Bull. Natl. Mus. Nat. Sci., Ser. B, **36**(2), pp. 61–64, May 22, 2010

Luteolinidin 5-*O*-glucoside from *Azolla* as a Stable Taxonomic Marker

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(Received 23 February 2010; accepted 24 March 2010)

Abstract A red pigment was isolated from the whole plants of *Azolla cristata* and identified as 3-deoxyanthocyanin, luteolinidin 5-*O*-glucoside by LC-MS, and ^{1}H and ^{13}C NMR spectra. It was also detected from two specimens of *Azolla pinnata*, which were collected in New Caledonia and Australia, by HPLC survey for the first time. Five *Azolla* species, including two species which were surveyed in this experiment, were surveyed for 3-deoxyanthocyanins until now, and luteolinidin 5-*O*-glucoside has been found in all species. Thus, 3-deoxyanthocyanin, luteolinidin 5-*O*-glucoside, was recognized as a stable taxonomic chemical marker of the genus *Azolla*.

Key words : *Azolla*, chemotaxonomy, 3-deoxyanthocyanin, luteolinidin 5-*O*-glucoside, NMR.

Introduction

The genus *Azolla* (Salviniaceae) consists of 6 or 7 species and is especially distributed from the tropical to temperate zone in the world (Iwatsuki, 1992; Mabberley, 1997). Though the whole plants are usually reddish green, they change to bright red in autumn and winter season. The red pigments have been surveyed in a few species. Two 3-deoxyanthocyanins have been isolated from *Azolla filiculoides* Lam. and *A. caroliniana* Willd. and partially characterized as luteolinidin and apigeninidin glycosides (Pieterse *et al*., 1977). As other flavonoids, the presence of proanthocyanidins has also been reported (Markham, 1988). A 3-deoxyanthocyanin has been isolated from *Azolla mexicana* Presl and identified as luteolinidin 5-*O*-glucoside by paper chromatography and UV-visible spectral survey (Holst, 1977). 3-Deoxyanthocyanin in *Azolla imbricata* Nakai and *A. japonica* Franch et Savat. has been surveyed by Ishikura (1982) and luteolinidin 5-*O*-glucoside was isolated together with several phenolic compounds, chlorogenic acid, aesculetin, caffeic acid 3,4-diglucoside, 6-(3′-glucosylcaffeoyl)-aesculetin, *p*-coumaroyl glucosyl ester, and 1,6-diesters of caffeic and chlorogenic acids. Thus, it has been shown that luteolinidin 5-*O*-glucoside is a red pigment in *A. mexicana*, *A. imbricata* and *A. japonica* (Holst, 1977; Ishikura, 1982). However, a major pigment in *A. cristata* has not been characterized. In this experiment, the red pigment in *A. cristata* was isolated and completely identified by LC-MS and ^{1}H and ^{13}C NMR. Moreover, the presence of luteolinidin 5-*O*-glucoside in *A. pinnata* R. Br., which is not surveyed for 3-deoxyanthocyanin, was shown by HPLC analysis.

Materials and Methods

Plant materials

Azolla cristata Kaulf. is natively growing in Tsukuba Botanical Garden, National Museum of Nature and Science, Tsukuba, Japan (TNS9546219, TNS9546462-9546464). The species was identified by one of the authors (S. Matsumoto). *Azolla pinnata* R. Br. was collected in Mts. Kogis, north

of Noumea, New Caledonia, 480 m alt., 4 Nov. 1997 (TNS9508992) by S. Matsumoto, and near Brisbane, Australia, 8 Aug. 1997 (TNS9527088) by Takehisa Nakamura. Voucher specimens were deposited in the herbarium of National Museum of Nature and Science, Tokyo (TNS).

Extraction and isolation of anthocyanin

Fresh whole plants (ca. 1 kg) of *A. cristata* were extracted with HCOOH/MeOH (8 : 92). The concentrated extracts were applied to preparative paper chromatography using solvent systems: BAW (*n*-BuOH/HOAc/H_2O=4 : 1 : 5, upper phase) and 15% HOAc. The anthocyanin bands was eluted with HCOOH/MeOH (8 : 92) and purified by Sephadex LH-20 column chromatography using solvent system: MeOH/H_2O/HCOOH (70 : 25 : 5).

Dry specimens of *A. pinnata* were extracted with HCOOH/MeOH (8 : 92). After concentration, the extracts were surveyed by HPLC for anthocyanin identification.

High performance liquid chromatography (HPLC)

HPLC was performed with Shimadzu HPLC systems using a Senshu Pak PEGASIL ODS column (I.D. 6.0×150 mm, Senshu Scientific Co. Ltd.), at a flow-rate of 1.0 ml min^{-1}. Detection was 500 nm and eluent was H_3PO_4/HOAc/MeCN/H_2O (3 : 8 : 6 : 83).

Liquid chromatograph-mass spectra (LC-MS)

LC-MS was performed with Shimadzu LC-MS systems using Senshu Pak PEGASIL ODS column (I.D. 2.0×150 mm, Senshu Scientific Co. Ltd.), at a flow-rate of 0.1 ml min^{-1}, ESI^+ 4.5 kV and ESI^- 3.5 kV, 250°C. The eluent was MeCN/H_2O/HCOOH (3 : 92 : 5).

Identification of anthocyanin

The isolated anthocyanin was identified by TLC, HPLC, UV-visible spectral survey, LC-MS, and ^1H and ^{13}C NMR spectra. Their data are as follows.

TLC: Rf 0.18 (BAW), 0.06 (1%HCl); visible—reddish orange, UV—dark purple. HPLC: *t*R 8.34 min. UV-visible spectra: λ max 0.1%MeOH-HCl 277, 496 nm; +$AlCl_3$ 276sh, 512, 545sh nm; E_{440}/E_{max} 20%. LC-MS: *m/z* 433 $[M]^+$ (luteolinidin+1 mol glucose), *m/z* 271 $[M-162]^+$ (luteolinidin). ^1H NMR (600 MHz, DMSO-d_6+TFA): δ 9.29 (1H, *d*, *J*=8.8 Hz, H-4), 8.35 (1H, *d*, *J*=8.9 Hz, H-3), 8.07 (1H, *dd*, *J*=2.1 and 8.7 Hz, H-6′), 7.94 (1H, *d*, *J*=1.3 Hz, H-2′), 7.27 (1H, *s*, H-8), 7.18 (1H, *d*, *J*=8.5 Hz, H-5′), 7.07 (1H, *d*, *J*=1.3 Hz, H-6), 5.19 (1H, *d*, *J*=7.7 Hz, glucosyl H-1), 3.81 (1H, *d*, *J*=10.5 Hz, glucosyl H-6a), 3.61 (1H, *dd*, *J*=5.3 and 12.0 Hz, glucosyl H-6b), 3.56 (1H, *m*, glucosyl H-5), 3.50 (1H, *m*, glucosyl H-2), 3.46 (1H, *m*, glucosyl H-3), 3.34 (1H, *m*, glucosyl H-4). ^{13}C NMR (150 MHz, DMSO-d_6+TFA): (luteolinidin) δ 171.4 (C-2), 111.4 (C-3), 148.1 (C-4), 156.5 (C-5), 103.7 (C-6), 169.9 (C-7), 96.9 (C-8), 158.0 (C-9), 112.0 (C-10), 120.2 (C-1′), 115.7 (C-2′), 147.2 (C-3′), 155.4 (C-4′), 117.3 (C-5′), 125.0 (C-6′); (glucose) δ 101.4 (C-1), 73.3 (C-2), 76.0 (C-3), 69.7 (C-4), 77.6 (C-5), 60.5 (C-6).

Results

A reddish orange pigment was obtained from the whole plants of *Azolla cristata*. The absorption maxima of the compound were 495 (Band I) and 277 (Band II) nm, showing that the pigment is 3-deoxyanthocyanin. In addition to $AlCl_3$, Band I bathochromically shifted, indicating the presence of B-ring *ortho*-dihydroxyl groups. Since the molecular ion peak, *m/z* 433 $[M]^+$ and a fragment ion peak, *m/z* 271 $[M-162]^+$ appeared by LC-MS, it was shown that the original glycoside is tetrahydroxyanthocyanin monohexoside. The presence of seven aromatic protons (H-3, H-4, H-6, H-8, H-2′, H-5′ and H-6′) was shown by ^1H NMR spectra. The coupling constant of the glucosyl anomeric proton signal (δ 5.19) was *J*=7.7 Hz, showing that the glucose is β-linkage to luteolinidin nucleus. Since HMBC correlation between glucosyl anomeric proton signal at δ 5.19 and C-5 carbon signal at δ 156.5 was recog-

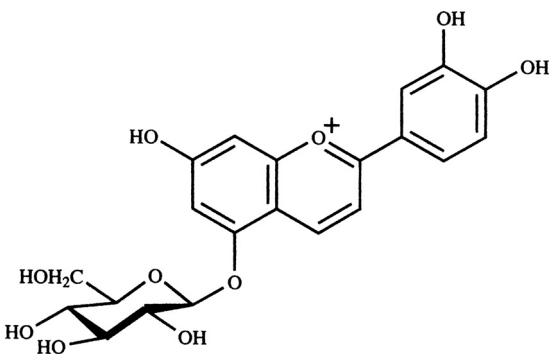

Fig. 1. Luteolinidin 5-*O*-glucoside.

nized, it was indicated that the glucose is attached to 5-position of luteolinidin. Thus, the 3-deoxyanthocyanin was identified as luteolinidin 5-*O*-*β*-glucopyranoside (Fig. 1).

Only one peak appeared by HPLC survey of *A. pinnata* extract and its retention time and UV spectral properties agreed with those of luteolinidin 5-*O*-glucoside from *A. cristata*. The presence of luteolinidin 5-*O*-glucoside in *A. cristata* and *A. pinnata* was reported in this paper for the first time.

Discussion

Almost anthocyanins of flowering plants are common anthocyanins, i.e. 3-oxyanthocyanins such as pelargonidin, cyanidin and delphinidin (Iwashina, 2000). On the other hand, the distribution of 3-deoxyanthocyanins such as apigeninidin and luteolinidin is extremely limited in flowering plants. They have been reported as flower pigments of bird-pollinated species in the family Gesneriaceae, e.g. *Alloplectus vittatus*, *Gesneria cuneifolia*, *Rechsteineria cardinalis*, *R. macrorhiza*, *Chrysothemis pulchella* and *Hypocyrta glabra* (Harborne, 1966, 1967). Apigeninidin and luteolinidin glycosides were also isolated from *Sorghum* spp. (Gramineae) as phytoalexins (Stafford, 1968; Nip and Burns, 1969, 1971; Nicholson *et al*., 1987, 1988; Hipskind *et al*., 1990; Snyder *et al*., 1991; Kouda-Bonafos *et al*., 1994; Pale *et al*., 1997).

Recently, the biosynthetic pathway of 3-deoxyanthocyanins was shown that lack of F3H activity allows action of the DFR/FNR on substrates and production of flavan-4-ols, and is then likely converted to 3-deoxyanthocyanins through the action of the ANS and subsequent glucosylation, by the examination of *Sinningia cardinalis* (Gesneriaceae) which produces luteolinidin and apigeninidin 5-*O*-glucosides (Winefield *et al*., 2005).

The occurrence of 3-deoxyanthocyanins has been reported from comparatively many species in ferns and bryophytes than in flowering plants. Luteolinidin 5-*O*-glucoside and 5-*O*-diglucoside were isolated from musci, *Bryum* spp. (Bendz and Mårtensson, 1961, 1963; Bendz *et al*., 1962). In ferns, luteolinidin 5-*O*-glucoside and other glycosides were found in *Adiantum veitchianum* and *A. pedatum* cv. "Klondyke", *Dryopteris erythrosora*, *Blechnum brasiliense* var. *corcovadense*, and three *Pteris* species, *P. longipinnula*, *P. quadriauria* and *P. vittata* (Harborne, 1966). Six luteolinidin and six apigeninidin glycosides, and acetylated luteolinidin 5-*O*-laminaribioside were isolated from two *Blechnum* species, *B. procerum* and *B. novae-zelandicae* (Crowden and Jarman, 1974; Swinny, 2001). Though their distribution is sporadic in ferns and bryophytes, the occurrence of 3-deoxyanthocyanins, especially luteolinidin 5-*O*-glucoside, in the genus *Azolla* is likely stable taxonomic chemical marker.

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