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Short communication

Identification of flavonoid 3′-hydroxylase in the yellow flower of *Delphinium zalil*

Taira Miyahara[a,*], Arisa Hamada[a], Mitsutoshi Okamoto[b], Yukio Hirose[c], Kimitoshi Sakaguchi[d], Shoji Hatano[d], Yoshihiro Ozeki[a]

[a] Department of Biotechnology, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan
[b] Ehime Research Institute of Citrus Fruits, Matsuyama, Ehime 799-3742, Japan
[c] Department of Agricultural Research, Ehime Research Institute of Agriculture, Forestry and Fisheries, Matsuyama, Ehime 799-2405, Japan
[d] Miyoshi & Co., Ltd., R&D Center, Hokuto, Yamanashi, 408-0041, Japan

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ABSTRACT

The flowers of delphinium cultivars owe their coloration to anthocyanins such as delphinidin or pelargonidin derivatives. To date, no delphinium cultivars have been found with red flowers due to the presence of cyanidin derivatives. This suggests that delphiniums do not have cyanidin biosynthesis ability because of the loss of function of flavonoid 3′ hydroxylase (F3′H). Here, we show that the wild delphinium species *Delphinium zalil* (synonym *semibarbatum*) can accumulate quercetin 3-glucosides in its sepals, presumably through F3′H activity. We isolated F3′H cDNA from *D. zalil* (*DzF3′H*) and produced a recombinant enzyme from a yeast transformant. The recombinant DzF3′H protein could convert naringenin, apigenin, dihydrokaempferol and kaempferol to eriodictyol, luteolin, dihydroquercetin and quercetin, respectively. An expression analysis confirmed that blue flowered *D. grandiflorum* does not express *F3′H*, and also showed that flavonoid 3′,5′-hydroxylase and anthocyanidin synthase do not function in *D. zalil* sepals. DzF3′H can act as a flavonoid hydroxylase to produce cyanidin accumulation. The introduction of the *DzF3′H* gene into other delphinium species by conventional breeding may enable development of cultivars with novel flower colors.

1. Introduction

The anthocyanin pigments that produce basic flower colors are dependent on the hydroxylation profiles of anthocyanidins at the B ring. For example, pelargonidin has a mono hydroxyl residue at the 4′ position, cyanidin has two such residues at the 3′ and 4′ positions, and delphinidin has three such residues at the 3′, 4′ and 5′ positions. Cyanidin, which produces red to purple flower coloration, is produced by the enzyme flavonoid 3′-hydroxylase (F3′H); delphinidin, which gives violet to dark blue coloration, is produced by flavonoid 3′,5′-hydroxylase (F3′5′H); pelargonidin, which gives orange to red coloration, is hydroxylated at the 4′ position but neither F3′H nor F3′5′H hydroxylase activity are involved (Davies and Gould, 2009). In delphiniums, considerable efforts have been made to produce novel flower color varieties. As such varieties would have different levels and contents of pigments, there has been much investigation of the molecular structures and characteristics of anthocyanins (Legro, 1961; Kondo et al., 1990, 1991; Saito et al., 1998; Hashimoto et al., 2002; Nishizaki et al., 2013, 2014; Miyagawa et al., 2014). Studies of anthocyanins have shown that the red, purple and blue colors of delphinium sepals are derived from the aglycones, delphinidin and pelargonidin; however, there have been no reports on the accumulation of cyanidin derivatives in the flowers of delphinium species. This reason for the lack of cyanidin derivatives accumulation may be that the wild species used as a breeding resource, such as *Delphinium grandiflorum*, *D. elatum*, *D. cardinale* and *D. nudicaule*, do not have F3′H activity. Therefore, to develop novel flower color varieties by cross-hybridization, it will important to identify species that have an active *F3′H* gene, as the introduction of this gene into established varieties would be expected to enable the F3′H activity necessary to produce the cyanidin necessary for red flowers. Here, we report the first identification of an active *F3′H* gene in the yellow flowered wild delphinium species *Delphinium zalil* and demonstrate the activity of F3′H.

Abbreviations: ANS, anthocyanidin synthase; DgF3′H, *Delphinium grandiflorum* F3′H; DgF3′5′H, *Delphinium grandiflorum* F3′5′H; DzF3′H, *Delphinium zalil* F3′H; DzF3′5′H, *Delphinium zalil* F3′5′H; DFR, dihydroflavonol 4-reductase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′ 5′-hydroxylase; HPLC, high-performance liquid chromatography; qRT-PCR, quantitative RT-PCR.

* Corresponding author at: Department of Biotechnology and Life Science, Faculty of Engineering, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan.
E-mail address: miyahara@cc.tuat.ac.jp (T. Miyahara).

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2. Materials and methods

2.1. Plant materials and chemicals

Delphinium zalil (synonym *semibarbatum*) and *D. grandiflorum* were gift from the Ehime Research Institute of Agriculture, Forestry, and Fisheries, and Miyoshi & Co., Ltd. Sepals from four different developmental stages were collected and immediately frozen in liquid nitrogen and stored at −80° C until use. Four flower developmental stages were selected: S1, green bud; S2, green and yellow bud; S3, early flower opening; S4, fully opened flower. For the F3′H enzyme reaction and authentication of the HPLC analysis, β-NADPH was purchased from Oriental Yeast Co., Ltd (Japan), and apigenin, luteolin, naringenin, eriodictyol, dihydrokaempferol, kaempferol and quercetin were obtained from Extrasynthase (France). Dihydroquercetin was purchased from Sigma-Aldrich (USA).

2.2. Isolation of full length DzF3′H cDNA

Total RNA was isolated from S3 sepals using an RNeasy Plant Mini Kit (Qiagen, USA). First-strand cDNA was synthesized as described previously (Nishizaki et al., 2013). Partial cDNA fragments for *DzF3′H* were amplified by RT-PCR using the degenerate primer set dgF3′H Fwd and dgF3′H Rv (primer nucleotide sequences used in this study are described in Supplemental Table 1) and first strand cDNAs prepared from sepal RNA of *D. zalil* as the template. The produced cDNA amplicons were inserted into the pMD20 vector (Takara Bio, Japan) to determine their nucleotide sequences. The 5′ and 3′ cDNA ends of *DzF3′H* cDNA were amplified with a SMARTer RACE cDNA Amplification Kit (Clontech Laboratories, USA) using DzF3′H Fwd, DzF3′H Rv2. The full-length *DzF3′H* cDNA nucleotide sequence in pMD20 was confirmed using M13 Fwd and M13 Rv.

2.3. Qualitative and quantitative RT-PCR

First strand cDNA prepared from *D. zalil* sepal RNA was used for qualitative RT-PCR to demonstrate gene expression. PrimeStar GXL DNA polymerase (Takara Bio) and sequence specific primers were used in the analyses: *F3′H* with F3′H RTFwd and F3′H RTRv; *F3′5′H* with F3′5′H RTFwd and F3′5′H RTRv; *DFR* with DFR RTFwd and DFR RTRv; *ANS* with ANS RTFwd and RTRv; and *Actin*, the reference gene, with Actin RTFwd and Actin RTRv (Nishizaki 2013, Miyagawa 2014). First strand cDNA of *D. grandiflorum* was used as a control for an anthocyanin synthesizing species that accumulates delphinidin derivatives. The following amplification conditions were employed: 2 min at 94 °C, then 30 cycles of 10 s at 98 °C, 15 s at 55 °C, and 15 s at 68 °C. Quantitative real-time PCR (qRT-PCR) was performed with the F3′H RTFwd and F3′H RTRv primer set for *F3′H* and the Actin RTFwd and Actin RTRev set for *Actin*. The analysis was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan) with a DNA Engine Opticone 2 (Bio-Rad Laboratories, USA). The conditions for the qRT-PCR were the same as described previously (Nishizaki et al., 2013).

2.4. Heterologous expression of DzF3′H protein in yeast and preparation of crude protein extract for detection of F3′H enzyme activity by the recombinant protein

The protein coding region of *DzF3′H* cDNA was amplified by PCR using the primer set F3′H-HindIII and F3′H-XbaI that have a *Hind*III and *Xba*I site at each end. The amplicon was introduced into a pYES2 (Invitrogen, USA) expression vector with a URA3 selection marker, and the resultant pYES2-DzF3′H was transferred into *Saccharomyces cerevisiae* strain INV*Sc*1. To support the F3′H reaction (Naur et al., 2003), the coding region of Arabidopsis NADPH-cytochrome P450 reductase, AtATR1 (At4g24520), was amplified by the primer set AtATR1-EcoRI and ATR1-NotI using leaf cDNA as a

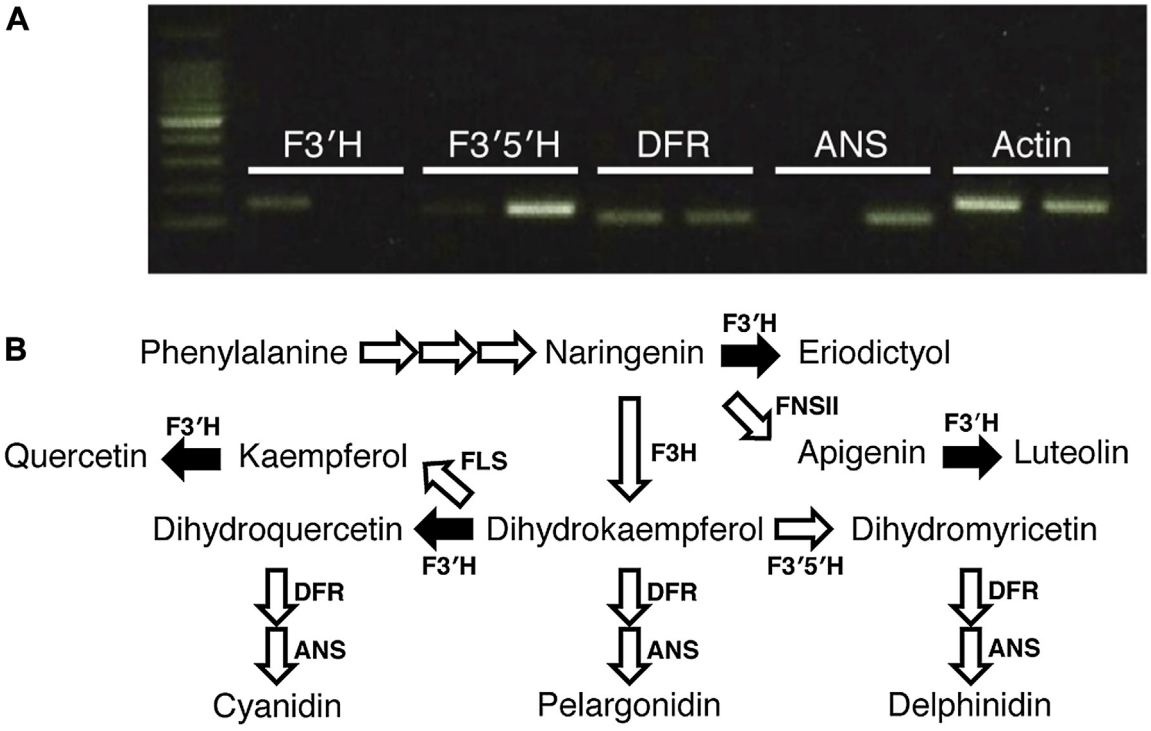

Fig. 1. Qualitative gene expression analysis of flavonoid synthetic enzymes and the metabolic pathway.
(A) PCR analysis for expression of *flavonoid 3′-hydroxylase (F3′H)*, *flavonoid 3′, 5′-hydroxylase (F3′5′H)*, *dihydroflavonol 4-reductase (DFR)*, *anthocyanidin synthase (ANS)* and *actin*. For each gene, the amplicon in the band on the left represents *D. zalil* cDNA and that on the right is *D. grandiflorum* cDNA. (B) Summary of the flavonoid metabolic pathway. Black arrows indicate the F3′H catalytic reaction.