Enhancing Petunia Varieties through Advanced Genetic Techniques: Modifying Flower Colors via Gene Editing and Gene Overexpression

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Enrolled Semester: Fall, 2023

B.S. in Biological Sciences, University of California, Davis, 2023.

Abstract

This report presents two innovative genetic modification strategies aimed at expanding and enhancing the color palette of petunia. Utilizing CRISPR-Cas9 technology, we target specific pigment-related genes for precise, transgene-free editing, while parallel efforts involve traditional gene overexpression to intensify and diversify floral colors. These methods support the development of enhanced petunia varieties that meet both aesthetic and commercial cultivation standards without relying on traditional approaches.

Introduction

Petunia is one of the most economically important ornamental plants worldwide, prized for its diverse flower colors and extended blooming period in gardens, containers and urban landscapes. Petunia contribute substantially to the horticultural sector's economic performance in the United States, representing a significant portion of the annual wholesale market for ornamental plants. According to the USDA's 2020 Floriculture Crops Summary, petunias sold in pots generated a wholesale value of over $55 million, with more than 26 million pots sold in 2020. Among the various petunia cultivars, the Supertunia series, especially Supertunia Vista Bubblegum, is particularly notable for its vigorous growth habit and exceptional flowering performance. However, introducing additional traits of interests such as flower color into these elite commercial varieties through the conventional hybridization and selection is challenging due to their highly heterozygous nature. Traditional breeding approaches risk disrupting existing desirable traits, necessitating innovative genetic strategies to enhance these varieties while preserving their superior characteristics.

Petunia flower coloration is primarily dictated by the biosynthesis of anthocyanins, a complex process involving several key enzymes and regulatory genes. This pathway includes crucial structural genes such as Chalcone Synthase (CHS) and Dihydroflavonol 4-Reductase (DFR), as well as various MYB transcription factors that regulate their expression (Fig.1). Our transgene-free gene editing project aims to manipulate this pathway by targeting specific genes to modify flower color. We will focus on editing MYB repressors that negatively regulate anthocyanin synthesis to enhance color intensity. Additionally, we plan to edit Flavonol Synthase (FLS) to potentially increase anthocyanin accumulation by redirecting the metabolic flux away from flavanol synthesis, thus intensifying pigmentation. Modification of DFR and Flavanone 3-Hydroxylase (F3H) could lead to white flowers by disrupting key steps in the anthocyanin biosynthesis pathway. By editing MYB genes that negatively regulates anthocyanin synthesis,

we aim to enhance color intensity. Furthermore, targeted editing of Flavonoid 3'-Hydroxylase (F3'H) and Flavonoid 3',5'-Hydroxylase (F3'5'H) can alter the hydroxylation patterns of anthocyanin molecules, potentially shifting the color spectrum and expanding the range of achievable flower colors.

To expand the color palette of petunia flowers, we are implementing strategic gene overexpression approaches. By heterologously overexpressing ROSEA1 and DELILA from snapdragon (*Antirrhinum majus*), we aim to enhance anthocyanin biosynthesis to develop purple and dark purple flower phenotypes. In parallel, overexpressing a synthetic RUBY gene from sugar beet (*Beta vulgaris*) will produce betalain, generating novel bright red and dark red coloration. Additionally, enhanced expression of F3'5'H will increase delphinidin accumulation, leading to more pronounced blue flower colors. These complementary genetic engineering strategies are designed to create a diverse spectrum of flower colors, significantly enhancing both the commercial and aesthetic appeal of petunia varieties.

Recent advancements in genetic engineering, particularly the development of CRISPR/Cas9 gene-editing technology, have provided promising new methods for enhancing petunia varieties. One advantage of CRISPR genome editing over traditional genetic engineering is that transgene-free modifications can be achieved.

Several transgene-free gene editing methods have been developed. For example, Ribonucleoprotein (RNP) delivery introduces pre-assembled CRISPR/Cas complexes directly into plant cells, particle bombardment uses high-velocity particles to deliver editing components into plant tissue, and virus-based vectors employ viral particles for efficient delivery of gene-editing tools. Despite these advancements, each method has drawbacks: RNP delivery often struggles with low editing efficiency, particle bombardment can cause cell damage and inconsistent delivery, and virus-based vectors face limitations in virus-host specificity and can trigger immune responses. Unlike other transgene free methods, our proposal offers a novel transgene-free approach with broad applicability across diverse plant species through targeted manipulation of DNA repair pathways. The pivotal component of our methodology is the strategic suppression of DNA Polymerase Theta (POLQ), a key enzyme in alternative DNA repair mechanisms. POLQ plays a dual role in plant genome modification: it mediates microhomology-mediated end joining (MMEJ) during DNA repair and facilitates T-DNA integration into host genomes.

Recent studies have shown that POLQ is essential for T-DNA integration through its involvement in double-strand break repair pathways. By transiently suppressing POLQ expression during CRISPR/Cas9-mediated genome editing, we can decrease the frequency of T-DNA insertion during the CRISPR/Cas9 editing process while maintaining efficient gene editing via the classical non-homologous end joining (NHEJ) pathway. This approach is particularly valuable for developing non-GMO edited crops, as it minimizes the risk of foreign DNA persistence in the plant genome.

The significance of this method will be exemplified in our current work with Supertunia Vista Bubblegum, an ornamental variety with a complex genetic background derived from multiple

interspecific hybridizations. Due to its highly heterozygous nature and exclusive vegetative propagation, traditional breeding approaches are ineffective for trait improvement while maintaining its unique horticultural characteristics. Our POLQ suppression strategy enables precise genetic modifications without transgene integration, thereby preserving the plant's non-GMO status. This approach not only satisfies regulatory requirements but also addresses market demands for non-GMO ornamental varieties while allowing for targeted trait enhancement. Furthermore, this methodology can be adapted for other vegetatively propagated crops where maintaining genetic fidelity while introducing beneficial traits is crucial.

These two projects are designed to harness this sophisticated technology to enhance the commercial viability and aesthetic appeal of petunia, ensuring these popular plants continue to thrive in gardens worldwide and maintain their competitive edge in the global market for ornamental plants.

Objectives

The goal of this project is to modify the flower color through genetic engineering approaches. Three objectives are proposed to achieve this goal.

- **To Modify Flower Color by Editing Structural Genes in Anthocyanin Pathway without Transgene Integration**

  Modifying flower pigmentation by targeting structural genes such as F3H, F3'5'H, F3'H, DFR, and FLS. Editing F3H and DFR can limit colored compound formation, while changes to F3'5'H and F3'H adjust hydroxylation, expanding hue diversity. Modifying FLS aims to reduce the production of flavonols, allowing greater anthocyanin accumulation.

- **To Intensify Flower Pigmentation by Knocking Out Transcription Factors That Act as Repressors in the Anthocyanin Biosynthesis Pathway**

  Intensifying flower pigmentation by regulating transcription factors such as MYB27, MYB-FL, and MYBx, which function as repressors in the anthocyanin biosynthesis pathway. Editing these transcription factors aims to alleviate their suppressive effects, enhancing the expression of downstream structural genes involved in anthocyanin production.

- **To Diversify Flower Coloration by Overexpressing Key Genes Involved in Pigment Production**

  Utilizing the pOX135 binary expression vector to overexpress genes such as ROSEA1, DELILA, and RUBY to intensify and diversify flower colors. Overexpressing ROSEA1 and DELILA aims to boost purple and dark purple hues by increasing anthocyanin synthesis. RUBY is targeted to enhance betalain production, introducing unique red and

dark red tones. Additionally, overexpressing F3'5'H will increase delphinidin production, leading to more pronounced bluish flower colors. This strategic overexpression complements the gene editing project and broadens the potential color palette.

Research Progress

Objective 1: To Enhance Flower Color by Editing Structural Genes in the Anthocyanin Pathway without Transgene Integration

- The construction of vectors PHN-DFR, PHN-FLS, and PHN-FLS-POLQ, for targeted editing of structural genes, has been completed. Stable transformations using PHN-DFR and PHN-FLS have been achieved in Mitchell, Carmine Velour, and Petunia Wave Pink varieties. We are currently observing these plants for phenotypic changes, with the aim of assessing the effects of structural gene knockouts on flower pigmentation. Additionally, the construction of vectors targeting the F3H, F3'H, and F3'5'H genes is ongoing.
- Regeneration protocols for *Supertunia Vista Bubblegum* are under optimization to support effective transformation. Upcoming steps involve using POLQ-mediated constructs in *Supertunia Vista Bubblegum* to ensure that edits are transgene-free, aligning with non-GMO standards.

Objective 2: To Intensify Flower Pigmentation by Knocking Out Transcription Factors That Act as Repressors in the Anthocyanin Biosynthesis Pathway

- Completed the construction of vectors targeting transcription factors MYB27, MYB-FL, and MYBx. These vectors have been stably transformed into *Mitchell*, *Carmine Velour*, and *Petunia Wave Pink*. Observations are ongoing to evaluate the impact of these gene knockouts on pigment intensity.

Objective 3: To Diversify Flower Coloration by Overexpressing Key Genes Involved in Pigment Production

- Successfully transformed pOX135 vectors containing ROSEA1, DELILA, RUBY, and F3'5'H into *Mitchell*, and F3'5'H into *Petunia Wave Pink*. Overexpression of these genes has resulted in notable color enhancement: ROSEA1 has intensified purple hues, while RUBY has introduced vibrant pink coloration. Additionally, F3'5'H overexpression has further enriched pink shades in *Petunia Wave Pink*, demonstrating the potential for expanded color diversity (Fig. 2).

Table: Key Genetic Targets and Project Progress

Target	Vector/Construct	Project Type	Transformed Varieties	Status	Remarks
MYB27, MYB-FL, MYBX	PHN-MYB27, PHN-MYBFL, PHN-MYBX, (both with and without POLQ)	Gene Editing	*Mitchell, Carmine Velour, Petunia Wave Pink*	Stable Transformation Completed	Awaiting phenotype observations
DFR, FLS	PHN-DFR, PHN-FLS, PHN-FLS-POLQ	Gene Editing	*Mitchell, Carmine Velour, Petunia Wave Pink*	Stable Transformation Completed	Awaiting phenotype observations
F3'H, F3'5'H	PHN-F3'H, PHN-F3'5'H (preparing to add POLQ)	Gene Editing	-	Plasmid construction	Awaiting ligation of POLQ to the plasmid
F3H	-	Gene Editing	-	gRNA design & sequencing	Awaiting gRNA design
ROSEA, RUBY, DEL	POX-ROSEA, POX-RUBY, POX-DEL	Overexpression	*Mitchell*	Successful transformation	Purple and pink colorations achieved in white flowers
F3'5'H	POX-F3’5’H	Overexpression	*Mitchell, Carmine Velour, Petunia Wave Pink*	Color changes observed, ongoing transformation	Intensified color observed in Petunia Wave Pink

Educational Achievements and Coursework

I obtained my bachelor's degree from the University of California, Davis, graduating with the highest honors and a GPA of 3.98 in Biological Sciences. This strong foundation has been vital in preparing me for advanced scientific inquiries and research.

At the University of Florida, I have furthered my education by completing several specialized courses that directly support my research in genetic engineering of horticultural crops. These courses include:

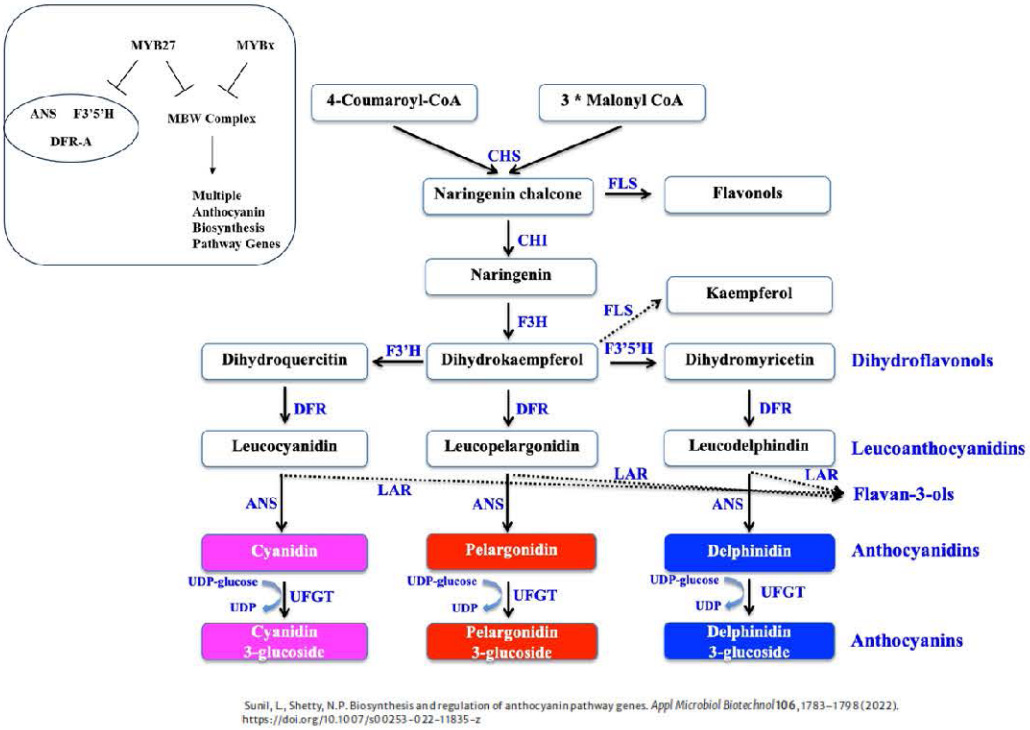
- PLS5222C (30398) - Propagation of Horticultural Crops
- AGR5307 (24456) - Molecular Genetics of Crop Improvement
- PCB5530 (16626) - Plant Molecular Biology and Genomics
- STA6093 (16790) - Introduction to Applied Statistics

I am currently enrolled in PLS5222C and maintain a 4.0 GPA, continuously applying learned concepts to my ongoing research on modifying flower coloration in petunia using advanced gene-editing technologies.

Timeline and Future Directions

I began my PhD studies in horticultural genetics at the University of Florida in August 2023, with an anticipated graduation date in the summer of 2028. My qualifying exam is expected to schedule in the summer of 2025. Over the next few years, my research will concentrate on advancing plant biotechnology to enhance color variations in petunia. This timeline is designed to efficiently manage my research goals and academic requirements, leading up to the preparation and defense of my dissertation in 2028.

Figures



Sunil, L., Shetty, N.P. Biosynthesis and regulation of anthocyanin pathway genes. *Appl Microbiol Biotechnol* 106, 1783–1798 (2022). https://doi.org/10.1007/s00253-022-11835-z

Fig. 1, Biosynthetic pathways of anthocyanins. CHS, chalcone synthase; FLS, flavonol synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin reductase; UFGT, flavonoid 3-O-glucosyltransferase; UDP-glucose, uridine diphosphate glucose; asterisk indicates multiplication (Sunil et al., 2022). MYB repressors may directly limit the expression of structural genes and disrupt the formation of the MBW complex, thereby repressing anthocyanin biosynthesis.

Fig. 2, Comparative display of Petunia flowers showing the wild type (left) with a standard pink hue and a genetically modified variant (right) with a deeper pink color due to the overexpression of the F3'5'H gene.

References

Albert, N.W., Davies, K.M., Lewis, D.H., Zhang, H., Montefiori, M., Brendolise, C., Boase, M.R., Ngo, H., Jameson, P.E., & Schwinn, K.E. (2014). A conserved network of transcriptional activators and repressors regulates anthocyanin pigmentation in eudicots. *The Plant Cell, 26*(3), 962–980. https://doi.org/10.1105/tpc.113.122069

Sunil, L., & Shetty, N.P. (2022). Biosynthesis and regulation of anthocyanin pathway genes. *Applied Microbiology and Biotechnology, 106*(5), 1783–1798. https://doi.org/10.1007/s00253-022-11835-z

USDA-NASS [U.S. Department of Agriculture-National Agricultural Statistics Service]. (2020). *USA sales of annual bedding plants and herbaceous perennials*. Washington, DC: USDA/NASS QuickStats Ad-hoc Query Tool.