Molecular Breeding **9:** 33–41, 2002.

Modification of flower color and fragrance by antisense suppression of the flavanone 3-hydroxylase gene

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Received 22 April 2001; accepted in revised form 15 October 2001

Key words: Anthocyanin, Carnation, Flavanone 3-hydroxylase, Flower color, Fragrance, Transgenic plants

Abstract

Anthocyanins are the major pigments contributing to carnation flower coloration. Most carnation varieties are sterile and hence molecular breeding is an attractive approach to creating novel colors in this commercially important crop. Characterization of anthocyanins in the flowers of the modern carnation cv. Eilat revealed that only the orange pelargonidin accumulates, due to a lack of both flavonoid 3′,5′-hydroxylase and flavonoid 3′-hydroxylase activities. To modify flower color in cv. Eilat, we used antisense suppression to block the expression of a gene encoding flavanone 3-hydroxylase, a key step in the anthocyanin pathway. The transgenic plants exhibited flower color modifications ranging from attenuation to complete loss of their original orange/reddish color. In the latter, only traces of pelargonidin were detected. Dramatic suppression of flavanone 3-hydroxylase level/activity in these transgenes was confirmed by northern blot, RT-PCR and enzymatic assays. The new phenotype has been stable for over 4 years of vegetative propagation. Moreover, transgenic plants with severe color modification were more fragrant than control plants. GC-MS headspace analyses revealed that transgenic anti-*f3h* flowers emit higher levels of methyl benzoate. The possible interrelation between pathways leading to anthocyanin and fragrance production is discussed.

Introduction

Flower color is one of the most important traits attracting consumer attention. As such, it is a critical factor for the commercial success of ornamental plants in the marketplace. Anthocyanins, carotenoids and betalain are the main flower pigments, of which the first have been the most studied (Forkmann 1993). The intense interest in anthocyanins, which derive from the general phenylpropanoid pathway, results from their wide distribution in many plants and their role in color determination (Mol et al. 1998; Shirley 1996). Anthocyanins are water-soluble compounds, accumulating in the vacuoles. Three major anthocyanin types contribute to flower color: pelargonidin-derived pigments are responsible for orange, pink or red colors, cyanidin-derived pigments for red or magenta, and delphinidin-derived pigments for purple or blue. Detailed biochemical and genetic analyses of anthocyanin production/accumulation (Holton and Cornish 1995) have brought about the development of two main strategies for altering flower color: introducing a foreign gene(s) to allow new branching in the anthocyanin biosynthetic pathway, and up/down-regulation of this pathway's native genes (Elomaa and Holton 1994). Several genes from the anthocyanin biosynthetic pathway have been used to manipulate flower color in numerous plants (Elomaa and Holton

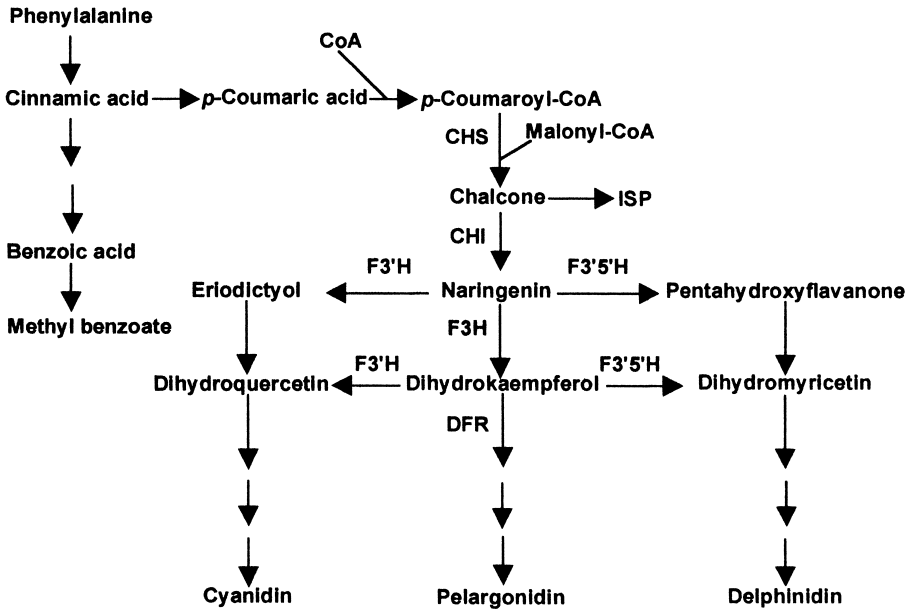

Figure 1. Schematic representation of anthocyanin and benzoic acid-derivative biosynthesis, originating from the general phenylpropanoid pathway. Abbreviations are indicated as follows: chalcone synthase (CHS); chalcone flavanone isomerase (CHI); flavanone 3-hydroxylase (F3H); dihydroflavonol 4-reductase (DFR); flavonoid 3′,5′-hydroxylase (F3′5′H); flavonoid 3′-hydroxylase (F3′H); ISP, isosalipurposide.

1994; Forkmann 1993; Zuker et al. 1998). Chalcone synthase (*chs)*, chalcone flavanone isomerase (*chi)* and flavanone 3-hydroxylase (*f3h)* are the first three genes encoding the early, unbranched segment of the flavonoid biosynthetic pathway (Figure 1). Although F3H is a key enzyme in this pathway (Pelletier and Shirley 1996) and its suppression, as opposed to that of CHS, should not block the production of the essential phytoalexins, isoflavonoids (Shirley 1996), it has never been harnessed for the genetic manipulation of flower color.

As one of the major contributors to the ornamental market (Jensen and Malter 1995), carnation (*Dianthus caryophyllus* L.) is an important target for the breeding of new varieties with novel characteristics. Carnation is a vegetatively propagated crop that is detrimentally affected by inbreeding (Galbally and Galbally 1997). Hence, controlled breeding is rather complicated and limited due to the fact that selection of a desired trait in the siblings is performed on the genetic background of their two parents, and due to the very high genetic variability among offspring. Furthermore, crosses within and between related species is limited by a rather small available gene pool for new traits.

In the present study, the flavonoid pathway in carnation was blocked by antisense suppression of the *f3h* gene. The transgenic plants exhibited various degrees of flower color modifications, ranging from partial to complete loss of their original orange/reddish color. In the latter, only traces of anthocyanins were detected. Interestingly, flowers with suppressed F3H activity were more fragrant than the controls.

Materials and methods

Plant material

Carnation (*Dianthus caryophyllus* L.) cv. Eilat, Coket, Desio and transgenic plants were grown under standard greenhouse conditions. Plants were propagated vegetatively by stem cuttings.

Gene construct and transformation of carnation plants

A partial *f3h* cDNA clone (1 kb) from carnation cv. Eilat was isolated by PCR of reverse-transcribed poly $(A)^+$ RNA, using specific primers (5′ CCC AAA ACG CTC ACT TCA CT 3′ and 5′ CCA AGC CCA TCT AAG CAA GT 3′) according to the sequence in GenBank (X70378). This fragment was subcloned in antisense orientation between the CaMV 35S promoter and *nos* terminator in pJD330 (Lavi 2001) to create pJD-anti-*f3h*. An *Xba*I fragment from pJD-anti-

f3h was then cloned into the binary vector pCGN1559 (Comai et al. 1990) to create pAM-anti-*f3h*. Carnation cDNA clones of *chs* and *dfr* were isolated, as described for *f3h*, by PCR using specific primers (5′ GGG CCG ATG GTC CTG CTA CTA T 3′ and 5′ ACG CGC TCG ACA TGT TCC CAA A 3′ for *chs*; 5′ TGT GAA TGT CGA AGC GAC TC 3′ and 5′ TTG AAT TTG GTG GGG ACA TT 3′ for *dfr*) according to their sequences in GenBank: *chs* (Z67982), *dfr* (Z67983). The pAM-anti-*f3h* gene construct was transferred to carnation cv. Eilat via co-cultivation of stem explants (Zuker et al. 1999) with *Agrobacterium tumefaciens* AGLO (Lazo et al. 1991) containing pAM-anti-*f3h.* Rooted putative transformants were removed from the *in vitro* rooting medium and hardened as previously described (Zuker et al. 1999). Hardened plants were grown in a greenhouse under standard growth conditions.

DNA and RNA analyses

For northern analysis, total RNA was extracted (Zuker et al. 2001) from petals of half-open flowers (Lavi 2001) and samples (10 μg) were electrophoresed on a 1.6% agarose gel using 1.2% formaldehyde (Maniatis et al. 1982). For RT-PCR analysis (de Lang et al. 1993), RNA samples treated with DNase were reverse-transcribed with M-MLV reverse transcriptase, using strand-specific *f3h* and *chs* (sense and antisense) primers (see above). The resultant cDNAs were PCR-amplified using a mixture of both sense and antisense primers. For Southern analyses, the cetyltrimethylammonium bromide (CTAB) DNA extraction procedure (Tzuri et al. 1991) was used. DNA (10 μg) from each line was digested with *Eco*RI or *Kpn*I. Digested DNA and RT-PCR products were electophoresed through a 1% agarose gel using Tris-borate buffer (containing 1.3 M Tris, 0.7 M boric acid and 24.5 mM EDTA, pH 8.4). All gels were stained with ethidium bromide and photographed under UV light. DNA and RNA were transferred from gels to a nylon membrane (Hybond N+, Amersham, UK) by capillary blotting as previously described (Maniatis et al. 1982). Blots were hybridized with PCR fragments of *f3h*, *dfr* or *chs*. Probe-labeling, pre-hybridization and hybridization of Southern and northern blots were performed as described (Tzuri et al. 1991; Vishnevesky et al. 1996). The blots were visualized by exposure to Agfa Curix PR2 film at −70 °C for 5 to 20 h with an intensifying screen.

Enzyme/flavonoid analyses

F3H and flavanone 3′-hydroxylase (F3′H) activity were assayed using petal extracts according to Britsch and Grisebach (Britsch and Grisebach 1986; Stotz et al. 1985), respectively. [^{14}C]naringenin (NAR) was used as a substrate for the F3H reaction; [^{14}C]dihydrokaempferol (DHK) was used as a substrate for the F3′H reaction. Products of the F3H and F3′H enzymatic reactions, DHK and dihydroquercetin (DHQ), respectively, were analyzed by thin-layer chromatography (TLC) followed by autoradiography. Cellulose plates (Merck) and chloroform/acetic acid/water (10:9:1) as the solvent system were used for TLC (Harborne 1967; Stotz et al. 1985). Feeding experiments (Deroles et al. 1998) were performed with detached petals, incubated for several hours in 1 mg ml^{-1} DHQ in 5% methanol. HPLC analyses of flavonoids were performed as described in Martens and Forkmann (1998).

Fragrance analyses

For fragrance analysis, flowers detached at anthesis were sealed in 35 -ml glass tubes for 30 min and volatile compounds were trapped for 10 min using a polydimethylsiloxane-coated solid-phase microextraction (SPME) fiber assembly (Supelco Inc., Bellefonte, PA). As a blank, trapping was performed in sealed tubes without flowers. Compounds were analyzed by SPME headspace gas chromatography-mass spectroscopy (GC-MS) (Helsper et al. 1998) (Varian Star 3400 CX GC equipped with a 30 m × 0.25 mm ID, DB-5MS column and interfaced with a Varian Saturn 3 MS). The fiber was manually placed into a 1077 splitless injector for 3 min (injector temperature was 210 °C). Helium served as the carrier gas and GC temperature was programmed as follows: 3 min at 40 °C, increased to 220 °C at 15 °C min^{-1} and then 5 min at 220 °C. Ionization energy was 70 eV. Each compound was tentatively identified (> 95% match) based on the Wiley library (Wiley Registry of Mass Spectral Data, 6th edition, F. W. McLafferty, 1994, J. Weiley & Sons, Inc). To confirm the identification of methyl benzoate and β-caryophyllene, authentic compounds (Sigma Chemical Co., St. Louis, MO) were used as standards to match GC retention times and MS spectra.

A paired-comparison fragrance test (O'Mahony 1986) was performed with 45 panelists. An intensity-category-scaling test (Meilgaard et al. 1991) (1–5

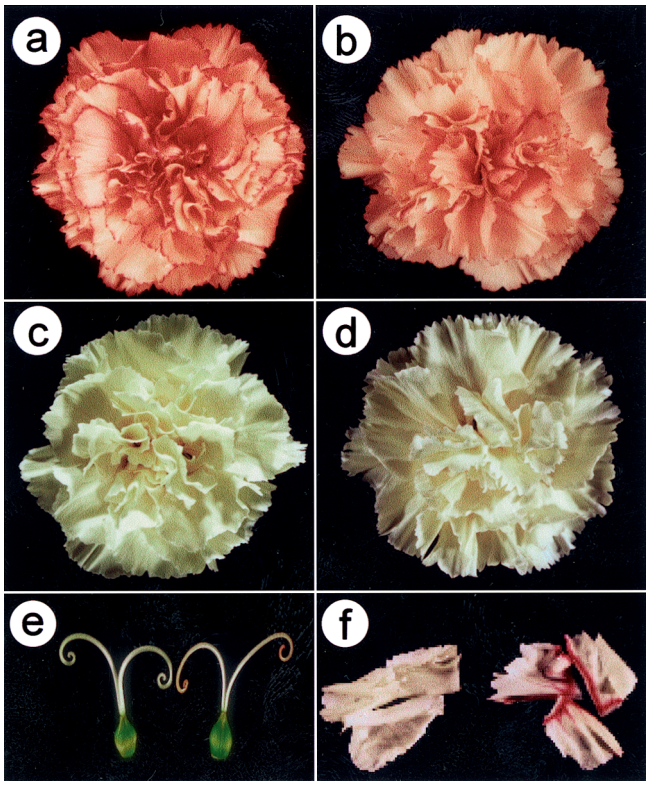

Figure 2. Flower-color modification of anti-*f3h* transgenic carnation. **a**. Flower of control, non-transformed cv. Eilat. **b-d**. Flowers of cv. Eilat transformed with antisense *f3h* (transgenic lines F3H-33, F3H-11, F3H-14, respectively). **e**. Anthers of control (right) and transgenic F3H-14 (left) flowers. **f**. Complementation of F3H suppression in the petals of line F3H-11, by feeding with (right) or without (left) dihydroquercetin.

scale, 1 for no fragrance, 5 for very intense fragrance) was performed with 20 panelists.

Results

The major pigments determining carnation flower color are cyanidin, pelargonidin and the yellow isosalipurposide (ISP) (Figure 1) (Forkmann 1991; Forkmann and Dangelmayr 1980). Carnation flower petals lack flavonoid 3′,5′-hydroxylase (F3′5′H) activity (Forkmann 1993). The flowers of carnation cv. Eilat have dark orange petals with reddish edges (Figure 2a). HPLC and TLC analyses of anthocyanin revealed that only pelargonidin, but not cyanidin, accumulates in this cultivar (data not shown). To study whether the lack of cyanidin accumulation in cv. Eilat is due to a deficiency in F3′H, we assessed this enzyme's activity. [C^{14}]DHK was used as a substrate and the reaction products were analyzed by TLC. No DHQ, the reaction product, was produced when a microsomal fraction of cv. Eilat was used (Figure 3a). In contrast, DHQ was detected when the reaction was performed with petals of the cyanidin-accumulating cv. Coket. These results suggest that in cv. Eilat, neither F3′5′H nor F3′H are active.

To modify carnation flower color, F3H activity was inhibited by antisense suppression of the corresponding gene: *f3h* was cloned in an antisense orientation under the regulation of the CaMV 35S promoter into a binary vector and used to transform carnation cv. Eilat plants. Following transformation, 14 individual transgenic plants were regenerated and grown to flowering in the greenhouse. All plants developed and flowered normally, and six of them exhibited flowers with color modifications ranging from attenuation (F3H-10 and F3H-33, Figure 2b) to complete loss (F3H-11 and F3H-14, Figure 2c and 2d) of their orange/reddish color. Flowers of lines F3H-11 and F3H-14 were yellow/cream-colored and accumulated only very low levels (2 to 4% of that in control cv. Eilat) of pelargonidin. In contrast, lines with slightly attenuated color, such as F3H-33, accumulated relatively high levels of pelargonidin (70% of

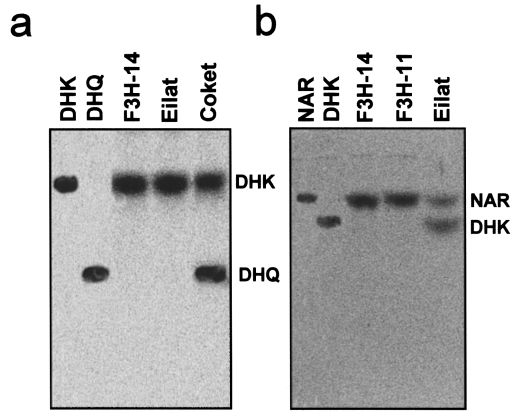

Figure 3. Analysis of enzyme activities in carnation flowers. **a**. F3′H enzyme activity in petal extracts of control cvs. Eilat and Coket and of anti-*f3h* transgenic F3H-14. Autoradiograph of TLC plate shows the conversion of [^{14}C]dihydrokaempferol (DHK) to dihydroquercetin (DHQ). **b**. F3H enzyme activity in petal extracts of control cv. Eilat and anti-*f3h* transgenic F3H-11 and F3H-14 plants. Autoradiograph of TLC plate shows conversion of [^{14}C]naringenin (NAR) to DHK. NAR, DHQ and DHK, used as standards, are presented.

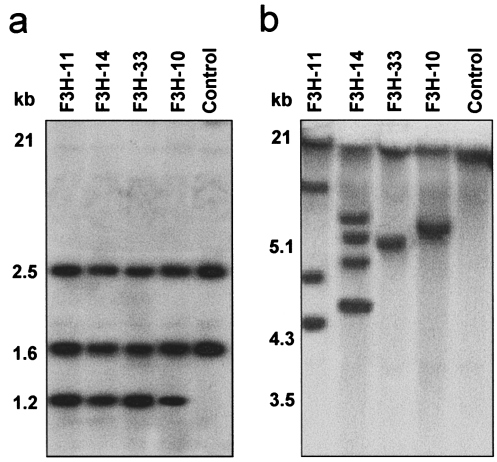

Figure 4. Southern blot analysis of DNA from anti-*f3h*-transgenic lines F3H-11, F3H-14, F3H-33 and F3H-10 and control, nontransformed cv. Eilat plants. Total DNA (10 μg) was digested with-*Eco*RI (a) and *Kpn*I (b) and hybridized with an *f3h* probe. *Eco*RI digestion confirms the presence of the transgene (1.2-kb fragment); digestion with *Kpn*I confirms the integration of the transgene into the genome of the transgenic plants.

the control). No significant change in the levels of naringenin and ISP was detected in anti-*f3h* transgenic plants. In lines with strong color modification, pigmentation was suppressed not only in the petals but also in the anthers (Figure 2e). Transformation of cv. Eilat with the identical vector carrying the *gus* reporter gene instead of *f3h* led only to transgenes with true-to-type flower color (Zuker et al. 1999). Since, like many commercial carnation varieties (Zuker et al. 1999), cv. Eilat is sterile, the inheritance of these color modifications could not be analyzed. Southern blot analysis of *Eco*RI-digested DNA confirmed the presence of the anti-*f3h* DNA fragment in selected transgenic lines (1.2 kb as predicted) but not in the non-transformed plants. In addition, two predicted (Dedio et al. 1995) fragments of the endogenous *f3h* gene were revealed (Figure 4a). Integration of the anti-*f3h* DNA fragment was confirmed in *Kpn*I-digested DNA, where one to four copies of the construct were revealed in the genome of transgenic lines (Figure 4b).

To evaluate the expression level of *f3h* in the transgenic plants, northern blot analyses were performed (Figure 5a). Dramatic suppression of *f3h* transcript levels was revealed in transgenes F3H-11 and F3H-14, in contrast to controls and transgene F3H-33. Reprobing of the same blots with *dfr* indicated no correlation between expression patterns of *f3h* and the downstream gene *dfr* (Figure 5a, lane F3H-11 vs. F3H-14). The reduced level of *dfr* transcript in transgene F3H-14 was not observed in other experiments and was probably due to physiological differences between petal samples, as carnation flower is composed of many petals at various developmental stages. Since *f3h* and anti-*f3h* transcripts were of similar sizes, to assess the severity of the suppression of endogenous *f3h* expression and to prove the expression of anti-*f3h* gene construct, highly sensitive RT-PCR analysis of the endogenous (sense) and transgenic (antisense) *f3h* transcripts was performed using strand-specific primers. Petals of F3H-11 transgenes, which exhibited a dramatically modified phenotype Figure 2c, had not accumulated detectable levels of sense *f3h* transcript (Figure 5b). In contrast, petals of transgenic line F3H-33, which showed only minor color alterations (Figure 2b), showed the accumulation of sense transcript. In control flowers, as expected, only sense transcript could be detected. Analysis of *chs* transcript, used as a control, yielded the expected results, i.e. sense transcripts were detected in all analyzed plants. Hence, the expression of *f3h* in line F3H-11 was almost completely suppressed, since *f3h* transcript could not even be detected by the very sensitive RT-PCR analysis. Analysis of F3H enzyme activity (converting flavanone to dihydroflavonol) in petals of F3H-11 and F3H-14 revealed no detectable activity, in contrast to strong F3H activity in control plants (Figure 3b). This block in the anthocyanin biosynthetic pathway could

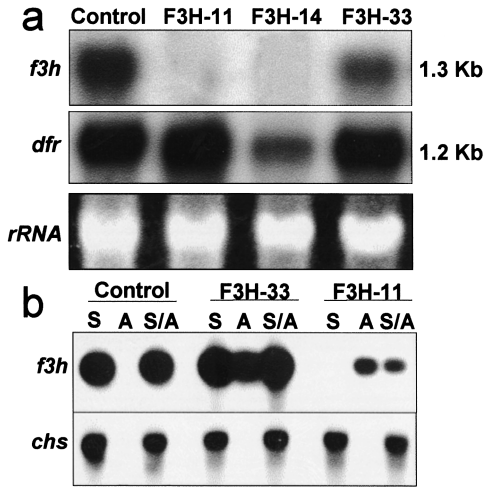

Figure 5. Analyses of *f3h* expression. **a**. Northern blot analysis of *f3h* transcript levels in petals of anti-*f3h* transgenic lines F3H-11, F3H-14 and F3H-33, and in control cv. Eilat petals. For comparison, analysis of *dfr* transcript is presented. **b**. Analysis of *f3h* sense and antisense transcript accumulation in petals of control, cv. Eilat and anti-*f3h* transgenic F3H-11 and F3H-33. Strand-specific primers were used to reverse-transcribe sense (S), antisense (A) and both (S/A) transcripts, followed by PCR amplification and Southern analysis. Analysis of *chs* was used as a control.

be overcome by feeding detached petals with the product of the F3H enzyme (Figure 2f). Thus suppression of F3H activity did not affect the downstream components of the anthocyanin biosynthetic pathway.

During the course of a 4-year field test, we observed that the flowers of transgenic plants F3H-11 and F3H-14 were more fragrant than non-transformed or GUS-transgenic controls. The enhanced fragrance was observed in flowers with strongly reduced anthocyanin accumulation, but not in F3H-33 or other transgenic lines exhibiting less dramatic reductions in anthocyanin level. In an attempt to confirm this observation, an evaluation with panelists, as is commonly practiced in the food and beverage industries, was performed. A paired-comparison fragrance test confirmed that the anti-*f3h* transgenes (F3H-11 and F3H-14) were more fragrant than controls. The overall fragrance intensity of transgenic F3H-11 flowers was significantly higher ($p = 0.001$) than that of control flowers. Furthermore, an intensity-category-scaling test revealed a highly significant difference ($p < 0.001$, paired t-test) in the intensity scores of transgenic F3H-11 (mean ± SE = 3.2 ± 0.3) vs. control (1.3 ± 0.2) flowers. Essentially identical results were found for transgene F3H-14.

In an attempt to characterize the scent of anti-*f3h* transgenic and control flowers, GC-MS headspace analyses were performed. Cv. Eilat and transgenic F3H-11 and F3H-14 plants were grown side-by-side in the greenhouse and flowers at anthesis were collected simultaneously and immediately analyzed. In all analyses performed during the 4-month flowering period, the level of methyl benzoate was higher in flowers of F3H-11 and F3H-14 relative to controls. While in control flowers methyl benzoate represented only 9% of total volatiles, in F3H-14 it represented 42% and in F3H-11 45% of total volatiles (means of four independent analyses performed on different dates, with four flowers per line). In contrast, the level of β-caryophyllene was not affected in transgenic flowers. The level of 2-hydroxy methyl benzoate, another derivative of benzoic acid, was also increased in F3H-11 and F3H-14 versus control cv. Eilat flowers (3.5% and 3% vs. 0.5% of total volatiles, respectively). Representative chromatograms of F3H-11 and control cv. Eilat are presented in Figure 6.

Discussion

Flower color, as well as fragrance, are essential for the attraction of pollinators and hence for the evolutionary success of plants. These two traits are equally important in terms of attracting consumers of ornamentals. Flower pigmentation has been intensively studied in the last several decades and today, the resultant deeper understanding of the underlying pathways has already been harnessed for crop improvement (Zuker et al. 1998). For example, anthocyanin biosynthetic genes, such as *chs*, *dfr*, *f3′h*, *f3′5′h* and flavonol synthase (*fls*), have been used to modify flower color in various transgenic plants (Brugliera et al. 1999; Elomaa and Holton 1994; Tanaka et al. 1998). On the other hand, the biochemistry of fragrance production and the mechanism regulating its emission remain sketchy (Dudareva and Pichersky 2000). At present, only a few genes involved in fragrance production in flowers have been cloned and characterized: their effects *in vivo* are currently under study (Dudareva et al. 1998, 2000; Dudareva and Pichersky 2000).

Carnation is a leading cut flower and as such is an important target for the molecular manipulation of ornamental traits. Here we show the modification of carnation flower color and fragrance by blockage of *f3h* gene expression. Analysis of anti-*f3h* transgenic plants with strongly modified flower color (F3H-11 and F3H-14) revealed that *f3h* transcript, as well as

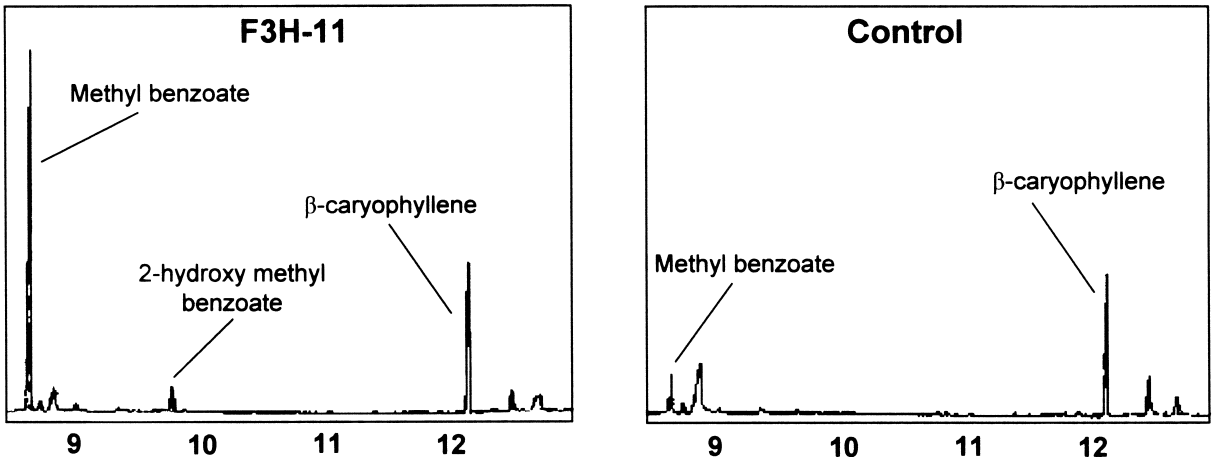

Figure 6. GC-MS headspace analysis of volatile compounds emitted from detached flowers of anti-*f3h* transgenic F3H-11 and control cv. Eilat plants. Representative GC chromatograms are presented.

enzyme activity, were almost completely abolished. The orange/reddish flowers of carnation cv. Eilat, due to the lack of F3′5′H and F3′H, accumulate pelargonidin, only traces of which were detected in transgenic plants F3H-11 and F3H-14. The high efficiency of *f3h* suppression in terms of color modification could be due to its being a single-copy gene (Dedio et al. 1995). The level of ISP, responsible for the yellow color of carnation flowers (Forkmann 1991; Forkmann and Dangelmayr 1980), was not increased in the anti-*f3h* transgenic plants (data not shown). Hence, the yellow/cream-color of F3H-11 and F3H-14 transgenic flowers may be due to unmasking of the yellow ISP as a result of the decrease in the pelargonidin levels. Suppression of *f3h*, as opposed to upstream genes such as *chs*, can be advantageous, as it should not suppress accumulation of the essential isoflavonoids involved in protection against pathogens (Shirley 1996). Furthermore, the levels of isoflavonoids in the anti-*f3h* transgenic plants may even increase due to the suppression of anthocyanin production. This aspect is currently being studied.

Flower fragrance is a highly important character for the ornamental industry in general and carnation in particular; nevertheless, it was never a target for breeders. Carnation fragrance is dominated by eugenol, β-caryophyllene and benzoic acid derivatives (Clery et al. 1999). While eugenol gives the spicy/clove character to the traditional carnation cultivars, the fragrance of modern cultivars, including cv. Eilat, is determined by β-caryophyllene and benzoic acid derivatives such as methyl benzoate (Clery et al. 1999; Lavi 2001). The level of these compounds increases during cv. Eilat flower development and coincides with an increase in flower fragrance (Lavi 2001). Transgenic anti-*f3h* flowers, with dramatically reduced anthocyanin levels, were significantly more fragrant than controls. Analyses of fragrance compounds in these transgenic plants revealed five- to sevenfold higher levels of the phenylpropanoids, methyl benzoate and 2-hydroxy methyl benzoate, as compared to controls. The level of other major fragrance compounds, such as the sesquiterpene β-caryophyllene (Croteau and Karp 1991), was not affected in the transgenes. Methyl benzoate is a known fragrance compound produced by many flowers (Knudsen et al. 1993), including carnation (Clery et al. 1999), and therefore it is not unreasonable to propose that this compound is responsible, at least in part, for the enhanced fragrance in the transgenic plants.

Recently, the developmental regulation of methyl benzoate biosynthesis and emission was detailed in snapdragon flowers (Dudareva and Pichersky 2000). This analysis revealed that the production of methyl benzoate is regulated by the level of benzoic acid. Thus, the observed enhanced fragrance in the transgenic carnation plants may be caused by diversion of metabolic flow from anthocyanin biosynthesis to benzoic acid production, both originating from the phenylpropanoid pathway (Figure 1). To further support this suggestion, quantitative GC-MS and detailed biochemical and molecular analyses are currently underway.

A major effort is currently being made to detail pathways leading to the production of fragrance compounds and to employ the knowledge to generate transgenic plants with novel scent (Dudareva and

Pichersky 2000; Zuker et al. 1998). The results presented in this paper reveal an alternative approach to the olfactory enhancement of flower fragrance. The finding that fragrance is affected by modulation of anthocyanin biosynthesis reveals an intriguing link between the two secondary metabolic pathways. From an evolutionary point of view, this interplay is probably less surprising, as both pathways are used by plants to attract pollinators and seed dispersers, thereby ensuring species survival.

Acknowledgements

This research was supported by BARD, by the Israeli Ministry of Agriculture, by the Israeli Ministry of Science (grant no. 1410) and by the Association of Israeli Flower Growers.

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