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Environmental regulation of floral anthocyanin synthesis in *Ipomoea purpurea*

YINGQING LU,* JIN DU,[1]*† JINGYU TANG,[1]*† FANG WANG,[1]*†, JIE ZHANG,[1]‡§ JINXIA HUANG,* WEIFENG LIANG* and LIANGSHENG WANG‡

*State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, 20 Nan Xin Cun, Beijing 100093, China, †Graduate School of the Chinese Academy of Sciences, Beijing 100049, China, ‡Beijing Botanical Garden, Institute of Botany, Chinese Academy of Sciences, 20 Nan Xin Cun, Beijing 100093, China, §College of Science, Northwest A&F University, Shaanxi 712100, China

Abstract

Responses of metabolites to environmental fluctuations may play large roles in biological adaptation, yet how these responses initiate in the natural environment and the molecular mechanisms remain unclear. Synthesis of floral anthocyanins, as typical examples of secondary metabolites, is known to respond to the physical environment and therefore an ideal system for understanding the process of the environmental regulation. Here, by simultaneous monitoring of six natural environmental variables and anthocyanin content of daily opening flowers throughout a natural flowering season (~50 days) of *Ipomoea purpurea*, we have identified significant and positive correlations of temperature (3-days ago) and ultraviolet (UV) light intensity (5-days ago) with the floral anthocyanin content. We sequenced all known (seven structural and three regulatory) anthocyanin genes in *I. purpurea* flowers and examined their transcript quantities in the natural environment across eight floral developmental stages (covering 0–96 h before anthesis). The anthocyanin gene expression patterns corroborated with the inferred effects from the time-series data, and further showed that the positive UV effect became negative on transcript levels about 36 h before anthesis. With falling natural temperature, content of the principal anthocyanin declined, whereas that of an alternative anthocyanin with fewer glucose and caffeic acid moieties increased. Our data suggest that environmental regulation of the anthocyanin pathway may account for more than half of the flux variation in the floral limb, and is influenced mainly by daily average temperature and UV light intensity that modulate anthocyanin transcript levels (most likely via *myb1*) at floral developmental stages.

Keywords: common morning glory, environmental regulation, HPLC-MS, real-time PCR, temperature effect, time-series, UV intensity

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Introduction

Biological adaptation to transient environments largely depends on specific metabolites and their modulations, owing to the sessile habit in plants (Wink 2003) or limited migration capability as in animals. The adaptation by metabolites has been implied in numerous species interactions (Agrawal 2001) and may form a basis for phenotypic plasticity of a relevant trait. Levels of metabolites, particularly those involved in plant defence and signalling, have been shown to respond to external biotic stimuli to protect plants or increase the fitness of plants (e.g., Steppuhn *et al.* 2008; Lankau & Kliebenstein 2009), but little is known about how the abiotic environment modifies an organism's metabolite production in nature. The question is not trivial. The fluctuating

Correspondence: Yingqing Lu, Fax: +86 10 62590843;
E-mail: yqlu@ibcas.ac.cn

[1]The authors contributed equally to the study

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atmospheric parameters on Earth (Zachos *et al.* 2001) may have long delineated the limits of physiological adaptations for living beings, as the physical environment has been the very first challenge for organisms since the dawn of life. How the external physical environment contributes to the endogenous metabolite fluctuation remains largely a black box, yet interesting because of its ubiquity among organisms. Plants are particularly suited for the explorations on environmental regulation because of easy tracking and sampling.

Plants have displayed a suite of changes in genomic expression involving primary and secondary metabolites when subject to controlled environmental alterations (Osuna *et al.* 2007; Sato *et al.* 2008; Usadel *et al.* 2008). Nonetheless, few insights have been gained on how external cues are processed by plants' endogenous systems to instigate subsequent changes on production of metabolites. As different categories of metabolites may be influenced by different sets of external signals and regulatory networks, investigations of environmental regulation in a related set of metabolites may offer an alternative perspective to that of the whole genome approach. Here, we choose floral anthocyanins in the common morning glory (*Ipomoea purpurea*) of Convolvulaceae as an example to address significant questions on environmental regulation of metabolites, i.e. which environmental parameters are the major effectors of the metabolites in nature? what is the magnitude of the regulation and how does the mechanism behind the regulation operate?

Anthocyanins are a class of flavonoids (Harborne & Williams 2000) capable of depositing from orange to blue colours to nearly all organs of plants (Grotewold 2006; Tanaka *et al.* 2008). In flowers, anthocyanins may influence pollinator behaviours, for instance, to render coloured flowers receiving more visits than white ones in *I. purpurea* (Brown & Clegg 1984). Transient changes in temperature may be directly sensed by flowers as seen in rose (Dela *et al.* 2003) or indirectly via leaves as in petunia (Moscovici *et al.* 1996). Light signals may also be perceived by flowers as in gerbera (Meng *et al.* 2004) and strongly modifies anthocyanin accumulation in plants (Weiss 2000; Chatterjee *et al.* 2006). In *Arabidopsis thaliana* seedlings, anthocyanin-related gene expression is stronger under ultraviolet (UV)-A than under UV-B, and under blue light than under red light (Cominelli *et al.* 2008). Different lines of evidence indicate that production of anthocyanins may respond to the physical environment, but little is known about how the natural environment modifies the productive process of floral anthocyanins.

Major genes leading to the production of anthocyanins have been characterized in several species (Winkel-Shirley 2001; Koes *et al.* 2005). In the corolla limbs of *Ipomoea*, seven structural genes respectively encoding chalcone synthase-D (CHS-D), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), dihydroflavonol 4-reductase -B (DFR-B), anthocyanidin synthase (ANS) and anthocyanidin 3-*O*-glucosyltransferase (3GT) have been cloned (Tiffin *et al.* 1998; Hoshino *et al.* 2003). These genes are known to be regulated by three regulatory genes—*myb1*, *bHLH2* and *WDR1* (Chang *et al.* 2005; Morita *et al.* 2006; Park *et al.* 2007). In *I. purpurea*, these anthocyanin pathway enzymes cause the synthesis of cyanidin-based anthocyanins (Saito *et al.* 1995) that render the wild-type flowers purple-blue colour (Clegg & Durbin 2003; Zufall & Rausher 2004). Dysfunction of F3′H in the species generates a commonly observed mutant showing reddish flowers (Hoshino *et al.* 2003) because of the production of pelargonidin-based anthocyanins (Saito *et al.* 1996). As environmental effects on anthocyanin production typically involve alterations in transcript expression (Christie *et al.* 1994; Shvarts *et al.* 1997; Takos *et al.* 2006; Ubi *et al.* 2006), the available sequences of the genes on the anthocyanin pathway make it possible to perform a detailed analysis of their transcript variations in *I. purpurea* flowers.

The common morning glory is a widely distributed weed with a blooming season from late summer to late fall when the natural environment rapidly fluctuates in the temperate zone, ideal for the assessment of how natural environmental factors affect the anthocyanin accumulations in the corolla. *Ipomoea* flowers open daily in the morning on a typically large individual during the anthesis, yet each flower has a short lifetime of often less than a half-day, which permits a continuous sampling of flowers on a daily basis with minimum disturbance to the plant. Monitoring anthocyanin accumulation in a natural environment is hence a distinct feature of this study, which helps to overcome at least two drawbacks of manipulated experiments. One is that transient environmental conditions are characteristic of the natural environment but difficult to mimic in a controlled laboratory; data from controlled experiments are consequently not suited for extrapolating patterns on environmental regulation, although anthocyanin production is known causally affected by light (Chappell & Hahlbrock 1984) and temperature (Shvarts *et al.* 1997). The other is known that anthocyanins typically accumulate under biotic and abiotic stressful conditions (Chalker-Scott 1999; Steyn *et al.* 2002) such as UV damage (Mendez *et al.* 1999), pathogen infection (Lo & Nicholson 1998), poor nutrition and senescence (Lillo *et al.* 2008; Peng *et al.* 2008); experimentally induced stress could confound the data of controlled experiments to an unknown degree.

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In this study, we started by sequencing all the major structural and regulatory genes of the anthocyanin pathway from *I. purpurea* individuals taken from different regions in China, where the species is considered naturalized. Then, on selected plants of known genotypes (including both purple-blue and pink flowered genets) grown in the open field, we estimated the transcript levels of each anthocyanin gene over floral developmental stages to detect its responsiveness to the physical environment. Over the entire anthesis, we further quantified the anthocyanin content of a daily opening flower to assess its correlation with the natural environment that had been simultaneously monitored. Correlation and time-series analyses of the data provided several insights to the key questions on the environmental regulation of anthocyanins. As to be shown, our results shed light on physiological adaptation of metabolites to the natural environment.

Materials and methods

Anthocyanin quantifications, hue measurements and gene expressions

Two sets of floral limb samples were collected in 2006. The first set included 32 random individuals covering floral colours of purple-blue, white and pink in the open fields of Beijing Botanical Garden, Institute of Botany, Chinese Academy of Sciences, Beijing, from 28 August to 25 September in 2006. The second sampling was taken on eight purple-blue flowered plants of distinct genotypes (collected randomly across China in the 2005 summer/fall) grown in an open-windowed greenhouse at the same garden from 30 September to 7 October in 2006. These two sets of samples were used to measure petal colour, identify anthocyanin compounds and quantify the total anthocyanin content to find out whether or not our measurement of anthocyanin content is relevant to the visual colour of *Ipomoea purpurea* flowers.

From early May 2007 to late October in 2007, selfed offsprings of seven genets (whose anthocyanin genes were sequenced in 2006 and detailed below) were germinated at 25 °C–28 °C in a growth chamber and sowed in the field ($n = 32$) and the open-windowed greenhouse ($n = 13$) respectively. The plants were fertilized with organic fertilizer at the time of planting. Floral limb samples were collected daily on these plants from late August to 21 October 2007. The 2007 samples were measured mainly for anthocyanin content and monitored for changes in anthocyanin components and aerial environmental variables. Seven plants, each representative of one of those seven genet families grown in the field, were chosen to provide samples of floral buds for later transcript assays.

Floral colour analysis

To study the visual colour of *I. purpurea* flowers, we evaluated random samples of flowers using the CIE-$L^*a^*b^*$ scale of International Commission on Illumination (CIE 1986). The colours of five fresh corollas per plant from the field in the 2006 summer were measured using a spectrophotometer NF333 (Nippon Denshoku Industries Co. Ltd.). For each flower, the upper epidermis of the corolla was measured at the same middle spot under the condition C (330–780 nm, 6774 K) and the viewing angle of 2°. Five measurements were taken for each flower and only the averages were used for data analysis. Within the CIE$L^*a^*b^*$ scale, L^* describes colour lightness from black ($L^* = 0$) to perfect white ($L^* = 100$); a^* is positive for reddish colours and negative for greenish ones, whereas b^* is positive for yellowish colours and negative for bluish ones. We also calculated chroma $C^* = (a^{*2} + b^{*2})^{0.5}$ and hue angle h (°, degree) = $\tan^{-1}(b^*/a^*)$ to further characterize floral colour. The colour parameters are integrated functions of the wavelengths reflected by the subject examined under the standard visible light source. The system is device-independent, making the colour parameters comparable across all light-reflecting objects.

High-performance liquid chromatography (HPLC)-DAD/ESI-MS analysis

After being taken the colour measurements in 2006, the corollas were immediately weighed following removal of the rays and ground in liquid nitrogen. Floral samples in 2007 were processed in the field and kept directly in liquid N_2 before being ground up for extraction in the laboratory. One millilitre of mixed solvents of methanol, water, formic acid and TFA (70:27:2:1; v/v) (Hashimoto *et al.* 2000) was added to each sample in a 1.5-mL tube. The extract was briefly vortexed and then kept at 4 °C for 24 h. After being filtered through a membrane filter (0.22 μm), 10 μL extract was injected onto a C18 column (ODS-80Ts QA, 150 mm × 4.6 mm i.d., Tosoh Co. Ltd.) mounted on an analytical HPLC system (TCC 100) equipped with a P680 pump, Dionex photodiode array detection (PDA-100) and the CHROMELEON 6.6 software (Dionex). Column temperature was set at 40 °C for the 2006 samples, but lowered to 35 °C for the 2007 samples to improve the efficiency of processing. The chromatographic conditions were kept the same in both years: 0.8 mL/min of flow rate, solution A of phosphoric acid and water (1.5:98.5; v/v) and solution B of phosphoric acid, formic acid, acetonitrile and water (1.5:20.0:25.0:53.5; v/v) (Wang *et al.* 2001). The elution profile was set as: 0 min, 40% B, 60% A; 35 min, 85% B, 15% A; 40 min, 40% B, 60% A. All fractions were

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monitored at 530 nm and 330 nm. The UV-visible light (Vis) absorption spectra, ranging 200–800 nm in steps of 2 nm, were recorded during the HPLC course. Content of each anthocyanin was quantified using malvidin 3, 5-di-*O*-glucoside as the standard (Extrasynthese).

The peak identity was confirmed by a HPLC system with an electrospray ionization interface connected to a mass spectrometry system (HPLC-ESI-MS) (Agilent 1100 LC/MSD Trap VL), which was operating in the positive ionization mode for monitoring of the protonated molecular ions under the following conditions: gas temperature 350 °C, flow rate 6.0 L/min, nebulizer pressure 241.3 kPa, octopole RF amplitude 150 vpp, skim 1 voltage 77.4 V, skim 2 voltage 6.0 V, capillary exit 178.7 V and cap exit offset 101.3 V. The mobile phase was made up of solvent A (0.1% TFA in water) and solvent B (HPLC grade methanol). The linear gradient profile was 20% B at 0 min, 55% B at 35 min and then returned to 20% B in 10 min. The LC/MSD TRAP 5.2 software provided complete control over the instrumentation.

Corolla cDNA sequencing

Ten accessions (including the previously mentioned eight genotypes of 2006) of *I. purpurea* from four regions of China (Yunnan, Guizhou, Shannxi and Beijing) were surveyed for expression of the anthocyanin pathway genes. Total RNAs were extracted from fresh corollas using the Trizol RNA extraction kit (Invitrogen). The first-strand cDNAs were synthesized by SuperScript III reverse transcriptase with an oligo dT primer, following the standard protocol (Invitrogen). Gene-specific primers (Table S1) were used in the polymerase chain reaction (PCR) amplification on the cDNAs in a volume of 25 μL using T-gradient thermocycler (Biometra). Fragments of expected sizes were cloned in pCR2.1 (Invitrogen), pGEM-T (Promega) and pEASY-T1 (TransGen). Positive inserts were sequenced using the ET Dye Terminator mix (Amersham Biosciences) processed by a MegaBACE 1000 automatic sequencer (Amersham Biosciences). At least four clones per transformation were sequenced in both directions. All sequences obtained were checked against their original chromatographs, and each allelic sequence was confirmed not only by multiple clones but also by multiple transformations to eliminate polymerase errors. We thus obtained sequences on seven structural (*CHS-D*, *CHI*, *F3H*, *F3'H*, *DFR-B*, *ANS* and *3GT*) and three regulatory genes (*myb1*, *bHLH2* and *WDR1*) on each of the 10 plants surveyed. Our survey focused mainly on those genes that contribute to the anthocyanin accumulation in the limb tissue, so we ignored other gene copies, such as *CHS-E*, that was previously shown to express mainly in the floral tube (Durbin *et al.* 1995). All newly obtained allelic sequences have been deposited in the GenBank (Table 1). The survey in 2006 led to a selection of seven distinct genets of *I. purpurea* on the principle of maximizing genotypic differences, whose offspring were used in the 2007 experiments.

Absolute quantifications of transcript levels

Among the 2007 field-grown *I. purpurea* and on seven plants (one each from self-fertilized progeny of seven genotypes, with the floral phenotypes being six purple-blue and one pink colour), floral buds were collected in triplicate at eight developmental stages (0, 12, 24, 36, 48, 60, 72 and 96 h before floral opening) and stored in RNase-free tubes in liquid nitrogen. Total RNAs were extracted and cDNAs were synthesized as described above. The synthesized solution was treated with 1.5 μL RNaseIf (New England BioLabs) following the manufacturer's instruction.

We developed a two-step procedure involving two dyes to quantify cDNAs by a Rotor-Gene 3000 machine (Corbett Research). Dye SYBR Green II (Molecular Probes, Inc.) binds to both single- and double-stranded nucleotides while dye PicoGreen (Molecular Probes) binds to double-stranded nucleotides only. As SYBR Green II detects DNA and RNA additively and linearly

Table 1 Anthocyanin gene sequences encountered in our samples of *Ipomoea purpurea*

Locus	Allele	Accession nos
CHS-D	*us1*	AF358659
	mex9	AF358655
CHI	*fl1*	AF028238
	fl2	EU032606
	fl3	EU032607
F3H	*fl1*	U74081
	fl2	EU032608
	fl3	EU032609
F3'H	*purp*	AB333419
	blue	EU032626
DFR-B	*fl1*	U90432
	fl2	AF028601
	kkfp39	AB018438
	sino1	EU032611
ANS	*c*	EU032612
	f	EU032614
3GT	*fl1*	AF028237
	b	EU032615
myb1	*Ipmyb1*	AB232769
bHLH2	*bh2b*	EU032619
	bh2c	EU032620
WDR1	*Ipwd1a*	AB232777
	Ipwd1b	EU032621

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in a solution with 0.5% sodium desoxycholate (Morozkin *et al*. 2003), we used the difference between the measurements of the same sample quantified with SYBR Green II and PicoGreen, respectively, to get an accurate quantification of the target gene transcript levels in the cDNAs. This step was needed since a small quantity of DNAs could be carried over from a RNA sample to cDNAs. Quantification of cDNA samples was conducted using the quantification program of ROTOR-GENE 6.0 software and a known quantity of lambda DNA as the reference.

To obtain the absolute transcript levels of the genes on the anthocyanin pathway, we first purified the fragments of coding sequences of 10 anthocyanin genes amplified from cDNAs by conventional PCRs and then quantified these solutions by Picogreen using the standard lambda DNA. These known fragments served as references to be included in each run with other qPCR reactions on unknown samples using Premix Ex Taq™ (TaKaRa) and SYBR Green I dye. The unknowns were subsequently obtained from the reference equation provided from a serially diluted standard for each allele. All primers were checked for their efficiencies to assure comparable results; in the case of *F3′H*, a second pair of primers targeting a different fragment of the coding region was used to check whether or not choices of primers would lead to varied reaction efficiencies. Random samples of amplified fragments were further sequenced to verify that the reactions were on target.

Environmental variables

In the pilot experiments in 2006, we measured air temperature, moisture and visible light intensity with a digital meter (TES 1332A, Taiwan) at the time of intermittent floral samplings in the field. In the 2007 flowering season, we started continuous environmental monitoring in August 2007 before *I. purpurea* blooming. This round of environmental survey added UV light (290–390 nm) intensity measured by TN 2340 (Taiwan) and increased monitoring of each environmental variable from a single measurement to three daily point estimates (8am–9am, 1pm–2pm, and 4pm–5pm) in the field and in the greenhouse until 21 October 2007.

Statistical analysis

Series of environmental and anthocyanin measures were ordered from 1 Sept 2007 to 21 Oct 2007. Eight out of 32 individuals planted in the field generated continuously long enough sample series for the time-series analysis. The eight series involve six parental genotypes (one for pink flower and five for purple-blue flower). Each series was checked with various smoothing models including auto regressive integrated moving average models to characterize the data. The best fitting model for each series was taken to provide the estimated values for absent measurements on dates (e.g. 12 October, 16 October and 17 October 2007) when anthocyanin measures were not taken. Residual cross-correlations were examined up to the rank of 12. Time-series models were then fit to the complete data series using the VARMAX procedure of SAS (version 8.01). All models were compared in terms of the significant levels of Granger Causality Wald test, *t*-tests on model parameters, Portmanteau test and univariate model for diagnostic checks. Information criteria and impulse response matrices in the transfer function were also considered. Five out of eight sample series allowed detections of significant effects, representing five genotypes. The best-fitted models were selected based on higher significant levels of Granger Causality Wald test, *t*-test and diagnostic checks.

To confirm the contributions of delayed effects of temperature and UV light on floral anthocyanin accumulation, we also examined the Pearson correlation coefficients between anthocyanin accumulation at time *t* and all environmental variables measured at different time lags from 1 day ago (*t*-1) to 7 days ago (*t*-7). The highest correlation coefficients were compared with the significant parameters identified by the time-series models.

As transcript levels were positively related to the floral anthocyanin content, we examined how the environmental factors including UV intensity and temperature associated with anthocyanin gene expression. This analysis was not affected much by the autocorrelations among the environmental variables as the floral samples were taken on random days for each developmental stage so that the sample independence could be assumed. Within-stage correlations were explored without corrections for multiple comparisons because the purpose was to guide a formation of hypothesis on the mechanism of the environmental regulation rather than a strict test on such a hypothesis.

Genotypic effect on the principal component of anthocyanins was explored using one-way ANOVA with genet considered as the only influencing factor and the rest as random errors.

Results

Anthocyanin content varied largely within flowering season and covaried with the floral hue in Ipomoea purpurea

For the first set of floral samples collected in 2006, chromatic parameters—*a** and *b** values—separated the flowers into three major colour groups including

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purple-blue, pink-red and white ones (Fig. S1). For the second set of floral samples, individuals of eight genotypes (all blue-purple flowered) differed mostly in a^* and b^* values (Fig. 1), and the total anthocyanin content in log scale was positively correlated with a^* (Pearson correlation coefficient $r = 0.362$, $P < 0.01$), but negatively associated with the lightness L^* ($r = -0.366$, $P < 0.01$) and the hue angle h ($r = -0.468$, $P < 0.01$).

In 2007, we examined the variation of anthocyanin content per flower throughout the flowering season among six genotypes of purple-blue flowers and one genotype of pink flowers. For purple-blue flowered genotypes, the largest range of cyanidin-based anthocyanin content per flower was from 1.14 to 7.92 μg/mg fresh corolla weight (FW) for a single plant and the smallest one was from 1.69 to 5.26 μg/mg FW. The average anthocyanin content was 3.31 (±0.17) to 5.01(±0.24) μg/mg FW among the genotypes. The pink-flowered genotype produced on average 1.08 (±0.05) μg/mg FW of pelargonidin-based anthocyanins.

The principal anthocyanin conjugates with three glucoses and five caffeic acids

Anthocyanin accumulation in *I. purpurea* floral buds became detectable about 96 h before flowers opened and increased rapidly in 48 h (Fig. 2). Combining HPLC and mass spectrometry data, we were able to identify the major anthocyanins in opening blue-purple flowers to be cyanidin 3-(2-glucosylcaffeoylglucosyl-6-caffeoylglucosylcaffeyl-glucoside)-5-glucoside (Cy3C5G), cyanidin 3-(2-caffeoylglucosyl-6-caffeoyl-glucoside)-5-glucoside (Cy2C3G), and cyanidin 3-(2-glucosylcaffeoylglucosyl-6-caffeoyl-glucoside)-5-glucoside (Cy2C4G). Similarly, pink flowers were found to contain pelargonidin 3-(2-glucosylcaffeoylglucosyl-6-caffeoylglucosylcaffeyl-glucoside)-5-glucoside (Pg3C5G), pelargonidin 3-(2-caffeoylglucosyl-6-caffeoyl-glucoside)-5-glucoside (Pg2C3G), pelargonidin 3-(2-glucosylcaffeoylglucosyl-6-caffeoyl-glucoside)-5-glucoside (Pg2C4G) and pelargonidin 3-(2-caffeoylglucosyl-6-caffeoylglucosylcaffeyl-glucoside)-5-glucoside (Pg3C4G). Table 2 lists their absorbance peaks with information including retention times, ultraviolet-visible (UV-Vis) spectrum and mass spectral characteristics. Our results confirmed the reports by Saito *et al.* (1995, 1996) and provided additional details below.

Three lines of evidence indicate that Cy3C5G is the primary storage form of anthocyanin in purple-blue flowers of *I. purpurea*. First, it appeared later than Cy2C3G and Cy2C4G during the floral bud development, suggesting that sequential additions of glucose and caffeic acid to the anthocyanin aglycone might be related to specific stages of floral bud development (Fig. 2a–c). Second, it was frequently the only pigment in opening flowers during the peak flowering season (Fig. 2d). A similar situation was also observed in pink flowers, where Pg3C5G was the major anthocyanin type (Fig. S2). Finally, Cy3C5G was the dominant anthocyanin molecule in mature floral limb throughout the floral season, taking about 58% of the total anthocyanins (Fig. 3).

Ten anthocyanin loci expressed as a functional unit to positively affect floral anthocyanin content

All loci except *myb1* showed two or more alleles among the accessions of *I. purpurea* examined in this study (Table 1). The total transcript level increased when floral development advanced towards anthesis. Along with the pattern, the anthocyanin content per flower also increased (Fig. 4). The expression of the structural genes followed closely the pattern of regulatory gene expression, suggesting a tight regulatory mechanism behind structural gene expression. A large variation, however, existed across the 10 loci, with the transcript level of *DFR-B* being the highest and that of *F3′H* the lowest (Fig. S3).

Environmental factors modulate anthocyanin-accumulation patterns between habitats

The 2007 field environment was characterized by higher intensities of UV (1556.3 ± 113.1 μW/cm^2) and visible light (403.0 ± 26.2 lux) than those (UV 303.8 ± 22.0 μW/cm^2; visible light 126.9 ± 8.6 lux) in the

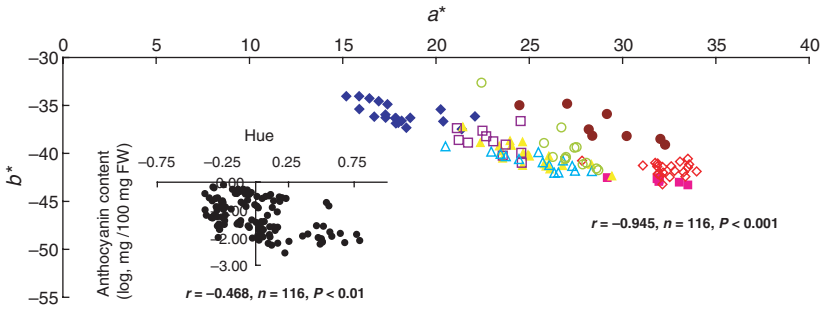

Fig. 1 Relationship between CIE$L^*a^*b^*$ parameters of purple-blue flowers on eight *Ipomoea purpurea* individuals (represented by colour-symbols). The nested figure shows the negative correlation between the total anthocyanin content [log scale in unit of mg per 100 mg fresh weight (FW)] and the hue of the flowers.

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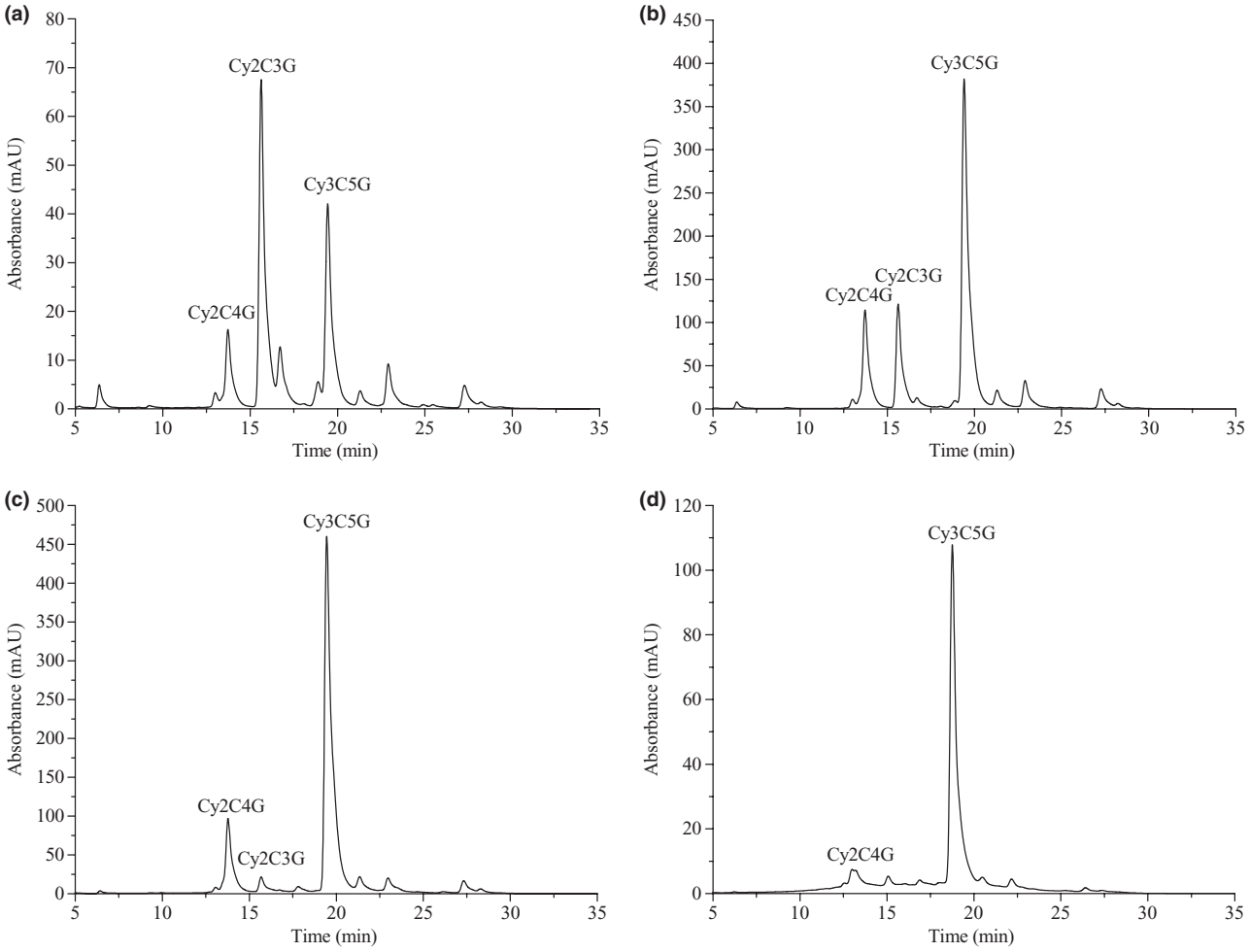

Fig. 2 HPLC detection of anthocyanins during *Ipomoea purpurea* floral development. The *x*-axis is the retention time (in minutes) and the *y*-axis is the absorbance in unit of mAU. Major peaks labelled are based on mass spectrometry data. Floral buds (purple-blue type) are shown at 60 h (a), 48h (b) and 12 h (c) before opening respectively. A case of opened flowers is displayed by (d).

Table 2 Properties of the major anthocyanins in *Ipomoea purpurea* flowers

Colour of floral limb	Anthocyanin component	HPLC retention time (min)		Maximum UV-Vis absorption (nm)	*m/z*
		2006	2007		
Purple-blue	Cy2C4G	12.75	13.43	283/324/528	287/449/935/1097/1259
	Cy2C3G	14.60	15.30	283/328/528	287/449//935/1097
	Cy3C5G	18.20	19.03	291/316/529	287/449/935/1097/1259/1421/1583
Pink	Pg2C4G	17.30	16.74	284/323/509	271/433/919/1081/1243
	Pg2C3G	19.23	18.89	286/316/515	271/433/919/1081
	Pg3C5G	22.69	22.06	287/317/512	271/433/919/1081/1243/1405/1568
	Pg3C4G	26.12	25.56	288/324/511	271/433/919/1081//1243/1405

The retention times between year 2006 and year 2007 correspond to the different operating temperatures (40 °C vs. 35 °C). The mass-to-charge ratio (*m/z*) is also listed for analysed fragments of each component.

greenhouse. Yet, the average temperature varied little between the two habitats (field 24.8 ± 0.9 °C; greenhouse 25.1 ± 0.6 °C) over the entire flowering season. The moisture was significantly higher in the greenhouse (55.1 ± 1.9%) than in the field (50.5 ± 1.9%) according to the *z*-test ($P = 0.008$).

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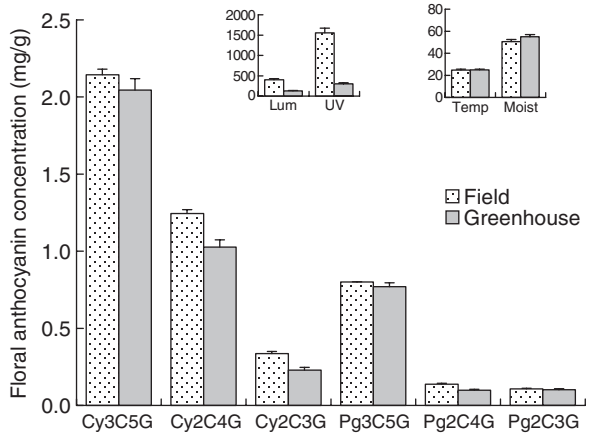

Fig. 3 Floral anthocyanins of *Ipomoea purpurea* in the field and the greenhouse in 2007. Cyanidin-based comparisons were from purple-blue flowers (greenhouse: $n = 180$; field: $n = 806$) of 24 individuals and pelargonidin-based pigments were from pink flowers (greenhouse: $n = 63$; field: $n = 270$) of eight individuals. The standard errors were shown in bars. The environmental variables (Temp, Moist, UV, Lum) were compared over the flowering season in the nested figures.

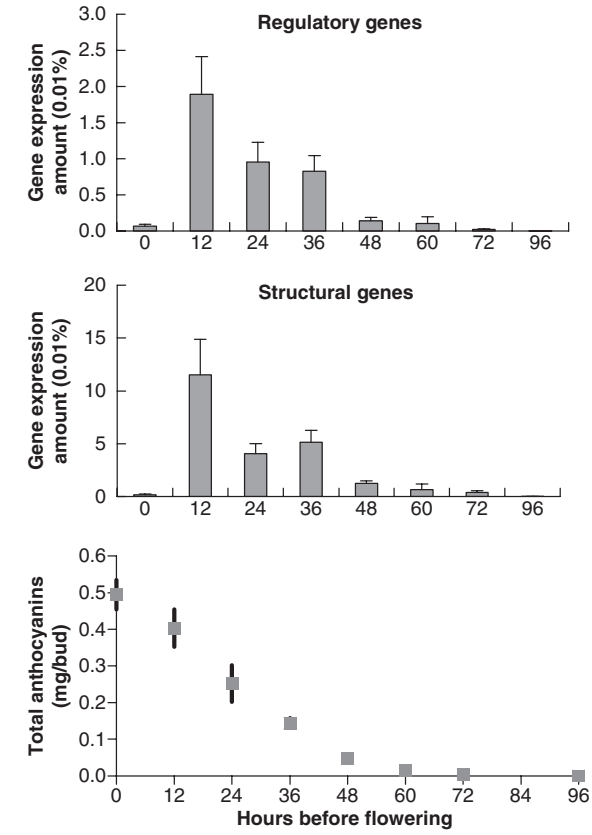

Fig. 4 Transcript levels (expressed as 0.01% cDNA in weight) of regulatory and structural genes and the corresponding anthocyanin content across eight developmental stages of *Ipomoea purpurea* flowers ($n = 21$). The standard error bar is shown for each stage.

As plants of the same genotypes were grown in the field and in the greenhouse at the same time, their anthocyanin accumulation patterns were directly comparable (Fig. 3). The total anthocyanin amount was significantly higher for plants grown in the field than for those in the greenhouse (z-test: $P < 0.001$ for cyanidin-based anthocyanins, and $P = 0.001$ for pelargonidin-based anthocyanins). Besides, a day-to-day pattern of anthocyanin accumulation was consistent between cyanidin- and pelargonidin-based anthocyanins (Fig 5a, b), suggesting that a common source of factor(s) had regulated the accumulation of anthocyanins in the floral limbs. A similar day-to-day pattern was also observed between sibling plants grown separately in the greenhouse and the field (Fig. S4).

Meanwhile, daily Cy2C4G production more or less traced the pattern of Cy3C5G ($r = 0.424$, $n = 51$, $P < 0.01$), but Cy2C3G had a tendency to increase towards the end of flowering season (Fig. 5c) when the declining temperature featured the environmental variables. For pelargonidin pigments, daily Pg2C4G production also followed the changing pattern of Pg3C5G closely ($r = 0.584$, $n = 48$, $P < 0.01$) while Pg2C3G behaved similar to that of Cy2C3G (Fig. S5). However, a higher Pearson correlation coefficient (-0.699, $n = 51$, $P < 0.01$) was found between Cy3C5G and Cy2C3G than that (-0.025, $n = 48$, $P > 0.10$) between Pg3C5G and Pg2C3G over the flowering season.

Modes and likely mechanism of environmental regulation of anthocyanins in I. purpurea

To identify which factors had significantly influenced anthocyanin accumulation among the environmental variables including temperature (Temp), moisture (Moist), UV light intensity (UV), visible light intensity (Lum), daily temperature difference and h of daily sunlight during the 2007 field season, we first examined the correlation matrix between floral anthocyanin content at flowering and these environmental variables from the time of 7 days ago up to the date when opened flowers were sampled. The maximum Pearson's correlation coefficient was 0.630 ($P < 0.0001$, $N = 804$) between Cy3C5G and temperature 3 days prior to anthesis [temp (t-3)], and 0.571($P < 0.0001$, $N = 269$) between Pg3C5G and temp (t-3); Likewise, the highest Pearson's correlation coefficient between light and anthocyanin variables was 0.350 ($P < 0.0001$, $N = 804$) between Cy3C5G and UV intensity 5 days prior to anthesis [UV (t-5)] and 0.461 ($P < 0.0001$, $N = 269$) between Pg3C5G and UV (t-5). Among all environmental parameters examined (Table 3), these correlations were more significant than others.

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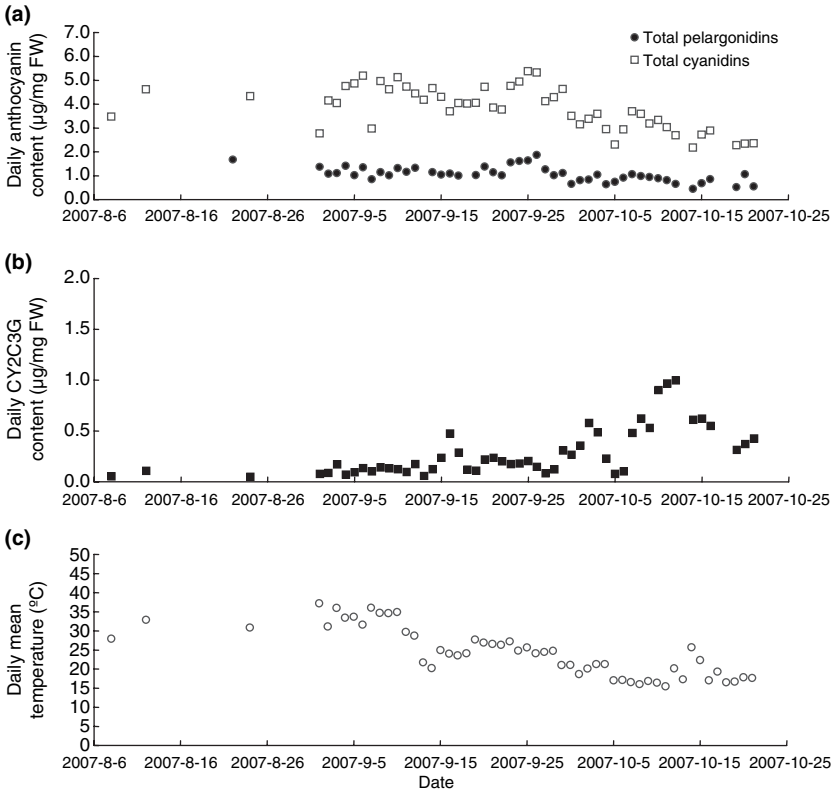

Fig. 5 Co-varying patterns of anthocyanins with the natural temperature. (a) The total daily anthocyanins between field-grown purple-blue-flowered individuals (n = 24) and pink-flowered individuals (n = 8). The Pearson correlation coefficient is 0.799 between the total content of cyanidins and that of pelargonidins over the flowering season (n = 48, $P < 0.01$). (b) Daily accumulation of Cy2C3G in the purple-blue-flowered individuals. (c) Daily mean natural temperature during the 2007 field flowering season.

To take the continuous nature of the data and their autocorrelation into account, we also applied a time-series model to the principal anthocyanin, considering the environmental factors as the predictor variables. Significant effects were detected for temp (*t*-3) and UV (*t*-5) (Table 4). The time-series models applied to Cy2C3G (or Cy2C4G, Pg2C3G, Pg2C4G) turned out to be statistically nonsignificant.

Detailed correlation analysis was conducted among the transcript numbers across the floral developmental stages (Fig. 6). Our results suggested that UV (*t*-5) positively related to enhanced expression of *myb1*; however, this relationship was not significant around 48–72 h before flowering and then became negative around 36 h before flowering (Fig. 6d). Meanwhile, high temperature significantly related to increased transcript levels of *myb1* (as well as *CHSD* and *3GT*) around 72 h before anthesis (Fig. 6f). A significant and positive impact of temperature on expression levels of regulatory and structural genes (except *F3′H*) also occurred at the stages of 12 h and 48 h prior to flowering (Fig. 6b, d), and *myb1*showed the maximum correlations (0.67, and 0.72, respectively) with temperature among the 10 loci examined at the two stages. Consistently, when environmental influence became significant, stronger correlations emerged between the structural and regulatory genes (Fig. 6a–f).

Table 3 Environmental factors and their highest Pearson correlation coefficient (r) with floral Cy3C5G content during the field sampling period from 1 September to 17 October of 2007 based on the average measurements of the variables

Environmental factors	Mean (SE)	Maximum	Minimum	*r* (day before anthesis)
Temperature (°C)	24.8 (0.9)	43.8	11.5	0.630 (day 3)
Moisture (%)	50.5 (1.9)	91.0	24.4	0.299 (day 6)
UV light intensity ($\mu W/cm^2$)	1556.3 (113.1)	5466.0	50.6	0.350 (day 5)
Visible light intensity (lux)	403.0 (26.2)	1120.6	13.1	0.300 (day 5)
*Daily temperature difference (°C)	9.8 (0.4)	15.5	3.7	0.274 (day 3)
*Daily sunlight (h)	6.4 (0.5)	11.0	0.0	0.278 (day 5)

*Data were from the local weather database of Beijing.

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Table 4 Significant factors in the time-series models on the major anthocyanin content

Dependent variable	Genotype	Model	Independent variables	Parameter	Estimate	SE	*T*-ratio	Prob	Significant variable	R^2	Granger
Cy3C5G	Blue2881	ARX(1,4)	Temp, UV, Moist, Lum	XL(3,1,1)	0.17	0.076	2.25	0.033	Temp (*t*-3)	0.732	<0.0001
	BlueSb1l	ARX(1,4)	Temp, UV, Moist, Lum	XL(3,1,1)	0.143	0.066	2.17	0.040	Temp (*t*-3)	0.666	0.005
	BlueSa91	ARX(1,5)	Temp, UV, Moist	XL(5,1,2)	0.00059	0.00026	2.26	0.033	UV (*t*-5)	0.660	0.009
	BlueG8l	ARX(1,5)	Temp, UV, Moist, Cy2C3G	XL(5,1,2)	0.00049	0.00018	2.81	0.011	UV (*t*-5)	0.910	<0.0001
				XL(5,1,4)	−2.533	0.780	−3.17	0.005	Cy2C3G (*t*-5)		
Pg3C5G	Pink07	ARX(1,4)	Temp, UV, Moist, Lum &Pg2C4G	XL(0,1,1)	−0.025	0.012	−2.15	0.044	Temp (*t*-0)	0.910	0.014
				XL(0,1,5)	1.996	0.632	3.16	0.005	Pg2C4G		
				XL(3,1,1)	0.037	0.013	2.87	0.010	Temp (*t*-3)		

Environmental variables included temperature (Temp), moisture (Moist), UV light intensity (UV) and visible light intensity (Lum). Granger is for the Granger Causality Wald test.

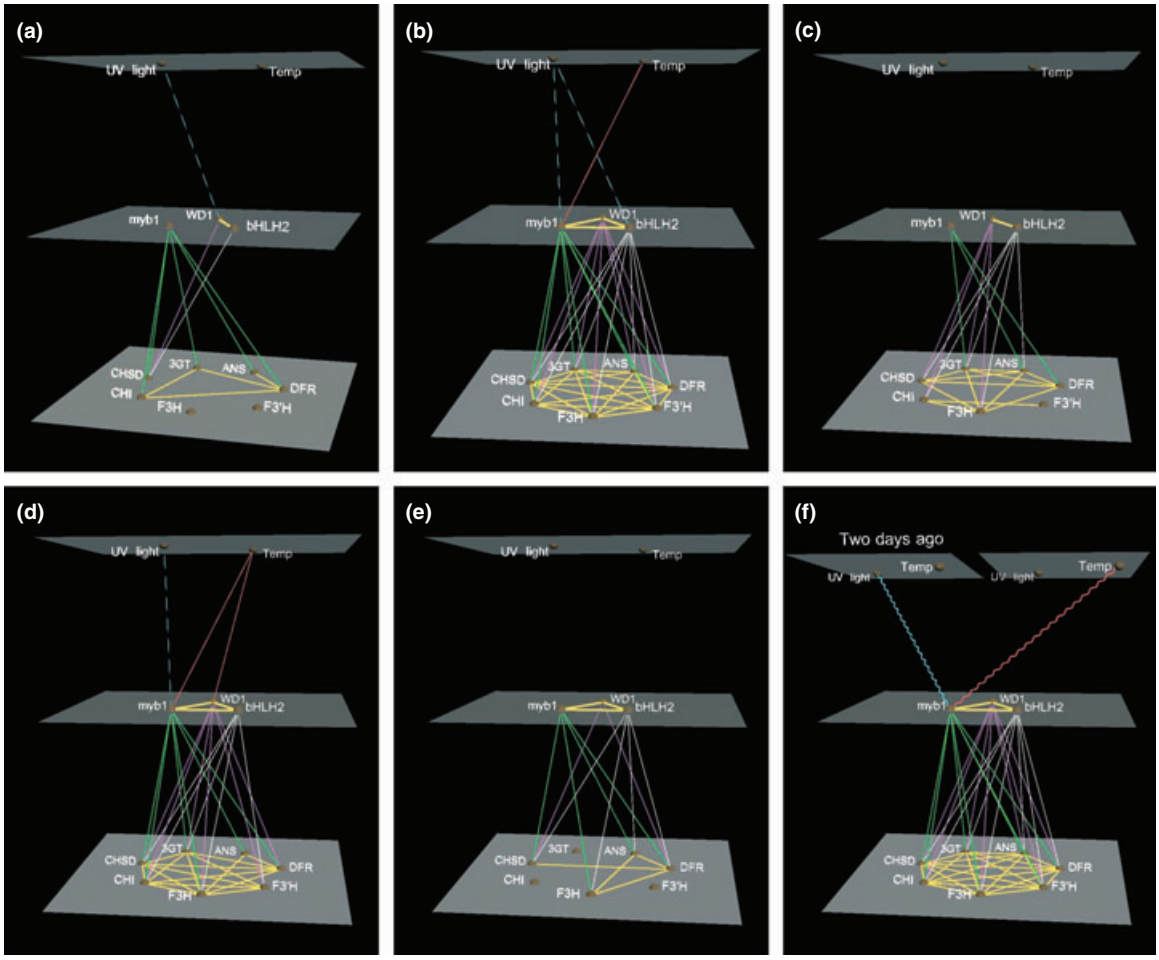

Fig. 6 Correlations of transcript levels among structural genes, between structural and regulatory genes and between regulatory genes and two environmental factors [UV light and temperature (Temp)]. The solid line indicates positive correlation (Pearson correlation coefficient, $P < 0.05$) while the dashed line shows negative correlation and the spiral line is for positive correlation with a significant level between 0.05 and 0.1. The relationships are shown for the stages at 0 h (a), 12 h (b), 24 h (c), 36 h (d), 48 h (e), and 72 h (f) before floral opening.

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Magnitudes of environmental and genotypic effects on anthocyanin accumulation

Genotypic effect on anthocyanin accumulation was evident among all sample sets collected in this study. The effect was significant in one-way ANOVA for the 2006 field samples ($P < 0.0001$, $R^2 = 0.32$, $n = 212$, days = 21) as well as for the 2006 greenhouse samples ($P < 0.0001$, $R^2 = 0.42$, $n = 115$, days = 8). In 2007, six genotypes significantly affected Cy3C5G content when considered alone (one-way-ANOVA, $P < 0.0001$, $R^2 = 0.093$, $n = 804$, days = 52), in the linear model with other parameters including Cy2C3G, UV and temp ($P < 0.0001$, $R^2 = 0.52$, $n = 804$), or in the linear model with date as a cofactor to account for day-to-day environmental fluctuation ($P < 0.0001$, $R^2 = 0.65$, $n = 804$).

Discussion

Environmental modification of metabolite production has been operating in nature probably since the origin of life, but kept more or less indiscernible to us. We show here in plants that one valid way of detecting the process is to expose individuals of known genotypes in the natural environment and take time-series data from them to extract information about environmental regulation of metabolites.

In the case of natural accumulation of floral anthocyanins in *Ipomoea purpurea*, we added a few links between genotypes and phenotypes of floral colours to materialize the process of the environmental regulation. First, the total amount of floral anthocyanins was shown to covary significantly with floral colour parameters (Fig. 1), particularly the hue of flowers in *Ipomoea purpurea*, hence the relevance of floral anthocyanin content to floral phenotype was established (although whether or not pollinators would respond to the phenotypic change remains to be explored). Correlations between anthocyanin components and colorimetric parameters were also observed in rose (Biolley & Jay 1993). We further revealed here, by profiling floral pigments over the floral developmental stages to identify the principal storage molecule (Fig. 2), that Cy3C5G was the ultimate molecule synthesized in the *I. purpurea* floral limb. This information helped pave the way for focused analyses on how the natural environment modifies the production of this molecule. Then, by showing that the anthocyanin content in the floral tissue closely follows the expressions of the anthocyanin pathway genes (Fig. 4), we suggest that the genes are directly responsible for the anthocyanin synthesis. Although the expression of most floral anthocyanin genes has been previously described by Northern blotting in *I. nil* (Morita *et al.* 2006) as well as by Reverse Transcription–PCR in *Aquilegia* (Whittall *et al.* 2006), our real-time assays of the expressions of 10 anthocyanin genes permitted analyses of quantitative relationships among environmental factors, anthocyanin content and transcript levels for the first time. Finally, our analyses on the time-series samples of floral limb provided convincing evidence on how natural environmental factors modified anthocyanin content in *I. purpurea*. These new observations led to formulation of a hypothesis on how the molecular mechanisms might operate in nature in floral pigment production.

Temperature has the largest effect on anthocyanin accumulation

Temperature has been known to affect accumulation of floral anthocyanins in controlled laboratory experiments (Shvarts *et al.* 1997; Dela *et al.* 2003), but the magnitude and mode of the effect in nature was unclear. In *I. purpurea*, the natural temperature effect of 3 days before floral opening was first suggested in this study by its highest correlation coefficient with Cy3C5G (Table 3), the time-series regressions (Table 4) and then by its correlation with transcript levels of the anthocyanin genes (Fig. 6f). The temperature effect in nature has two features. First, it is not genotype-specific, because a similar influence of temperature was found for different genotypes of *I. purpurea* (Fig. 5a). Second, the magnitude of the effect is larger than those of other factors considered (Table 3). The comparisons of floral anthocyanin accumulating patterns between the greenhouse and the field (Fig. 3) suggest that the two habitats differed mostly in intensities of visible light and UV; nevertheless, anthocyanins changed in magnitudes mostly comparable with those of temperature variation.

We suspect that the mode of temperature effect is not restricted to morning glories. In rose, the most sensitive time for temperature effect is at the stage when the petals are barely visible (Dela *et al.* 2003), which is approximately the stage of flowers 3 days before anthesis for *I. purpurea*. Compared to the effects of the maximum and minimum daily temperatures as well as daily temperature difference, the average daily temperature explained more variation of the anthocyanin content in our analysis. The effect of average temperature on floral coloration was also noticed in *Chrysanthemum morifolium* (Nozaki *et al.* 2006).

The optimal temperature for anthocyanin accumulation, however, seems to differ among species. It could be 17 °C in apple skin (Ubi *et al.* 2006;), around 20 °C in *Chrysanthemum morifolium* (Nozaki *et al.* 2006) and about 25 °C for *Vitis vinifera* cv. Cabernet Sauvignon (Mori *et al.* 2007). Our data suggest that it is near 30 °C

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in *I. purpurea* (Fig. 5). The interspecific variation may hold hidden information on historical adaptation of each species to its original habitat. For instance, *I. purpurea* is believed to be native to central Mexico (Clegg & Durbin 2003). Although distributed into the Far East, the species may still maintain features of its physiological adaptation acquired long before its secondary distribution.

UV effect may reverse its sign on anthocyanin synthesis

After the demonstration of the UV light induction of CHS expression in parsley cells (Chappell & Hahlbrock 1984), detailed analysis in *Arabidopsis* has established the role of UV light in anthocyanin pathway gene expression (Jenkins *et al.* 2001; Cominelli *et al.* 2008), but no evaluation has been given to compare the effect of this environmental signal with those of others in nature. In our observations on *I. purpurea*, UV light (from 290–390 nm) represents the second strongest environmental signal, indicated by its correlation with the anthocyanin content (Table 3), its significant term in the time-series analysis (Table 4) and its correlations with the transcript levels (Fig. 6f). As our measurements made no distinction between UV-A and UV-B light, they collectively accounted for the slightly reduced average anthocyanin contents in the greenhouse where the light intensities were significantly lower than those in the field (Fig. 3).

The effect of UV light has a temporal action mode different from that of temperature—a 5-day delay on the anthocyanin accumulation of *I. purpurea* grown in the field. In *Phaseolus vulgaris*, a controlled experiment has shown that the effect of UV-B treatment may last up to 6 days (Diara *et al.* 2007). Our analysis examined the correlations between floral anthocyanin content and UV radiance of up to 7 days prior to floral sampling and showed that UV intensity at day 5 before anthesis had the largest impact on the final anthocyanin content (Tables 3 and 4). As visible anthocyanin accumulation starts about 4 days before anthesis (Fig. 4), the regulatory genes are more likely than the structural genes to become the target of this UV action. Consistently, we observed a positive correlation at a level of border significance between *myb1* transcript level at 72 h before flowering and UV light of 2 days before the sampling (Fig. 6f). As anthocyanin transcript levels were already low at 96 h before floral opening, we did not attempt quantifying the anthocyanin transcripts at the stage of 120 h before floral opening (e.g., *t*-5) to make an instantaneous connection between transcripts and UV intensity, because large errors were expected to be associated with the estimates.

An unexpected turn of UV action was later exposed in our transcript analysis (Fig. 6). With the approaching of floral opening in *I. purpurea*, the effect of UV light becomes negative (Fig. 6b, d) on all anthocyanin gene expressions, while the effect of temperature remains simultaneously positive. Previously unseen, these reversed effects of UV intensity do not appear to be associated with drastic changes in fluence rate according to our records, rather, they might be the results of engagements of two different signalling pathway during the floral development. Little has been known about these signalling processes so far.

Responses of anthocyanins to seasonal changes in I. purpurea

Besides Cy3C5G, we followed variations of other anthocyanins in flowers of *I. purpurea* and documented an increasing pattern of Cy2C3G towards the end of flowering season in the field (Fig. 5c). This tendency accompanied the declining of Cy3C5G ($r = -0.70$, $n = 51$, $P < 0.01$), suggesting that anthocyanin accumulation in morning glory flowers could take different forms in response to the environmental alterations. The highest Pearson correlation coefficient between Cy2C3G and the environmental variables was -0.73 ($n = 51$, $P < 0.01$), with the temperature of 3 days ago. The timing of this highest correlation concurs with that of Cy3C5G and temperature, implying that the reverse relationship between Cy3C5G and Cy2C3G was probably because of the same mechanism of temperature regulation.

Increasing amount of Cy2C3G towards the end of flowering season indicates that less Cy3C5G was produced as the ultimate storage anthocyanin, presumably because of reduced flux levels of photosynthetic metabolites in leaves and consequently a shrinking supply of sugar and caffeic acid to the floral limb. Another possibility for increased Cy2C3G is active breakdown of Cy3C5G in response to the declining temperature in fall. Although there is no evidence for breaking down of Cy3C5G in the common morning glory, anthocyanin degradation has been shown to be a process independent from senescence in *Brunfelsia calycina* (Vaknin *et al.* 2005). In our field experiment, the most likely source of stress in the autumn is associated with declining temperature in the soil and air, because patterns of light intensities and moisture level remained steady near the end of the sampling period. As sugar levels (including glucose) may increase as a response to low temperatures as seen in a number of species (Moalem-Beno *et al.* 1997; Teng *et al.* 2005), reduced attachment of glucose to anthocyanin aglycone may be part of the stress resistance mechanism in flowers.

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Magnitude of environmental regulation

Depending on genotypes included and lengths of sampling period, about 9% to 43% of the variance of the anthocyanin content in *I. purpurea* may be explained by genotypes alone in different environments. The environmental effect on anthocyanin content, however, may surpass that of genotypes, especially in the context of the entire growth season. Among the six distinct genotypes in 2007, a plant may have its floral anthocyanin content varying seven-fold within the flowering season, while the largest difference in average anthocyanin content was less than two folds between the genotypes. Also, while the genotypes alone accounted for about 9% of the total variation in floral Cy3C5G content in the 2007 field experiment, the daily environmental fluctuation significantly improved the fitting of the model to explain 65% of the total variance of floral Cy3C5G content. Despite that the linear regression results should be taken with caution, individual-based analysis with time-series models also suggests more than half of the variation in the major anthocyanin component could be interpreted by the fluctuating environment (Table 4).

In addition, the correlation coefficients (Table 3) suggest that about 40% or 12% of the Cy3C5G variance may be explained by temperature three days ago or UV light 5 days ago, respectively. As these two environmental factors appear to act independently, their collective influence may account for 52% of the Cy3C5G variance. Obviously, the environmental regulation on anthocyanin accumulation is highly prominent in *I. purpurea*.

Molecular mechanism for the environmental regulation of anthocyanin accumulation

Little has been known about the molecular mechanism of temperature effect. Artificially induced temperature change may trigger a wide-range response mechanisms that may instruct/modify the regulatory genes of the anthocyanin pathway, as seen in *Arabidopsis thaliana* (Usadel *et al.* 2008), and sucrose may induce anthocyanin accumulation as well (Teng *et al.* 2005). The previous results seem to imply direct and indirect effects of temperature on anthocyanin content. Our data suggest that these effects may be by the regulation of the regulatory genes on the anthocyanin pathway as the transcript level of *myb1* holds the largest correlation (0.72) with temperature, when compared with other anthocyanin transcripts.

The influence of UV light, both positive and negative, appears to be *myb1*-related, too (Fig. 6). The effect of *myb1* on floral colour was demonstrated in both *I. purpurea* (Chang *et al.* 2005) and *I. nil* (Park *et al.* 2007). In these cases, *myb1* appears to be the ortholog of *An2* in petunia whose function in anthocyanin synthesis has been well defined (Quattrocchio *et al.* 1999). Periodically tightened correlations among regulatory and structural genes seemed to co-occur when an environmental impact was unleashed (Fig. 6b, d, f) and the expression of *myb1* was always correlated with the impact. In *Arabidopsis*, the expression of *PAP1* (also a myb gene) preceded the expression of structural genes under light induction (Cominelli *et al.* 2008), supporting the role of myb in transporting the signal of UV light on the anthocyanin gene expression. The circumstantial evidence calls for more specific data on the internal regulation of the anthocyanin genes.

Conclusion

We are just at the beginning of understanding how the physical environment contributes to physiological adaptation of organisms. Atmospheric temperature and radiation are proven to be the leading factors shaping anthocyanin synthesis in nature, but how plants receive and transport the signals require further investigations. The information obtained here suggests that genotype by environment interactions could be placed under a new light that emphasizes different magnitudes of responses of genotypes under the same environment and across different conditions. Phenotypic plasticity in nature may be the consequence of both the responses to environmental stimuli and developmental chaos. In the case of anthocyanin accumulation, as environmental stimuli may also induce perturbations in gibberelin, abscisic acid or sugar level *in vivo*, which may alter anthocyanin accumulation in their ways (Weiss 2000; Loreti *et al.* 2008). Those indirect influences may constitute developmental chaos to anthocyanin accumulation and appear to be secondary in magnitude at least in *Ipomoea purpurea*.

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References

Agrawal AA (2001) Phenotypic plasticity in the interactions and evolution of species. *Science*, **294**, 321–326.

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Biolley J, Jay M (1993) Anthocyanins in modern roses—chemical and colorimetric features in relation to the color range. *Journal of Experimental Botany*, **44**, 1725–1734.

Brown BA, Clegg MT (1984) Influence of flower color polymorphism on genetic transmission in a natural population of the common morning glory, *Ipomoea purpurea*. *Evolution*, **38**, 796–803.

Chalker-Scott L (1999) Environmental significance of anthocyanins in plant stress responses. *Photochemistry and Photobiology*, **70**, 1–9.

Chang S-M, Lu Y, Rausher MD (2005) Neutral evolution of the nonbinding region of the anthocyanin regulatory gene *Ipmyb1* in *Ipomoea*. *Genetics*, **170**, 1967–1978.

Chappell J, Hahlbrock K (1984) Transcription of plant defence genes in response to UV light or fungal elicitor. *Nature*, **311**, 76–78.

Chatterjee M, Sharma P, Khurana JP (2006) Cryptochrome 1 from *Brassica napus* is up-regulated by blue light and controls hypocotyl/stem growth and anthocyanin accumulation. *Plant Physiology*, **141**, 61–74.

Christie PJ, Alfenito MR, Walbot V (1994) Impact of low-temperature stress on general phenylpropaniod and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta*, **194**, 541–549.

CIE (1986) *Recommendations on Uniform Color Spaces, Color Difference Evaluations and Psychometric Color Terms*, 2nd edn. CIE Central Bureau, Colorimetry, Commission Internationale de l'Éclairage, Vienna, Austria.

Clegg MT, Durbin ML (2003) Tracing floral adaptations from ecology to molecules. *Nature Reviews Genetics*, **4**, 206–215.

Cominelli E, Gusmaroli G, Allegra D *et al.* (2008) Expression analysis of anthocyanin regulatory genes in response to different light qualities in *Arabidopsis thaliana*. *Journal of Plant Physiology*, **165**, 886–894.

Dela G, Or E, Ovadia R, Nissim-Levi A, Weiss D, Oren-Shamir M (2003) Changes in anthocyanin concentration and composition in 'Jaguar'rose flowers due to transient high-temperature conditions. *Plant Science*, **164**, 333–340.

Diara C, Vernieri P, Gorini S, Horton P, Lercari B (2007) Damaging and non-damaging effects of UV-B in South American bean genotypes under controlled environment conditions. *Advances in Horticultural Science*, **21**, 28–34.

Durbin ML, Learn GH Jr, Huttley GA, Clegg MT (1995) Evolution of the chalcone synthase gene family in the genus *Ipomoea*. *Proceedings of the National Academy of Sciences, USA*, **92**, 3338–3342.

Grotewold E (2006) The genetics and biochemistry of floral pigments. *Annual Review of Plant Biology*, **57**, 761–780.

Harborne JB, Williams CA (2000) Advances in flavonoid research since 1992. *Phytochemistry*, **55**, 481–504.

Hashimoto F, Tanaka M, Maeda H, Shimizu K, Sakata Y (2000) Characterization of cyanic flower color of *Delphinium* cultivars. *Japanese Society of Horticultural Science*, **69**, 428–434.

Hoshino A, Morita Y, Choi J-D *et al.* (2003) Spontaneous mutations of the flavonoid 3'-hydroxylase gene conferring reddish flowers in the three morning glory species. *Plant, Cell & Physiology*, **44**, 990–1001.

Jenkins GI, Long JC, Wade HK, Shenton MR, Bibikova TN (2001) UV and blue light signalling: pathways regulating chalcone synthase gene expression in *Arabidopsis*. *New Phytologist*, **151**, 121–131.

Koes R, Verweij W, Quattrocchio F (2005) Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends in Plant Science*, **10**, 236–242.

Lankau RA, Kliebenstein DJ (2009) Competition, herbivory and genetics interact to determine the accumulation and fitness consequences of a defence metabolite. *Journal of Ecology*, **97**, 78–88.

Lillo C, Lea US, Ruoff P (2008) Nutrient depletion as a key factor for manipulating gene expression and product formation in different branches of the flavonoid pathway. *Plant, Cell & Environment*, **31**, 587–601.

Lo S-CC, Nicholson RL (1998) Reduction of light-Induced anthocyanin accumulation in inoculated *Sorghum* mesocotyls. *Plant Physiology*, **116**, 979–989.

Loreti E, Povero G, Novi G *et al.* (2008) Gibberellins, jasmonate and abscisic acid modulate the sucrose-induced expression of anthocyanin biosynthetic genes in *Arabidopsis*. *New Phytologist*, **179**, 1004–1016.

Mendez M, Jones DG, Manetas Y (1999) Enhanced UV-B radiation under field conditions increases anthocyanin and reduces the risk of photoinhibition but does not affect growth in the carnivorous plant *Pinguicula vulgaris*. *New Phytologist*, **144**, 275–282.

Meng X, Xing T, Wang X (2004) The role of light in the regulation of anthocyanin accumulation in *Gerbera hybrida*. *Plant Growth Regulation*, **44**, 243–250.

Moalem-Beno D, Tamari G, Leitner-Dagan Y, Borochov A, Weiss D (1997) Sugar-dependent gibberellin-induced chalcone synthase gene expression in petunia corollas. *Plant Physiology*, **113**, 419–424.

Mori K, Goto-Yamamoto N, Kitayama M, Hashizume K (2007) Loss of anthocyanins in red-wine grape under high temperature. *Journal of Experimental Botany*, **58**, 1935–1945.

Morita Y, Saitoh M, Hoshino A, Nitasaka E, Iida S (2006) Isolation of cDNAs for R2R3-MYB, bHLH and WDR transcriptional regulators and identification of *c* and *ca* mutations conferring white flowers in the Japanese morning glory. *Plant Cell Physiology*, **47**, 457–470.

Morozkin ES, Laktionov PP, Rykova EY, Vlassov VV (2003) Fluorometric quantification of RNA and DNA in solutions containing both nucleic acids. *Analytical Biochemistry*, **322**, 48–50.

Moscovici S, Moalem-Beno D, Weiss D (1996) Leaf-mediated light response in petunia flowers. *Plant Physiology*, **110**, 1275–1282.

Nozaki K, Takamura T, Fukai S (2006) Effects of high temperature on flower colour and anthocyanin content in pink flower genotypes of greenhouse chrysanthemum (*Chrysanthemum morifolium* Ramat.). *Journal of Horticultural Science & Biotechnology*, **81**, 728–734.

Osuna D, Usadel B, Morcuende R *et al.* (2007) Temporal responses of transcripts, enzyme activities and metabolites after adding sucrose to carbon-deprived *Arabidopsis* seedlings. *Plant Journal*, **49**, 463–491.

Park K-I, Ishikawa N, Morita Y *et al.* (2007) A bHLH regulatory gene in the common morning glory, *Ipomoea purpurea*, controls anthocyanin biosynthesis in flowers, proanthocyanidin and phytomelanin pigmentation in seeds, and seed trichome formation. *Plant Journal*, **49**, 641–654.

Peng M, Hudson D, Schofield A *et al.* (2008) Adaptation of *Arabidopsis* to nitrogen limitation involves induction of

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anthocyanin synthesis which is controlled by the NLA gene. *Journal of Experimental Botany*, **59**, 2933–2944.
Quattrocchio F, Wing J, van der Woude K *et al.* (1999) Molecular analysis of the anthocyanin2 gene of *Petunia* and its role in the evolution of flower color. *Plant Cell*, **11**, 1433–1444.
Saito N, Tatsuzawa F, Yoda K *et al.* (1995) Acylated cyanidin glycosides in the violet-blue flowers of *Ipomoea purpurea*. *Phytochemistry*, **40**, 1283–1289.
Saito N, Tatsuzawa F, Yokoi M *et al.* (1996) Acylated pelargonidin glycosides in red-purple flowers of *Ipomoea purpurea*. *Phytochemistry*, **43**, 1365–1370.
Sato S, Arita M, Soga T, Nishioka T, Tomita M (2008) Time-resolved metabolomics reveals metabolic modulation in rice foliage. *BMC Systems Biology*, **2**, 51.
Shvarts M, Borochov A, Weiss D (1997) Low temperature enhances petunia flower pigmentation and induces chalcone synthase gene expression. *Physiologia Plantarum*, **99**, 67–72.
Steppuhn A, Schuman MC, Baldwin IT (2008) Silencing jasmonate signalling and jasmonate-mediated defences reveals different survival strategies between two *Nicotiana attenuata* accessions. *Molecular Ecology*, **17**, 3717–3732.
Steyn WJ, Wand SJE, Holcroft DM, Jacobs G (2002) Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. *New Phytologist*, **155**, 347–361.
Takos AM, Jaffe FW, Jacob SR, Bogs J, Robinson SP, Walker AR (2006) Light-Induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. *Plant Physiology*, **142**, 1216–1232.
Tanaka Y, Sasaki N, Ohmiya A (2008) Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant Journal*, **54**, 733–749.
Teng S, Keurentjes J, Bentsink L, Koornneef M, Smeekens S (2005) Sucrose-specific induction of anthocyanin biosynthesis in *Arabidopsis* requires the MYB75/PAP1 gene. *Plant Physiology*, **139**, 1840–1852.
Tiffin P, Miller RE, Rausher MD (1998) Control of expression patterns of anthocyanin structural genes by two loci in the common morning glory. *Genes & Genetic System*, **73**, 105–110.
Ubi BE, Honda C, Bessho H *et al.* (2006) Expression analysis of anthocyanin biosynthetic genes in apple skin: effect of UV-B and temperature. *Plant Science*, **170**, 571–578.
Usadel B, Blaesing OE, Gibon Y *et al.* (2008) Multilevel genomic analysis of the response of transcripts, enzyme activities and metabolites in *Arabidopsis* rosettes to a progressive decrease of temperature in the non-freezing range. *Plant, Cell & Environment*, **31**, 518–543.
Vaknin H, Bar-Akiva A, Ovadia R *et al.* (2005) Active anthocyanin degradation in *Brunfelsia calycina* (yesterday–today–tomorrow) flowers. *Planta*, **222**, 19–26.
Wang LS, Hashimoto F, Shiraishi A et al. (2001) Phenetics in tree peony species from China by flower pigment cluster analysis. *Journal of Plant Research*, **114**, 213–221.
Weiss D (2000) Regulation of flower pigmentation and growth: multiple signaling pathways control anthocyanin synthesis in expanding petals. *Physiologia Plantarum*, **110**, 152–157.
Whittall JB, Voelckel C, Kliebenstein DJ, Hodges SA (2006) Convergence, constraint and the role of gene expression during adaptive radiation: floral anthocyanins in Aquilegia. *Molecular Ecology*, **15**, 4645–4657.
Wink M (2003) Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry*, **64**, 3–19.
Winkel-Shirley B (2001) It takes a garden. how work on diverse plant species has contributed to an understanding of flavonoid metabolism. *Plant Physiology*, **127**, 1399–1404.
Zachos J, Pagani M, Sloan L, Thomas E, Billups K (2001) Trends, rhythms, and aberrations in global climate 65 Ma to present. *Science*, **292**, 686–693.
Zufall RA, Rausher MD (2004) Genetic changes associated with floral adaptation restrict future evolutionary potential. *Nature*, **428**, 847–850.

Y. Lu is interested in ecological and evolutionary genomics, who designed the experiments, developed the real-time method, participated in data collection, and performed data analysis and writing. As Master students, J. Du worked on the natural variation of anthocyanin content, F. Wang participated in gene sequencing and genotyping, and J. Tang collected data on real-time quantification of gene expression. J. Zhang was a second year PhD student when performed 2006 experiments. W. Liang and J. Huang helped with the earlier planning and sequencing. L. Wang is interested in horticulture of floral colours and applications of secondary compounds, and assisted in both the HPLC and the fieldwork.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Primer sequences used in the amplifications of the anthocyanin genes in cDNAs

Fig. S1 Distribution of colour parameters on the CIE*L*a*b** scale among 295 flowers of *Ipomoea purpurea*.

Fig. S2 Anthocyanins in *I. purpurea* pink flowers during floral development.

Fig. S3 Individual gene expressions in the floral cDNAs.

Fig. S4 Consistent pattern of anthocyanin accumulation between habitats (greenhouse and field) in *Ipomoea purpurea*.

Fig. S5 Increasing Pg2C3G with declining environmental temperature in *Ipomoea purpurea*.

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