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Characteristics of Green Hairy Roots of *Hyoscyamus albus* Transformed with *Agrobacterium rhizogenes* Strain A4

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A transformed root culture of *Hyoscyamus albus* L. induced by *Agrobacterium rhizogenes* strain A4 was employed to investigate the tropane alkaloid producition and some characteristics of light-grown roots. Modified Woody Plant liquid media which consisted of half strength inorganic elements containing 25-40 mM KNO_3 effectively enhanced the alkaloid yields. The hairy roots turned palegreen when cultured in half-strength Murashige and Skoog liquid medium under 16 h/d light at 25°C. Chlorophyll contents of hairy roots in Gamborg B5 medium containing an additional 4 mM $(NH_4)_2SO_4$ was comparable to that of the roots in half-strength Murashige and Skoog medium although the hairy root in the modified Gamborg B5 medium showed slow growth. Ammonium ion may be one of the factors which control chlorophyll content. Electron microscopy revealed that the green hairy roots contained two types of plastids, immature chloroplasts and amyloplast-like ones which had both large granules and some thylakoid.

Introduction

Root cultures are expected to be advantageous to obtain secondary metabolites normally synthesized in the roots of plants. Those established by transformation with *Agrobacterium rhizogenes*, widely known as hairy roots, are particularly useful materials because of their rapid growth in culture media without phytohormones.

Hairy roots of several solanaceous plants including *Atropa, Datura, Duboisia, Hyoscyamus*, and *Scopolia* have been established and their tropane alkaloid production investigated[1]. Non-transformed adventitious root cultures and hairy root cultures of *Hyoscyamus albus* have been established and studied to determine the production of tropane alkaloids using *A. rhizogenes* strain 15834, MAFF 03-01724 and A4[2-6]. In this report, we describe further investigations with hairy roots transformed with *A. rhizogenes* A4.

Among the root cultures of *H. albus* mentioned above, the only one which was transformed with strain A4 turned pale green in colour when cultured in the light. Greening of hairy roots has been reported for *Bidens sulphureus, Tagetes patula*[7,8], *Digitalis purpurea*[9], *D. lanata*[10], *Lippia dulcis*[11], and *Amsonia elliptica*[12]. Some green hairy roots yielded remarkably higher amounts of the secondary metabolites than those cultured in the dark[9-11]. We also report the characteristics of the green

hairy roots of *H. albus*.

Materials and Methods

Hairy root culture.

Establishment of the hairy root culture was described previously[6]. *Hyoscyamus albus* L. was infected with *Agrobacterium rhizogenes* harbouring the Ri plasmid (pRiA4) by the leaf-disc method and the induced hairy roots were cultured on Murashige and Skoog (MS) solid medium[13] containing 0.5 g/*l* Claforan (Hoechst Japan Ltd.) to eliminate bacteria. The transformation was proved by an opine assay with paper electrophoresis[14].

For every experiment, hairy roots were pre-cultured in 50 m*l* (100 m*l* Erlenmeyer flask) of culture medium supplemented with 3% sucrose at 25°C on a rotary shaker at 100 rpm for 3 weeks. Two root tipe (about 20 mg fresh weight) from the conditioned hairy roots were inoculated into the same fresh medium and cultured under the same conditions.

Determination of tropane alkaloids.

Hairy roots were harvested 19 days after inoculation and the fresh weight (fw), and the dry weight (dw) after lyophilization, were determined individually. About 50 mg (dw) of each sample were extracted with 5 m*l* $CHCl_3$-MeOH-28% NH_4OH (15 : 5 : 1, w/w/w) as described by Kamada *et al.*[15]. The alkaloid fraction dissolved in MeOH was injected into ODS 120A column (4.6×250 mm, TOSOH, Japan) and eluted with MeCN-10 mM SDS solution (adjusted to pH 3.3 with phosphoric acid) (2 : 3)[3]. For quantitative analysis, authentic samples were used as the external standards. 7β-Hydroxyhyoscyamine was isolated previously from *H. albus* hairy roots[2]. 6β-Hydroxyhyoscyamine, scopolamine, and hyoscyamine were purchased from Sigma Chemical Co., USA. Littorine was synthesized from tropine and phenyllacetic acid by the same procedure described for atropine[16].

Chlorophyll determination.

Chlorophylls (Chls) were extracted with acetone-2.5 mM potassium phosphate (pH 7.8) (4 : 1) and determined with spectrophotometer as described Porra *et al.*[17].

Observation of ultrastructure

Green hairy roots were excised into small segments (2 mm length), and fixed with 2.5% glutaraldehyde in 50 mM phosphate buffer (pH 7.5) for two hours at room temperature. After rinse in the same buffer, samples were post-fixed with 1% OsO_4 in the same buffer for one hour at 4°C, dehydrated in a graded ethanol series and embedded in Spurr's resin. Ultrathin sections were made by ultramicrotome (Sorval MT-1), stained with uranyl acetate and lead citrate successively, and examined with an electron microscope (JEOL, JEM2000EX).

Results and Discussion

Effects of potassium nitrate addition on tropane alkaloid production

Sauerwein and Shimomura[5] demonstrated that hairy roots of *H. albus* transformed with *A. rhizogenes* MAFF 03-01724 exhibited remarkable growth and high productivity of tropane alkaloids in McCown's Woody Plant (WP) medium[18] supplemented with 15 mM KNO_3. On the other hand, Christen *et al.*[6] showed that alkaloid productivity in hairy roots transformed with another strain, *A. rhizogenes* A4 were not affected by addition of KNO_3 to WP medium. They also investigated the effects of several metal ions and reported that the growth and alkaloid production depended on the concentrations of Ca^{2+}, Zn^{2+} and Cu^{2+}. Their result implies that the growth and alkaloid production were the best when the concentrations of these three cations in WP medium were about a half. **Fig.**

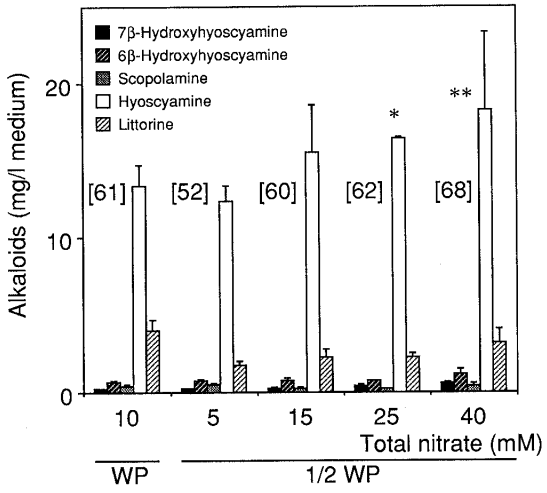

Fig. 1 Alkaloid production in *H. albus* hairy roots cultured in half-strength WP liquid medium with 3% sucrose and additional KNO_3 for 19 days.
WP medium originally contains 10 mM NO_3^-. Numbers in brackets show the fresh weight (g) per litre medium. Vertical bars denote the standard error ($n=3$). * $p<0.05$, ** $p<0.01$

1 shows the effcets of the addition of KNO_3 to a modified WP medium which consisted of half strength inorganic elements (1/2 WP) on the growth and the tropane alkaloid production of the hairy roots. The modified media containing 25 and 40 mM nitrate significantly enhanced the alkaloid yield. These results and those reported previously[6] suggested that a high concentration of nitrate with appropriate concentrations of inorganic elements was effective for promoting growth and alkaloid prodution. The inorganic elements might have some effect on the uptake and/or the metabolism of nitrate.

Effects of culture media on chlorophyll contents in the green hairy roots

Culturing in the light can make some kinds of hairy roots green and, in some cases, enhance the production of secondary metabolites as described in the introduction. The detailed characteristics of green hairy roots have not been reported in spite of their importance. We determined chlorophylls *a* and *b* in the green hairy roots cultured in various media. Christen *et al.*[6] reported that in the light *H. albus* hairy roots, which were also used in this study, showed a high yield of tropane alkaloids in Gamborg B5 (B5) medium[19]. However, the chlorophyll contents of the green hairy roots cultured in the medium were very low while culturing in 1/2 MS medium gave relatively high chlorophyll contents as shown in **Fig. 2**. We added ammonium salts to B5 medium, which originally contains 2 mM NH_4^+, to make the concentration of NH_4^+ equal to that of 1/2 MS medium (10 mM). Supplementation with $(NH_4)_2SO_4$ remarkably enhanced the chlorophyll contents while NH_4NO_3 was less effective (**Fig. 2**). It is unlikely that SO_4^{2-} increased the amount chlorophyll because B5 medium contains more SO_4^{2-} (2 mM) than 1/2 MS medium (1. 6 mM). We presume that NH_4^+ might have been the significant element which enhanced the chlorophyll contents. Nitrate ion might have some inhibitory effect on the chlorophyll contents. However, the growth of hairy roots in modified B5 media (B5+$(NH_4)_2SO_4$ or NH_4NO_3) was significantly lower than those cultured in 1/2 MS or B5 medium. Although relatively high chlorophyll content was observed with 1/2 MS medium, this phenomenon can not be explained by the high concentration of NH_4^+ alone.

Growth and alkaloid production of the green hairy in 1/2 MS medium were not significantly

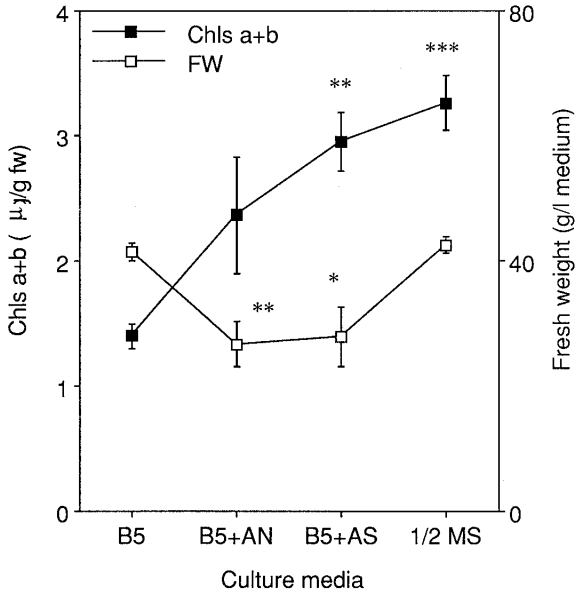

Fig. 2 Effect of culture media on chlorophyll content in *H. albus* hairy roots.
The culture media tested were Gamborg B5 medium (B5), B5 medium supplemented with 8 mM ammonium nitrate (B5+AN) or 4 mM ammonium sulfate (B5+AS), and 1/2 MS medium. Vertical bars denote the standard error (n=3-5). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

different from those of dark-grown hairy roots in the same medium (data not shown).

Ultrastructure of plastids in the green hairy roots

Observation by an optical microscope previously suggested that hairy roots of *Bidens sulphureus* and *Tagetes patula* cultured in the light had chloroplasts, although the activity of photosynthesis was not proved[7]. Generally the function of a plastid correlates with the ultrastructure. Normal chloroplasts in green photosynthetic tissues are characterized by the developed thylakoid membrane and

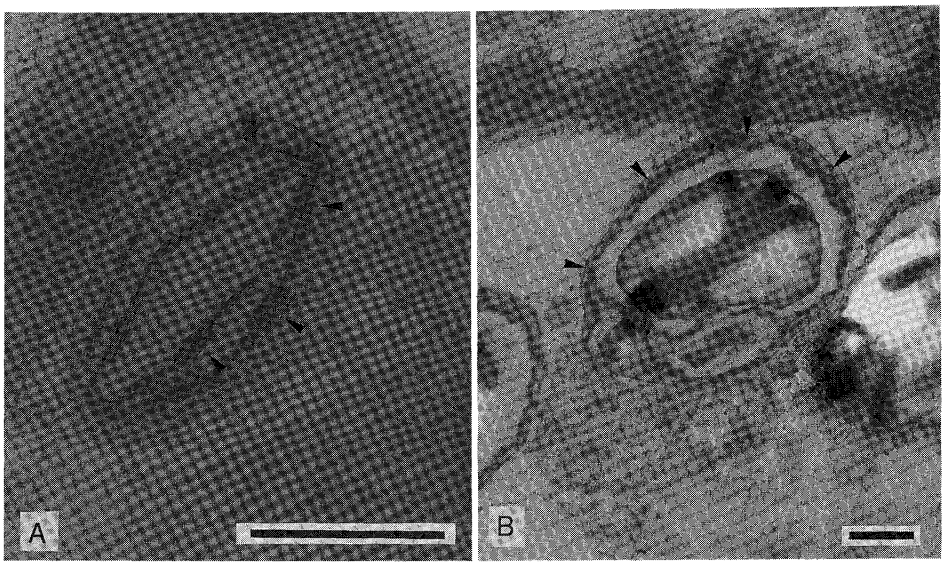

Fig. 3 Ultrastructure of different types of plastids in the light-grown hairy roots of *H. albus*.
Scale bars represent 1 μm. A: An immature chloroplast-like plastid containing poorly developed grana stack (arrow heads). B: An amyloplast-like plastid with some thylakoid in the marginal part (arrow heads).

the stacking of grana thylakoid. On the other hand, dark-adapted roots often contain leucoplasts, proteoplasts or amyloplasts, which have few thylakoid.

We observed the ultrastructure of *H. albus* green hairy roots. **Fig. 3** shows the different types of plastids observed by electron microscopy. The chloroplast-like structure shown in **Fig. 3-A** implied somewhat photosynthetic activity although the plastids had poor thylakoid and grana stack compared with normal chloroplasts in light-grown leaves. The other type of plastids shown in **Fig. 3-B** have large granules, which are generally observed in amyloplasts, and some thylakoid in the marginal area of the plastid. The plastids of this type were 3-5 times as large as those of the chloroplast-type. A single cell contained the same type of plastids even though the different types were observed in the green hairy roots as shown in **Fig. 3**. The ultratructures suggest that these plastids have different functions from those in underground roots or chloroplasts in normal green leaves. Further study is required to clarify the significance of greening and the physiological role of plastids in hairy roots.

Acknowledgements

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References

1) Tepfer, D., 1990. Physiol. Plant., **79** : 140-146.
2) Ishimaru, K., K. Shimomura, 1989. Phytochemistry, **28** : 3507-3510.
3) Shimomura, K., M. Sauerwein, K. Ishimaru, 1991. Phytochemistry, **30** : 2275-2278.
4) Sauerwein, M., K. Ishimaru, K. Shimomura, 1991c. Phytochemistry, **30** : 2977-2978.
5) Sauerwein, M., K. Shimomura, 1991. Phytochemistry, **30** : 3277-3280.
6) Christen, P., T. Aoki, K. Shimomura, 1992. Plant Cell Reprots (in press).
7) Flores, H. E., J. J. Pickard, M. W. Hoy, 1988. In "Chemistry and Biology Naturally Occurring Acetylenes and Related Compounds" (ed. by Lam, J., H. Breteler), p. 233-254, Elsevier, The Netherlands.
8) Flores, H. E., M. W. Hoy, J. J. Pckard, 1989. TIBTECH, **5** : 64-69.
9) Satito, K., M. Yamazaki, K. Shimomura, K. Yoshimatsu, I. Murakoshi, 1990. Plant Cell Reports, 8 : 121-124.
10) Yoshimatsu, K., M. Satake, K. Shimomura, J. Sawada, T. Terao, 1990. J. Nat. Prod., **53** : 1498-1502.
11) Sauerwein, M., T. Yamazaki, K. Shimomura, 1991a. Plant Cell Reports, **9** : 579-581.
12) Sauerwein, M., K. Ishimaru, K. Shimomura, 1991b. Phytochemistry, **30** : 1153-1155.
13) Murashige, T., F. Skoog, 1962. Physiol. Plant., **15** : 473-497.
14) Petit, A., C. David, G. A. Dahl, J. G. Ellis, P. Guyon, F. Casse-Delbart, J. Tempé, 1983. Mol. Gen. Genet., **190** : 204-214.
15) Kamada, H., N. Okamura, M. Satake, H. Harada, K. Shimomura, 1986. Plant Cell Reports, **5** : 239-242.
16) Schmidt, G.C., T. E. Eling, J. C. Drach, 1967. J. Pharm. Sci., **56** : 215-221.
17) Porra, R. J., W. A. Thompson, P. E. Kriedemann, 1989. Biochim. Biophys. Acta, **975** : 384-394.
18) Lloyd, G. B., B. H. McCown, 1980. Inter. Plant Prop. Soc., **30** : 421-427.
19) Gamborg, O. L., R. A. Miller, K. Ojima, 1968. Exp. Cell Res., **50** : 151-158.

《和文要約》

Agrobacterium rhizogenes A4 株を用いて形質転換した *Hyoscyamus albus* の緑色毛状根の性質

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Agrobacterium rhizogenes A4 株を用いて形質転換した *Hyoscyamus albus* L. の毛状根を用いて，トロパンアルカロイドの生産および光照射下で培養した根の特質を調べた．無機要素を 1/2 にし 25-40 mM の KNO_3 を添加した Woody Plant 液体培地において良好なアルカロイドの生産が得られた．また，本毛状根を 1/2 MS 液体培地において 16 時間照明下で培養すると淡い緑色を呈した．B5 液体培地に 4 mM の硫酸アンモニウムを添加すると，1/2 MS 培地を用いた場合と同程度のクロロフィル含量が得られたが生育は改善されなかった．アンモニウムイオンがクロロフィル含量に影響を与えていることが考えられる．本緑色毛状根を電子顕微鏡で観察した結果，未成熟な葉緑体に近いものとアミロプラストに近いものとの二つのタイプの色素体が見いだされた．