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Ton-Phuc Huynh
United Graduate School of Agricultural Sciences Kagoshima University
Chihiro Motoyama
Kagoshima University
Hiroshi Oshima
Kagoshima University
Naoko Kozai
United Graduate School of Agricultural Sciences Kagoshima University
Yamate Nagisa
Kagoshima University
Fumio Hashimoto
United Graduate School of Agricultural Sciences Kagoshima University
Keiichi Shimizu
k3705864@kadai.jp

United Graduate School of Agricultural Sciences Kagoshima University

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Ectopic expression of the *flavonoid 3′,5′-hydroxylase* gene in *Ipomoea nil* induces the accumulation of delphinidin-based anthocyanins in the petals and inhibits their opening

Ton-Phuc Huynh[1, 2], Chihiro Motoyama[2], Hiroshi Oshima[2], Naoko Kozai[1, 2], Yamate Nagisa[2], Fumio Hashimoto[1, 2], Keiichi Shimizu[1, 2*]

[1]*United Graduate School of Agricultural Sciences Kagoshima University, Kagoshima 890-0065, Japan*

[2]*Faculty of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan*

[*]Corresponding author:

K. Shimizu

Faculty of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan

E-mail: k3705864@kadai.jp

Tel: +81-99-285-8561

ORCID: 0000-0003-0132-654X

Abstract

Background: Many commercially important ornamental species, such as rose, carnation, and chrysanthemum, lack blue flower varieties due to the absence of the flavonoid 3′,5′-hydroxylase (*F3′5′H*) gene, which is essential for delphinidin (Dp) biosynthesis. Heterologous *F3′5′H* genes have been introduced into these species to enable Dp accumulation. This study aimed to investigate the potential of *Ipomoea nil*, a diploid species with a short life cycle and high transformation efficiency, as a model plant for anthocyanin biosynthesis study. As *I. nil* naturally lacks a functional *F3′5′H* gene, this study aimed to engineer Dp production via ectopic expression of *F3′5′H* genes from four different plant species.

Result: This study represents the first successful engineering of Dp-based anthocyanins (Dps) biosynthesis in the genus *Ipomoea*. Transgenic *I. nil* lines expressing *F3′5′H* from *Eustoma grandiflorum*, *Campanula medium*, and *Viola* × *wittrockiana* showed high Dps content, whereas the gene from *Gentiana triflora* resulted in low content. High levels of Dps accumulation were strongly correlated with a notable physiological phenotype wherein petal cell expansion was inhibited during flowering, preventing the flowers from being fully open. To investigate the underlying mechanism of this inhibition, RNA-Seq analysis was conducted on petal tissues to reveal the significant transcriptomic changes associated with the inhibition. Gene Ontology enrichment analysis revealed differential changes in genes involved in salicylic acid signaling pathway,

anthocyanin-related transport, oxidative stress response, and senescence-associated processes. These findings suggest that high-level accumulation of metabolites associated with *F3′5′H* expression may trigger complex stress or defense responses, which could interfere with normal petal opening.

Conclusion: This study suggests that *Ipomoea nil* cv. Violet can serve as a useful system for functional analysis of *F3′5′H*. In addition, high-level expression of the *F3′5′H* gene and the resulting high content of Dps were correlated with inhibited petal opening in this cultivar. RNA-Seq analysis revealed that inhibited petals showed elevated expression of stress-responsive genes, anthocyanin-related transporters, and oxidative stress-associated and senescence-related genes. These findings provide important insights into the mechanisms underlying the inhibition of petal opening caused by altered pigment accumulation.

Keywords: *Agrobacterium*, anthocyanin, biosynthesis, Campanula, Eustoma, flower, pigment, petal opening inhibition, transcriptome

Background

The colors of several flowers are primarily determined by anthocyanins. The colors of these pigments are derived from their central chromophores, known as the anthocyanidin. Anthocyanidins are classified into three main types based on their B-ring

hydroxylation pattern: orange-red pelargonidin (Pg), red-magenta cyanidin (Cy), and blue-violet delphinidin (Dp). An alteration, such as methylation, converts Cy into peonidin (Pn) and Dp into petunidin (Pt) and malvidin (Mv) [1]. Dp-based anthocyanins (Dps), such as Dp, Pt, and Mv, are collectively responsible for most of the purple and blue floral pigmentations.

Some economically important ornamental plants, such as roses, chrysanthemums, lilies, and carnations, naturally lack the ability to produce blue flowers due to the absence of a functional *F3′5′H* gene that encodes the key enzyme required for the biosynthesis of Dp [1, 2]. Genetic engineering has become a primary strategy for developing novel, blue-flowered cultivars. The introduction of heterologous *F3′5′H* genes has led to the successful commercialization of Dp-accumulating transgenic carnations, phalaenopsis, and roses. However, the approach is often limited because the degree of Dp accumulation, which varies significantly, depends on the functional compatibility between the introduced transgene and the metabolism of the host plant [3, 4]. Moreover, the precise molecular mechanisms underlying this compatibility are not yet fully understood, making the breeding of blue flowers a persistent challenge [5].

Ipomea nil (Japanese morning glory) is an annual climbing herb that exhibits several advantages as a research material, owing to its short life cycle, efficient *Agrobacterium*-mediated transformation, and extensive genetic resources including a fully

sequenced genome [6–10]. Although *I. nil* lacks an endogenous *F3′5′H* gene, it possesses flavonoid 3′-hydroxylase (*F3′H*), which competes with *F3′5′H* for the same substrate [11]. These characteristics suggest that if an exogenous *F3′5′H* gene could accumulate Dp in *I. nil*, it could serve as an ideal candidate for evaluating the function and compatibility of the gene.

To avoid competition and create a clear pathway for functional analysis, *I. nil* cv. Violet was used in this study. The suitability of *I. nil* as a model plant for Dps flower color engineering was evaluated by introducing *F3′5′H* genes from four different species. Although *I. nil* can exhibit blue coloration through Cy-based anthocyanins (Cys), it remains unclear how its phenotype responds to the novel biosynthesis of Dps. The expression of these transgenes resulted in an unexpected and notable phenotypic change: that is, petal cell expansion during flowering was inhibited in the transformants with high levels of Dps accumulation, thereby preventing bud opening. To elucidate the molecular basis of this inhibition, a comparative transcriptome analysis of developing petals was performed and the induction of defense-related responses in petals with high Dps content was identified.

Methods

Plant Materials of Host *I. nil*

I. nil cv. Violet seeds were procured from Marutane Trading Co. Ltd. (Kyoto, Japan). The plant was cultivated in pots for seed

production under a 16h light / 8h dark cycle at 25±3°C with fluorescent lighting (27μmol·m^{-2}·s^{-1} PPFD). Suspension calli were initiated from immature embryos of the plant according to the protocol described by Shimizu et al [12].

Construction of transgenic vector

Sequence data for the binary vectors constructed in this study (pKANre3AH, pEg, pEg-ADH^{-}, pVw, pCm, and pGT) are provided as Supplementary Material 1 in formats that are compatible with SnapGene Viewer and ApE (A plasmid Editor). First, the delphinidin accumulation ability of three vectors containing the *E. grandiflorum F3′5′H* gene (*EgF3′5′H*) were compared. A schematic representation of the T-DNA region in the vector used in this study is shown in Figure 1. To construct pEg, a binary vector designated as pKANre3AH was used. This vector is based on the pKAN43Ag8-GFP backbone [13] (Addgene #178182), from which the GFP sequence was removed, and in which the translational enhancer and terminator were replaced with elements from pRI201AN (Takara Bio Inc., Otsu, Japan).

The *EgF3′5′H* gene was amplified via PCR from cDNA isolated from the RV strain of *E. grandiflorum* (Takatori et al., 2022[14]) using KOD-Plus-Ver.2 polymerase (TOYOBO Co., Ltd., Osaka, Japan) and the primers listed in Supplementary Table S1. The amplified fragment was then inserted into the NdeI/SalI-digested pKANre3AH vector using the In-Fusion HD Cloning Kit (Takara Bio Inc.),

resulting in the construction of pEg. The pEg-ADH$^-$ construct was generated by inserting *EgF3′5′H* into XbaI/SalI-digested pKANre3AH using the same method as that for pEg construction, except that a different forward primer was used (see Table S1). The *F3′5′H* gene in pB237 [4] had already been incorporated into the vector.

To compare the Dp accumulation ability of *F3′5′H* genes from different plant species, the *F3′5′H* gene from *Campanula medium*, *Viola* × *wittrockiana*, and *Gentiana triflora* were used. The resulting constructs were named pCm, pVw, and pGt, respectively and the *F3′5′H* gene in them were derived from plasmids pB244, pB247 and pB242 [4], respectively. The *F3′5′H* genes amplified from these vectors were integrated into pKANre3AH using the same method described above for pEg.

Transformation

Transformation of *I. nil* was carried out following the protocol described by Takatori et al. [7] with minor modifications. The *Agrobacterium* strain AGL0 harboring the vectors described above was used to infect the calli of *I. nil* cv. Violet. Transgenic calli were selected on a medium containing 120 mg/L G-418 and One tablet/L Augmentin250RS (GlaxoSmithKline K.K., Tokyo, Japan). After 3–4 weeks, the developing embryoids were transferred to EMG medium to promote shoot formation and root elongation. Regenerated plantlets were acclimatized in vermiculite for approximately one

week before being moved to the soil. To confirm successful transformation, the regenerated plants were sprayed with a 300 mg/L kanamycin solution to test for resistance. Transgenic plants were grown for analysis and seed production under the same conditions as those for the wild type.

High-Performance Liquid Chromatography (HPLC) Analysis

Anthocyanidin content was analyzed using HPLC, as described by Hashimoto et al. [15, 16] and Liu et al. [13]. Petals that had reached the developmental stage that corresponds to full bloom were subjected to freezing at −80°C, followed by pulverization in a 1% HCl/methanol solution for a duration of three days at 4°C. Hydrolysis was performed using 2 N HCl at 100°C for two hours. Extracts were filtered (0.45 μm) and analyzed with a Jasco HPLC system with a TSKgel ODS-80Ts QA Column. The mobile phase consisted of solvent A (1.5% phosphoric acid) and solvent B (1.5% phosphoric acid, 20% formic acid, 25% acetonitrile, and 5% tetrahydrofuran [THF]), with a gradient shift of over 35 min. Anthocyanin was detected at 525 nm using a UV-Vis detector to ensure precise quantification of the pigment concentration. In this study, the retention times varied among the HPLC runs; therefore, peak identification was performed by co-injection with authentic anthocyanidin standards.

RNA Extraction, cDNA Synthesis, RNA sequence, and Quantitative Real-Time RT-PCR (qPCR)

RNA was extracted from frozen plant tissues using the Total RNA Extraction Kit (RBC Bioscience) and then treated with DNase. PCR was used to confirm the complete digestion of the somatic DNA. cDNA was synthesized using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa bio). Quantitative PCR (qPCR) was conducted using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and an Applied Biosystems StepOnePlus™ Real-Time PCR System. A total of 10 μL reaction mixture was prepared for each reaction, containing 1 μL cDNA and 0.3 μL of specific primers (Supplementary Table 3). Relative expression levels were calculated using the ΔΔCt method. The transcript levels were normalized to the mitochondrial ATP synthase (*InATP*) gene as an internal reference [17, 18]. All the qRT-PCRs for each sample were performed in 3 biological replicates. Melting curve analysis of the amplicons was used to confirm the specificity of the PCR.

The RNA sequences were sent to the Seibutsugiken Bioengineering Laboratory for RNA sequencing to analyze changes in gene expression in *I. nil*. Total RNA quality was assessed using a Quantus Fluorometer (Promega) and a Fragment Analyzer System (Agilent Technologies). Libraries were prepared using the MGIEasy RNA Directional Library Prep Set (MGITech) and quantified using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific). Sequencing was performed on a DNBSEQ-T7 platform with a paired-end 150 bp read length. The raw reads were subjected to adapter trimming and quality filtering using Cutadapt and Sickle, followed by mapping to

the *I. nil* reference genome using STAR. Expression levels were quantified using featureCounts and normalized using Transcripts Per Million (TPM). Differential gene expressions were analyzed using Python, and functional enrichment analysis was performed using GO annotations from the Asagao Database (Shigen Inc.) criteria of DEG with q.value < 0.05 and m.value >1. To validate the RNA-seq results, we selected nine DEGs and performed qRT-PCR using primers designed from the sequences obtained from the database (http://viewer.shigen.info/asagao/) (Table S6), following the procedure described above.

Observation of petal cells

The *I. nil* petals were harvested, trimmed, and embedded in 5% agarose. The cross-section was then cut using a thin blade and placed on a clean glass slide with water. A cover slip was placed over the section and examined under a light microscope at a magnification that allowed clear visualization of the cells.

Statistical analysis

Statistical analyses was performed using the Tukey-Kramer multiple comparison test. Data are presented as mean ± standard deviation (SD). The numbers of biological replicates are indicated in the figure legends. Different letters above the bars indicate statistically significant differences at $p < 0.05$ based on Tukey-Kramer's test. Relative expression of *F3′5′H* gene RT-qPCR was indicated as statistically significant different at $p < 0.05$ based on

ANOVA

Results

Vectors configuration is suitable for transgenic introduction of *F3′5′H* to *I. nil*

Transformation with pEg yielded 40 independent kanamycin-resistant plants, and HPLC analysis of petal anthocyanins revealed Dps accumulation in 23 of these individuals (Table 1). As shown in Figure S1A, the pEg with 22 transformants accumulated up to six anthocyanidins, such as Pg, Cy, Dp, Pn, Pt, and Mv and one transformant with three anthocyanidins (Figures S1A and B). The highest Dps were 77.4% and 56.6%, respectively (Table 1). Notably, transformants with high Dp accumulation exhibited inhibition of petal opening (Figure S1A). Furthermore, despite these petal abnormalities, no severe defects, such as morphological abnormalities in other floral organs or sterility, were observed.

In the pEg-ADH$^-$ lines, a total of six transformants were generated, five of which accumulated Dps and also accumulated six anthocyanidins (Figure S1C). The maximum Dps reached 64%, with an average of 56.6% (Table 1). A similar petal inhibition was also observed (Figure S1C).

The Dps contents in pEg and pEg-ADH$^-$ transformants were significantly higher than those in the pB237 line, with 12 plants out of 18 transformants accumulating Dps with a maximum of 35% and an average of 14.5% (Table 1). Only three types of anthocyanidins

(Pg, Pn, and Mv) were accumulated, with Pg being the major anthocyanidins, and no petal inhibition was observed (Figure S1D). This result indicates that the combination of the *CaMV 35S* and HT vector configuration is effective for transgenic introduction of *F3′5′H* into *I. nil* for Dps accumulation, therefore pEg will be employed to construct a vector for subsequent experiments.

Table 1. Accumulation of Dps in petals of transgenic *I. nil* plants expressing the *Eustoma Grandiflorum F3′5′H* gene with various vector configuration

Vector	No. of Regenerated Plant	No. of Dps-producing plants	Best (%)	Average (%)	SE
pEg	40	23	77.4	54.3 a	12.6
pEg-ADH$^-$	6	5	64	56.6 a	11.2
pB237*	16	12	36.7	14.5 b	8.7

Percentage of Dps in total anthocyanins in petals. [a,b]Different letters in the column indicate significantly different means ($p < 0.05$), as determined by the Tukey's test.

Different plant species *F3′5′H* and Dps content in *I. nil*.

Based on the above results, we constructed three additional types of vectors, pCm, pVw, and pGt, in which the *F3′5′H* gene of pEg was replaced with the corresponding *F3′5′H* derived from *C. medium*, *V.* × *wittrockiana*, or *G. trifloral*. These vectors were then introduced into *I. nil* through genetic transformation, and their effects were directly compared to result of pEg, as shown in Table 1.

As shown in Table 2* [19], Dps content varies depending on the *F3′5′H* source; hence, individuals with accumulated Dps in each line were measure for comparison.

The pCm line had the highest Dps content (86%, $p < 0.05$, n=8), followed by *EgF3′5′H* (77.4%, $p < 0.05$, n=22) and then *VwF3′5′H* (83.8%, $p < 0.05$, n=10). These three lines had Dps as their primary pigment. In contrast, *GtF3′5′H* accumulated the lowest content (7%, $p < 0.05$, n=7), with Pg as the primary pigment (Table 2) and no petal inhibition observed. These findings are similar to those of previous studies, which demonstrated species-dependent Dps variation in *F3′5′H* genes in transgenic chrysanthemum and rose [4, 5].

Transformants in the pCm line showed varied anthocyanidin compositions, ranging from five to six types of anthocyanidins (Figures S1E and S1F); however, the pVw transformant composition was similar to that of the pEg results, with most transformants accumulating the six types (Figure S1G) and only one transformant accumulating three anthocyanidins (Figure S1H). Notably, most pEg, pCm, and pVw transformants accumulated all six anthocyanidins, whereas only three anthocyanidins were detected in the pGt line (Figure S1I).

Table 2. Accumulation of Dps in petals of transgenic *I. nil* plants expressing the *F3′5′H* gene from various plant species

Vector	No. of Regenerated plant	No. of Dps-producing plants	Best (%)	Average (%) b	SE
pEg	40	23	77.4	54.3 b	16.3
pCm	16	8	94.2	85.9 a	7.9
pVw	21	10	83.8	52.2 b	29.2
pGt	12	7	10.8	7.1 c	3.2

Percentage of Dps in total anthocyanins in petals. [a, b,] [c]Different letters in the column indicate significantly different means ($p < 0.05$), as determined by the Tukey's test

* This table was previously used in Huynh et al., 2023 [19]. The data are slightly different and updated for this paper.

F3′5′H **gene expression and anthocyanidin content**

To ensure stable inheritance and reliable evaluation of the transgene and Dps accumulation, analyses were conducted on the progeny. The presence of the transgene in the progeny of the transformants was confirmed via kanamycin spraying. Three independent lines that inhibited petal opening were selected as the pEg, pVw, and pCm transformants. For pGt, analyses were conducted on flowers from lines in which the presence of Dps was detected using HPLC. Transcript levels were calculated as the relative abundance normalized to *InATP* by setting the internal control expression level to 1.0.

The HPLC profiles of the T1 plants are shown in Figure 2A. In T0 transformants, the Dp content in pCm was significantly different

from that in pEg, pVw, and pGt (Table 1*). The progeny Dps content rate in Figure S3B shows slightly different results; pCm (90.6%) is significantly higher than either pEg (64.5%) or pGt (14.2%) but not pVw (85.5%). Furthermore, pGt (14.2%) showed the lowest value, which was similar to that observed in the T0 generation (Figure 2D). In each transgenic progeny line, no substantial differences were observed among the individuals analyzed in terms of appearance or HPLC profiles (Figure S2). Significant variation in transgene expression was observed among individual plants within the pEg, pWv, and pGt lines (Figure 2B). Significant differences in Dps content were observed among the pEg, pCm, and pGt lines (Figure 2D). Significant differences in Dps content were detected among individuals in the pWv, pCm, and pGt lines (Figure 2C). In these lines, transgene expression and Dps accumulation showed a moderate degree of association, and a significant correlation was detected, particularly in pVw (Figure S3B). In contrast, Dp occupancy did not correlate with either of these variables.

Inhibition of petal opening and transgene expression

Microscopic cross-sectional analysis was conducted on the non-transgenic cv. Violet and Dps-accumulated-inhibited pEg transgenic *I. nil* petals before and after bud opening (Figures 3A–D). Conical epidermal cells exhibit normal physiological development in non-transgenic host plants. These cells underwent significant expansion that resulted in a substantial increase in cellular volume and the formation of a conical shape (Figures 3A–B), indicating petal

opening [20, 21]. Conversely, the high Dp-accumulating line presented a peculiar phenotype characterized by non-expansion and non-opening of the petals. The epidermal cells in this line remained densely round with different pigment colors and failed to undergo the characteristic cellular expansion observed in the cv. Violet line (Figures 3C–D). This developmental inhibition suggests that the accumulation of Dps correlates with petal opening inhibition in transgenic *I. nil*.

In the majority of the progeny from selected individuals of the pEg, pCm, and pVw lines, the petals exhibited the expected phenotypes of altered pigmentation and inhibited petal opening. In the progeny population of pEg transformants, fully expanded petals with distinct colors were occasionally observed on otherwise inhibited plants (Figure 3E). Conversely, although the pCm and pVw lines produced some slightly open petals, they remained partially impaired, and no fully open petals were observed.

Fully opened petals from three kanamycin-resistant pEg individuals were selected for expression and anthocyanin content analyses (Figures 3E and 3F). As expected, these fully-open petals showed relatively lower expression of the *F3′5′H* and significantly lower Dps content gene compared to inhibited petals. In these samples, the major anthocyanidin was Pg, similar to that in the host *I. nil* cv. Violet, and Dp-type Mv were detected only at trace levels.

Transcriptome comparison of opening and inhibiting petals

Both inhibited and opened petals were observed in the progeny of the pEg transformants, as described above. RNA sequencing of these petals revealed 913 differentially expressed genes (DEGs) between inhibited and opened petals. (Figure 5A). Gene Ontology (GO) term enrichment analysis showed significant alterations, particularly the upregulation of terms related to salicylic acid (SA) biosynthesis (Figure 4), suggesting a potential stress response [22, 23]. The highly upregulated genes were *SARD1-SAR DEFICIENT 1* (INIL01g04886 and INIL03g37226), *CBP60-Calmodulin-binding protein 60 A* (INIL03g20446), and *Calmodulin-binding protein 60 B* (INIL06g29912).

Based on these findings, we further analyzed the gene families previously implicated in the SA pathway activation in other plant species, including the *Pathogenesis-Related Protein (PR)* family [24–26] and the *WRKY* co-transcription factor gene family (Van Verk MC et al., 2011[27]). Our results revealed four upregulated *PR* genes (Figure 5C) and 14 upregulated *WRKY* genes (Figure 5B), indicating a potentially strong activation of defense responses mediated by the SA pathway.

In addition, we assessed gene families associated with flower opening in *I. nil*. Aquaporins, which facilitate water transport required for cell expansion and petal movement [28, 29], were represented by 78 annotated genes, of which only one, *INIL05g21764* (*NIP5-1*), was differentially expressed (Figure 5F).

This gene was significantly upregulated in inhibited petals, with a $\log_2$ fold change exceeding 5, suggesting its role in altered petal hydration dynamics under inhibited opening conditions.

Thereafter, 96 *expansin* genes were analyzed along with the cell wall–modifying *pectinesterase* genes. Although canonical *expansin* genes were not differentially expressed (Figure 5D), one upregulated gene encoding an EG45-like domain protein was observed. This protein may contribute to cell wall remodeling, which is critical for petal opening [30, 31]. Although several *pectinesterase* genes were also upregulated, *pectinesterase inhibitor* genes were downregulated by over four-fold, further supporting active cell wall loosening in the inhibited petals (Figure 5D).

Dps might be considered as the primary metabolic compound affected in the inhibited petals; hence, we analyzed the expression profiles of key anthocyanin-related genes, including *UGTs*, *GSTs/TT19-like*, *MATE/ABCC* transporters, *O-methyltransferases*, and *acyltransferases*. Nineteen genes related to anthocyanin transport and modification were differentially expressed (Figure 5E; Supplementary Table S4), indicating an increased effort to sequester anthocyanins in the vacuole. The potential toxicity of reactive oxygen species (ROS)-related genes was also evaluated. Five ROS-associated genes were upregulated (Table S5), suggesting the possible induction of somatic toxicity.

Finally, the gene families involved in structured petal

development, such as *SPIRAL1-like (SP1L)* genes [32] and *Mixta*, a subfamily of *MYB* co-transcription factors implicated in petal cell differentiation [33], were investigated. No *MYB* genes were significantly down-regulated or up-regulated in the inhibited petals (Figure 5G). However, *INIL05g23997 (SP1L2)*, a member of the *SP1L* family, was differentially expressed (Figure 5H). As *SP1L* genes are essential for microtubule organization and directional cell growth [34], this suggests a possible role in modulating petal structure under inhibited conditions. Several genes related to petal inhibition were selected for validation using qRT-PCR, as shown in Figure S5. The qRT-PCR results showed that the selected genes were upregulated.

Top upregulated DEGs and comparison with petal development databases

To identify transcriptomic changes between opening and inhibited petals in great detail, the top 20 upregulated DEGs were compared (Figure 5A) using the interactive web-based database ipomoeanil.nibb.ac.jp/fpkm/ that visualize the temporal transcriptomes of *I. nil* petals, created by Nakagawa et al. [29]. Among the top 20 upregulated DEGs in inhibited petals, most genes maintained very low levels throughout the development of petals, except for the unidentified *INIL11g26672* gene (Table S3). Within these top 20 upregulated genes, several candidates were considered to be potentially associated with the inhibition of petal opening, including the INIL12g08473 (Mate) transporter and INIL06g37875

(SAG39) Senescence-Associated Gene 39. Several genes presumed to be involved in petal inhibition were selected for validation using qRT-PCR, and the results are shown in Figure S5.

Discussion

Vector design for Dp accumulation in transgenic *I. nil*

Appropriate promoter selection is critical for achieving robust transgene expression. The cauliflower mosaic virus (CaMV) 35S promoter is widely used for constitutive gene expression in plant transformation [35]; however, its efficiency can be species-dependent. For example, a report showed that the 35S promoter is ineffective in some ornamental species such as chrysanthemum and gentian. In chrysanthemum, the native *flavonone 3-hydroxylase* (*F3H*) promoter was found to be significantly more effective than the 35S promoter in driving petal-specific expression [4].

In the present study, the vector constructs containing the 35S promoter (pEg and pEg-ADH^{-}) were more effective in promoting Dp-based anthocyanin accumulation in *I. nil* than the pB237 vector, which contains the chrysanthemum *F3H* promoter. These constructs also differ in their terminator and other regulatory regions; therefore, enhanced expression cannot be attributed solely to the promoter. The result of this study, combined with several previous reports on successful flower color modification in *I. nil* using the 35S promoter [7, 9, 18, 36] suggests that it is a highly effective promoter for modifying pigments in this species.

Relative *F3′5′H* expression level and anthocyanin composition in transgenic plant

Noda et al. [4] reported that the high proportion of delphinidin in chrysanthemum ray florets was associated with the upregulation of the campanula *F3′5′H* transgene. This study revealed a significant positive correlation between transgene expression levels and Dps accumulation in petals of *I. nil* transformed with the *F3′5′H* gene from *V.* × *wittrockiana*. A similar, though not statistically significant, trend was seen for *I. nil* expressing *F3′5′H* genes derived from *E. grandiflorum* and *C. medium*. However, no correlation was detected between the transgene expression and Dps content (%) in any of the transgenic lines. Katsumoto et al [37] demonstrated that overexpressing *F3'5'H* was not enough to increase Dp content in rose metabolic engineering. This observation suggests that the proportion of Dp in transgenic *I. nil* is influenced not only by the expression level of the transgene but also by sequence-dependent functional properties of the *F3′5′H* variants.

***F3′5′H* gene origins and complex metabolic flux in different host system regulate anthocyanin composition**

Engineering of novel flower colors via heterologous gene expression is highly dependent on the functional compatibility between the introduced exogenous gene and the host plant environment. The function of *F3′5′H* gene can vary with different species. For instance, *F3′5′H* genes from *V.* × *wittrockiana* and

cineraria enabled moderate delphinidin accumulation in chrysanthemum, but the gene from campanula proved superior, yielding delphinidin levels as high as 95% [4]. Similarly, compared to other sources, the *C. medium*-derived *F3′5′H* was the most effective at generating Dps, when expressed in tobacco or rose [38, 39]. In this study, T0 showed significantly higher Dps content rate than either pEg, pVw. and pGt. However, analysis of T1 progeny revealed that pCm is still significantly higher than pEg and pGt but not significantly higher than pVw (Figure 2D), and pGt progeny was similar to pGt T0, which was observed to still be the significantly lowest among all the lines.

This variation suggests that functional compatibility is governed by complex factors beyond simple gene expression, possibly including enzyme kinetics, substrate affinity, protein stability, and potential interactions within multi-enzyme complexes or metabolons. Furthermore, anthocyanidin composition is not solely affected by the activity of the introduced *F3′5′H*. The substrate specificity of downstream enzymes, particularly dihydroflavonol 4-reductase (DFR), plays an important role. The DFR-B isozyme, primarily responsible for floral pigmentation in *I. nil*, is an asparagine (Asn)-type DFR, a class known for its broad substrate utility [40]. Previous studies have confirmed this critical role, as its knockout results in the complete loss of floral anthocyanins [8, 9]. This study demonstrates for the first time that the *I. nil* DFR-B efficiently reduces not only its native substrates dihydrokaempferol

519 (DHK) and dihydroquercetin (DHQ) but also dihydromyricetin
520 (DHM), the product of *F3′5′H* activity. This broad substrate
521 acceptance explains why transgenic lines, such as those expressing
522 the *E. grandiflorum F3′5′H*, were able to produce a full spectrum of
523 anthocyanidins, including those derived from pelargonidin and
524 cyanidin, alongside the novel delphinidin-based pigments.

525 ## ***Ipomoea nil*** **as a model for anthocyanin biosynthesis studies**

526 Although established organisms such as *A. thaliana* [41] and
527 *N. tabacum* [42] have been widely used in anthocyanin biosynthesis
528 pathway, *I. nil* offers a unique and powerful combination of
529 advantages. Similar to other model systems, this system provides
530 essential tools for genetic research, including a well-characterized
531 genome, efficient transformation protocols, and a short lifecycle that
532 accelerates experimental timelines. *I. nil* has a critical advantage
533 over other models in the specific study of B-ring hydroxylation. The
534 cv. Violet used in this study lacks a functional endogenous *F3′H*, and
535 the species naturally lacks *F3′5′H*. This genetic background provides
536 an ideal host for competition with native hydroxylases. In contrast,
537 no available cultivated lines of *N. tabacum* lack both *F3'H* and *F3'5'H*
538 functions, making it difficult to assess the precise contribution of an
539 introduced transgene without metabolic interference [43–45].
540 Although some *Petunia* mutants lack hydroxylase functions, the
541 cultivars are limited in terms of efficient genetic transformation.
542 Furthermore, the small and unpigmented flowers of *A. thalia*, which

543 is a widely adapted model for molecular genetics, make it unsuitable
544 for research on visible pigment phenotypes.

545 **Petals opening inhibition in transgenic petals.**

546 A significant and unexpected outcome of this study was the
547 severe inhibition of petal opening in the transgenic *I. nil* lines with
548 high delphinidin accumulation (Figure 3). Microscopic observations
549 revealed that, unlike in wild-type plants, the petal cells in these
550 transgenic lines failed to expand, causing impairment of the petal
551 opening. Furthermore, in individuals of the transgenic progeny,
552 where petal opening was no longer inhibited, transgene expression
553 was suppressed and Dps content was reduced, as mentioned above.
554 These observations clearly indicate that the inhibition of petal
555 opening is likely associated the expression of *F3′5′H* and the
556 resulting accumulation of 3′,5′-hydroxylated flavonoids.

557 Aberrant accumulation of metabolic compounds, which leads
558 to petal abnormalities, has been reported for other species. For
559 instance, the accumulation of delphinidin 3-glucoside causes petal
560 shrinkage in *Petunia × hybrida* [46], and an excess of chalcone in a
561 chalcone isomerase (*CHI*) mutant of *I. nil* also results in shrunken
562 petals [18]. Similarly, petal malformation in Torenia has been linked
563 to the accumulation of cyanidin-type anthocyanins or their metabolic
564 precursors [47]. It is possible that the high level of delphinidin or
565 related metabolic compounds in transgenic *I. nil* in this study
566 triggers a physiological stress or defense response that possibly

disrupted the normal cellular processes required for petal opening. Although the inhibition correlated with high Dps content, it is possible that other 3′,5′-hydroxylated flavonoids that were biosynthesized by *F3′5′H*, such as the flavanol myricetin, also accumulated and contributed to this effect. The findings in this study may show a clear connection between the engineered accumulation of delphinidin-pathway flavonoids and the disruption of floral opening. However, the precise causative compound is yet to be identified.

Gene expression changes in inhibited petals

Salicylic acid (SA) is well known for its role in regulating plant defense mechanisms. In this study, GO enrichment analysis revealed that SA-associated processes were upregulated in petals with inhibited opening compared to petals with normal openings. Recent findings revealed the involvement of SA in cell wall remodeling during immune responses, as observed in *Pseudomonas*-infected plants, in which localized immune activation triggers structural changes in the cell wall [48]. Additionally, in phosphorus-deficient rice, SA mediates phosphorus remobilization by acting upstream of auxin and nitric oxide signaling [49].

The upregulation of genes associated with anthocyanin modification, transporters, and ROS may indicate cellular stress or efforts to sequester potentially harmful compounds in the vacuoles. Additionally, an anthocyanin-related MATE transporter gene was

included among the top DEGs. In *A. thalia*, the MATE transporter *TRANSPARENT TESTA12* (*TT12*) functions as a proton-driven flavonoid importer into the vacuole [50, 51]. The expression of such genes in the inhibited petals may reflect an effort to safely compartmentalize newly synthesized or misprocessed flavonoids. Transgenic petals may attempt to conjugate and vacuole-sequester delphinidin (Dp)-type anthocyanins, which are compounds not naturally produced in *I. nil*. This suggests the presence of bottlenecks in delphinidin glycosylation, acylation, or transport processes. The accumulation of aglycones or aberrantly conjugated anthocyanin forms in the cytosol could lead to stress and then disruption of normal developmental processes.

This stress response likely activates key transcription factors, such as *SARD1* and *CBP60*, which in turn promote the biosynthesis of salicylic acid. Elevated SA levels can subsequently trigger the expression of *WRKY* and *PR* genes, which are known markers of SA-induced defense pathways. These genes were also significantly upregulated in this study, indicating a potential link between stress-induced defense activation and inhibition of petal opening. However, further studies are required to fully elucidate these triggering mechanisms.

Among the top 20 upregulated DEGs, two were classical stress responders. *HIPP26*, which responds to heavy metal (cadmium) exposure and drought stress [52], suggests the presence of abiotic

or biotic stress signals in the inhibited petals.

Other significantly upregulated genes included those encoding MATE transporters and glycosyltransferases, further suggesting an attempt by plant cells to process and export cytosolic flavonoid pathway intermediates. Taken together, these observations suggest that the accumulation of non-native compounds such as delphinidin-related metabolites may trigger a cellular defense response. This response potentially disrupts developmental programs and inhibits petal opening. Further investigations are needed to determine the underlying molecular mechanisms and develop strategies to allow *I. nil* petals to open normally under transgenic conditions.

Conclusion

In this study, multiple *F3′5′H* genes derived from different species were successfully expressed in *I. nil*, enabling the production of Dps that do not naturally occur in this species. These findings demonstrate that *I. nil* is a promising model system for investigating the functional role of *F3′5′H* in anthocyanin biosynthesis. The species origin of *F3'5'H* significantly influenced the anthocyanin composition of transgenic plants, indicating that evolutionary divergence of *F3′5′H* contributes to distinct flavonoid metabolic outcomes. Notably, transgenic plants with high Dps content exhibited restricted petal cell expansion during flowering. RNA sequencing results revealed the activation of detoxification- and defense-related pathways, suggesting that excessive accumulation of delphinidin-pathway

flavonoids may trigger stress responses that interfere with normal petal development. Together, these findings provide new insights into the regulatory and physiological consequences of engineering delphinidin biosynthesis in ornamental plants.

Abbreviations

35S	Cauliflower mosaic virus 35S promoter
ANS	Anthocyanidin synthase
Asn	Asparagine
AtADH	5′untranslated region of the *Arabidopsis thaliana* alcohol dehydrogenase gene
cDNA	Complementary DNA
CHI	Chalcone isomerase
Cm	Campanula medium
Cy	Cyanidin
Cys	Cyanidin-based anthocyanins
DEGs	Differentially expressed genes
DFR	Dihydroflavonol 4-reductase
DHK	Dihydrokaempferol
DHM	Dihydromyricetin
DHQ	Dihydroquercetin
Dp	Delphinidin
Dps	Delphinidin-based anthocyanins
EC	Inhibited petal cells (Experimental Condition/Inhibited)
Eg	*Eustoma grandiflorum*
EO	Opening petal cells (Experimental Condition/Opening)

F3H Flavanone 3-hydroxylase

F3′H Flavonoid 3′-hydroxylase

F3′5′H Flavonoid 3′,5′-hydroxylase

Gt *Gentiana triflora*

HCl Hydrochloric acid

HPLC High-Performance Liquid Chromatography

HT Arabidopsis heat shock protein 18.2 gene terminator

Mv Malvidin

NOS Nopaline synthase gene terminator

NtADH 5′untranslated region of the *Nicotiana tabacum* alcohol dehydrogenase gene

Pg Pelargonidin

Pn Peonidin

PR Pathogenesis-Related Protein

Pt Petunidin

qPCR Quantitative real-time reverse transcription-polymerase chain reaction

RNA Ribonucleic acid

ROS Reactive oxygen species

SA Salicylic acid

SP1L SPIRAL1-like

THF Tetrahydrofuran

TPM Transcripts Per Million

UV Ultraviolet

Vw *Viola* × *wittrockiana*

Declarations

This manuscript is an extended and substantially revised version of our conference paper published in Acta Horticulturae (Huynh et al., 2023) [19]. This submission includes additional experiments, expanded datasets, and new analyses that were not part of the original publication.

Ethics approval and consent to participate.

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors contributions

Design and supervision of experiment: K. Shimizu, F. Hashimoto, and N. Kozai.

Transformation and vector construction: T.-P. Huynh, C. Motoyama, and H. Oshima.

Pigment analysis: T.-P. Huynh, C. Motoyama, and H. Oshima.

RNA-Seq data analysis: T.-P. Huynh.

RT-PCR: T.-P. Huynh and N. Yamate.

Writing: T.-P. Huynh and K. Shimizu.

All authors read and approved the final manuscript.

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Authors' information

Supplementary information

Supplementary material 1

Additional file 1.zip: File containing the sequence of binary vectors constructed in this study. After decompression, the sequence was

opened using SnapGene Viewer or ApE (A plasmid Editor).

pKANre3AH.dna

pEg.dna

pEg-ADH-.dna

pVw.dna

pCm.dna

pGt.dna

Supplementary material 2

Additional file 2.ppt: Supplemental figure and table.

Figure S1. Phenotype pictures and HPLC chromatograms showing anthocyanidin profiles in the petals of T0 transformant. The detected anthocyanidins are labeled as follows: Dp, delphinidin; Cy, cyanidin; Pn, peonidin; Pg, pelargonidin; Pt, petunidin; and Mv, malvidin. T0 of pEg has two varied HPLC compositions: (A) one that accumulated six types of anthocyanidins and (B) one that accumulated three types of anthocyanidins. T0 of pCm have two varied HPLC compositions: (E) one that accumulated five types of anthocyanidins and (F) one that accumulated three types of anthocyanidins. T0 of pWm have two varied HPLC compositions: (G) one that accumulated six types of anthocyanidins and (H) one that accumulated three types of anthocyanidins. T0 of pGt have one HPLC composition: (I) one that accumulated three types of anthocyanidins. Each peak was identified by co-injection with authentic anthocyanidin standards.

Figure S2. HPLC chromatograms showing anthocyanidin profiles in

757 the petals of T1 progeny. The detected anthocyanidins are labeled
758 as follows: Dp, delphinidin; Cy, cyanidin; Pn, peonidin; Pg,
759 pelargonidin; Pt, petunidin; and Mv, malvidin. (A), (B), and (C)
760 chromatograms represent the corresponding three individuals of
761 pEg progeny lines. (D), (E), and (F) chromatograms represent the
762 corresponding three individuals of pVm progeny lines. (G), (H), and
763 (I) chromatograms represent the corresponding three individuals of
764 pCm progeny lines. (J), (K), and (L) chromatograms represent the
765 corresponding three individuals of pGt progeny lines. Each peak was
766 identified by co-injection with authentic anthocyanidin standards. X-
767 axis indicate intensity (μV), whereas U-axis indicate retention time.

768 **Figure S3.** Correlation analysis between relative *F3'5'H* expression
769 and Dp concentration. Scattered plots showing the linear regression
770 between the relative transcript levels of *F3'5'H* (x-axis) and the
771 concentration of Dps (mg/g fresh weight) (y-axis). The analysis was
772 performed for four different constructs/lines: (A) pEg, (B) pVw, (C)
773 pCm, and (D) pGt. The dotted blue lines represent the linear trend.
774 The coefficient of determination (R^2), t-value, and *p*-value are
775 indicated within each panel. (B) indicate that the correlation of pVw
776 is significantly different.

777 **Figure S4.** Differentially expressed genes (DEGs) connected to
778 anthocyanidin in inhibited (close) and open petals. Category-specific
779 distribution of DEGs between open and closed petals of *I. nil*. The
780 bar plot summarizes the number of DEGs classified into three major

functional categories: reactive oxygen species (ROS)/redox-related genes, transcription factors, and anthocyanin transport/modification genes. Bars to the right (positive x-axis) represent genes that were upregulated in closed petals, whereas bars to the left (negative x-axis) represent genes that were downregulated in closed petals. Red for downregulated and blue for upregulated in inhibited (close) petal.

Figure S5. Validation of differentially expressed genes (DEGs) between opening and inhibited petal. (A) Comparison of expression levels for selected *I. nil* genes as determined by RNA-Seq (blue bars) and quantitative real-time PCR (orange bars). The x-axis represents the specific gene identifiers, and the y-axis represents the relative expression level and m.value $\log_2$ fold change. (B) The plot shows the correlation between qRT-PCR and RNA-seq validation results, with a linear regression line indicating the relationship between the two datasets and R^2 representing the coefficients of determination, that is, the degree of correlation between two measurements.

Table S1. Specific primers for amplification and Infusion cloning of *F3′5′H* from *E. grandiflorum*, *C. medium*, *V. × wittrockiana*, and *G. trifloral* for the construction of transgenic vectors.

Table S2. Primers used for qPCR analysis of transgenic *F3′5′H* expression level.

Table S3. Top 20 upregulated genes and their comparison with

804 temporal gene expression database of *I. nil.* ↑ (Upregulated), =
805 (Unchanged)

806 **Table S4.** Possible anthocyanin modification and transporter
807 related gene in inhibited (close) and open petals**.**

808 **Table S5. Reactive oxygen species (**ROS)-related DEGs in
809 inhibited (close) and open petals.

810 **Table S6.** Primer list used for qRT-PCR validation of RNA-seq data.

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FIGURE LEGENDS

Figure 1. Schematic representation of the binary vectors containing *F3′5′H* used for *I. nil* transformation. Each vector contains one *F3′5′H* gene from a specific species ((*E. grandiflorum*, *C. medium*, *V. × wittrockiana*, or *G. triflora*). The T-DNA region of the binary vectors extend from the left border (LB) to the right border (RB) and includes the nopaline synthase gene promoter of *Agrobacterium* (NosP); a kanamycin resistance selectable marker (NPTII, KmR); and an expression cassette comparing a tetrameric enhancer array fused to the CaMV 35S promoter (4×35S), a 5′-UTR module (AtADH 5′-UTR or NtADH 5′-UTR), the Flavonoid 3',5'-Hydroxylase (*F3′5′H*) coding region, and a terminator (either Heat Shock Protein terminator (HT) or nopaline synthase gene terminator of Agrobacterium (NosT)). Three vector backbones were used: pEg (carrying *E. grandiflorum* F3′5′H), pEg-ADH^{-} (as *EgF3′5′H* but lacking the *A. thaliana* ADH 5′-UTR), and pB237 [4]. Elements outside the T-DNA include the *E. coli* origin of replication (Ori) and AmpR for bacterial selection.
* The *F3′5′H* coding region was derived from one of the following species: *E. grandiflorum*, *C. medium*, *V. × wittrockiana*, or *G. triflora*.

Figure 2. Characterization of transgenic *I. nil* T1 progeny lines. (A) Flower phenotypes and High-Performance Liquid Chromatography (HPLC) profiles of anthocyanins in representative transgenic *I. nil* T1 progeny lines. Each line was transformed with an *F3′5′H* gene from one of four different species: *E. grandiflorum*, *C. medium*, *V. ×*

wittrockiana, or *G. triflora*. The chromatograms show peaks that correspond to various anthocyanidins: Dp (Delphinidin), Cy (Cyanidin), Pt (Petunidin), Pn (Peonidin), Mv (Malvidin), and Pg (Pelargonidin). Each peak was identified by co-injection with authentic anthocyanidin standards. (B) Relative expression levels of the introduced *F3′5′H* gene in three progeny individuals of each line. Expression levels are shown for lines pEg (*EgF3'5'H*), pWm (*VwF3'5'H*), pCm (*CmF3'5'H*), and pGt (*GtF3'5'H*). Expression levels were normalized to the internal control gene *InATP*, which was set to 1 for relative expression level analysis, and it is presented as relative transcript abundance. Data are presented as mean ± standard deviation (SD). Statistically significant difference between individuals within the same transgenic line was tested using Tukey HSD (n=3, significant $p < 0.05$). (C) Accumulation of delphinidin-based anthocyanins (Dps) in the petals of the transgenic *I. nil* T1 progeny lines described in (B). The concentration of Dps is expressed as mg/g fresh weight. Data are presented as mean ± standard deviation (SD) for each individual in line. Statistically significant difference between individuals within the same transgenic line group was evaluated using Tukey's HSD test ($p < 0.05$). Different letters indicate significantly different means ($p < 0.05$), as determined by the Tukey's test. (D) Bar chart displays the Dp content (%) in progeny of lines pEg, pWv, pCm, and pGt. Data are presented as mean ± standard deviation (SD). Different lowercase letters above individual bars indicate significant differences between lines

within the same construct, as determined using Tukey HSD test ($p < 0.05$). Letters situated above the brackets indicate significant differences between the overall means of the groups tested with Tukey HSD ($p < 0.05$).

Figure 3. Microscopic analysis of petal cross-sections from a Delphinidin (Dp)-accumulating *Ipomoea nil* line across different developmental stages, including an inhibited flower opening phenotype. Red Bar = 0.03mm. The figure displays: (A) One day before flower opening, with the corresponding petal cross-section showing initial pigment accumulation primarily in the epidermal cells. (B)* A fully open flower, with its petal cross-section revealing intense pinky-purple (Pg) pigmentation concentrated in the epidermal cells and clearly observed cells expansion. (C) A more developed but still closed bud, with its petal cross-section showing significant blue-purple pigmentation (Dps) in the epidermal cells. (D)* An inhibited bud, with its petal cross-section displaying blue-purple pigmented (Dps) epidermal cells. The cross-sections highlight the distribution and intensity of anthocyanin pigments within the petal cells, but no sign of cell expansion has been observed. (E) Phenotype images and anthocyanidin profiles of opening and inhibited petals. (F) Relative expression level of *EgF3′5′H* in inhibited and open petals. The x-axis indicates relative expression level. Different lowercase letters above individual bars indicate significant differences between lines within the same construct, as determined using Turkey HSD test ($p < 0.05$).

* This study is an extension of a report by Huynh et al 2023 [18]. Figure 3B and 3D in this paper were obtained from the report in Figure 2.

Figure 4. Gene Ontology (GO) term enrichment analysis of transgenic *Ipomoea nil* open and inhibited petals of T1 progeny. Gene Ontology (GO) enrichment analysis was performed on differentially expressed genes (DEGs) between open and inhibited petals of transgenic *I. nil*. The top 20 enriched GO terms are in three categories across Biological Process, Molecular Function, and Cellular Component. The x-axis is the Gene Ratio (number of DEGs annotated to a term ÷ all DEGs), and terms are listed on the y-axis. Bubble size corresponds to the number of DEGs mapped to each term, and bubble color indicates enrichment significance as $-\log_{10}$(*P*-value) (darker = more significant). Abbreviation: DEG, differentially expressed gene.

Figure 5. Transcriptomic analysis of differentially expressed genes between *I. nil* open and inhibited petals. Differentially gene expression was analyzed based on $\log_2$ fold change (x-axis) and statistical significance ($-\log_{10}$ adjusted *P*-value; y-axis). (A) Volcano plot showing the genome-wide distribution of differentially expressed genes (DEGs), (B) WRKY transcription factor family; (C) Pathogenesis-related (PR) genes; (D) Cell wall-modifying genes, including expansin, EG45-like, and pectinesterase; (E) Genes involved in anthocyanin transport and modification; (F) Aquaporin

genes; (G) MYB transcription factors; (H) SPL1 gene family. Red dots indicate significantly upregulated genes, blue dots represent significantly downregulated genes, and gray dots denote non-significant changes.

Figure 6. Heatmap and physiological characterization of gene expression in open and inhibited petals of *Ipomoea nil*. Heatmap illustrates the expression profiles of the top 20 upregulated differentially expressed genes (DEGs) identified in inhibited petals of *I. nil* compared to open petals. Each row represents one gene, labeled by transcript ID and putative annotation (where available). Columns represent biological replicates for mean value of open (n=3) and inhibited (closed, n=3) petal samples. Color intensity indicates raw expression levels (read counts), with the color scale ranging from low (white) to high (dark red). (E) Photographs of open and inhibited petals and their corresponding HPLC anthocyanin profiles. (F) Relative *F3′5′H* gene expression levels of inhibited and open petals. Data for gene expressions are presented as mean ± standard deviation (SD). Different lowercase letters (a, b) indicate significantly different means ($p < 0.05$), as determined by Tukey's test. (G) Delphinidin-based anthocyanin (Dps) content (%) in inhibited and open petals. Data for gene expressions are presented as mean ± standard deviation (SD). Different lowercase letters (a, b) indicate significantly different means ($p < 0.05$), as determined by the Tukey's test.