Flavonoid Biosynthesis in Pink-flowered Cultivars Derived from ‘William Sim’ Carnation
(Dianthus caryophyllus)

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Summary

Analyses for anthocyanin concentration and flavonoid composition of flowers of two white,
four light-pink, four deep-pink, and three red cultivars derived from ‘William Sim’ carnation
revealed that anthocyanin content increased from the light-pink flowered cultivars, to the deep-
pink flowered cultivars, and to the red-flowered cultivars. Simultaneously, examination of gene
expression of enzymes involved in anthocyanin biosynthesis showed that transcriptional levels
of dihydroflavonol 4-reductase gene (DFR) and anthocyanidin synthase gene (ANS) in the deep-
pink flowered cultivars were correspondingly lower in the red-flowered cultivars, whereas those
of ANS was much lower in the light-pink flowered cultivars except in ‘Lolyta’. In light-pink-
flowered cultivars, transcripts of flavanone 3-hydroxylase gene (F3H) in ‘Lolyta’ and ANS in
‘Dusty’ were undetectable on the Northern blots, but both transcripts were detected on reverse
transcription-polymerase chain reaction (RT-PCR). Low anthocyanin accumulation in pink-
flowered cultivars seems to be regulated at different steps in anthocyanin biosynthesis.

Key Words: anthocyanidin synthase, Dianthus caryophyllus, dihydroflavonol 4-reductase, flavanone 3-hydroxylase, pink flower.

Introduction

A primary goal in the breeding of ornamental plants is
to obtain a wide range of flower colors. Recently, there
has been a great demand for the neutral tint flowers,
such as pink, but there are only a few investigations on
the genetic regulation of anthocyanin biosynthesis in
such flowers.

The anthocyanin content in pink flowers was lower
than that of red flowers in Dendranthema moriforum
(Kawase and Tsukamoto, 1974), Rosa sp. (Yokoi et al.,
1979), and Cosmos bipinnatus (Samata and Inazu,
1983). The relationship between pink coloration and
anthocyanin biosynthesis has been investigated exten-
sively in Dianthus caryophyllus. Anthocyanin biosyn-
thesis involves several enzymes as shown in Fig. 1. Pink
flower color in D. caryophyllus seems to result from
the regulation at different steps of anthocyanin biosynthesis.
Reduced translation and transcription of F3H are
responsible for the pink color of ‘Nora’ and ‘Aladdin’pet-
tals, respectively (Dedio et al., 1995; Forkman et al.,
1995). Chalcone synthase (CHS) activity was extremely
low in very pale pink flowers (Geissman and Mehliquist,
1947; Forkman et al., 1995).

\[
\begin{align*}
\text{p-Coumaroyl-CoA} & \quad 3 \times \text{Malonyl-CoA} \\
\uparrow & \quad \text{CHS} \\
\downarrow & \quad \text{CHI} \\
\downarrow & \quad \text{FS} \\
\downarrow & \quad \text{F3H} \\
\downarrow & \quad \text{FLS} \\
\downarrow & \quad \text{Dihydroflavonol} \\
\downarrow & \quad \text{Flavone} \\
\downarrow & \quad \text{DFR} \\
\downarrow & \quad \text{Flavan-3,4-diyl} \\
\downarrow & \quad \text{ANS} \\
\downarrow & \quad \text{Anthocyanidin} \\
\downarrow & \quad \text{UFGT} \\
\end{align*}
\]

Fig. 1. Pathway for flavonoid synthesis. CHS: chalcone syn-
thease, CHI: chalcone flavanone isomerase, FS: flavone
synthase, F3H: flavanone 3-hydroxylase, FLS: flavonol
synthase, DFR: dihydroflavonol 4-reductase, ANS:
anthocyanidin synthase.

Received; April 28, 2000. Accepted; October 13, 2000
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The purpose of this study was to investigate the transcriptional regulation of anthocyanin biosynthesis in pink-flowered carnations. The ‘Sim’ strains of carnations, which originated from ‘William Sim’ include about 400 bud mutants of various petal colors. In this study, we, a) analyzed flavonoid composition and content by HPLC, b) identified several anthocyanin biosynthetic genes by Northern blot, and c) concluded RT-PCR in 13 cultivars ranging in color from white, light pink, dark pink, and red.

**Materials and Methods**

**Plant materials**

Thirteen experimental cultivars derived from ‘William Sim’ were (Fig. 2): ‘U Conn Sim’ and ‘White Sim’ (white-flowered cultivars); ‘Crowley’s Sim’, ‘Lolita’, ‘Dusty’, and ‘Keefer’s Cherry Sim’ (light pink-flowered cultivars); ‘Shocking Pink Sim’, ‘Framingo Sim’, ‘Dark Lena’, and ‘Lenah’ (deep pink-flowered cultivars); and ‘Red Lena’, ‘Scania’, and ‘William Sim’ (red-flowered cultivars). Flower buds were harvested when exposed part of petals reached 5 mm long at (2000).

**Isolation of RNA and construction of cDNA**

Total RNAs were prepared from the petals as described by Mato et al. (2000). Poly(A)^+ RNA was purified from the total RNA by oligo (dT)-latex affinity chromatography using Oligo TM-dT30 <Super> (Takara), according to the manufacture. A cDNA library was constructed from the poly(A)^+ RNA using the λ ZAPII-cDNA/Gigapack III Gold cloning kit (Stratagene), except for an additional reaction with AMV reverse transcriptase with MMLV-RT (Mato et al., 1998).

**RNA blot analysis**

Carnation cDNA clones of phenylalanine ammonia lyase (PAL), chalcone synthase, flavanone 3-hydroxylase, dihydroflavonol 4-reductase, anthocyanidin synthase (Mato et al., 1998) and actin were labeled with DIG-High Prime (Boehringer - Mannheim) and used as probes for Northern blot analysis. Electrophoresis of 1 μg of poly(A)^+ RNA was carried out in a denatured formaldehyde - agarose gel and blotted onto a nylon membrane filter (Nytran - plus). Membranes were prehybridized for 1 hr and hybridized overnight in 5 × SSC (Saline sodium citrate), 50% deionized formamide, 0.1% N-lauroyl sarcosine, 0.02% SDS, and 2% blocking reagent (Boehhringer - Mannheim) at 42 °C. The membranes were washed twice in 2 × SSC/0.1% SDS for 10 min at room temperature, twice in 0.1 × SSC/0.1% SDS for 10 min at room temperature, and twice in 0.1 × SSC/0.1% SDS for 30 min at 68 °C. The signals were made visible with DIG - DNA Detection Kit (Boehringer - Mannheim) according to the manufacturer’s instructions; the blots were exposed onto X-ray film.

**RT - PCR analysis**

Transcripts of flavanone 3-hydroxylase and anthocyanidin synthase were detected by RT-PCR using an RNA PCR Kit, AMV Ver 2.1 (Takara) with sets (Mato et al., 2000). Poly(A)^+ RNA samples were treated with RNase-free DNase at 25 °C for 15 min, then extracted with phenol and chloroform before RT-PCR.

**Results and Discussion**

Anthocyanin content was the highest in the red-flowered carnations, intermediate in the deep-pink-flowered cultivars, and lowest in the light-pink-flowered cultivars (Table 1). HPLC analysis of flavonoid showed that all cultivars except ‘Lolita’ contained predominant, peaks 1 and 2 (Fig. 3a). These peaks were identified as kaempferol glycosides found in white-flowered ‘U Conn Sim’ and ‘White Sim’ and red-flowered ‘Scania’ (Mato et al., 2000; Onozaki et al., 1999). Tracing of ‘Lolita’ exhibited peaks 3 and 4 (Fig. 3b), which are naringenin glycosides which exist in pure - white-flowered ‘Kaly’ (Mato et al., 2000; Onozaki et

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**Fig. 2.** Photographs of experimental ‘Sim’ carnations. Upper left: ‘White Sim’. Upper right: ‘Lolita’. Lower left: ‘Dusty’. Lower right: ‘Scania’.

which stage the anthocyanin content and expression of the DFR and ANS genes increased (Mato et al., 2000).

**Extraction and analysis of flavonoids**

A 1 g sample of fresh petal was blended with 10 ml of 1% HCl - methanol at room temperature. After filtration, the absorbance of the filtrate at 530 nm was compared with that of ‘Scania’ (100%) to yield the relative anthocyanin content. Flavonoids were extracted from 1 g of fresh petals in methanol. The filtrate was partitioned with petroleum ether to remove fat-soluble substances; the residual was analyzed by high performance liquid chromatography (HPLC) as described by Mato et al.
Table 1. Carnation cultivars derived from ‘William Sim’ and their relative anthocyanin content.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Relative absorbance at 530 nm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White - flowered cultivars</td>
<td></td>
</tr>
<tr>
<td>U Conn Sim</td>
<td>0</td>
</tr>
<tr>
<td>White Sim</td>
<td>0</td>
</tr>
<tr>
<td>Light - pink - flowered cultivars</td>
<td></td>
</tr>
<tr>
<td>Crowley’s Sim</td>
<td>7.1</td>
</tr>
<tr>
<td>Lolyta</td>
<td>8.3</td>
</tr>
<tr>
<td>Dusty</td>
<td>9.7</td>
</tr>
<tr>
<td>Keefer’s Cherry Sim</td>
<td>18.4</td>
</tr>
<tr>
<td>Deep - pink - flowered cultivars</td>
<td></td>
</tr>
<tr>
<td>Shocking Pink Sim</td>
<td>48.8</td>
</tr>
<tr>
<td>Flamingo Sim</td>
<td>56.1</td>
</tr>
<tr>
<td>Dark Lena</td>
<td>41.6</td>
</tr>
<tr>
<td>Lena</td>
<td>39.4</td>
</tr>
<tr>
<td>Red - flowered cultivars</td>
<td></td>
</tr>
<tr>
<td>Red Lena</td>
<td>77.8</td>
</tr>
<tr>
<td>Scania</td>
<td>100</td>
</tr>
<tr>
<td>William Sim</td>
<td>58.9</td>
</tr>
</tbody>
</table>

*Anthocyanin content defined the percentage of absorbance of ‘Scania’.


al., 1999). These results indicate that the anthocyanin biosynthetic pathway is blocked by flavanone 3-hydroxylase in ‘Lolyta’ and by dihydroflavonol 4-reductase in other pink cultivars.

In the Northern blot analysis (Fig. 4), all cultivars had high expression of PAL. Expression of CHS was high in all cultivars except in the white ones. DFR and ANS mRNAs were high in the red–flowered cultivars, low in the deep–pink–flowered cultivars, and undetectable in the white–flowered ones. The expression of ANS was much lower in the light–pink–flowered cultivars except in ‘Lolyta’. The accumulation of flavonol and decreased transcription of DFR and ANS in the deep–pink–flowered cultivars suggest that reduced activity of dihydroflavonol 4-reductase and anthocyanidin synthase disrupts the accumulation of anthocyanin. The lack of anthocyanins in light–pink–flowered cultivars except ‘Lolyta’ is attributable to the further transcriptional reduction of ANS.

In Northern blot analysis, F3H mRNA in ‘Lolyta’ and ANS mRNA in ‘Dusty’ were not detected (Fig. 4). Transcription of F3H was reduced in ‘Aladdin’ carnation which has flowers with thin red stripes on a pink background (Dedio et al., 1995; Forkmann et al., 1995), whereas the transcription of F3H was defective in ‘Kaly’ which has nearly pure white flowers (Mato et al., 2000). ‘Lolyta’ and ‘Dusty’ were not expected to possess defective F3H and ANS, respectively, so that their RT–PCR analyses were conducted with ‘Keefer’s Cherry Sim’ as a standard for comparison. The analyses identified F3H cDNA in ‘Lolyta’ and ANS cDNA in ‘Dusty’ (Fig. 5) which suggest that transcription of these genes is reduced markedly and not lacking in these cultivars. We assume that the decreased transcription of F3H leads to the reduced anthocyanin concentration and the accumulation of flavanone of ‘Lolyta’. These reductions in transcription differ from the reduced translation that gives rise to the light–pink–flowered ‘Nora’ (Dedio et al., 1995) and from the defective transcription of F3H in the nearly pure white–flowered ‘Kaly’ (Mato et al., 2000). Thus, there are decreased and defective transcrip-
tions and the reduced translation in the regulation of $F3H$. The reduced anthocyanin level in ‘Dusty’ seems to result from marked reduction in the transcription of $ANS$ which was previously postulated for pink flowered carnation cultivars (Geissman and Mehlquist, 1947; Forkmann et al., 1995).

We have no explanation as to why ‘Lolyta’ and ‘Dusty’ have high anthocyanin content in comparison with ‘Crowley’s Sim’, in spite of very low expressions of $F3H$ in ‘Lolyta’ and $ANS$ in ‘Dusty’. Likewise, the small difference in anthocyanin contents between deep-pink-flowered ‘Flamingo Sim’ and red-flowered ‘William Sim’ is inexplicable, despite their large difference in $DFR$ and $ANS$ expressions. The variance in absorbance between these cultivars might be influenced by the kinds of anthocyanin rather than by their concentrations. $\beta$-ring hydroxylated and glucosylated of anthocyanin seem to influence the quality of the pink and red colors in flowers (Spr bible and forkmann, 1982; Terahara and Yamaguchi, 1986; Gonnet and Hieu, 1992; Bloor, 1997). Therefore, the steps in flavonoid 3′-hydroxylation and glucosylation in the biosynthetic pathway need to be investigated in the future.

**Literature Cited**


シム系カーネーションピンク花品種におけるフラボノイド合成

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摘 要

シム系カーネーションの白色花2品種、薄桃色花4品種、濃桃色花4品種および赤色花3品種の花弁
が5 mm出たつぼみを用い、カーネーションの桃色花品種におけるフラボノイド生成経路を調査した。
吸光度およびHPLCによるフラボノイド分析の結果、薄桃色花品種「ロリータ」を除くすべての品種はフラボ
ノイドが重要なフラボノイドであった。アントシアニン
含量は赤色花品種でもっとも高く、濃桃色花品種、薄桃
花品種の順に減少し、白色花品種からアントシアニンは
検出されなかった。「ロリータ」ではフラパノンが主要色
素であった。フラボノイド合成酵素遺伝子であるフェ
ニルアラニンアンモニアリアーゼ（PAL）、カルコシン
ターゼ（CHS）、フラパノン3-ヒドロキシラーゼ（F3H）、
ジヒドロフラパノール4-レダクターゼ（DFR）、アントシ
アニンシンターゼ（ANS）遺伝子のノーザンハイブリダイ
セーションを行った結果、濃桃花品種ではDFRおよび
ANSの発現量が赤色花品種より低かった。一方、薄桃色
花のうち「ロリータ」ではF3Hの発現が、また「グリセ
リー」ではANS遺伝子の発現がノーザンレベルで検出さ
れない程度まで低下し、RT-PCRでのみ検出された。そ
の他の薄桃色花品種では、濃桃色花品種よりANSの発現
量が低かった。これらの結果より、桃色花品種は、今回
調べたアントシアニン生成のすべての段階の遺伝子が発
現するものの、一部の遺伝子の発現が抑制されているた
めにアントシアニン含量が低下することが示唆された。

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