スギ苗のアミノ酸代謝におけるシトルリンの役割とオルニチンサイクル

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論　文

The Role of Citrulline in Amino Acid Metabolism and the Ornithine Cycle in *Cryptomeria japonica* Seedlings*

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MORI, Tokunori: **The role of citrulline in amino acid metabolism and the ornithine cycle in *Cryptomeria japonica* seedlings J. Jap. For. Soc. 58: 328～333, 1976** Uniformly labeled ^{14}C-ornithine and ^{14}C-arginine were used to estimate the biosynthesis, translocation, and conversion of citrulline in *Cryptomeria* seedlings which were grown under aseptic conditions. The main products from ornithine were citrulline, arginine, glutamic acid, and proline, and those from arginine were glutamic acid, glutamine, citrulline, γ-guanidinobutyric acid, and an unidentified arginine derivative. Ornithine was converted to citrulline to a larger extent than arginine. When radioactive ornithine and arginine were applied to the roots, major labeled compounds in the shoots were citrulline and glutamic acid, and citrulline was accumulated markedly. In contrast, when arginine was applied directly to the shoots, citrulline level was very low. Translocation of the large amount of citrulline and the marked accumulation of citrulline prove the suggestion of the previous studies that the roots of *Cryptomeria* seedlings export citrulline to the shoots and that the shoots pool it as a soluble reserve. The ornithine cycle may operate as a complete cycle in *Cryptomeria* seedlings, but conversion from arginine to ornithine is thought to take place at a low level.

森　徳典：**スギ苗のアミノ酸代謝におけるシトルリンの役割とオルニチンサイクル　日林誌 58: 328～333, 1976**　無菌状態で育てたスギ芽ばえにシトルリンの前駆物質として，オルニチン-^{14}C(U) とアルギニン-^{14}C(U) を与えた後のシトルリンの合成，転流，集積等について調べた。オルニチンからシトルリンへの転換は非常に多かったが，アルギニンからのそれは少なかった。根部に標識化合物を与えた場合，地上部の各アミノ酸への ^{14}C の分配は根部とは明らかに異なるとともに，また地上部に直接標識化合物が与えられた場合とも異なり，シトルリンの放射能の割合が高かった。地上部ではまたシトルリンの顕著な集積がみられた。これらのことは根から地上部へ送られるアミノ酸は主としてシトルリンであり，地上部でそれが集積されやすいとした前報の結果を裏付けた。オルニチンおよびアルギニンから各アミノ酸への ^{14}C の分配だけからみると，オルニチンはオルニチンサイクルによってすみやかにシトルリン，アルギニンに代謝されるが，アルギニンからオルニチンへの転換は前者とくらべてやや不活発であるように思われる。

I. Introduction

In *Cryptomeria* shoots, citrulline is one of the major free amino acids during the growing season, occupying about 20 to 40% of the total free amino acid(*7*). Its level is affected markedly by the nitrogenous nutrient conditions; the high level of nitrogen in the culture solution resulted in remarkable increase in citrulline level, and the shoots which have a high concentration of total nitrogen contain a large amount of citrulline(*9*). When the seedlings become nitrogen deficient, the citrulline level shows the greatest decline(*9*), and the rate of citrulline degradation in the shoots is also higher at the low free amino acid pool than at the high free amino acid one(*10*). These findings lead to the assumption that citrulline plays an important role as a soluble reserve in the shoots. The previous study also indicates that citrulline may be biosynthesized in the roots and then translocated readily to the shoots in the transpiration stream in the xylem(*8*).

In the present study special attention was given to the synthesis of citrulline in the roots and the fate of citrulline in the shoots. In addition, the

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metabolic changes of ornithine and arginine which were applied independently to the seedlings were discussed, as there has been no general agreement that the KREBS-HENSELEIT or ornithine cycle operates in the same way in trees as in animals (*1, 2, 4, 6*).

II. Materials and Methods

1. Plant materials

Germinating *Cryptomeria japonica* seeds were transplanted on agar in large test tubes with the following nutrients(mg/*l*); $(NH_4)_2SO_4$: 189, $NaNO_3$: 182, $Ca(NO_3)_2 \cdot 4H_2O$: 84, KH_2PO_4: 94, KCl: 52, $CaCl_2 \cdot 2H_2O$: 51, $MgSO_4 \cdot 7H_2O$: 123, $MnSO_4 \cdot 4H_2O$: 10, Fe-FDTA salt: 5, $ZnSO_4 \cdot 7H_2O$: 4, KI: 1.6, H_3BO_3: 3, $NaMoO_4 \cdot 2H_2O$: 0.02, $CuSO_4 \cdot 7H_2O$: 0.002, $CoCl_2 \cdot 6H_2O$: 0.002, and sucrose: 2,000. The seedlings were left in a growth cabinet at 24°C, 4,500 lux with a 14 hour light period for about 100 days. Before 15 days of radioisotope application, light intensity was raised to 25,000 lux. All procedures were carried out under sterile conditions and test tubes contaminated with fungi were discarded. Average height and fresh weight were about 5 cm and 100 mg per seedling.

2. Application of ^{14}C-ornithine to intact seedlings

Aseptic 1/20 M phosphate buffer solution (pH 5.8) containing 3×10^{-4} M $(NH_4)_2SO_4$ and [U-^{14}C]-L-ornithine (261 mCi/mmol, NE) was applied to the roots under the same conditions mentioned above. After 24 hours, the seedlings were transferred into non-radioactive nutrient solution. Six to eight seedlings were sampled at each testing.

3. Application of ^{14}C-arginine to intact and rootless seedlings

The solution with [U-^{14}C]-L-arginine (240 mCi/mmol, NE) was fed to the roots for 8 hours, and then the seedlings were transferred to the non-radioactive solution. After start of radioisotope application, the seedlings were kept under continuous light and the other conditions were the same as above. In the experiment with the rootless seedlings, the radioactive arginine was absorbed from the cut end of the stem in the same manner as the intact seedlings.

4. Extraction and determination of ^{14}C-compounds

The seedlings were weighed immediately after they were washed and sorted into roots and shoots, which were separately homogenized in 80% ethanol by an Ultra-Turrax homogenizer for 20 sec. The homogenate was centrifuged at 9,500 g for 10 min. The precipitate was again homogenized and centrifuged, followed by two washings with 80% ethanol. All of the supernatant were combined and then evaporated to dryness *in vacuo* below 40°C. The dried extract was dissolved in 20 m*l* water, and the solution was fractionated on Dowex 50 W×8 column into cationic and anionic plus neutral fractions. Radioactivity of these fractions was determined by the procedure described previously(*8*).

The cationic fraction was again evaporated to a small volume by the method mentioned above. Amino acids were separated by two dimensional paper chromatography with *n*-butanol: acetic acid: water (90: 15: 29, v/v) and phenol: water (80: 20, v/v, in ammonia gas atmosphere). After preparation of autoradiography, each amino acid spot on the paper was cut off and combusted by a Packard 503 sample oxidizer. $^{14}CO_2$ was dissolved into Carbosorb™-Permaflour V (Packard Inst. Co., Inc.) scintillator, and the radioactivity was determined by liquid scintillation spectrophotometry.

Paper chromatographical determination was done three times, and one of these was used for identification of amino acids by the color reaction with ninhydrine-, SAKAGUCHI-, and EHLRICH-reagents. The residues extracted by alcohol were hydrolyzed with 6 N HCl *in vacuo* at 110°C for 20 hours to determine of radioactivity in proteins. After removal of HCl from hydrolyzate, amino acids were separated on Dowex 50 W×8 column from anionic and neutral substances. Protein amino acids were determined by paper chromatography.

III. Results

1. Ornithine metabolism in intact seedlings

Incorporation of ^{14}C into cationic- and anionic plus neutral-fractions and proteins is shown in Table 1. The main ^{14}C-labeled compounds in the cationic fraction in the roots at 2 hours are shown

Table 1. Incorporation of ^{14}C derived from [U-^{14}C]-L-ornithine into soluble fractions and proteins. ^{14}C-labeled ornithine was applied to the roots of *Cryptomeria* seedlings for 24 hr.

Part	Roots						Shoots					
Sampling time (hr)	2	4	8	24	48	72	2	4	8	24	48	72
Cationic fraction (dpm×10^{-4}/g, fresh weight)	219	313	601	579	458	402	5	8	21	41	53	86
Anionic plus neutral fraction (dpm×10^{-3}/g, fresh weight)	217	344	833	1058	1214	1230	2	29	119	142	353	475
Proteins (dpm×10^{-5}/g, dry weight of the residues)	98	192	472	752	1150	1135	1	3	7	17	53	143

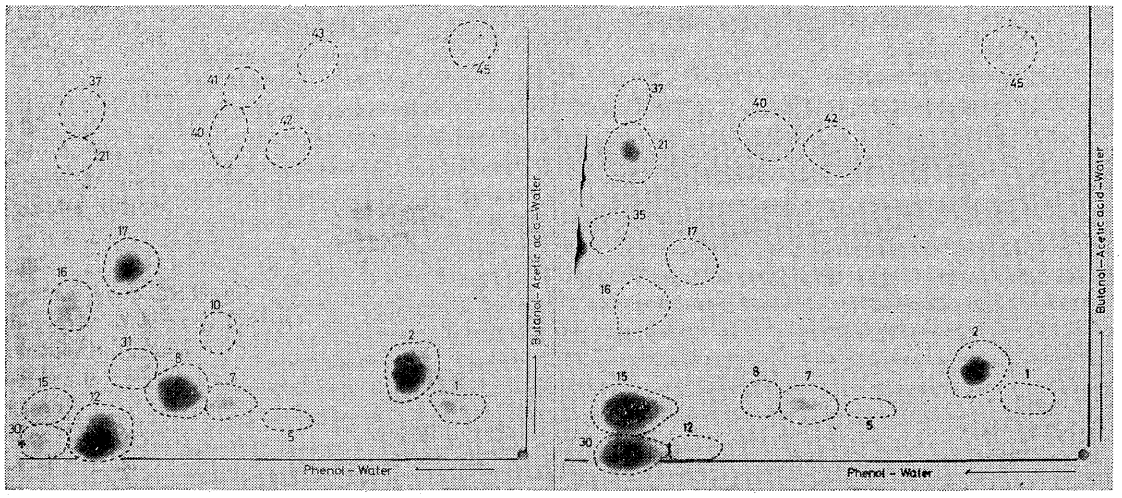

Fig. 1. Autoradiograms of cationic fractions

Left: labeled compounds derived from [U-^{14}C]-L-ornithine in the roots at 2 hr. Right: labeled compounds derived from [U-^{14}C]-L-arginine in the roots at 2 hr. Numbers in the figures indicate the following compounds; 1: aspartic acid, 2: glutamic acid, 5: asparagine, 7: glutamine, 8: citrulline, 10: alanine, 12: ornithine, 15: arginine, 16: proline, 17: γ-aminobutyric acid, 21: γ-guanidinobutyric acid (?), and 30-45: unidentified compounds.

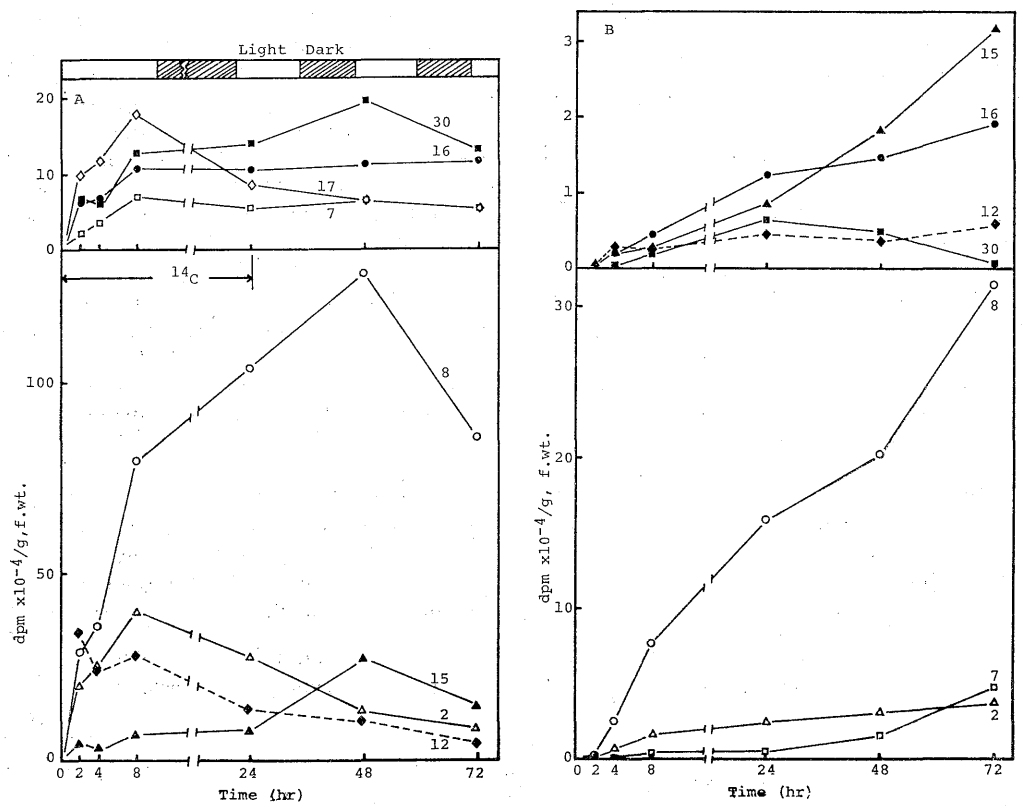

Fig. 2. Changes in radioactivity of main amino compounds derived from [U-^{14}C]-L-ornithine in the roots (A) and in the shoots (B)

^{14}C-ornithine was fed to the roots for 24 hr. The numbers in the figures indicate the same compounds as shown in Fig. 1.

Table 2. Distribution of ^{14}C in protein amino acids after application of [U-^{14}C]-L-ornithine to the roots

Part	Roots		Shoots
Sampling time (hr)	2	8	8
Aps.	3.1*	1.6	4.4
Glu.	7.3	8.1	10.6
Ser.	2.3	0.9	2.3
Ala.	1.8	1.5	2.7
Hyd-pro.	3.7	4.8	ND**
Arg.	41.7	46.3	50.9
Pro.	14.7	19.6	13.3

* Percentages of the total radioactivity on the paper
** No determination

in Fig. 1. Compound 30, as well as arginine, reacted strongly with the SAKAGUCHI reagent. Figure 2 shows the changes of radioactivity incorporated into main amino compounds in the roots and the shoots. In the roots, ^{14}C from ornithine was derived rapidly to citrulline, glutamic acid, and their relatives. Citrulline and its related compounds such as arginine and compound 30 increased to 48 hours, but glutamic acid and γ-aminobutyric acid reached maxima at an early sampling time (Fig. 2 A). In the shoots, almost all ^{14}C-labeled compounds increased to the end of the experiment, but citrulline accumulated an extremly large amount, and its radioactivity always occupied around 50 to 60% of the total radioactivity on the paper (Fig. 2 B). Amino acids incorporated into proteins were mainly arginine, proline, and glutamic acid (Table 2).

2. Arginine metabolism in intact and rootless seedlings

Incorporation of ^{14}C from arginine to soluble fractions and proteins is shown in Table 3. The main products of arginine in the cationic fraction were glutamic acid, glutamine, citrulline, and several unidentified compounds such as 21, 30, 42, and 45 (Fig. 1). Compounds 21 and 30 reacted with the SAKAGUCHI reagent and, judging from cochromatographic determination with standard chemical (Sigma Chem. Co.), the former compound appears to be γ-guanidinobutyric acid. Arginine was converted to citrulline much less than ornithine was, whereas glutamic acid, glutamine, and compounds 21 and 30 showed strong radioactivity in both roots and excised shoots (rootless seedlings, Fig. 3 A, 4). In contrast, there were large differences in the labeled compounds between the intact shoots and the excised shoots. When ^{14}C-arginine was applied to the roots, the shoots had high levels of glutamic acid and citrulline and low levels of arginine and its derivatives (compound 21 and 30, Fig. 3 B). On the other hand, when ^{14}C-arginine was applied directly to the shoots, the excised shoots had high levels of arginine and its derivatives as well as

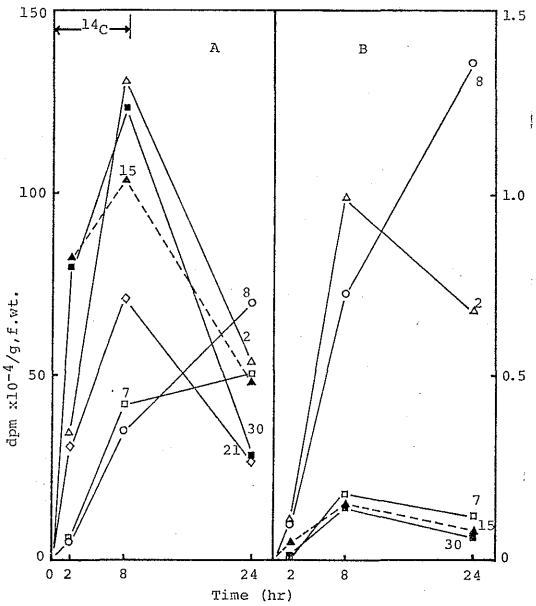

Fig. 3. Changes in radioactivity of main amino compounds derived from [U-^{14}C]-L-arginine in the roots (A) and in the shoots (B)

^{14}C-arginine was fed to the roots for 8 hr. The numbers in the figures indicate the same compounds as shown in Fig. 1.

Table 3. Incorporation of ^{14}C derived from [U-^{14}C]-L-arginine into soluble fractions and proteins*

Part	Intact seedlings						Rootless seedlings		
	Roots			Shoots			Shoots		
Sampling time (hr)	2	8	24	2	8	24	2	8	24
Cationic fraction (dpm x 10^{-4}/g, fresh weight)	700	1537	650	3	19	12	18	112	54
Anionic plus neutral fraction (dpm x 10^{-3}/g, fresh weight)	244	1267	2806	10	230	444	34	181	325
Proteins (dpm x 10^{-5}/g, dry weight of the residues)	101	495	ND**	2	7	26	25	172	281

* ^{14}C-labeled arginine was applied to the roots (intact seedlings) and the cut end of the stem (rootless seedlings) of *Cryptomeria* seedlings for 8 hr.
** No determination

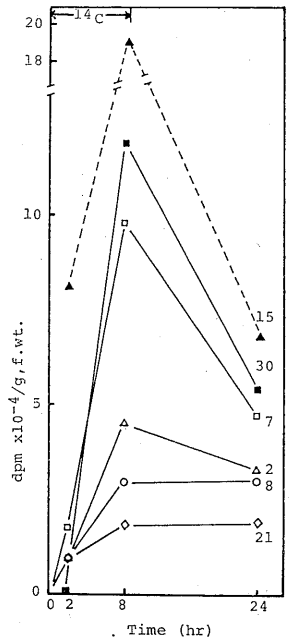

Fig. 4. Changes in radioactivity of main amino compounds derived from [U-^{14}C]-L-arginine in the rootless seedlings

^{14}C-arginine was fed to the cut end of the stem for 8 hr. The numbers in the figure indicate the same compounds as shown in Fig. 1.

Table 4. Distribution of ^{14}C in protein amino acids after application of [U-^{14}C]-L-arginine to the roots (intact seedlings) and to the cut end of the stem (rootless seedlings) for 8 hr

Part	Intact seedlings			Rootless seedlings	
	Roots		Shoots	Shoots	
Sampling time (hr)	2	8	8	2	8
Asp.	6.9*	2.3	10.0	0.7	1.4
Glu.	11.3	6.7	14.6	2.8	4.5
Ser.	2.0	0.6	0.7	0.6	0.5
Ala.	1.8	0.3	1.6	0.7	1.0
Arg.	39.2	56.6	44.5	86.6	81.6
Pro.	10.4	12.5	10.7	7.9	7.6

* Percentages of the total radioactivity on the paper

glutamic acid, but a lower level of citrulline (Fig. 4). In either case, the accumulation of citrulline was observed, and the incorporation of ^{14}C into protein amino acids did not show much difference from the result of ornithine application (Table 4).

IV. Discussion

When radioactive ornithine and arginine were applied to the roots, their levels in the shoots were very low while the levels of citrulline and glutamic acid were high even at the early sampling time (Fig. 2 B, 3 B). Furthermore, if exogenously applied arginine was transported directly to the shoots, such a large difference would not be observed in the distribution of radioactivity between the intact and excised shoots (Fig. 3 B, 4). Therefore, ornithine and arginine absorbed by the roots should be not transported to the shoots until they are converted to suitable forms for translocating, i.e. citrulline and glutamic acid(*8*). High radioactivity of citrulline in the shoots is also brought about by its accumulation. This is proven by the fact that after removing radioactive ornithine and arginine, citrulline continued to increase, but certain amino acids such as glutamic acid and arginine decreased or did not change markedly.

These results support the previously reported suggestion regarding the function of citrulline in *Cryptomeria* seedlings; citrulline plays a role of nitrogen translocation from root to shoot and is pooled as a reserve amino acid in the shoot. PATE(*11*) has described similar findings that roots of the field pea export a fairly restricted range of amino acids to the shoots, and that in the shoots certain amino acids are incorporated mostly into soluble reserves, while others are effective as immediate donor of nitrogen and carbon to synthesis of protein.

The pathway from ornithine to proline and glutamic acid via glutamic-γ-semialdehyde has been reported in various tree species(*1, 2, 4, 6*), and the findings in this experiment are in agreement with those results. Another pathway from ornithine, which is from ornithine to citrulline and arginine in the ornithine cycle, is also working actively in *Cryptomeria* seedlings (Fig. 1, Table 2). However, the incorporation of ^{14}C from arginine to citrulline was considerably lower than that of ^{14}C from ornithine (Fig. 2 A and Fig. 3 A). In contrast, substances which reacted with the SAKAGUCHI reagent, such as γ-guanidinobutyric acid and compound 30, had very strong radioactivity levels and the changes in radioactivity were comparable with the change in arginine. Therefore, those compounds appear to be derived from arginine by a pathway other than the ornithine cycle.

Previously BARNES(*1, 2*) reported that it was uncertain whether or not the ornithine cycle was functioning as a complete cycle in roots and needles of pine. DURZAN(*3, 4*) observed recently in white spruce buds that although the ornithine cycle is present at low level, arginine is converted to monosubstituted guanidino compounds to a large extent. Arginase was not found in pine needles(*1*) although it was detected in conifer seeds(*5*). On the other hand, HILL-COTTINGHAM and LLOYD-

JONES (6) found that arginine, ornithine, and citrulline, were interconverted with each other by the ornithine cycle after application of these labeled amino acids singly to apple stem internodes throughout the year. The incorporation of ^{14}C into urea and guanidino compounds from carbamyl-^{14}C-citrulline and guanidino-^{14}C-arginine was not found. They concluded that no detection of labeled urea was due to the metabolization of urea as fast as it was formed.

When carbamyl-^{14}C-citrulline was fed to the shoots of one-month-old (10) and two-year-old (unpublished data) *Cryptomeria* seedlings, labeled arginine, arginosuccinic acid, and urea were found, but no monosubstituted guanidino compounds were detected. A trace of ^{14}C-urea was also found in the experiment of arginine application (no data indicated). Although the significance of the two metabolic pathways remains obscure, (i. e. the pathways from arginine to ornithine and to the guanidino compounds which may decompose to glutamic acid), the experiment shows that arginine converted in both directions and arginase activity may be low in *Cryptomeria* seedlings.

Acknowledgement

The author thanks Mr. H. ISIKAWA, Mr. T. SATO, and Mr. K. ODANI for the technical advice and assistance in the cultivation of the seedlings under sterile conditions.

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Errata

In the article entitled "Effect of nitrogenous nutrition on citrulline accumulation in *Cryptomeria japonica* shoots," by Tokunori MORI, which appeared in this journal **58**(1): 15～19, 1976, there are two errors in printing.

(1) Read "H_2O" for "N_2O" in the second column of Table 2 on page 16.

(2) Read "High nitrogen level of the culture solution brought about high total nitrogen and……." for "High nitrogen level of the culture solution brought about total nitrogen and……." in the first paragraph of IV. Discussion on page 18.