CRISPR/Cas9-mediated targeted mutation reveals a role for AN4 rather than DPL in regulating venation formation in the corolla tube of Petunia hybrida

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Abstract
Venation is a common anthocyanin pattern displayed in flowers that confers important ornamental traits to plants. An anthocyanin-related R2R3-MYB transcription factor, DPL, has been proposed to regulate corolla tube venation in petunia plants. Here, however, we provide evidence redefining the role of DPL in petunia. A CRISPR/Cas9-mediated mutation of DPL resulted in the absence of the vein-associated anthocyanin pattern above the abaxial surface of the flower bud, but not corolla tube venation, thus indicating that DPL did not regulate the formation of corolla tube venation. Alternately, quantitative real-time PCR analysis demonstrated that the spatiotemporal expression pattern of another R2R3-MYB gene, AN4, coincided with the formation of corolla tube venation in petunia. Furthermore, overexpression of AN4 promoted anthocyanin accumulation by increasing the expression of anthocyanin biosynthesis genes. CRISPR/Cas9-mediated mutation of AN4 led to an absence of corolla tube venation, suggesting that this gene in fact determines this key plant trait. Taken together, the results presented here redefine the prime regulator of corolla tube venation, paving the way for further studies on the molecular mechanisms underlying the various venation patterns in petunia.

Introduction
Anthocyanin pigments contribute to the diverse colors and pigmentation patterns of flowers, which are important ornamental characteristics of horticultural plants. Anthocyanins are derived from the flavonoid biosynthetic pathway, involving a series of enzymes encoded by several structural genes. In dicot plants, these structural genes can be subdivided into early biosynthetic genes (EBGs), including chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), and flavonoid 3′hydroxylase (F3′H), and late biosynthetic genes (LBGs), such as flavonoid 3′5′-hydroxylase (F3′5′H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and glutathione-S-transferase (GST). Expression of these EBGs is regulated by R2R3-MYB transcription factors, while the expression of the LBGs is regulated by the ternary complex MYB–bHLH–WD40 consisting of R2R3-MYB, a basic helix-loop-helix (bHLH), and WD40 transcription factors. Among these transcription factors, R2R3-MYB genes play major roles in providing the required specificity for LBG expression and consequently determining the spatiotemporal accumulation of anthocyanin in plants.

Venation patterning arises from pigmented stripes overlying veins and is a common anthocyanin pattern seen in flowers. In Antirrhinum, the bHLH gene Delila is expressed in its petal epidermis, and Venosa, an R2R3-MYB gene, is specifically expressed in cells above the vascular tissues, with venation arising in the overlapping expression domains of the R2R3-MYB and bHLH genes. Since the bHLH and WD40 genes are usually expressed constitutively, the formation of venation patterning...
depends mainly on $R2R3$-$MYB$, which provides vein specificity. For example, in *venosa* mutants, no venation is displayed in their petals\(^{14}\), and silencing of *PeMYB12*, an $R2R3$-$MYB$ gene, induces the loss of venation patterning in sepals/petals of *Phalaenopsis* spp.\(^{15}\). In addition to venation, $R2R3$-$MYB$ is also the key regulator of two other anthocyanin patterns: blotch\(^{16}\) and spot\(^{17,18}\).

*Petunia hybrida*, distinguished by its diverse flower colors and pigmentation patterns, is one of the most popular bedding plants\(^ {19}\). In such petunia plants, diverse types of venation patterns have been developed in the corolla of some cultivars to improve their ornamental value. Several genetic loci reportedly control the various patterns of venation in the corolla of petunias. For example, individuals harboring the *Ve1* locus display reticulate venation of the corolla tube, whereas *ve1* individuals exhibit a few longitudinal pigmented stripes over the veins; *Ve2* and *Ve3* loci determine the extension of venation from the corolla throat to the outer edge of the limb; and the *Fn* locus controls the thickness of venation in the corolla limb\(^ {20}\). A previous study proposed that an anthocyanin-related $R2R3$-$MYB$ gene, *DEEP PURPLE* (*DPL*), may reside at the *Ve1* locus\(^ {21}\). The role of *DPL* in the regulation of venation patterning in corolla tubes has since been cited widely in many recent publications\(^ {12,15,22–24}\). In addition to *DPL*, another anthocyanin-related $R2R3$-$MYB$ gene, *ANTHOCYANIN4* (*AN4*), is capable of associating with these *Ve* and *Fn* loci according to genetic analyses. For example, a stronger venation is associated with the presence of the dominant allele of *AN4* and the recessive *ve2* allele\(^ {20,25}\). We know that *AN4* can regulate the anthocyanin accumulation in anthers\(^ {26}\), but whether this gene also determines venation patterning remains unknown.

In this work, we reinvestigated the respective roles of *DPL* and *AN4* in flower development, mainly by using CRISPR/Cas9 targeted mutation technology. We found that venation patterning in petunia corolla tubes is actually regulated by *AN4*, not the proposed *DPL*. Our results also revealed that *DPL*’s true role in petunia flower petals is to regulate their vein-associated anthocyanin patterns above the abaxial surface.

**Results**

**CRISPR/Cas9-induced mutation of *DPL* in MD**

To investigate the specific role of *DPL* in petunia, we mutated *DPL* in the *P. hybrida* inbred line “Mitchell Diploid” (MD) by using the CRISPR/Cas9 system. One target site in the second exon was selected as the sgRNA complementary site (Fig. 1a), and three *DPL* biallelic mutants (*dpl-M3*, -M4, and -M5) in MD were obtained. In the *dpl-M5* line, one allele of *DPL* exhibited a deletion of 18 bp, which introduced a new stop codon in *DPL*, leading to premature terminations. The deletion in the second *DPL* allele of *dpl-M5* and the indel mutations in the *DPL* alleles of *dpl-M3* and *dpl-M4* mutants interrupted the
reading frame, leading to frameshift mutations (Fig. 1b). Considering that the target sequence of DPL is highly similar to that in AN4I and AN4II, a potential off-target site was detected in AN4I and AN4II of the dpl-M3, -M4, and -M5 lines; however, no mutations could be found in the off-target site of these lines (Fig. S3a). Therefore, dpl-M3, -M4, and -M5 were dpl-knockout mutants and were used for further investigations.

No significant changes in corolla tube venation patterning were detected between dpl mutants and MD (Fig. S1a–d). However, vein-associated anthocyanin deposition above the abaxial surface of the flower bud was lost in dpl mutants (Fig. 1c, d and Fig. S1e–g). In the T1 lines of dpl-M3, -M4, and -M5, this mutated phenotype was observed. Vein-associated anthocyanin patterning initially emerged at the flower bud abaxial surface, but gradually faded as the flower opened (Fig. S1h). Furthermore, this anthocyanin patterning became more obvious in flower buds of MD, when these plants with flower buds were treated at 12 ± 2 °C for 2 weeks, but it was always absent in those of dpl-knockout mutants (Fig. 1e). These results indicated that DPL determines vein-associated anthocyanin occurrence above the abaxial epidermis of petunia flower buds rather than the venation patterning of its corolla tube.

Positive correlation between the expression patterns of AN4 and the formation of corolla tube venation

Because venation patterning was not affected in dpl mutants, we next investigated whether other MYB genes were perhaps involved in regulating petunia venation patterning. qPCR analysis showed that the mRNA abundance of AN4 was much higher in the corolla tube than in the anther or limb, whereas the mRNA abundance of DPL was much higher in the limb and corolla tube than in the anther (Fig. 2a). Notably, within the corolla tube, the transcript level of AN4 was higher on the venation side than on the nonvenation side after the opening of the flower (Fig. 2b). The transcript level of DPL was higher on the venation side than on the nonvenation side and was higher than the expression of AN4 on the nonvenation side (Fig. 2b).

Going further, we then investigated the AN4 and DPL expression levels during the flower development. Based on corolla tube length (0.5, 1.5, 2.5, and 3.5 cm), flower development was divided into stage 1 (S1), stage 2 (S2), stage 3 (S3), and stage 4 (S4; Fig. 2c). During flower development, the venation pattern was gradually accentuated by the presence of anthocyanin. The mRNA abundance of DPL continuously decreased from S1 to S4.

![Fig. 2 AN4 and DPL expression patterns. a, b Spatial expression pattern of AN4 and DPL in the opening flower of MD. c Annotation of flower developmental stages in MD; scale bar = 1 cm. d Temporal expression patterns of AN4 and DPL during flower development in MD. SAND was used as the reference gene. Shown are the means ± SDs, n = 3. Asterisks indicate a significant difference: *P < 0.05; **P < 0.01; nonsignificant (NS). e Annotation of different parts of the flower in MD; scale bar = 1 cm.](image)
(Fig. 2d). However, the expression level of AN4 continuously increased from S1 to S4 (Fig. 2d), in accordance with the emergence of venation. These results indicated that AN4 could determine corolla tube venation in MD.

**AN4 promotes anthocyanin biosynthesis in MD**

To confirm that AN4MD encoded a functional R2R3-MYB transcription factor in MD, the transcript of AN4 was isolated from this plant’s corolla tube. Sequence analysis revealed that the open reading frame of AN4MD was highly similar to that of AN4V30, which has been identified as an activator of anthocyanin biosynthesis in the V30 line27. The deduced amino acid sequence of AN4MD, along with other anthocyanin-related R2R3-MYBs in petunia, contained the conserved [D/E]Lx2[R/K]x3Lx6Lx3R motif in its R3 domain (Fig. 3), as required for interacting with the AN1 (bHLH) protein21. Outside the conserved R2 and R3 domains, several substitutions of amino acids were present in AN4MD compared with AN4V30 (Fig. 3).

To verify the function of AN4MD in MD, transgenic lines expressing AN4MD under the control of the CaMV35S promoter were generated in the MD background. Twelve independent transgenic lines (AN4OE) with pigmented leaves, stems, anthers, and corolla limbs were obtained (Fig. 4a, b). Although anthocyanins had accumulated in the anthers of these transgenic lines, their pollen was still yellow (Fig. 4c). The mRNA abundance of AN4 in the five transgenic lines was assessed by qPCR. As expected, the transcription of AN4 in these lines was higher than that in MD, and correspondingly, their anthocyanin content was markedly increased (Fig. S2a, b). We next investigated the effect of overexpressing AN4MD on the mRNA abundance of anthocyanin biosynthetic genes. These qPCR results revealed that the expression levels of most of these genes, including CHSA, CHSI, F3H, F3’H, F3’5’H, DFR, ANS, 3RT, 5GT, and GST but not F3’H, were strongly upregulated in AN4OE transgenic plants compared with those in wild-type plants (Fig. 4d). These results indicated that AN4MD functions as a transcriptional activator of the anthocyanin biosynthetic pathway in MD.

**AN4 determines corolla tube venation patterning**

To identify the function of AN4 in regulating corolla tube venation, the CRISPR/Cas9 system was applied to generate an AN4 mutant. Referring to the genome sequence of Petunia axillaris19, two AN4 genes, AN4I and AN4II, containing the same gene structure but different promoters were isolated from the MD genome (Fig. 5a). One sgRNA was designed to simultaneously target the second exon of both AN4I and AN4II (Fig. 5a); in this way, seven transgenic lines without venation patterning in their corolla tube were obtained. Three nonvenation lines (an4-M1, -M2,
and -M6) were randomly selected to detect the mutation types in the AN4I and AN4II loci. In the an4-M1 and an4-M6 lines, biallelic mutations occurred in the AN4I and AN4II loci, and the an4-M2 line harbored a biallelic mutation of AN4II and a monoallelic mutation of AN4I (Fig. 5b). The indel mutations in these mutants resulted in frameshift mutations, rendering the AN4 protein inactive.

In the T1 lines of an4-M1, -M2, and -M6, venation patterning disappeared in the corolla tube as well. Considering that the target sequences of AN4 and DPL were highly similar to each other, a potential off-target site was detected in the DPL locus of the an4-M1, -M2, and -M6 lines; however, no mutations could be found in the off-target site of these lines (Fig. S3b).

To investigate the role of AN4 in regulating the expression of EBGs and LBGs, qPCR was carried out to detect the relative expression levels of anthocyanin biosynthetic genes in the an4-M1, -M2, and -M6 lines. These results showed that transcript levels of EBGs (CHSA, CHSI, F3H, and F3′H) went unchanged, but those of LBGs (F3′5′H, DFR, ANS, 3RT, 5GT, and GST) were significantly downregulated in the an4-M1, -M2, and -M6 lines compared with those in MD; however, no mutations could be found in the off-target site of these lines (Fig. S3b).

**Discussion**

Petunia is a classical model system for studying the biosynthesis and regulation of flower pigments. Some pigmentation patterns in petunia flowers have been well characterized. For example, the spot pattern is the result of an unstable transposon insertion in the ANTHOCYANIN3 locus, and star-type and picotee patterns are induced by RNA silencing of CHS. In this paper, we revealed that venation patterning in the corolla tube is determined by the R2R3-MYB transcription factor AN4, but not DPL.

Formerly, according to patterns of gene expression, segregation analysis, and vein-associated promoter activity, DPL was proposed to govern venation patterning in MD flower tubes. However, the lines of evidence for this were indirect and inconclusive. First, the mRNA abundance of DPL gradually decreased during flower development, coinciding with the disappearance of vein-associated anthocyanin on the abaxial epidermis of the flower bud, whereas the venation patterning gradually emerged during flower development, coinciding with AN4 expression. Second, despite the cosegregation of DPL and venation patterning, the latter’s formation may have been driven by AN4 instead of DPL in the segregation analysis, because AN4 and DPL are on the same chromosome, lying just 60 kb apart. Third, loss-of-function mutants or gene-silenced lines of DPL, which are important for verifying the specific roles of DPL in petunia, were lacking. Hence, overall, DPL might not determine venation...
patterning in petunia. By relying on the CRISPR/Cas9 system, we were able to obtain sought-after dpl mutants. In these, vein-associated anthocyanins on the abaxial epidermis of flower buds disappeared rather than venation patterning, which unchanged (Figs. 1d and S1). Combining the vein-associated promoter activity of DPL and the phenotype of CRISPR/Cas9-induced DPL mutants suggests that DPL’s role is to determine vein-associated anthocyanin patterning on the abaxial epidermis of flower buds. Nonetheless, vein-associated anthocyanin patterning was more pronounced under cold conditions (Fig. 1e) and matched well to DPLpro-directed GUS localization in the corolla21, thus suggesting that DPL may respond to cold stress in petunia.

To date, it has been reported that nine MYB genes—AN4, AN2, DPL, PURPLE HAZE (PHZ), MYB27, MYBs, ANTHOCYANIN SYNTHESIS REGULATOR 1 (ASR1), ASR2, and ASR3—regulate anthocyanin biosynthesis in petunia. These genes act as transcriptional activators or repressors of anthocyanin biosynthesis by positively or negatively regulating the expression of anthocyanin biosynthetic genes. Previous research has demonstrated that AN4 determines the pigmentation of anthers in petunia, so AN4 is primarily regarded as having recessive alleles in some petunia lines with acyanic anthers, such as those of MD, V26, R27, and W138 (refs. 26,28). However, we found that AN4 is a functional activator of anthocyanin biosynthesis genes in MD plants. The expression levels of most EBGs were upregulated in transgenic plants overexpressing AN4 (Fig. 4d), but were not affected in CRISPR/Cas9-induced an4 mutants (Fig. 5c). Similarly, the transcript levels of EBGs were not changed in the an2 mutant26. In Arabidopsis, EBGs are activated by flavonol-specific R2R3-MYBs, including MYB11, MYB12, and MYB111 (refs. 37,38). The expression of EBGs in the an2 mutant and CRISPR/Cas9-induced an4 mutants might be activated by the orthologs of AtMYB11, AtMYB12, and AtMYB111, which have not been isolated in petunia. Therefore, it remains challenging to improve the regulatory mechanism controlling the expression of EBGs and LBGs in petunias.

During flower development, the spatiotemporal expression pattern of AN4 was positively associated with the formation of venation patterning in the corolla tube (Fig. 2a, b, d). Furthermore, CRISPR/Cas9-induced AN4 mutation resulted in the complete absence of venation patterning in MD corolla tubes (Fig. 5b). Together, these results indicate that AN4 determines venation patterning in the corolla tube of MD flowers. In addition to regulating corolla tube venation, AN4 also regulates anther pigmentation. Similar to AN4, PeMYB12 determines not only venation patterning in sepal/petals, but also full pigmentation in the central lobe of the lip in Phalaenopsis spp. Chia et al. indicated that the dual functions of PeMYB12 may arise from a differential regulatory mechanism that exists in different floral organs. In the MD line, the
expression of AN4 was activated in its corolla tube but
inactivated in its anthers (Fig. 2a), which indicates that AN4
may be regulated via different mechanisms in the anther
versus corolla tubes. The same expression pattern of AN4
was also observed in the V/26 line. The inactivation of AN4
in the anthers of V/26 was believed to result from the
methylation that occurred in the AN4 coding sequence
and promoter, but the mechanism activating the expression of
AN4 in the corolla tube remains unclear. Additional
efforts are therefore required to dissect the upstream reg-
ulatory mechanism of AN4, which should be helpful for a
better understanding of how AN4 activity is governed to
form venation patterning.

In addition to AN4, Ve1 also participates in the reg-
ulation of corolla tube venation patterning in petunia.
Unlike the phenotype of the an4 mutants, a few lon-
titudinal pigmented stripes appeared in the corolla tube
of ve1 individuals, pointing to other as-of-yet unclear
factors involved in determining corolla tube venation.
Given the associations between AN4 and Ve loci uncov-
ered in genetic analysis, elucidating the molecular
nature of these Ve loci would provide insight into the
mechanism underlying various venation patterns in petu-
ния, making it a promising area of future research.

CRISPR/Cas9 is a powerful tool for creating null
mutants and dissecting the functions of genes. To date, it
has been used successfully to re-evaluate and redefine plant
gene functions in a few cases, from which novel
insights were obtained. In the present work, the func-
tioning of DPL and AN4 was re-evaluated in petunia using
the CRISPR/Cas9 system. The obtained results strongly
suggest that DPL regulates vein-associated anthocyanin
patterning on the abaxial epidermis of flower buds, while
AN4 determines corolla tube venation in petunia. These
findings refine the regulatory framework of flower
anthocyanin patterns in petunia and can facilitate further
understanding of the mechanism underpinning these
various patterns in petunias and perhaps other flowers.

Materials and methods

Plant materials and growth conditions

The petunia (P. hybrida) inbred species MD was used in
this study. Transgenic and MD plants were grown under
greenhouse conditions (16 h light/8 h dark photoperiod,
200–250 μmol m−2 s−1 light intensity, at 25 °C). For the
cold treatment, the MD line and the dpl-M3, -M4, and
-M5 mutants were cultured under greenhouse conditions
until flowering, and the plants were placed in a growth
chamber at 12 ± 2 °C for 2 weeks with a 16 h light/8 h dark
photoperiod and 120 μmol m−2 s−1 light intensity.

Vector construction and transformation of petunia

To isolate the AN4 cDNA from MD, the gene-specific
primers AN4MD-F/R were designed according to the
sequence of AN4V30 (accession number HQ428105).
Total RNA extraction and cDNA synthesis were per-
formed, as previously described. The complete coding
sequence of AN4MD was isolated from the cDNA of MD
petals. The PCR products were cloned into a pMD19-T
vector (Takara, Dalian, China) and then sequenced by
Tsingke Biological Technology (Chongqing, China). To
overexpress AN4 in MD, the p35S:AN4 vector was gen-
erated by cloning the coding sequence of AN4 into a
modified pGreenII0229 vector between the 35S promoter
and 35S terminator. To generate AN4-knockout mutants,
the genomic sequences of AN4I and AN4II were amplified
from MD according to the genomic sequence of P. axil-
laris, and then, sgRNA targeting the border region of AN4
exons was designed using the CRISPR-GE web tool
(skl.scau.edu.cn). The sgRNA cassette was synthesized and
cloned into a CRISPR/Cas9 construct to generate the
pGGEAN4 vector, as described by Zhang et al. Likewise,
to generate DPL-knockout mutants, a corresponding
pGGEDPL vector was constructed as above. All the prim-
ners used for gene amplification or the construction of
gene-editing vectors can be found in Supplementary
Table 1. These recombinant vectors were introduced into
Agrobacterium tumefaciens strain GV3101 by electro-
poration. The Agrobacterium-mediated transformation of
petunia was performed according to Zhang et al.

Detection of mutations

Genomic DNA was first extracted from transgenic lines
via the CTAB method and then used as a template to
amplify DNA fragments harboring the designed target
sites with gene-specific primers (Supplementary Table 1).
PCR was conducted using TopTag DNA polymerase
(Trans, Beijing, China) in a 25 μl volume. The amplifica-
tion reaction was performed as follows: 94 °C for 5 min,
followed by 28–30 cycles of 94 °C for 30 s, 55 °C for 30 s,
and 72 °C for 2 min, and then 72 °C for 10 min. The PCR
products were separated by 1% agarose gel electrophoresis
directly sequenced by Sanger sequencing. Then, the
sequencing chromatograms were decoded into allelic
sequences to identify the mutations using the CRISPR-GE
web tool (skl.scau.edu.cn). The sequencing chromatograms
are presented in Fig. S3.

Real-time PCR analysis

Total RNA extraction and cDNA synthesis were carried
out as previously described. Quantitative real-time PCR
was performed with Ssofast EvaGreen Supermix (Bio-Rad,
Hercules, CA, USA) on a CFX96 Real-time PCR System
(Bio-Rad, Hercules, CA, USA). Primers for anthocyanin
biosynthetic genes were designed according to Albert
et al. The SAND gene was used to normalize the sam-
ple. Relative gene expression values were calculated
using the methodology of Schmittgen and Livak. The
qPCR data are presented here as the mean ± SE of three biological replicates. All primer sequences used for qPCR are listed in Supplementary Table 1.

**Anthocyanin measurement**

Extraction of anthocyanins was performed, as described by Viola et al. Briefly, 0.15 g fresh samples were ground into powder using liquid nitrogen and then incubated in 15 mL of 1% HCl–methanol (v/v) at 4 °C overnight in the dark. After centrifuging each sample, the absorbance of the resulting supernatant was measured at 530 and 657 nm on a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher, Waltham, MA, USA). The relative anthocyanin content was then determined as \((A_{530} - 0.25 \times A_{657})\) per gram fresh weight.

**Statistical analysis**

The significance of differences among the data were determined by Student’s *t* test using SPSS 17.0 software. Statistically significant differences are indicated by asterisks * \((P < 0.05)\) or ** \((P < 0.01)\).

**Accession numbers**

The sequences of the genes described in this work can be found in GenBank: *DPI*, HQ116169; *AN4*, HQ428104. The genome sequences of *AN4* and *AN4III* can be obtained from the Sol Genomic Network (https://solgenomics.net/) under the accession numbers Peaxi162Scf00578g00008.1 and Peaxi162Scf00578g00007.1, respectively.

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**Author contributions**

Y.G. designed the experiments. B.Z., X.X., and S.Y. performed the experiments. B.Z. and R.H. analyzed the data. M.L. and Y.G. contributed materials and reagents. Y.G. and B.Z. wrote and edited the manuscript.

**Conflict of interest**

The authors declare no competing interests.

**Supplementary information**

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