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Allelic variants from *Dahlia variabilis* encode flavonoid 3′-hydroxylases with functional differences in chalcone 3-hydroxylase activity ☆

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ABSTRACT

In the petals of *Dahlia variabilis*, hydroxylation of chalcones at position 3 can be detected, except the well-known flavonoid 3′-hydroxylation. Although the reaction is well characterized at the enzymatic level, it remained unclear whether it is catalyzed by a flavonoid 3′-hydroxylase (F3′H, EC1.14.13.21, CYP75B) with broad substrate specificity. Two novel allelic variants of F3′H were cloned from *D. variabilis*, which differ only in three amino acids within their 508 residues. The corresponding recombinant enzymes show significant differences in their chalcone 3-hydroxylase (CH3H) activity. A substitution of alanine at position 425 with valine enables CH3H activity, whereas the reciprocal substitution leads to a loss of CH3H activity. Interaction of the valine at position 425 with not yet identified structural properties seems to be decisive for chalcone acceptance. This is the first identification of an F3′H which is able to catalyze chalcone 3-hydroxylation to a physiologically relevant extent from any plant species.

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Introduction

We have recently shown that hydroxylation of chalcones in position 3 is not a general property of flavonoid 3′-hydroxylase (F3′H,[1] EC 1.14.13.21, CYP75B) [1]. This was unexpected because F3′Hs generally show a broad substrate specificity [2] and the same position in the B-ring of flavonoids and chalcones is affected (Fig. 1) [3]. However, studies with recombinant F3′Hs from 11 plant species clearly indicated the involvement of a more specific enzyme in chalcone 3-hydroxylation [1]. Chalcones are intermediates in the biosynthesis of flavonoids, which are important secondary metabolites in the plant kingdom. However, chalcones are rarely accumulated in plants as they are rapidly converted to flavanones, which are precursors for all other flavonoid classes (Fig. 2) [2]. In addition, some ornamental plants accumulate the rare, but more stable 6′-deoxychalcones as yellow flower pigments. This particularly includes members of the Asteraceae family, e.g., *Bidens ferulifolia*, *Cosmos sulphureus*, *Coreopsis grandiflora*, and *Dahlia variabilis* [4].

The previous studies included an F3′H from *D. variabilis*, which owes its bright flower coloration to the presence of both 4′- and 3′,4′-hydroxylated flavonoids and 6′-deoxychalcones showing a corresponding 4- and 3,4-hydroxylation pattern [5]. Whereas anthocyanins (derivatives of the 4′-hydroxylated pelargonidin and the 3′,4′-hydroxylated cyanidin) are responsible for the formation of red, magenta, and orange hues, 6′-deoxychalcones (derivatives of 4-hydroxylated isoliquiritigenin and 3,4-hydroxylated butein) are the exclusive yellow pigments in dahlia flowers [5–9]. Although both 6′-deoxychalcones and flavonoids present in *D. variabilis* show a corresponding B-ring hydroxylation pattern, the recombinant F3′H from *D. variabilis* cv. Chat noir (Accession No. FJ216428) does not accept 6′-deoxychalcones as substrates [1]. Therefore, another enzyme must be involved in chalcone 3-hydroxylation in *D. variabilis*.

Earlier studies demonstrated the presence of a CH3H activity in *D. variabilis* petals, which could be identified as a cytochrome P450-dependent monooxygenase [1,10]. However, the question remained whether CH3H activity is performed by an as yet unidentified F3′H isoenzyme or an unknown independent enzyme. Cytochrome P450-dependent monooxygenases form one of the largest protein families in higher plants [11]. Nelson et al. [12] have set up a common system for the nomenclature of cytochrome P450 genes, which is based on their amino acid sequence identity. According to this nomenclature, members of a family share more than 40% identity, whereas members of a subfamily share more than 55% amino acid identity. If sequences share more than 97% homology, they are regarded as allelic variants.

We here report on the first cloning and heterologous expression of an F3′H from *D. variabilis* which can catalyze chalcone 3-hydroxylation. The molecular basis for the divergent substrate acceptance of this F3′H variant was assessed via site-directed mutagenesis.

☆ Nucleotide sequences reported in this paper have been submitted to GenBank database under GenBank Accession Nos. GQ281058 and GQ281059.

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[1] *Abbreviations used:* F3′H, flavonoid 3′-hydroxylase; CH3H, chalcone 3-hydroxylase; ISO, isoliquiritigenin; NAR, naringenin; DHK, dihydrokaempferol; KAM, kaempferol; API, apigenin; SRS1, substrate recognition site 1.

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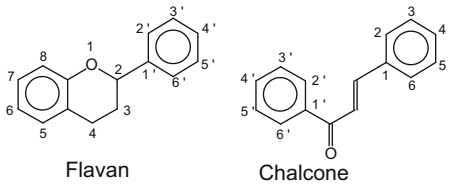

Fig. 1. The basic structures of flavan and chalcone. Please note the divergent ring numbering.

Materials and methods

Plant material

Studies were performed with petals of *D. variabilis*, cv. Auroras kiss (Haslhofer, St. Pankraz, Austria), which were collected during summer 2008, frozen immediately in liquid nitrogen and stored at −80 °C.

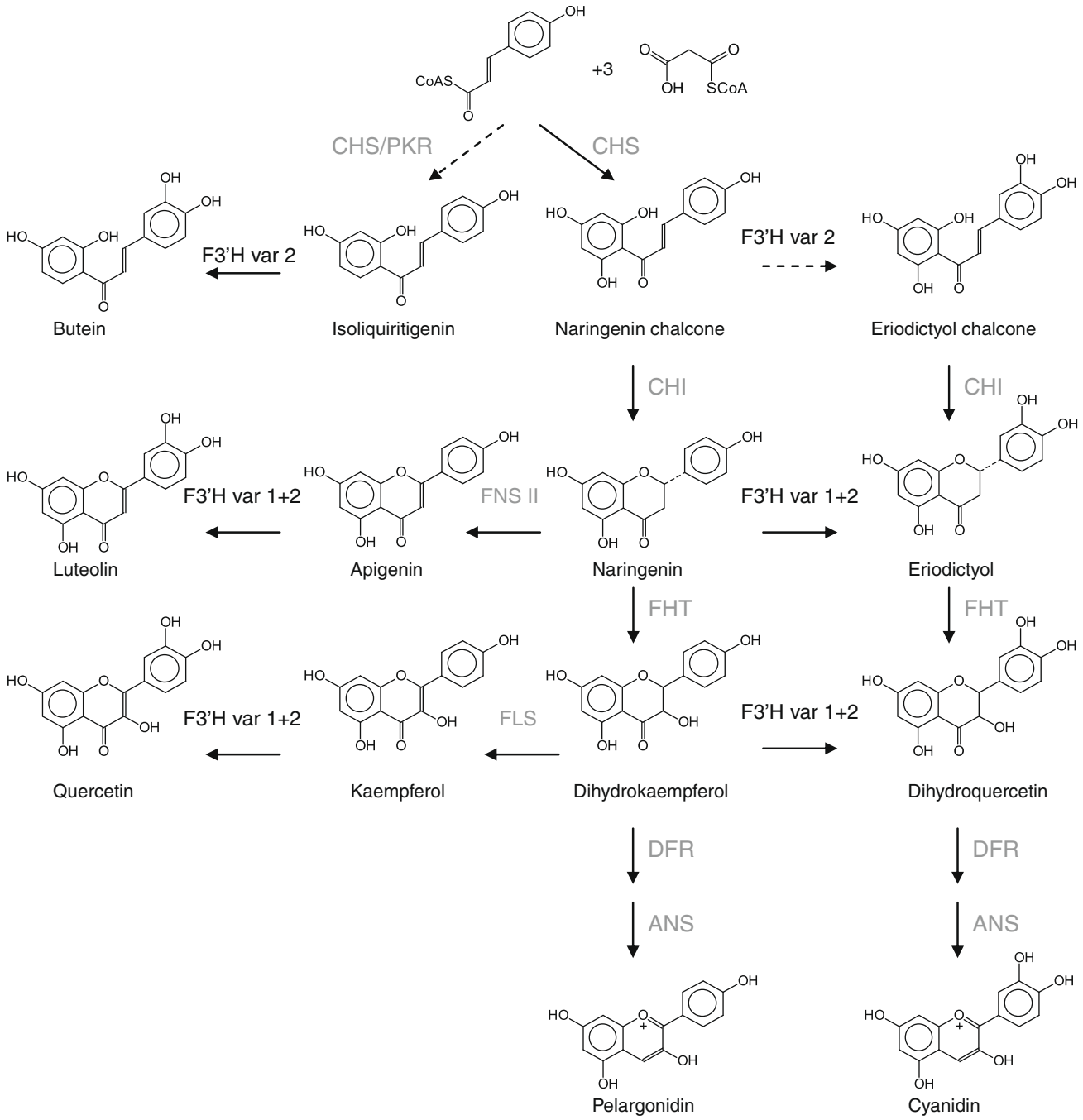

Fig. 2. The updated flavonoid pathway in *D. variabilis* modified from [9]. The enzymes demonstrated in this study are shown in black, previously known enzymes are shown in gray. Dashed arrows indicate as yet undemonstrated steps. Abbreviations: ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3′H, flavonoid 3′-hydroxylase; FHT, flavanone 3-hydroxylase; FNSII, flavone synthase II; PKR, polyketide reductase.

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