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Molecular characterization of the flavonoid biosynthesis of *Verbena hybrida* and the functional analysis of verbena and *Clitoria ternatea F3′5′H* genes in transgenic verbena

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Abstract Homologues of the flavonoid 3′,5′-hydroxylase (*F3′5′H*) gene, a key gene determining flower color, were obtained from a *Verbena hybrida* (verbena) cultivar Temari Violet, verbena cultivar Tapien Pink, and *Clitoria ternatea* (butterfly pea). The expression of the Temari and butterfly pea homologues in yeast confirmed that they encoded F3′5′H. The two genes under the control of an enhanced cauliflower mosaic virus 35S promoter were introduced into verbena Temari Sakura. Some of the transgenic verbena plants had elevated delphinidin contents and flower color altered toward violet. Interestingly, the butterfly pea *F3′5′H* gene yielded more delphinidin and gave clearer flower color change than the verbena Temari gene in the transgenic verbena plants. The results indicate that the choice of the gene source should be considered to obtain strong phenotypic changes, even if the genes encode the same enzymatic activity. We also cloned some flavonoid biosynthetic genes from verbenas. The potential usefulness of verbena in the phytomonitoring of environmental pollutants is also discussed.

Key words: Anthocyanin, *Clitoria ternatea*, flavonoid, flavonoid 3′,5′-hydroxylase, verbena.

Flower color is largely determined by three classes of pigments: flavonoids, carotenoids, and betalains (Tanaka et al. 2005). Among them, flavonoids and their colored class of compounds, anthocyanins, contribute to the flower colors of *Verbena hybrida* (verbena), which is a popular ornamental plant with violet, red, pink and white flowers. *Verbena hybrida* petals are known to accumulate anthocyanidin 3-(6-acetylglucoside)-5-glucoside and anthocyanidin 3,5-di -(6-acetylglucoside) (Toki et al. 1991). The flowers also contain flavones and flavonols (Figure 1).

The anthocyanin color shifts towards blue due to the increased hydroxylation of the B-ring. Most violet/blue flowers, including violet verbenas, contain delphinidin-based anthocyanins (3′,4′,5′-hydroxy anthocyanins), while red and magenta flowers contain pelargonidin- and cyanidin-based anthocyanins (4′ and 3′,4′-hydroxy anthocyanins, respectively). The key enzymes that determine the hydroxylation pattern are flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H), which are cytochrome P450 enzymes (Forkmann and Heller 1999; Tanaka et al. 2005). These genes have been isolated from many plant species.

The F3′5′H activity was demonstrated in the microsomal fraction of verbena flowers for the first time (Stotz and Forkmann 1981). Hung (2003) isolated the verbena *F3′5′H* genes (AY604727, AY566988) and showed that the expression of the genes correlated with delphinidin production in verbena. The activity of flavone synthase II (FNSII), a cytochrome P450 catalyzing the flavone synthesis from flavanones, was also demonstrated in verbena (Forkmann and Heller 1999). Anthocyanin 5-glucosyltransferase and flavonol 3-glucoside-6″-malonyltransferase cDNAs have been obtained and characterized (Yamazaki et al. 1999 and Suzuki et al. 2004, respectively). However, many of the flavonoid biosynthetic genes have not been isolated from verbena, and flower color modification of verbena by genetic transformation has not yet been achieved.

The expression of a cloned gene in transgenic plants is

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Abbreviations: ANS, anthocyanidin synthase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; F3H, flavanone 3′-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; FNSII, flavone synthase II.
This article can be found at http://www.jspcmb.jp/

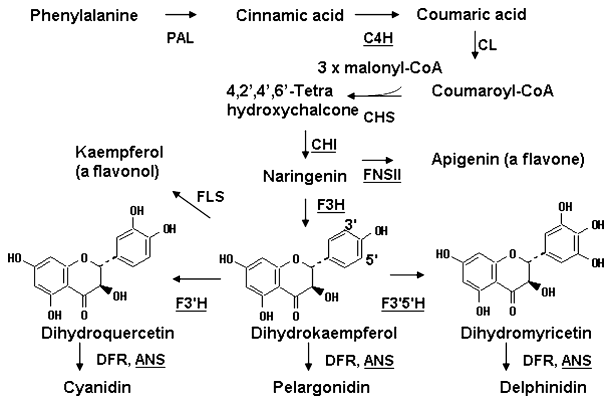

Figure 1. Putative flavonoid biosynthetic pathway in verbena. The genes for the underlined enzymes were cloned in this study. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; CL, hydroxycinnamate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; FNSII, flavone synthase II; FLS, flavonol synthase.

often unpredictable, and it is not always easy to achieve the proper level of expression of the gene in the target species. Genes encoding the same enzymatic activity from different plant species perform differently in transgenic plant species. The maize gene encoding phytoene synthase performed better than the daffodil gene encoding the same enzyme in transgenic rice (Paine et al. 2005). *Viola F3′5′H* genes functioned in *Rosa hybrida*, while petunia and some plant *F3′5′H* genes did not (Brugliera et al. 2004). Campanula F3′5′H performed better than petunia F3′5′H to convert cyanidin to delphinidin in transgenic tobacco (Okinaka et al. 2003).

We previously developed its transformation system with GFP as a marker protein (Tamura et al. 2003). In this study, we isolated verbena and *Clitoria ternatea* (butterfly pea) *F3′5′H* genes and comparatively expressed them in verbena to achieve its flower color modification with genetic transformation. We also isolated a few flavonoid biosynthetic genes from verbena that are useful molecular tools to modify flower color.

Materials and methods

Plant materials and flavonoid analysis

Verbena hybrida cultivar Temari Violet, Temari Sakura, and Tapien Pink are products of Suntory Flowers Ltd. (Japan). Temari and Tapien originated from *Verbena peruviana* and *Verbena tenera*, respectively, and are morphologically different (Tamura et al. 2003). *Petunia hybrida* Skr4 xSw63 was previously described (Holton et al. 1993). Butterfly pea seeds were kindly provided by Dr. Tachibana. These plants are grown in containment glasshouses that are compatible with the genetically modified plants. Flavonoids were analyzed as previously described (Murakami et al. 2004).

Cloning of the genes involved in flavonoid biosynthesis

Molecular biological procedures have been described previously (Fukuchi-Mizutani et al. 2003), except that all DNA labeling and detection procedures were carried out with the DIG system (Roche) following the manufacturer's protocols. Petals of the buds of Temari Violet, Tapien Pink and butterfly pea were subjected to RNA preparation. The petal cDNA library derived from them was constructed using the directional λ ZAP-cDNA synthesis kit (Stratagene). These libraries were screened with a petunia *F3′5′H* (Hf1) cDNA (Holton et al. 1993). The two verbena libraries were screened with petunia *F3′H* (Brugliera et al. 1999) and snapdragon *FNS II* (Akashi et al. 1999) cDNAs. The Temari library was screened with flavanone 3-hydroxylase (*F3H*, *Holton* unpublished results; the sequence was the same as that reported in Britsch et al. 1992). The Tapien library was screened with cinnamic acid 4-hydroxylase (*C4H*) and torenia anthocyanidin (*ANS*) cDNAs (Mizutani et al. 1997 and Nakajima et al. 2000, respectively). An LA PCR *in vitro* Cloning Kit (TaKaRa) was used to obtain the 5′non coding region and N-terminal coding sequence Tapien F3′H gene with the use of the primer 5′- CCTGATAGTTATACGCCACG-3′ and the *Hin*dIII-digested genomic DNA.

A chalcone isomerase gene fragment was amplified from the cDNA derived from the Temari violet petal with the use of the pair of primers 5′-TT(T/C)(A/G)TIAA(A/G)TT(T/C)ACIGCIAT-3′ and 5′-CCIGGIAA(A/G)CT(T/C)TT(T/C)(A/G)A-3′. These sequences were designed on the basis of the conserved amino acid sequences (FSKFTAI and GPFEKF, respectively) of a chalcone isomerase (CHI). The amplified fragment was used for library screening to obtain a full-length cDNA clone.

Expression of Temari and butterfly pea F3′5′H cDNA in yeast

The plasmid, pHVF7, containing the putative full-length Temari *F3′5′H* cDNA, was digested with *Eco*RI and *Xho*I. The obtained fragment of about 1.8 kb was cloned into pYE22m (Tanaka et al. 1996), a yeast expression vector in which the inserted cDNA is regulated under the control of the glyceraldehyde 3-phosphate dehydrogenase promoter, to yield pYHVF7.

The obtained butterfly pea *F3′5′H* cDNA in pBHF2 seemed to lack two bases (AT) to have the initiation codon (ATG); therefore, the two bases along with a *Bam*HI restriction site were added to the cDNA clone using a synthetic primer, 5′-GGGATCCAACAATG-TTCCTTCTAAGAGAAAT-3′ (*Bam*HI site and the initiation codon are underlined), as described previously

Table 1. Flavonoid biosynthetic genes cloned in this study.

Plasmid	Species	Putative function	CYP number	Accession number
pBHF2	Butterfly pea	*F3′5′H**	CYP75A24	AB234897
pHVF7	Verbena Temari	*F3′5′H**	CYP75A19v2	AB234898
pSPB1408	Verbena Tapien	*F3′5′H*	CYP75A23	AB234899
	Verbena Tapien	F3'H	CYP75B26	AB234900, AB234901
pSPB588	Verbena Tapien	C4H	CYP73A46	AB234902
pHVFN2	Verbena Temari	FNSII	CYP93B9v1	AB234910
pSPB590	Verbena Tapien	FNSII	CYP93B9v2	AB234903
pSPB1411	Verbena Tapien	P450	CYP76S2	AB234904
pSPB9	Verbena Temari	F3H	—	AB234905
pSPB598	Verbena Tapien	ANS	—	AB234906
pHVCHI	Verbena Temari	CHI	—	AB234907
pSPB11	Verbena Temari	BEBT	—	AB234908
pSPB595	Verbena Tapien	GLOBOSA	—	AB234909

See text for details. * The function was demonstrated in this study. BEBT, benzoyl-CoA: benzoic acid benzoyl transferase. CYP numbers were kindly provided by Dr. Nelson (Nelson 1999).

(Yonekura-Sakakibara et al. 2000). The resultant fragment was digested with the restriction endonucleases *Bam*HI and *Pst*I, and the subsequent DNA fragment of about 200 bp was recovered. The DNA fragment was ligated with a 3.3 kb fragment of *Bam*HI/*Pst*I-digested pBHF2 to yield pBHF2F. The DNA sequence was confirmed to exclude errors made during PCR. The constructed plasmid, pBHF2F, was digested with *Bam*HI and *Xho*I, and the resultant fragment was inserted into pYE22m to yield pYBHF2F. A yeast strain G-1315 (Tanaka et al. 1996) was transformed with pYHVF7 and pYBHF2F. The transformants were subjected to the F3′5′H assay using naringenin as the substrate, and the reaction mixtures were analyzed using HPLC with a photodiode array attached as described previously (Tanaka et al. 1996).

Construction of binary vectors

El235S, an enhanced cauliflower mosaic virus 35S promoter from pBE2113-GUS (Mitsuhara et al. 1996), was used to transcribe *F3′5′H* in transgenic plants. A *Bam*HI linker was inserted into the SnaBI site of pBE2113-GUS. This plasmid was digested with *Sac*I, blunted, and ligated with an *Xho*I linker. The resultant plasmid was digested with *Eco*RI-*Hin*dIII, and this fragment was inserted into the *Eco*RI-*Hin*dIII site of pBinPLUS (van Engelen et al. 1995) to yield pSPB176. A fragment of pHVF7 and pBHF2F of about 1.8kb *Bam*HI-*Xho*I containing the coding region *F3′5′H* was ligated with a 12 kb *Bam*HI-*Xho*I fragment of pSPB176 to yield pSPB1431 and pSPB748, respectively.

Transformation of verbena

Verbena Temari Pink was transformed with pSPB1431 and pSPB748, as described previously (Tamura et al. 2003), to modify the flower color. The pair of primers 5′-ATCTAACAGCGGCAATCC-3′ and 5′-CATAACAATGTGGAGGCAAC-3′ was used to detect the transcripts of the verbena *F3′5′H* gene in the leaves of verbena plants transformed with pSPB1431 to confirm that they were transgenic. The pair of primers 5′-AGCTCGTGCATTCCTCAAAACC-3′ and 5′-TCGATTCCGAACCCTTTGTCTC-3′ was used to detect the transgene and transcripts of the butterfly pea *F3′5′H* gene in the leaves of verbena plants transformed with pSPB748. The transgenic verbena plants were also subjected to Southern blot analysis using the neomycin phosphotransferase gene as the probe.

Results and discussion

Isolation of cDNA encoding structural genes involved in flavonoid biosynthesis

The list of genes obtained in this study is shown in Table 1. Among the enzymes in the flavonoid biosynthetic pathway, F3′H, F3′5′H, FNSII, and C4H belong to the cytochrome P450 family (Forkmann and Heller 1999). In addition to these cDNAs, Tapien cDNA library screening with the petunia *F3′H* gene yielded one cytochrome P450 cDNA (pSPB1411, Table 1). Full-length cDNAs were not obtained for the *F3′H* and *FNSII* genes. The 5′-noncoding region and 5′-end portion of the coding region of the Tapien *F3′H* gene were amplified from the genomic DNA, and the entire coding sequence was deduced. The 5′-noncoding region sequence contained *Myb*- and *Myc* (*bHLH*)-binding sequences. *Myb* and *Myc* (*bHLH*) proteins are known to regulate flavonoid biosynthesis transcriptionally (Koes et al. 2005). F3′H catalyzes the formation of the 3′-hydroxy group on flavonoids, but it has been suggested that, in verbena, some of the 3′,4′-flavonoids are derived from caffeoyl CoA (Stotz et al. 1984). However, Temari Sakura and Tapien Pink almost exclusively contained pelargonidin (Table 2) and a small amount of cyanidin (Table 2). The contribution of the pathway from caffeoyl CoA is not clear. The presence of the *F3′H* gene in Tapien may suggest that F3′H also contributes to the 3′-hydroxylation of the flavonoid.

Table 2. Anthocyanidin analysis of host and transgenic verbena plants.

Line	Delphinidin content(%)	Pelargonidin mg g^{-1} petal	Cyanidin mg g^{-1} petal	Delphinidin mg g^{-1} petal
Temari Sakura	0	0.415	0.048	0
Line 748-1	31.9	0.240	0.115	0.166
Line 748-2	7.1	0.364	0.070	0.033
Line 748-4	3.7	0.250	0.040	0.011
Line 748-5	47.9	0.111	0.167	0.256
Line 748-6	44.0	0.125	0.171	0.233
Line 1431-1	13.4	0.495	0.060	0.086
Line 1431-2	11.0	0.274	0.033	0.038
Line 1431-3	1.7	0.308	0.042	0.006
Line 1431-4	10.5	0.339	0.038	0.044
Line 1431-5	12.0	0.359	0.044	0.055
Temari Violet	87.6	0.039	0.176	1.518

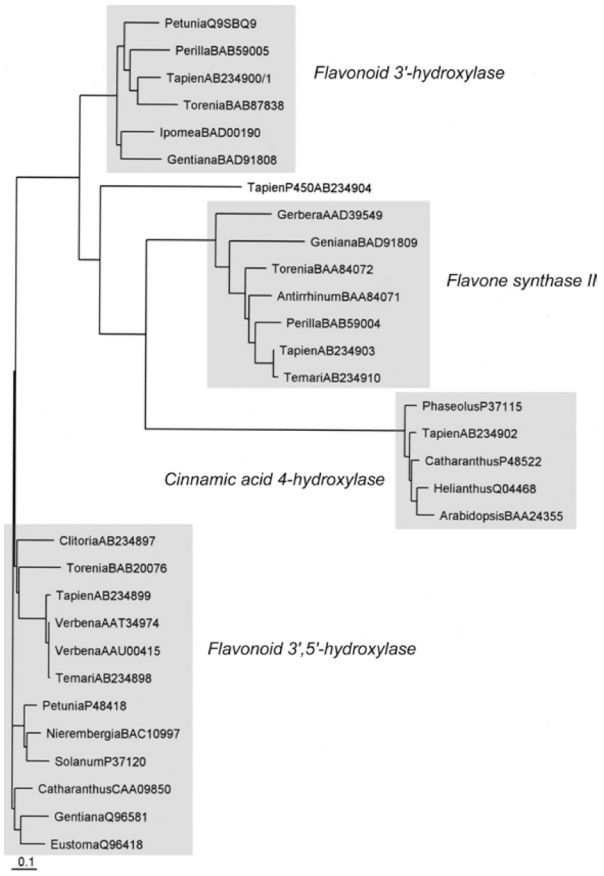

Figure 2. Phylogenetic relationship of F3′H, F3′5′H, C4H and FNSII. All of them belong to the cytochrome P450 superfamily. The genus or common names and accession number in the databases are shown in the figure. The amino acid sequences were aligned using the CLUSTALW (Thompson et al. 1994) program, and the tree was constructed using the TREEVIW (Page 1996) program.

Figure 2 shows a phylogenetic tree consisting of F3′H, F3′5′H, FNSII and C4H. The tree indicates that *F3′H*, *F3′5′H*, *FNSII* and *C4H* genes were diverted before speciation of the species shown in Figure 2, as reported previously (Ueyama et al. 2002). The deduced amino acid sequences obtained in this study had the typical features of cytochrome P450 enzymes, including the conserved and essential heme-binding motif (Schuler and Werck-Reichhart 2003). The amino acid sequences encoded by the two verbena *F3′5′H* genes cloned in this study and the two previously reported verbena *F3′5′H* genes (Hung 2003) were very similar (Figure 2). Since Temari and Tapien F3′5′Hs are very similar in their sequence, Temari F3′5′H was used for further study.

F3H and ANS belong to the 2-oxoglutarate-dependent dioxygenase family, which requires 2-oxoglutarate and ferrous iron in their activities (Forkmann and Heller 1999). The crystal structure of *Arabidopsis* ANS indicates the involvement of His-232, Asp-234 and His-288 residues and 2-oxoglutarate in the binding of iron in the active site (Wilmouth et al. 2002). The corresponding residues in *verbena* F3H and ANS are also conserved.

We also obtained homologues of CHI (Forkmann and Heller, 1999), benzoyl-CoA: benzoic acid benzoyl transferase, which is involved in the floral scent (D'Auria et al. 2002) and *GLOBOSA*, which is a homeotic gene whose mutants show sepaloid petals and carpelloid stamens in snapdragon (Trobner et al. 1992, Table 1).

Expression of F3′5′H genes in yeast

We expressed Temari and butterfly pea *F3′5′H* homologues in yeast to confirm their function. The HPLC profiles of the *F3′5′H* assay are shown in Figure 3. Two new peaks were generated from naringenin (4′,5,7-trihydroxyflavanone). They had spectral features of flavanones (data not shown). One of them had the same retention time as eriodictyol (3′,4′,5,7-tetrahydroxyflavanone), and the other was presumably 3′,4′,5,5′,7-pentahydroxyflavanone. Although we do not have authentic 3′, 4′, 5, 5′, 7-pentahydroxyflavanone, the retention time of the peak matched that of the peak generated from naringenin by the microsomal fraction yeast expressing petunia *F3′5′H* (data not shown). We concluded that Temari and butterfly pea *F3′5′H* homologues encoded functional F3′5′H.

Modification of flower color by overexpressing F3′5′H *genes in verbena*

In order to reveal if flower color modification is feasible

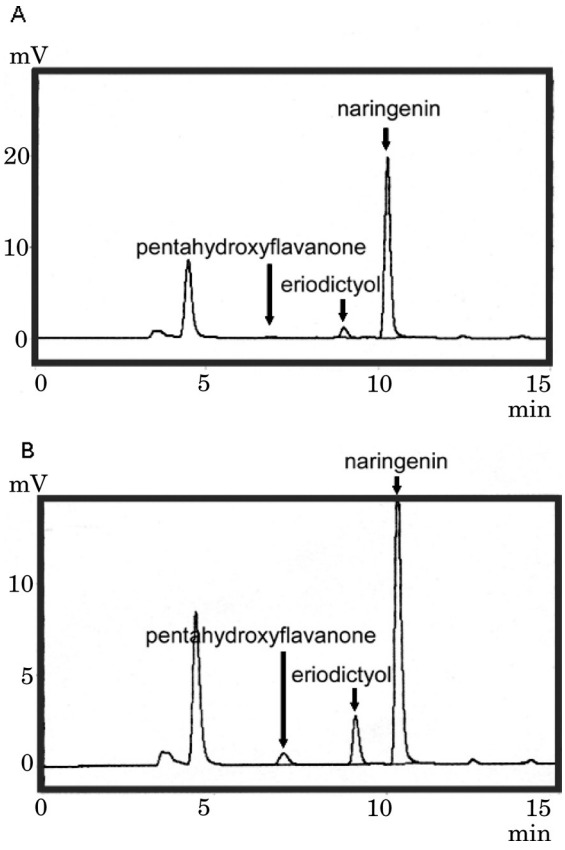

Figure 3. HPLC profiles of flavonoid 3′,5′-hydroxylase activity assay of the Temari (A) and butterfly pea (B) F3′5′H homologues. The homologues were expressed in yeast, and the crude yeast extracts were subjected to the assay using naringenin as the substrate. The arrows indicate naringenin, eriodictyol and pentahydroxyflavanone. They are shown to encode F3′5′H.

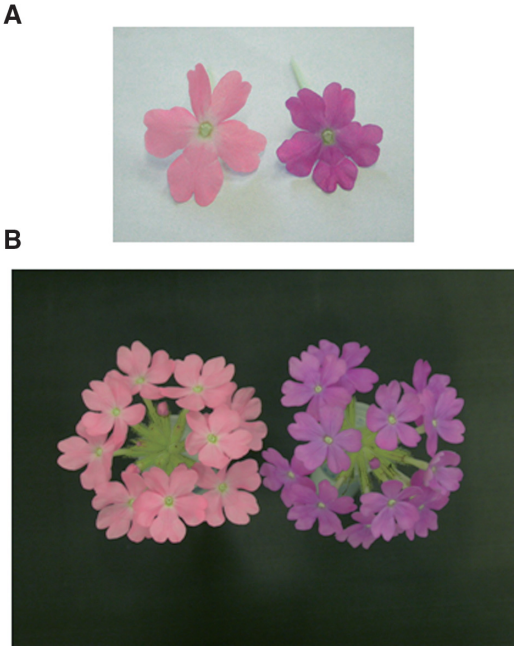

Figure 4. A. Flowers of Temari Sakura (left) and the transgenic Temari Sakura (Line 748-5) expressing the butterfly pea *F3′5′H* gene (right). A similar flower color change was observed for Line 748-6. B. Influorescence of Temari Sakura (left) and the transgenic Temari Sakura (Line 748-5, right).

in verbena and if there are differences in delphinidin production by the *F3′5′H* genes derived from verbena and butterfly pea, we transformed Temari Sakura with pSPB1431 and pSPB748. Five independent transgenic verbena plants of each type were obtained, and the transcripts of the two *F3′5′H* genes were detected through RT-PCR analysis of the leaves (data not shown). Genomic Southern blot analysis with the use of the neomycin phosphotransferase gene as the probe confirmed that the transgenes were integrated in the genome (Data not shown). The number of transgenic verbena is still limited, as the transformation frequency is not high.

A few transgenic verbena harboring pSPB748 had an elevated delphinidin content (up to 47.9%, Table 2) and altered flower color from pink to violet (Figure 4). Those with pSPB1431 showed a slight increase in delphinidin (Table 2) and slight flower color change (data not shown). This, to our knowledge, is the first report of flower color modification of verbena by genetic engineering. Successful flower color modification by genetic engineering has been reported for roses, carnations, and many plants, as reviewed (Tanaka et al. 2005). Interestingly, butterfly pea *F3′5′H* gene yielded more delphinidin and stronger phenotypic changes than the verbena *F3′5′H* gene in verbena. This may be due to the transcriptional expression level of the two *F3′5′H* genes or the difference in the kinetic characteristics between the two F3′5′Hs. Further analysis of transgenic verbena will be necessary to determine the reasons.

Some verbena varieties, such as the Temari Violet, contain almost exclusively delphinidin. Although we used a strong promoter in this study (Mitsuhara et al. 1996), the delphinidin amount was lower than that in the Temari Violet. Petal-specific promoters, such as the promoters of the flavonoid biosynthetic gene, may be suitable to achieve higher expression.

This study shows that the expression of the transgenes and the resultant phenotypes varies depending on the gene source and that the host gene is not always suitable. Choice of the gene source is important to achieve the desirable phenotype in transgenic plants.

Future perspectives

We have been developing transgenic ornamental plants that change their flower colors in response to dioxins in soils, in order to detect them on site. Verbena Temari and Tapien are suitable ornamental plants for this purpose because they spread over the soil and vigorously generate new roots from the stems. Inui et al. (2005) have developed a dioxin inducible gene expression system in transgenic plants with the use of an aryl hydrocarbon receptor. The combination of the inducible gene

expression system and the flower color modification achieved in this study may lead to transgenic verbena plants that can change their flower color in response to dioxins. Such phytomonitoring should be useful to detect the pollution on site and also contribute to acceptance of the genetically modified plants by the public.

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