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CRISPR-Guided Functional Genomics of Anthocyanin Biosynthesis Pathways in Strawberry

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ABSTRACT

Anthocyanins are flavonoid-derived secondary metabolites responsible for the characteristic red pigmentation and antioxidant potential of strawberry fruits. In cultivated strawberry, *Fragaria* × *ananassa*, anthocyanin accumulation significantly influences fruit quality, nutritional value, postharvest shelf life, and consumer acceptance. Recent advances in genome editing technologies, particularly Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas systems, have revolutionized functional genomics and crop improvement strategies. This study presents a comprehensive investigation of anthocyanin biosynthesis pathways in strawberry using CRISPR-guided functional genomics approaches. Candidate structural and regulatory genes, including *FaCHS*, *FaCHI*, *FaF3H*, *FaDFR*, *FaANS*, *FaUFGT*, and transcription factors such as *FaMYB10* and *FabHLH3*, were targeted through CRISPR/Cas9-mediated editing. Functional validation was performed using Agrobacterium-mediated transformation, targeted mutagenesis, transcriptome profiling, and metabolomic analyses. Gene-edited strawberry lines exhibited significant alterations in fruit pigmentation, anthocyanin concentration, and expression profiles of flavonoid pathway genes. Knockout of *FaMYB10* resulted in white-fruited phenotypes with reduced pelargonidin accumulation, whereas activation or promoter editing enhanced anthocyanin biosynthesis and fruit coloration. Transcriptomic analyses revealed extensive regulatory networks involving light-responsive and stress-associated pathways. The study demonstrates the effectiveness of CRISPR-guided functional genomics for dissecting complex metabolic pathways in polyploid crops and highlights its potential for precision breeding of nutritionally

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enhanced strawberry cultivars. These findings contribute to the understanding of anthocyanin regulation and provide a molecular framework for future improvement of fruit quality traits in strawberry breeding programs.

Keywords: CRISPR/Cas9, Strawberry, Anthocyanin Biosynthesis, Functional Genomics, Genome Editing, Flavonoid Pathway, *Fragaria* × *Ananassa*, Transcription Factors

INTRODUCTION

Strawberry (*Fragaria* × *ananassa*) is one of the most commercially important berry crops worldwide and is widely appreciated for its distinctive flavor, bright red coloration, nutritional value, and health-promoting properties. The global demand for strawberry fruits has increased substantially over the last decade due to growing consumer awareness regarding functional foods rich in antioxidants, vitamins, and bioactive phytochemicals. Among the various quality attributes of strawberry fruit, coloration is considered a major determinant of consumer preference and market value (Newerli-Guz et al., 2023). The characteristic red pigmentation of strawberry fruits primarily results from the accumulation of anthocyanins, a class of water-soluble flavonoid compounds synthesized through the phenylpropanoid pathway. In addition to their role in fruit aesthetics, anthocyanins contribute significantly to plant defense mechanisms against environmental stresses and provide important health benefits owing to their antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. Anthocyanins are secondary metabolites belonging to the flavonoid family and are widely distributed in higher plants (Crespo, 2010). In strawberries, pelargonidin-3-glucoside is the predominant anthocyanin responsible for the bright red coloration, while cyanidin derivatives contribute to variations in fruit pigmentation ranging from pink to dark red hues. The biosynthesis and accumulation of anthocyanins are highly dynamic processes regulated by developmental, environmental, and genetic factors. Environmental stimuli such as temperature, light intensity, ultraviolet radiation, and nutrient availability significantly influence anthocyanin biosynthesis in strawberry fruits. Similarly, endogenous hormonal signals including abscisic acid, jasmonic acid, and auxins are involved in the modulation of anthocyanin accumulation during fruit ripening. Due to their direct influence on fruit quality and nutritional composition, anthocyanins have become major targets for genetic improvement in strawberry breeding programs (Charoenwoodhipong et al., 2025).

The regulation of anthocyanin biosynthesis is controlled not only by structural genes but also by a complex transcriptional regulatory network. Central to this regulatory mechanism is the MYB-bHLH-WD40 (MBW) transcriptional complex, which activates the expression of anthocyanin biosynthetic genes. Among these transcription factors, R2R3-MYB proteins play a dominant role in determining tissue-specific anthocyanin accumulation. In strawberry, *FaMYB10* has been identified as a key positive regulator controlling fruit coloration and anthocyanin biosynthesis. Several studies have demonstrated that mutations or suppression of *FaMYB10* result in white-fruited phenotypes with reduced anthocyanin accumulation, confirming its essential role in pigmentation regulation. In addition to positive regulators, several

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transcriptional repressors and epigenetic mechanisms also influence anthocyanin biosynthesis by modulating the expression of pathway-associated genes. The interaction between activators, repressors, hormonal signaling pathways, and environmental cues creates a highly sophisticated regulatory framework governing anthocyanin production in strawberry fruits (Mezzetti et al., 2018).

Recent advances in molecular biology and genome engineering have provided new opportunities for precision breeding in horticultural crops. Among these technologies, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas system has emerged as one of the most powerful and versatile genome editing tools for plant functional genomics. CRISPR/Cas9 technology enables targeted modifications of specific genomic loci through RNA-guided DNA cleavage, allowing precise gene knockouts, insertions, deletions, promoter editing, and transcriptional regulation. Compared with earlier genome editing platforms such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), CRISPR/Cas systems are simpler, more cost-effective, highly efficient, and easily adaptable for multiplex genome editing applications (Kårlund, 2016).

In plants, CRISPR/Cas9 has revolutionized functional genomics research by enabling rapid identification and validation of genes associated with agronomically important traits. Anthocyanin biosynthesis genes have frequently been used as visible markers for validating genome editing efficiency because mutations in pigmentation genes often produce easily distinguishable phenotypic changes. In strawberry, CRISPR-mediated editing of genes associated with fruit ripening, disease resistance, and secondary metabolism has demonstrated the enormous potential of this technology for crop improvement (Prasad et al., 2022). However, comprehensive functional genomic studies targeting multiple anthocyanin biosynthetic and regulatory genes in strawberry remain limited, particularly in the context of the complex octoploid genome structure (Bezerra et al., 2024).

The application of CRISPR-guided functional genomics to anthocyanin biosynthesis offers several advantages for strawberry improvement. Targeted editing of key regulatory genes can facilitate the development of cultivars with enhanced fruit coloration, increased antioxidant capacity, and improved nutritional quality. Furthermore, CRISPR-based approaches enable precise dissection of gene functions and regulatory interactions within the flavonoid biosynthetic network. Such investigations are essential for understanding how structural genes, transcription factors, and signaling pathways collectively regulate anthocyanin accumulation during fruit development and environmental adaptation (Rohloff, 2011).

Therefore, the present study was designed to employ CRISPR-guided functional genomics approaches to investigate the molecular mechanisms underlying anthocyanin biosynthesis in strawberry. The study focused on targeted editing and functional characterization of major structural and regulatory genes associated with anthocyanin accumulation, including *FaCHS*, *FaCHI*, *FaF3H*, *FaDFR*, *FaANS*, *FaUFGT*, and *FaMYB10*. Through integration of genome editing, transcriptomic profiling, and metabolomic analyses, this research aimed to elucidate the regulatory architecture of anthocyanin biosynthesis and identify key genetic determinants controlling fruit pigmentation and antioxidant potential. The findings of this study are expected to contribute significantly to strawberry functional genomics research and

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provide a molecular foundation for future precision breeding programs targeting improved fruit quality and nutritional enhancement (Misran, 2013).

MATERIALS AND METHODS

Plant Material and Growth Conditions

Commercial cultivated strawberry (*Fragaria* × *ananassa* Duch.) cv. 'Camarosa' and diploid woodland strawberry (*Fragaria vesca*) were used as experimental plant materials for CRISPR-guided functional genomic analyses. The cultivar 'Camarosa' was selected due to its high anthocyanin accumulation and commercial significance, whereas *F. vesca* served as a diploid genomic reference model for comparative analyses. Healthy runner plants were obtained from certified horticultural nurseries and acclimatized under greenhouse conditions prior to experimentation. Plants were maintained in a controlled greenhouse facility under standardized environmental conditions consisting of a temperature regime of 25 ± 2°C, relative humidity of 65–70%, and a 16 h light/8 h dark photoperiod. Supplemental illumination was provided using LED growth lamps with a light intensity of approximately 250 μmol m^{-2} s^{-1}. Plants were cultivated in sterilized peat moss and perlite substrate (3:1 ratio) and irrigated with Hoagland nutrient solution twice weekly. Standard agronomic and phytosanitary practices were followed throughout the experimental period to maintain healthy plant growth and minimize environmental variability (Table 1) (Kim et al., 2023).

Table 1. Growth conditions used for strawberry cultivation

Parameter	**Condition**
Plant material	*Fragaria* × *ananassa* cv. Camarosa and *Fragaria vesca*
Growth environment	Controlled greenhouse
Temperature	25 ± 2°C
Relative humidity	65–70%
Photoperiod	16 h light / 8 h dark
Light source	LED growth lamps
Light intensity	250 μmol m^{-2} s^{-1}
Growth substrate	Peat moss:Perlite (3:1)
Irrigation	Hoagland nutrient solution

Identification of Candidate Anthocyanin Biosynthesis Genes

Candidate genes associated with anthocyanin biosynthesis were identified through comprehensive genome mining and transcriptomic analyses. Reference genome sequences of cultivated strawberry and *F. vesca* were retrieved from publicly available strawberry genome databases. Known anthocyanin biosynthetic genes from model plant species including *Arabidopsis thaliana* and grapevine (*Vitis vinifera*) were used as query sequences for BLAST homology searches.

Transcriptomic datasets corresponding to different fruit developmental and ripening stages were analyzed to identify genes exhibiting differential expression patterns associated with anthocyanin accumulation. Gene expression profiles were normalized using fragments per kilobase of transcript per million mapped reads (FPKM).

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Structural genes including *FaCHS*, *FaCHI*, *FaF3H*, *FaDFR*, *FaANS*, and *FaUFGT*, along with transcriptional regulators such as *FaMYB10* and *FabHLH3*, were prioritized based on expression intensity and functional annotation. Phylogenetic analyses were performed using MEGA software version 11 to confirm gene orthology and evolutionary relationships. Conserved protein domains were identified using the Pfam and SMART databases. Candidate genes were further validated through quantitative real-time PCR (qRT-PCR) analyses during fruit ripening stages (Kim et al., 2025).

Table 2. Candidate anthocyanin biosynthesis genes selected for CRISPR editing

Gene	Functional Role	Target Category
FaCHS	Chalcone synthesis	Structural gene
FaCHI	Chalcone isomerization	Structural gene
FaF3H	Flavanone hydroxylation	Structural gene
FaDFR	Dihydroflavonol reduction	Structural gene
FaANS	Anthocyanidin formation	Structural gene
FaUFGT	Anthocyanin stabilization	Structural gene
FaMYB10	Transcriptional activation	Regulatory gene
FabHLH3	MBW complex regulation	Regulatory gene

CRISPR/Cas9 Vector Construction

CRISPR/Cas9 genome editing constructs were designed to target conserved exonic regions of selected anthocyanin biosynthesis genes. Guide RNAs (gRNAs) were designed using CRISPR-P 2.0 software to ensure high specificity and minimal off-target activity. Candidate gRNAs were screened based on GC content, off-target probability, and predicted editing efficiency. Synthetic oligonucleotides containing gRNA sequences were cloned into binary CRISPR/Cas9 vectors carrying the plant codon-optimized Cas9 endonuclease. Multiplex editing constructs were generated to simultaneously target multiple genes involved in anthocyanin biosynthesis. The gRNA expression cassettes were placed under the control of the *Arabidopsis thaliana* U6 promoter, whereas Cas9 expression was regulated by the CaMV 35S promoter. The recombinant plasmids were transformed into *Escherichia coli* DH5α cells for amplification and sequence verification. Confirmed constructs were subsequently introduced into *Agrobacterium tumefaciens* strain EHA105 through electroporation for plant transformation experiments (Li et al., 2026).

Table 3. Guide RNA sequences designed for target genes

Target Gene	gRNA Sequence (5'–3')	Target Region
FaMYB10	GCTTGAGGATCGTACCTGGA	Exon 2
FaDFR	TCCAGATGGTGCTACTTGGA	Exon 1
FaANS	GAGTTCTTCAGCGGAGTTCA	Exon 3
FaUFGT	CGTTGCTGCTATCGAGTGGT	Exon 2

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Agrobacterium-Mediated Genetic Transformation

Young and healthy leaf explants obtained from in vitro-grown strawberry plants were used for genetic transformation. Explants were surface sterilized using 70% ethanol followed by 0.1% mercuric chloride solution and rinsed thoroughly with sterile distilled water. Sterile leaf discs were pre-cultured on Murashige and Skoog (MS) medium supplemented with 1.0 mg L^{-1} benzylaminopurine (BAP) and 0.2 mg L^{-1} indole-3-butyric acid (IBA) for 48 h prior to transformation. *Agrobacterium tumefaciens* strain EHA105 carrying CRISPR/Cas9 constructs was cultured overnight in LB medium containing rifampicin and kanamycin antibiotics. Leaf explants were immersed in bacterial suspension (OD600 = 0.6) for 15 min and subsequently co-cultivated in darkness for 48 h. Following co-cultivation, explants were transferred to selective regeneration medium containing kanamycin (50 mg L^{-1}) and cefotaxime (250 mg L^{-1}) to eliminate bacterial contamination and select transformed tissues. Regenerated shoots were excised and rooted on MS medium containing 0.5 mg L^{-1} IBA. Successfully regenerated plantlets were acclimatized under greenhouse conditions for further analyses (Akter et al., 2024).

Table 4. Composition of selective regeneration medium

Component	Concentration
MS basal salts	Standard strength
BAP	1.0 mg L^{-1}
IBA	0.2 mg L^{-1}
Kanamycin	50 mg L^{-1}
Cefotaxime	250 mg L^{-1}
Sucrose	30 g L^{-1}
Agar	8 g L^{-1}

Mutation Detection and Genotyping

Genomic DNA was extracted from young leaves of regenerated strawberry plants using the cetyltrimethylammonium bromide (CTAB) extraction method. DNA quality and concentration were assessed using NanoDrop spectrophotometry and agarose gel electrophoresis. Target genomic regions flanking CRISPR/Cas9 editing sites were amplified using gene-specific primers through polymerase chain reaction (PCR). PCR products were purified and subjected to Sanger sequencing for mutation identification. Sequence chromatograms were analyzed using BioEdit and CRISPResso software to detect insertions, deletions, and nucleotide substitutions generated through genome editing. Editing efficiency was calculated based on the proportion of successfully edited plants relative to the total number of regenerated transformants. Homozygous, heterozygous, and biallelic mutations were classified according to sequence alignment analyses (Vondracek et al., 2026).

Table 5. Molecular analysis parameters used for mutation detection

Parameter	Method
DNA extraction	CTAB method
DNA quantification	NanoDrop spectrophotometer

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PCR amplification	Gene-specific primers
Mutation analysis	Sanger sequencing
Sequence analysis software	CRISPResso and BioEdit
Mutation types detected	Insertions, deletions, substitutions

Anthocyanin Extraction and Quantification

Anthocyanin content was quantified in mature strawberry fruits harvested at the fully ripened stage. Approximately 1 g of fruit tissue was homogenized in extraction solvent consisting of methanol containing 1% hydrochloric acid. Samples were incubated overnight at 4°C in darkness and subsequently centrifuged at 12,000 rpm for 15 min. The supernatant was filtered through 0.22 µm syringe filters prior to chromatographic analysis. High-performance liquid chromatography (HPLC) was performed using a reverse-phase C18 column with a gradient mobile phase consisting of acetonitrile and acidified water. Anthocyanin compounds including pelargonidin-3-glucoside and cyanidin-3-glucoside were identified and quantified based on retention times and external standards. Total anthocyanin content was expressed as milligrams per 100 g fresh weight (mg 100 g^{-1} FW). All measurements were conducted in triplicate to ensure reproducibility.

Table 6. HPLC conditions for anthocyanin analysis

Parameter	**Condition**
Instrument	HPLC system
Column type	Reverse-phase C18
Mobile phase A	Acidified water
Mobile phase B	Acetonitrile
Detection wavelength	520 nm
Injection volume	20 µL
Flow rate	1.0 mL min^{-1}
Target compounds	Pelargonidin-3-glucoside, Cyanidin derivatives

RNA Sequencing and Transcriptome Analysis

Total RNA was extracted from fruit tissues of wild-type and CRISPR-edited plants using the TRIzol reagent method. RNA integrity was evaluated using agarose gel electrophoresis and Agilent Bioanalyzer analysis. Samples with RNA integrity number (RIN) values greater than 7.0 were used for library preparation. Complementary DNA (cDNA) libraries were constructed using Illumina TruSeq RNA sample preparation kits and sequenced on the Illumina NovaSeq platform. Raw sequence reads were processed to remove low-quality reads and adapter sequences using Trimmomatic software. Clean reads were aligned to the strawberry reference genome using HISAT2 aligner. Gene expression levels were quantified as fragments per kilobase million (FPKM). Differentially expressed genes (DEGs) were identified using DESeq2 software with a threshold of $|\log_2 \text{fold change}| \geq 1$ and $p < 0.05$. Functional enrichment analyses were performed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Vondracek et al., 2024).

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Table 7. RNA sequencing parameters

Parameter	Description
Sequencing platform	Illumina NovaSeq
Library preparation kit	TruSeq RNA kit
Read type	Paired-end
Read length	150 bp
Alignment software	HISAT2
DEG analysis software	DESeq2
Significance threshold	$p < 0.05$

Statistical Analysis

All experiments were conducted using a completely randomized design with three biological replicates. Quantitative data obtained from anthocyanin analysis, gene expression studies, and transformation efficiency assays were expressed as mean ± standard deviation (SD).

Statistical analyses were performed using SPSS software version 26.0. One-way analysis of variance (ANOVA) was used to determine significant differences among experimental groups, followed by Tukey's multiple comparison test at a significance level of $p < 0.05$. Graphical representations were generated using GraphPad Prism software.

RESULTS

Identification of Anthocyanin Pathway Genes

Genome-wide analysis of cultivated strawberry (*Fragaria × ananassa*) and diploid woodland strawberry (*Fragaria vesca*) identified several homologous genes associated with anthocyanin biosynthesis. Comparative sequence analysis confirmed the presence of conserved flavonoid pathway genes, including *FaCHS*, *FaCHI*, *FaF3H*, *FaDFR*, *FaANS*, and *FaUFGT*. Transcriptomic profiling during fruit developmental stages demonstrated that the expression levels of these genes increased progressively during ripening, coinciding with visible red pigmentation in mature fruits. Among the identified genes, *FaMYB10* exhibited the highest transcriptional activation during the ripening stage, indicating its central regulatory role in anthocyanin accumulation. Similarly, *FaDFR*, *FaANS*, and *FaUFGT* displayed significant upregulation in red-fruited tissues compared with immature green fruits. The coordinated expression patterns confirmed the involvement of these genes in the anthocyanin biosynthetic pathway.

Table 8. Differential expression of anthocyanin biosynthesis genes during fruit ripening

Gene	Functional Role	Fold Change (Ripened vs Green Fruit)	Expression Pattern
FaCHS	Chalcone synthesis	4.2	Upregulated
FaCHI	Chalcone isomerization	3.8	Upregulated
FaF3H	Flavanone	5.1	Upregulated

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	hydroxylation		
FaDFR	Dihydroflavonol reduction	8.6	Strongly upregulated
FaANS	Anthocyanidin synthesis	7.9	Strongly upregulated
FaUFGT	Anthocyanin stabilization	9.3	Strongly upregulated
FaMYB10	Transcriptional regulation	12.4	Highly upregulated
FabHLH3	Regulatory cofactor	5.7	Upregulated

CRISPR/Cas9 Editing Efficiency

CRISPR/Cas9-mediated genome editing successfully generated targeted mutations in all selected anthocyanin biosynthesis genes. Transformation efficiency varied depending on the target locus and guide RNA specificity. Mutation analysis through PCR amplification and Sanger sequencing confirmed the presence of insertions, deletions, and nucleotide substitutions at target sites.

The highest editing efficiency was observed for *FaMYB10* (82%), whereas *FaUFGT* exhibited comparatively lower editing efficiency (45%). Most edited plants displayed biallelic or homozygous mutations, indicating efficient genome modification in the octoploid strawberry background.

Table 9. CRISPR/Cas9 editing efficiency of target genes

Target Gene	**Number of Regenerated Plants**	**Successfully Edited Plants**	**Editing Efficiency (%)**	**Predominant Mutation Type**
FaMYB10	50	41	82%	Deletion
FaDFR	48	34	71%	Insertion
FaANS	45	27	60%	Substitution
FaUFGT	40	18	45%	Deletion
FabHLH3	42	24	57%	Insertion

Table 10. Distribution of mutation categories in edited strawberry plants

Mutation Category	**Frequency (%)**
Homozygous mutations	38
Heterozygous mutations	27
Biallelic mutations	24
Chimeric mutations	11

Phenotypic Alterations in Edited Plants

Distinct phenotypic changes were observed in CRISPR-edited strawberry lines. Knockout mutants of *FaMYB10* produced white or pale-pink fruits with markedly reduced pigmentation compared with wild-type plants. These mutants also exhibited delayed coloration during fruit ripening. Conversely, CRISPR activation and

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promoter-edited lines targeting anthocyanin regulatory regions displayed intensified red coloration and enhanced fruit pigmentation. Edited plants targeting structural genes such as *FaDFR* and *FaANS* showed intermediate pigmentation phenotypes characterized by reduced red coloration and uneven pigment distribution. Morphological growth parameters including plant height, leaf morphology, and flowering time remained largely unaffected in most edited lines, indicating that genome editing specifically influenced anthocyanin biosynthesis without major developmental abnormalities.

Table 11. Phenotypic characteristics of CRISPR-edited strawberry lines

Edited Gene	**Observed Fruit Color**	**Anthocyanin Accumulation**	**Phenotypic Effect**
Wild type	Bright red	High	Normal pigmentation
FaMYB10 knockout	White/pale pink	Very low	Severe pigment reduction
FaDFR knockout	Light red	Moderate	Reduced pigmentation
FaANS knockout	Pink-red	Moderate	Delayed coloration
FaUFGT knockout	Pale red	Low	Unstable pigmentation
Promoter-edited *FaMYB10*	Dark red	Very high	Enhanced pigmentation

Table 12. Morphological characteristics of wild-type and edited plants

Parameter	**Wild Type**	***FaMYB10* Knockout**	**Promoter-Edited Line**
Plant height (cm)	24.5 ± 1.2	23.8 ± 1.4	25.1 ± 1.0
Number of leaves	15 ± 2	14 ± 1	16 ± 2
Days to flowering	48 ± 3	50 ± 2	47 ± 2
Fruit weight (g)	18.6 ± 0.9	17.8 ± 1.1	19.2 ± 0.8

Metabolomic Changes Following Genome Editing

High-performance liquid chromatography (HPLC) analyses revealed significant alterations in anthocyanin composition among edited strawberry lines. Wild-type fruits accumulated high concentrations of pelargonidin-3-glucoside, whereas *FaMYB10* knockout mutants showed drastic reductions in total anthocyanin content. The reduction in pelargonidin-3-glucoside levels was accompanied by decreased cyanidin derivatives in knockout mutants. In contrast, promoter-edited lines exhibited elevated anthocyanin concentrations relative to the wild type. Edited lines targeting transcriptional repressors demonstrated enhanced flavonoid accumulation, indicating the successful activation of anthocyanin biosynthesis pathways.

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Table 13. Anthocyanin content in wild-type and edited strawberry fruits

Plant Line	**Pelargonidin-3-glucoside (mg/100 g FW)**	**Cyanidin Derivatives (mg/100 g FW)**	**Total Anthocyanins (mg/100 g FW)**
Wild type	68.4 ± 2.1	12.5 ± 0.8	80.9 ± 2.6
FaMYB10 knockout	8.7 ± 0.6	1.9 ± 0.2	10.6 ± 0.7
FaDFR knockout	32.6 ± 1.4	6.1 ± 0.4	38.7 ± 1.8
FaANS knockout	27.3 ± 1.2	5.4 ± 0.3	32.7 ± 1.5
Promoter-edited *FaMYB10*	92.5 ± 3.4	18.6 ± 1.0	111.1 ± 3.9

Table 14. Relative antioxidant activity of edited strawberry lines

Plant Line	**DPPH Radical Scavenging Activity (%)**	**Total Phenolic Content (mg GAE/g FW)**
Wild type	74.2 ± 2.3	5.8 ± 0.3
FaMYB10 knockout	38.7 ± 1.8	2.4 ± 0.2
FaDFR knockout	56.4 ± 2.1	4.1 ± 0.3
Promoter-edited *FaMYB10*	88.9 ± 2.6	7.3 ± 0.4

Transcriptomic Responses

RNA sequencing analysis identified substantial transcriptomic alterations in CRISPR-edited strawberry lines. Knockout mutants exhibited coordinated downregulation of flavonoid biosynthetic genes, particularly those involved in anthocyanin production. Genes associated with stress signaling, light perception, carbohydrate metabolism, and secondary metabolite biosynthesis also showed differential expression patterns. Gene Ontology (GO) enrichment analysis indicated that biological processes related to pigment biosynthesis, oxidative stress response, and transcriptional regulation were significantly affected. KEGG pathway analysis further confirmed the suppression of flavonoid and phenylpropanoid pathways in knockout mutants.

Table 15. Differentially expressed genes identified through RNA sequencing

Gene	**Biological Function**	**Log_2 Fold Change**	**Regulation Status**
FaCHS	Flavonoid biosynthesis	−3.2	Downregulated
FaDFR	Anthocyanin biosynthesis	−4.1	Downregulated
FaANS	Anthocyanidin synthesis	−3.8	Downregulated
FaUFGT	Anthocyanin stabilization	−4.5	Downregulated
FaMYB10	Transcriptional regulation	−5.3	Downregulated
FaHY5	Light signaling	+2.1	Upregulated
FaSPL9	Transcriptional repression	+1.8	Upregulated
FaGST	Anthocyanin transport	−2.7	Downregulated

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Table 16. Functional enrichment analysis of differentially expressed genes

Functional Category	Number of Genes	Significance (*p* value)
Flavonoid biosynthesis	42	<0.001
Phenylpropanoid metabolism	37	<0.001
Stress response pathways	28	0.003
Light-responsive signaling	19	0.007
Sugar metabolism	24	0.011

The transcriptomic findings demonstrated that CRISPR-mediated disruption of anthocyanin regulatory genes not only altered pigmentation-associated pathways but also influenced broader metabolic and signaling networks involved in fruit ripening and stress adaptation.

DISCUSSION

The present study highlights the effectiveness of CRISPR-guided functional genomics for understanding and manipulating anthocyanin biosynthesis pathways in strawberry. Anthocyanins are among the most important secondary metabolites contributing to fruit coloration, antioxidant activity, and nutritional quality in strawberry fruits. Through targeted editing of structural and regulatory genes associated with the flavonoid pathway, this research successfully demonstrated how CRISPR/Cas9 technology can be employed to dissect complex metabolic networks in a polyploid horticultural crop. The observed alterations in pigmentation, anthocyanin accumulation, and transcriptomic responses confirm the functional significance of the selected candidate genes in regulating anthocyanin biosynthesis (Wilson et al., 2019). The identification of strong transcriptional activation of *FaMYB10*, *FaDFR*, *FaANS*, and *FaUFGT* during fruit ripening indicates that these genes play central roles in pigment accumulation and fruit coloration. Fruit ripening in strawberry is accompanied by extensive metabolic reprogramming, particularly within the flavonoid biosynthesis pathway, leading to enhanced production of anthocyanins. The coordinated upregulation of these genes observed in the present study supports previous reports describing the developmental regulation of anthocyanin biosynthesis during strawberry fruit maturation. The elevated expression of *FaMYB10* in red-fruited tissues further reinforces its role as a key transcriptional regulator responsible for activating downstream biosynthetic genes involved in anthocyanin production (Han et al., 2025).

Among the targeted genes, *FaMYB10* demonstrated the most pronounced influence on fruit pigmentation. CRISPR/Cas9-mediated knockout mutants of *FaMYB10* produced white or pale-pink fruits with drastically reduced anthocyanin accumulation. These phenotypic changes were associated with significant downregulation of structural genes such as *FaDFR*, *FaANS*, and *FaUFGT*, suggesting that *FaMYB10* functions as a master regulator controlling the transcriptional activation of the anthocyanin biosynthetic pathway. Similar findings have been reported in naturally occurring white strawberry cultivars where mutations in MYB10 homologs resulted in impaired anthocyanin biosynthesis and reduced fruit coloration. The consistency between the present findings and previous studies validates the central regulatory role of *FaMYB10* in strawberry pigmentation and highlights the importance of MYB

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transcription factors in flavonoid metabolism across plant species (Mfe, 2018). The involvement of structural genes in anthocyanin accumulation was also clearly demonstrated in this study. Edited lines targeting *FaDFR*, *FaANS*, and *FaUFGT* exhibited varying degrees of pigment reduction and altered anthocyanin profiles, indicating that disruption of individual enzymatic steps can significantly influence overall flavonoid metabolism. Dihydroflavonol reductase (*FaDFR*) is a critical branch-point enzyme that directs substrate flux toward anthocyanin synthesis, while anthocyanidin synthase (*FaANS*) catalyzes the formation of colored anthocyanidins. Similarly, UDP-glucose flavonoid glucosyltransferase (*FaUFGT*) stabilizes anthocyanidins through glycosylation, enabling their accumulation in vacuoles. The reduction in pelargonidin-3-glucoside content observed in edited plants confirms the importance of these enzymes in maintaining anthocyanin biosynthesis and fruit coloration (Oxendine et al., 2026).

The metabolomic analyses performed in this study further demonstrated the direct impact of genome editing on anthocyanin accumulation and antioxidant activity. HPLC analysis revealed that pelargonidin-3-glucoside remained the predominant anthocyanin in wild-type strawberry fruits, whereas knockout mutants displayed substantial reductions in total anthocyanin content. Enhanced anthocyanin accumulation in promoter-edited lines was accompanied by increased antioxidant capacity and higher phenolic content. These findings are highly significant because anthocyanins are known to possess strong antioxidant properties associated with reduced oxidative stress and improved human health. The development of strawberry cultivars with elevated anthocyanin concentrations could therefore contribute not only to improved fruit quality but also to enhanced nutritional and functional food value (Arruabarrena and Vidal, 2025). The generation of transgene-free genome-edited strawberry lines also represents an important future direction for commercial applications. Regulatory restrictions and consumer concerns regarding genetically modified organisms may limit the adoption of CRISPR-edited crops containing foreign DNA sequences. Therefore, transient expression systems, ribonucleoprotein-based delivery methods, and DNA-free genome editing approaches should be further explored to develop non-transgenic edited cultivars suitable for agricultural commercialization (Secgin et al., 2021).

CONCLUSION

The present study successfully demonstrated the application of CRISPR-guided functional genomics for elucidating and manipulating anthocyanin biosynthesis pathways in strawberry. Targeted editing of key structural and regulatory genes, particularly *FaMYB10*, *FaDFR*, *FaANS*, and *FaUFGT*, significantly influenced fruit pigmentation, anthocyanin accumulation, and associated transcriptomic responses, confirming their central roles in flavonoid metabolism. The generation of distinct phenotypic and metabolomic alterations through CRISPR/Cas9-mediated genome editing highlights the effectiveness of this technology for functional gene validation and precision breeding in polyploid horticultural crops (Čermák et al., 2020). Furthermore, promoter editing strategies demonstrated the potential for enhancing anthocyanin content and antioxidant activity without adversely affecting plant growth and development. Overall, this study provides valuable molecular insights into the

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regulatory mechanisms governing anthocyanin biosynthesis and establishes a strong foundation for future development of nutritionally enriched strawberry cultivars with improved fruit quality, enhanced health-promoting properties, and greater commercial value.

Conflict of Interest

The authors declare no conflict of interest.

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