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Evidence of ammonium assimilation via the glutamine synthetase-glutamate synthase system in rice seedling roots

Yasuhiro Arima and Kikuo Kumazawa

Department of Agricultural Chemistry, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan

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When rice seedling roots were fed ^{15}N-ammonium for 1 hr, the amide nitrogen of glutamine showed the highest ^{15}N abundance. Moreover, glutamine amino, glutamic acid, aspartic acid and alanine showed higher ^{15}N abundance than ammonium did.

In roots whose GS activity was inhibited with MS, both the amount of ammonium and its ^{15}N abundance were increased. In contrast, both the amount of all examined amino acids containing glutamic acid and their ^{15}N abundance decreased in roots whose GS activity was inhibited. From these results, it could be concluded that the first step of ammonium assimilation in rice seedling roots was mainly glutamine synthesis by GS and the second was glutamic acid formation by the GOGAT system.

The results of an experiment using ^{15}N-labelled glutamine also supported this conclusion.

Cocking and Yemm showed (*4*) that in intact roots of barley seedlings fed with ^{15}N-labelled ammonium phosphate, the amide and amino nitrogen of glutamine were labelled most rapidly, and suggested that glutamine occupied the key position in ammonium assimilation.

Recently, we have shown by the ^{15}N tracer method (*2*) that glutamine is a primary product of ammonium assimilation and is synthesized from glutamic acid and newly absorbed ammonium. These facts and the presence of glutamine: 2-oxoglutarate aminotransferase ($NADPH_2$ oxidising) (glutamate synthase, EC 2.6.1.53) (GOGAT) in several plants (*5*, *7*, *8*, *10*) suggest that the glutamine synthetase-glutamate synthase system is a main route of entry of ammonium into amino acids and amides under certain conditions.

Investigation of L-glutamate : ammonia ligase (ADP) (glutamine synthetase, EC 6.3.1.2) (GS) in plant tissues (*3*, *9*) showed that glutamine synthesis was closely related to the external ammonium concentration. In those experiments, GS activity was reversibly repressed by increasing the external ammonium concentration.

Moreover, the GS activity of crude extract from rice seedling roots grown with a high ammonium concentration was increased by the addition of α-ketoglutaric acid (*3*). Rhodes et al. recently demonstrated that GOGAT activity showed a

Abbreviations: GOGAT, glutamate synthase; GS, glutamine synthetase; MS, L-methionine DL-sulfoximine; GDH, glutamate dehydrogenase.

similar response to external ammonium concentration (*10*). On the other hand, the universal existence of L-glutamate : NAD(P) oxidoreductase (glutamate dehydrogenase, EC 1.4.1.2–4) (GDH) in plants is well known, but the physiological significance of this enzyme is still not clear, especially as regards its high *Km* to ammonium.

The present study was carried out to detect the presence and the function of GS-GOGAT system and GDH dependent glutamic acid synthesis during ammonium assimilation in intact rice seedling roots using the ^{15}N tracer method.

Materials and methods

Plant: Rice seedlings (*Oryza sativa L.* cv Koshihikari japonica type) were grown under natural light at 30°C (day)–25°C (night) for 2 weeks after germination in tapwater. Seedlings were transplanted to ammonium sulfate medium (N 0.357 eq per liter) and grown for 2 days under the same conditions. The culture solution was renewed every day and also 3 hr before GS inhibition treatment or ^{15}N absorption.
GS inhibition: After washing with tapwater, intact roots were immersed in a 5 mM solution of L-methionine-DL-sulfoximine (MS), an inhibitor of GS. In the case of crude extract, GS inhibition was performed by addition of a concentrated MS solution after pretreatment with ATP as described by Ronzio et al. (*12*).
Preparation of crude GS: For preparation of GS and GDH, 40 mM Tris-HCl (pH 7.4) and 0.3 M Tris-HCl (pH 8.3) were used, respectively. Washed, excised roots were homogenized with each buffer solution and filtrated through gauze. The filtrates were centrifuged at $500 \times g$ for 10 min. All procedures were done at 4°C.
Measurement of GS and GDH activity: GS activity was determined by a modification of the method of Elliott (*6*). GDH activity was measured by the O.D. at 340 nm of $NADH_2$ or $NADPH_2$.
Feeding with ^{15}N-labelled ammonium or glutamine: After washing with tapwater, MS-treated and untreated plants were transplanted into ^{15}N-mediums containing;

A: 0.357 meq/liter ^{15}N-ammonium sulfate
B: 5 mM ^{15}N(amido)-glutamine
C: 0.357 meq/liter ^{15}N-ammonium sulfate and 5 mM ^{14}N-glutamine
D: 0.357 meq/liter ^{14}N-ammonium and 5 mM ^{15}N(amido)-glutamine.

Separation of amino acids or ammonium: After feeding with ^{15}N-labelled compounds for 1 hr, plants were washed with tapwater. Then the roots were harvested and homogenized with hot 80% ethanol followed by filtration. Ammonium was separated from the extract by the micro air-distillation method (*1*) at 40°C and pH 10.0. Amino acids were separated by 2-dimensional silica gel thin layer chromatography (TLC) using phenol-water (4 : 1 v/v) and butanol-acetic acid-water (4 : 1 : 1 v/v/v) respectively.

For determination of the ^{15}N abundances of glutamine and asparagine amino nitrogen, the amide fraction was collected by 1-dimensional TLC. After hydrolysis of the amide fraction, amino nitrogen of glutamine and asparagine were separated each other and recovered as glutamic or aspartic acid by 2-dimensional TLC (*2*).
Determination of ^{15}N: The ^{15}N abundances of ammonium and amino acids were determined by the emission spectrographic method given by Yoneyama et al. (*13*).

Results and discussion

The inhibition of GS activity in the root extract by MS is given in Table 1, which shows that treatment with 5 mM MS inhibited GS activity up to 90% within 2 min and 100% within 5 min. After preincubation with Tris-HCl alone, GS activity decreased only slightly after MS-treatment. It seems that the inhibition of GS by MS requires preincubation with ATP and Mg^{++}. The same trend was shown in sheep brain GS by Ronzio et al. (*12*).

Table 2 shows the inhibition of GS activity by MS in intact roots. GS activity of intact roots treated with 5 mM MS for 10 min decreased markedly. When plants were placed in a medium without MS for 70 min after 8 min MS-treatment, the inhibition of GS activity increased rather than decreased. According to these results, we decided to give 10 min MS-pretreatment in subsequent ^{15}N tracer experiments.

The effects of MS treatment on GDH activities in intact roots were determined.

Table 1 *GS inhibition in root extract[a] by MS*

1-1

	MS treatment (min)					
		1	2	3	4	5
GS activity[b]	0.025	0.007	0.003	0.002	0.001	0.000
Inhibition %	70	72	88	92	96	100

The MS concentration was 5 mM.

1-2

	MS concentration (mM)					
	0	0.1	0.5	1.0	2.0	5.0
GS activity[b]	0.027	0.020	0.010	0.009 (0.020)[c]	0.006	0.004
Inhibition %	0	26	63	69 (26)[c]	80	85

MS treatment was for 2 min.

[a] All samples except C were preincubated for 10 min at 37°C with 10 mM Na-ATP, 20 mM $MgSO_4$ and 40 mM Tris-HCl (pH 7.40) before MS-treatment.

[b] O. D. 540 nm.

[c] Preincubated only with 40 mM Tris-HCl (pH 7.40) before MS treatment.

Table 2 *Inhibition of GS in intact root by MS*

	MS treatment time (min)					
	0	1	2	4	8	10
GS activity	0.036	0.031	0.032	0.025	0.013 (0.003)[a]	0.007
Inhibition %	0	14	11	31	64 (92)[a]	81

[a] 8 min with MS and 70 min without MS.

Table 3 *Effect of MS treatment on GDH activity of intact roots*

	Coenzyme	
	$NADH_2$	$NADPH_2$
Control	0.042	0.010
MS-treated	0.042	0.010

Activities are expressed as decrease in absorbance at 340 nm of $NAD(P)H_2$ per min.

The results in Table 3 show that GDH activities were not affected by MS-treatment. The GDH activity of crude extract was not affected by the addition of concentrated MS either.

The results of ^{15}N tracer experiments are shown in Tables 4–8. The ^{15}N abundances of amino acids and ammonium in roots supplied with medium A are given in Table 4, and the quantities of amino acids and ammonium are shown in Table 5. In roots without MS-pretreatment, the amido nitrogen of glutamine showed the highest ^{15}N abundance, followed by glutamic acid, glutamine amino, aspartic acid and alanine. It was characteristic that the ^{15}N enrichment of amido and amino nitrogen were higher than that of ammonium. These results are inexplicable without the assumption that there are different compartments containing ammonium as one of their components in rice seedling roots. And at least one of these com-

Table 4 *The ^{15}N abundances of free amino acids and ammonium in rice seedling roots after absorption of ^{15}N-ammonium[a] for 1 hr*

	−MS pretreatment		+MS pretreatment		
	(A) atom % ex.	index[b]	(B) atom % ex.	index[b]	$\frac{(B)}{(A)}\times 100$
Ammonium	23.5	75	50.4	697	214
Glutamic acid	31.4	100	7.23	100	23
Aspartic acid	25.4	81	6.27	87	25
Glutamine amido	42.7	136	4.08	56	10
Glutamine amino	28.8	92	1.65	23	6
Asparagine amido	15.7	50	2.10	29	13
Asparagine amino	11.9	38	0.54	7	5
Alanine	24.5	78	3.43	47	14
Serine	9.36	30	1.39	19	15
γ-Amino butyric acid	7.62	24	0.68	9	9
Threonine	3.76	12	0.49	7	13
Proline	2.82	9	0.21	3	7
Tyrosine	1.71	5	0.17	2	10
Cysteic acid	2.45	8	0.14	2	6
Valine	3.45	11	0.07	1	2
Leucine, Isoleucine	1.02	3	0.03	0	3

[a] ^{15}N 69.6 atom% excess.

[b] Glutamic acid = 100.

Table 5 *Quantities of free amino acids and ammonium in rice seedling roots treated and untreated with MS*

	(A) −MS pretreatment (nmole/plant)	(B) +MS pretreatment (nmole/plant)	$\frac{(B)}{(A)} \times 100$
Ammonium	46.3	113.3	240
Glutamic acid	70.8	54.8	77
Aspartic acid	39.5	29.0	73
Glutamine	118.3	11.8	10
Asparagine	42.0	24.2	58
Alanine	23.8	14.0	59
Serine	16.0	12.9	80
γ-Amino butyric acid	4.2	1.5	36
Threonine	1.9	1.2	65
Valine	6.6	4.9	73
Leucine	1.2	1.1	92
Isoleucine	1.2	1.0	87

partments must be composed of ammonium which has a higher ^{15}N abundance than any amino or amido nitrogen.

Asparagine, especially its amino nitrogen, showed low ^{15}N value. Amido nitrogens of glutamine and asparagine showed remarkably higher ^{15}N value than their amino nitrogens.

In roots pretreated with MS, in spite of the decrease in total uptake of ammonium (Table 6), the quantity and ^{15}N enrichment of ammonium distinctly increased compared to roots not pretreated with MS. We have proposed the hypothesis (*1*, *2*) that most of the glutamine is synthesized adjacent to the outer membrane or plasmalemma of root cells, through which ammonium with a high ^{15}N abundance permeates from the external solution. Accordingly it can be considered that the increase in the ^{15}N abundance of ammonium after pretreatment with MS might be due to enlargement of this ammonium compartment near the membrane.

On the other hand, MS does not inhibit GDH, but both the quantity (Table 5) and the ^{15}N abundance (Table 4) of glutamic acid decrease, in contrast to the case of ammonium, suggesting that the main route of glutamic acid synthesis is not from ammonium but from glutamine. Two cases are possible for glutamine-dependent glutamic acid synthesis. In the first case, glutamic acid synthesis requires the amido radical of glutamine as a direct nitrogen source. In the second case, glut-

Table 6 *Effect of MS pretreatment on ammonium uptake by rice seedlings*

Control	MS-pretreated
10.2	5.2

MS-pretreatment was carried out with 5 mM MS for 10 min. After the pretreatment, plants were placed in a 5 ppm ammonium nitrogen medium for 1 hr. Uptake amounts are expressed as N μg per plant·1 hr.

amine has the role of transporting ammonium to the site of glutamic acid synthesis by GDH. In this case, the site of glutamic acid synthesis is separated from the site of glutamine synthesis, and glutamic acid must be carried back to the site of glutamine synthesis as its precursor. Considering the active synthesis of glutamine under certain conditions and the low permiability of glutamic acid, it is not likely that the second case is dominant in the glutamine-dependent glutamic acid synthesis observed here. The ^{15}N abundances of glutamic and aspartic acid does not decrease as much as it does in the case of amido nitrogen after MS pretreatment (Table 4), suggesting that glutamic acid synthesis partially depends on the GDH system using ammonium with a high ^{15}N abundance in roots pretreated with MS. Consequently, it could be concluded that the GS-GOGAT system is the dominant path of glutamic acid synthesis in rice seedling roots and, at least under certain conditions such as high ammonium concentration in the roots due to MS-treatment, the GDH system partially contributes to its synthesis.

The ^{15}N abundances of amino nitrogen of glutamine and asparagine remarkably decreased after pretreatment with MS. This is probably due to the inhibition of glutamine synthesis and decrease of the ^{15}N abundances of glutamic and aspartic acid. Moreover, it is noteworty that the ^{15}N abundance of asparagine amido decreased remarkably after pretreatment with MS, in spite of the increase of the ^{15}N abundance of ammonium (Table 4). This suggests that glutamine has a certain role in asparagine synthesis. It has already been shown (*2*) that, in rice seedling roots, incorporation of ^{15}N-labelled ammonium into asparagine had a lag-time whereas incorporation into glutamine and ammonium did not. Hence, it is thought that asparagine is synthesized getting its amido nitrogen mainly from glutamine amido directly. It is conspicuous that the ^{15}N abundances of many amino acids became much lower than that of glutamic acid after pretreatment with MS.

Table 7 shows the ^{15}N abundances of free amino acids and ammonium in rice seedling roots after 1 hr absorption of ^{15}N(amido)-glutamine. As shown in Table 7, asparagine amido has much a higher ^{15}N abundance than that of ammonium in roots not pretreated with MS. Although in roots pretreated with MS the ^{15}N abundance of glutamine amido decreased, these of asparagine amido and ammonium were 11% and 138% higher respectively than in the case of untreated roots. These results also suggest the predominance of glutamine-dependent asparagine synthesis and a minor synthesis depending on ammonium. Accordingly, it could be assumed that L-aspartate : L-glutamine amido-ligase (EC 6.3.5.4) exists in rice seedlings. Rognes has recently demonstrated its existence in *Lupinus luteus* (*11*).

Moreover, Table 7 shows that, in roots not pretreated with MS, the ^{15}N abundances of glutamic acid, aspartic acid, asparagine amido, alanine and glutamine amino were higher than that of ammonium. In this experiment, external ammonium was not supplied. In this case, glutamine-dependent glutamic acid synthesis might play a more important role in supplying the amino nitrogen to other amino acids than in the case of ammonium-feeding experiments. Data presented in Table 7 show that the ^{15}N abundance of glutamic acid is more than 2 times higher than that of ammonium, and this ratio is larger than that in the ammonium-feeding experiment in Table 4. From these considerations also, it could be concluded that glutamic acid synthesis is mainly GOGAT-dependent.

Table 7 *The ^{15}N abundances of free amino acids and ammonium in rice seedling roots after 1 hr absorption of ^{15}N (amido)-glutamine*[a]

	−MS pretreatment		+MS pretreatment		
	(A) atom%ex.	index[b]	(B) atom%ex.	index[b]	$\frac{(B)}{(A)}\times 100$
Ammonium	2.50	44	5.95	139	238
Glutamic acid	5.64	100	4.28	100	76
Aspartic acid	5.30	94	3.62	85	68
Glutamine amido	9.24	164	7.93	185	86
Glutamine amino	2.21	39	0.30	7	14
Asparagine amido	3.79	67	4.21	98	111
Asparagine amino	1.03	23	0.57	13	55
Alanine	3.65	65	1.81	42	50
Serine	1.56	28	1.07	25	69
γ-Amino butyric acid	0.94	17	N.D.	—	—
Threonine	0.63	11	0.51	12	81
Proline	0.58	10	0.21	5	36
Tyrosine	0.37	7	0.07	2	19
Cysteic acid	0.65	12	N.D.	—	—
Valine	0.39	7	0.10	2	26
Leucine, Isoleucine	0.28	5	0.12	3	43

[a] ^{15}N 49.6 atom% excess in amido nitrogen.
[b] Glutamic acid=100.

Table 7 showed that, in roots pretreated with MS, ammonium had a much higher ^{15}N abundance than in untreated roots, suggesting active release from glutamine amido and reincorporation into it. All amino acids except asparagine amido and ammonium in roots pretreated with MS showed lower ^{15}N abundances than in untreated roots. But the decrease in these ^{15}N abundances after MS-treatment (Table 7) was less than that in the ammonium-fed experiment (Table 4). Hence, exogenous glutamine could be assumed to partially compensate for the decrease of glutamine synthesis after MS-treatment.

The results of experiments in which plants were supplied with ^{15}N-ammonium and ^{14}N-glutamine (medium C), and ^{14}N-ammonium and ^{15}N(amido)-glutamine (medium D) are given in Table 8. The chemical composition of both media were equal; hence the nitrogen metabolism should be the same.

In roots unpretreated with MS, the ^{15}N abundances of all examined nitrogeneous compounds were higher in roots fed medium C, showing that the ammonium is absorbed more easily than glutamine.

Generally, the pattern of ^{15}N abundance indexes (glutamic acid=100) are similar for roots unpretreated with MS in both media, indicating that ammonium and glutamine amido nitrogen are mainly metabolized through the same pathway. In roots fed medium C, a remarkable increase in the ^{15}N abundance of ammonium compared to other amino acids, as well as a relatively small decrease in the ^{15}N abundances of glutamic and aspartic acids after MS-pretreatment were observed. In roots fed medium D, the ^{15}N abundance of ammonium decreased, and those of

Table 8 *The ^{15}N abundances of free amino acids and ammonium in rice seedling roots after 1 hr simultaneous absorption of ammonium and glutamine*

	−MS pretreatment				+MS pretreatment			
	Medium C [a]		Medium D [b]		Medium C [a]		Medium D [b]	
	atom%ex.	index [c]	atom%ex.	index [c]	atom%ex.	index [c]	atom%ex.	index [c]
Ammonium	24.5	108	3.14	167	49.8	540	1.71	67
Glutamic acid	22.6	100	1.88	100	9.21	100	2.56	100
Aspartic acid	20.1	89	1.55	82	7.93	86	2.00	78
Glutamine {amido	28.0	124	4.32	230	5.12	56	4.81	188
Glutamine {amino	19.8	88	1.60	85	2.41	26	0.15	6
Asparagine {amido	13.5	60	1.43	76	2.99	32	2.16	84
Asparagine {amino	7.10	31	0.53	28	1.19	13	0.20	8
Alanine	14.5	64	1.13	60	4.66	51	1.65	64
Serine	6.81	30	0.42	22	1.62	18	0.60	23
γ-Amino butyric acid	3.47	15	0.86	46	0.93	10	0.57	22
Threonine	N.D.	—	N.D.	—	0.84	9	0.36	14
Proline	2.22	10	N.D.	—	0.50	5	0.16	6
Tyrosine	0.96	4	N.D.	—	0.07	1	0.09	4
Cysteic acid	1.58	7	N.D.	—	0.52	6	0.25	10
Valine	3.49	15	0.88	47	0.11	1	0.11	4
Leucine, Isoleucine	0.94	4	0.08	4	0.00	0	0.07	3

[a] Medium C contained ^{15}N-ammonium (^{15}N: 69.6 atom% excess) and ^{14}N-glutamine.
[b] Medium D contained ^{14}N-ammonium and ^{15}N(amido)-glutamine (^{15}N: 49.6 atom% excess in amido nitrogen).
[c] Glutamic acid=100.

amino acids, except γ-aminobutyric acid, valine and amino nitrogen of glutamine and asparagine, increased after MS-pretreatment. This might indicate that the ^{14}N-ammonium in the roots increased, and the incorporation of ^{14}N-ammonium into amino acids via the GS-GOGAT pathway decreased due to GS inhibition.

References

(*1*) Arima, Y.: Rapid incorporation of ^{15}N into amide nitrogen of rice seedling roots from $(^{15}NH_4)_2SO_4$ (Part 1). Physiological significance of glutamine on nitrogen absorption and assimilation in plants. *J. Sci. Soil and Manure, Japan* 45: 509–512 (1974).

(*2*) Arima, Y. and K. Kumazawa: A kinetic study of amide and amino acid synthesis in rice seedling roots fed with ^{15}N labelled ammonium (Part 2). Physiological significance of glutamine on nitrogen absorption and assimilation in plants. ibid. 46: 355–361 (1975).

(*3*) Arima, Y., T. Horinouchi and K. Kumazawa: Variation and regulation of glutamine synthetase activity in rice seedlings fed ammonium and nitrate (Part 4). Physiological significance of glutamine on nitrogen absorption and assimilation in plants. ibid. 47: 198–203 (1976).

(*4*) Cocking, E. C. and E. W. Yemm: Synthesis of amino acids and proteins in barley seedlings. *New Phytol.* 60: 103–116 (1961).

(*5*) Dougall, D. K.: Evidence for the presence of glutamate synthase in extracts of carrot cell cultures. *Biochem. Biophys. Res. Commun.* 58: 639–646 (1974).

(*6*) Elliott, W. H.: Isolation of glutamine synthetase and glutamo transferase from green peas. *J. Biol. Chem.* 201: 661–672 (1953).

(*7*) Fowler, M. W., W. Jessup and G. K. Sarkissian: Glutamate synthase type activity in higher plants. *Febs. Lett.* 46: 340–342 (1974).

(*8*) Lea, P. J. and B. J. Miflin: An alternative route for nitrogen assimilation in higher plants. *Nature* 251: 614–616 (1974).

(*9*) Rhodes, D., G. A. Rendon and G. R. Stewart: The control of glutamine synthetase level in *Lemna minor* L. *Planta* 125: 201–211 (1975).

(*10*) Rhodes, D., G. A. Rendon and G. R. Stewart: The regulation of ammonia assimilating enzymes in *Lemna minor*. ibid. 129: 203–210 (1976).

(*11*) Rognes, S. E.: Glutamine-dependent asparagine synthetase from *Lupinus loteus*. *Phytochemistry* 14: 1975–1982 (1975).

(*12*) Ronzio, R., W. B. Rowe and A. Meister: Studies on the mechanism of inhibition of glutamine synthetase by methionine sulfoximine. *Biochemistry* 8: 1066–1075 (1969).

(*13*) Yoneyama, T. and K. Kumazawa: A kinetic study of the assimilation of ^{15}N-labelled ammonium in rice seedling roots. *Plant & Cell Physiol.* 15: 655–661 (1974).