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Protein synthesis during the germination of turion in *Spirodela polyrrhiza*

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To evaluate protein synthesis during germination of turions (dormant body), the turions were induced by nitrogen-limiting medium from the greater duckweed, *Spirodela polyrrhiza*. During germination of the turions, fresh weight and intracellular components such as amino acids, proteins, RNA, and DNA were estimated. Effects of several antimetabolites added at various germination stages on the germination and the incorporation of radioactive amino acid into proteins were estimated.

These results indicate that the dormant turions should preserve long-lived messenger RNAs which are required for early protein synthesis because protein synthesis was not inhibited by cordycepin and 2-thiouracil. Pulse labeling experiments with ^{35}S-methionine showed that the proteins synthesized after 48 h of the germination appeared to be dependent upon newly transcribed mRNAs, since protein bands in SDS-polyacryamide gel electrophoresis and autoradiogram were markedly different between before 24 h and after 48 h of the germination.

In the greater duckweed, *Spirodela polyrrhiza*, dormant bodies called turions can be induced by low nitrogen levels, high concentrations of sucrose, high light intensities, and low temperatures (JACOBS, 1947). The turions produced by nitrogen deficiency were classified into young (Y) and old (O) types, which were induced at the early and the late stages, respectively (SIBASAKI and ODA, 1979). O-Type turions germinate rapidly in the presence of nitrogen and Y-type can germinate only after a definite dormant period. Y-Type turions can be transformed into O-type by exposure to low temperature for 2 weeks (SIBASAKI and ODA, 1979; MALEK and ODA, 1980).

It has been shown that various dry seeds and embryos preserve long-lived mRNAs by which early protein synthesis are sustained (WEEKS and MARCUS, 1971; GOLDEN and PAYNE, 1976). However, in wheat and radish embryos, it has been reported that half-life of the long-lived mRNAs is as short as about 2 h in the early germination and that its role is rapidly taken over by newly transcribed mRNAs (DELSENY *et al.*, 1977; CAERS *et al.*, 1979).

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In this paper, quantitative changes in amino acids, proteins, and nucleic acids, effects of several antimetabolites on germination and protein synthesis, and *in vivo* protein synthesis during the germination in O-type turions of *S. polyrrhiza* are described.

Materials and Methods

Plant material

Spirodela polyrrhiza L. Schleiden used in this experiment was provided by Dr. Y. ISHIGURI (Institute for Agricultural Research, Tohoku University). *S. polyrrhiza* was cultured aseptically in 1/2 nitrate Hoagland's medium containing 1% sucrose. Cultures were placed in a growth cabinet which was controlled at 26°C during a 16 h light and 8 h dark cycle. The light intensity in the growth cabinet was approximately 10,000 lux.

To induce turion (dormant body), frond colonies of *S. polyrrhiza* were transferred to 1/30 nitrate Hoagland's medium containing 3% sucrose, and cultured under continuous light at 26°C.

Germination of the turions was started by transferring them to the 1/2 nitrate Hoagland's medium containing 1% sucrose.

Extraction of amino acids, nucleic acids, and proteins

Amino acids, nucleic acids, and proteins were extracted by the STS method (SCHMIDT and THANNHAUSER, 1945; SCHNEIDER, 1946) with some modifications. Fifty turions were homogenized with 10% PCA at 0°C using a glass homogenizer. After centrifugation at 12,000×*g* for 10 min, the pellet was re-extract with cold 5% PCA, which was combined with the first PCA extract and subjected to determination of amino acids. After successive washing with 95% ethanol, ethanol-ether (1 : 1) at 40°C, and the same ethanol -ether at 0°C, the residue was suspended in 0.3 N KOH and stored at 30°C for 18 h. The suspension was chilled, neutralized with 6 N HCl, and acidified with 60% PCA. After centrifugation, the residue was again extracted with cold 5% PCA. The extracts were combined and subjected to RNA assay. The residue was suspended in 5% PCA and heated at 70°C for 15 min. After centrifugation, the residue was re-extracted with cold 5% PCA and the extracts obtained were combined with the hot PCA extract. The combined extracts were used for estimation of DNA. The resultant residue was extracted with 1 N NaOH at 50°C for 30 min, and re-extracted with cold 1 N NaOH. Both extracts were combined and subjected to estimation of protein content.

Assay

Amino acids and proteins were estimated by the method of YEMM and

COCKING (1954) and of LOWRY *et al.* (1970), respectively.

RNA and DNA were determined by measuring the absorbance at 260 nm.

Effects of antimetabolites

To investigate the effect of antimetabolites on the germination of turions, 10^{-4} to 5×10^{-4} M of 5-fluorouracil (5-FU), 2-thiouracil (2-TU), cordycepin, puromycin, and cycloheximide were added to the culture media at various stages of the germination.

The effect of the antimetabolites on protein synthesis was studied as follows: turions were incubated in culture media containing the respective antimetabolites and 100 μCi/m*l* of ^{3}H-leucine (200 μCi/mmol) for 1 to 3 h. Amino acids and proteins were extracted by the method of MARSH *et al.* (1982) with minor modification. One m*l* of the final extracts with 1 N NaOH was mixed with 4 m*l* of 25% TCA and the resultant precipitate was collected on a glass-fiber filter. The filter was rinsed with 5% TCA, dried, and placed in toluene-based scintillation fluid. Radioactivity was measured in a Beckmann liquid scintillation system.

In vivo protein synthesis

Soluble proteins were extracted by the methods of HANLEY-BOWDOIN and LANE (1983) and of HIGGINS *et al.* (1982). Fifty turions were homogenized with extraction buffer consisting of 100 mM potassium acetate, 3 mM magnesium acetate, 20 mM HEPES-KOH (pH 7.5) and 1 mM dithiothreitol. The homogenate was centrifuged at 27,000$\times g$ for 10 min and the supernatant was mixed with 4 vol of cold acetone (−20°C). The mixture was centrifuged at 10,000$\times g$ for 10 min, the precipitate was dissolved in sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% sucrose, and 0.002% Bromophenol Blue. The solution was heated at 95°C for 5 min and loaded onto the gel. SDS-polyacrylamide (12.5% acrylamide) gel electrophoresis (SDS-PAGE) was carried out according to LAEMMLI (1970). After electrophoresis, the gels were stained with 0.05% Coomassie brilliant blue, destained, and dried.

For pulse labeling studies, 2 μCi/m*l* of ^{35}S-methionine (1255 Ci/mmole) was added to the culture medium at different stages and the cultures were further incubated for 5 h in a growth cabinet. Extraction of soluble proteins was as described above.

Results

Changes in fresh weight and amounts of cellular components

As shown in Fig. 1A, the fresh weight of the turions increased rapidly at the eighth day of germination. Degrees of the germination were 70% at 3-

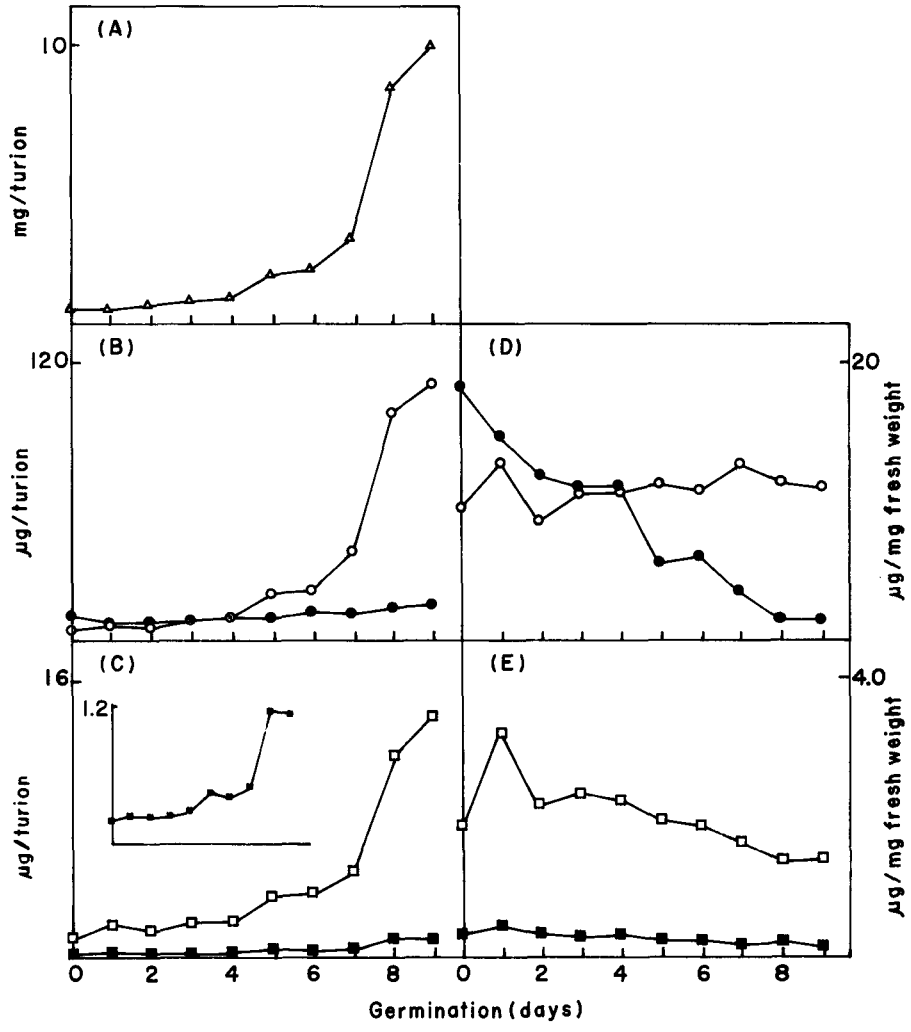

Fig. 1. Changes in fresh weight, amino acids, proteins, RNA and DNA of turions during germination. Extraction and estimation were carried out as described in **Materials and Methods**. Fresh weight (△—△), amino acids (●—●), proteins (○—○), RNA (□—□) and DNA (■—■).

day and 100% at 5-day (data not shown). Proteins, RNA, and DNA per turion increased by similar manner as fresh weight (Fig. 1B and 1C), accordingly their contents per fresh weight were little changed throughout the germination process (Fig. 1D and 1E). Conversely, amount of free amino acids per turion was not changed, but the contents per fresh weight decreased progressively (Fig. 1B and 1D).

Effects of antimetabolites on germination

When the turions were exposed to each of several antimetabolites from the start to the seventh day of the germination, all antimetabolites examined inhibited the germination (Table 1). When the turions were exposed to the respective antimetabolites for 24 h at divers time between the start and the fourth day of the germination, degrees of the germination of of the turions are shown in Table 1. When the turions were exposed to 5-FU, puromycin, and cycloheximide, the germination was definitely inhibited, but not by 2-TU and cordycepin. These results suggest that protein synthesis are essential to the early germination stage of the turions, and that some pre-existed mRNAs are operating for the protein synthesis.

Effects of antimetabolites on protein synthesis

The rates of uptake of ^{3}H-leucine by 0- and 2-day turions were similar (Fig. 2). The uptake of radioactivity was not influenced by addition of antimetabolites. Incorporation of ^{3}H-leucine into protein in the 0-day turions occurred a low level, but in the 2-day turions it was increased about 3-fold. Incorporation of the radioactivity was not affected by 2-TU and cordycepin in the 0- and 2-day turions, but completely inhibited by cyclohexi-

Table 1. Effects of antimetabolites on the germination of turions in *S. polyrrhiza*

		Stages for Addition (day)				
		0 - 7	0 - 1	1 - 2	2 - 3	3 - 4
5-Fluorouracil	5×10^{-4}M	+	+	+	±	−
2-Thiouracil	5×10^{-4}M	+	±	−	−	−
Cordycepin	10^{-4}M	+	−	−		
Puromycin	10^{-4}M	+	+			
Cycloheximide	10^{-4}M	+	+	+	+	+

After turions were germinated in media containing antimetabolites from the start to the seventh day or for 24 h between the start and the fourth day of germination, the effects were obserbed at the seventh or the fourth day of germination, respectively.
+ : inhibition, − : no effect.

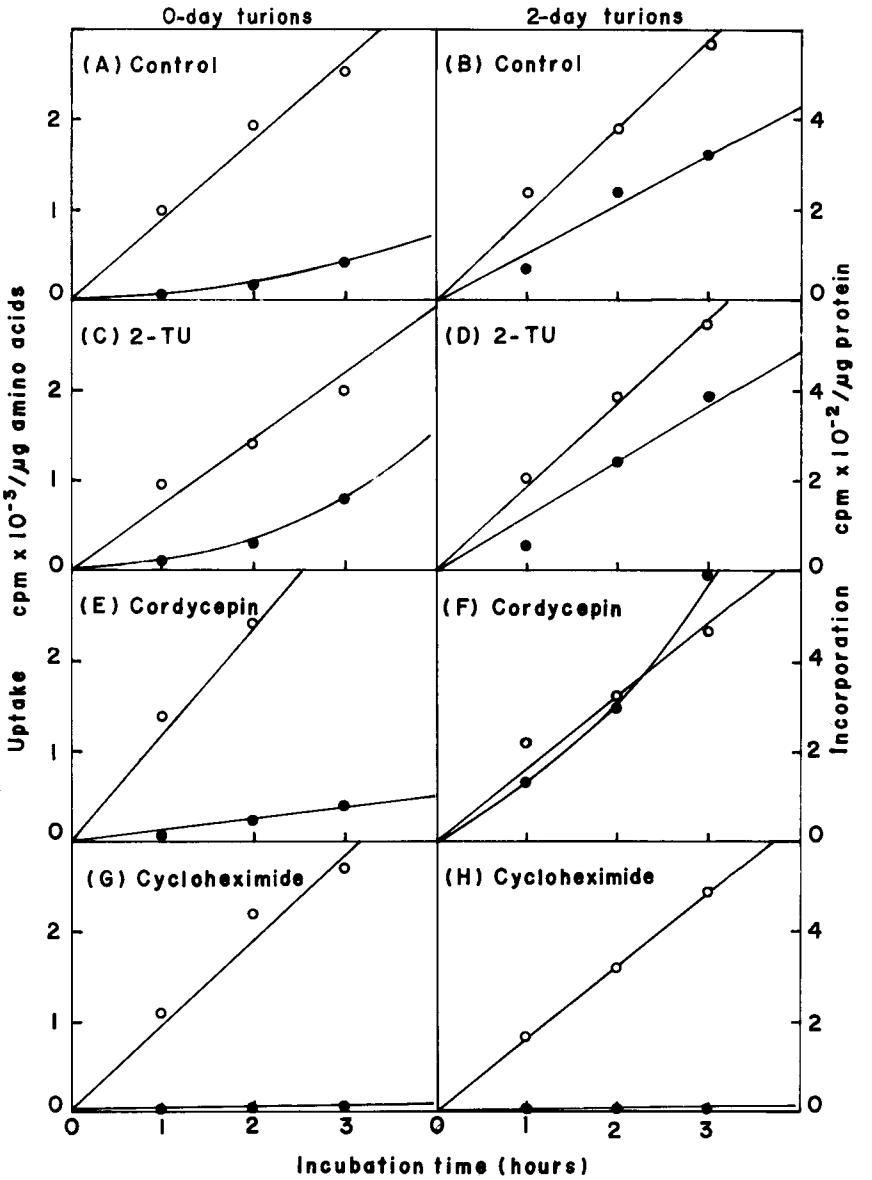

Fig. 2. Effects of antimetabolites on the uptake of ^{3}H-leucine and its incorporation into proteins in 0-and 2-day turiouns. Turions were incubated in 1/2 nitrate Hoagland's medium with or without antimetabolites. Assay counditions were the same as in **Materials and Methods**. Uptake (○—○) and incorporation into proteins (●—●).

mide, suggesting that the 0- and 2-day turions preserve long-lived mRNAs which may be mobilized during early protein synthesis.

In vivo protein synthesis

In vivo protein synthesis was assayed by incorporation of ^{35}S-methionine into turions during germination (Fig. 3). Soluble proteins in the turions were extracted and subjected for SDS-PAGE. After electrophoresis, one set of the gels were stained with Coomassie brilliant blue, and another set of the gels were subjected to autoradiography. As shown in Fig. 3, ungerminated turions contained only a small amount of soluble proteins and newly synthesized proteins. Among the newly synthesized proteins, about 58 kDa polypeptide was a major product and synthesized most actively throughout the germination period. Although quantity of the soluble proteins was considerably increased after 12 and 24 h germination, compositions of the newly synthesized proteins at the 12 h and 24 h samples were almost the same as the 0 time turions. However, in the 48 and 96 h turions, it was found that many different sizes of proteins were synthesized and the patterns became resemble those of the soluble proteins.

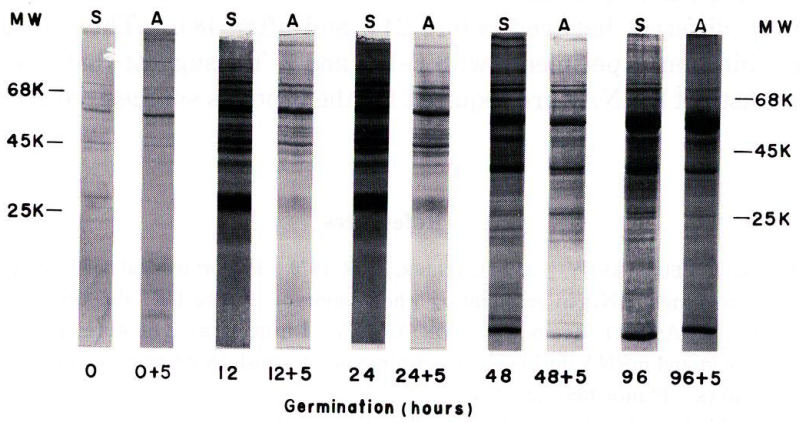

Fig. 3. Analysis of soluble proteins and *in vivo* labeled proteins in germinating turions by SDS-polyacrylamide gel electrophoresis. Figures show: Coomassie brilliant blue stained (Track S) and autoradiographs (Track A). Assay conditions were described in **Materials and Methods**.

Discussion

Proteins, RNA, and DNA increased during germination of the turions of *Spirodela polyrrhiza* as similar increasing manner as fresh weight of the turions, that is, they increased markedly during the eighth day of the germination (Fig. 1A-1C), when new fronds were produced from the turions (data not shown). Therefore, their contents per fresh weight of the turions were little changed during the germination period, but the amounts of amino acids per fresh weight decreased with the germination period (Fig. 1D and 1E). Thus, it seems likely that most newly synthesized amino acids are directly utilized for protein synthesis and did not increase the size of free amino acid pool in the turions.

As seen in Table 1, puromycin and cycloheximide inhibited the germination of the turions, but 2-TU and cordycepin did not. Furthermore, as shown in Fig. 2, incorporation of ^{3}H-leucine into proteins was inhibited by cycloheximide, but not by 2-TU and cordycepin. These results suggest that the dormant turions have pre-existed long-lived mRNAs which play an important role during early germination.

However, both profiles of the electrophoresis and autoradiogram were obviously different between before 24 h and after 48 h. These results and above inhibition experiments with 5-FU and 2-TU suggest that newly transcribed distinct mRNAs are required for the proteins synthesized after about 48 h.

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