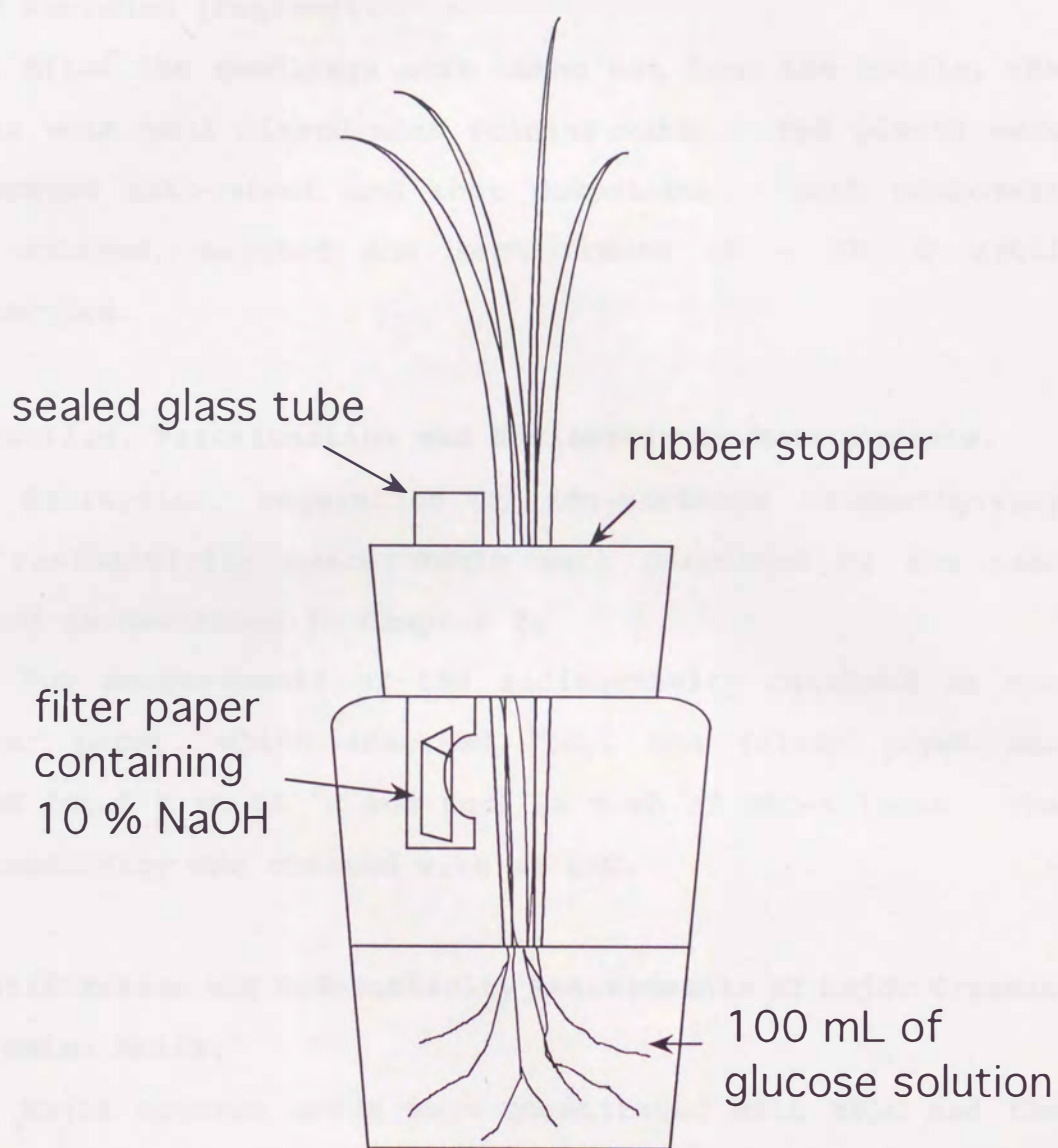


Fig. 5-1. Wide-mouth bottle (250mL)
for ^{14}C -glucose feeding.

recovered to filter paper containing 10 % NaOH. Each treatment was duplicated. D-[U-¹⁴C] glucose was purchased from Amersham (England).

After the seedlings were taken out from the bottle, the roots were well rinsed with running water. The plants were separated into shoot and root components. Each component was excised, weighed and kept frozen at - 70 °C until extraction.

Extraction, Fractionation and Radioactivity Measurements.

Extraction, separation by ion-exchange chromatography and radioactivity measurements were performed by the same method as described in Chapter 2.

For measurements of the radioactivity retained at the filter paper, which absorbed ¹⁴CO₂, the filter paper was dried for 6 h at 50 °C and sunk in 5 mL of PPO-toluene. The radioactivity was counted with an LSC.

Quantification and Radioactivity Measurements of Major Organic and Amino Acids.

Major organic acids were quantitated with HPLC and the radioactivity in separated organic acids was determined as described in Chapter 2.

Major amino acids were quantitated as PTC-amino acids with HPLC as described in Chapter 2.

The radioactivity in major amino acids was determined after the separation by thin layer chromatography. An

aliquot of the basic fraction was dried by evaporation and the dried sample was resuspended in 100 μ L of 50 % ethanol. Three μ L of cold amino acid solution (5 mM Asp, Asn, Glu, Gln and Ala) and 3 μ L of the sample was spotted on a silica gel plate (Silica gel 60, 20 cm $\times$ 20 cm $\times$ 0.25 mm, Merck, Germany). The plate was primarily developed with phenol-water (4:1 [v/v]) and subsequently developed with 1-butanol-acetic acid-water (4:1:1 [v/v/v]). The plate was dried by heating and then sprayed with a solution (80 mg o-phthalaldehyde, 1 mL of ethanol and 0.2 mL of 2-mercaptoethanol per 100 mL of 0.4 M boric acid [pH 9]). The spots were individually collected into a vial with a small amount of water. The radioactivity was determined with the sample cocktail with an LSC.

5.3 Results and Discussion

The substrates for the dark carbon fixation are PEP and bicarbonate. As shown in Chapter 2, when the dark carbon fixation was stimulated in roots by the supply of ammonium, the utilization of PEP was considered to be stimulated as well. In this chapter, the metabolism of glucose in wheat roots differing in nitrogen nutrition was investigated using 14 C-glucose as a precursor of PEP.

In the present study, glucose was exogenously supplied to wheat roots but the amount of glucose taken up by roots

was different among the nitrogen treatments (data not shown). Fig. 5-2 shows the distribution of the radioactivity derived from ^{14}C -glucose in the plants. When nitrate was the nitrogen source, the distribution of ^{14}C was not affected by the length of nitrate nutrition. When ammonium was supplied, the distribution of metabolites in the root-soluble fraction was greater in the plants grown on ammonium media than those on N-free media, while the distribution in the root-insoluble fraction was less in the plants grown on ammonium media for 1 d. In plants which were treated with MSX prior to the supply of ammonium, the radioactivity in the root-soluble fraction did not increase even in plants grown on ammonium media for 1 d. The release of $^{14}\text{CO}_2$ from roots was small in plants grown on ammonium media for 1 d.

The root-soluble fraction was further investigated since this fraction contained a major part of metabolites (Table 5-1). When nitrogen sources were supplied to plants grown on N-free media, the incorporation of ^{14}C into basic fraction was increased. It was slightly larger for the supply of ammonium than for the supply of nitrate although the dark carbon fixation was not yet stimulated by the supply of ammonium for 3 h. The incorporation of ^{14}C into basic fraction was greatly increased in plants grown on ammonium media for 1 d. In plants which were treated with MSX prior to the supply of ammonium, ammonium nutrition did not increase the formation of amino acids from glucose, indicating that ammonium assimilation is required for the

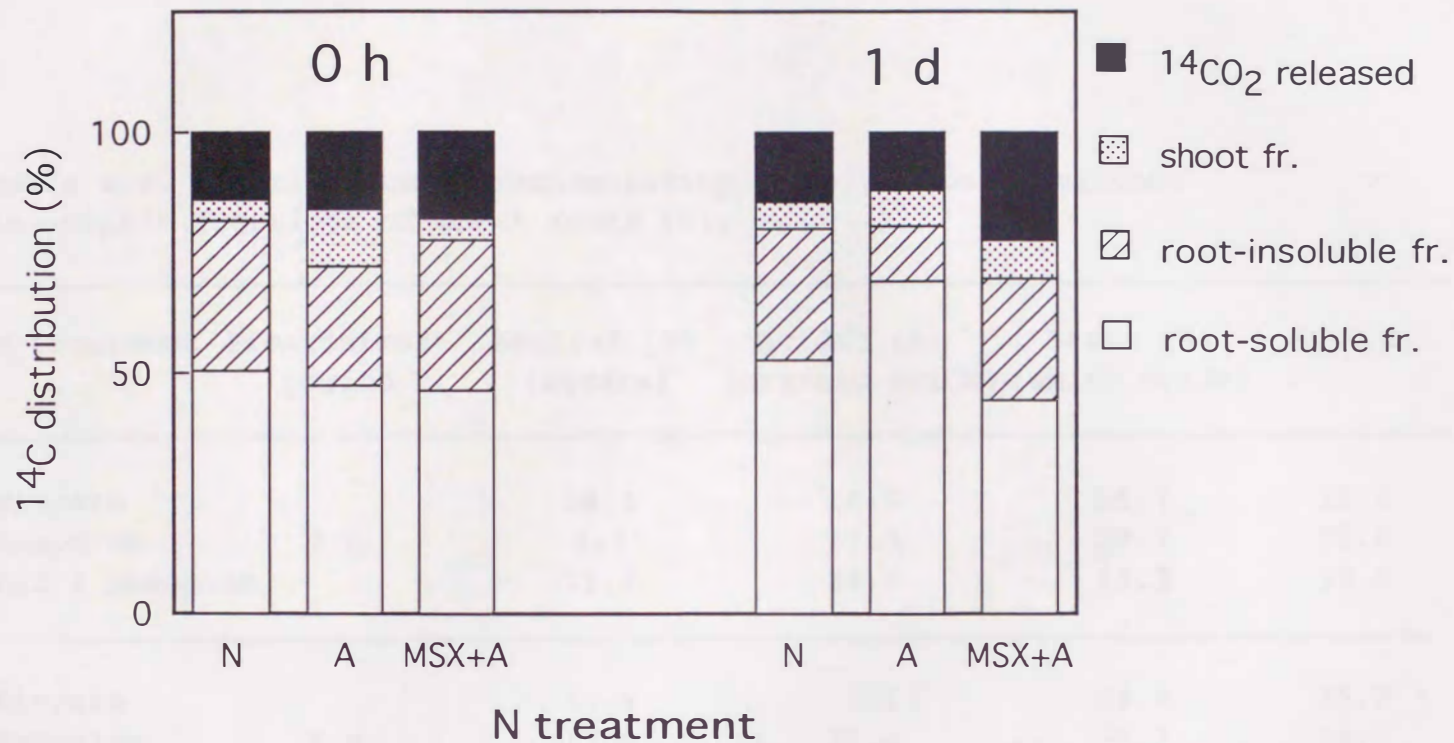


Fig. 5-2. Distribution of radioactivity from ^{14}C -glucose within wheat plants.

N : nitrate A : ammonium

MSX+A : ammonium after MSX pretreatment

Table 5-1. Distribution of radioactivity from ^{14}C -labeled glucose in soluble fractions of wheat roots (%).

N treatment	Pretreatment period	Neutral (N) (sugars)	Acidic (A) (organic acids)	Basic (B) (amino acids)	(A)+(B)
Nitrate	0 h	18.1	16.6	15.7	32.3
Ammonium		9.3	17.3	20.7	38.0
MSX + Ammonium		11.3	24.8	10.2	35.0
Nitrate	1 d	16.9	11.4	24.4	35.8
Ammonium		17.6	12.0	39.7	51.7
MSX + Ammonium		15.5	16.6	12.2	28.8

^{14}C -glucose was fed for 3 h after N pretreatment.

stimulation of amino acid synthesis from glucose.

When wheat plants were grown on ammonium media for 1 d, the concentration of citrate and malate was greatly decreased in the roots (Table 5-2). The similar results were already described in Chapter 2. The pretreatment with MSX for 3 h failed to decrease the content of citrate and malate but moderately increased succinate content. The radioactivity derived from ^{14}C -glucose was mainly found in citrate and malate among major organic acids. The radioactivity in succinate was relatively low despite its sizable abundance and fumarate was not labeled. The specific radioactivity in malate was substantially increased in the plants supplied with ammonium for 1 d.

The concentrations of Asp, Asn, Glu and Gln in the roots were increased by the supply of nitrate or ammonium for 1 d (Table 5-3). Above all, asparagine concentration was remarkably increased by the supply of ammonium. The radioactivity in individual amino acids was measured for Asp, Asn, Glu, Gln and Ala. The proportions of these five amino acids in basic fraction was 33 % and 41 % after the supply of nitrate and ammonium for 3 h, respectively. The proportions in basic fraction were 39 % and 63 % in nitrate- and ammonium-grown plants for 1 d, respectively. When nitrate was supplied, the specific radioactivity in aspartate and glutamate was relatively high (Table 5-3). Therefore, nitrate-grown plants are considered to preferentially consume the carbon skeleton from glucose for the synthesis of

Table 5-2. Concentration, radioactivity and specific radioactivity of major organic acids in acidic fractions in wheat roots.

N treatment	Pretreatment period	Concentration				Radioactivity				Specific radioactivity			
		cit	mal	suc	fum	cit	mal	suc	fum	cit	mal	suc	fum
		$\mu\text{mol g}^{-1} \text{FW}$				$\text{Bq g}^{-1} \text{FW}$				$\text{Bq } \mu\text{mol}^{-1}$			
Nitrate	0h	4.4	13.8	5.5	0.07	156	387	44	N.D.	35.2	28.1	8.1	N.D.
	1d	3.2	6.2	6.2	0.05	80	127	17	N.D.	25.0	20.4	2.7	N.D.
Ammonium	0h	4.6	9.5	4.8	0.07	116	199	28	N.D.	25.3	20.9	5.8	N.D.
	1d	1.5	1.7	2.6	0.02	33	126	14	N.D.	22.5	74.0	5.5	N.D.
MSX + Ammonium	0h	5.2	18.6	6.8	0.07	220	598	65	N.D.	42.3	32.2	9.6	N.D.
	1d	5.1	19.0	9.2	0.10	120	430	43	N.D.	23.7	22.7	4.7	N.D.

cit : citrate mal : malate suc : succinate fum : fumarate N.D. : Not detected

^{14}C -glucose was fed for 3 h.

N source was supplied during the pretreatment and ^{14}C -glucose feeding.

Table 5-3. Concentration, radioactivity and specific radioactivity of major amino acids in basic fractions in wheat roots.

N treatment	Pretreatment period	Concentration				Radioactivity					Specific radioactivity			
		Asp	Glu	Asn	Gln	Asp	Glu	Asn	Gln	Ala	Asp	Glu	Asn	Gln
		$\mu\text{mol g}^{-1} \text{FW}$				$\text{Bq g}^{-1} \text{FW}$					$\text{Bq } \mu\text{mol}^{-1}$			
Nitrate	0h	0.20	0.26	0.52	0.06	96	250	47	50	112	480	961	90	833
	1d	0.27	0.33	1.11	0.12	277	505	277	72	233	1026	1533	250	600
Ammonium	0h	0.19	0.23	0.50	0.26	75	149	59	389	261	395	648	118	1496
	1d	0.33	0.57	5.66	0.66	171	307	2582	231	323	518	539	456	350

^{14}C -glucose was fed for 3 h.

N source was supplied during the pretreatment and ^{14}C -glucose feeding.

aspartate and glutamate rather than for amide synthesis. In plants supplied with ammonium for 3 h, the specific radioactivity was higher in glutamine than in asparagine. The incorporation of ^{14}C into asparagine was markedly increased in plants grown with ammonium for 1 d. Therefore, carbon skeletons coming from glucose were utilized for glutamine synthesis immediately after the supply of ammonium whereas the carbon skeletons were directed toward asparagine synthesis with a prolonged period of the supply of ammonium. Alanine is synthesized from pyruvate without the carboxylation (Joy 1988) but the labeling of alanine was little affected by the supply of ammonium (Fig. 5-3).

In only plants grown with ammonium for 1 d, the sum of acidic and basic fractions retained more than 50 % of total radioactivity absorbed in the plants (Table 5-1). In addition, asparagine was much labeled from ^{14}C -glucose in these plants (Table 5-3). Therefore, it is assumed that organic acids and amino acids in these fractions were synthesized via PEP from glucose, suggesting that the degradation of glucose to PEP is activated when the dark carbon fixation is stimulated in roots by the supply of ammonium.

If 2-OG synthesis is required for ammonium assimilation, it is predicted that the release of CO_2 from roots will be more active in ammonium-fed plants than in nitrate-fed ones, because in the breakdown of ^{14}C -glucose to $^{14}\text{CO}_2$, pyruvate derived from glucose is oxidized in the TCA cycle to produce

CO₂ (Bryce and Hill 1993). However, there was no significant difference in the release of ¹⁴CO₂ among the nitrogen treatments (Fig. 5-2). Therefore, it is presumed that 2-OG is of lower importance as the carbon skeleton for ammonium assimilation in wheat roots. Actually, ¹⁴C from glucose is utilized for asparagine synthesis rather than glutamine synthesis in wheat roots (Table 5-3). Since asparagine is synthesized from aspartate by the action of AS (Joy 1988 ; Oaks 1994), OAA appears more important than 2-OG as the carbon skeleton when ammonium is supplied to wheat roots.

5.4 Summary

It was supposed that when the dark carbon fixation in roots was stimulated by the supply of ammonium, the provision of PEP from hexoses was also stimulated. Hence, the metabolism of exogenously supplied ¹⁴C-glucose was studied in roots of young wheat plants which were supplied with nitrate or ammonium.

When nitrogen sources were supplied for 1 d, the plants grown with ammonium, which showed the stimulated dark carbon fixation, contained more ¹⁴C from glucose in soluble materials and less ¹⁴C in insoluble materials than the plants grown with nitrate. More ¹⁴C from ¹⁴C-glucose was incorporated into amino acid fraction by the supply of ammonium compared to the supply of nitrate and the pretreatment with MSX.

In wheat roots, asparagine was strongly labeled with ^{14}C from glucose when ammonium was previously supplied for 1 d. At this time, the incorporation of ^{14}C into succinate and fumarate was very low or undetectable. In addition, it was shown that there was no significant difference in $^{14}\text{CO}_2$ release by respiration in the roots, regardless of the nitrogen treatments. These results suggested that OAA produced from glucose was directly utilized for asparagine synthesis during ammonium assimilation in wheat roots.

Chapter 6

TRANSLOCATION OF PHOTOSYNTHATES TO ROOTS DURING AMMONIUM NUTRITION

6.1 Introduction

Most of nitrate absorbed by roots is transported to shoots without reduction and assimilation (Riens and Heldt 1992 ; Sechley et al. 1992 ; Sivasankar and Oaks 1996). On the other hand, ammonium is mostly assimilated in roots and the assimilates are subsequently transported to shoots in a form of amides (Givan 1979 ; Oaks 1994). Chapter 2 showed that the dark carbon fixation was stimulated in roots to replenish carbon skeletons for ammonium assimilation. The carbon skeletons necessary for ammonium assimilation in roots are ultimately dependent on the photosynthates translocated from shoots (Cramer and Lewis 1993). Furthermore, unlike isolated plant cells and algal cells, higher plants involve the long-distance transport. Generally C fixed by

photosynthesis is translocated to roots in a form of sucrose, depending on the sink-source relationship. Therefore, it is possible that there is mutual integration between nitrogen nutrition and translocation of photosynthates to roots.

It can be considered that ammonium nutrition requires the provision of more carbon skeletons from shoots to roots. This chapter was undertaken to understand the translocation of photosynthetically fixed C to roots during ammonium nutrition.

6.2 Materials and Methods

Plant Growth.

Wheat plants (*Triticum aestivum* L. var Saitama) were hydroponically grown with 1/8 strength modified Hoagland solution (pH 6, Table 2-1) containing 2 mM NaNO_3 in a glasshouse following germination and subsequent sand culture for several days. The 2-week-old plants were supplied with 1/4 strength nutrient solution lacking N for 3 d. Then the plants were supplied with 1/4 strength modified Hoagland solution containing either 4 mM NaNO_3 or 2 mM $(\text{NH}_4)_2\text{SO}_4$. Each treatment was duplicated.

Photosynthetic $^{14}\text{CO}_2$ Pulse-labeling.

In pulse-labeling experiments, the plants grown on N-free media for 3 d and the plants supplied with nitrate or

ammonium for 1 d were used. Three plants were transferred in a 100 mL flask containing 100 mL of the 1/4 strength nutrient solution containing 4 mM NaNO_3 , 2 mM $(\text{NH}_4)_2\text{SO}_4$ or no nitrogen source and were supported with a rubber stopper. In order to prevent $^{14}\text{CO}_2$ gas from dissolution into the nutrient solution, the space at a rubber stopper was sealed with wheat flour wad.

Prior to $^{14}\text{CO}_2$ pulse-labeling, fresh air was introduced to the $^{14}\text{CO}_2$ -labeling apparatus illustrated in Fig.6-1. $^{14}\text{CO}_2$ gas was released by acidifying $\text{NaH}^{14}\text{CO}_3$ (5.92 MBq, 1.92 GBq mmol^{-1}) aqueous solution with 2 mL of 50 % lactic acid in the $^{14}\text{CO}_2$ gas generator and then mixed in the reservoir with a fan. Initial concentration of CO_2 was about 390 $\mu\text{L L}^{-1}$. Subsequently, the plants in eight flasks were placed in the pulse-labeling box. The plants were pulse-labeled with $^{14}\text{CO}_2$ for 20 min by the circulation of the $^{14}\text{CO}_2$ gas from the reservoir to the pulse-labeling box. The plants were illuminated with a metalhalide lamp. Light intensity was 580 $\mu\text{E m}^{-2} \text{ s}^{-1}$ at shoot height and the temperature in the box was near 20 °C. The concentration of CO_2 was monitored with an infrared gas analyzer (VIA-300 model, HORIBA). At the end of pulse-labeling, the lamp was turned off and the $^{14}\text{CO}_2$ gas was recovered through a soda lime trap until the CO_2 concentration decreased to zero (for 15 min). These flasks were taken out from the box. The plants in the flask were supported with cottons during chase periods.

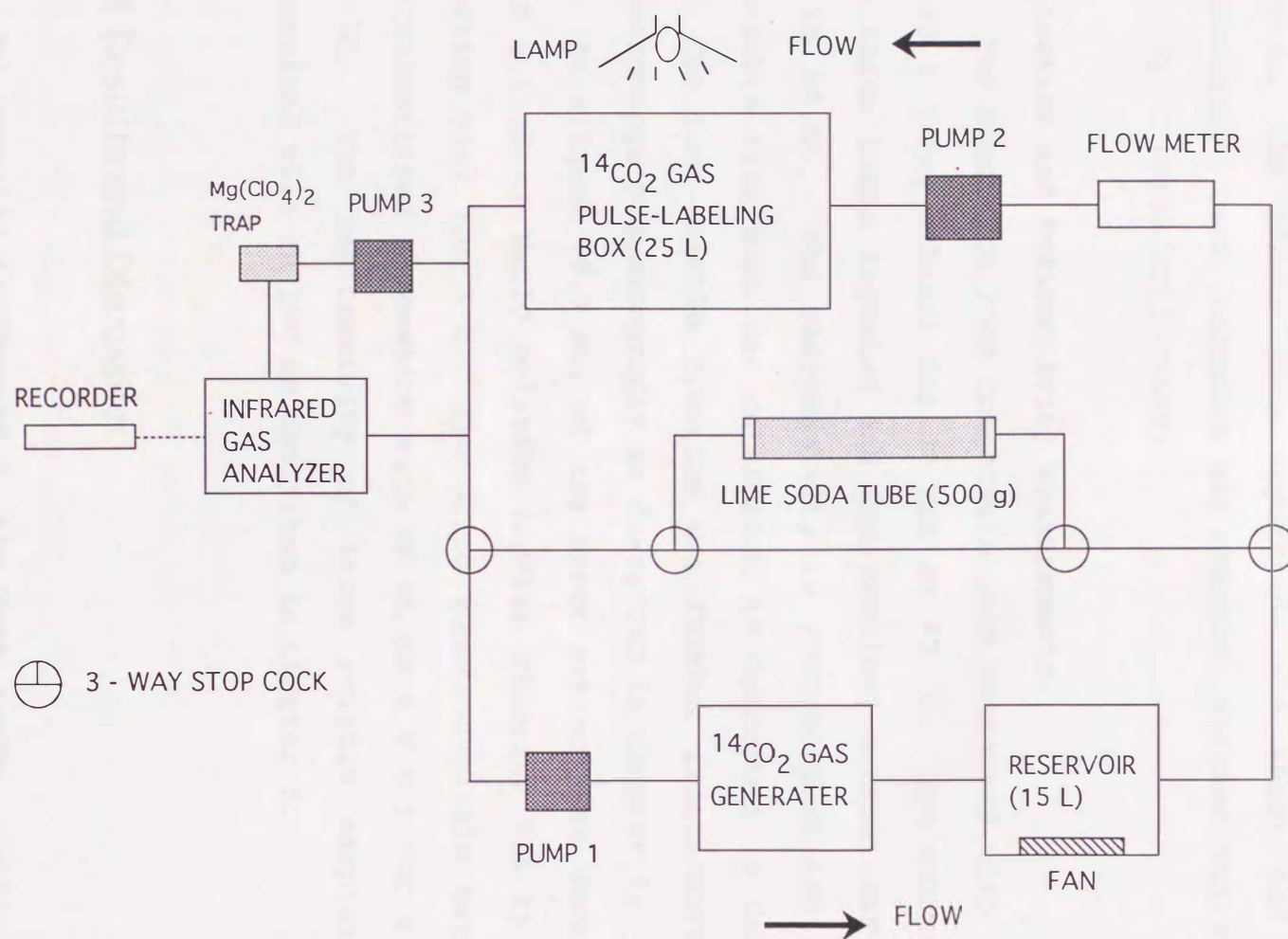


Fig. 6-1. Apparatus for photosynthetic $^{14}\text{CO}_2$ pulse-labeling.

Chasing Conditions.

The plants were kept with shaking (100 rpm) for 3 or 12 h in a darkened chamber (22 °C). At decided times of the harvest, the plants were separated into shoot and root components. Each component was chopped, weighed and stored at - 70 °C until extraction.

Extraction and Radioactivity Measurements.

The shoot and root components were extracted with 15 mL of 80 % (v/v) ethanol for 30 min at 80 °C. The extraction was three times repeated and the combined extract was made up to 50 mL. The radioactivity in root-soluble and root-insoluble fractions was determined as described in Chapter 2. The root-soluble fraction was further fractionated by ion-exchange chromatography as described in Chapter 2.

An aliquot (0.5 mL) of the shoot extract was decolorized with 0.1 mL of NaClO solution (active chlorine 8.5 %) in a counting vial for 3 h. The dried shoot-insoluble material was solubilized by heating with 20 mL of 6 N HCl for 6 h at 80 °C. The radioactivity of those soluble samples was determined with an LSC as described in Chapter 2.

6.3 Results and Discussion

As revealed in Chapter 2, the dark carbon fixation was stimulated in wheat roots by the supply of ammonium. In

other words, the replenishment of carbon skeletons was accelerated for ammonium assimilation in the roots. Since the carbon skeletons expended in roots mostly originate from the photosynthates, the translocation of photosynthates to roots was investigated by pulse-chase experiments with $^{14}\text{CO}_2$ in wheat plants supplied with different nitrogen sources.

Although eight plants were simultaneously pulse-labeled with $^{14}\text{CO}_2$ in a pulse-labeling box, the different radioactivity was found in each plant even in duplicate. It seemed to be due to different position of each plant in the box. However, the proportions of the distribution of ^{14}C were almost same in the duplicate. In every treatment, judging from changes in the concentrations of CO_2 in the apparatus, 30 - 40 % of the radioactivity which was fed as $^{14}\text{CO}_2$ was incorporated into the plants during a 20 min pulse-labeling (data not shown).

As shown in Fig. 6-2, the translocation of photosynthates from shoot to roots was affected by the nitrogen treatments employed in this study. Less than 15 % of ^{14}C fixed by photosynthesis was found in the roots in every treatment when the plants were chased for both 3 and 12 h in the dark (Fig. 6-2). When the plants were chased for 3 h, more photosynthates were translocated to roots in the plants supplied with ammonium for 1 d (14.5 %) compared to -N plants (10.5 %) and nitrate-supplied plants (9.4 %). After a 12 h chase period, ^{14}C retained in the roots was slightly larger in ammonium-supplied plants than -N plants and nitrate-

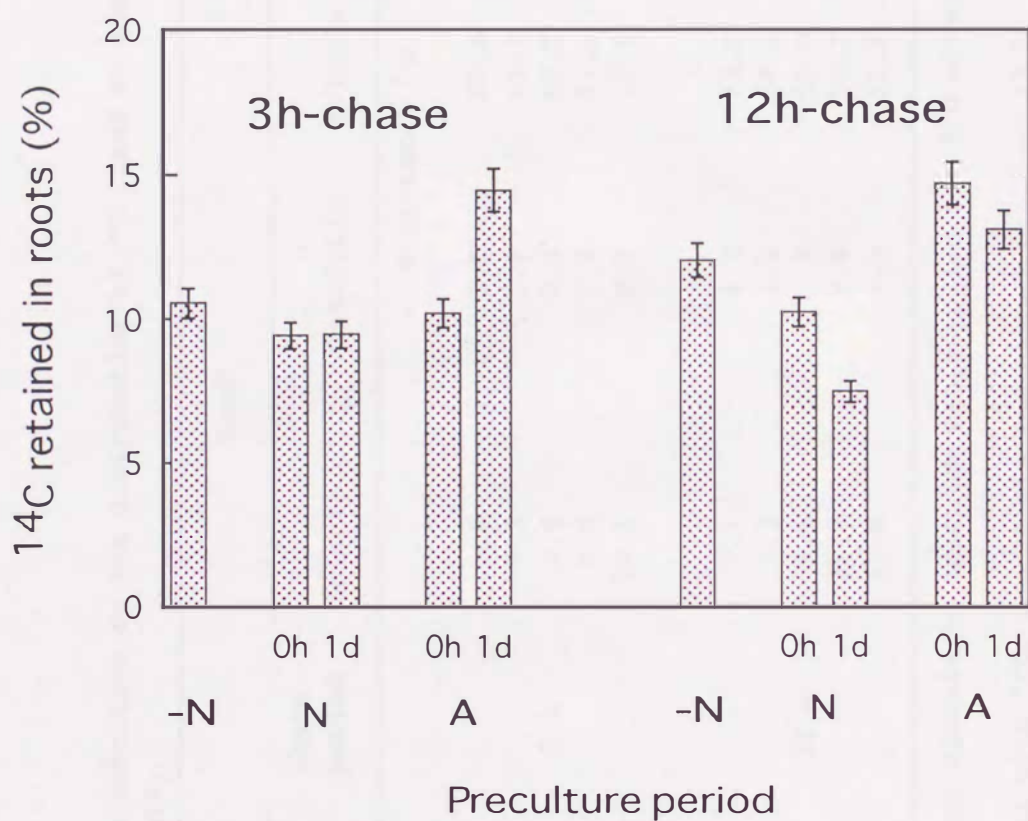


Fig. 6-2. Effect of nitrogen nutrition on the translocation of photosynthates to roots during chase periods in the dark. Nitrate (N), ammonium (A) or no N (-N) was supplied during preculture and chasing.

Table 6-1. Effect of nitrogen nutrition on the distribution of ^{14}C fixed by photosynthesis during chase periods in the dark.

N source			Root		Shoot	
Preculture period	Pulse-chase period	Chase period	Soluble	Insoluble	Soluble	Insoluble
% of total ^{14}C						
no N	no N	3 h	9.0	1.5	67.8	21.7
no N	Nitrate		8.3	1.1	69.7	20.9
Nitrate	Nitrate		8.6	0.9	67.2	23.4
no N	Ammonium		9.0	1.2	74.5	15.4
Ammonium	Ammonium		13.5	0.9	62.6	22.9
no N	no N	12 h	7.7	4.3	53.6	34.4
no N	Nitrate		7.3	3.0	54.1	35.7
Nitrate	Nitrate		5.6	1.8	57.0	35.6
no N	Ammonium		11.7	2.9	52.7	32.6
Ammonium	Ammonium		11.6	1.5	51.8	35.1

During preculture period, either nitrate or ammonium was supplied for 1 d after the N-free culture for 3 d.

Wheat plants were pulse-labeled with $^{14}\text{CO}_2$ for 20 min and chased for 3 or 12 h.

supplied plants. It has been reported that the supply of ammonium increased the translocation of the photosynthates to roots in soybean plants (Rabie et al. 1980) and wheat plants (Lewis et al. 1987). Therefore, it was conceivable that the root system became a strong sink for photosynthetic C during ammonium assimilation. Nevertheless, when the plants were supplied with ammonium, the translocation of photosynthates was not increased after a 3 h chase period (10.2 %) but increased after a 12 h period (14.7 %), indicating that the translocation was affected slowly by the supply of ammonium (Fig. 6-2). In contrast to the supply of ammonium, the supply of nitrate resulted in a decreased translocation of photosynthates compared to -N plants after a 12 h chase period (Fig. 6-2). Since the main site of reduction and assimilation of nitrate is a shoot (Sechley et al. 1992), this result may be the consequence of the site at which nitrate was assimilated. The effects of a prolonged chase period were not found on the partitioning of ^{14}C into roots but found on increased incorporation of ^{14}C into the insoluble materials of each organ (Table 6-1)

Fig. 6-3 shows that the supply of ammonium caused the increased incorporation of ^{14}C originating from the photosynthates into amino acid fraction in wheat roots. When periods of both preculture and chasing were longer, more photosynthates were utilized for amino acid synthesis by the supply of ammonium. Similar trends were observed in nitrate-treated plants to a smaller extent. Cramer and

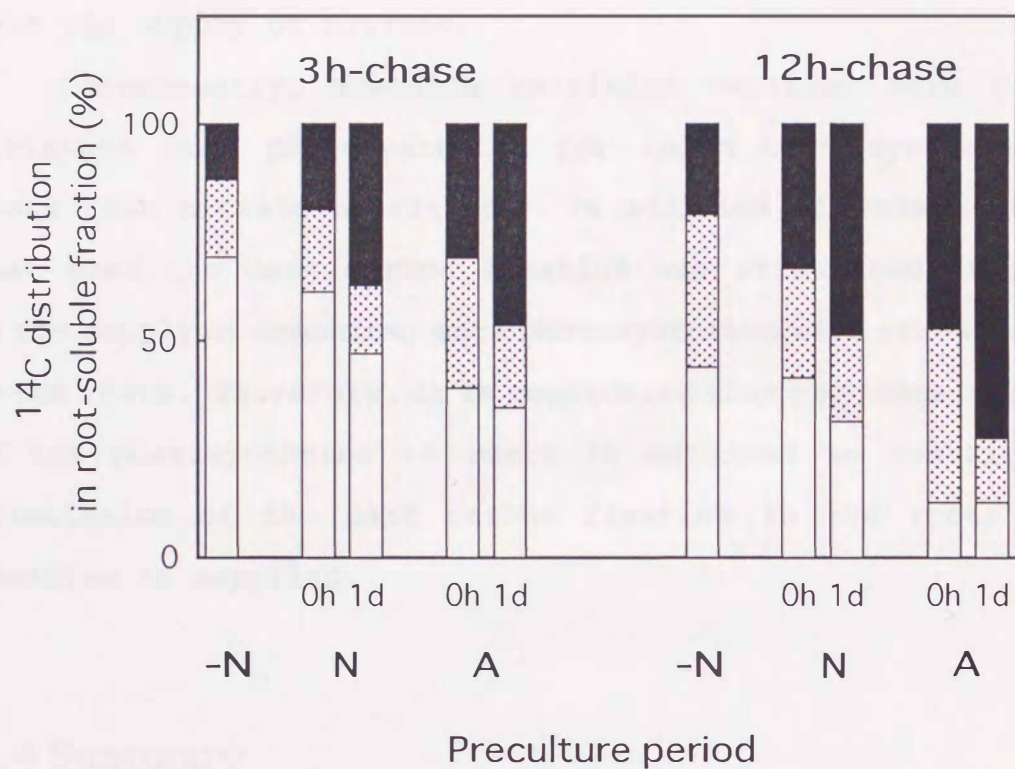


Fig. 6-3. Effect of nitrogen nutrition on the distribution of ^{14}C fixed by photosynthesis and translocated to roots in three soluble fractions during chase period in the dark. Nitrate (N), ammonium (A) or no N (-N) was supplied during preculture and chasing.

- basic (amino acids) fraction
- ▤ acidic (organic acids) fraction
- neutral (sugars) fraction

Lewis (1993) showed that the larger amount of ^{14}C from photosynthates was located in amino acid fraction in roots of wheat and maize plants by the supply of ammonium rather than the supply of nitrate.

Consequently, ammonium nutrition required more carbon skeletons from photosynthates for amino acid synthesis in roots than nitrate nutrition. In addition, it was revealed that when the dark carbon fixation was stimulated in roots by the supply of ammonium, more photosynthates were translocated to the roots. Therefore, it is considered that the translocation of the photosynthates to roots is enhanced to sustain the stimulation of the dark carbon fixation in the roots when ammonium is supplied.

6.4 Summary

The translocation of photosynthates to roots was investigated in young wheat plants which had been grown with different nitrogen sources, since the roots of ammonium-fed plants were considered to demand more carbon skeletons for ammonium assimilation.

In plants grown with nitrate, the translocation of photosynthates was not affected after a 3 h chase period but decreased after a 12 h chase period. In ammonium-fed plants, more photosynthates were translocated to roots compared with -N plants and nitrate-fed plants, indicating that the

root was an important sink organ for photosynthetic C during ammonium assimilation. Consequently, the translocation of photosynthates to roots was slowly stimulated in response to the supply of ammonium as observed in the dark carbon fixation in roots of ammonium-fed plants. When ammonium was supplied, more photosynthates translocated from shoot were utilized for amino acid synthesis in roots compared to -N plants and nitrate-fed plants. It is concluded that when ammonium is the nitrogen source more photosynthates are translocated to roots because more amino acids are actively synthesized in roots to detoxify ammonium.

Chapter 7

GENERAL DISCUSSION AND CONCLUSIONS

Ammonium is a main component of nitrogen fertilizers. It has a quick effect on the growth of crop plants compared to nitrate, which has to be reduced before its assimilation. When ammonium is supplied, the plants are compelled to assimilate ammonium into amides and amino acids immediately after the entry of ammonium into roots to prevent its toxicity to cells (Givan 1978 ; Joy 1988 ; Mehrer and Mohr 1989 ; Sechley et al. 1992). Therefore, it can be readily considered that a continuous supply of ammonium requires plenty of carbon skeletons to accept ammonium. In this dissertation, the replenishment of carbon skeletons utilized for imperative assimilation of ammonium was studied using the roots at which ammonium is absorbed and assimilated.

Plants receiving ammonium accumulate amides such as asparagine and glutamine in roots. Oxaloacetate and 2-OG, members of the TCA cycle, are essential carbon skeletons in the primary ammonium assimilation (Lea 1993 ; Engels and

Merschner 1995). The precursor of 2-OG is citrate, which is oxidized to 2-OG either in mitochondria or in cytoplasm. When 2-OG is consumed for the synthesis of glutamate and glutamine (C_5 compounds), a level of OAA that is used for citrate synthesis declines. On the other hand, the production of aspartate and asparagine (C_4 compounds) directly leads to a decreased level of OAA. As long as ammonium nitrogen continues to enter into roots and to be assimilated there, C_4 - and/or C_5 -dicarboxylic acids have to be supplied as the acceptors of ammonium nitrogen. Ultimately for the efficient provision of OAA, plants need anaplerotic β -carboxylation of PEP in roots during ammonium nutrition (Fig. 1-1).

The rate of dark carbon fixation was first investigated in wheat roots, which were treated with different nitrogen sources. When ammonium was supplied at a concentration of 4 mM, the rate of dark carbon fixation was slowly but consistently increased in the roots. The rates in plants supplied with ammonium for 3 h and 1 d were twice and six times as high as the rate in the control plants, respectively. It has been reported that the dark carbon fixation is not stimulated by the supply of nitrate in rice and tomato roots (Ikeda et al. 1992), maize roots (Cramer et al. 1993) and *Acer* cells (Goodchild and Givan 1991). Therefore, these results suggest that the stimulation of dark carbon fixation is a specific phenomenon that is caused by continuous ammonium nutrition.

The supply of ammonium resulted in the decrease in concentration and labeling of citrate and malate in roots

while it led to the great increase in concentration and labeling of asparagine. From the heavy labeling of asparagine with ^{14}C from ^{14}C -bicarbonate, it was made clear that the stimulated dark carbon fixation in roots is an inevitable reaction to replenish carbon skeletons for ammonium assimilation. MSX-pretreatment experiment strongly supports the above interpretation because the plants pretreated with MSX did not show the stimulation at all and did not cause the incorporation of ^{14}C into amino acid fraction. Furthermore, the MSX-pretreatment experiment indicates that the entry of ammonium into roots itself cannot be a trigger of the stimulation of dark carbon fixation but that the assimilation of ammonium is an essential requisite for the stimulation.

As a consequence, it is clarified that the dark carbon fixation in roots is stimulated by the supply of ammonium so that the carbon skeletons for ammonium assimilation are effectively provided. Based on the hypothesis that the stimulation of dark carbon fixation is attributed to the increase in *in vivo* activity of root PEPC and/or the enhanced provision of the substrate necessary for the dark carbon fixation, the mechanisms by which the dark carbon fixation is controlled in roots in response to ammonium were examined.

The reaction of dark carbon fixation is mostly mediated by PEPC. By the supply of 4 mM NH_4^+ , the extractable activity of PEPC was gradually increased in wheat, barley and tomato roots. At 7 d after the onset of the N

treatment, the activity in the plants supplied with ammonium was 2- to 2.5-fold higher than that in the plants supplied with nitrate. In addition, Western blot analyses indicated that the increased extractable activity of PEPC was due to *de novo* synthesis of PEPC proteins in tomato roots. The positive effects of ammonium on the accumulation of proteins and mRNAs were already reported for GS from soybean roots (Hirel et al. 1987) and NADH-dependent GOGAT from rice roots (Yamaya et al. 1995). It is of great interest that not only ammonium-assimilating enzymes such as GS and GOGAT but also PEPC responsible for the replenishment of TCA cycle intermediates respond to the supply of ammonium in roots.

Even though wheat plants were supplied with ammonium, both activity and amounts of root PEPC were not increased in the presence of MSX, which inhibits the primary assimilation of ammonium in roots. Therefore, it is supposed that the assimilates of ammonium or their metabolites are of great importance to the induction of root PEPC. In maize leaves (Sugiharto et al. 1992) and wheat leaves (Manh et al. 1993), glutamine is suggested to be the most likely metabolite for controlling the N-dependent expression of PEPC. Consistent with these reports, the exogenous supply of glutamine increased the activity and amounts of PEPC in wheat roots. The exogenous supply of asparagine was also effective on the increase of both activity and amounts of PEPC in wheat roots to a similar extent to the supply of glutamine. In fact, when wheat plants were supplied with ammonium, the roots

accumulated a considerable amount of asparagine besides glutamine. It is thus postulated that amides such as asparagine and glutamine may function as a inducer to PEPC during ammonium assimilation in roots of plants that accumulate asparagine as well as wheat plants.

PEPC activity in roots was regulated in response to ammonium at a transcriptional or translational level. The increase in extractable PEPC activity depending on *de novo* protein synthesis was observed in a gradual manner as seen in the stimulation of the dark carbon fixation in roots receiving ammonium. Moreover, the plants treated with MSX failed to stimulate the dark carbon fixation and to induce the expression of PEPC in roots. Therefore, it is strongly suggested that the increase in extractable PEPC activity substantially contributes to the stimulated dark carbon fixation to provide sufficient carbon skeletons for ammonium assimilation.

In addition to *de novo* synthesis of PEPC protein, there is a possibility that *in vivo* activity of PEPC will be enhanced through metabolite effects on this enzyme. The metabolite effects on PEPC activity are well investigated in C₄ and CAM leaves (Winter 1982 ; Doncaster and Leegood 1987 ; Gupta et al. 1994), C₃ leaves (Gupta et al. 1994 ; Krömer et al. 1996), root nodules (Schuller et al. 1990b ; Schuller and Werner 1993) and green algae (Schuller et al. 1990a ; Rivoal et al. 1996) whereas there are few reports on root enzyme because of low abundance of the enzyme in roots and

the difficulty in purification. PEPC was purified from tomato roots to get the specific activity of 10.3 units mg^{-1} protein using a combination of the modified chromatographic procedures. The regulatory properties were examined using this preparation.

Tomato root PEPC was severely inhibited by organic acids such as citrate and malate and acidic amino acids. Particularly, the inhibition by malate was most conspicuous among the inhibitions by those acids. The supply of ammonium markedly decreased malate content in tomato and wheat roots. Turpin et al. (1990) estimated the change in intracellular concentration of various metabolites in a green alga and reported that malate concentration decreased from 5.8 mM to 0.8 mM by the supply of ammonium. It is presumed that the decrease in concentration of organic acids, especially malate, alleviates the inhibition of PEPC by malate and consequently increases *in vivo* activity of PEPC in roots.

Malate concentration and the rate of dark carbon fixation in roots were not affected within a few hours by the supply of ammonium. At 1 d after the onset of the supply, however, the concentration of malate was markedly decreased and the dark carbon fixation was greatly stimulated. Moreover, in plants treated with MSX, the failure to decrease malate content coincided with failing to stimulate the dark carbon fixation throughout the supply of ammonium. The evidence enables us to consider that the stimulated dark

carbon fixation to replenish the carbon skeletons for ammonium assimilation was partially attributable to the increased *in vivo* PEPC activity caused by the decreased concentration of organic acids.

As mentioned above, PEPC activity in roots seems to be a key factor controlling the rate of dark carbon fixation. The increase in PEPC activity in quantity and quality must help the efficient provision of the TCA cycle intermediates for ammonium assimilation.

The stimulation of dark carbon fixation by the supply of ammonium is probably concomitant with more utilization of PEP as the substrate for the reaction. In wheat roots receiving ammonium, more ^{14}C -glucose was metabolized to organic acids and amino acids compared to the roots receiving nitrate or treated with MSX. In addition, asparagine was heavily labeled with ^{14}C from ^{14}C -glucose when ammonium was fed. Thus, it can be estimated that the degradation of glucose to PEP in glycolysis is enhanced in roots during ammonium assimilation. Huppe and Turpin (1994) pointed out that the flow of C through glycolytic pathway is accelerated during nitrogen assimilation. This is partially explained by the activation of phosphofructokinase caused by the decrease in its inhibitors such as PEP and 3-phosphoglycerate (Botha and Turpin 1990 ; Huppe and Turpin 1994). It is needless to say that the provision of PEP plays a significant role in the dark carbon fixation and thereby in ammonium assimilation. Further work is necessary to investigate how

the conversion of carbohydrates to PEP in roots is regulated in relation to ammonium assimilation.

The pulse-chase experiment with $^{14}\text{CO}_2$ revealed that the translocation of photosynthates to roots was more active in ammonium-supplied wheat plants than in nitrate-supplied wheat plants. The translocated carbon was shown to be utilized for amino acid synthesis in roots, suggesting that the translocation of photosynthates to roots is deeply involved in ammonium assimilation in roots. However, it is difficult to state that the translocation itself limits the rate of overall reaction to replenish carbon skeletons. The reason is that the translocation basically follows such a physical law as the gradient of sucrose concentration. It is likely that the roots are a strong sink organ for photosynthetic C in the course of ammonium assimilation.

In conclusion, the plants receiving ammonium increase the activity of root PEPC in quantity and quality and thereby stimulate the dark carbon fixation in roots to cope with the large demands for carbon skeletons in ammonium assimilation. In addition, these plants promote the translocation of photosynthates to roots and the degradation of carbohydrates in roots in order to maintain the provision of the substrates to be required in the dark carbon fixation. Ammonium assimilation in roots can be sustained by the replenishment of carbon skeletons, which needs a strengthened activity of dark carbon fixation based on increased PEPC activity and a supply of PEP originating from photosynthates.

Summary

Ammonium is one important form of inorganic nitrogen nutrients for plants. Because ammonium is cytotoxic, absorbed ammonium is immediately assimilated and detoxified in roots. On a continuous supply of ammonium, the assimilates of ammonium are exported from roots to shoots. Therefore, necessary carbon skeletons must be supplied to continue the ammonium assimilation in roots. This work deals with the processes in the replenishment of carbon skeletons consumed for ammonium assimilation in roots.

Oxaloacetate and 2-oxoglutarate, TCA cycle intermediates, are important carbon skeletons for the primary assimilation of ammonium. The rate of dark carbon fixation responsible for the synthesis of C_4 -dicarboxylic acids from C_3 compounds was determined in roots of wheat plants. The rate in ammonium-fed plants was about six times as high as the rate in plants grown in N-free media. However, when ammonium assimilation was inhibited by the glutamine synthetase inhibitor (MSX), the stimulation of the dark carbon fixation did not occur despite the accumulation of ammonium in the roots. These results indicate that the dark carbon fixation plays an important role in the replenishment of carbon skeletons for ammonium assimilation in roots.

In wheat, barley and tomato plants, extractable PEPC activities in roots of ammonium-fed plants were gradually increased and reached 2- to 2.5-fold higher values than

those in roots of nitrate-fed plants at 7 d after the N supply. Western blot analysis indicated that the increase in the PEPC activity was caused by *de novo* synthesis of PEPC protein in tomato roots. In addition, tomato root PEPC was inhibited by organic acids and acidic amino acids and activated by hexose phosphates. Above all, malate was a potent inhibitor for the root PEPC. The concentration of malate in roots was markedly decreased by the supply of ammonium. Hence, it is considered that the possible increase in *in vivo* PEPC activity regulated by effective metabolites contributes to the stimulation of the dark carbon fixation in roots.

The breakdown of exogenous ^{14}C -glucose to organic acids and amino acids in wheat roots was greater in ammonium nutrition than in nitrate nutrition. Asparagine was heavily labeled in the metabolites of ^{14}C -glucose. These results suggest that the conversion of carbohydrates to PEP is also stimulated in roots by the supply of ammonium. Photosynthates were actively translocated to roots in wheat plants supplied with ammonium. It seems that a root system becomes a strong sink organ for photosynthetic C during ammonium nutrition.

Plants increased root PEPC activity both in quality and in quantity to stimulate the dark carbon fixation in roots in response to the supply of ammonium. At the same time, the provision of the substrate necessary for the dark carbon fixation was also enhanced by the supply of ammonium to efficiently sustain the dark carbon fixation.

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