

Article

# The Regulation of Floral Colour Change in *Pleroma raddianum* (DC.) Gardner

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## SUPPLEMENTAL MATERIAL

**Figure S1.** Agarose gel (0.8%) showing the cloned fragments. The abbreviations indicate: PHENYLALANINE AMMONIUM LYASE (PAL), CINAMMATE 4-HYDROXYLASE (C4H), CHALCONE SYNTHASE (CHS), FLAVONOL SYNTHASE (FLS) and ANTHOCYANIDIN SYNTHASE (ANS), ELONGATION FACTOR 1- $\alpha$  (EF1) and RIBOSOMAL PROTEIN S13 (RPS) genes.

**Figure S2.** Phenograms for PHENYLALANINE AMMONIUM LYASE (PAL) (A), CINAMMATE 4-HYDROXYLASE (C4H) (B), CHALCONE SYNTHASE (CHS) (C), FLAVONOL SYNTHASE (FLS) (D), and ANTHOCYANIDIN SYNTHASE (ANS) (E) amino acid sequences. Only sequences from species with full sequenced genomes were included in the analyses: *Eucalyptus grandis* (Eucgr), *Arabidopsis thaliana* (AT), *Brassica rapa* (Brara), *Medicago trunculata* (Medtr), *Trifolium pratense* (Tp) and *Solanum lycopersicum* (Soly). Trees were constructed using the following parameters: Neighbor-joining, Bootstrap of 1,000 replicates and the best model test for each analysis. Grey boxes show the clusters for *Pleroma raddianum* (T.pulchra) and *E. grandis* sequences.

**Figure S3.** *Pleroma raddianum* flowers need light to turn from white to purple colour. Plants were maintained indoor under low light irradiance. After 24 h (A to B) white flowers did not homogenously turn to pink (D) and fell down to following day (B to C). (E). Normal pink flower at S3 stage.

**Figure S4.** Alignments used for primer design to clone the partial gene sequences of PHENYLALANINE AMMONIUM LYASE (PAL) (A), CINAMMATE 4-HYDROXYLASE (C4H) (B), CHALCONE SYNTHASE (CHS) (C), FLAVONOL SYNTHASE (FLS) (D) and ANTHOCYANIDIN SYNTHASE (ANS) (E). Boxes indicate the primers sequences.

**Figure S5.** RNA integrity. Agarose gel (1%) for analysis of RNA integrity, approximately 500  $\mu$ g of each sample was loaded. The numbers indicate the biological replicates (1 to 5) for each stage (S1 to S4). S1–buds (day 0), S2–white flowers (day 1), S3–pink (day 2), S4–purple (day 3).

**Table S1.** Sugar, organic acids and phenolic acids analysed by GC-EIMS in each developmental stage of *Pleroma raddianum* flowers. S1–buds (day 0), S2–white flowers (day 1), S3–pink (day 2), S4–purple (day 3).

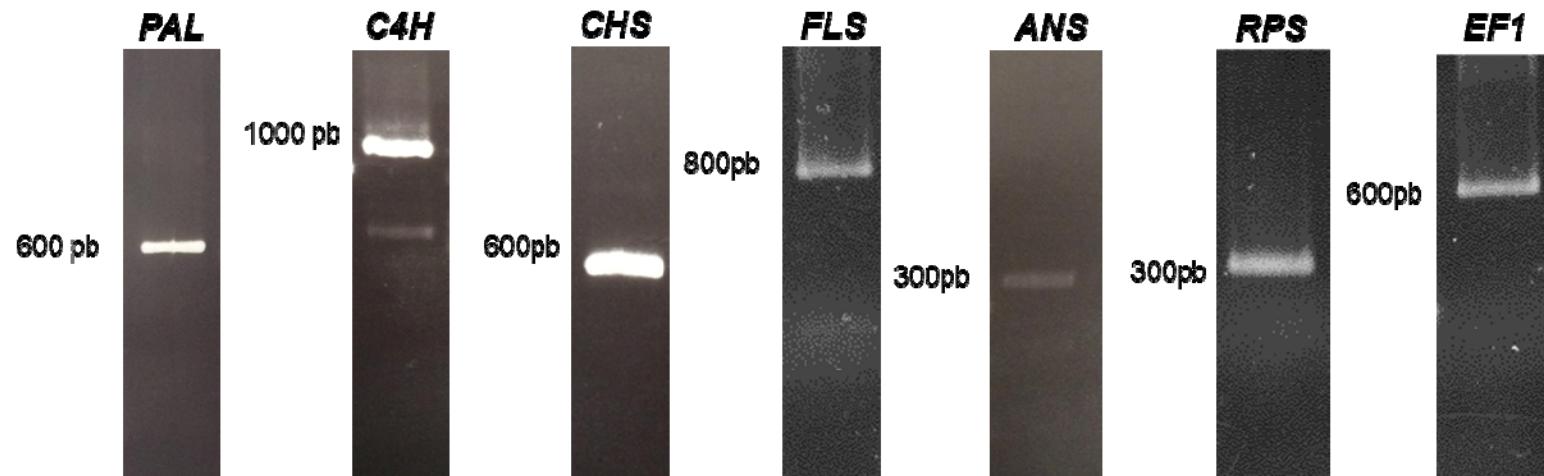
**Table S2.** Pigment profile along *Pleroma raddianum* flower development. S1–buds (day 0), S2–white flowers (day 1), S3–pink (day 2), S4–purple (day 3).

**Table S3.** Identity of obtained cDNA fragments from *Pleroma raddianum* with *Eucalyptus grandis* sequences.

**Table S4.** Standard curves parameters.

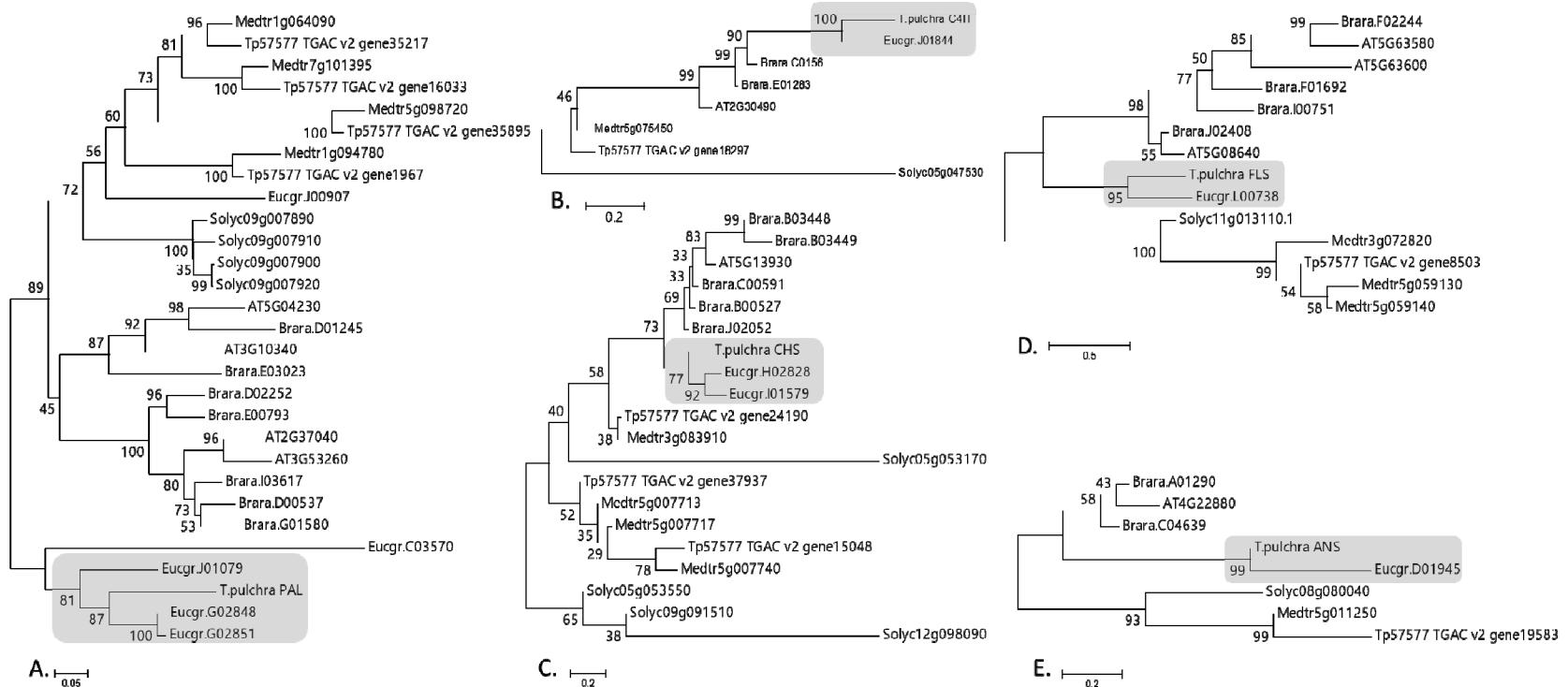
**Table S5.** Primers used for gene cloning and RT-qPCR.

**Table S6.** RNA quantification by nanodrop. The numbers indicate the biological replicates (1 to 5) for each stage (S1 to S4). S1–buds (day 0), S2–white flowers (day 1), S3–pink (day 2), S4–purple (day 3).



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3 *CINAMMATE 4-HYDROXYLASE (C4H)*, *CHALCONE SYNTHASE (CHS)*, *FLAVONOL SYNTHASE (FLS)* and *ANTHOCYANIDIN SYNTHASE (ANS)*, *ELONGATION FACTOR 1- $\alpha$  (EF1)*  
4 *and RIBOSOMAL PROTEIN S13 (RPS) genes.*

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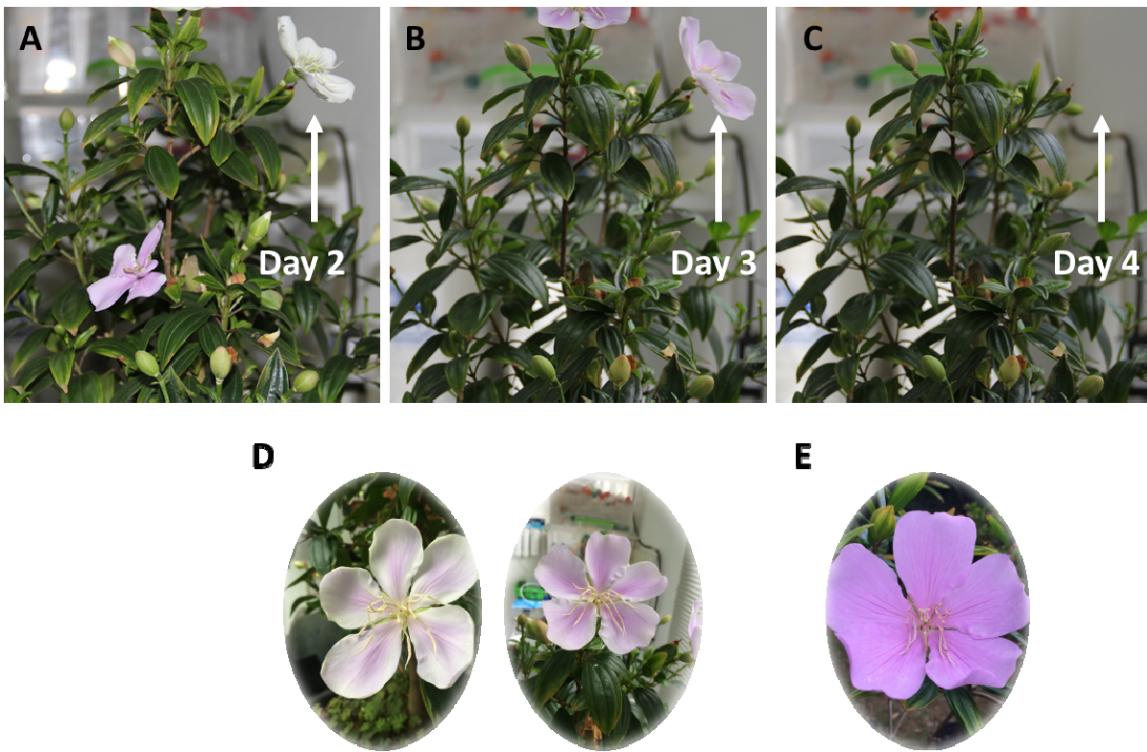
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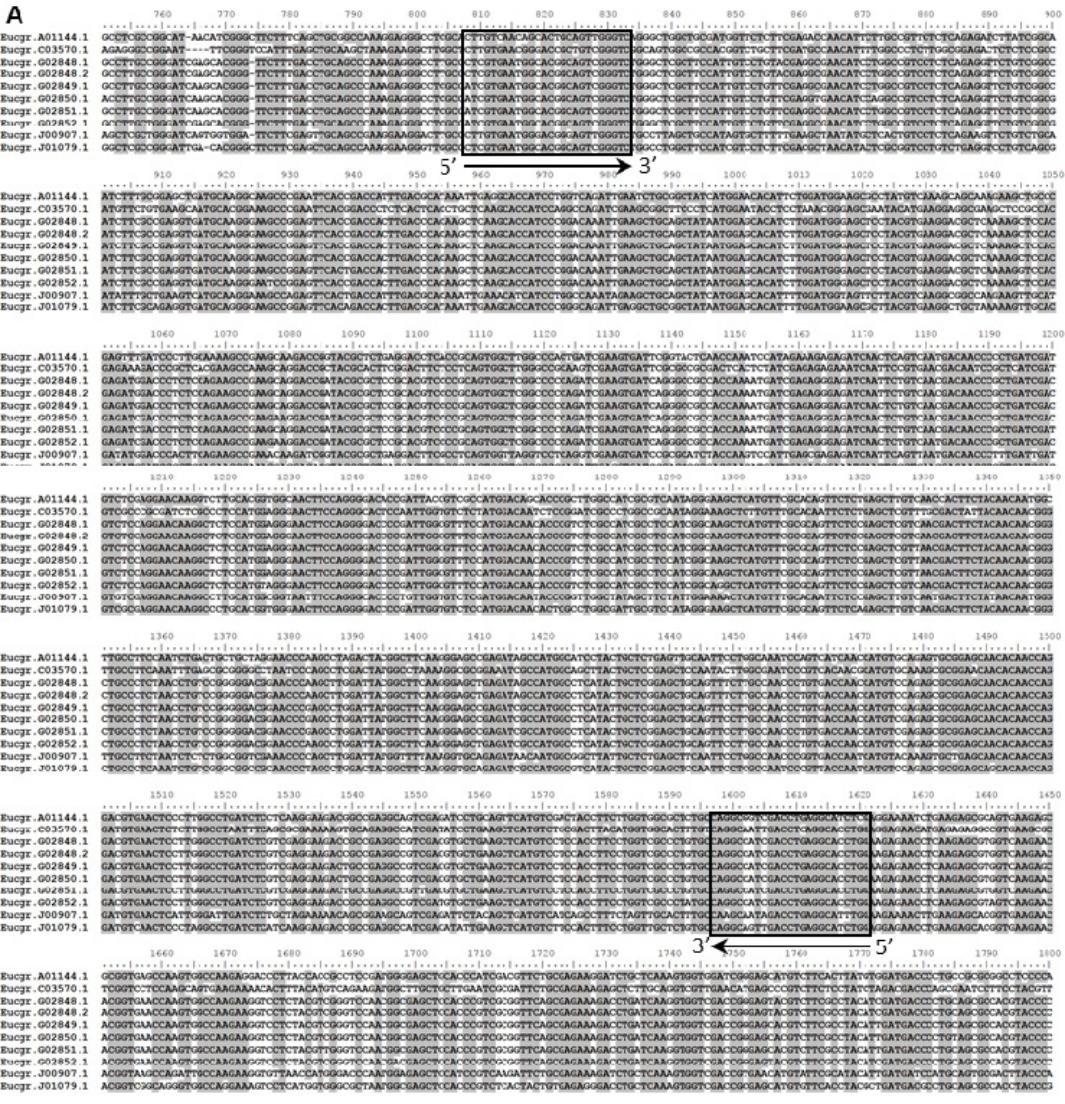


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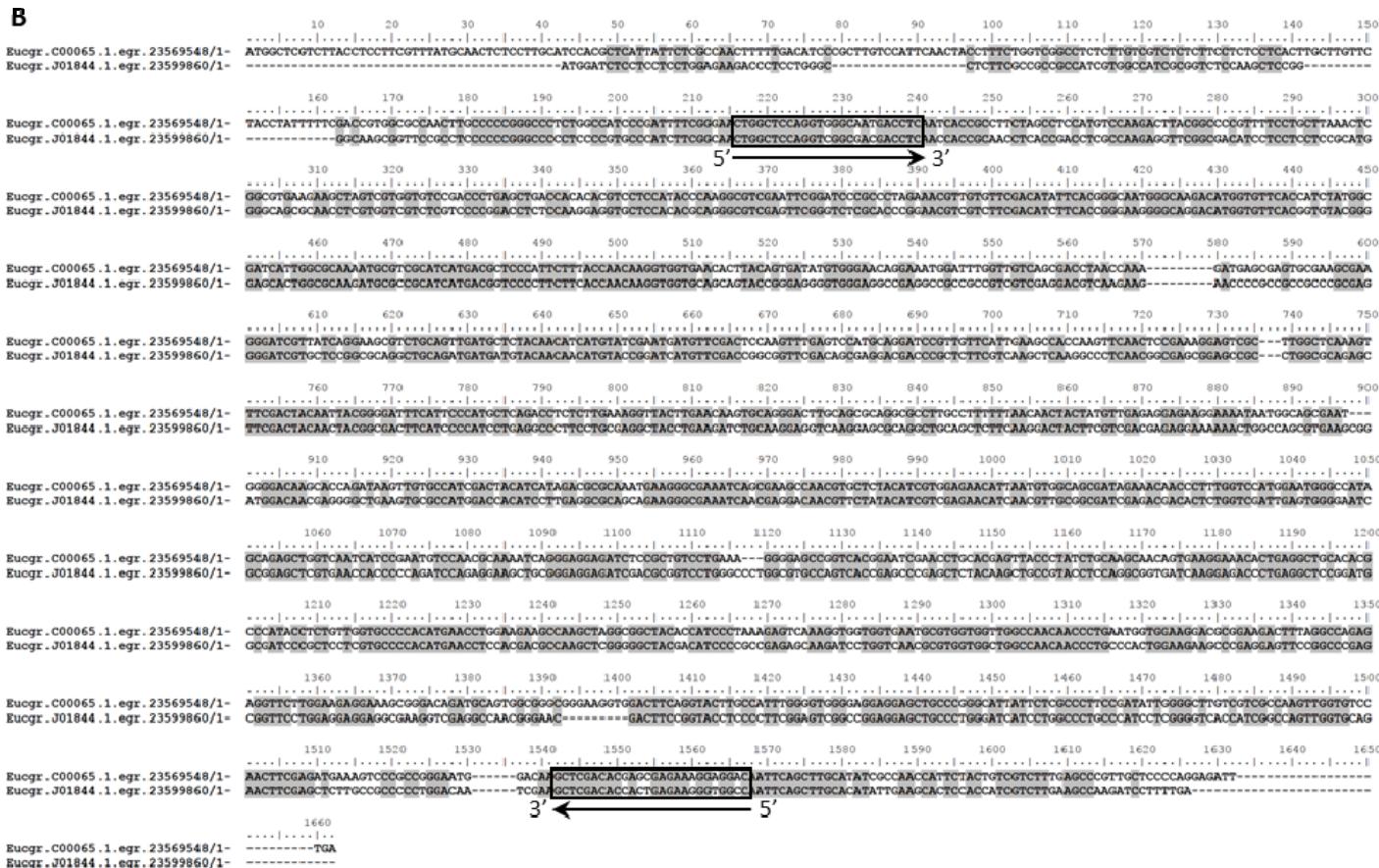
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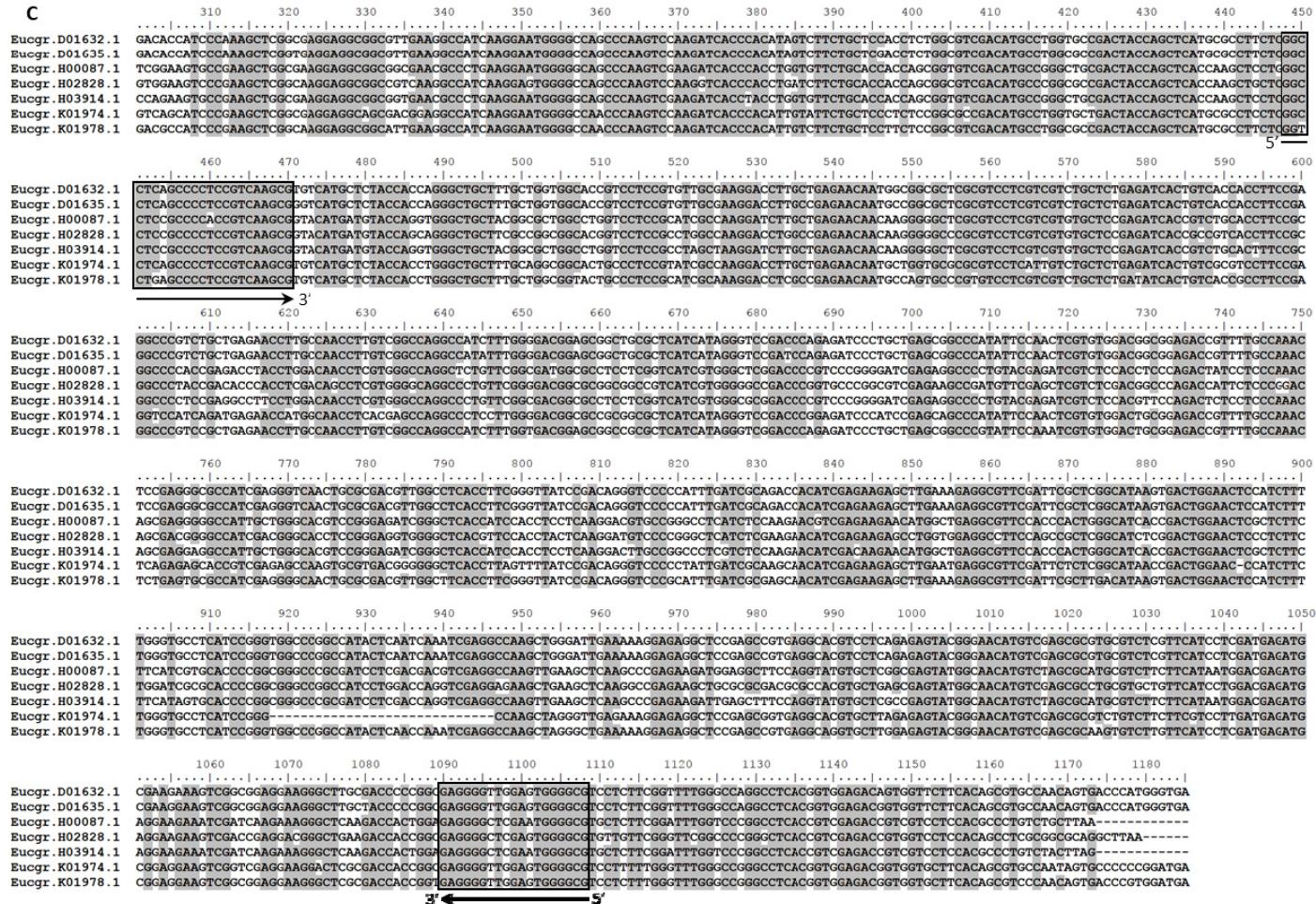
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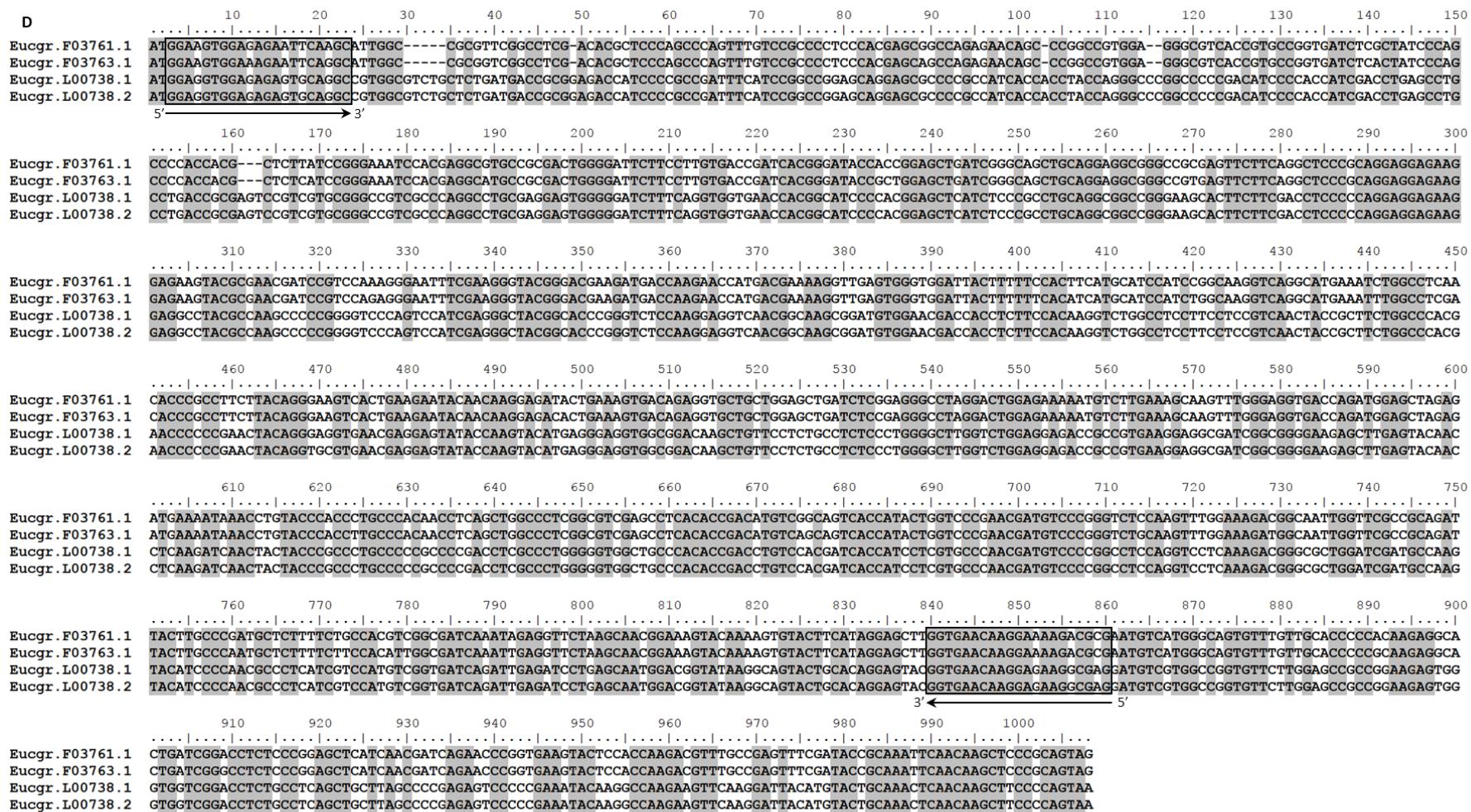
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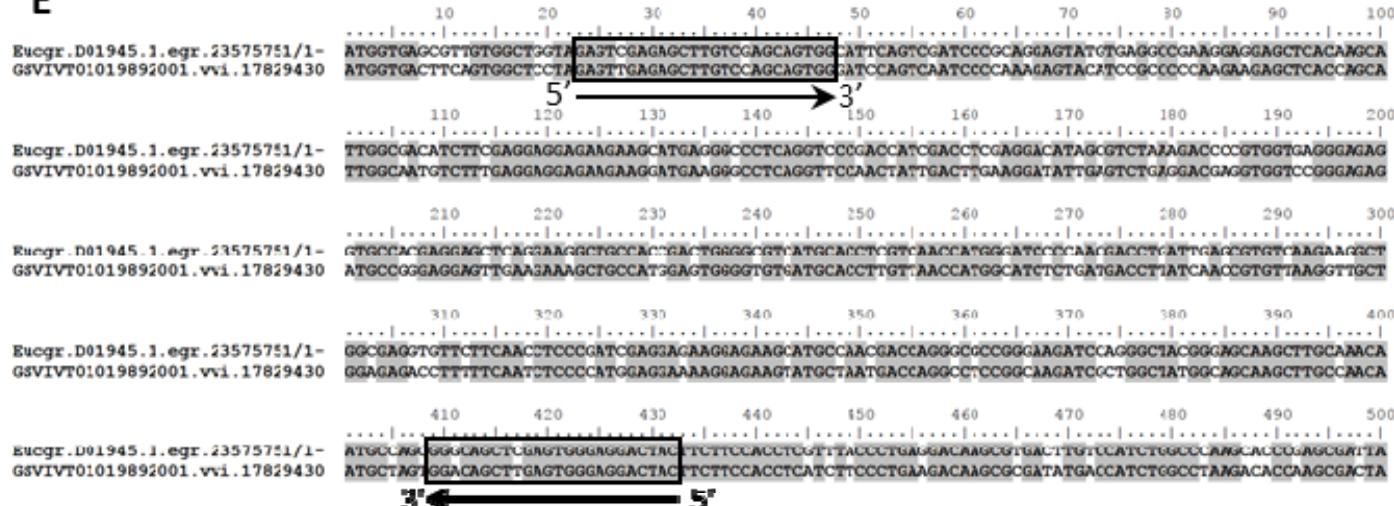
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**Figure S4.** Alignments used for primer design to clone the partial gene sequences of PHENYLALANINE AMMONIUM LYASE (PAL) (A), CINAMMATE 4-HYDROXYLASE (C4H) (B), CHALCONE SYNTHASE (CHS) (C), FLAVONOL SYNTHASE (FLS) (D) and ANTHOCYANIDIN SYNTHASE (ANS) (E). Boxes indicate the primers sequences.





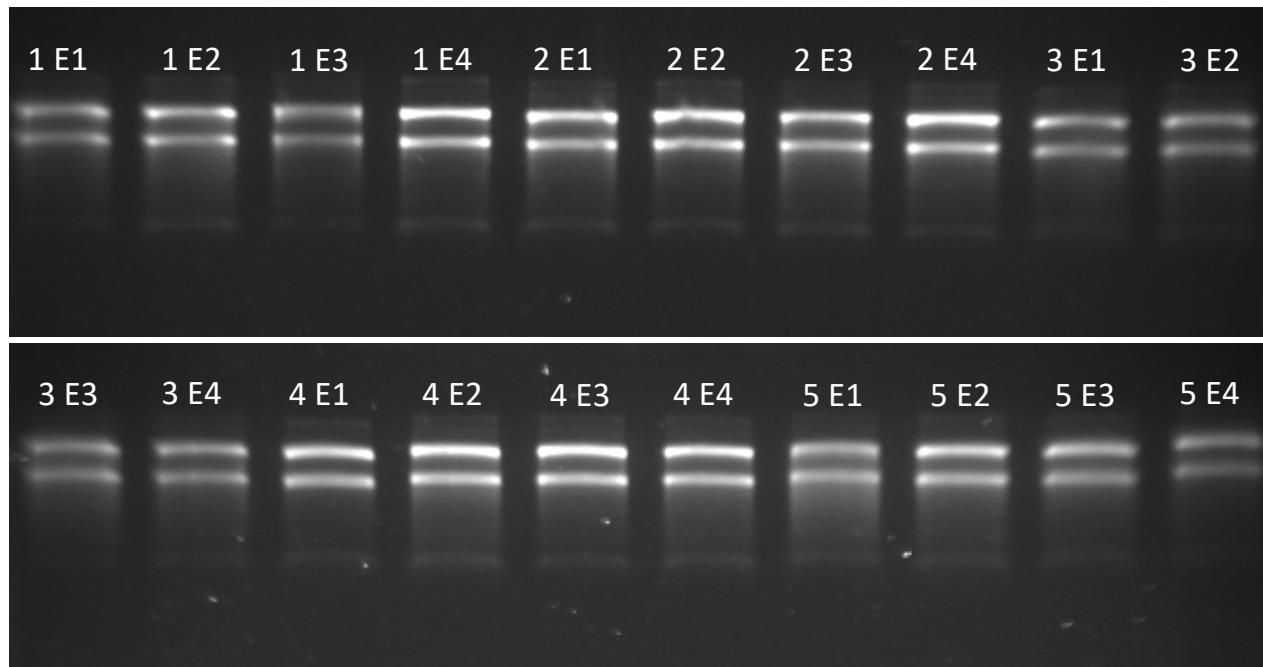


**E**

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Figure S4. (Continued)



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**Figure S5.** RNA integrity. Agarose gel (1%) for analysis of RNA integrity, approximately 500 µg of each sample was loaded. The numbers indicate the biological replicates (1 to 5) for each stage (S1 to S4). S1–buds (day 0), S2–white flowers (day 1), S3–pink (day 2), S4–purple (day 3).

35           **TableS1.** Sugar, organic acids and phenolic acids analysed by GC-EIMS in each developmental stage  
 36           of *Pleroma raddianum* flowers. S1–buds (day 0), S2–white flowers (day 1), S3–pink (day 2), S4–purple  
 37           (day 3).

Carbohydrates	S1 (mg g <sup>-1</sup> DW)	S2 (mg g <sup>-1</sup> DW)	S3 (mg g <sup>-1</sup> DW)	S4 (mg g <sup>-1</sup> DW)
1- Glycerol	4.34 ± 0.59	2.98 ± 1.49	2.76 ± 0.74	3.78 ± 1.05
<b>2- Myo-inositol</b>	<b>25.00 ± 2.76<sup>a</sup></b>	<b>21.93 ± 4.51<sup>ab</sup></b>	<b>19.52 ± 4.03<sup>ab</sup></b>	<b>16.59 ± 1.83<sup>b</sup></b>
<b>Total sugar alcohol<sup>np</sup></b>	<b>22.61 ± 13.28<sup>a</sup></b>	<b>24.91 ± 3.58<sup>a</sup></b>	<b>22.28 ± 3.42<sup>ab</sup></b>	<b>20.37 ± 1.42<sup>b</sup></b>
3- Ribose	0.42 ± 0.12	1.33 ± 0.10	0.71 ± 0.38	0.92 ± 0.70
<b>4- Xylose<sup>np</sup></b>	<b>2.79 ± 1.25<sup>a</sup></b>	<b>6.85 ± 0.44<sup>b</sup></b>	<b>3.02 ± 2.13<sup>a</sup></b>	<b>5.72 ± 1.40<sup>ab</sup></b>
<b>5- Fructose</b>	<b>199.58 ± 60.88<sup>b</sup></b>	<b>500.06 ± 61.65<sup>a</sup></b>	<b>474.17 ± 85.72<sup>a</sup></b>	<b>492.13 ± 124.60<sup>a</sup></b>
<b>6- Glucose<sup>np</sup></b>	<b>25.07 ± 12.99<sup>b</sup></b>	<b>75.91 ± 2.53<sup>ab</sup></b>	<b>82.75 ± 2.41<sup>a</sup></b>	<b>72.66 ± 3.58<sup>a</sup></b>
<b>7- Galactose</b>	<b>112.08 ± 44.44<sup>b</sup></b>	<b>324.82 ± 35.46<sup>a</sup></b>	<b>294.86 ± 52.55<sup>a</sup></b>	<b>279.70 ± 62.15<sup>a</sup></b>
<b>Total soluble sugar</b>	<b>361.92 ± 112.79<sup>b</sup></b>	<b>902.71 ± 81.55<sup>a</sup></b>	<b>876.29 ± 133.88<sup>a</sup></b>	<b>857.64 ± 209.19<sup>a</sup></b>
Organic acids	S1 (mg g <sup>-1</sup> DW)	S2 (mg g <sup>-1</sup> DW)	S3 (mg g <sup>-1</sup> DW)	S4 (mg g <sup>-1</sup> DW)
<b>8- Succinic acid</b>	<b>0.08 ± 0.04<sup>b</sup></b>	<b>0.24 ± 0.05<sup>a</sup></b>	<b>0.28 ± 0.03<sup>a</sup></b>	<b>0.22 ± 0.05<sup>a</sup></b>
<b>9- Malic acid</b>	<b>20.83 ± 12.97<sup>c</sup></b>	<b>92.52 ± 18.00<sup>b</sup></b>	<b>116.66 ± 16.51<sup>ab</sup></b>	<b>129.41 ± 27.11<sup>a</sup></b>
10- Citric acid	4.71 ± 2.46	5.81 ± 1.16	6.40 ± 0.63	6.96 ± 0.73
<b>Total</b>	<b>25.60 ± 14.87<sup>c</sup></b>	<b>98.48 ± 18.41<sup>b</sup></b>	<b>121.68 ± 17.89<sup>ab</sup></b>	<b>136.55 ± 26.81<sup>a</sup></b>

38 DW–Dry weight. Compounds highlighted in bold showed statistically significant differences between stages.

39 Letters indicate statistically significant different values. Parameters indicated with a superscript *np* (<sup>np</sup>)  
 40 had non-parametrical distribution and the Kruskal-Wallis test was applied.

**Table S2.** Pigment profile along *Pleroma raddianum* flower development. S1–buds (day 0), S2–white flowers (day 1), S3–pink (day 2), S4–purple (day 3).

Phenolic acids	S1 (mg g <sup>-1</sup> DW)	S2 (mg g <sup>-1</sup> DW)	S3 (mg g <sup>-1</sup> DW)	S4 (mg g <sup>-1</sup> DW)
1- Phenolic acid	4.43 ± 2.96	5.9 ± 2.67	3.76 ± 2.46	7.6 ± 3.08
2- Cinnamic acid derivative <sup>np</sup>	3.89 ± 2.28	4.18 ± 3.85	6.16 ± 1.50	3.53 ± 3.41
3- Phenolic acid <sup>np</sup>	0.84 ± 0.91	1.55 ± 0.17	1.85 ± 0.20	1.92 ± 0.21
4- Phenolic acid <sup>np</sup>	0.38 ± 0.75	0.14 ± 0.22	0.14 ± 0.21	0.15 ± 0.20
5- Cinnamic acid derivative <sup>np</sup>	0.23 ± 0.23	0.57 ± 0.62	0.22 ± 0.16	0.26 ± 0.27
<b>6- Phenolic acid</b>	<b>0.90 ± 0.61<sup>b</sup></b>	<b>5.24 ± 1.33<sup>a</sup></b>	<b>4.19 ± 1.08<sup>a</sup></b>	<b>4.23 ± 0.94<sup>a</sup></b>
<b>Total phenolic acids</b>	<b>10.69 ± 3.43<sup>b</sup></b>	<b>17.58 ± 2.66<sup>a</sup></b>	<b>16.39 ± 2.24<sup>a</sup></b>	<b>17.69 ± 0.49<sup>a</sup></b>
Flavonols	S1 (mg g <sup>-1</sup> DW)	S2 (mg g <sup>-1</sup> DW)	S3 (mg g <sup>-1</sup> DW)	S4 (mg g <sup>-1</sup> DW)
7- N.I.	0.40 ± 0.41	0.70 ± 0.13	0.49 ± 0.40	0.57 ± 0.34
8- Myricetin hexoside	0.77 ± 0.47	0.56 ± 0.19	0.56 ± 0.08	0.58 ± 0.18
9- Myricetin hexoside	0.29 ± 0.21	0.22 ± 0.13	0.23 ± 0.07	0.27 ± 0.06
10- Quercetin galloylhexoside	0.41 ± 0.30	0.22 ± 0.10	0.23 ± 0.06	0.23 ± 0.16
11- Quercetin hexoside	1.26 ± 0.65	1.02 ± 0.37	0.98 ± 0.19	0.94 ± 0.16
12- Quercetin glucuronide <sup>np</sup>	0.16 ± 0.07	0.06 ± 0.03	0.07 ± 0.04	0.11 ± 0.06
13- Kaempferol galloylhexoside <sup>np</sup>	1.61 ± 0.85	1.36 ± 0.61	1.34 ± 0.22	1.24 ± 0.33
14- N.I. <sup>np</sup>	0.19 ± 0.12	0.14 ± 0.08	0.14 ± 0.08	0.17 ± 0.09
15- Kaempferol hexoside	3.40 ± 1.30	3.20 ± 0.91	3.12 ± 0.55	2.92 ± 0.42
16- Kaempferol galloylhexoside <sup>np</sup>	2.16 ± 0.94	1.78 ± 0.72	1.78 ± 0.46	1.70 ± 0.23
17- Kaempferol 3-O-β-D-glucopyranoside/ kaempferol-(2''-O-methyl)-4'-O-α-D-glucopyranoside	6.48 ± 1.51	6.32 ± 0.85	6.44 ± 0.50	6.21 ± 0.47
18- Kaempferol pentoside	2.75 ± 1.43	2.41 ± 1.07	2.23 ± 0.63	1.96 ± 0.30
19- Kaempferol galloylhexoside	0.24 ± 0.14	0.22 ± 0.11	0.18 ± 0.07	0.16 ± 0.04
20- Kaempferol pentoside	0.23 ± 0.15	0.15 ± 0.10	0.14 ± 0.07	0.11 ± 0.05
21- Kaempferol <i>p</i> -coumaroylhexoside	0.53 ± 0.29	0.46 ± 0.26	0.40 ± 0.18	0.29 ± 0.07
23- Kaempferol 3-O-glucuronide-6"-O-methylester	0.28 ± 0.15	0.29 ± 0.09	0.21 ± 0.01	0.17 ± 0.05

**Table S2.** (Continued)

Flavonols	S1 (mg g <sup>-1</sup> DW)	S2 (mg g <sup>-1</sup> DW)	S3 (mg g <sup>-1</sup> DW)	S4 (mg g <sup>-1</sup> DW)
25- Quercetin 3-O-(6"-O- <i>p</i> -coumaroyl)- $\beta$ -D-glucopyranoside <sup>np</sup>	0.68 ± 0.38	0.52 ± 0.19	0.44 ± 0.08	0.40 ± 0.05
26- N.I.	0.43 ± 0.21	0.35 ± 0.20	0.31 ± 0.13	0.26 ± 0.04
27-Kaempferol 3-O-(6"-O- <i>p</i> -coumaroyl)- $\beta$ -D-glucopyranoside <sup>np</sup>	1.44 ± 0.65	1.20 ± 0.46	1.05 ± 0.00	0.94 ± 0.12
28- Kaempferol <i>p</i> -coumaroylhexaside	0.19 ± 0.09	0.16 ± 0.06	0.10 ± 0.03	0.08 ± 0.02
29- Kaempferol	0.94 ± 0.48	1.05 ± 0.44	0.83 ± 0.20	0.71 ± 0.08
<b>30- Kaempferol <i>p</i>-coumaroylhexaside<sup>np</sup></b>	<b>0.00 ± 0.00<sup>b</sup></b>	<b>0.08 ± 0.00<sup>b</sup></b>	<b>0.13 ± 0.03<sup>a</sup></b>	<b>0.13 ± 0.03<sup>a</sup></b>
Total Flavonols	24.99 ± 10.31	22.17 ± 6.66	21.25 ± 3.13	19.94 ± 2.64
Anthocyanins	S1 (μg g <sup>-1</sup> DW)	S2 (μg g <sup>-1</sup> DW)	S3 (μg g <sup>-1</sup> DW)	S4 (μg g <sup>-1</sup> DW)
22- Petunidin <i>p</i> -coumaroylhexaside acetylpentoside <sup>np</sup>	0.00 ± 0.00 <sup>c</sup>	0.00 ± 0.00 <sup>c</sup>	8.43 ± 2.21 <sup>b</sup>	22.42 ± 8.00 <sup>a</sup>
24- Malvidin <i>p</i> -coumaroylhexaside acetylpentoside <sup>np</sup>	0.00 ± 0.00 <sup>c</sup>	0.00 ± 0.00 <sup>c</sup>	19.87 ± 5.28 <sup>b</sup>	53.72 ± 20.18 <sup>a</sup>
<b>Total Anthocyanins<sup>np</sup></b>	<b>0.00 ± 0.00<sup>c</sup></b>	<b>0.00 ± 0.00<sup>c</sup></b>	<b>28.29 ± 7.47<sup>b</sup></b>	<b>76.14 ± 28.11<sup>a</sup></b>

DW-Dry weight. NI- not identified. Compounds highlighted in bold showed statistically significant differences between stages. Letters indicate statistically significant different values in each line. Parameters indicated with a superscript np (<sup>np</sup>) had non-parametrical distribution and the Kruskal-Wallis test was applied.

**Table S3.** Identity of obtained cDNA fragments from *Pleroma raddianum* with *Eucalyptus grandis* sequences.

Gene	Enzyme	Nucleotides number in partial sequence of <i>P. raddianum</i>	Ammino acids number in partial sequence of <i>P. raddianum</i>	Identity of ammino acids (%) with <i>E. grandis</i>
PAL	PHENYLALANINE AMMONIA-LYASE	633	206	95
C4H	CINNAMATE 4-HYDROXYLASE	1,073	357	87
CHS	CHALCONE SYNTHASE	621	207	88
FLS	FLAVONOL SYNTHASE	817	272	69
ANS	ANTHOCYANIDIN SYNTHASE	361	120	83
RPS	RIBOSOMAL PROTEIN S13	371	123	98
EF1	ELONGATION FACTOR 1 $\alpha$	694	231	93

**Table S4.** Standard curves parameters.

Compound	R <sup>2</sup>	equation
p-coumaric	0.99	y=12955x
kaempferol	0.97	y=49285x
cyanidin	0.99	y=364963x

**Table S5.** Primers used for gene cloning and RT-qPCR.

Gene	Description	Forward	Melting temperature	Reverse	Melting temperature	Amplicon	Use
<i>PAL</i>	flavonoid biosynthesis	MTYGTSAAYRGCACBGSWGTYGGTC	65.3°C	CSRTGCCTSAGGTCDAYBGCYTG	63.4°C	813	Cloning
<i>C4H</i>	flavonoid biosynthesis	CTGGCTCCAGGTSGCRAYGACCTC	65.4°C	GKCCWCCYTTCTCRSTSGTGTGAGC	64.3°C	1277	Cloning
<i>CSH</i>	flavonoid biosynthesis	GGYCTSMGCCCCWCCGTCAAG	65.0°C	CGCCCCAYTCSARCCCCCTC	63.5°C	661	Cloning
<i>FLS</i>	flavonoid biosynthesis	GGARGTGGARAGARTKCARGC	58.1°C	GACATYCKCGYCTTKTCCTGTTCAC	56.2°C	863	Cloning
<i>ANS</i>	flavonoid biosynthesis	GAGTYGAGAGCTTGTCSAGCAGTGG	62.7°C	GTAGTCCTCCCACTRAGCTGSCC	64.7°C	385	Cloning
<i>EF1α</i>	reference gene	GGGTAARGARAAGGTTCACATC	54.9°C	CCRATACCACCAATCTTGASAC	53.7°C	740	Cloning
<i>RPS13</i>	reference gene	GTCGCATGCACAGYCGMGG	61.9°C	CCARACRGWGKGAGCTTCTTGG	58.1°C	412	Cloning
<i>PAL</i>	flavonoid biosynthesis	GCACGAGATGGACCCGTTGC	61.3°C	GTGCCTGGAAATTCCCGCC	59.6°C	158	RT-qPCR
<i>C4H</i>	flavonoid biosynthesis	GGCGAGATCAACGAGGACAACGTCC	63.2°C	GAGGTTCATGTGGGGCACGAGG	62.4°C	229	RT-qPCR
<i>CSH</i>	flavonoid biosynthesis	GAACAACAAGGGGGCTCGTGTCC	62.4°C	CCGAGTCAGGCAGGATGGTCTG	61.6°C	168	RT-qPCR
<i>FLS</i>	flavonoid biosynthesis	GCTGACCACGAGGGATTGGTGCG	64.5°C	CTTCTGGAGCCGAGTCCCGTAACC	64.5°C	166	RT-qPCR
<i>ANS</i>	flavonoid biosynthesis	CGGTTGTGCGTGAGACCTGCC	63.3°C	GCCTTGGATCTTCCCCGACCCCC	64.3°C	150	RT-qPCR
<i>EF1α</i>	reference gene	GAGGAGCGTCACGGGAAGCAAG	62.3°C	GTAGTAGCGAGCCAGCCTGTGG	61.8°C	142	RT-qPCR
<i>RPS13</i>	reference gene	GGATGGACAGACCCGTGAGCACG	63.8°C	GGGACAAACGGGATCTTCTCGGGG	63.3°C	155	RT-qPCR

**Table S6.** RNA quantification by nanodrop. The numbers indicate the biological replicates (1 to 5) for each stage (S1 to S4). S1- buds (day 0), S2- white flowers (day 1), S3- pink (day 2), S4- purple (day 3).

Stage	Biological replicate	ng/ $\mu$ L	260/280	260/230
S1	1	875.70	2.11	2.30
	2	494.40	2.07	1.93
	3	629.80	2.15	1.78
	4	3,173.30	2.12	2.23
	5	418.70	2.10	2.21
S2	1	1,012.80	2.13	2.25
	2	847.70	2.10	2.01
	3	499.30	2.10	1.96
	4	1,039.80	2.13	2.32
	5	1,135.40	2.13	2.30
S3	1	888.20	2.11	2.20
	2	1,136.90	2.12	2.37
	3	890.30	2.13	2.36
	4	1,026.60	2.12	2.29
	5	702.70	2.16	2.21
S4	1	1,030.30	2.13	1.93
	2	534.20	2.15	2.08
	3	1,262.70	2.14	2.35
	4	1,565.40	2.12	2.31
	5	954.20	2.12	1.92