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ANTHOCYANIN PRODUCTION OF *GLEHNIA LITTORALIS* CALLUS CULTURES

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Key Word Index—*Glehnia littoralis*; Umbelliferae; callus culture; acylated anthocyanin; cyanidin 3-*O*-(6-*O*-(6-*O*-(*E*)-feruloyl-β-D-glucopyranosyl)-2-*O*-β-D-xylopyranosyl-β-D-glucopyranoside).

Abstract—A stable callus line that produces anthocyanins was established from callus derived from a petiole of a *Glehnia littoralis* seedling and subcultured in the dark. The major anthocyanin which made up about 60% of the total anthocyanins was determined as cyanidin 3-*O*-(6-*O*-(6-*O*-(*E*)-feruloyl-β-D-glucopyranosyl)-2-*O*-β-D-xylopyranosyl-β-D-glucopyranoside) by chemical and spectroscopic analyses. Anthocyanin contents in the cells cultured on B5 basal medium containing NAA (1 mg l^{-1}), kinetin (0.01 mg l^{-1}) and 3% sucrose reached 14% (dry wt basis) and the productivity has been sustained for 5 years.

INTRODUCTION

Glehnia littoralis (Umbelliferae) is a perennial herb, indigenous to the seashore of Japan, Korea and China. The young buds are edible and the roots and rhizomes are used as the Chinese traditional medicine "bai sha shen" as an antipyretic, anodyne and diaphora [1]. Wang [2] and Sasaki *et al.* [3] reported the constituents of this medicine as a coumarin glucoside and its derivatives, such as osthenol-7-*O*-β-gentiobioside. The surface of *G. littoralis* petioles is partially reddish-purple, which is especially deep during the edible stage of growth. Nothing is known of anthocyanine in this plant. During our study of the micropropagation of *G. littoralis* through petiole-derived callus cultures, we maintained the white-yellow callus in the dark for about one year. The callus in which a reddish-purple spot appeared was selected as a stable anthocyanin producer. In this report we describe production of anthocyanin by the callus and structural elucidation of the major anthocyanin.

RESULTS AND DISCUSSION

Establishment of a pigment producing-callus line

Callus cultures were induced from a petiole of a *G. littoralis* seedling on MS basal agar medium supplemented with 2,4-D (1 mg l^{-1}), kinetin (0.01 mg l^{-1}) and 3% sucrose, and subcultured on the same medium at 25±1° in the dark at 1-month intervals. After 1 year of subculture, a reddish-purple spot appeared in the white calli. The pigmented parts were isolated mechanically and subcultured under the same culture conditions. The most intensively coloured parts of the calli were repeatedly selected by visual discrimination more than seven times. Finally we established a stable, very dark purple callus line. Growth of the purple callus was similar to that of colourless callus both on solid and in liquid media (Fig. 1). In the following experiments, we determined the optimal culture conditions required to produce anthocyanins.

Effect of culture conditions on cell growth and pigment production

Carbon sources. The effects of eight sugars were investigated and we found that sucrose was the best carbon source for both cell growth and anthocyanin production by *G. littoralis* callus (Fig. 2). Glucose, mannose and galactose were next in descending order and lactose as well as xylose gave very poor cell growth. The optimal sucrose concentration (determined at 1, 2, 3 and 5%) was 2–3%. The anthocyanin yield in the presence of 1% sucrose was 40% of that at 3% sucrose. Yamamoto [4] reported that maltose was optimal for growth and flavonoid production by

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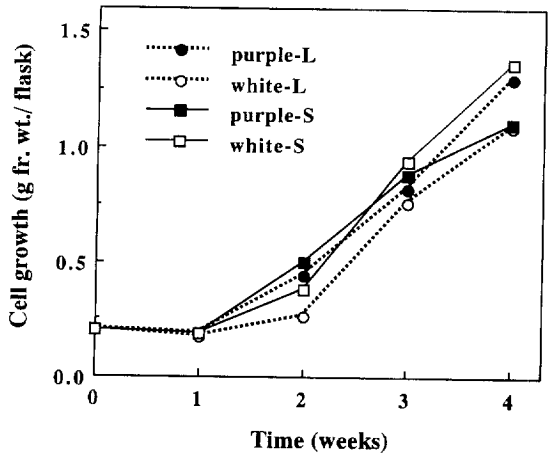

Fig. 1. Growth curves of pigment producing (purple) and non-producing (white) cells of *G. littoralis* cultured on solid (S) and in liquid (L) media, respectively. Callus (initial fresh wt 200 mg) was inoculated onto fresh MS medium containing 2,4-D (1 mg l^{-1}) and kinetin (0.01 mg l^{-1}). Each point represents the mean of duplicates.

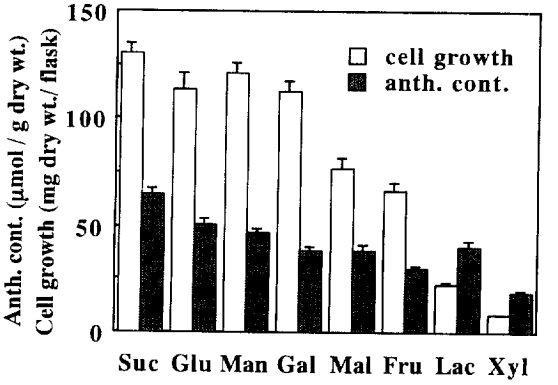

Fig. 2. The effects of various sugars on cell growth and anthocyanin production by *G. littoralis* cells. Callus (initial fresh wt, 200 mg; dry wt, 6.9 mg) was inoculated onto MS medium as described in the legend to Fig. 1 and harvested 4 weeks later. Each value represents the mean ± s.e. of triplicates.

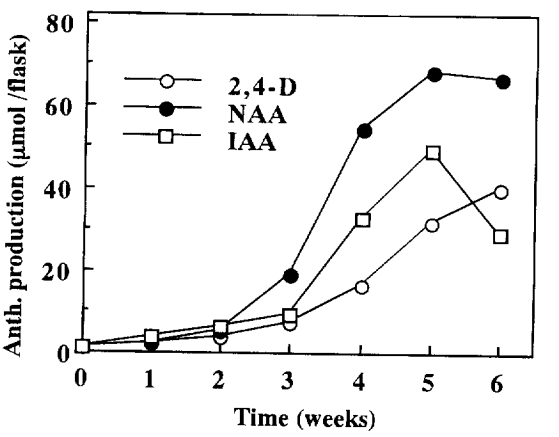

Fig. 3. Anthocyanin production by *G. littoralis* cells cultured on MS medium containing auxin (NAA, IAA or 2,4-D) and kinetin. Each value represents the mean of duplicates. Initial fresh and dry wts were described in the legend to Fig. 2.

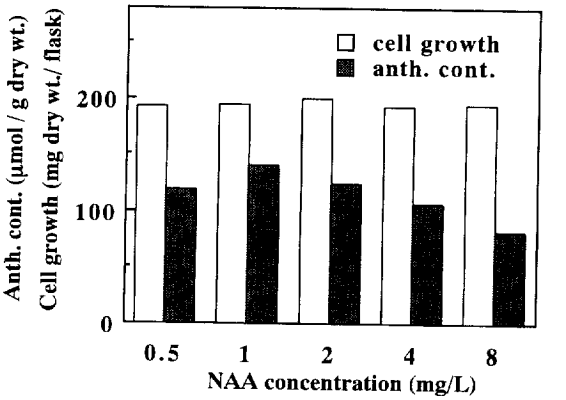

Fig. 4. The effect of the NAA concentration on cell growth and anthocyanin production by *G. littoralis* cells. Each value represents the mean of duplicates. Initial fresh and dry wts were described in the legend to Fig. 2 and the culture period was 4 weeks.

Scutellaria baicalensis callus; however it was not so effective for *G. littoralis* callus to produce anthocyanin. Sucrose is commonly used as a carbon source for anthocyanin production by cultured cells [5–7]; our results agree.

Phytohormones. To determine the effect of auxin on callus growth and pigment production, three auxins, 2,4-D, NAA and IAA, were added at a concentration of 1 mg l^{-1}. After 2 weeks in culture, all calli started to produce anthocyanin, but to very different degrees, depending upon the auxin (Fig. 3). Callus cultured in a medium containing NAA proliferated well and with the most anthocyanin productivity; the cells were uniform in size and their colour was fine and deep purple. On the other hand, callus cultured in a medium containing IAA grew poorly, had a hard texture and decreased anthocyanin productivity after 5 weeks in culture. In a medium containing 2,4-D white callus was occasionally scattered and the purple colour was lighter than that in the presence of NAA. Anthocyanin productivity with 2,4-D or IAA was almost half of that with NAA after 6 weeks.

The effects of various concentrations of NAA (0.5, 1, 2, 4 and 8 mg l^{-1}) were then assayed. It was found that the cell growth was similar between different NAA concentrations, but that anthocyanin productivity was best at 1 mg l^{-1} (Fig. 4). The anthocyanin content decreased with increasing NAA concentration. Combinations of NAA (1 mg l^{-1}) and kinetin (0, 0.01 and 0.1 mg l^{-1}) were also examined, showing 0.01 mg l^{-1} of kinetin was optimal for both growth and anthocyanin production (data not shown). 2,4-D is best for anthocyanin production by the roselle callus cultured under light [7], whereas NAA was superior for cultured *Aralia cordata* cells in

the dark, but 2,4-D was better for the same cells in the light [4]. Here NAA exerted positive effects on *G. littoralis* cells cultured in the dark, alone and in combination with kinetin.

Others. The anthocyanin level produced by the cells increased 10% in B5 basal medium in comparison with that in MS basal medium, though there were no apparent differences between them with respect to cell growth. Using B5 basal medium without KNO_3, we examined the effect of the KNO_3 concentration (0–2500 mg l^{-1}) and found that cell growth was very poor at levels below 250 mg l^{-1} of KNO_3, but there was no difference when over 500 mg l^{-1} of KNO_3 was included in the medium. The anthocyanin content of the cells cultured in a medium containing 250 mg l^{-1} of KNO_3 was highest among concentrations tested and not much difference was observed between 0–2500 mg l^{-1} except 250 mg l^{-1} (data not shown). Considering both the cell growth and the anthocyanin content, the usual KNO_3 concentration in B5 basal medium was used. The importance of the ratio of NO_3^- and NH_4^+ for anthocyanin has been reported with *Aralia* [5] and rossele [8], but we did not pursue this in detail.

These results obtained above indicated that we should grow calli in B5 basal medium with NAA (1 mg l^{-1}), kinetin (0.01 mg l^{-1}) and 3% sucrose to produce anthocyanin in the dark. Under these conditions, the number of cells increased about 30-fold and anthocyanin production reached 154 μmol g^{-1} dry wt during 4 weeks in culture when the anthocyanin (M_r 919) content was calculated as cyanidin 3-*O*-glucoside (log ε, 4.47); the content was 14.2% g^{-1} dry wt. This growth and productivity has remained stable for 5 years.

Anthocyanins can be produced in roselle calus under light, but not in the dark [7]. The growth rate of pigment producing cells is lower than that of non-producing cells of maize [9]. Anthocyanin-producing cells of *Ajuga reptans* are unstable [10]. The *G. littoralis* cell line established here proliferated well, produced high levels of anthocyanin in the dark and were stable.

Structural elucidation

Dark purple calli of *G. littoralis* (150 g) were ground and extracted with 50% HOAc to give crude pigments. The crude pigments contained five major anthocyanins indicating retention times of 2.35, 3.23, 4.31 (**1**), 4.57, and 5.55 min at the levels of 14, 4, 62, 9, and 6%, respectively, on HPLC analyses. Then, successive purification of crude pigments by an HP-20 resin column, LH-20 column and prep. HPLC were carried out to afford one component. Of the five pigments, **1** was isolated as red powder (TFA salt). The four remaining minor pigments are not discussed here.

Chemical analyses of **1** were performed according to the usual method [11]. Pigment **1** give cyanidin (Cy) as aglycone, D-glucose (Glc) and D-xylose (Xyl) as sugars and ferulic acid (Fer) as an acylating acid. In the IR spectra of **1**, the presence of the conjugated ester carbonyl band at 1678 cm^{-1} suggested ferulic acid acylation. The presence of ferulic acid in **1** is also supported by a characteristic and strong absorption at 330 nm. The number of ferulic acid residues in **1** was estimated to be 1 mol from the ratio E_{acyl}/E_{vismax} (67%) of the absorbance at acyl peak (λ_{acyl}) and the absorbance at visible maxima (λ_{vismax}) [12]. The presence of Cy as aglycone was supported by bathochromic shift by addition of $AlCl_3$. ESI-MS spectrum of **1** showed molecular ion peak $[M]^+$ at *m/z* 919 corresponding to $C_{42}H_{47}O_{23}^+$ and a fragment ion peak at *m/z* 287 corresponding to $[Cy]^+$. These data suggest **1** is a cyanidin feruloyldiglucosylmonoxyloside.

The ^1H NMR spectrum of **1** showed the presence of a Cy, and feruloyl group with large coupling constant (J = 15.6 Hz) and two glucopyranose and one xylopyranose, exhibiting constants of anomeric protons with J values of 7.2–7.9 Hz. The connecting positions of aglycone, two glucose moieties, xylose moiety, and a feruloyl group of **1** were confirmed by the HMBC technique. The correlation of **1** was observed between the anomeric proton signal (δ 5.34) of glucose A (Glc A) and the carbon signal (δ 144.0) at 3-position of Cy, the anomeric proton signal (δ 4.32) of glucose B (Glc B) and the carbon signal (δ 70.9) at 6-position of Glc A, the proton signal (δ 4.21) at the 2-position of Glc A and the carbon signal (δ 105.1) at the 1-position of Xyl, the proton signals (δ 4.82 and 4.15) at 6-position of Glc A and the carbon signal (δ 104.9) at 1-position of Glc B, the anomeric proton signal (δ 4.63) of Xyl and carbon signal (δ 79.3) at 2-position of Glc A, and the proton signals (δ 4.82 and 4.15) at 6-position of Glc B and the carbonyl carbon signal (δ 167.0) of feruloyl group, in the HMBC spectrum. Hence **1** is cyanidin 3-*O*-(6-*O*-(6-*O*-(E)-feruloyl-β-D-glucopyranosyl)-2-*O*-β-D-xylopyranosyl-β-D-glucopyranoside), (Fig. 5). This is the first anthocyanin to be identified in *G. littoralis* and **1** is a novel compound.

EXPERIMENTAL

Plant material and culture methods

Callus was originally derived from a petiole of a seedling of *Glehnia littoralis* cultivated in the Medicinal Plant Garden attached to School of Pharmaceutical Sciences, Nagasaki University. The callus culture conditions and establishment of a pigment producing-callus line were described in the text. The optimal culture conditions suitable to both cell growth and pigment production were then defined. The carbon sources, maltose (Mal), sucrose (Suc), fructose (Fru), mannose (Man), lactose (Lac), galactose (Gal), glucose (Glu) and xylose (Xyl) were used. The plant hormones, indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin were added at various concentrations. The calli were cultured in Murashige-Skoog (MS) [13] and Gamborg B5 (B5) [14] basal media, and

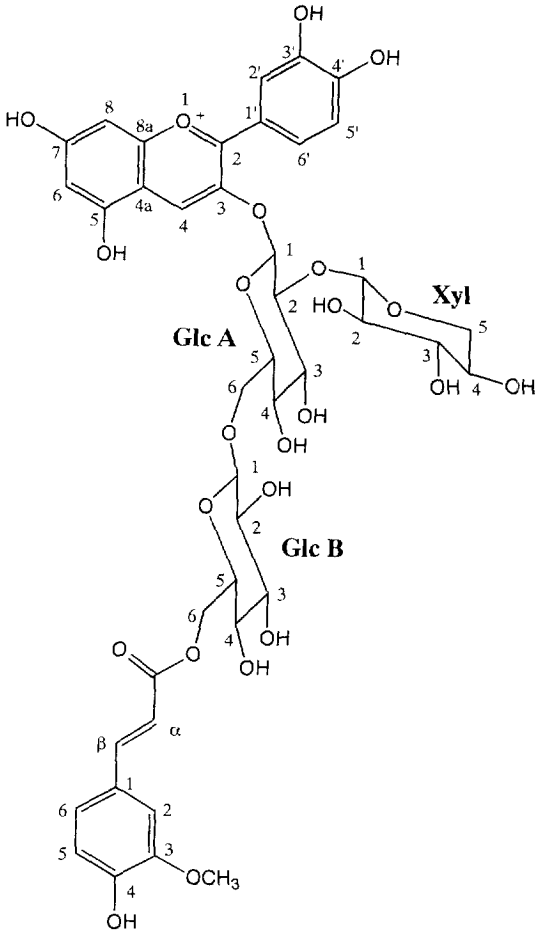

Fig. 5. Chemical structure of the acylated anthocyanin isolated from *G. littoralis* cells.

the concentration of KNO_3 in B5 was changed from 0 to 2500 mg l^{-1}.

Anthocyanin content

Freeze dried cells were homogenized with MeOH containing 0.1% HCl, extracted overnight at 4° and centrifuged (560 g, 10 min). The absorbance of the supernatant containing anthocyaninis was measured at 535 nm. The anthocyanin content was calculated using log ε 4.47 for cyanidin 3-monoglucoside [15].

Spectroscopic analyses

LC-ESI-MS (positive) analyses were performed on a VG BIOTECH Platform spectrometer. The sample dissolved in 0.05% TFA solution was directly injected. The IR and UV-VIS spectral analyses were performed on a JASCO FT/IR-350 (in KBr) and Shimadzu UV-260 (in 0.01% HCl–MeOH) spectrometer, respectively. ^{1}H- and ^{13}C-NMR spectra were measured in DMSO-d_6–TFA-d_1 (9:1) with TMS as an internal standard on a JEOL A-600 spectrometer at 303 K. Assignments of proton and carbon signals of **1** were confirmed by DQF-COSY, HMQC and HMBC analyses.

HPLC

Analytical HPLC was performed on an Develosil ODS-UG-3 (4.6 $\phi \times 150$ mm, Nomura–Kagaku) column at 40° with detection at 520 nm. The solvent systems used for gradient elution at a flow rate 0.8 ml min^{-1} were as follows: solvent A (CH_3CN–0.1% TFA, 5:95), and B (CH_3CN). The gradient conditions were as follows: 0–10 min, a linear gradient from 10 to 50% solvent B in solvent A. The retention time of **1** was 4.31 min. Preparative HPLC was performed on an Develosil ODS-5 (20 $\phi \times 250$ mm, Nomura Chemical) column and L-column ODS (10 $\phi \times 250$ mm, Chemical Inspection and Testing Institute, Japan) with 4–6 ml min^{-1} by isocratic elution using a mixture of 13 or 15% CH_3CN in 0.2 M phosphate buffer (pH 2.0).

Isolation of anthocyanin

Calli (150 g) were ground and steeped in 50% HOAc solution (300 ml × 3) overnight and filtrated. The reddish purple extract was diluted with water and applied to an HP-20 (Diaion) resin column (80 $\phi \times 150$ mm). The column was washed with 1% HOAc and eluted with 5% HOAc in 80% EtOH. The pigment eluent was evaporated and applied to a Sephadex LH-20 column (32 $\phi \times 460$ mm), 10% HOAc in 70% EtOH was used as a solvent. The pigment eluent was separated and purified by prep. HPLC. The major anthocyanin fraction was evaporated to dryness, dissolved in a small amount of TFA, and precipitated with ether to give TFA salt of **1** (43 mg) as red powder.

Pigment **1**. IR ν_{max}^{KBr} cm^{-1}: 3416 (O—H), 1678 (C=O), 1636 (aromatic C=C), 1600 (aromatic C=C); UV-VIS λ = max 0.01% HCl–MeOH nm: 537 (bathochromically shifted with $AlCl_3$), 330, 285, $E_{acyl}/E_{vismax} = E_{330}/E_{537} = 0.67$, $E_{440}/E_{537} = 0.22$, ^{1}H NMR δ ppm: 8.58 (*s*, Cy-4), 6.70 (*s*, Cy-6), 6.73 (*s*, Cy-8), 8.02 (*d*, $J = 2.4$ Hz, Cy-2'), 7.08 (*d*, $J = 7.8$ Hz, Cy-5'), 8.16 (*dd*, $J = 7.8$, 2.4 Hz, Cy-6'), 5.34 (*d*, $J = 7.2$ Hz, Glc A-1), 4.21 (*m*, Glc A-2), 3.91 (*dd*, $J = 9.0$, 8.6 Hz, Glc A-3), 3.85 (*t*, $J = 9.0$ Hz, Glc A-4), 4.20 (*m*, Glc A-5), 3.80 (*dd*, $J = 2.2$, 9.0 Hz, Glc A-6a), 3.88 (*m*, Glc A-6b), 4.32 (*d*, $J = 7.2$ Hz, Glc B-1), 3.13 (*dd*, $J = 7.2$, 8.3 Hz, Glc B-2), 3.22 (*m*, Glc B-3), 3.40 (*m*, Glc B-4 and Glc B-5), 4.82 (*brd*, $J = 11.4$ Hz, Glc B-6a), 4.15 (*brd*, $J = 11.4$ Hz, Glc B-6b), 4.63 (*d*, $J = 7.9$ Hz, Xyl-1), 3.03 (*t*, $J = 7.9$ Hz, Xyl-2), 3.13 (*dd*, $J = 7.9$, 9.6 Hz, Xyl-3), 3.22 (*m*, Xyl-4), 3.36 (*dd*, $J = 10.8$, 5.7 Hz, Xyl-5a), 2.79 (*t*, $J = 10.8$ Hz, Xyl-5b), 6.92 (*s*, feruloyl-2), 6.54 (*d*, $J = 7.8$ Hz, feruloyl-5), 6.83 (*d*, $J = 7.8$ Hz, feruloyl-6), 7.40 (*d*, $J = 15.6$ Hz, feruloyl-β), 6.33 (*d*, $J = 15.6$ Hz, feruloyl-α) and 3.67 (*s*, feruloyl-OCH_3). ^{13}C-NMR δ ppm: 161.5 (Cy-2), 144.0 (Cy-3), 132.6 (Cy-4), 112.4 (Cy-4a), 157.5

(Cy-5), 93.9 (Cy-6), 168.0 (Cy-7), 102.4 (Cy-8), 155.4 (Cy-8a), 119.7 (Cy-1′), 117.9 (Cy-2′), 146.2 (Cy-3′), 154.4 (Cy-4′), 116.7 (Cy-5′), 126.9 (Cy-6′), 100.4 (Glc A-1), 79.3 (Glc A-2), 73.5 (Glc A-3), 68.3 (Glc A-4), 75.1 (Glc A-5), 70.9 (Glc A-6), 104.9 (Glc B-1), 73.2 (Glc B-2), 76.5 (Glc B-3), 69.5 (Glc B-4), 74.0 (Glc B-5), 61.7 (Glc B-6), 105.1 (Xyl-1), 74.8 (Xyl-2), 76.6 (Xyl-3), 69.7 (Xyl-4), 66.0 (Xyl-5), 125.4 (feruloyl-1), 109.9 (feruloyl-2), 147.9 (feruloyl-3), 149.5 (feruloyl-4), 115.0 (feruloyl-5), 123.2 (feruloyl-6), 114.7 (feruloyl-α), 145.7 (feruloyl-β), 167.0 (carbonyl), 55.4 (feruloyl–OCH_3).

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