Changes in the Contents of Free and Protein Amino Acids in Hiproly Barley Callus during Differentiation

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Changes in the levels of free and protein amino acids in the callus of Hiproly barley were studied during differentiation. Adventitious roots were formed in the callus after 90 days of cultivation on modified White's medium containing 1 μ M indoleacetic acid (IAA) and 200 μ M adenine sulfate, and callus placed on Murashige-Skoog's medium without plant hormones formed adventitious roots after 30 days of cultivation. During differentiation, protein amino acids in the callus decreased, then increased, without an appreciable compositional change in the protein amino acids. The amount of free amino acids in the callus increased with root formation. The major free amino acids during differentiation were glutamine, asparagine, alanine, and proline. Glutamine increased until roots were found in the callus after cultivation. Asparagine gradually increased during differentiation.

Qualitative changes of enzyme proteins occur in a short period during differentiation of callus,¹⁾ and changes in the levels of soluble protein occur in the callus throughout differentiation.²⁾ These changes have been thought to be a reflection of differentiation.

Free and protein amino acids must be actively utilized in a callus throughout differentiation. In spite of the extensive studies on the changes in free and protein amino acids during differentiation, only a little is known about changes of nitrogenous constituents, and in particular, about the role of free and protein amino acids in differentiation. The earlier works of Sekiya and Yamada showed that protein in tobacco callus changes during differentiation of callus concomitant with roots and leaves.³⁾ Lamport reported compositional changes in hydroxyproline between rose callus and its organ tissues.⁴⁾ Ammirato reported that the presence of reduced nitrogen such as glutamine was especially important for differentiation in cultures of the yam.⁵⁾

On the basis of these studies, we have investigated how amino acids in Hiproly callus are changed throughout differentiation. This paper describes the changes of the contents in free and protein amino acids of Hiproly callus during differentiation.

MATERIALS AND METHODS

Material and chemicals. Hiproly barley was used in the experiment. Indoleacetic acid, adenine sulfate, and amino acid standard solution were obtained from Wako Pure Chemicals., 2,4-dichlorophenoxyacetic acid (2,4-D) was obtained from the Tokyo Kasei Co. White's medium and Murashige-Skoog's medium were obtained from Flow Laboratories.

Induction and growth of Hiproly callus. The seeds of Hiproly barley were sterilized by soaking in an ethanol-hydrogen peroxide mixture (1:1, v/v) for 10 min, and for germination they were put on modified White's medium containing 0.9% agar and 100 μ M 2,4-D in silicone capped test tubes at 26°C for one month in the dark. A white friable callus was produced on most root sections within one month.

FIG. 1. Formation of Adventitious Roots on Hiproly Callus.

A) Hiproly callus differentiated adventitious roots at 90th day of cultivation on modified White's medium containing $1\text{ }\mu\text{M}$ indoleacetic acid and $200\text{ }\mu\text{M}$ adenine sulfate.

B) Hiproly callus differentiated adventitious roots at 90th day of cultivation on Murashige and Skoog's medium without plant hormones.

The callus was transferred to modified White's medium containing 0.9% agar and $10\text{ }\mu\text{M}$ 2,4-D in an Erlenmeyer flask. The callus was kept in logarithmic growth phase by transferring in the same medium every two months.

Formation of adventitious root from the callus. For differentiation, Hiproly callus put on a modified White's medium containing 0.9% agar, $1\text{ }\mu\text{M}$ indoleacetic acid and $200\text{ }\mu\text{M}$ adenine sulfate or on Murashige-Skoog's medium without plant hormones in an Erlenmeyer flask. The callus was kept at 26°C in the dark, and formed roots.

Analytical procedures for amino acids. Each lot of callus at a different stage of organ differentiation was extracted with 80% ethanol at 80°C for 30 min. This process was repeated 3 times. The extracts were dried *in vacuo* at 40°C and the residues, dissolved in 0.02 N HCl, were used for the free amino acid analysis with an auto amino acid analyzer (Hitachi-835).

The plant materials insoluble in 80% ethanol were hydrolyzed with 6 N HCl at 110°C for 24 hr. The content and composition of the protein amino acids were calculated from the amount of amino acids.

RESULTS

1) Formation and growth of adventitious roots on the callus

Formation and growth of adventitious roots on the callus are shown in Fig. 1. Adventitious roots were formed in the callus after 90 days of incubation, cultivating on the modified White's medium with $1\text{ }\mu\text{M}$ indoleacetic acid and $200\text{ }\mu\text{M}$ adenine sulfate. On the other hand,

the callus placed on Murashige-Skoog's medium without plant hormones also formed an adventitious root at the 30th day of cultivation. Photographs A and B show that adventitious roots grew on both media.

The addition of amino acids (glutamine and asparagine) in modified White's medium stimulated the root formation of Hiproly callus, a weak effect compared with the effect of adenine sulfate or Murashige-Skoog's medium containing inorganic reduced nitrogen such as $\text{NH}_3\text{-N}$.

2) Changes in free and protein amino acids in the callus during differentiation

Amount of free amino acids in the callus increased throughout the organ differentiation (Fig. 2). The accumulation of free amino acids increased with adventitious root formation. On the other hand, the level of protein amino acids in the callus decreased, then subsequently increased during the formation of adventitious roots (Fig. 2). When the callus differentiated, the level of protein amino acids declined and rose again.

3) Changes in composition of free and protein amino acids in the callus during differentiation

The composition of protein amino acids in

FIG. 2. Changes in the Levels of Free and Protein Amino Acids in Callus on Murashige-Skoog's Medium or Modified White's Medium during Differentiation.

Free form: △, Murashige and Skoog's medium, ▲, modified White's medium.

Protein form: ○, Murashige and Skoog's medium; ●, modified White's medium.

the callus at different stages of organ differentiation were investigated. No significant change in the amino acid composition of the callus protein was observed during differentiation.

Changes in the contents of free amino acids in the callus during organ differentiation are shown in Tables I and II. Appreciable compositional changes of glutamine, asparagine, alanine, and proline in free amino acids of callus were observed during differentiation. At an early stage of development, these four amino acids were the major free amino acids in the callus. At a latter stage of development, glutamine and asparagine were the major free amino acids. Glutamine increased in the callus associated with root formation. Asparagine gradually increased during differentiation.

Table III shows the levels of free amino acids in undifferentiated Hiproly callus. Increases in glutamine and asparagine in free amino acids were not observed during subculture.

DISCUSSION

Gamborg *et al.*⁶⁾ and Neumann *et al.*⁷⁾ reported that protein amino acids in calluses had almost the same amino acid composition which its organ tissue had. On the other hand, the works of Besenber,¹⁾ Bajaj *et al.*,²⁾ Sekiya *et al.*³⁾ and Lamport⁴⁾ suggest that the content of protein amino acids changes in calluses, along with an compositional change in protein amino acids during differentiation.

The content of protein amino acids in

TABLE I. LEVELS OF FREE AMINO ACIDS IN CALLUS AT DIFFERENT STAGES OF DIFFERENTIATION ON MURASHIGE AND SKOOG'S MEDIUM ($\mu\text{mol}/\text{callus} \cdot \text{g}$)

Amino acid	Cultivation time (day)				
	0	30	60	90	120
Aspartic acid	0.48	0.40	0.86	0.95	1.47
Threonine	0.18	0.19	0.50	0.35	0.41
Serine	0.79	0.74	0.93	1.11	1.56
Asparagine	1.98	1.98	2.51	5.68	65.88
Glutamic acid	1.36	0.89	1.49	1.83	4.21
Glutamine	2.40	9.27	12.83	17.82	44.73
Proline	1.10	1.22	2.35	3.70	5.21
Glycine	0.15	0.14	0.31	0.65	0.60
Alanine	2.25	2.29	0.65	1.61	2.71
Valine	0.33	0.29	0.21	0.44	1.12
Cystine	0.01	0.02	0.14	0.09	0.11
Methionine	0.02	0.04	0.21	0.09	0.10
Isoleucine	0.08	0.09	0.29	0.22	0.25
Leucine	0.15	0.17	0.43	0.32	0.38
Tyrosine	0.05	0.09	0.14	0.16	0.14
Phenylalanine	0.06	0.02	0.04	0.12	0.18
β -Alanine	0.04	+	+	+	+
γ -Aminobutyric acid	0.61	0.71	0.14	0.74	0.95
Ornithine	+	0.02	0.03	0.05	0.02
Tryptophan	0.02	+	+	+	0.05
Lysine	0.08	0.15	0.36	0.69	0.45
1-Methylhistidine	0.04	0.04	0.29	1.11	0.54
Histidine	0.06	0.08	0.14	0.30	0.33
Arginine	0.07	0.45	1.49	2.07	1.35

Symbol: +, trace.

TABLE II. LEVELS OF FREE AMINO ACIDS IN CALLUS AT DIFFERENT STAGES OF DIFFERENTIATION ON MODIFIED WHITE'S MEDIUM ($\mu\text{mol}/\text{callus} \cdot \text{g}$)

Amino acid	Cultivation time (day)				
	0	30	60	90	150
Aspartic acid	0.48	0.48	0.45	0.71	0.29
Threonine	0.18	0.16	0.18	0.29	0.31
Serine	0.79	0.71	1.45	1.08	0.64
Asparagine	1.98	3.08	3.98	5.05	10.91
Glutamic acid	1.36	1.13	1.70	1.47	1.14
Glutamine	2.40	4.60	5.61	13.03	9.83
Proline	1.10	5.58	4.22	0.68	2.22
Glycine	0.15	0.10	0.22	0.12	0.24
Alanine	2.25	2.46	2.56	2.12	0.59
Valine	0.33	0.31	0.38	0.51	0.33
Cystine	0.01	0.01	0.01	0.02	0.01
Methionine	0.02	0.03	0.02	0.02	0.02
Isoleucine	0.08	0.08	0.09	0.14	0.10
Leucine	0.15	0.12	0.13	0.29	0.10
Tyrosine	0.05	0.06	0.05	0.06	0.05
Phenylalanine	0.06	0.07	0.09	0.09	0.03
β -Alanine	0.04	0.06	0.03	0.03	+
γ -Aminobutyric acid	0.61	0.90	0.35	0.53	0.03
Ornithine	+	+	0.01	0.01	0.02
Tryptophan	0.02	0.02	0.01	0.01	+
Lysine	0.08	0.07	0.13	0.14	0.17
1-Methylhistidine	0.04	0.05	0.11	0.04	0.30
Histidine	0.06	0.06	0.09	0.08	0.16
Arginine	0.07	0.13	0.13	0.17	0.51

Symbol: +, trace.

Hiproy callus decreased then subsequently increased, without appreciable compositional change in the protein amino acids throughout root formation. Composition of protein amino acids in Hiproy callus naturally is almost the same as its origin tissue, root, has. This will affect decreases or increases in the content of protein amino acids without an appreciable compositional change (in the protein amino acids) during the differentiation.

During the organ differentiation of Hiproy callus, the amount of protein amino acids in Hiproy callus decreased, then increased, and the amount of free amino acids in the callus increased. This result suggests that the storage materials for nitrogen, protein amino acids, were hydrolyzed to free amino acids, and free amino acids were utilized for organ formation.

A decrease in the content of protein amino acids would be most important to the root formation of Hiproy callus.

Ammirato⁵⁾ reported that the presence of reduced nitrogen such as glutamine was especially important for differentiation in cultures of the yam. Kamata and Harada⁸⁾ reported that the addition of alanine or glutamine in Murashige-Skoog's medium without ammonium nitrate strongly stimulated somatic embryogenesis, glutamine and arginine were accumulated in cells during the maturation stage of somatic embryos, and the activity of nitrate reductase was enhanced by the application of alanine and glutamine.

During the differentiation of Hiproy callus endogenous free amino acids were especially important. The qualitative composition of free

TABLE III. LEVELS OF FREE AMINO ACIDS IN UNDIFFERENTIATED HIPLY CALLUS AFTER CULTIVATION ON MURASHIGE AND SKOOG'S MEDIUM WITH $10 \mu\text{M}$ 2,4-D ($\mu\text{mol/callus} \cdot \text{g}$)

Amino acid	Cultivation time (day)	
	60	120
Aspartic acid	0.35	0.35
Threonine	0.14	0.17
Serine	1.60	1.48
Asparagine	0.71	0.60
Glutamic acid	0.69	0.82
Glutamine	4.19	4.34
Proline	1.39	0.65
Glycine	0.14	0.19
Alanine	7.10	7.54
Valine	0.45	0.49
Cystine	+	+
Methionine	0.02	0.03
Isoleucine	0.05	0.07
Leucine	0.14	0.17
Tyrosine	0.03	0.07
Phenylalanine	0.03	0.05
β -Alanine	0.09	0.16
γ -Aminobutyric acid	5.14	9.93
Ornithine	0.01	+
Tryptophan	0.02	+
Lysine	0.10	0.10
1-Methylhistidine	0.06	0.07
Histidine	0.05	0.07
Arginine	0.12	0.09

Symbol: +, trace.

amino acids drastically change in Hiproly callus throughout organ differentiation. Alanine, proline, glutamine, and asparagine were the major free amino acids in the callus. Glutamine and asparagine increased in the callus associated with the organ differentiation. Accumulation of glutamine and asparagine were not observed in undifferentiated Hiproly callus. Although there is a need to discuss how the accumulation of glutamine or asparagine is related to differentiation, the change of physiological conditions in which glutamine and asparagine were accumulated, in particular, is significant for the organ differentiation of Hiproly callus.⁹⁾

These findings point out the importance of the changes in respiration during the organ formation. The relationship between differen-

tiation and respiration has been investigated in detail. There is evidence that catabolic reactions are geared to meet the energy requirements associated with organ differentiation. Thorpe¹⁰⁾ observed that requirements for ATP and NADPH are very high in the organ formation process, and differentiated callus frequently exhibits much higher rates of respiration than nondifferentiated callus, implying a coupling between differentiation and respiration.¹¹⁻¹³⁾

During root formation of Hiproly callus, an increase in the levels of glutamine and asparagine or a decrease in the levels of alanine and proline in the free amino acids will be much more closely related to the changes in respiration with the organ differentiation, along with the enhancement of the pentose phosphate pathway and malate metabolism, and the activities of the oxidation of pyruvate in the tricarboxylic acid cycle and concomitant oxidative phosphorylation.¹⁰⁾ The changes in these free amino acids therefore point out the enhancement of energy requirements in Hiproly callus associated with organ differentiation.

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