Supplementary Materials

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Anthocyanin synthesis potential in betalain-producing Caryophyllales plants

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Table S1. Primer sequences used in this study

Genotyping	
ANS-GT-L	GATTGTTTTACTAAAGAAAACAAA
ANS-GT-R	GTTAAAGCGCTTACATCGGTG
Complementation analysis	
spiANS-gw-F	CACCATGACGGCCACAATTGCTC
spiANS-gw-R	TTATGGATCTTGAGCTTCC
perANS-gw-F	CACCATGGTTACGCGTGCAATGGG
perANS-gw-R	CTAGTACGTTGGCTTCTCAT
ANS-F1	CGCGAAGTGTCTTCGTTTG
ANS-R3	GAAAAGCTGCAAACCCGGAA
spiA	TAGTCAGCAGGAGTCTTAGG
perANS-F	GATTTTATCAGTCCTATCAATCGGG
Actin-F1	GTGAAGGCTGGATTTGCAGGA
Actin-R1	AACCACCGATCCAGGCACTGT
Promoter-GUS transgenic lines	
SoANSs2	GCAAGCTTAGAGATATGATAGAGAT
SoANSas2	GAAGAAATATGGCTTGACTAAAGC
Transient expression assay	
AtPAP-S-Sal I	ATGCGTCGACATGGAGGGTTCGTCCAAAGG
AtPAP-AS-Not I	GTCAGCGGCCGCTCTAATCAAATTTCACAGT
Isolation of PAP homologs from the Caryophyllales	
PAPlikesearch1F	GG(A/G)GTGAG(A/G)AAAGGTGCATGGAC
PAPlikesearch1R	CCAGTA(A/G)TT(C/T)TTGACATCATT
PAPlikesearch2F	AA(A/G)AG(C/T)TG(C/T)AGATT(A/G)AG(A/G)TGG
PAPlikesearch2R	TTGACATCATTAGCGGTCC(G/T)(A/T)CC
TAIL-PCR/AD2	NGTCGA(G/C)(A/T)GANA(A/T)GAA
TAIL-PCR/AD5	(G/C) (G/C)TGG(G/C)TANAT(A/T)AT(A/T)CT
PgPAP5'TAIL1	CCCGGATGCCTACTAGCTATGAGTG
PgPAP5'TAIL2	GGATCAACACCCAAGGTAGGCAGG
PgPAP5'TAIL3	GGAGGAAACTAGCTAGCTAGTGTG
PgPAP3'TAIL1	GGGAGCTTCACCAATGAGGAGGTGG
PgPAP3'TAIL2	CACACTAGCTAGCTAGTTTCCTCC
PgPAP3'TAIL3	CCTGCCTACCTTGGGTGTTGATCC
PgPAP5'TAIL4	CCACCTCCTCATTGGTGAAGCTCCC
PgPAP5'TAIL5	GTTGTTAGAACGGATTTAATTTGCC
PgPAP3'TAIL4	GTAGGTGGTGTTTCTATCACTACTG
PgPAP5'TAIL6	GTTTGATGATGTGTTCCACCTCC
PgPAP3'TAIL5	GGAGGTGGAACACATCATCAAAC
PgPAP5'TAIL7	GAGCTTGTGAAGTTTGATGATGTG
PgPAP5'TAIL8	CATGCAACATGGGAACTTTGGAG
3', 5'-RACE	
ODTA	CACGCGTATCGATGTTTTTTTTTTTTTTTTTTTT
AUAP(dG)	GGCCACGCGTCGACTAGTACGGGGGGGGGGGGGG
BaPAPlike3'RACE 1	GATTGCTAGTAGGCTACCAGGGA
BaPAPlike3'RACE 2	CAGGGAGAACAGCAAATGATG
BaPAPlike5'RACE 1	GACCACCTATTGCCGTTGAG
BaPAPlike5'RACE 2	CGATGATATGTTCGACCTCCTC

BaPAP-F-SalI	ATGCGTCGACATGGGAGGAGTTGCATG
BaPAP-R-NotI	GTCAGCGGCCGCTCACAAAGACTGGG
MzPAPlike3'RACE 1	AAGAGTTGTAGATTGAGGTGGTT
MzPAPlike3'RACE 2	GGCAATAGGTGGTCACTCATAGCT
MzPAPlike5'RACE 1	GACCACCTATTGCCATAGAG
MzPAPlike5'RACE 2	TGATGATGTGTTCAACTTCCTC
MzPAP-F-SalI	ATGCGTCGACATGGGAGGAGTTGCATGGAC
MzPAP-R-NotI	GTCAGCGGCCGCTCAGAAAGAATCAGTCCATAG

RT-PCR

PgPAP-ACTIN-F	TCGAGACATTCAATGTCCCTGC
PgPAP-ACTIN-R	ACCTCTCAGCTCCGATAGTGAT
RT-PCR_PgPAP-F	GAGGAGTTGCCTGGACTGAA
RT-PCR_PgPAP-R	TCACAGAGAGTTAGTCCACAATTCC

Point mutation

V4GF	GAGGTGCCTGGACTGAAGAGGAA
V4GR	CTCCCATGTCGACTGTAATTG
F47LF	GGTTGAACTATTTACGTCCTAAC
F47LR	ATCTCAATCTGCAACTTTTTCGACAC
H66LF	CTCATCATCAAACTTCACAAGCTC
H66LR	TTCCACCTCCTCATTGGTGAA
K69RF	CATCAGGCTTCACAAGCTCTATGG
K69RR	ATGTGTTCCACCTCCTCATTGG
Y74LF	GCTCTTAGGAAATAGGTGGTCAC
Y74LR	TTGTGAAGTTTGATGATGTGTTCC
S83GF	GGTAGGCTACCGGGGAGGACAG
S83GR	AGCTATGAGTGACCACCTATTTCC

Table S2. List of genes in phylogenetic analysis

LjTT2a	BAG12893
LjTT2b	BAG12894
LjTT2c	BAG12895
AtTT2	Q9FJA2
ZmC1	AAA33482
ZmPL	AAB67721
PmMBF1	AAA82943
AmMIXTA	CAA55725
PhMYB1	CAA78386
AtMYB12	AAC83586
ZmP	AAC49394
AtGL1	BAA86879
AtWER	Q9SEI0
VvMYB5A	AAS68190
PhPH4	AAY51377
AtPAP1	AAG42001
AtPAP2	AAG42002
VvMYBA1	BAD18977
LeAN1	AAQ55181
PhAN2	AAF66727
BvMYB1	AET43457
PgPAP	MW192234
MzPAP	MW192235
BaPAP	MW192236

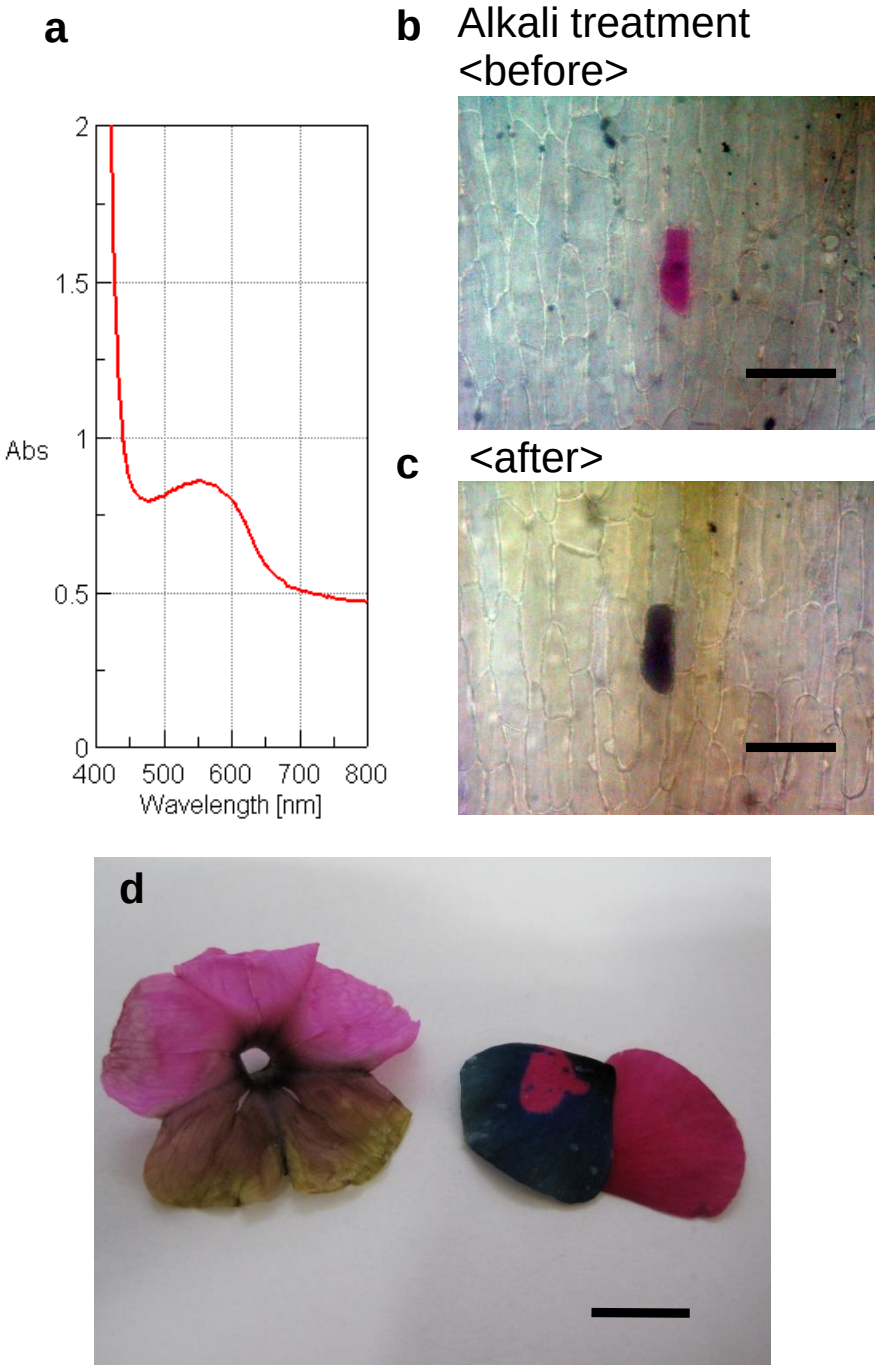


Fig. S1 **a** The absorption spectra of red cells were measured in the visible region (400–800 nm). Colors of transformed cells before (**b**) and after (**c**) alkali treatment with 25% ammonia water. Bars represent 50 μm. **d** Color change showing betacyanin accumulation in *Mirabilis jalapa* petals (left) and anthocyanin accumulation in *Rosa* sp. (right). Some petals were treated with 25% ammonia water. The bar represents 10 mm.

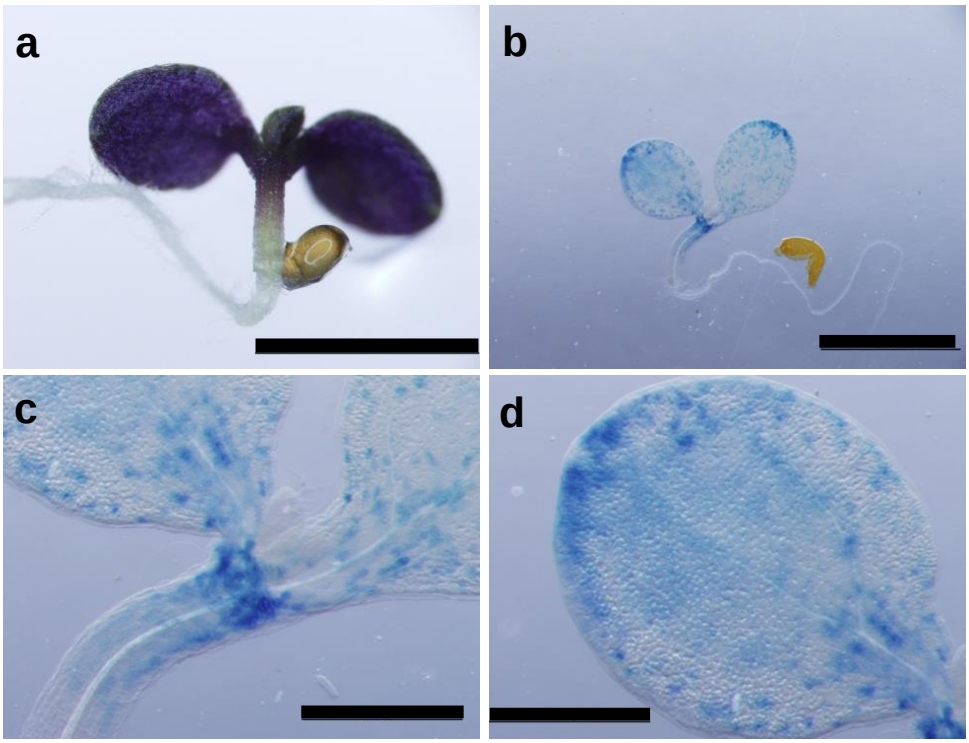


Fig. S2 **a** Anthocyanin-accumulating seedlings at 3 days after germination on 1/2 MS agar medium containing 5% sucrose. *SoANS promoter*::GUS expression pattern in (**b**) whole seedlings, (**c**) basilar cotyledons, and (**d**) cotyledons. Bars in **a** and **b** represent 2.0 mm. Bars in **c** and **d** represent 500 μm.

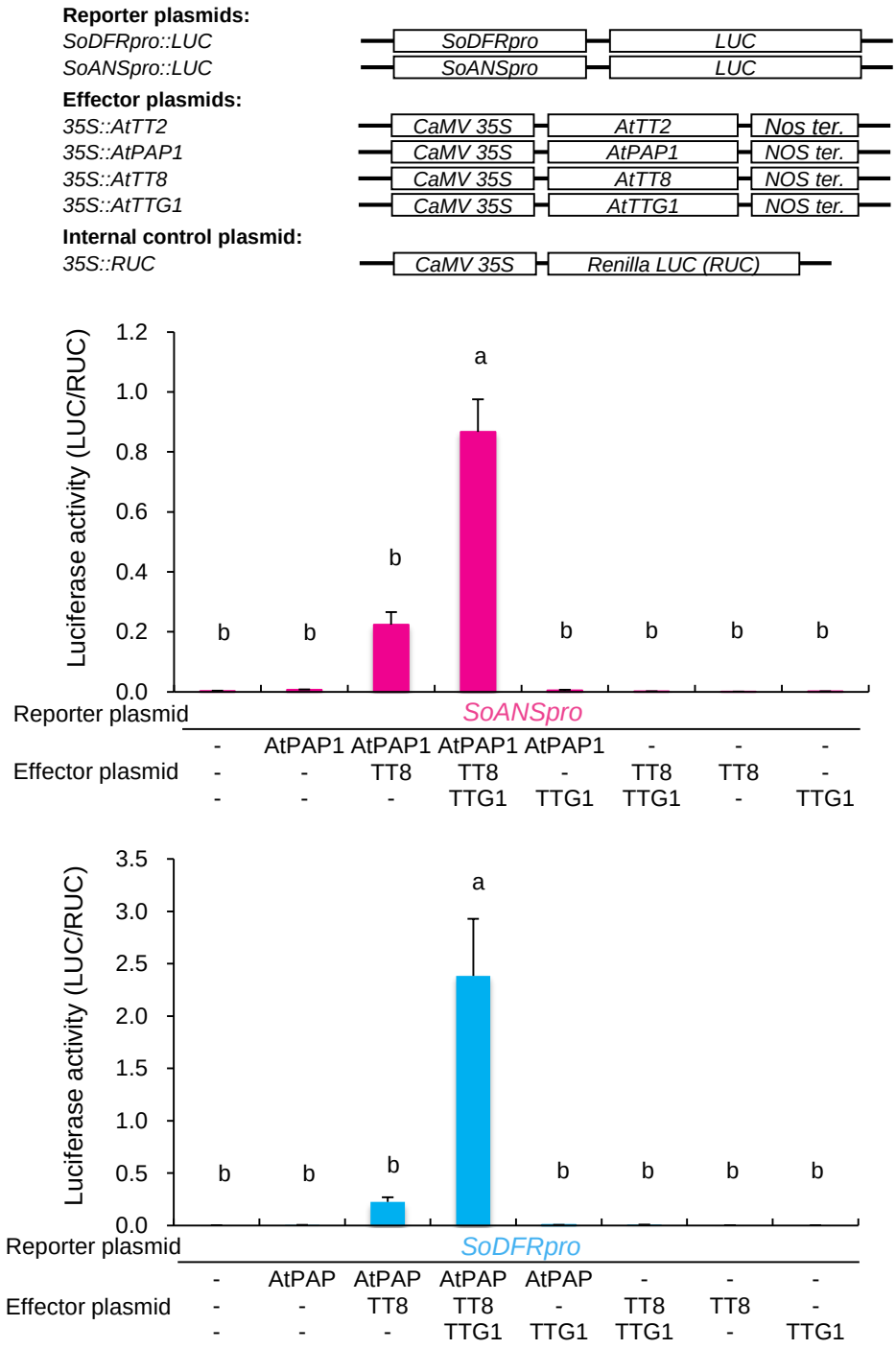
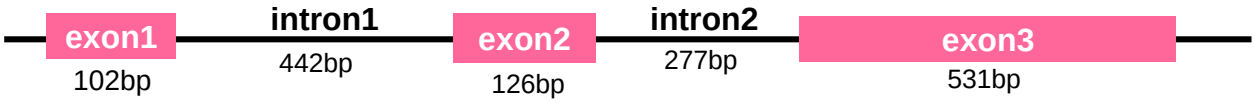


Fig. S3 Transient expression of the reporter constructs of *SoANSpro* and *SoDFRpro*, which contain *LUC* as a reporter gene, with or without expression of *Arabidopsis* transcription factors PAP1/TT8/TTG1 in particle-bombarded *Arabidopsis* leaves. Reporter gene activity, which was measured in the LUC enzyme, is expressed in arbitrary units after normalization to RUC activity expressed from a co-bombarded inner control plasmid, *35S-RUC* (mean ± s.d, $P < 0.05$ by Tukey's test, n = 3). The reporter and effector plasmid constructs (described in Materials and Methods) are shown in the upper part of the figure.

a



b

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-836 GGCCCAATCAATGAAATCACAATGAAATACCCAAACATTTTTTCATATTTTATATTTATT -777

-776 TTCAAAAAAAATGATTCACCTTTTCAATTAAAATATAATATCCCACCACTTTCTAACCTA -717
                                                              MRE
-716 ACACAACTAAATCAATCTAAAATTTCTAACCTAACTTAACTTAAAACTATAGCAATTTAA -657
                                    MRE
-656 ACAACTCACTAAAAGAACATGGTAATTAATAAGAAATTGGGTCTTTAATCTTTTTTTTAT -597
                                    POLLEN1LELAT52
-596 TATTATGGAAGAGTCTTTAATCTTAATAGCACATTTGAATCAATACCTTCTAGGTTTACT -537

-536 TCTTAATCATTTCAGTACTATAAAAATAATACTGATCATCAATAATCGTCTCGCAATATA -477

-476 TTTTCAACACACACATGCATAGATATTTGTTAATGACTTAAACAAGCAGAAACCCCATAT -417
                    RY          GARE  Skn-1      POLLEN1LELAT52
-416 ATAATGATTTGCTAATTATAAGTAAGTGACGAAAGGGCCAGCAAAGTGCATAGTACAAGT -357
                                TGACG-motif
-356 CAACCTGTAGATAGCATGCAGAAGCAGAGGGACAAGAGTACTAGAGAAATAAAAAGTGGT -297
                    RY                          POLLEN1LELAT52
-296 GGAGGCCCGGATTTGTGTTTTGGTGCCTTCCCTTTTATCAAGTTGATAGCCTCCAAAATC -237

-236 TAAAGTACTCCTCATTATTGCTCACTGGAATCCCAAATCTGAGTGAGAAAATTAAAGTAC -177
                                                POLLEN1LELAT52
-176 AACCCACATTGAATAAATAGCTAGCTAGCTATTGAGATCTGTGCAACAATCATCACTGAT -117

-116 ATATCTTCTTCAATTCAGTACCCAGGTACACAAGGAAGAATTGTTACGTTATTCACAAGT -57

 -56 GTATGATCGAGCAAGCAAACTAATAATAATCTGGAGCACAGCTGCGCTAGCAAACGATG  +3
                                                              M
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Fig. S4 PgPAP genome sequence **a** Genomic organization of the PgPAP gene (GenBank accession No. MW192237). Numbers under the lines and boxes indicate the number of base pairs in each intron and exon, respectively. **b** Sequences of promoter of *P. grandiflora* ANS. The putative translation initiation codon (ATG) is underlined. In the promoter, four POLLEN1LELAT52, which is involved in pollen-specific transcription; two MREs, which are involved in light regulation; and two RY motifs, which are conserved in seed specific promoters, were found. Skn-1, which is involved in endosperm formation; a gibberellin responsive element (GARE); and a methyl jasmonate responsive motif (TCAGC), were found.

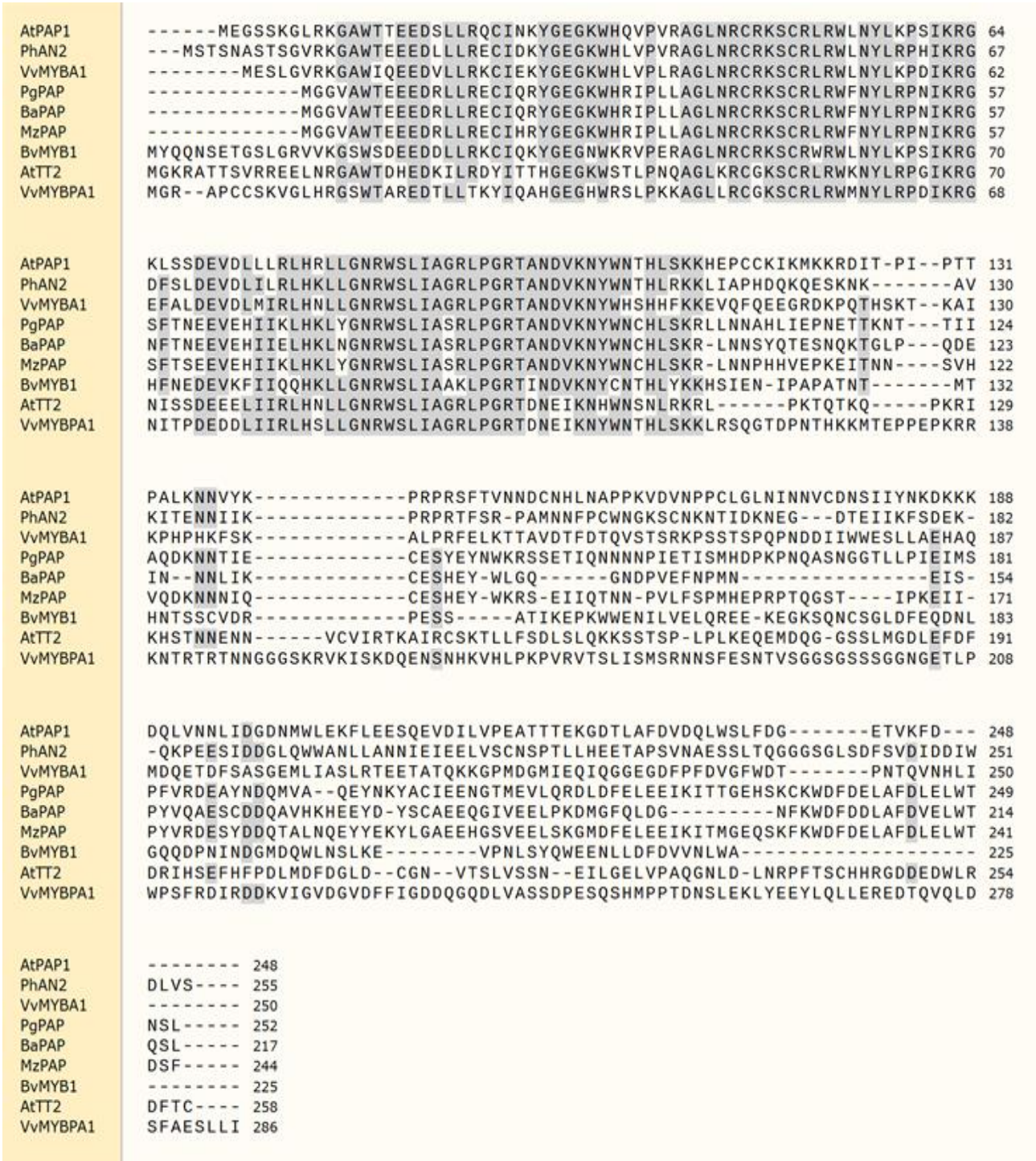

Fig. S5 Comparison of the amino acid sequences of PAP and TT2 homorogs. Accession numbers of genes are listed in Supplementary Table S2.

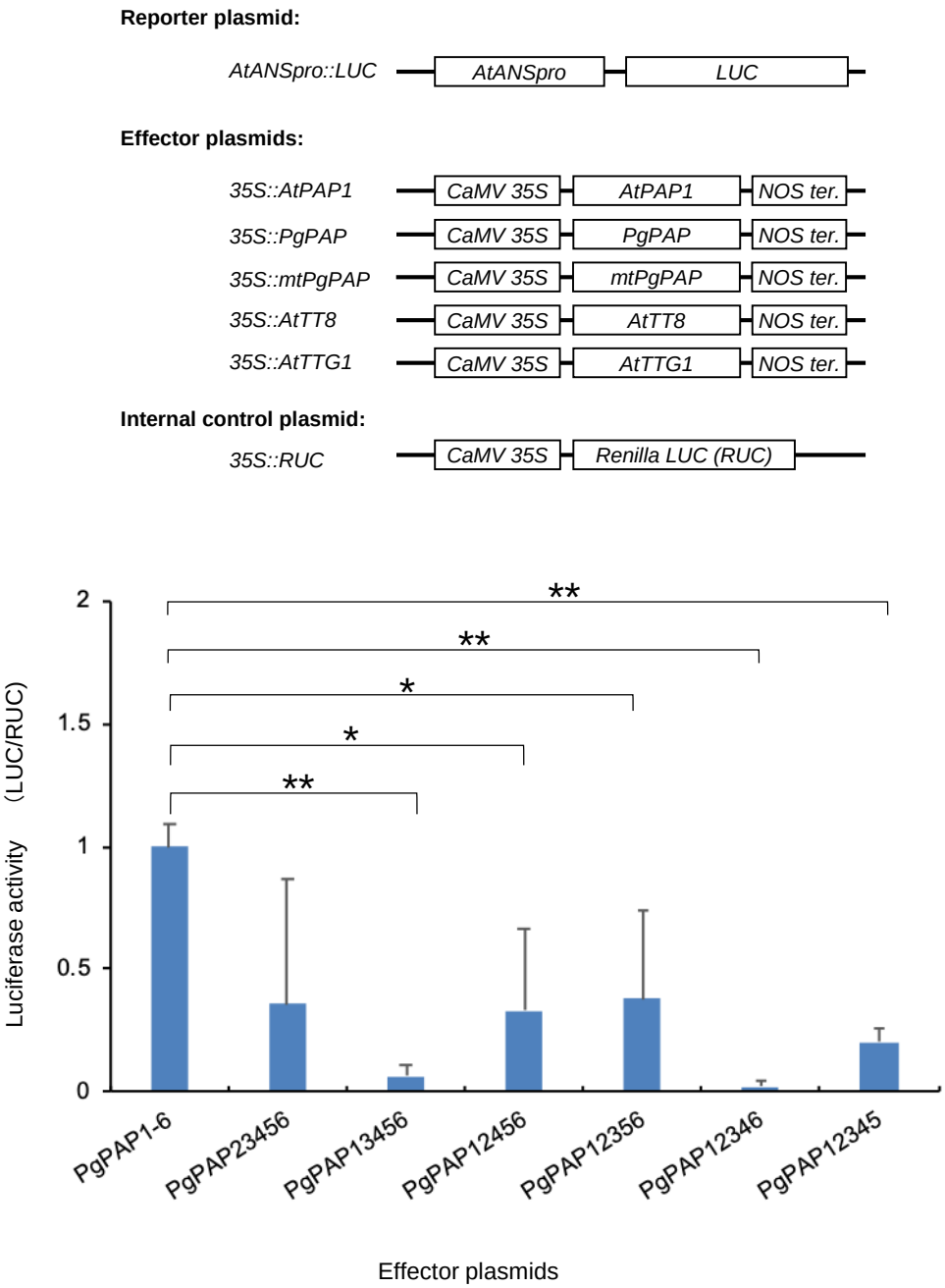

Fig. S6 Transactivation of *AtANS promoter* by PgPAP1-6 (mtPgPAP) replaced five of six amino acids common among PAPs of anthocyanin-producing plants. The upper panel shows the reporter and effector plasmid constructs (mean ± s.d., $^{**}P < 0.01$, $^{*}P < 0.05$ by Student's t-test, n = 3).