Bull. Natl. Mus. Nat. Sci., Ser. B, 45(4), pp. 165–168, November 22, 2019

Reconstruction of Flower Color of *Amherstia nobilis* by *in vitro* Examination

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(Received 17 July 2019; accepted 25 September 2019)

Abstract Flower anthocyanin of *Amherstia nobilis* has been reported as pelargonidin 3-*O*-pentoside. However, they were more recently identified as cyanidin 3-*O*-glucoside and 3-*O*-xyloside, and peonidin 3-*O*-glucoside, together with eight flavonol glycosides, i.e. isorhamnetin 3-*O*-glucoside, 7-*O*-glucoside, 3,7-di-*O*-glucoside and 3-*O*-rutinoside, quercetin 3-*O*-rutinoside and 3-*O*-glucoside, and kaempferol 3-*O*-rutinoside and 3-*O*-glucoside. Color of cyanidin 3-*O*-glucoside which is major anthocyanin in the flowers is originally red but not reddish orange. We presumed that color of anthocyanins is hypsochromically shifted under lower pH, and measured pH of the pressed juice of the petals. As the results, it was proved that pH of the petals is lower (pH 3.3) and in spite of flower pigments are cyanidin glycosides, the flowers of *A. nobilis* express reddish orange. This was followed by *in vitro* examination.

Keywords: *Amherstia nobilis*, cell sap pH, cyanidin 3-*O*-glucoside, *in vitro* examination, reddish orange flower color.

Introduction

The genus *Amherstia* (Leguminosae) consists of only one species, *A. nobilis* Wall. and is endemic to Myanmar. Although the species is growing in some botanical gardens in tropical area and greenhouses, its wild population in Myanmar is not known. *A. nobilis* is generally regarded as one of the most beautiful of all flowering trees (Fig. 1) (Bisby *et al.*, 1994). Its flower anthocyanin has been reported to be pelargonidin 3-*O*-pentoside without chemical data (Beale *et al.*, 1941). More recently, three anthocyanins and eight flavonols were isolated from the reddish orange flowers of this species. However, anthocyanins were identified as cyanidin 3-*O*-glucoside and 3-*O*-xyloside, and peonidin 3-*O*-glucoside (Iwashina *et al.*, 2019). On the other hand, flavonols were characterized as isorhamnetin 3-*O*-glucoside, 7-*O*-glucoside, 3,7-di-*O*-glucoside and 3-*O*-rutinoside, quercetin 3-*O*-rutinoside and 3-*O*-glucoside, and kaempferol 3-*O*-rutinoside and 3-*O*-glucoside (Iwashina *et al.*, 2019). Although major anthocyanin was cyanidin 3-*O*-glucoside, its flower color was reddish orange which is such as due to pelargonidin and/or carotenoids. In this survey, we clarified the coloring mechanism of reddish orange flowers by pH measurement of the petals and *in vitro* examination.

Fig. 1. Flowers of *Amherstia nobilis* (photographed in Bogor Botanic Garden, Indonesia by one of the author, T. Iwashina in 1 July 2016).

Materials and Methods

Plant materials

The flowers were collected in the greenhouse of the Tsukuba Botanical Garden, National Museum of Nature and Science, Tsukuba, Japan in 21 January 2016.

Isolation and identification

Flowers were extracted with MeOH/HCOOH (92 : 8). Anthocyanins and flavonols in crude extracts were characterized by HPLC comparisons with authentic samples which were obtained from the flowers of this species (Iwashina *et al.*, 2019). HPLC measurement was performed using Shimadzu HPLC system (Shimadzu Co., Ltd., Kyoto,), Inertsil ODS-4 column (I.D. 6.0 × 150 mm, GL Sciences Inc., Tokyo), at flow-rate of 1.0 ml min^{-1}, detection wave length of 530 nm for anthocyanins and 350 nm for flavonols, and eluents were MeCN/HOAc/H_2O/H_3PO_4 (10 : 8 : 79 : 3) for anthocyanins and MeCN/H_2O/H_3PO_4 (20 : 80 : 0.2) for flavonols.

pH measurement

The pH values of the pressed juice from the flower tissues were measured using a handhold pH meter (Twin pH; AS-212, Horiba Instruments, Kyoto). The reddish orange epidermis was carefully peeled and placed between glasses, and pressed to get the pressed juice. The pH of the pressed juice prepared was immediately measured. Measurement were triplicated.

In vitro examination

The pH effect of cyanidin 3-*O*-glucoside (5×10^{-4} M) was determined at different pH values (pH 2.6, 3.3, 4.3, 5.3 and 6.3) of Mellvaine buffer prepared by mixing 0.1 M citric acid and 0.2 M Na_2HPO_4. The absorption spectra of the solution were measured immediately (0 min) and after 30 min.

Results and Discussion

Flower color of *Amherstia nobilis* was reddish orange, presumed that it is due to carotenoid and/

or anthocyanin, especially pelargonidin. Practically, pelargonidin 3-*O*-pentoside has been reported as flower pigment without chemical data (Beale *et al.*, 1941). However, cyanidin 3-*O*-glucoside was found in the flowers of this species as a major pigment, together with minor cyanidin 3-*O*-xyloside and peonidin 3-*O*-glucoside, and eight flavonol glycosides, instead of pelargonidin glycoside and carotenoids (Iwashina *et al.*, 2019). Although cyanidin glycosides commonly express red color in almost flowers except for a few plants, e.g. *Meconopsis* spp. (Takeda *et al.*, 1996; Tanaka *et al.*, 2001; Yoshida *et al.*, 2006) and *Centaurea cyanus* L. (Takeda *et al.*, 2005; Shiono *et al.*, 2005), reddish orange color was expressed by cyanidin 3-*O*-glucoside in *A. nobilis* flowers. Such the fact was presumed that other factors are changed the pigment color (red) to reddish orange in this flowers. Generally, because color of anthocyanins is hypsochromically shifted under lower pH (acidity), pH of the pressed juice of the flowers was measured. As the results, it was shown that pH of the petals is lower (pH 3.3) and in spite of major flower pigment is cyanidin 3-*O*-glucoside, the flowers of *A. nobilis* expressed reddish orange. It was followed by *in vitro* examination (Table 1 and Fig. 2). Absorption maxima of intact petals was λmax 511 nm (Fig. 2A). In *in vitro* examination, absorption maxima of cyanidin 3-*O*-glucoside were measured in conditions of pH 2.6, 3.3, 4.3, 5.3 and 6.3 (Table 1 and Fig. 2). In lower acidic solutions (pH 2.6 and 3.3), their absorption maxima showed λmax 511 and 512 nm without additional absorption peaks and shoulders, which was the same with that of intact petals. On the other hand, absorption maxima, λmax 519 nm was shown in pH 4.3. Moreover, absorption maxima appeared as two peaks (434 and 529 nm, and 439 and 535 nm, respectively) in pH 5.3 and 6.3 (Fig. 2B). Although absorption maxima were measured after 30 min, those of pH 2.6 and 3.3 were very stable. However, those of pH 5.3 and 6.3 were unstable (Fig. 2C). Thus, it was proved that reddish orange color of *A. nobilis* flowers is due to lower acidic condition in cell sap. Accompanyingly, although absorption maxima of cyanidin 3-*O*-glucoside were measured in addition to isorhamnetin 3-*O*-glucoside which was one of the major flavonols in the flowers of *A. nobilis*, the bathochromic and also hypsochromic shifts were

Table 1. Absorption maxima of cyanidin 3-*O*-glucoside with various pH

pH	λ max (nm)	
	0 min	30 min
2.6	511	511
3.3	512	511
4.3	519	510
5.3	434, 529	514
6.3	439, 535	438, 533

λmax of fresh petal of *Amherstia nobilis* was shown at 511 nm.

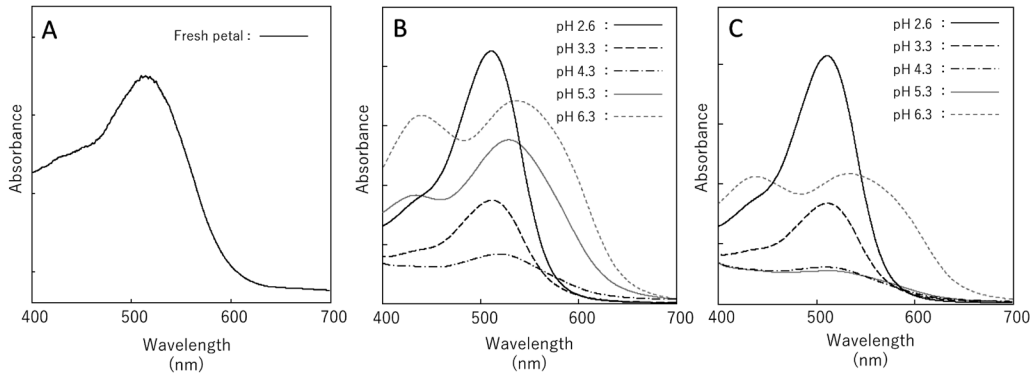

Fig. 2. Absorption spectra of intact petal (A), cyanidin 3-*O*-glucoside with various pH (0 min, B, and after 30 min C).

hardly occurred (data not shown).

The pH of flower cell sap is generally slightly acidic (Shibata *et al.*, 1949). However, pH of the pigmented epidermal cell sap of *A. nobilis* was found to be extremely lower. The pH of epidermis of the blue and greenish blue petals of morning glory cv. Heavenly Blue and jade vain (*Strongylodon macrobotrys* A. Gray) were reported to be slightly alkali (pH 7.5) (Stewart *et al.*, 1975) and pH 7.9 (Takeda *et al.*, 2010), respectively. By contraries, pH of the flower cells of *Begonia* cv. Orange Schwabenland and Azalea cv. Gloria were lower pH (2.5 and 3.1, respectively). Their flower colors were strong red-orange and light yellowish pink in spite of their anthocyanins are cyanidin glycosides (Stewart *et al.*, 1975). Although major pigment of *A. nobilis* was cyanidin 3-*O*-glucoside, its flower color is changed to reddish orange due to lower acidity of cell saps. There is close correlation between flower color and pollinators (Harborne and Grayer, 1994). Flavonol glycosides directly do not influence the flower color of *A. nobilis*. However, since insects and birds can discriminate UV range, flavonol glycosides may be useful as pollinator attractants. Although wild population of *A. nobilis* in Myanmar is unknown, we presume that unknown insects, birds or animals, which were tempted to reddish orange color, visit to this flowers.

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