Expression of chimeric P450 genes encoding flavonoid-3′,5′-hydroxylase in transgenic tobacco and petunia plants[1]

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Abstract Flavonoid-3′,5′-hydroxylase (F3′5′H), a member of the cytochrome P450 family, is the key enzyme in the synthesis of 3′,5′-hydroxylated anthocyanins, which are generally required for blue or purple flowers. A full-length cDNA, TG1, was isolated from prairie gentian by heterologous hybridization with a petunia cDNA, AK14, which encodes F3′5′H. To investigate the in vivo function of TG1 and AK14, they were subcloned into a plant expression vector and expressed under the control of the CaMV35S promoter in transgenic tobacco or petunia, both of which originally lack the enzyme. Transgenic petunia plants had a dramatic change in flower color from pink to magenta with a high content of 3′,5′-hydroxylated anthocyanins. In contrast, transgenic tobacco plants had minimal color change with at most 35% 3′,5′-hydroxylated anthocyanin content. These results indicate that the products of TG1 and AK14 have F3′5′H activity in planta and that interspecific gene transfer alters anthocyanin pigment synthesis. The difference in apparent F3′5′H activity between tobacco and petunia is discussed.

Key words: Anthocyanin biosynthesis; Flower color; Flavonoid-3′,5′-hydroxylase; P450; Transgenic plant; Electron transport system for P450

1. Introduction

Flavonoid-3′,5′-hydroxylase (F3′5′H) is the key enzyme that synthesizes 3′,5′-hydroxylated anthocyanidins (e.g. delphinidin), which are usually a prerequisite for the expression of blue or purple flower color (Fig. 1). The enzyme is considered to be a member of the cytochrome P450 family from its biochemical characteristics such as association with microsomal membrane fraction, dependence on NADPH and O_2, and sensitivity to inhibitors [1,2]. However, it has been difficult to purify and isolate the enzyme in sufficient amounts to carry out further investigations, such as amino acid sequencing and cDNA isolation.

Petunia (*Petunia hybrida*) has two F3′5′H encoding genes, *Hf1* and *Hf2* [3]. *Hf1* has a greater effect on flower color expression than *Hf2* because *Hf1* affects both the corolla limb and tube whereas *Hf2* affects only the limb. We (see [17]) and other groups succeeded in cloning F3′5′H cDNA from *P. hybrida* in 1993. Holton et al. [4] isolated pCGP176 and pCGP175, cDNA clones encoding F3′5′H from petunia. They detected F3′5′H activity in vitro using a yeast expression system but presented a limited transgenic study. Sequences have also been reported for petunia [5], egg plant [6], gentian [7] and lisianthus [8]. Tanaka et al. [7] also reported F3′5′H activity using a yeast expression system. There are, however, few studies of their in vivo activities or pigment synthesis using transgenic plants, in contrast to a number of reports of cDNA clones related to the petunia sequence.

Here, we present the isolation of F3′,5′H cDNA, TG1, from *Eustoma russellianum* (prairie gentian), by hybridization with the petunia *F3′,5′H* cDNA, AK14, which corresponds to *Hf1*. TG1 was subcloned into a plant expression vector and transformed to pink tobacco (*Nicotiana tabacum* cv. Petit Havana SR1), which originally lacks the enzyme. We also subcloned AK14 into a plant expression vector and transformed it to pink tobacco (*N. tabacum* cv. Petit Havana SR1) and pink petunia (var. Falcon), both of which originally lack the enzyme. The effects of the transgene on anthocyanin composition and flower color were investigated, especially in comparison between interspecific and intraspecific transformation.

2. Materials and methods

2.1. Plant materials and petunia F3′5′H cDNA

Petunia (*P. hybrida* var. Falcon) seeds were from Sakata Seeds Co, Ltd. (Yokohama, Japan). Plants to be used for transformation were grown aseptically on half strength Murashige and Skoog's medium [9] supplemented with 1.5% sucrose. Petunia F3′5′H cDNA, AK14 (DDBJ, EMBL and GenBank database accession number D14588) was isolated through a PCR-based strategy (see [17]), which had the same ORF sequence as that of pC176 reported by Holton et al [4].

Flower buds of *E. russellianum* var. royal-light-purple (Takii Seeds, Kyoto) were obtained from a local market.

2.2. Construction and screening of a cDNA library from E. russellianum

cDNA was prepared from flower buds of *E. russellianum* as described previously [10]. A cDNA library was constructed with the λ gt22 vector (Amersham) and GigapackII Gold Packaging Extracts (Stratagene) according to the manufacturers' instructions. The full-length cDNA fragment of petunia F3′5′H, AK14, was used as a probe to screen the cDNA library by hybridization as described previously [11]. Blots were washed with 2×SSC at 50°C and subjected to autoradiography. Six positive cDNA clones were isolated and the two longest clones were chosen for nucleotide sequence determination. Since the ORF sequences of the two cDNAs were identical, one of

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[1] The nucleotide sequence data in this paper have been submitted to the EMBL, GenBank and DDBJ databases (CYP75A7, accession number D14589).

Abbreviations: F3′5′H, flavonoid-3′,5′-hydroxylase; F3′H, flavonoid-3′-hydroxylase; PCR, polymerase chain reaction

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them was selected and designated TG1. It was excised from the λ gt22 vector with *Sal*I *and Not*I and subcloned between the *Sal*I and *Not*I sites of pBluescript II KS(+) for subsequent analysis.

2.3. DNA sequence analysis

DNA sequences were determined with an automated DNA sequencer (Model 373A DNA Sequencing System, ABI) according to the manufacturer's instructions. The nucleotide sequence was analyzed with GENETYX-Mac (Software Development Co., Ltd., Tokyo).

2.4. Plant expression vector and transgenic plant production

AK14 cDNA was excised from a λ gt10 vector with *Hin*dIII, followed by treatment with BAL31 nuclease S to truncate the 5′ and 3′ non-coding regions. The resulting DNA fragments were subcloned into the *Sma*I site of pUC19 to be excised with *Sac*I and *Bam*HI. The AK14 fragment, which has a 45-bp 5′ non-coding sequence, was selected and cloned between the *Sac*I and *Bam*HI sites of the plant expression vector pBI121 in exchange for the *GUS* gene, yielding pB853. TG1 was excised from pBluescript II KS(+) with *Sal*I and *Sac*I, and then subcloned between the *Sma*I and *Sac*I sites of the plant expression vector pBI121, in exchange for the *GUS* gene, yielding pTI201. In these constructs AK14 and TG1 should be expressed under the control of the CaMV35S promoter. The construct was transformed to tobacco (*N. tabacum* L. cv. Petit Havana SR1) or petunia (var. Falcon Pink Vein, Falcon Rose) via *Agrobacterium tumefaciens* LBA4404 using the leaf disc method [12]. After kanamycin-resistant plants had been regenerated, they were transferred to pots and grown in a growth chamber at 28°C. Integration of the chimeric construct in the genome was confirmed by PCR with the CaMV35S forward primer (5′ GAT GTG ATA TCT CCA CTG ACG TAA GGG 3′) and the AK14 reverse primer (5′ TAG GAG CTC ACT TGT CCG ATC AT 3′) or the TG1 reverse primer (5′ CCT GAT TCA GCC ATT GCA TAG AGC 3′) as described previously [13].

2.5. Pigment extraction and analysis of anthocyanins

Petal anthocyanins were extracted and converted to anthocyanidins to be analyzed according to the method of Harborne [14]. Cellulose thin layer plate chromatography (TLC) was performed according to the method of Harborne [14]. HPLC analysis was performed basically according to the method of Wilkinson et al. [15]. An ODS column (YMC-Pack ODS-A, 250×4.6 mm, S-5 120 Å) was used with a solvent system of 69% water, 10% acetic acid and 21% methanol at a flow rate of 1 ml/min. Commercially obtained cyanidin and delphinidin (Extrasynthese Ltd.), peonidin, and malvidin (Apin Chemicals Ltd.) were used as standards. Petunidin was extracted from petunia flowers (var. Cascade Royal) and used as a standard after purification with TLC according to the method of Harborne [14].

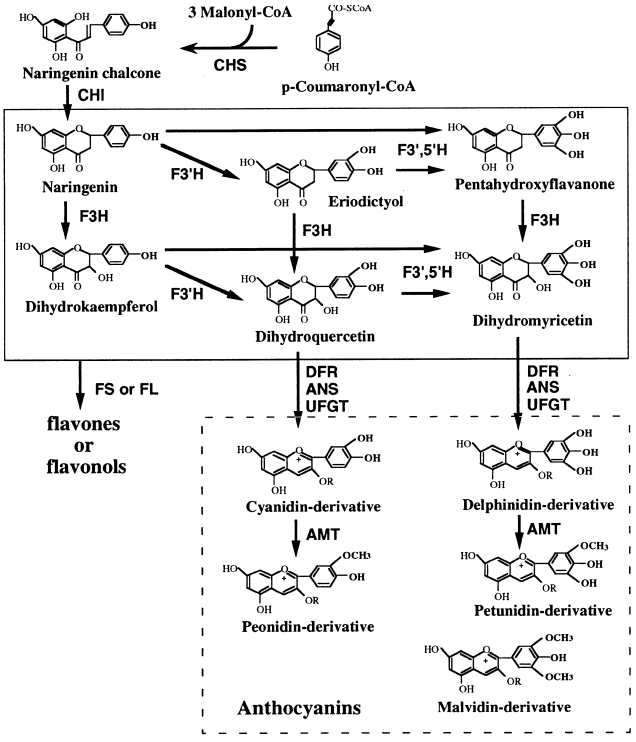

Fig. 1. Simplified representation of the flavonoid biosynthetic pathway. Of these compounds, naringenin chalcone and the anthocyanins are pigmented. Since *N. tabacum* cv. Petit Havana SR1 lacks both F3′5′H and anthocyanin methyltransferase (AMT), the cyanidin derivatives are the most abundant anthocyanins in the petals. Pink or red petunia varieties mainly accumulate cyanidin or peonidin derivatives in the petals. Blue petunia varieties mainly accumulate delphinidin, petunidin and malvidin derivatives. Flavonoid-3′-hydroxylase (F3′H) converts naringenin and dihydrokaempferol to eriodictyol and dihydroquercetin, whereas F3′,5′H converts naringenin, dihydrokaempferol, eriodictyol and dihydroquercetin to pentahydroxyflavanone or dihydromyricetin. These six compounds can also be substrates for flavone synthase and flavonol synthases, which produce flavones and flavonols respectively. Other enzymes involved in the pathway are indicated as follows: F3H, flavanone-3-hydroxylase; UFGT, UDP-glucose:3-*O*-flavonoid glucosyl transferase; DFR, dihydroflavonol-4-reductase; CHS, chalcone synthase; CHI, chalcone isomerase; ANS, anthocyanin synthase. R represents a sugar chain.

3. Results and discussion

A full-length cDNA, TG1, was isolated from a cDNA library prepared from flower buds of *E. russellianum* by heterologous hybridization with AK14 (CYP75A1), a previously isolated cDNA of F3′5′H from petunia. TG1 was 2174 bp (nucleotide sequence not shown) and contained an ORF encoding a polypeptide of 510 amino acid residues. The deduced amino acid sequence had characteristics of P450s [16]. The amino acid sequence of the TG1 product and related sequences are listed in Fig. 2. TG1 shares 98% homology with a lisianthus clone (*E. grandiflorum*) [8], 77% with a gentian clone (*Gentiana triflora*) [7], 74% with petunia *Hf1* [4,17], 73% with petunia *Hf2* [4], and 71% with egg plant (*Solanum melongena*) [5]. Although *E. russellianum* and *E. grandiflorum* are not strictly distinguished systematically, we found significant genetic variation between TG1 and a previously reported lisianthus clone: there are nine substitutions of amino acid residues. All these related sequences belong to a subfamily of the P450 superfamily, CYP75A [18].

In contrast to a number of reports on cDNA sequences, which were isolated by hybridization with the petunia sequence, only a few studies present information on their enzymatic activities. To demonstrate the function of the TG1 product in planta, TG1 was subcloned into pBI121 in exchange for the *GUS* structural gene to produce pTG201. It was transformed to tobacco via *A. tumefaciens* by leaf disk transformation. Twenty transgenic plants were isolated and flowered. Anthocyanin pigments were extracted from the petals of transgenic plants, chemically converted to anthocyanidins and analyzed by HPLC. All transgenic plants accumulated delphinidin derivatives, whereas all 20 control plants transformed with pBI121 only accumulated cyanidin derivatives. Table 1 shows the results of the pigment analysis for transgenic plant No. 6. In the transgenic plant with pTG201, 23% of the total anthocyanin was delphinidin derivatives, whereas in the transgenic plant with pBI121 all the anthocyanin was cyanidin derivatives. The flower color of the transgenic plant was magenta instead of pink (Fig. 3A). No phenotypes other than flower color were affected by the

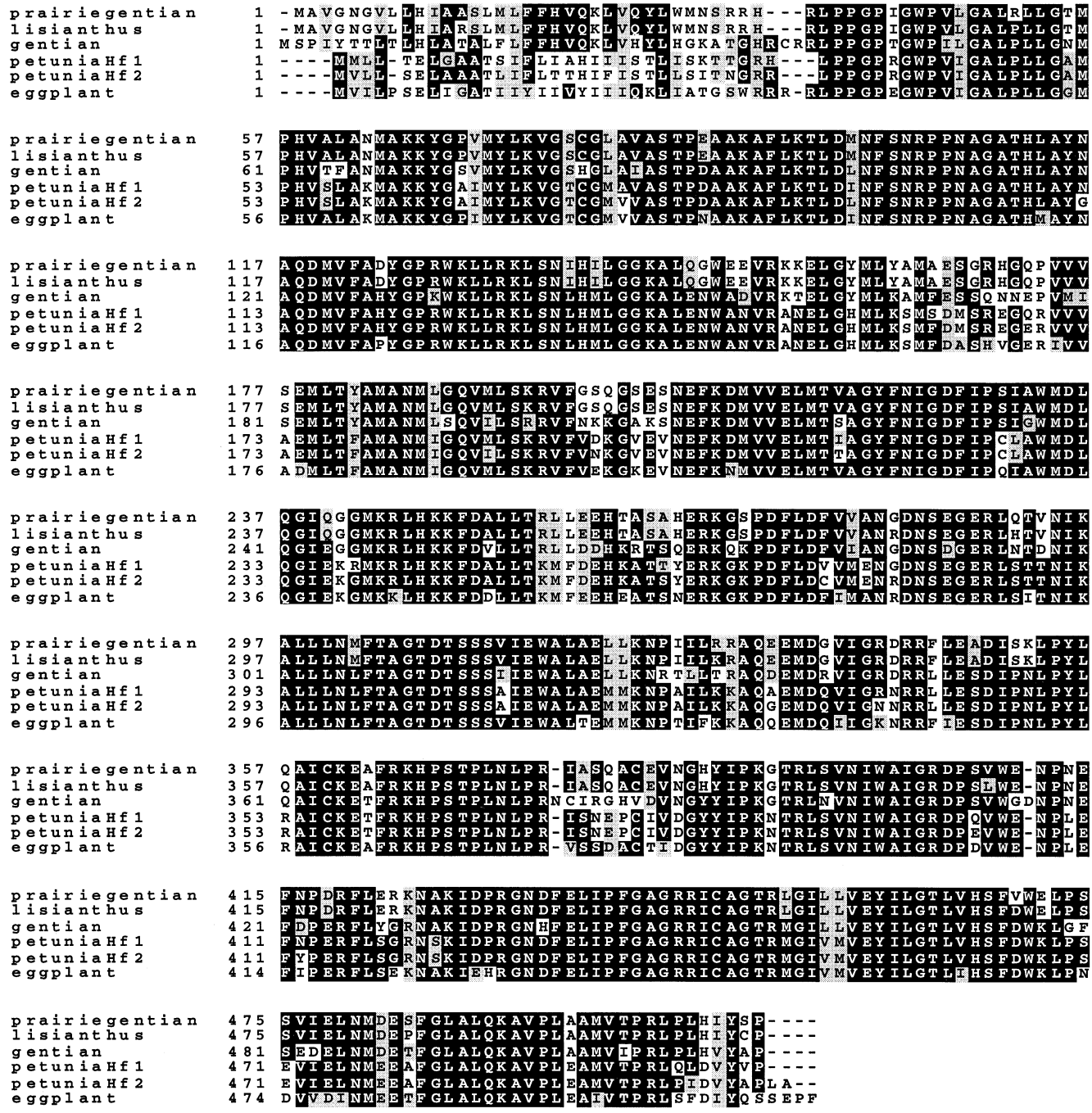

Fig. 2. Sequence alignment of F3′,5′H cDNAs from different species. The amino acid sequences were aligned using ClustalW on the server at NCBI (National Center for Biotechnology Information) and shaded using the program Boxshade on the server at EMB net (European Molecular Biology network). Identical amino acid residues are shown with reverse contrast characters and conserved residues with hatching.

transgene. The delphinidin-producing phenotype cosegregated with kanamycin resistance in the progeny plants (data not shown). Based upon these observations, we conclude that TG1 encodes F3′5′H. The delphinidin content varied from 2% to 23% in the transgenic plants. The flower color changed in proportion with the delphinidin content. This variation was probably due both to positional effects and to the copy number of the transgene.

In comparison with the results of transgenic plants with pTG201, transgenic tobacco and petunia plants were produced with pB853, in which petunia *F3′5′H* was controlled by the CaMV35S promoter. More than 100 T1 (first generation) transgenic tobacco plants were isolated and allowed to flower. Transgenic plant No. 4, which had the deepest flower color and accumulated the most delphinidin, as confirmed by TLC analysis, was chosen for further analysis. The flower color of the transgenic plant was magenta instead of pink (Fig. 3A). No apparent phenotype other than flower color was affected. The T1 plant of transgenic strain No. 4 was self-pollinated and the T2 generation was produced to exam-

Table 1
Anthocyanidin contents of transgenic tobacco plants

	Cyanidin	Delphinidin	Total (mg/gfw)
pBI121	242 (100)	n.d.	242
pTG210	191 (77)	57 (23)	248
pB853	152 (65)	82 (35)	234

Data are means of five flowers. The numbers in parentheses are the percentage of each anthocyanidin. n.d., not detected; gfw, gram fresh weight.

ine the co-segregation of F3′5′H with Km^r. The delphinidin-producing phenotype cosegregated with the Km^r phenotype at a single locus in the T2 progeny plants (data not shown). Anthocyanidin was extracted from a T2 plant of strain No. 4 and analyzed using HPLC (Table 1). 35% of the total anthocyanidins was delphinidin in the transgenic T2 plant.

We also transformed pB853 intraspecifically into pink flowering petunia varieties, Falcon Pink Vein and Falcon Rose, which lack 3′,5′-hydroxylated anthocyanidins (*hf1hf1* genotype). Of 12 independently isolated transgenic plants, the flower color was altered from pink to magenta in 11 plants. A flower from transgenic plant No. 18 derived from Falcon Pink Vein is shown in Fig. 3B. Anthocyanidins were extracted from flowers of this plant and analyzed by HPLC (Table 2). As Falcon Pink Vein has leaky F3′5′H activity, 15% of the total anthocyanins were hydroxylated at the 3′ and 5′ positions (petunidin and malvidin) in the control transformant with pBI121. In contrast, 94% of the anthocyanins were hydroxylated at the 3′ and 5′ positions (delphinidin, petunidin and malvidin) in the transgenic plant with pB853. All of the transgenic petunia plants that had color alterations accumulated more than 90% 3′,5′-hydroxylated anthocyanidins. There is, therefore, a marked difference between the results in transgenic tobacco and petunia plants: the transgene caused a significant alteration in anthocyanin composition and flower color in petunia, whereas it caused a limited alteration in tobacco. The result of transgenic tobacco plants with the petunia clone was consistent with that of the prairie gentian clone presented above.

There are a number of possible explanations for why tobacco did not accumulate more delphinidin. It could either be due to intrinsic properties of interspecific transgene or to the fact that the transgene was under the control of the CaMV35S promoter, even though we used the same construct, pB853, to compare these near relative species. The physiological environment of the host plant could be another possible explanation. Many different components and factors determine flavonoid accumulation. Among them, their composition is determined by the balance of many enzymes. It should be noted that F3′5′H competes with at least five enzymes for substrates (Fig. 1). The balance of these enzymes could be a mechanism to explain the low delphinidin content in transgenic tobacco. For example, if the substrates of F3′5′H (naringenin, dihydrokaempferol, eriodictyol and dihydroquercetin) are less abundant in tobacco cells than in petunia cells because of the higher flavanone-3-hydroxylase and dihydroflavonol-4-reductase activities in tobacco than in petunia, then F3′5′H should be apparently less catalytic in tobacco cells than in the petunia cells, since eriodictyol and dihydroquercetin are rapidly converted into cyanidin derivatives in tobacco. Suppressing competitive enzymes such as F3′H by antisense or co-suppression may increase the delphinidin content in transgenic tobacco plants.

Fig. 3. Flower phenotypes of transgenic plants. A: Flower phenotypes of transgenic tobacco (cv. Petit Havana SR1) transformed with pBI121 (left), pTI201 (middle) and pB853 (right). B: Flower phenotypes of transgenic petunia (var. Falcon Pink Vein) transformed with pBI121 (left), and pB853 (right).

Very recently, de Vetten et al. reported in vivo evidence that full activity of F3′5′H in petunia was supported by a specific cytochrome b_5 encoded by *difF* [19]. Cytochrome b_5 is known to enhance the activities of some P450s in reconstituted membrane vesicles [19], although its physiological function is not yet clear. On the other hand, plants are known to possess multiple P450 reductases, in contrast to the P450 systems in mammals and fungi. For example, even *Arabidopsis thaliana* has two distantly related P450 reductases [20,21]. However, we know little about the relationship of P450 reductases with the specificity of P450s directly or in combination with a specific cytochrome b_5. The transgenic tobacco lines presented here could be used to test components to support higher F3′5′H activity in vivo. Since the in vivo activity of F3′5′H can be easily monitored by flower color, this may be ideal for characterizing and understanding the P450 electron transport system in higher plants.

The biochemical mechanisms to determine flavonoid bio-

Table 2
Anthocyanidin contents of transgenic Falcon Pink Vein

	3′-Hydroxylated		3′,5′-Hydroxylated			Total (mg/gfw)
	Cyanidin	Peonidin	Delphinidin	Petunidin	Malvidin	
pBI121	54	170	n.d.	4	36	264
pB853	4	12	45	21	192	275

Anthocyanidins were extracted from petals of T1 transgenic plant No. 18 (transformed with pB853) or a control T1 transgenic plant (transformed with pBI121). Data are means of three flowers. n.d., not detected; gfw, gram fresh weight.

synthesis are not yet clearly understood. Enzyme complementation and competitive studies using transgenic plants may present new and valuable insights into flavonoid biosynthesis and the P450 electron transport system.

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