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Supplementary Materials for

Generation of blue chrysanthemums by anthocyanin B-ring hydroxylation and glucosylation and its coloration mechanism

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Supplementary Materials and Methods

1. Construction of binary vectors for transgene expression.

Independent transgene cassettes were tandemly connected on a T-DNA binary vector by a modification system using eight-base recognition restriction endonuclease sites (*31*). For the co-expression of multiple transgenes, we constructed two T-DNA binary vectors containing the *CmF3H*p:*GUS*:*NOS*t cassette, pBI121 HANS-CmF3Hp1k-S (*6*) and pBI121-FASS-CmF3H1k. The eight-base recognition restriction endonuclease sites *Asc*I, *Not*I and *Swa*I, upstream of the *CmF3H* promoter of pBI121 HANS-CmF3Hp1k-S were changed to *Fse*I, *Asc*I, *Stu*I and *Swa*I sites in pBI121-FASS-CmF3H1k. The first gene was directly subcloned in place of the *GUS* gene in the binary vectors, and the additional gene cassettes were subcloned into the *Asc*I/*Fse*I and *Swa*I sites. The second and third cassettes were constructed in entry vector pMCE5-2 containing the *CmF3H*p:*GUS*:*AtHSP*t (*Arabidopsis* heat shock protein 18.2 gene terminator) (*32*) cassette. The transgenes were expressed under the control of *NtADH*-5′-UTR as a translational enhancer (*33*). The transgene was cloned in place of the *GUS* gene in the entry vector, which was then digested with *Asc*I/*Pme*I or *Fse*I/*Pme*I to remove the *CmF3H*p:gene:*AtHSP*t cassette for subcloning into the *Asc*I/*Swa*I or *Fse*I/*Swa*I sites of the binary vectors. The primer sets used in vector construction are listed in supplementary table S3.

To obtain entry vector pMCE5, first, we amplified 1047 bp of the *CmF3H* promoter region (GenBank accession number FW570860) with primer set 1 and cloned it into pGEM-T easy (Promega). The resulting plasmid was digested with *Hin*dIII/*Spe*I, and *CmF3H*p was subcloned into the *Hin*dIII/*Xba*I sites of pBI221. Next, we amplified *NOS*t with primer set 2 and TA-cloned it into pCR2.1 (Thermo Fisher Scientific). The *Sac*I/*Eco*RI-digested *NOS*t fragment was subcloned into the *Sac*I/*Eco*RI sites of plasmid pBI221 containing *CmF3H*p, which was then digested with *Hin*dIII and *Eco*RI to obtain the *CmF3H*p:*GUS*:*NOS*t cassette. The cassette was then subcloned into the *Hin*dIII/*Eco*RI sites of pSPORT2, and the resulting

plasmid was named pMCE5. We constructed the pMCE5-2 entry vector as follows. The 5′-restriction enzyme sites of *CmF3H*p in pMCE5 were changed to *Hin*dIII, *Fse*I, *Asc*I, *Stu*I and *Swa*I. The *CmF3H*p fragment was amplified with primer set 3 and subcloned into pCR-BluntII-TOPO (Thermo Fisher Scientific) as pCR-FASS-CmF3Hpro1k. We then subcloned the *Hin*dIII/*Nhe*I *CmF3H*p digest of pCR-FASS-CmF3Hpro1k into the *Hin*dIII/*Nhe*I sites of pMCE5 as pMCE5-FASS. Next, *AtHSP*t was amplified with primer set 4 and cloned into pCR-BluntII-TOPO. The *AtHSP*t-containing pCR-BluntII-TOPO vector was digested with *Sac*I/*Kpn*I, and the resulting *AtHSP*t fragment was subcloned into the *Sac*I/*Kpn*I sites of pMCE5-FASS in place of *NOS*t. The resulting plasmid (pMCE5-2) contained the *CmF3H*p:*GUS*:*AtHSP*t cassette.

The binary vector pB428 contained cassettes for overexpressing Canterbury bells (*Campanula medium*) *F3′5′H* (*CamF3′5′H*, GenBank accession number FW570877) and butterfly pea (*Clitoria ternatea*) *A3′5′GT* (*CtA3′5′GT, UGT78K8*, GenBank accession number AB115560), and an RNAi cassette for down-regulation of *CmF3′H*. We constructed the *CmF3′H* down-regulation cassette as follows. *CmF3′H* was amplified from chrysanthemum ‘Arietta’ petal cDNA by PCR with primer set 5, and cloned into pCR2.1 (pCR-CmF3′H). The RNAi trigger sequence was amplified from pCR-CmF3′H with primer set 6, and cloned into pENTR-D/TOPO (pENTR-CmF3′H-C). We transferred the RNAi trigger sequence from pENTR-CmF3'H-C to pANDA35K (*34*) by LR reaction (pANDA-CmF3'H-C IR). The inverted repeat of the *CmF3′H* fragment with *GUS* spacer was removed from pANDA-CmF3'H-C IR by digestion with *Xba*I/*Eco*ICRI, and subcloned into the *Spe*I/*Eco*ICRI sites of pBI121-FASS-CmF3H1k (pB319, pBF3′H-Ci). pCR ADHNF-CamF3′5′H (*6*) containing *NtADH*-5′-UTR-fused *CamF3′5′H* was digested with *Kpn*I, blunt-ended, and then digested with *Xba*I. We ligated the resulting fragment into the *Nhe*I/*Eco*ICRI digest of pMCE5-2 and named it pMCE5-2 ADHNF-CamF3′5′H. The *CmF3H*p:*NtADH*-5′*UTR:CamF3′5′H*:*AtHSP*t cassette

was removed from pMCE5-2 ADHNF-CamF3′5′H by digestion with *Fse*I/*Pme*I, and subcloned into the *Fse*I/*Swa*I sites of pB319 (pB332, pBF3′H-Ci:CamF3′5′H).

To create the *NtADH*-5′-UTR-fused *CtA3′5′GT* construct, first, we amplified two primary PCR products from pBCtBGT1DB24 containing *CtA3′5′GT* cDNA by primer set 7, and from pBI221 ADH-221 by primer set 8. A DNA fragment in which the *NtADH*-5′UTR was directly coupled to the start codon of *CtA3′5′GT* was obtained by PCR using two primary amplified DNA fragments as templates and primer set 9. This DNA fragment was cloned into pCR2.1-TA and then digested with *Xba*I and *Hin*dIII, and the resulting DNA fragment was ligated into *Xba*I/*Hin*dIII-digested pBluescriptII SK($^{+}$) to obtain a pBSII-ADH-5′-Ct3′5′GT-HindIII plasmid. We cloned the *Hin*dIII/*Xho*I insert fragment of pBSII-ADH-5′-Ct3′5′GT-HindIII into the *Hin*dIII/*Xho*I site of pBCtBGT1DB24 (pBSII-ADH-CtA3'5'GT). The *NtADH*-5′UTR:*CtA3′5′GT* fragment obtained from pBSII-ADH-CtA3'5'GT by *Nhe*I/*Eco*ICRI digestion was subcloned into the *Nhe*I/*Eco*ICRI sites of pMCE5-2 (pMCE5-2 ADHNF-CtA3'5'GT). The *CmF3H*p:*NtADH-5'UTR:CtA3'5'GT*:*AtHSP*t cassette was removed from pMCE5-2 ADHNF-CtA3'5'GT with *Fse*I/*Pme*I, and subcloned into the *Fse*I/*Swa*I sites of pB332 (pB428, pBF3′H-Ci:CamF3′5′H:CtA3′5′GT, fig. S2B). The sequence of the *CmF3′H* RNAi cassette has been registered in DDBJ/ENA/GenBank under accession code LC222468.

We constructed the binary vector pB423 for co-expression of *CamF3′5′H* and *CtA3′5′GT* as follows. The *NtADH*-5′UTR fused to *CtA3′5′GT* was amplified from pBSII-ADH-CtA3'5'GT with primer set 10, and ligated into pCR-BluntII-TOPO (Thermo Fisher Scientific). The *NtADH*-5′UTR-*CtA3′5′GT* DNA fragment from the *Nhe*I/*Eco*ICRI digest was cloned into pBI121 HANS-CmF3Hp1k-S (pB249, fig.S2B). The *CmF3H*p:*NtADH*-5′*UTR:CamF3'5'H*:*AtHSP*t cassette from the *Asc*I/*Pme*I digest of pMCE5-2 ADHNF-CamF3′5′H was subcloned into the *Asc*I/*Swa*I sites of pB249 (pB423, CtA3′5′GT:CamF3′5′H;

fig. S2, A and B). We registered the sequence of the *CtA3′5′GT* and *CamF3′5′H* cassettes in DDBJ/ENA/GenBank under accession code LC222467.

2. Compound characterization data.

A3: cyanidin 3-*O*-(6″-*O*-malonyl)glucoside-3′-*O*-glucoside; ^{1}H NMR (600 MHz, DMSO-d_6-TFA): δ 8.86 (*s*, 1H), 8.39 (*d*, *J* = 9.0 Hz, 1H), 8.19 (*br s*, 1H), 7.03 (*d*, *J* = 8.3 Hz, 1H), 6.96 (*s*, 1H), 6.70 (*s*, 1H), 5.24 (*d*, *J* = 7.2 Hz, 1H), 4.95 (*d*, *J* = 7.2 Hz, 1H), 4.44 (*d*, *J* = 12.0, 1H), 4.11 (*dd*, *J* = 11.7 Hz, 7.5 Hz, 1H), 3.81 (*d*, *J* = 11.4 Hz, 1H), 3.73 (*t*, *J* = 8.4 Hz, 1H), 3.59 (*dd*, *J* = 2.7, 11.4 Hz, 1H), 3.49 (*t*, *J* = 8.4 Hz, 3H), 3.37–3.43 (*m*, 5H), 3.25 (*t*, *J* = 8.7 Hz, 2H); HRMS (*m/z*): $[M]^+$ calcd. for $C_{30}H_{33}O_{19}$, 697.1616037; found 697.1605, $[M-H+Na]^+$ calcd. for $C_{30}H_{32}O_{19}Na$, 719.1435487; found 719.1781.

A8: delphinidin 3-*O*-(6″-*O*-malonyl)glucoside-3′,5′-*O*-diglucoside (ternatin C5) (*17*); ^{1}H NMR (600 MHz, CD_3OD-TFA): δ 9.00 (*s*, 1H), 8.23 (*s*, 1H), 7.03 (*br s*, 1H), 6.86 (*br s*, 1H), 5.25 (*d*, *J* = 7.2 Hz, 1H), 5.08 (*d*, *J* = 6.0 Hz, 2H), 4.53 (*br d*, *J* = 11.4 Hz, 1H), 4.29 (*dd*, *J* = 7.5, 11.7 Hz), 3.96 (*d*, *J* = 12.0 Hz, 2H), 3.91 (*m*, 1H), 3.78 (*dd*, *J* = 5.7, 12.3 Hz, 2H), 3.72 (*t*, *J* = 7.8 Hz, 2H), 3.56–3.63 (*m*, 6H), 3.54 (*t*, *J* = 9.3 Hz, 1H), 3.46 (*t*, *J* = 9.3 Hz, 2H); HRMS (*m/z*): $[M]^+$ calcd. for $C_{36}H_{43}O_{25}$, 875.2093417; found 875.2628, $[M-H+Na]^+$ calcd. for $C_{36}H_{42}O_{25}Na$, 897.1912867; found 897.1888.

C1: luteolin 7-*O*-(6″-*O*-malonyl)glucoside (*21*, *22*); ^{1}H-NMR (600 MHz, DMSO-d_6): δ 6.71 (*s*, 1H, H-3 of aglycone), 6.43(*d*, *J* = 1.4, 1H, H-6), 6.75 (*d*, *J* = 1.4 Hz, 1H, H-8), 7.43 (*s*, 1H, H-2′), 6.89 (*d*, *J* = 8.4 Hz, 1H, H-5′), 7.43 (*d*, *J* = 8.4 Hz, 1H, H-6′), 5.10 (*d*, *J* = 7.6 Hz, 1H, H-1″ of glucosyl), 3.28 (*m*, 1H, H-2″), 3.31 (*m*, 1H, H-3″), 3.19 (*t*, *J* = 9.3 Hz, 1H, H-4″), 3.74 (*t*, *J* = 7.8 Hz, 1H, H-5″), 4.37 (*d*, *J* = 11.0 Hz, 1H, H-6″-a), 4.13 (*dd*, *J* =11.7 Hz, 6.7 Hz, 1H, H-6″-b), 3.32 (*d*, *J* = 15.6 Hz, 1H, H-2‴-a of malonyl), 3.38 (*d*, *J* = 15.6 Hz, 1H, H-2‴-b); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 164.8 (C-2 of aglycone), 103.4 (C-3), 182.2 (C-4), 161.4 (C-5), 99.8 (C-6), 162.9 (C-7), 94.9 (C-8), 157.2 (C-9), 105.7 (C-10), 121.7 (C-10.4 (aglycone), 113.8 (C-2′), 146.1 (C-3′), 150.2 (C-4′), 116.2 (C-5′), 119.3 (C-6′), 99.9 (C-1″ of

glucosyl), 73.3 (C-2″), 76.4 (C-3″), 69.8 (C-4″), 74.1 (C-5″), 64.3 (C-6″), 167.1 (C-1‴ of malonyl), 168.0 (C-3‴), 41.7 (C-2‴); HRMS (*m/z*): [M+H]+ calcd. for $C_{24}H_{23}O_{14}$, 535.1087803; found 535.1046, $[M+Na]^+$ calcd. for $C_{24}H_{22}O_{14}Na$, 557.0907253; found 557.1089.

C2: tricetin 7-*O*-(6″-*O*-malonyl)glucoside; ^{1}H-NMR (600 MHz, DMSO-d_6): δ 6.59 (*s*, 1H, H-3 of aglycone), 6.44 (*d*, *J* = 2.1, 1H, H-6), 6.70 (*d*, *J* = 2.1 Hz, 1H, H-8), 6.98 (*s*, 2H, H-2′ & 6′ of aglycone), 5.10 (*d*, *J* = 7.6 Hz, 1H, H-1″ of glucosyl), 3.28 (*t*, *J* = 8.1 Hz, 1H, H-2″), 3.33 (*t*, *J* = 8.7 Hz, 1H, H-3″), 3.20 (*t*, *J* = 9.0 Hz, 1H, H-4″), 3.74 (*dif.t*, *J* = 9.3 Hz, 1H, H-5″), 4.33 (*d*, *J* = 10.3 Hz, 1H, H-6″-a), 4.17 (*dd*, *J* =12.1 Hz, 6.5 Hz, 1H, H-6″-b), 3.34 (*d*, *J* = 15.6 Hz, 1H, H-2‴-a of malonyl), 3.39 (*d*, *J* = 15.6 Hz, 1H, H-2‴-b); ^{13}C-NMR (150 MHz, DMSO-d_6): δ 165.0 (C-2 of aglycone), 103.5 (C-3), 182.1 (C-4), 161.5 (C-5), 99.8 (C-6), 162.9 (C-7), 95.0 (C-8), 157.2 (C-9), 105.7 (C-10), 120.6 (C-1′ of aglycone), 106.2 (C-2′), 146.6 (C-3′), 138.3 (C-4′), 146.6 (C-5′), 106.2 (C-6′), 99.7 (C-1″ of glucosyl), 73.3 (C-2″), 76.4 (C-3″), 69.8 (C-4″), 74.0 (C-5″), 64.3 (C-6″), 167.1 (C-1‴ of malonyl), 168.1 (C-3‴), 41.6 (C-2‴); HRMS (*m/z*): $[M+H]^+$ calcd. for $C_{24}H_{23}O_{15}$, 551.1036949; found 551.1034. $[M+Na]^+$ calcd. for $C_{24}H_{22}O_{15}Na$, 573.0856399; found 573.0919.

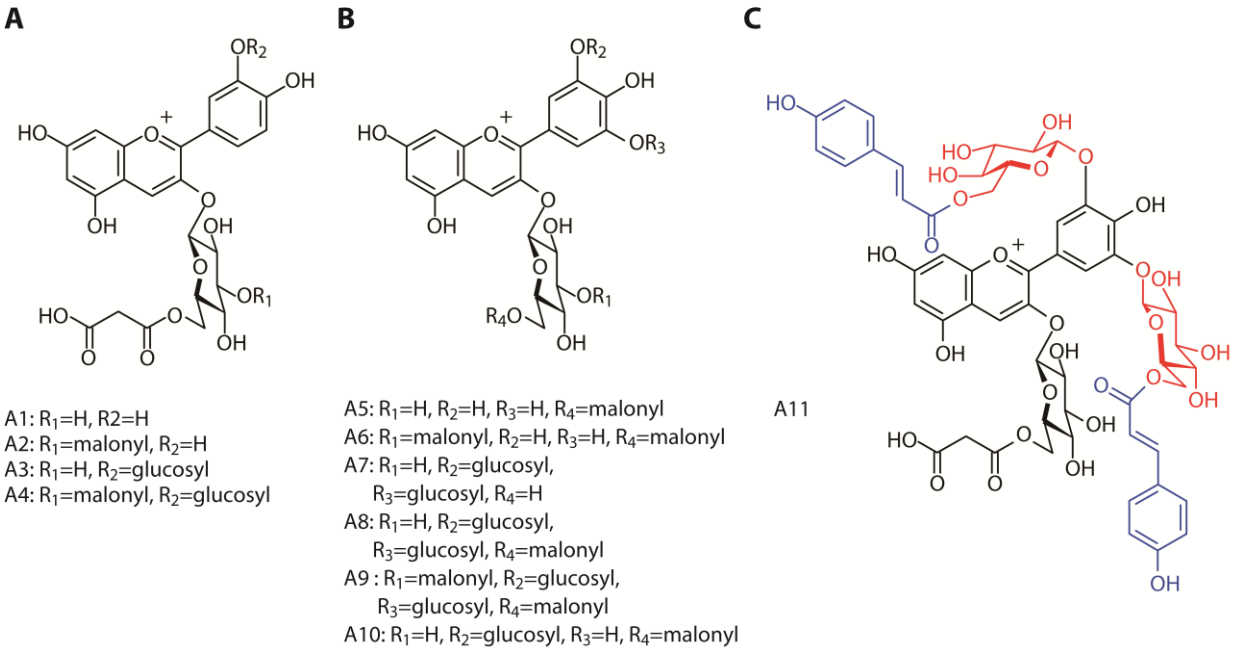

fig. S1. Structures of anthocyanins. (**A**) Cyanidin-based anthocyanins. (**B**) Delphinidin-based anthocyanins. The A3 and A8 structures were determined by ^{1}H-NMR and NOESY spectra. The NOESY spectra of A3 and A8 indicated that glucose was connected at aglycon-3-OH, 3′-OH, and/or 5′-OH through a glycosyl bond. The structures of A1–A2, A4–A7 and A9–A10 were putatively identified by LC-MS/MS spectra as follows: A1, cyanidin 3-(6′′-malonyl)glucoside; A2, cyanidin 3-(3′′,6′′-dimalonyl)glucoside; A3, cyanidin 3-(6′′-malonyl)glucoside-3′-glucoside; A4, cyanidin 3-(3′′,6′′-dimalonyl)glucoside-3'-glucoside; A5, delphinidin 3-(6′′-malonyl)glucoside; A6, delphinidin 3-(3′′,6′′-dimalonyl)glucoside; A7, delphinidin 3,3′,5′-triglucoside (preternatin C5); A8, delphinidin 3-(6′′-malonyl)glucoside-3′,5′-diglucoside (ternatin C5); A9, delphinidin 3-(3′′,6′′-dimalonyl)glucoside-3′,5′-diglucoside; A10, delphinidin 3-(6′′-malonyl)glucoside-3'-glucoside. (**C**) A11, delphinidin 3-(6′′-malonyl)glucoside-3′,5′-di-(6′′′,6′′′′-*p*-coumaroyl)glucoside (ternatin D3).

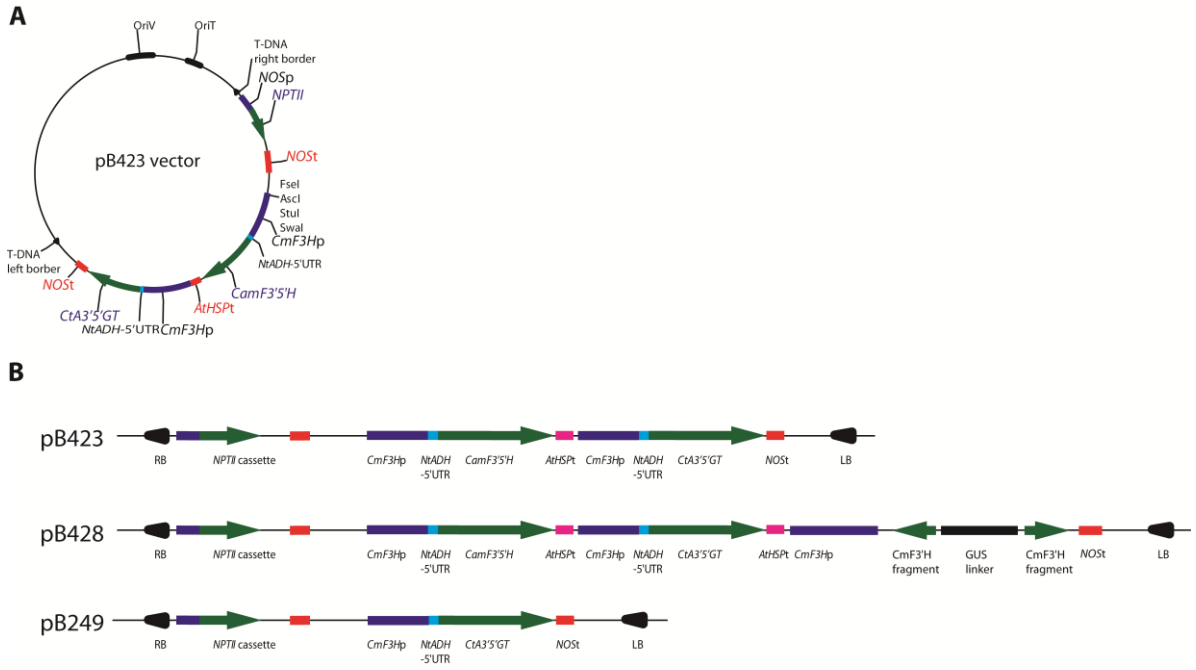

fig. S2. Schematic representation of binary vectors used for chrysanthemum transformation. (**A**) pB423 vector contains *CamF3′5′H* and *CtA3′5′GT* expression cassettes in pBI121. (**B**) T-DNA components of pB423, pB428, and pB249 vectors. RB, right border; *NPTII*, neomycin phosphotransferase II gene; *CmF3H*p, chrysanthemum flavanone 3-hydroxylase gene promoter; *NtADH*-5′UTR, tobacco alcohol dehydrogenase gene-5′UTR; *CamF3′5′H*, *Campanula* flavonoid 3′,5′-hydroxylase gene; *AtHSP*t, *Arabidopsis* heat shock protein 18.2 gene terminator; *NOS*t, agrobacterium nopaline synthase gene terminator; *CtA3′5′GT*, butterfly pea UDP-glucose:anthocyanin 3′,5′-*O*-glucosyltransferase gene; *CmF3′H*, chrysanthemum flavonoid 3′-hydroxylase gene; GUS, β-glucuronidase gene; LB, left border.

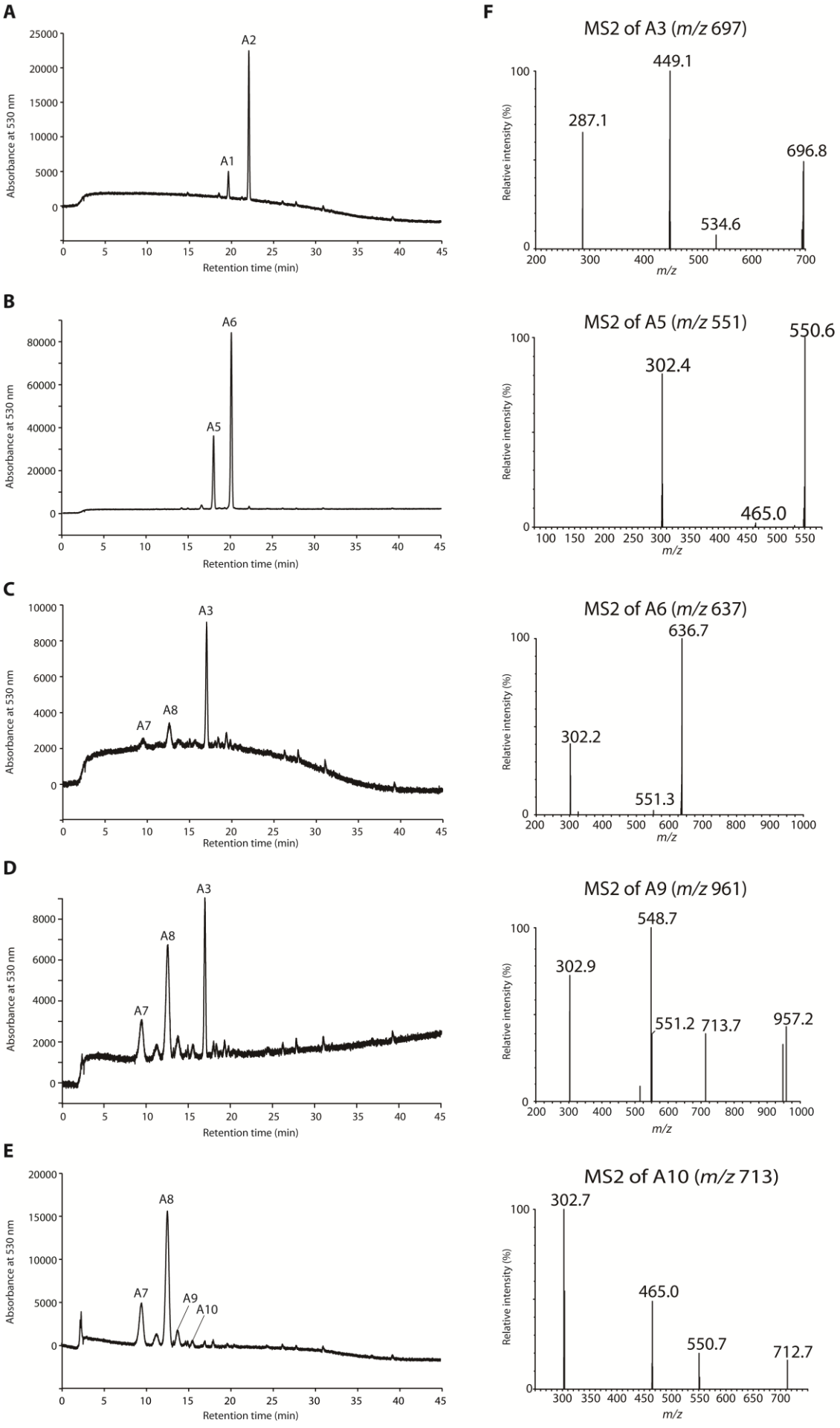
A
Absorbance at 530 nm
Retention time (min)
A1
A2
B
A5
A6
C
A3
A7
A8
D
A3
A7
A8
E
A7
A8
A9
A10
F
MS2 of A3 (m/z 697)
449.1
287.1
696.8
534.6
MS2 of A5 (m/z 551)
550.6
302.4
465.0
MS2 of A6 (m/z 637)
636.7
302.2
551.3
MS2 of A9 (m/z 961)
548.7
302.9
551.2
713.7
957.2
MS2 of A10 (m/z 713)
302.7
465.0
550.7
712.7
Relative intensity (%)
m/z

fig. S3. Anthocyanin profiles of representative 'Sei Arabella' transgenic lines. (**A-E**) HPLC chromatograms monitored at 530 nm. For structures A1-A10, see fig. S1. (A) 'Sei Arabella' wild type with pink flowers mainly accumulated A1 and A2. (B) Transgenic 1916-10 line with purple-violet flowers, in which only *CamF3′5′H* was expressed, mainly accumulated A5 and A6. (C) Transgenic 1916-25 line with pale purple flowers, in which only *CtA3′5′GT* was expressed, mainly accumulated A3. (D) Transgenic 1916-12 line with violet-blue flowers, in which *CamF3′5′H* and *CtA3′5′GT* were expressed, mainly accumulated A7 and A8 with a small amount of A3. (E) Transgenic 1916-23 line with blue flowers, in which *CamF3′5′H* and *CtA3′5′GT* were expressed, mainly accumulated A7 and A8. (F) MS/MS (MS2) fragmentation spectra of these major anthocyanins. Structures of A1-A10 anthocyanins (precursor ion $[M]^+$): A1, cyanidin 3-(6″-malonyl)glucoside; A2, cyanidin 3-(3″,6″-dimalonyl)glucoside; A3, cyanidin 3-(6″-malonyl)glucoside-3′-glucoside (*m/z* 697), A5, delphinidin 3-(6″-malonyl)glucoside (*m/z* 551); A6, delphinidin 3-(3″,6″-dimalonyl)glucoside (*m/z* 637); A7, delphinidin 3,3′,5′-triglucoside (preternatin C5)(*m/z* 789); A8, delphinidin 3-(6″-malonyl)glucoside-3′,5′-diglucoside (ternatin C5) (*m/z* 875); A9, delphinidin 3-(3″,6″-dimalonyl)glucoside-3′,5′-diglucoside (*m/z* 961); A10, delphinidin 3-(6″-malonyl)glucoside-3′-glucoside (*m/z* 713).

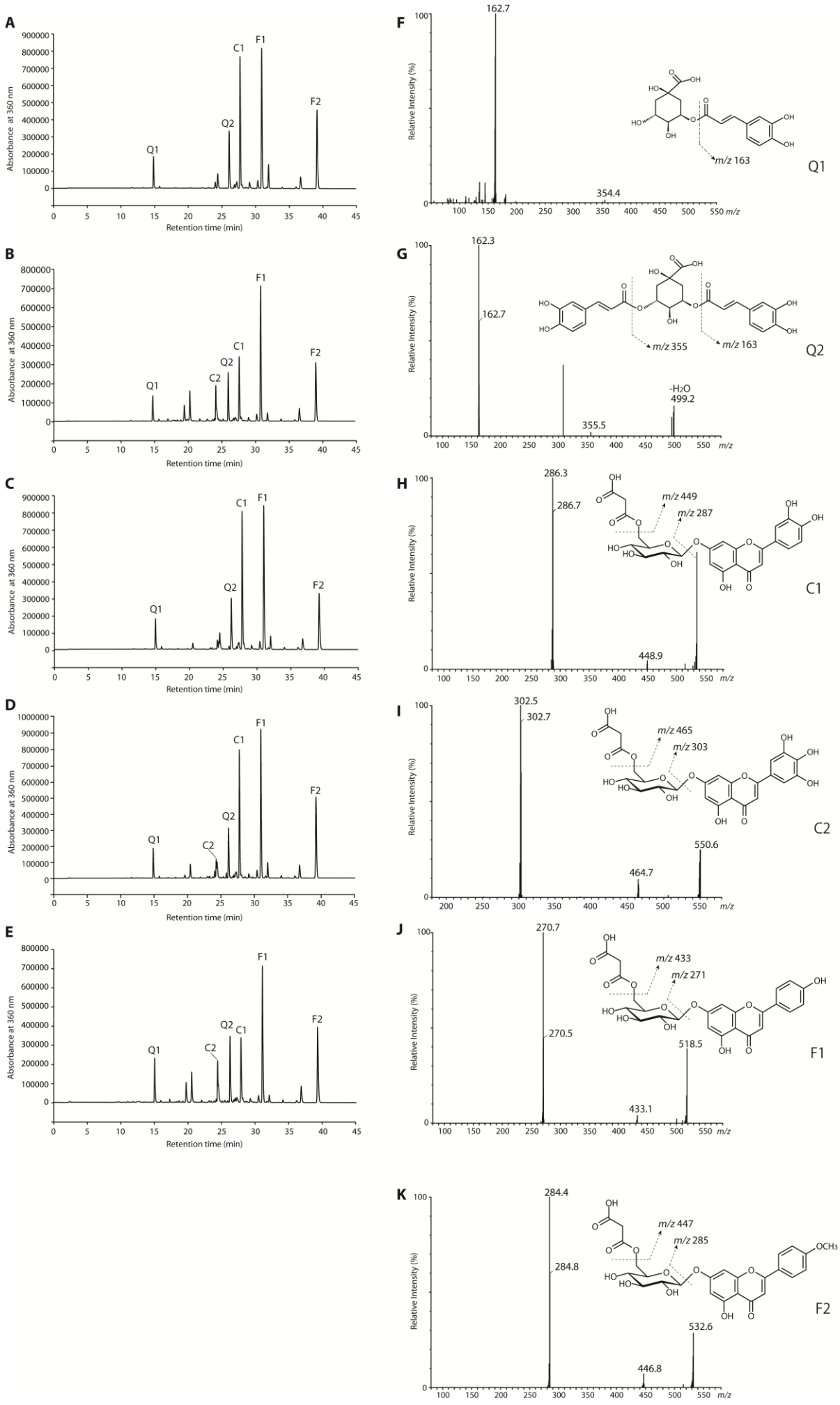
A
B
C
D
E
F
G
H
I
J
K
Absorbance at 360 nm
Retention time (min)
Q1
Q2
C1
C2
F1
F2
Relative Intensity (%)
m/z
162.7
354.4
m/z 163
162.3
355.5
-H2O
499.2
m/z 355
286.3
286.7
448.9
m/z 449
m/z 287
302.5
302.7
464.7
550.6
m/z 465
m/z 303
270.7
270.5
433.1
518.5
m/z 433
m/z 271
284.4
284.8
446.8
532.6
m/z 447
m/z 285
OCH3

fig. S4. Flavonoids and caffeoylquinates profiles of representative 'Sei Arabella' transgenic lines. (**A–E**) HPLC chromatograms monitored at 360 nm. (A, C) Wild type and transgenic 1916-25 line accumulating cyanidin-based anthocyanins have almost same chromatogram. (B, D, E) Transgenic 1916-10, 12, 23 lines accumulating delphinidin-base anthocyanins contained C2 flavone and some unknown compounds. (**F–K**) ESI-MS/MS fragmentation of main peaks. Structures of Q1–Q2, C1–C2 and F1–F2 (precursor ion $[M]^+$): Q1, 3-caffeoylquinic acid (*m/z* 355); Q2, 3,5-dicaffeoylquinic acid (*m/z* 517); C1, luteolin 7-(6″-malonyl)glucoside (*m/z* 535); C2, tricetin 7-(6-malonyl)glucoside (*m/z* 551); F1, apigenin 7-(6″-malonyl)glucoside (*m/z* 519); F2, acacetin 7-(6″-malonyl)glucoside (*m/z* 533).

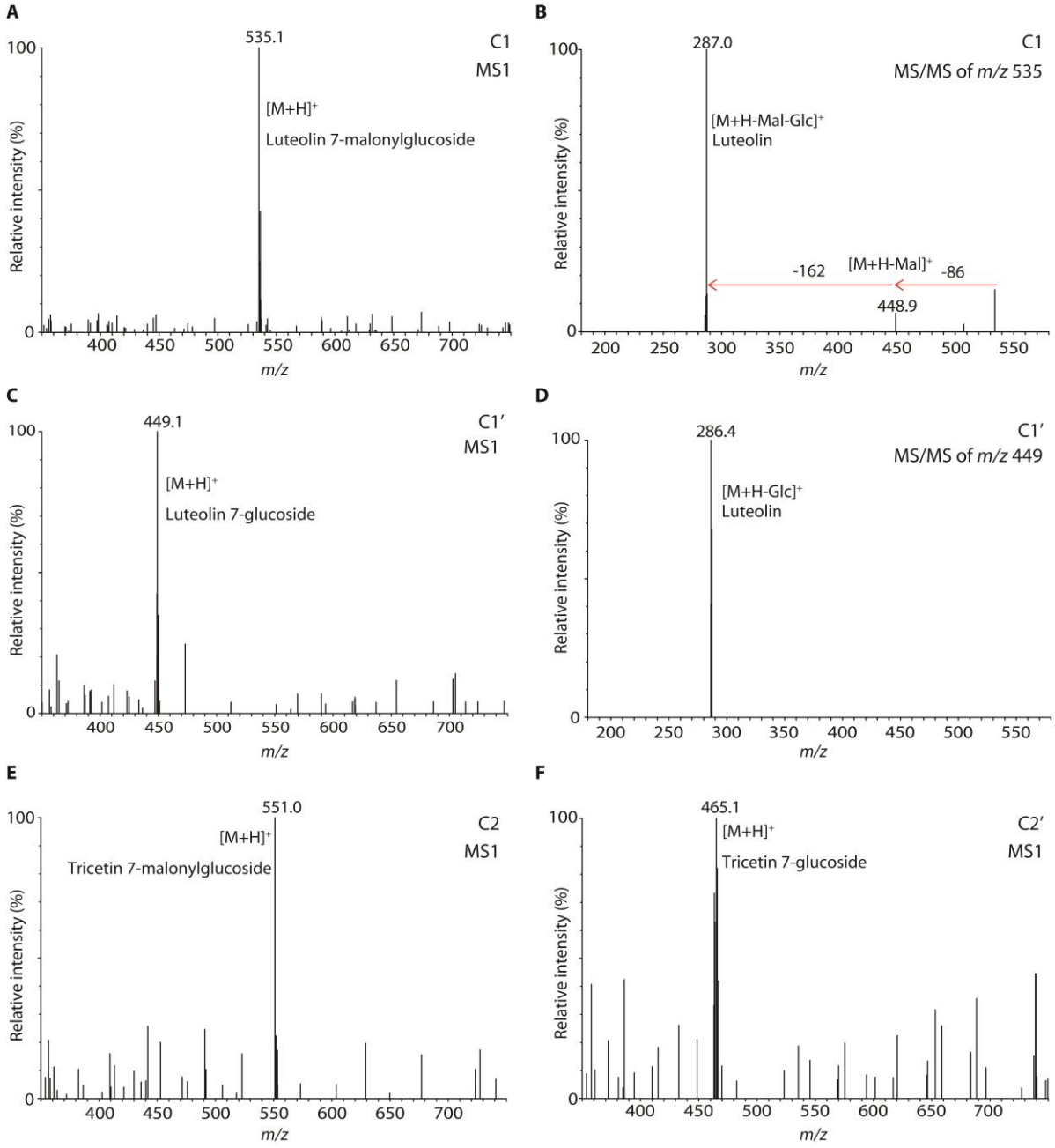

fig. S5. LC-MS analysis of copigment extract from a cellulose TLC plate. (**A**) MS spectrum of C1 in extract from band X. (**B**) MS/MS fragmentation spectrum of C1 (*m/z* 535). (**C**) MS spectrum of C1′ in extract from band X. (**D**) MS/MS fragmentation spectrum of C1′ (*m/z* 449). (**E**) MS spectrum of C2 in extract from band Y. (**F**) MS spectrum of C2′ in extract from band Y. Compound C1′ and C2′ were predicted to be the demalonylated degradation product of C1 and C2, respectively. C1′, luteolin 7-glucoside; C2′, tricetin 7-glucoside; C1, luteolin 7-malonylglucoside; C2, tricetin 7-malonylglucoside. Glc, glucosyl, Mal, malonyl.

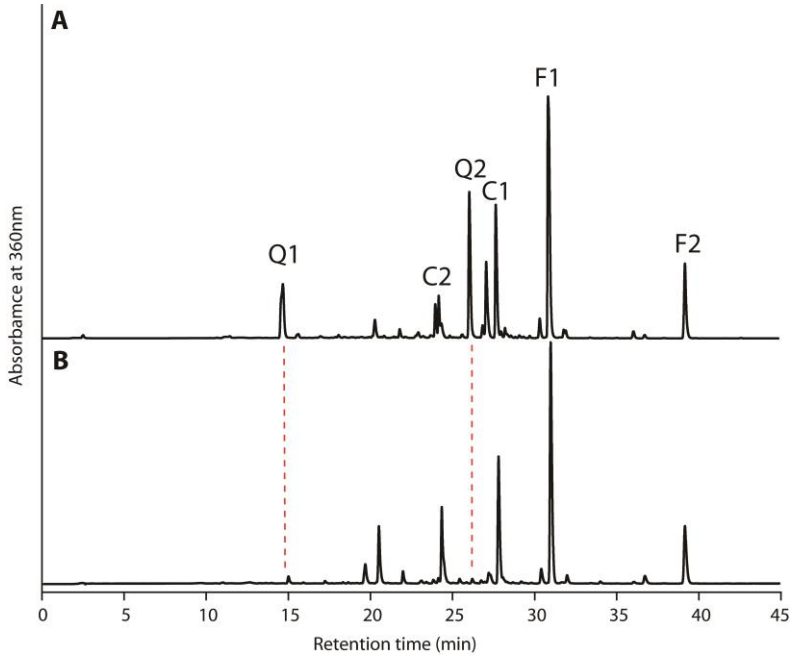

fig. S6. Flavonoids and caffeoylquinates profiles of whole and epidermal petal tissue from a blue transgenic line. HPLC chromatograms monitored at 360 nm. For structures, see fig.S4. (**A**) Whole petal extract. (**B**) Adaxial epidermal tissues extract. The epidermal tissues were obtained by using tweezers. Caffeoylquinates Q1 and Q2 were trace levels in epidermal tissues containing anthocyanins. Q1, 3-caffeoylquinic acid; Q2, 3,5-dicaffeoylquinic acid; C1, luteolin 7-malonylglucoside; C2, tricetin 7-malonylglucoside; F1, apigenin 7-malonylglucoside; F2, acacetin 7-malonylglucoside.

table S1. Flower color changes due to expression of *CamF3'5'H* and/or *CtA3'5'GT*.

Host	Plasmid	Tansgene cassettes (RB to LB direction of T-DNA)	Transgenic line name	No. of trangenic lines	No. of purple/violet/blue trangenic lines	(%)	No. of trangenic lines with blue flowers of RHSCC Blue/Violet-Blue group	(%)
		For 3',5'-hydroxylation and glucosylation*						
Taihei	pB423	CtA3'5'GT : CamF3'5'H	1728	32	21	(66)	19	(59)
Sei Arabella	pB423	CtA3'5'GT : CamF3'5'H	1916	26	21	(81)	16	(62)
T37	pB423	CtA3'5'GT : CamF3'5'H	1921, 1939	10	10	(100)	9	(90)
Sei Arabella	pB428	CmF3'H-RNAi : CamF3'5'H : CtA3'5'GT	1804	3	3	(100)	2	(67)
T27	pB428	CmF3'H-RNAi : CamF3'5'H : CtA3'5'GT	1805, 1872	10	8	(80)	4	(40)
Sei Shawl	pB428	CmF3'H-RNAi : CamF3'5'H : CtA3'5'GT	1870, 1891	10	6	(60)	3	(30)
T57	pB428	CmF3'H-RNAi : CamF3'5'H : CtA3'5'GT	1890	1	1	(100)	1	(100)
		For 3'-glucosylation						
940-0765	pB249	CtA3'5'GT	1356	25	0	(0)	0	(0)
Taihei	pB249	CtA3'5'GT	1979	25	0	(0)	0	(0)

*The chrysanthemum *F3H* promoter (*CmF3H*p) was used for all gene cassettes. The *NOS* terminator (*NOS*t) from *Agrobacterium tumefaciens* was used for the first gene cassette, and *Arabidopsis HSP* terminator (*AtHSP*t) was used for subsequent cassettes.

Abbreviations: *Ct*, *Clitoria ternatea*; *Cam*, *Campanula medium*; *Cm*, *Chrysanthemum morifolium*; A3'5'GT, UDP-glucose:anthocyanin 3',5'-*O*-glucosyltransferase; F3'5'H, flavonoid 3',5'-hydroxylase; F3'H, flavonoid 3'-hydroxylase; RB, right T-DNA border; LB, left T-DNA border; RHSCC, Royal Horticultural Society Colour Charts.

table S2. Anthocyanin and flavone content and their ratios in petals of 'Sei Arabella' and blue/violet-blue–colored transgenic chrysanthemums.

Line	RHS colour charts		Anthocyanin contents (nmol/mg)*									Total anthocyanin content (nmol/mg)	Flavone contents (nmol/mg)†				Total flavone content (nmol/mg)	Total flavone /Total anthocyanin	C1 /A7-A9	C2 /A7-9
			Cyanidin type				Delphinidin type													
			A1	A2	B-ring glucosyl-type		A5	B-ring glucosyl-type												
					A3	A4		A7	A8	A9	A10		C1	C2	F1	F2				
WT	Red-Purple	73A-B	0.16	0.84	-	-	-	-	-	-	-	1.00	2.53	-	2.87	1.97	7.37	7.4	-	-
1916-01	Violet-Blue	97A	-	-	0.10	-	0.05	0.24	0.62	0.11	0.06	1.19	1.57	0.58	2.52	1.90	6.56	5.5	1.6	0.6
1916-02	Violet-Blue	97A	-	-	-	-	-	0.40	1.12	0.13	-	1.65	0.86	0.54	1.78	1.07	4.25	2.6	0.5	0.3
1916-04	Violet-Blue	95C	-	-	-	-	0.06	0.56	1.85	0.21	0.07	2.75	1.34	0.77	2.93	1.14	6.17	2.2	0.5	0.3
1916-05	Violet-Blue	92B	0.06	0.06	0.29	0.07	0.07	0.21	0.49	0.11	0.09	1.44	2.62	0.20	3.06	1.51	7.39	5.1	3.3	0.3
1916-06	Violet-Blue	92B-C	-	-	0.38	-	-	0.19	0.49	0.09	-	1.15	2.64	0.25	3.48	1.92	8.30	7.2	3.4	0.3
1916-12	Violet-Blue	94B	-	-	0.41	-	-	0.28	0.68	0.11	0.05	1.53	2.75	0.30	3.36	2.23	8.64	5.6	2.6	0.3
1916-14	Blue	100D	-	-	-	-	-	0.21	0.57	0.08	-	0.87	0.64	0.52	1.26	0.99	3.42	3.9	0.7	0.6
1916-15	Violet-Blue	97A-B	-	-	0.16	-	0.07	0.34	0.86	0.14	0.09	1.65	2.11	0.46	3.56	1.94	8.07	4.9	1.6	0.3
1916-17	Violet-Blue	96D	-	-	-	-	-	0.43	1.14	0.12	-	1.69	1.06	0.47	2.28	1.03	4.84	2.9	0.6	0.3
1916-19	Violet-Blue	95C	-	-	0.00	-	0.08	0.33	0.78	0.13	0.09	1.42	0.82	0.54	1.72	0.76	3.85	2.7	0.7	0.4
1916-20	Violet-Blue	95C-D	-	-	0.08	-	-	0.23	0.60	0.08	-	1.00	1.76	0.30	3.36	1.45	6.87	6.9	1.9	0.3
1916-22	Violet-Blue	95C-D	-	-	-	-	0.08	0.30	0.12	0.16	0.11	0.76	1.39	0.54	3.43	1.60	6.97	9.2	2.4	0.9
1916-23	Blue	100B-C	-	-	-	-	-	0.53	1.46	0.14	-	2.13	0.89	0.46	1.98	1.38	4.71	2.2	0.4	0.2
1916-24	Violet-Blue	92C	-	-	0.15	-	-	0.15	0.30	0.07	0.07	0.74	2.14	0.17	3.46	1.95	7.73	10.4	4.1	0.3
1916-27	Violet-Blue	96D	-	-	-	-	-	0.51	1.41	0.16	0.05	2.13	1.26	0.87	3.10	1.72	6.95	3.3	0.6	0.4
Average																		5.0	1.7	0.4

*A1, cyanidin 3-(6"-malonyl)glucoside; A2, cyanidin 3-(3",6"-dimalonyl)glucoside; A3, cyanidin 3-(6"-malonyl)glucoside-3'-glucoside; A4, cyanidin 3-(3",6"-dimalonyl)glucoside-3'-glucoside; A5, delphinidin 3-(6"-malonyl)glucoside; A7, delphinidin 3,3'5'-triglucoside (preternatin C5); A8, delphinidin 3-(6"-malonyl)glucoside-3',5'-diglucoside (ternatin C5); A9, delphinidin 3-(3",6"-dimalonyl)glucoside-3',5'-diglucoside; A10, delphinidin 3-(6"-malonyl)glucoside-3'-glucoside.†C1, luteolin 7-(6"-malonyl)glucoside; C2, tricetin 7-(6"-malonyl)glucoside; F1, apigenin 7-(6"-malonyl)glucoside; F2, acacetin 7-(6"-malonyl)glucoside.

table S3. Primer sets used in binary vector construction.

Set No.	Direction	Name	Sequence (5'-3')	Restriction enzyme cleavage sites in underlined	Target
Primers					
1	Fd	HANS-*F3H*pro1k-Fd	CCAAGCTTGGCGCGCCGCGGCCGCATTTAAATTTACAAAACCATGTGCAAGAATG	HindIII, AscI, NotI, SwaI	*CmF3H*p
	Rv	SNM-*F3H*pro-Rv	ACTAGTGCTAGCACGCGTTTTTTATTTTTTCTTCACACACTTG	SpeI, NheI, MluI	
2	Fd	SSS-*NOS*ter-Fd	GAGCTCACTAGTGTCGACGATCGTTCAAACATTTGGCAATAAAG	SacI, EcoICRI (Ecl136II), SpeI, SalI	*NOS*t
	Rv	ESP-*NOS*ter-Rv	CGAATTCAGGCCTGTTTAAACGATCTAGTAACATAGATGACAC	EcoRI, SrfI, PmeI	
3	Fd	hFAStSw-pro*CmF3H*-Fd	AAGCTTGGCCGGCCTAGGCGCGCCAGGCCTATTTAAATTTACAAAACCATGTGCAAGAATG	HindIII, FseI, AscI, StuI, SwaI	*CmF3H*p
	Rv	SNM-*F3H*pro-Rv	ACTAGTGCTAGCACGCGTTTTTTATTTTTTCTTCACACACTTG		
4	Fd	SSS-ter*HSP*-Fd	GAGCTCACTAGTGTCGACATATGAAGATGAAGATGAAAT	EcoICRI, SacI, SpeI, SalI	*AtHSP*t
	Rv	KESP-ter*HSP*-Rv	GGTACCGGTCCGGAATTCGTTTAAACGCCCGGGCCTTATCTTTAATCATATTCCATAGTCC	KpnI, PmeI, SmaI, SrfI	
5	Fd	*CmF3'H*_full_ORF_F	ATGAACATTTTACCTTTCGTATTTTATG		*F3'H* ORF
	Rv	*CmF3'H*_full_ORF_R	TTAAATACTTTCATATACGTGGG		
6	Fd	*CmF3'H*_3'-Fd for dsRNA	CACCCCGAACTCATTCGTCATCCAC		*F3'H* RNAi trigger
	Rv	*CmF3'H*_3'-Rv for dsRNA	TCAATCCATACGCTTCTTCCATG		
7	Fd	ADH-*3'5'GT*-Fd	CAAGAAAAATAAATGGAAAACAATAAGCATGTC		*Ct3'5'GT*-5'

	Rv	Hind-*Ct3'5'GT*-Rv	AAGCTTGCGTTTTTAGCATCATTC	HindIII	
8	Fd	XbaI-*ADH*-Fd	ACGCGTTCTAGAGTCTATTTAACTCAGTATTC	XbaI	*NtADH*-5'UTR
	Rv	*Ct3'5'GT-ADH*-Rv	ATTGTTTTCCATTTATTTTTCTTGATTTCCTTCAC		
9	Fd	XbaI-*ADH*-Fd	ACGCGTTCTAGAGTCTATTTAACTCAGTATTC	XbaI	*NtADH*-5'UTR fused *Ct3'5'GT*-5'
	Rv	Hind-*Ct3'5'GT*-Rv	AAGCTTGCGTTTTTAGCATCATTC	HindIII	
10	Fd	NheI-*ADH*-Fd2	GCTAGCGTCTATTTAACTCAGTATTCAGAAAC	NheI	
	Rv	*Ct3'5'GT*-SacI-Rv	GAGCTCTTAGCTAGAGGAAATCATTTCCAC	SalI	