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yanling Hao

schuanedu@126.com

Chengdu Agricultural College

Miao Yang

Sichuan Agricultural University

Jing Meng

Yunnan Agricultural University

Stephen Maciejewski

The Gesneriad Society Gesneriad Conservation Center of China (GCCC)

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Genetic Characteristics and Formation Mechanisms of Distinctive Traits in Two Types of *Sinningia speciosa* Corolla Tubes Based on Complete Diallel Cross and Multi-Generation Selfing

Hao Yanling^{1*}, Yang Miao², Meng Jing³, Stephen Maciejewski⁴ ...

¹ Department of Agronomy and Horticulture, Chengdu Agricultural College, Chengdu, Sichuan, 611130, China;

² College of Agriculture, Sichuan Agricultural University, Chengdu, Sichuan, 611130, China;

³ College of Landscape Architecture and Horticulture, Yunnan Agricultural University, Kunming, Yunnan, 650201, China.

⁴ The Gesneriad Society (Gesneriad Conservation Center of China (GCCC)), Seattle, 90876, USA.

Abstract: [Methods] Based on two corolla tube types (L-T, C-T), 256 F₁ individuals from a complete diallel cross involving, 5000 F₂ individuals and 100 S₆ (selfed for six generations) populations. Phenotypic traits, cytology, qRT-PCR, and CRISPR/Cas9 assays were conducted to examine genetic differential expression between the two corolla types. **[Results]** The order of dominance in hybrids was labiate > campanulate; For shared typical traits, the dominance order was angled > pubescent > veined. Dorsal and ventral cells of the L-T differed significantly, exhibiting thin walls, fewer stomata, and more trichomes; Whereas the C-T displayed uniformly arranged cells, thicker walls, and more stomata. Gene analysis revealed that 20 genes from five classes (A, B, C, E, and AGL6 subfamily) of the MIKCC-type MADS-box family, 30–32 genes from the TCP family, 8 genes from the YABBY family, and 12 genes from the WOX family transcription factors were involved in regulating typical corolla tube traits. Continuous selfing for six generations did not cause significant changes in the related regulatory genes, with the L-T exhibiting higher stability than the C-T. Both corolla tube types underwent six distinct developmental stages. The L-T was primarily activated by CYC/DICH genes, while the C-T was activated by RAD genes, leading to global expression of MADS-box identity transcription factors. The regulatory patterns, genes involved, and formation mechanisms differed significantly. **[Conclusion]** The corolla tube of *Sinningia speciosa* exhibits high genetic stability, and the formation of its typical traits demonstrates significant representativeness and classification characteristics.

Keywords: *Sinningia speciosa*, corolla tube, epigenomics, cytology, genetic variation.

Introduction

The corolla tube, as the core structure of the *Sinningia speciosa* floral organ, directly influences pollination efficiency, seed setting rate, and ornamental traits through its morphological characteristics (e.g., length, width, etc.), making it one of the key traits determining its commercial value. The morphological differentiation of the corolla tube represents an important feature of plant adaptive evolution and is closely related to pollinator selection and reproductive success [1-2].

At the molecular level, the MADS-box gene family plays a central role in floral meristem determination, organ differentiation, and morphogenesis. The classical "ABC model" and its extended "ABCDE model" elucidate how MADS-box genes combinatorially regulate the development of floral organs such as petals. For instance, B-class genes like *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) influence corolla tube morphology by regulating petal and stamen development [3]. Meanwhile, genes such as *CYCLOIDEA* (*CYC*) shape floral forms by regulating dorsoventral symmetry of the corolla [4]. However, as a representative species exhibiting both zygomorphic and actinomorphic flowers, the genetic regulatory network underlying corolla tube morphology in *S. speciosa* has not been systematically elucidated.

In recent years, epigenomic technologies have provided new approaches for deciphering the genetic basis of complex traits. Chen et al., by integrating transcriptomic and phenomic data, revealed associations between corolla tube elongation in *Petunia hybrida* and the expression of genes related to cell expansion [5-6]. They suggested that selecting for shorter and wider corolla tubes through hybridization during breeding could improve pollination rates and commercial value. The fused-base trait of the corolla tube relies on cell expansion and oriented division [7]. However, current functional gene sequencing studies remain limited, and systematic research on the genetic regulation of corolla tubes and the epigenetic regulation of specific typical traits is still lacking. Regarding corolla tube color, studies have indicated that Gesneriaceae plants often exhibit complex pigment deposition in the corolla tube, with color intensity significantly correlated with methylation levels of genes in the flavonoid biosynthesis pathway [8]. Anthocyanin enrichment at the tube base forms "nectar guides" [9], serving as contrasting coloration, while chloroplast DNA may influence pollinator selection through corolla tube color [10]. These findings suggest that morphological differences in the corolla tube of *S. speciosa* may be regulated by the coordinated action of multiple genes, with epigenetic modifications potentially involved. Based on the above background, this study utilizes campanulate and labiate corolla tube types of *S. speciosa* as materials. Through phenotypic comparison and qRT-PCR analysis of key MADS-box gene expression patterns, we aim to elucidate the genetic regulatory mechanisms underlying corolla tube morphological differences, providing a theoretical basis for molecular-assisted breeding in *S. speciosa*.

1.2 Experimental Methods

1.2.1 Phenotypic Data Collection and Analysis

Sixteen individuals with distinct typical traits were selected for a complete diallel cross. Trait variations were observed in 256 F₁ progeny. Subsequently, nearly 5000 F₂ individuals were obtained by selfing, from which those with typical traits were selected for six consecutive generations of selfing, and their trait changes were measured. The length and width of the corolla tube, floral throat diameter, and corolla diameter were measured using a digital vernier caliper (Mitutoyo, precision 0.01 mm), with the average value taken from three repeated measurements per flower. The outer wall color and ridging features were quantified through visual assessment combined with ImageJ software (NIH, Version 1.53) analysis of petal RGB values and morphological parameters [12]. The number of flowers per plant was counted as the cumulative value over 30 consecutive days. Pollination and seed setting rates were determined using the artificial pollination method referenced from Zhang et al. [13], recorded as the proportion of successfully set seeds among the pollinated flowers per plant.

1.2.2 Cellular and Related Parameter Analysis

Optical microscopy observation, paraffin sectioning, scanning electron microscopy, and epidermal imprint methods, combined with ImageJ image processing, were employed to systematically measure morphological, arrangement, and surface structure parameters of corolla epidermal cells, quantitatively distinguishing differences between labiate and campanulate corolla tubes. Each method involved at least three biological replicates, with over 30 cells measured per replicate to ensure data reliability and statistical significance [14-15].

1.2.3 Determination of Endogenous Hormones and Substrate Enzymes

Enzyme-linked immunosorbent assay (ELISA) was used to determine the content of endogenous hormones in the corolla tube, primarily including IAA, CTK, JA, GA, ETH, and BRs. Various substrate enzymes were measured using methods such as "substrate synthase assays via

spectrophotometry, isotope labeling, enzyme-linked immunosorbent assay, and fluorescence analysis" [16-18].

1.2.4 RNA Extraction and qRT-PCR Analysis

Based on publicly available plant MADS-box gene databases, the target species genome was aligned using BLAST. Fluorescence quantitative qRT-PCR technology was applied to analyze the relative expression levels of MADS genes in different floral corolla tubes, followed by comparative phenotypic analysis after CRISPR/Cas9-mediated gene knockout. The Plant-TFDB plant transcription factor database (planttfdb.gao-lab.org) was utilized to retrieve the MADS-box family. Known control-related sequences were used to design primers. Corolla tube tissues from both types (collected on the 3rd day post-anthesis, $n=5$) were flash-frozen in liquid nitrogen and stored at -80°C . Total RNA was extracted using the TRIzol reagent (Invitrogen) method. The sequencing of the internal reference gene SsACTIN was performed by Invitrogen, and primer synthesis was primarily conducted by Sangon Biotech. CRISPR/Cas9 technology was used to precisely edit homologous divergent base pairs in *S. speciosa*, adding CCT- to the front and -GGA to the end of the target sequence, followed by observation of phenotypic changes. The recombinant vector was transformed into *Agrobacterium* and introduced into plant tissues via the floral dip method. PCR was used to detect the integration of Cas9 and sgRNA into the corolla. Primers were designed to amplify DNA fragments (200-500 bp) containing the target site. Sanger sequencing was performed on the PCR products for direct sequence alignment and comparison. Phenotypic changes were observed, leading to final inferences [19-20].

1.3 Data Statistics and Analysis

SPSS 26.0 was used for statistical analysis, including comparisons of hybrid progeny traits and cellular parameters, as well as qRT-PCR-related analyses, with the significance level set at $P < 0.05$. Gene expression differences were visualized using the "ggplot2" package in R language (Version 4.1.2) [21]. Relative gene expression levels were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method [22]. Sequencing results were aligned and analyzed using the bioinformatics software BioEdit. Principal Component Analysis (PCA) was employed to comprehensively assess the correlation between corolla tube traits and gene expression, following the method referenced by Jolliffe [23]. Comparisons of gene content between different corolla tube types were conducted using one-way/two-way Analysis of Variance (ANOVA) and multiple comparison tests (Duncan's/LSD method, $\alpha=0.05$). R (ggplot2 package), GraphPad Prism, and Origin software were used for comparative analysis of endogenous hormone and various substrate enzyme contents [24].

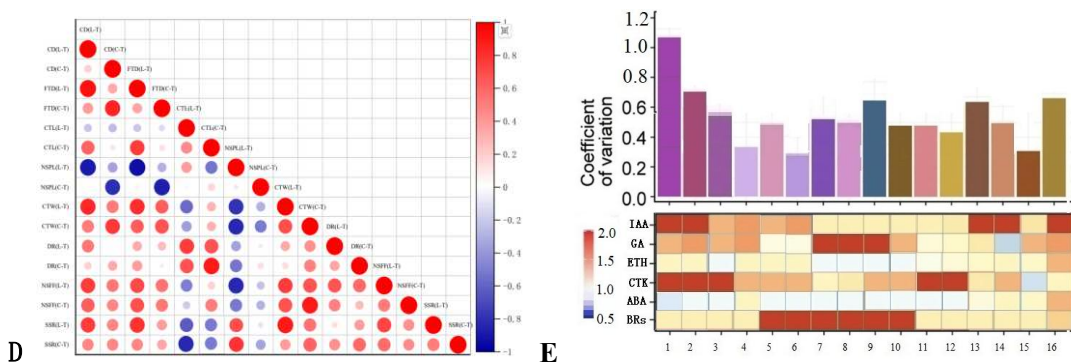
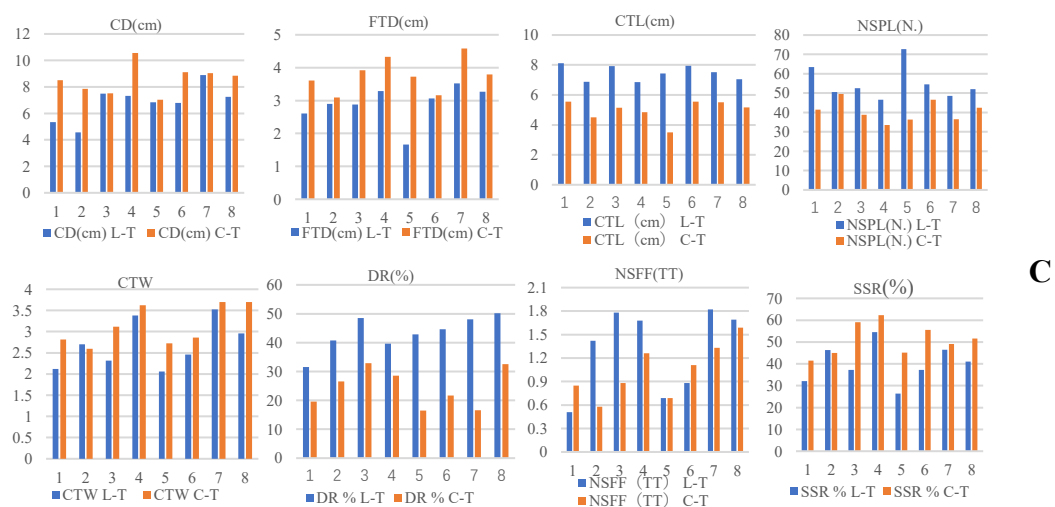
1. Results and Analysis

2.1 Population Construction and Phenotypic Analysis of Two Corolla Tube Types

Figure 1A-B illustrates the eight representative types each of the labiate (L-T) and campanulate (C-T) corolla tubes. Using crosses such as Labiate $\times$ Campanulate, Labiate 1 $\times$ Labiate 2, and Labiate Single-petal $\times$ Labiate Double-petal as examples, observations of F_1 and F_2 progeny individuals indicate that the labiate corolla tube exhibits dominant inheritance, whereas the campanulate type shows semi-dominant inheritance. Single-petal is dominant over double-petal, pubescence is recessive, and the ridged trait is dominant. Analysis based on Table 1 and Figure 3 reveals that the combining ability and heritability for corolla tube length, floral throat diameter, and corolla diameter are relatively high, followed by the number of flowers per plant and the seed setting rate. Regarding typical trait analysis, the size of the saccate structure in labiate corollas varies by cultivar and is characteristic of this type. For traits such as ridges, veins, and the degree of

pubescence on the outer wall of the corolla tube, general combining ability and narrow-sense heritability analysis conducted via complete diallel cross indicate the following dominance order: ridged > pubescent > veined; labiate > campanulate; single-petal > double-petal. However, the dominance relationship for corolla tube width is not clearly evident. Based on the characteristics of typical traits in the hybrid progeny populations, it was further observed that the outer walls of corolla tubes also differ in the presence or absence of coloration. The outer walls of labiate corolla tubes (IOWCT) often possess ridges, whereas campanulate corolla tubes exhibit greater variation in width, and veins of different colors. The corolla tube diameter (CTW) is widest in the campanulate type, consequently leading to the largest floral throat diameter (FTD) and corolla diameter (CD) in this type, alongside the lowest number of flowers per plant (NSPL). Conversely, the corolla tube length (CTL) is longest in the labiate type, which also exhibits the highest number of flowers per plant. Correlation coefficient analysis presented in Figure 4 indicates that corolla tube width (CTW) is significantly negatively correlated with the number of flowers per plant (NSPL) and significantly positively correlated with the seed setting rate (SSR). Corolla tube length (CTL) shows a significant negative correlation with the number of seeds set and a significant positive correlation with the receptacle abscission rate (DR); longer tubes are more prone to abscission from the pedicel, resulting in lower seed set. Positive correlations are observed among CTW, CD, and FTD. As shown in Figure 1E, analysis of endogenous hormones in different corolla tube types reveals that IAA, CTK, and BRs are the most abundant in both types. Their concentrations differ significantly from those of other endogenous hormones ($p < 0.05$).





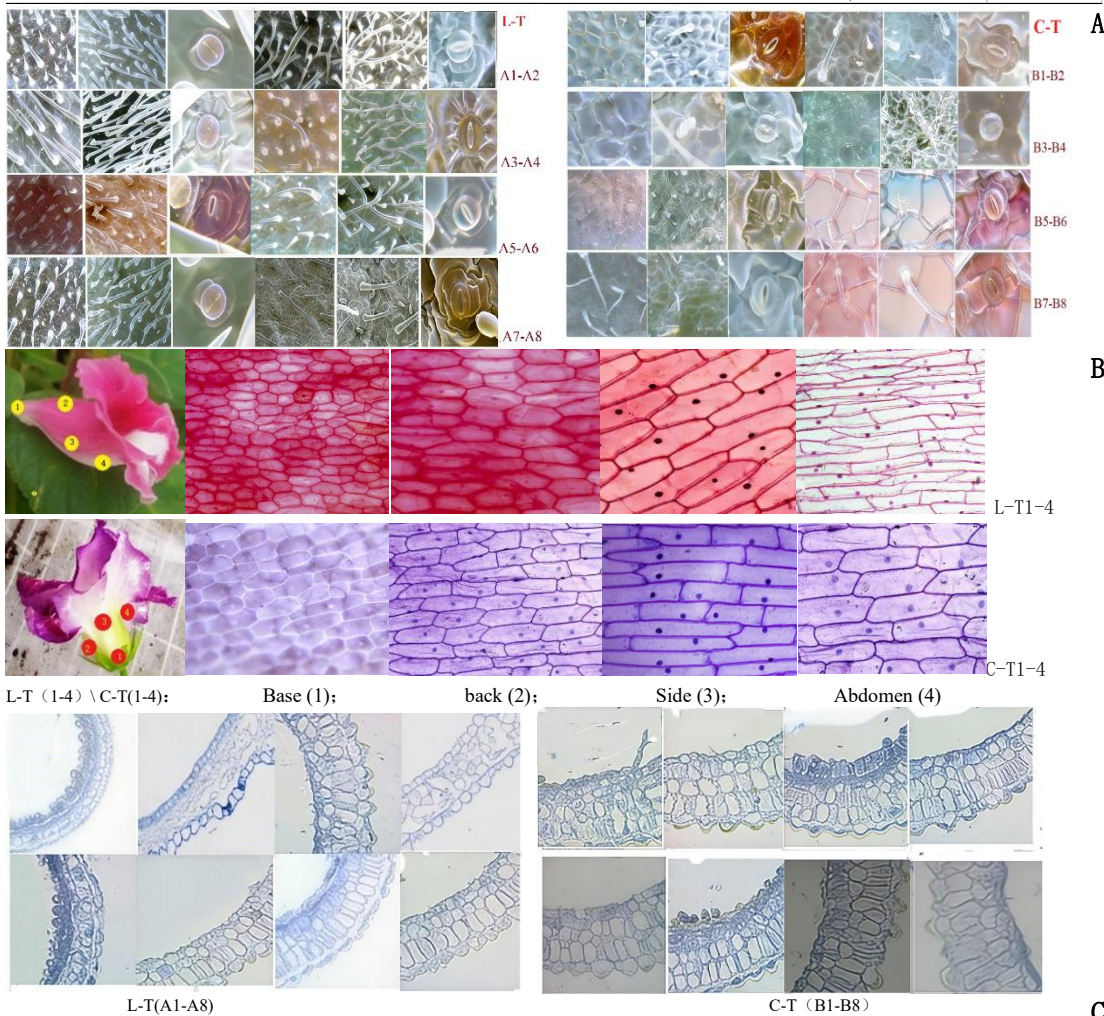
C	CTEW				CTL		CTLTH				CD		FTD				FSR%				SSR%				NFPP			
	AR	VP	SR	CH	GCA	h ^a	GCA	h ^a	GCA	h ^a	GCA	h ^a	GCA	h ^a	GCA	h ^a	GCA	h ^a	GCA	h ^a	GCA	h ^a	GCA	h ^a	GCA	h ^a	GCA	h ^a
	LT	CT	LT	CT	LT	CT	LT	CT	LT	CT	LT	CT	LT	CT	LT	CT	LT	CT	LT	CT	LT	CT	LT	CT	LT	CT	LT	CT
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8		*	*	*	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***

Table 1, Comparison of Various Parameters in Hybrid Progeny. Figure 1A, Examples of hybridization of different corolla tube types; Figure 1B, Typical traits from the hybrid population offspring (same as the hybrid parents); Figure 1C and D, Comparison and changes of typical traits in hybrid offspring and correlation analysis. Figure 1E, Analysis of the distribution of endogenous hormones in different corolla tubes. Note: CD, Corolla diameter (cm); FTD, Flower throat diameter (cm); NSPL, The number of single petallobes; CTL, Corolla tube length (cm); FD, Flowering period; NFPP, Number of flowers per plant; PH, Plant height; PL, Pedicel Length; Campanulate type(C-T) and Labiate type(L-T); CTEW, Corolla tube Exterior wall; AR, angular ridge; VP, venation pattern; SR, sunken rib; CH, Cover hair; FSR, Flower shedding rate; SSR, seed setting rate; CTLTH, thickness of the corolla tube.

2.1 Cytological Analysis of Hybrid Progeny Populations

Table 2, Cell Parameter Measurement Table.

C	CL (μm)	CW (μm)	CA (μm ²)	CR	CD /mm ²	CSTμm	ISμm	OSμm	SND
L-T	58.2±12.5	15.3±3.2	456±112	0.31±0.08	920±85	152±18	L: 28.5±3.2; S: 15.8±2.1	L: 28.5±3.2; S: 15.8±2.1	125±18
C-T	32.5±8.7	22.1±4.5	589±145	0.68±0.12	650±70	185±22	L: 19.8±2.5; S: 11.2±1.6	L: 22.3±2.8 ; S: 12.5±1.8	142±20



2ABC: Figure 2A, Surface structure of the corolla tube and the arrangement of stomata of 16 different corolla tubes; Figure 2B, the size and distribution direction of epidermal cells in different parts of the two types of corolla tubes; Figure 2C, Two cross-sectional views of corolla tubes. Note: Every three images represent the upper part, lower part, and the stomatal image of the outer wall of a corolla tube respectively. CL, cell length; CW, cell width; CA, cell area; CR, circularity ratio; CD, cell density; CST, cross-section thickness; IS, inner wall stoma; OS, outer wall stoma; SND, stoma number (density).

As shown in Figure 2A and B, analysis of epidermal cells, cross-sections of the corolla tube

wall, and various parameters revealed significant differences between the upper and lower parts of the labiate corolla tube. The length and density of trichomes were lower in the upper part compared to the lower part. Cells in the upper region were more densely arranged than those in the lower region, while stomata in the lower part were larger than those in the upper part. For the campanulate corolla tube, no significant difference in cell size was observed between the upper and lower parts. However, the lower epidermal cells of types B1, B3, and B7 exhibited an uneven surface, although stomatal size showed no variation. Compared the two corolla tube types, labiate corolla tubes generally possessed more trichomes and larger stomata than campanulate tubes ($P < 0.05$). Per unit area, the number of stomata was higher in campanulate corolla tubes. In terms of cellular arrangement, cells in the campanulate corolla tube were primarily oriented along the longitudinal axis. The epidermal cells at the base of the tube were slightly smaller, with a uniform increase in size observed in the remaining regions ($P < 0.05$). In contrast, cell size in the labiate corolla tube differed significantly across four distinct regions, following the order: base < apex < lateral region < ventral region. Table 2 Cellular parameters of different corolla tube types. Cells in the labiate corolla tube were predominantly elongated spindle-shaped (length-to-width ratio > 3), whereas cells in the campanulate corolla tube were more square-shaped (length-to-width ratio < 2). Cell arrangement was tighter in the labiate corolla, with a higher average cell density and a smaller corolla tube cross-sectional thickness ($P < 0.05$). Cells in the dorsal petal and ventral corolla regions were oriented longitudinally along the corolla tube axis, while cells in the lateral regions exhibited transverse or oblique orientations. In contrast, cells in the campanulate corolla were more loosely arranged, all aligning longitudinally along the main axis, resulting in a larger cross-sectional width. The epidermis of the labiate corolla often featured various trichomes and patterned ornamentation with relatively uniform color distribution, while the campanulate corolla surface was mostly granular or smooth with more prominent venation patterns.

2.2 Genetic Assay and Analysis

2.2.1 Analysis of Color Regulation Mechanisms in the Corolla Tube

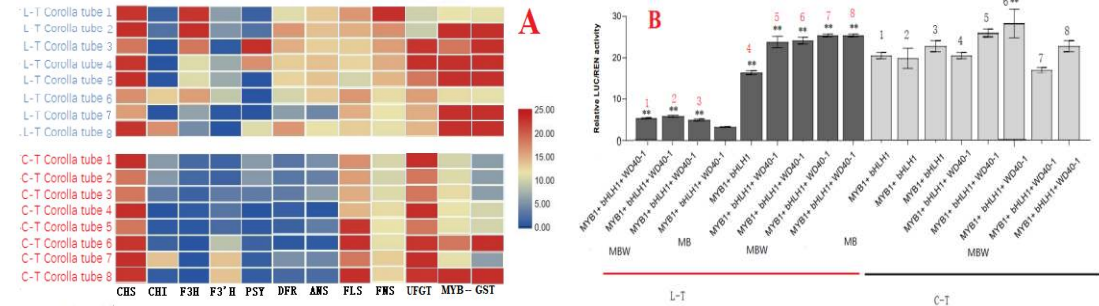


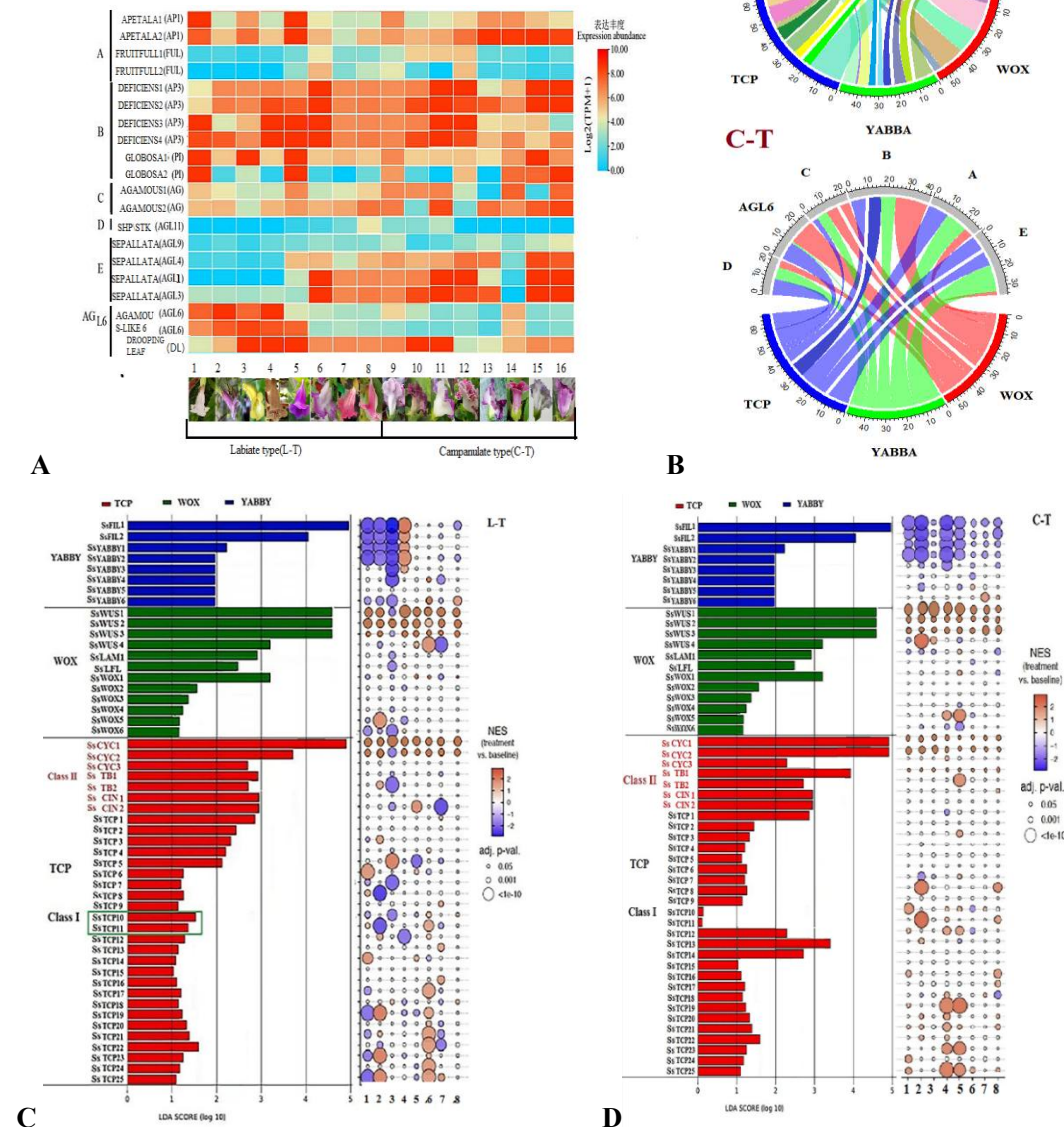
Figure 3A, Expression of key regulatory genes for anthocyanin formation; Figure 3B, Regulation modes of MYB-bHLH-WD40 (MBW) Analysis of key color framework regulatory genes, as shown in Figure 3A and B, revealed that pigment-related genes CHS, FLS, FNS, and UFGT were all highly expressed in both corolla tube types. The contents of DFR, ANS, FLS, FNS, and UFGT were also elevated in the labiate corolla. The structural genes responsible for anthocyanin biosynthesis, particularly "MYB-", were minimally present in corolla tube types A6, B1, B2, B3, B4, B5, and B7 (all featuring white outer walls), correlating with the white coloration observed. Conversely, glutathione S-transferase (GST) was most abundant in types A2, A3, A4, A5, A7, A8, B6, and B8, indicating that GST likely binds to anthocyanins, facilitating their transport into vacuoles for pigment deposition. qRT-PCR analysis of the MYB-bHLH-WD40 (MBW) complex indicated that among the 16 corolla tube types, four were regulated by an MYB1+bHLH1 complex, while the coloration of the remaining types involved

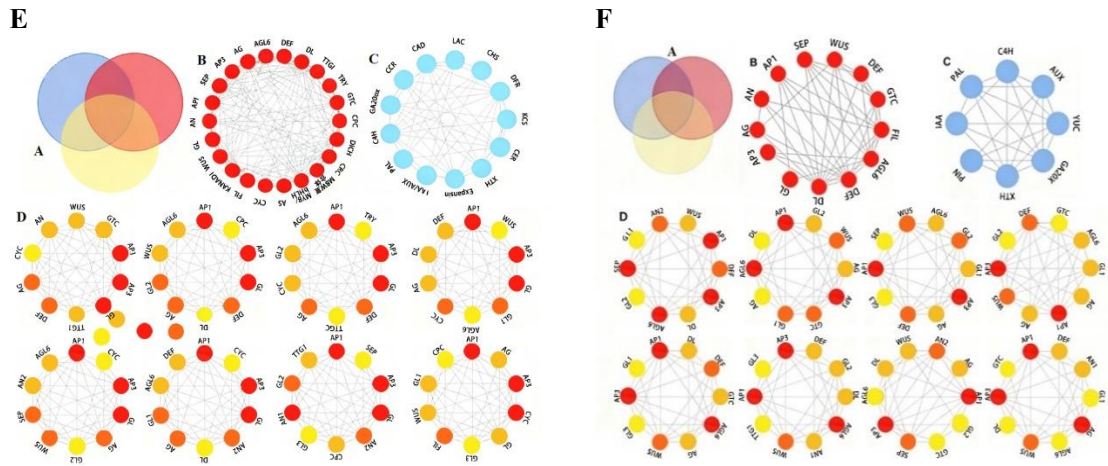
the full MBW complex. This demonstrates that the color of the corolla tube outer wall is influenced by multiple factors.

2.2.2 Genetic Assay and Network Regulation Analysis of Morphology

As illustrated in Figure 4A-D, MADS-box gene analysis identified 20 homologous genes from five classes (A, B, C, E, and the AGL6 subfamily) within the MIKC^C type associated with the development of the 16 sampled corolla tubes. These included A-class genes *SsAP1* (1-2) and *FUL1-2*; B-class genes *SsGLO1-2* (PI homologs) and *DEF1-4* (AP3 homologs); C-class genes *AG1-2*; E-class genes *SEP* (1-3); and AGL6 subfamily genes *SsAGL6* (1-2) and *SsDL*. These genes collectively participate in the phenotypic morphogenesis regulation of the entire corolla tube. D-class genes *SHP* and *STK* (AGL11) exhibited the lowest expression throughout corolla tube morphological changes, whereas B-class genes showed the highest overall expression. The regulatory patterns of MADS-box genes were entirely distinct for each corolla tube type.

Figure 4A: Expression of MADS-box-related genes in 16 types of corolla tubes; Figure 4B: Regulatory chord diagram of MADS-box and three families of transcription factor genes; Figure 4C: Regulatory gene enrichment diagram and bubble diagram of three transcription factor families; Figure 4E: Regulatory patterns of major genes for 16 different corolla tube traits.





Note: Figure 4E (L-T) \ F (C-T), Venn diagram (A), major gene(B), minor gene(C), typical trait genes(D) from A1\B1 - A1\B8

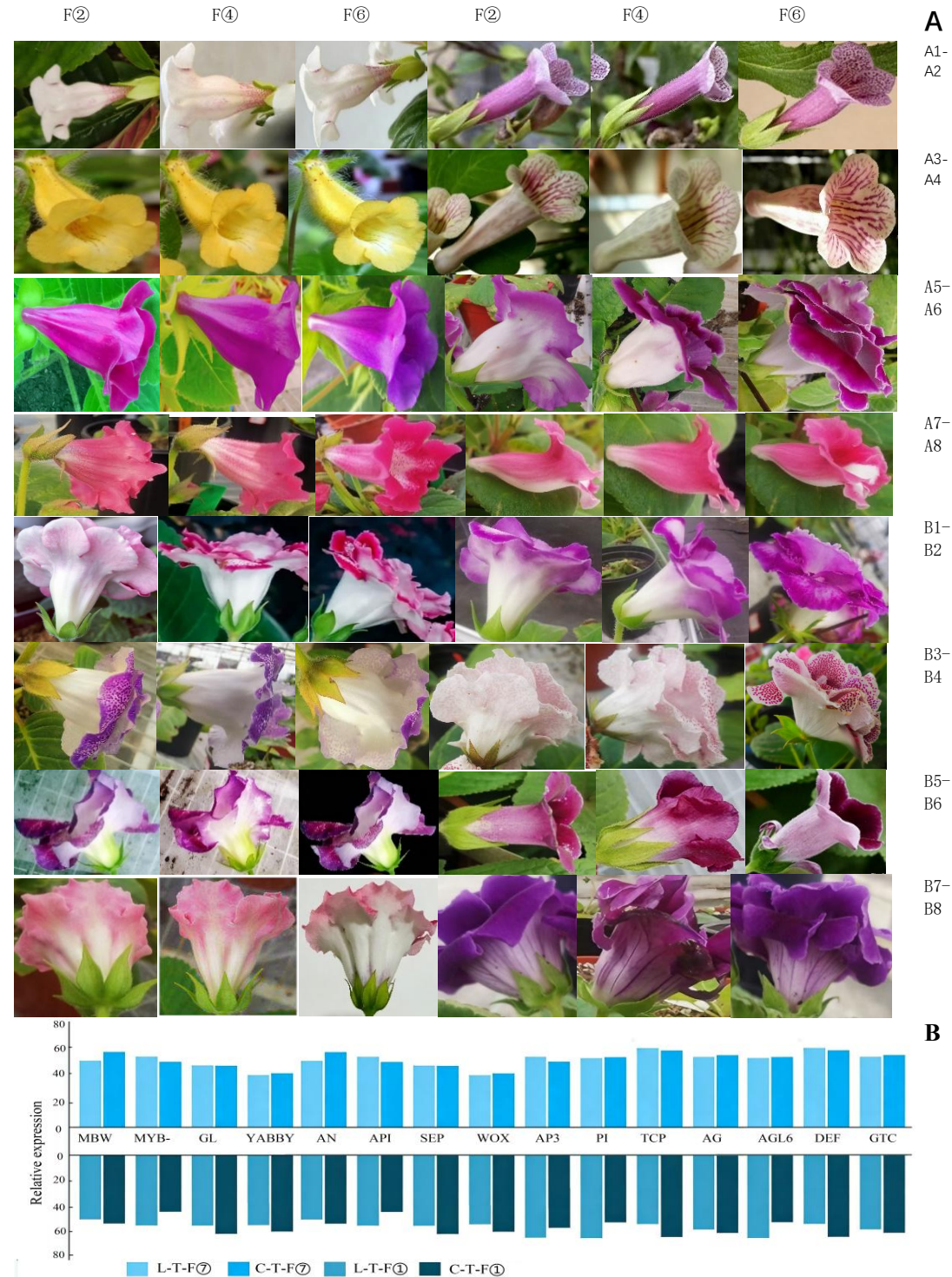
Additionally, 30–32 TCP family genes (with 32 regulatory genes in the labiate corolla, two more than in the campanulate type, specifically TCP10–11), 8 YABBY family genes, and 12 WOX family transcription factors were respectively involved in the corolla tube morphogenetic regulatory network, as visualized in the chord diagram (Fig. 4B, C). The gene regulatory network for the labiate corolla tube was more complex, with significantly more intricate gene connections between MADS-box and these three families compared to the campanulate type, and distinct arrangements among the genes. Among these, SsCYC (TCP Class II), SsFIL (YABBY family), and SsWUS (WOX family) were the most abundant and played a key role in determining corolla tube morphology.

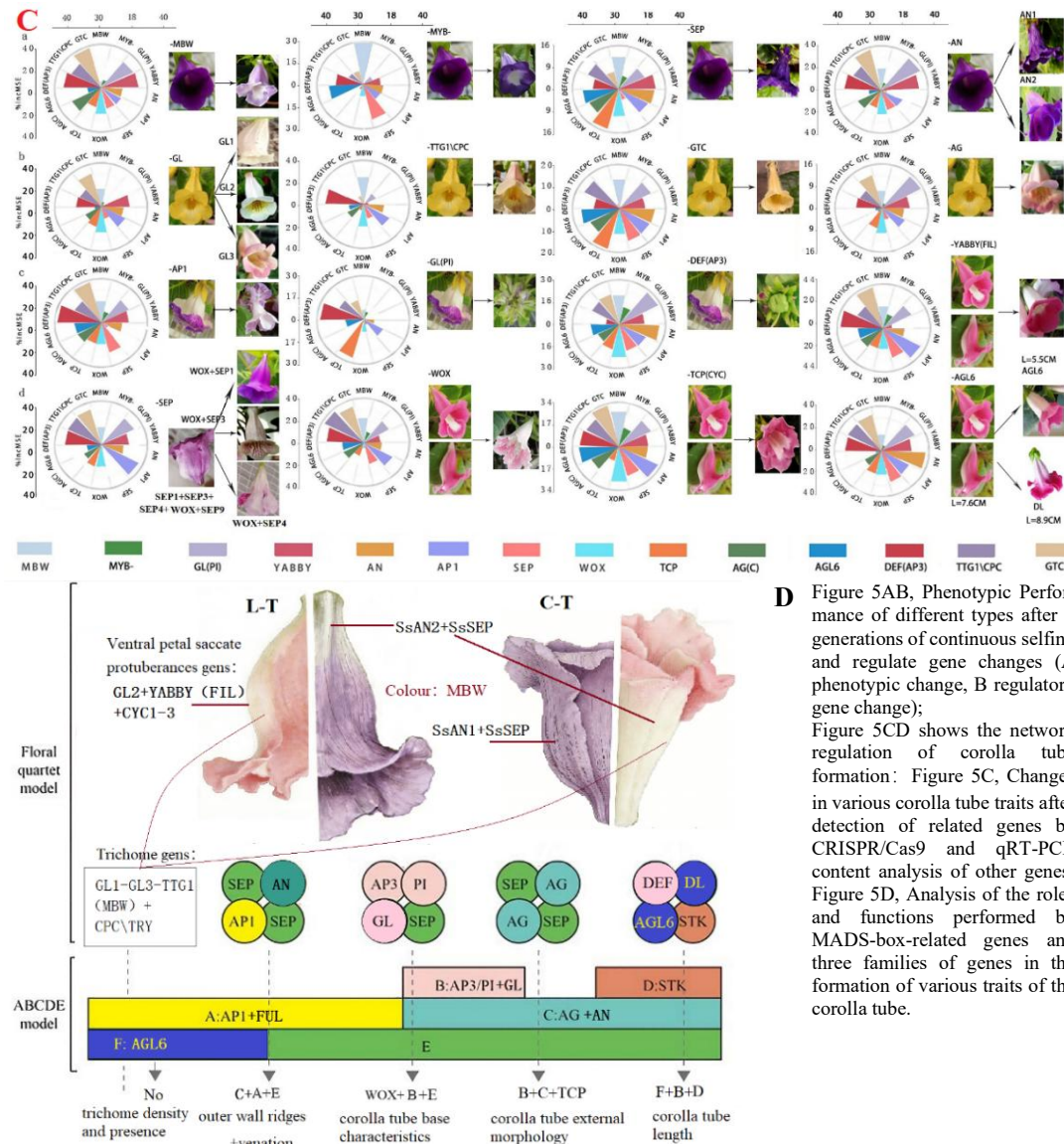
qRT-PCR analysis targeting regulatory factors for the 16 different corolla tube traits (Fig. 4E, F) revealed that SsCYC and SsGL genes were most abundant in labiate corollas, while SsAGL6 and SsAUS genes were highest in campanulate corollas. The presence of SsAN2 and SsSEP genes was notably associated with ridged individuals, whereas SsAN1 and SsSEP presence led to a significant increase in corolla tube venation. Individuals with high trichome density exhibited significant presence of SsGL3, SsTTG1, and SsCPC, whereas the presence of SsGL1 and SsGTC (core pathway genes) was clearly linked to a marked reduction in corolla tube pubescence.

2.2.3 Trait Stability Expression and qRT-PCR Analysis

Figure 5A, B. Analysis of trait changes across six consecutive generations of selfing revealed that the apical width of the corolla tube in labiate type A6 and campanulate types B3, B4, B6, and B7 varied with a reduction in petal number. However, this difference did not reach a highly significant level ($p=0.01$), and changes in length were not pronounced. All other morphological traits remained relatively stable, exhibiting minimal variation in selfed progeny, and were therefore classified as qualitative traits. Figure 5C, D. Based on observations of actual corolla tube samples following single-gene knockout via CRISPR/Cas9 technology, it was determined that, in addition to the MBW complex and MYB- genes regulating corolla tube color, MADS-box MIKC^C-type homologous genes from six classes (A, B, C, E) were involved in corolla tube development. Among these, changes associated with the A-class gene SsAP1 and E-class SEP genes showed a significant correlation with alterations in the ridging of the outer tube wall. The B-class genes SsGL/AP3 were found to regulate overall corolla shape, while the AGL6 family gene SsDL interacted with the B-class gene SsDEF to influence corolla tube length and width. Combined with the transcriptional regulators from three gene families—TCP (SsCYC), YABBY (SsFIL), and WOX (SsWUS)—and integrated with data from Figures 4A-F and 5A-C, a comprehensive summary (Figure 5D) was generated, delineating the control of 12 gene classes over 16 distinct corolla tube traits. The results

indicate: ① The combination of GL2 (B-class) + YABBY (FIL) + CYC1-3 (TCP) governs the formation of the ventral saccate protrusion; ② AN1-2 (C-class) + AP1 (A-class) + SEP (E-class) regulate outer wall ridges (AN1 primarily for ridges) and venation patterns (AN2); ③ The WOX family (WUS1-6) + GL3 (B-class) + AP3 (B-class) control basal tube characteristics; ④ GL2 (B-class) + AP3 (B-class) + CYC (TCP) + AG1-2 (C-class) coordinately regulate the external morphology; ⑤ AGL6 + DEF1-4 (B-class) + DL (AGL6 subfamily) + STK (D-class) modulate corolla tube length and width; ⑥ GL1-GL3-TTG1 (components of the MBW complex) + CPC/TRY (R3-MYB) + GTC (core pathway gene) determine the presence and density of trichomes on the outer corolla tube wall; ⑦ The MBW complex and MYB1 collectively regulate color distribution.





D Figure 5AB, Phenotypic Performance of different types after 6 generations of continuous selfing and regulate gene changes (A phenotypic change, B regulatory gene change); Figure 5CD shows the network regulation of corolla tube formation: Figure 5C, Changes in various corolla tube traits after detection of related genes by CRISPR/Cas9 and qRT-PCR content analysis of other genes; Figure 5D, Analysis of the roles and functions performed by MADS-box-related genes and three families of genes in the formation of various traits of the corolla tube.

2.3 Analysis of the Formation Mechanisms for Different Traits in Two Corolla Tube Types

Both corolla tube types undergo six developmental stages from primordium to maturity. Although each stage involves similar chemical processes, the participating substrate enzymes and regulatory genes are entirely different (Fig. 6). The formation of these characteristic and typical traits is manifested at each developmental stage. The core distinction lies in the zygomorphic (bilateral symmetry) labiate corolla versus the actinomorphic (radial symmetry) campanulate corolla, leading to divergent regulatory processes. Campanulate Corolla:① The RAD gene (core regulator of radial symmetry) is activated, which successfully represses CYC/DICH (genes for bilateral symmetry). This results in uniform distribution of endogenous hormones (IAA, CK, JA), while MADS-box/identity transcription factors are globally and uniformly expressed, ultimately conferring an "identical identity" to all petal primordia.

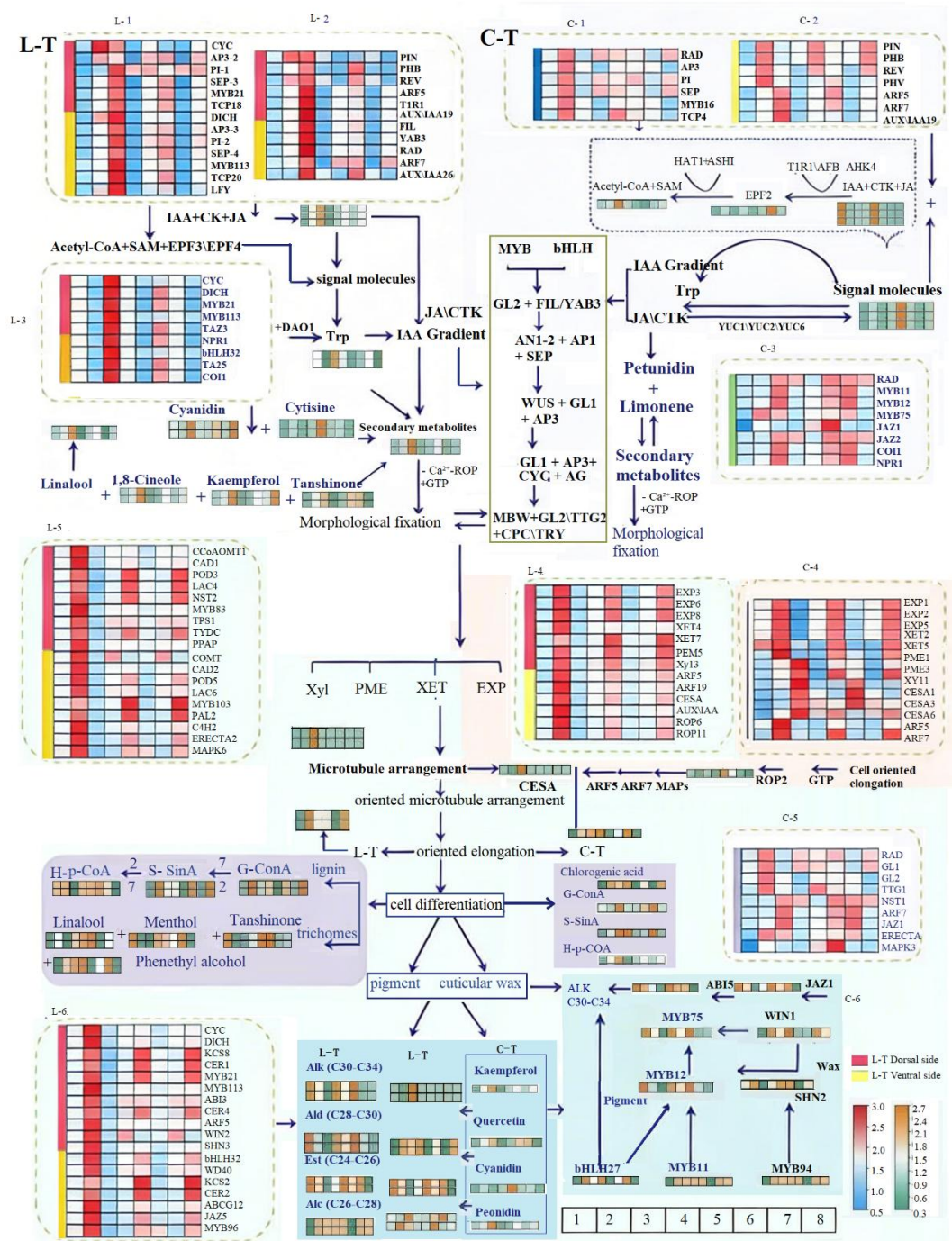


Figure 6, The formation process of two types of corolla tubes and the mechanism of action of related regulatory genes

Note: Abbreviation of core regulatory genes: IALLY SYMMETRIC (RAD, AT1G09540), CYCLOIDEA (CYC, X83998), DICHOTOMA (DICH, AJ277521), APETALA3 (AP3), PISTILLATA (PI), SEPALLATA (SEP), MYB DOMAIN PROTEIN 21 (MYB21), MYB DOMAIN PROTEIN 113 (MYB113), TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR 4 (TCP4), LEAFY (LFY), TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR 18 (TCP18), PHABULOSA (PHB), REVOLUTA (REV), FILAMENTOUS FLOWER (FIL), YUCCA (YUC), PIN-FORMED (PIN) AUXIN RESPONSE FACTOR 5/7 (ARF5/7), CORONATINE INSENSITIVE 1 (COI1), NON-EXPRESSOR OF PR GENES 1 (NPR1), GLABRA3 (GL3), TRANSPARENT TESTA GLABRA1 (TTG1), NAC SECONDARY WALL THICKENING PROMOTING FACTOR 2 (NST2), WRKY DNA-BINDING PROTEIN 70 (WRKY70), MYC DOMAIN PROTEIN 2 (MYC2). Abbreviations of key compounds: Indole-3-acetic acid (IAA), Cytokinin (CK), Jasmonic acid (JA), Salicylic acid (SA), Abscisic acid (ABA), S-adenosylmethionine (SAM), Coniferyl alcohol (G-S), Sinapyl alcohol (S-S), p-Coumaryl alcohol (H-S), Long-chain alkanes (C30-C34), (C28-C32) Short-chain esters, C24-C26, Aldehydes, C28-C30, Alcohols, C26-C28.

② Polarity genes (PHB/REV/PIN) are uniformly expressed throughout, leading to no regional differences in auxin synthesis (YUC) or transport (PIN), and thus a homogeneous distribution. The global signaling pathway for the entire corolla tube is activated, promoting radially symmetric development of petal primordia. ③ Metabolic transcription factors (MYB, bHLH) are expressed uniformly, resulting in no regional differences in secondary metabolite synthesis. This global metabolic activation leads to uniform distribution of compounds (adaptation for generalist pollination/defense). ④ Cell wall loosening enzymes and microtubule-associated proteins are uniformly expressed, causing consistent cell loosening/elongation rates across all petals, ultimately forming a radially symmetric corolla tube. ⑤ Lignification enzymes and trichome-related transcription factors are expressed uniformly, leading to homogeneous lignification and random trichome distribution. Consequently, compounds (lignin, phenolic acids) accumulate uniformly (adaptation for generalist mechanical protection). ⑥ Pigment and wax-related transcription factors are expressed uniformly, resulting in homogeneous deposition of pigments/waxes. This ensures consistent distribution of compounds (e.g., anthocyanins, long-chain alkanes) across all petals.

Labiata Corolla: ① The CYC/DICH genes (core regulators of bilateral symmetry) are activated, thereby repressing RAD gene development. This leads to asymmetrical hormone distribution (IAA high dorsally, CK high laterally, JA high ventrally), causing dorsoventral-specific expression of MADS-box/identity transcription factors. Consequently, petal primordia differentiate into three distinct identities: dorsal, lateral, and ventral. ② Polarity genes exhibit dorsoventral-specific expression (e.g., high PHB/REV dorsally, polar localization of PIN1 ventrally). High IAA synthesis coupled with directional transport establishes a dorsoventral gradient. Local signal activation (e.g., high ARF activity dorsally) stimulates dorsoventrally differentiated development of petal primordia. ③ Metabolic transcription factors show localized, specific expression, leading to partitioned synthesis of secondary metabolites along the dorsoventral axis. This localized metabolic activation results in regionalized enrichment of compounds (adaptation for directed pollination/precise defense). ④ Asymmetric expression of cell wall loosening enzymes and microtubule proteins occurs. Consequently, dorsal cells exhibit more pronounced loosening and elongation, while ventral cell expansion is suppressed, ultimately leading to the differentiation of the characteristic "upper short, lower long" lip morphology (bilateral symmetry). ⑤ Lignification enzymes and trichome transcription factors show dorsoventral-specific expression. This leads to dorsal enrichment of lignification and ventral specialization of trichomes, causing partitioned accumulation of compounds (lignin dorsally, alkaloids/volatile oils ventrally) (adaptation for structural support in directed pollination + precise defense). ⑥ Pigment and wax-related transcription factors are expressed in a dorsoventral-specific manner, leading to partitioned deposition of pigments and waxes. This results in regionalized enrichment of compounds (e.g., flavonoids dorsally, anthocyanins ventrally; long-chain alkanes dorsally, esters ventrally) (adaptation for directed pollination signaling + environmental adaptation), culminating in the formation of the labiate corolla tube.

3. Discussion

This study established a comprehensive genetic analysis system by performing a complete diallel cross on 16 different corolla tube traits of *Sinningia speciosa*, constructing a substantial framework comprising 256 F₁ individuals, nearly 5000 F₂ individuals, and 100 S₆ populations. This scale far exceeds most previous genetic studies on *S. speciosa* traits, providing a solid material foundation for systematically dissecting the genetic architecture of the complex corolla tube trait.

The research not only confirmed the high genetic stability of corolla tube traits in *S. speciosa* but, more importantly, systematically revealed the formation mechanisms underlying the differentiation of labiate and campanulate corolla tube types from multiple perspectives—including cytology, developmental dynamics, transcriptional regulatory networks, and endogenous hormone distribution—for the first time. This provides a novel theoretical framework and a candidate gene pool for the molecular design breeding of Gesneriaceae ornamental plants. It is more in line with the actual needs of "multi-phenotypic differentiation" in the artificial breeding of ornamental plants. The multi-phenotypic genetic system further revealed by our research is more consistent with the genetic characteristics of complex traits in the artificial breeding of ornamental flowers.

The dominance patterns identified in this study (labiate > campanulate, single-petal > double-petal, ridged > pubescent > veined) offer direct guidance for early selection of ornamental traits. The lack of clear dominance for corolla tube width suggests these traits are controlled by polygenes with minor effects, necessitating multi-generation directional selection strategies in breeding. This finding aligns with and is corroborated by a recent large-scale genetic analysis of corolla markings in *S. speciosa* [25], which also indicated high heritability for traits like spots and stripes, while highlighting a significant association between floral tube length and floral type (labiate/campanulate). Our study further refines this association: the expression of relevant regulatory genes showed no significant change after six generations of continuous selfing, and the labiate corolla tube exhibited higher stability. This indicates that stable superior lines can be efficiently obtained through hybridization and selfing purification.

Previous research on specific plant traits has often been limited to studying single gene families. For instance, some papers focused on MADS-box genes regulating corolla morphology in *Antirrhinum*, without addressing the synergistic roles of families like TCP or YABBY [26]. While some papers mentioned the association of MYB genes with flower color in African violets, they did not extend this to the regulation of corolla tube structural traits [27]. In contrast, our study clearly demonstrates that 19 genes from the MIKC^C-type MADS-box family (classes A, B, C, E, and the AGL6 subfamily), 30-32 TCP family genes, 8 YABBY family genes (SsFIL), and 12 WOX family transcription factors (SsWUS) collectively participate in regulating corolla tube morphology and typical traits. We also confirmed the dominant role of the MBW complex and other MYB genes in regulating outer wall color, constructing a comprehensive network of multi-gene family co-regulation, which offers a more plausible explanation for the complexity of genetic regulation.

This experiment is the first to identify A/B/C/D/E class MADS-box gene combinations (e.g., SsCYC-like, SsGLO, SsLAP1) associated with corolla tube morphology in *S. speciosa*, overcoming the limitations of the traditional "ABC model" in Gesneriaceae research [27]. Specifically, the association of SsLAP1-2 (A-class) with outer wall ridges and SsLAP1-2 (A-class) with venation color reveals novel functions of A-class genes in corolla tube morphogenesis. This discovery contrasts with the mechanism where the LAP1 gene regulates petal margin serration in *petunia* [28], suggesting functional divergence of A-class genes across species. Furthermore, the high expression of B-class genes SsGLO1-2 in the corolla tube (Fig. 4) supports the hypothesis that B-class genes regulate corolla tube width by promoting petal fusion [29]. The dominant allele SsGLO1-A2 drives the formation of tubular flowers by regulating the expression of B-class MADS-box genes [30]. Our study found that B-class genes SsCYC1-2 and SsGLO1-2, together with YABBY (FIL) and WOX family (WUS) genes, regulate corolla tube shape. Moreover, the AGL6 subfamily gene DL is closely associated with the D-class gene SsDEF1-2, exhibiting significant regulatory effects on corolla tube

length and width—a finding that is unprecedented in floral tube research across all flowering plants, further revealing the innovative mechanism of multiple genes in floral diversification. Additionally, SscCYC regulates floral symmetry in *S. speciosa*, consistent with its functional expression in symmetrical varieties of *Tengia* and *Saintpaulia ionantha* [31-32]. The regulation of corolla tube color and pubescence by the MBW complex aligns with previous studies in *petunia* [33], indicating the broad conservation of this pathway in anthocyanin synthesis. Our findings suggest that D-class genes SsIDEF/SscDEF may regulate corolla tube length and width by interacting (e.g., via protein dimerization) with B-class MADS-box genes, similar to the function of the DEF-GLO complex in *Antirrhinum* [34]. Furthermore, the phenotypic plasticity of ridges on the labiate corolla tube outer wall (presence/absence) hints at the molecular regulatory mechanism underlying the initiation of fused corolla tubes [35].

This study innovatively proposes that the formation of both corolla tube types involves six consecutive developmental stages but with different intrinsic drivers. The development of the labiate corolla tube is initiated by CYC/DICH genes, which guide the asymmetric distribution of auxin (IAA), cytokinin (CK), and jasmonic acid (JA) along the dorsoventral axis of the tube. This drives unequal cell division and differentiation, ultimately forming the dorsally distinctive labiate structure, a model validated cytologically (thin dorsal cell walls, fewer stomata, more trichomes). In contrast, the campanulate corolla tube is dominated by the RAD gene, establishing a more uniform endogenous hormone field that promotes uniform cell expansion and thickening. This cascade regulation model—"key transcription factors → spatial distribution of endogenous hormones → cell-specific differentiation → final morphological establishment"—provides a dynamic, integrated new perspective for understanding the diverse development of corolla tubes. Based on the identified key genes (e.g., SsFIL, SsWUS, and specific MADS-box subfamily members), functional molecular markers can be developed for early and precise screening of superior individuals with target corolla tube types (e.g., deep-throated campanulate or highly ridged labiate).

Despite a series of innovative findings, this study has certain limitations. First, the analysis of interaction networks among core genes requires further depth to clarify the synergistic regulatory relationships among different gene families. For example, specific members within the TCP and YABBY families that play leading roles and their upstream/downstream target gene networks need further mapping. Second, future research can utilize the populations constructed and the genetic transformation system established in this study to conduct in-depth functional analysis of single genes and synthetic biology design, aiming for precise and predictable engineering of corolla tube traits in *S. speciosa*. Third, the development and application of molecular markers are still preliminary, requiring further screening for highly efficient and stable specific markers.

4. Conclusion

Through the construction of large-scale genetic populations and integrated multi-omics analysis, this study systematically elucidated the genetic patterns and formation mechanisms of typical corolla tube traits in *Sinningia speciosa*, leading to the following core conclusions: Genetic Patterns: The primary morphological and microscopic traits of the *S. speciosa* corolla tube exhibit high genetic stability. Labiate type is dominant over campanulate. Morphological features of the corolla tube (e.g., ridges, pubescence) show significant associations with overall floral traits (e.g., floral throat diameter, flower number). These patterns provide clear guidance for parental selection in hybridization breeding and early-stage selection. Cytological Basis: The differentiation between labiate and campanulate corolla tubes stems from fundamental differences in their cell proliferation

and differentiation patterns. The asymmetry of the labiate type originates from the heterogeneity of dorsal and ventral cells, while the uniformity of the campanulate type results from homogeneous cell growth. Molecular Mechanisms: Corolla tube development is controlled by a complex transcriptional regulatory network. MADS-box genes determine the foundational identity of floral organs, while TCP and YABBY family genes are key regulators of morphological differentiation. Flower color is regulated by gene modules such as the MBW complex. The divergence between labiate and campanulate types initiates from the differential activation of CYC/DICH versus RAD genes, which subsequently regulate the spatial distribution of endogenous hormones (IAA, CK, JA) to guide cell fate, ultimately forming two distinct corolla tube structures.

In summary, this study not only systematically dissected the genetic patterns and formation mechanisms of distinctive corolla tube traits in *S. speciosa* but also established a solid theoretical and material foundation for subsequent molecular breeding and germplasm innovation through the constructed large genetic populations, identified key genes, and clarified trait-gene associations. Future work can utilize technologies like CRISPR/Cas9 for functional validation and editing of these key genes, enabling precise design breeding for floral organ morphology in *S. speciosa* and related species.

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