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Induction of ultrastructural specialization for ureide metabolism in non-nodule soybean tissues cultured *in vitro*

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ABSTRACT

Root nodules of tropical legumes, such as beans and soybeans, produce ureides from nitrogen fixed by bacterial symbionts. Ultrastructural specialization for ureide production, including the appearance of abundant tubular ER and marked enlargement of peroxisomes, is known to occur in uninfected cells in the root nodule. Here we have investigated the capability of non-nodule tissues of soybean to specialize for ureide metabolism. Soybean embryonic axes excised from imbibed seeds were cultured *in vitro* on media with various nitrogen sources. The axes grew well on media containing urate or allantoin as the sole nitrogen source, but grew poorly on media with asparagine or glutamine as the sole nitrogen source. Root tissue and callus induced from segments of soybean embryonic axes also grew well on media containing urate or allantoin as the major nitrogen source. Numerous peroxisomes were observed in the root tissue and in proliferating callus cells growing on media containing urate as the major nitrogen source, whereas abundant ER was observed in the root tissue and callus cells growing on media containing allantoin. The capacity of non-nodule tissue cells to ultrastructurally differentiate for metabolizing externally supplied urate or allantoin was demonstrated.

1. Introduction

Many leguminous plants, including important crops, produce root nodules in which biological nitrogen fixation takes place. Organic nitrogenous compounds formed by N_2 fixation can be transported to the upper parts of the plant either as amides or ureides (allantoin and allantoic acid), so that legume nodules are classified as amide exporters or ureide exporters according to the compounds used for the mobilization of fixed nitrogen [1]. Soybeans develop globular nodules and synthesize ureides from fixed nitrogen. It is known that the uninfected cells in the nodules play an important role in the final steps of ureide biogenesis. For this purpose, they undergo ultrastructural differentiation, which includes the appearance of abundant tubular endoplasmic reticulum (ER) and a marked enlargement of peroxisomes [2–4]. Urate derived from xanthine dehydrogenase action in infected cells in the nodules moves to the uninfected cells where it is oxidized by uricase and catalase in the peroxisomes, eventually forming allantoin [5]. Allantoinase (EC 3.5.2.5) catalyzes the hydrolysis of allantoin to form allantoic acid, which is a key reaction for biogenesis and the degradation of ureides. Localization of nodule specific uricase in the peroxisomes has been shown immunocytochemically [4,6]. Since allantoinase was found in the microsomal fraction [7], it is assumed to be localized in the abundant tubular ER in the uninfected cells. Recently, the gene coding for allantoinase has been identified in Arabidopsis and Robinia [8] and allantoinase was shown to be localized in the ER in Arabidopsis [9]. However, cellular specialization of these tissues has not been observed at the ultrastructural level.

In order to better understand the mechanisms which furnish the capacity to produce and utilize ureides and the accompanying cellular differentiation outside of nodules, callus and root cultures of soybean were initiated from embryonic axis segments grown on media containing either urate or allantoin as the major nitrogen source and the resulting ultrastructural differentiation of these tissues was investigated.

2. Materials and methods

2.1. Culture of embryonic axes

Seeds of *Glycine max* (L.) Merr. cv. Mikawashima and *Pisum sativum* L. cv. Denko 30 nichi were disinfected in 70% (v/v) ethanol for 10 min, 1.2% sodium hypochlorite for 15 min and then washed

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Abbreviations: BA, benzyladenine; DAB, diaminobenzidine; ER, endoplasmic reticulum; NAA, naphthaleneacetic acid; TEM, transmission electron microscope.

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in sterile distilled water six times. Embryonic axes were excised from the sterilized seeds and cultured on MS [10] based media (pH 5.7) with the respective nitrogen source and kept under continuous light (15 W m^{-2}, FL40S fluorescent lamps, Toshiba, Tokyo, Japan) at 25 ± 2 °C. The media used in this experiment were MS medium, MS without nitrogen source, and MS with one of the following as the sole organic nitrogen source: asparagine, glutamine, xanthine, urate or allantoin at a concentration of 12 mM each. All media also contained 3% sucrose and 0.8% agar.

2.2. Root culture from embryonic axes

In order to identify the cells with specialized structure for ureide metabolism, we tried to establish a simpler culture system producing only root or callus. However, as consistent results were difficult to obtain with the media for embryonic axes, we compromised and adopted the following media for this purpose. The embryonic axes were excised from the disinfected *G. max* cv. Mikawashima seeds (as described above) and sliced into 1 mm thick segments. The segments were cultured on B5 [11] medium (pH 5.7) in which the inorganic nitrogen (KNO_3, $(NH_4)_2SO_4$) was reduced to 1/20 of its original content and the organic nitrogen sources 4 mM urate or 4 mM allantoin (modified from Shetty et al. [12]) with 0.01 ppm NAA, 0.001 ppm BA, 3% sucrose and 0.2% gelrite were added. The culture was maintained at 25 ± 2 °C under continuous light as above. Root segments for observation were taken 7 days after initiation of culture.

2.3. Callus induction from embryonic axes

The embryonic axes were excised from the disinfected *G. max* cv. Mikawashima seeds (as described above) and sliced into 1 mm thick segments. The segments were cultured on B5 medium (pH 5.7) with exactly the same replacement of nitrogen sources, etc, as above, but with 1 ppm NAA and 0.01 ppm BA. The culture was maintained at 25 ± 2 °C under continuous light as above. Induced calli were subcultured every two weeks with the same media. Calli for observation were taken after three successive subcultures.

2.4. Processing for light and electron microscopy

The root segments were cut with a razor blade and the callus was divided into small pieces (less than 2 mm in diameter), which were fixed with 2% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 6.8) for 2 h at room temperature and at 4 °C overnight. They were rinsed with the same buffer and post-fixed in 2% OsO_4 in 0.05 M potassium phosphate buffer (pH 6.8) for 2 h at room temperature, dehydrated in an acetone series and embedded in Spurr's resin. For light microscopy, sections about 0.5 μm in thickness were stained with a mixture of 0.05% toluidine blue and 1% sodium borate. For TEM, ultrathin sections (silver–gold) were stained with aqueous 2% uranyl acetate for 10 min followed by lead citrate for 5 min and observed with a Hitachi H-7500 TEM at an accelerating voltage of 100 kV.

2.5. Uricase cytochemistry with DAB

We used DAB for the cytochemical localization of uricase (EC 1.7.3.3) according to the method of Yokota and Nagata [13] as modified by Kaneko and Newcomb [3].

The callus and root cultures grown on urate containing medium were fixed in 2% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 6.8) for 30 min at 0 °C and rinsed in the same buffer for 30 min at 0 °C. The standard incubation medium consisted of 6 ml of 0.1 M Tris–HCl buffer (pH 9.6), 4 ml of a solution of sodium urate (5 mg/ml) and 10 mg of DAB (3,3′ diaminobenzidine, Sigma Chemical Company). The segments were incubated for 90 min at 30 °C in a reciprocal incubator at 60 rpm. In order to ensure aeration, the segments were placed in 30 ml beakers with a small amount (1.5 ml) of medium so that the surface of the segments made contact with air frequently during incubation. Control incubation was carried out in medium lacking urate. Following incubation, the segments were rinsed in 0.05 M potassium phosphate buffer (pH 6.8) and then post-fixed in 2% OsO_4 in 0.05 M potassium phosphate buffer (pH 6.8) for 1 h at room temperature, dehydrated in an acetone series and embedded in Spurr's resin. Ultrathin sections were observed with a Hitachi H-7500 TEM at an accelerating voltage of 100 kV.

2.6. Statistical Analysis

For statistical analysis, one-way ANOVA, Holm-Sidak method was performed using the SigmaStat program.

3. Results

3.1. Effect of different nitrogen sources on the growth of soybean and pea embryonic axes in vitro

Growth of *G. max* and *P. sativum* embryonic axes cultured *in vitro* with various nitrogen sources was compared. The media used were MS based media in which nitrogen compounds had been replaced by a single organic nitrogen source, either asparagine, glutamine, urate, allantoin, or xanthine. A typical result of five

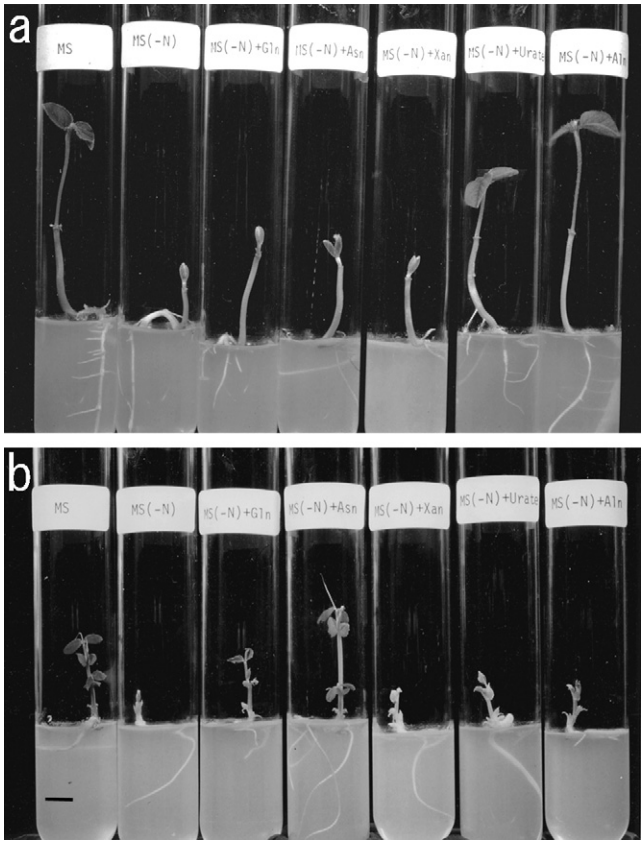

Fig. 1. (a) Soybean embryonic axes cultured for 12 days and (b) pea embryonic axes cultured for 18 days on MS, MS without inorganic nitrogen, MS without inorganic nitrogen but with either glutamine, asparagine, xanthine, urate, or allantoin (from left to right). Bar, 1 cm.