Note

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Isolation and Characterization of a cDNA for Phenylalanine Ammonia-Lyase (PAL) from *Dianthus caryophyllus* (carnation)

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Abstract

A nearly full-length PAL cDNA clone was isolated from petals of carnation (*Dianthus caryophyllus*). Northern analysis revealed that the cDNA obtained here as a probe gave a strong signal corresponding to PAL transcripts prepared from petals, but weak signals in young and old leaves, stems and roots. The amount of PAL transcripts in petals increased with development from small buds to opening flowers, along with the accumulation of anthocyanin in petals. This is the first report of a PAL cDNA clone derived from petals.

Carnation (*Dianthus caryophyllus*) petals come in several colors including light pastel, yellow, white, pink and red. The pink and red pigments in the petals consist of anthocyanin, which is synthesized by the phenylpropanoid pathway followed by the flavonoid (anthocyanin) pathway (Geissmann *et al.*, 1947; Terahara *et al.*, 1986; Forkmann *et al.*, 1995).

Most molecular biological analyses on anthocyanin pigmentation in petals have focused on the genes encoding enzymes involved in the flavonoid pathway, e.g., chalcone synthase (CHS), flavanone 3-hydroxylase, dihidrofravonol 3-reductase, anthocyanindin synthase, and UDP-glucosyltransferases (reviewed by Van Tunen and Mol, 1991). Some of the transcription regulatory factors in the gene expression of the above enzymes have been identified (Avila *et al.*, 1993; de Vetten *et al.*, 1997; Uimari and Strommer 1997; Elomaa *et al.*, 1998; Quattrocchio *et al.*, 1998, 1999). Molecular engineering to obtain new coloration in petals has made use of sense and anti-sense technology with the genes involved in the flavonoid pathway (van der Krol *et al.*, 1988; Courtney-Gutterson 1994; Gutterson, 1995; Mol *et al.*, 1995). Little attention, however, has been paid to the expression of genes of enzymes involved in the phenylpropanoid pathway during the development of petal colors. Clearly, an increase in the activity of enzymes involved in the phenylpropanoid pathway is needed to supply *p*-coumaroyl-CoA, which is an important substrate of CHS, the first enzyme of the flavonoid pathway (Hahlbrock *et al.*, 1989). The gene expression of these enzymes during the pigmentation by anthocyanin in petals should be examined.

Because the induction of phenylalanine ammonia-lyase (PAL) gene expression plays an important role in defense response, recent approaches to investigate the expression have focused on the induction by environmental stresses. Most cDNAs for PAL have been obtained from elicitor-treated, injured, or ultraviolet (UV) light exposed cells, organs and tissues (reviewed by Hahlbrock *et al.*, 1989; Lamb, 1994). There has been no report of PAL cDNA cloning from petals. As a first step in studying the role of the phenylpropanoid pathway in petals accumulating anthocyanin, a cDNA for PAL transcribed in carnation petals was obtained and the expression patterns of the PAL transcripts corresponding to a PAL cDNA obtained here were examined.

To prepare of cDNA libraries, total RNA was extracted from 5 g fresh weight of petals of carnation (*Dianthus caryophyllus* cv. Symphony Rose) at stage 3 (see **Fig. 5 A**) using a modified phenol method followed by CsCl-ultracentrifugation (Ozeki *et al.*, 1990). Poly(A)$^+$ RNA was prepared from total RNA using oligotex-dT30 <Super>(TaKaRa, Otsu) as described in the manual from the supplier.

A cDNA library was constructed in λZAPII using ZAP-cDNA Synthesis Kit (Stratagene, La Jolla).

The phage clones containing cDNAs for PAL were screened using carrot PAL cDNA (Ozeki *et al.*, 1990) as a probe. To determine the double strand nucleotide sequence, deletion mutants were prepared by Exonuclease III/ mung bean nuclease digestion. The nucleotide sequences were determined using an automated DNA sequencer (LI-COR model 4000).

The genomic DNA was prepared from leaves of carnation using the CTAB method (Murray and Thompson 1980). *Bgl*II-, *Eco*RI- and *Hind*III-digested genomic DNAs (4 μg each) were separated by 0.8 % agarose gel electrophoresis and stained with ethidium bromide to visualize a molecular weight marker. The DNAs were blotted and fixed on a nylon membrane filter then hybridized with the ^{32}P-labeled PAL cDNA of carnation at 60 ℃. The filter was washed twice at room temperature with 2 x SSC, 0.5% SDS solution for 15 min, then twice at room temperature with 0.5 x SSC, 0.1% SDS for 10 min, and finally twice at 55℃ in 0.5 x SSC, 0.1% SDS for 30 min. The filters were then exposed to X-ray film (Kodak) at -80 ℃.

Total RNA (20 μg) was prepared using a modified phenol method and separated by a 1.5% formaldehyde-denaturing agarose gel electrophoresis and stained with ethidium bromide to visualize ribosomal RNA. RNA was blotted and fixed on a nylon membrane filter then hybridized with the ^{32}P-labeled PAL cDNA of carnation at 65 ℃. The filters were washed twice at room temperature with 2 x SSC, 0.5% SDS solution for 15 min, then twice at room temperature with 0.1 x SSC, 0.1% SDS for 10 min, and finally twice at 65 ℃ with 0.1 x SSC, 0.1% SDS for 30 min. The filters were then exposed to X-ray film at -80 ℃.

Anthocyanin was extracted from fresh tissues (0.1 to 0.5 g) using methanol (1 m*l*) containing 1% HCl in the dark at 4 ℃ overnight. The absorbance of the extract was measured at 530 nm and the amount of anthocyanin was presented as A_{530} per gram tissue fresh weight.

One positive clone for carrot PAL cDNA as a probe under the mild hybridization condition (hybridization at 55 ℃, final wash at 55 ℃ with 0.5 x SSC) was obtained from 2.2 x 10^4 independent phage clones of the carnation cDNA library. The clone was 2090 bp long (DDBJ accession number AB041361) and encoded a 618-amino acid polypeptide (**Fig.1**). The cDNA displayed 73.4% simi-

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1    GGATAGTATGAATAAAGGGACAGATAGTTATGGTGTAACTACTGGGTTTGGTGCCACTTCTCACCGTAGAACTAAGCAAGGTGGTGCTCTTCAAAAGGAACTCATTAGATTCTTGAACGC  120
       D  S  M  N  K  G  T  D  S  Y  G  V  T  T  G  F  G  A  T  S  H  R  R  T  K  Q  G  G  A  L  Q  K  E  L  I  R  F  L  N  A

121  TGGAGTTTTCGGCAATGGAACAGAAACAAGCCACACATTGCCACACACGGCATCAAGGGCGGCAATGTTGGTTAGAATTAACACCCTTCTACAAGGGTACTCCGGTATTCGTTGGGAAAT  240
       G  V  F  G  N  G  T  E  T  S  H  T  L  P  H  T  A  S  R  A  A  M  L  V  R  I  N  T  L  L  Q  G  Y  S  G  I  R  W  E  I

241  CCTCGAAGCGATCACTAGCCTCCTTAACCACGACGTCACTCCTTGCCTTCCTCTGCGCGGAACAATCACCGCGTCAGGTGACTTGGTCCCGCTTTCCTACATTGCTGGGCTTTTAACTGG  360
       L  E  A  I  T  S  L  L  N  H  D  V  T  P  C  L  P  L  R  G  T  I  T  A  S  G  D  L  V  P  L  S  Y  I  A  G  L  L  T  G

361  CAGGCCCAACGCCAAAGCCGCGGGCCCCAACGGTGAGATCCTCACCGCTGAGGAAGCCTTCAAGGCCGCCAAAATTGAGTCCCCATTTTTTGAGCTCCAGCCTAAGGAGGGACTTGCGAT  480
       R  P  N  A  K  A  A  G  P  N  G  E  I  L  T  A  E  E  A  F  K  A  A  K  I  E  S  P  F  F  E  L  Q  P  K  E  G  L  A  M

481  GGTGAACGGTACTGCCGTGGGGTCAGGCATGGCCTCGATTGTCCTCTACGAAGCGAACATTTTAGCTGTTCTGGCTGAGGTCATATCAGCAGTGTTTGCTGAAGTTATGAACGGAAAACC  600
       V  N  G  T  A  V  G  S  G  M  A  S  I  V  L  Y  E  A  N  I  L  A  V  L  A  E  V  I  S  A  V  F  A  E  V  M  N  G  K  P

601  CGAGTTCACGGACCACTTGACCCACAAATTGAAGCACCACCCAGGTCAAATTGAGGCTGCTGCAATAATGGAGCATATATTAGATGGAAGCTCGTACATGAAAGCGGCCAAGGAGTTACA  720
       E  F  T  D  H  L  T  H  K  L  K  H  H  P  G  Q  I  E  A  A  A  I  M  E  H  I  L  D  G  S  S  Y  M  K  A  A  K  E  L  H

721  CGAAATGGATCCGCTCCAGAAGCCTAAGCAGGATAGGTATGCCCTGAGGACGTCGCCACAGTGCGTTGGGCCACAAATTGAGGTCATTCGTTGGGCGACTAAGTCCATCGAGCGTGAGAT  840
       E  M  D  P  L  Q  K  P  K  Q  D  R  Y  A  L  R  T  S  P  Q  C  V  G  P  Q  I  E  V  I  R  W  A  T  K  S  I  E  R  E  I

841  TAACTCTGTCAACGATAATCCTCTTATTGATGTTTCTCGTAACAAGGCCTTGCACGGTGGGAACTTCCAGGGTACGCCCATTGGAGTGTCCATGGATAATACCCGTTTAGCTATTTCCGC  960
       N  S  V  N  D  N  P  L  I  D  V  S  R  N  K  A  L  H  G  G  N  F  Q  G  T  P  I  G  V  S  M  D  N  T  R  L  A  I  S  A

961  TATTGGGAAGCTGTTGTTTGCGCAGTTCTCTGAACTAGTTAACGACTTTTACAACAATGGTCTGCCATCAAACTTGACTGCAAGCCGAGACCCTAGCTTGGACTATGGTTTCAAAGGTGC  1080
       I  G  K  L  L  F  A  Q  F  S  E  L  V  N  D  F  Y  N  N  G  L  P  S  N  L  T  A  S  R  D  P  S  L  D  Y  G  F  K  G  A

1081 GGAAATCGCTATGGCATCTTACTGTTCTGAGCTTCAGTTTTTGGGGAACCCTGTGACGAACCATGTGCAGAGCGCTGAACAACATAACCAGGACGTCAACTCTTTGGGACTCATCTCTGC  1200
       E  I  A  M  A  S  Y  C  S  E  L  Q  F  L  G  N  P  V  T  N  H  V  Q  S  A  E  Q  H  N  Q  D  V  N  S  L  G  L  I  S  A

1201 GCGTAAGACATTTGAGGCGGTCGAGATTCTGAAACTCATGTCGTCCACCTTCCTTGTCGCTCTCTGCCAGGCAGTCGACCTGAGGCACATCGAGGAGAACCTTCAGAGCGCGGTGAAAAA  1320
       R  K  T  F  E  A  V  E  I  L  K  L  M  S  S  T  F  L  V  A  L  C  Q  A  V  D  L  R  H  I  E  E  N  L  Q  S  A  V  K  N

1321 CACAGTGAGCCAAGTCTGCAAACGGGTCCTGATCACTGGCGTAAAAGGGGAGCTTCACCCAGGACGATTCTGCGAGAAGGATCTGATCAGAGTGGTCGAGAGAGAGCACGTCTTCACCTA  1440
       T  V  S  Q  V  C  K  R  V  L  I  T  G  V  K  G  E  L  H  P  G  R  F  C  E  K  D  L  I  R  V  V  E  R  E  H  V  F  T  Y

1441 CGCAGACGACCCCTGCAGCTCAACCTACCCACTACTCCAAAAGCTCCGACAAGTCCTCGTGGACCAAGCTCTCGTAAATGGAGACGCCGAGAAGGTCGCAACCACTTCCATCTCCCAAAA  1560
       A  D  D  P  C  S  S  T  Y  P  L  L  Q  K  L  R  Q  V  L  V  D  Q  A  L  V  N  G  D  A  E  K  V  A  T  T  S  I  S  Q  K

1561 GATTGGTGCGTTCGAAGAAGAGCTCAAGGCACGGCTCCCTAAGGAAATCGAGGCTGTCAGATGCGCAGTGGAGAACGGTTCTGCTACAATCCCAAACAGAATCAAAGAGTGCAGGTCTTA  1680
       I  G  A  F  E  E  E  L  K  A  R  L  P  K  E  I  E  A  V  R  C  A  V  E  N  G  S  A  T  I  P  N  R  I  K  E  C  R  S  Y

1681 CCCGCTGTACAAGTTCGTGAGGGAAGTGCTTAAGACCGACTTGTTGACAGGCGAGGGAGTAAGGTCACCAGGTGAGGAAATCGACAAGGTGTTCACGGCACTTAACGAAGGGAAGATAGT  1800
       P  L  Y  K  F  V  R  E  V  L  K  T  D  L  L  T  G  E  G  V  R  S  P  G  E  E  I  D  K  V  F  T  A  L  N  E  G  K  I  V

1801 CGATCCTCTACTGGAGTGTCTTCAGGAGTGGAATGGTGCTCCTCTACCAATTTGTTGATAACAGCTTACAGTGGCCTTATATTGTGTTACTACAGACTACAGTAATATGTTTAATTTTCT  1920
       D  P  L  L  E  C  L  Q  E  W  N  G  A  P  L  P  I  C  *

1920 CAGAACTAATGTTGTTCAGAAATTGTTTTTGTTTTATCAAACTGTGTTTAGAGTACTGGACTGTTTTTTGTGTTTATGTTTTTGTGTTGTTAATGTAATCATCATATGTTTATTTTTTGA  2040

2041 AATGAGAAATTAAAGTCATCTATAAAAATAAAAAAAAAAAAAAAAAAAAA  2090
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Fig. 1. The nucleotide and deduced amino acid sequences of the PAL cDNA obtained from *Dianthus caryophyllus* (carnation).

larity to carrot PAL cDNA (Ozeki *et al.*, 1990), but was not full-length. In comparison with the other PAL cDNAs previously reported, this cDNA might lack longer than 200 bp of the 5' end.

A phylogenetic relationship of *Dianthus* and previously reported 30 PAL genes was inferred using the PROTDIST, NEIGHBOR, SEQBOOT, and CONSENSE programs in the PHYLIP version 3.572c software package (Felsenstein, 1995). Nucleotide sequences of these PAL genes were aligned using the program CLUSTAL W (Thompson *et al.*, 1994). Gap positions were removed, and the evolutionary distances were calculated using the 1787 nucleotides with the program PROTDIST under the Dayhoff and PAM matrix model (Dayhoff, 1978). The gene tree was constructed with the Neighbor-Joining method (Saitou and Nei, 1987) by the NEIGHBOR program. Statistical support for internal branches was estimated by bootstrap analysis with 100 replications using the SEQBOOT and CONSENSE programs. The PAL gene tree shows that PAL genes are not extensively duplicated during the course of seed plant evolution, and that *Dianthus caryophyllus* PAL gene is orthologous to other seed plant PAL genes (**Fig.2**).

Genomic DNA from leaf tissue of carnation was digested with restriction endonucleases, *Bgl*II, *Hin*dIII and *Eco*RI, all which do not digest the PAL

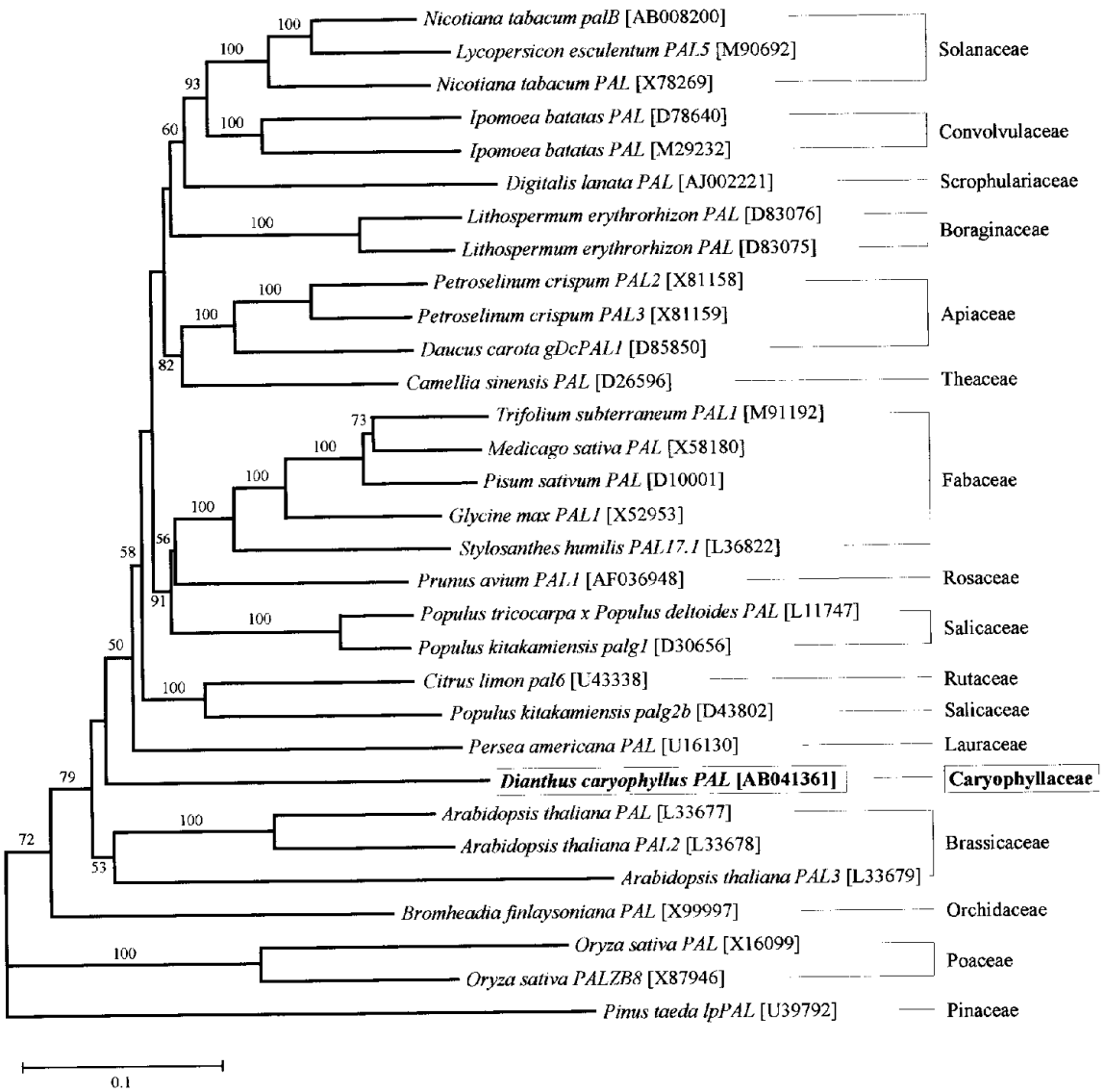

Fig. 2 A Neighbor-Joining tree of 31 seed plant PAL genes with nucleotide sequences. The 1787 nucleotides corresponding to positions 1 to 136, 151 to 288, 292 to 436, 443 to 1331, 1333 to 1341, 1344 to 1360, 1375 to 1387, 1395 to 1516, 1521 to 1529, 1534 to 1536, 1539 to 1542, 1548 to 1577, 1579 to 1599, 1602 to 1621, 1623 to 1645, 1647 to 1698, 1702 to 1840, 1842 to 1855 in the *Dianthus caryophyllus* PAL cDNA coordinates (Fig. 1) were used to calculate the evolutionary distances. Bootstrap values are indicated for branches supported by more than 50% of 100 bootstrap replicates. The conifer *Pinus taeda* PAL gene is used as an outgroup. The scale bar corresponds to 0.1 amino acid substitution per residue. Accession numbers in the databases are shown in parenthesis.

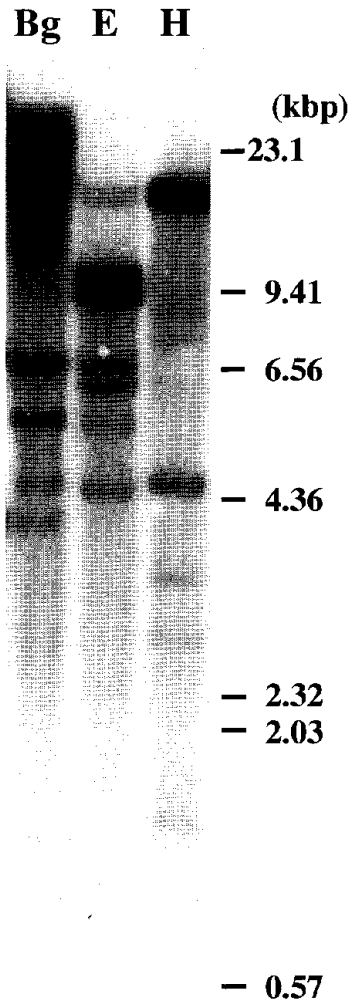

Fig. 3. Genomic southern blot hybridization of carnation with the PAL cDNA probe. The genomic DNA (4 μg each) of carnation was digested with various endnucleases and separated by 0.8 % agarose gel electrophoresis. The DNAs were blotted on a nylon membrane filter then hybridized with the ^{32}P-labeled PAL cDNA as a probe. Lane Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII

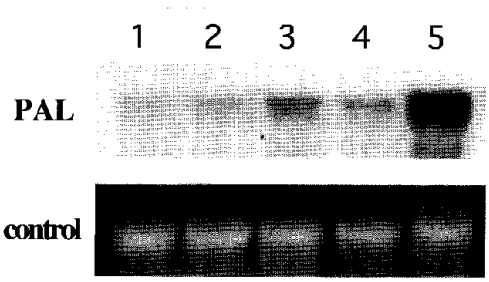

Fig. 4. Northern blot analysis of PAL mRNA expression in organs. 1, young leaves; 2, old leaves; 3, stems; 4, roots; 5, petals. RNA (20 μg) was loaded onto the gel and hybridization was carried out using PAL cDNA obtained in this work as a probe. Control shows 25S ribosomal RNA stained by ethidium bromide before blotting.

cDNA of carnation, and analyzed by southern blotting under the mild hybridization condition (**Fig.3**). PAL genes have been known to comprise a small multi-gene family in several plant species (Cramer *et al.*, 1989; Ohl *et al.*,1990; Logemann *et al.*, 1995; Fukasawa-Akada *et al.*, 1996). Southern hybridization patterns (**Fig. 3**) suggested that carnation has at least five PAL genes as a multi-gene family, because at least five bands with strong signal strength were observed on the lane of *Eco*RI digested genomic DNA and more than five bands with various signal strength were done on that of *Bgl*II or *Hin*dIII digested one.

To analyze the expression of the PAL gene corresponding to the cDNA obtained here, PAL transcripts were detected in young leaves, old leaves, stems, roots and petals by northern hybridization using the cDNA as a probe under the stringent condition (**Fig. 4**). A strong signal corresponding to PAL transcripts prepared from petals was observed, but only weak signals from young and old leaves, stems and roots.

During flower development in carnation, anthocyanin is not synthesized in petals of young buds (**Fig. 5A** and **B, stage 1** and **2**). It is, however, rapidly accumulated in mature buds up until the flowers open (**Fig. 5A** and **B, stage 3-5**). At stage 1, the PAL transcripts were almost undetectable (**Fig. 5 C**). Nevertheless, the PAL gene expression level increased gradually from stage 2 to 5, along with the amount of anthocyanin in petals.

These expression patterns suggested that the PAL cDNA obtained here is involved in the petal-specific expression in carnation. The importance of genetic manipulation of the phenylpropanoid pathway has been proposed to improve the defense responses via the production of phytoalaxins (Dixon *et al.*, 1996, 1998). Much attention should be paid to the expression ofgenes of the enzymes involved in the phenylpropanoid pathway in flowers, as well as those of the flavonoid pathway. Metabolic engineering of the phenylpropanoid pathway in flowers will give us novel tints and tones of petal colors.

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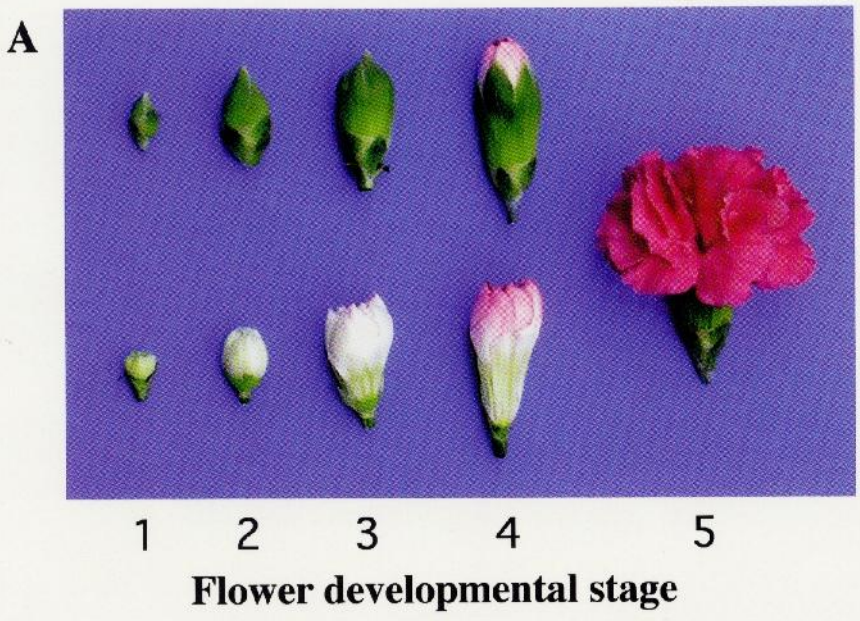

Fig. 5 Accumulation of anthocyanin and PAL transcripts during the development of carnation flowers. **A**, flower developmental stages. Sepals of buds are peeled to show the accumulation of anthocyanin in petals at stage 1-4. **B** and **C**, accumulation of anthocyanin and PAL transcripts, respectively, at stages 1-5.

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