THESIS

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USE OF CDDP MARKERS TO FOLLOW THE ANTHOCYANIN BIOSYNTHESIS IN PEPPER

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1. INTRODUCTION AND OBJECTIVES

Anthocyanins play a pivotal role in conferring vibrant colors and health-promoting properties to numerous fruits and vegetables, including peppers. In peppers, these pigments are crucial not only for their visual appeal but also for their functional properties as antioxidants, which significantly contribute to human health by scavenging harmful free radicals, as Mazza and Miniati (1993) observed. Gould (2004) states that anthocyanins also serve protective functions in plants, such as shielding against UV radiation, deterring herbivores, and attracting pollinators. The biosynthesis of anthocyanins in plants is a highly regulated process, influenced by both genetic and environmental factors. Stintzing and Carle (2007) found that, in peppers, this biosynthesis varies across species and cultivars and is affected by genetic background, maturity, environmental conditions, and agricultural practices.

Beyond their roles in plant biology, anthocyanins have been extensively studied for their health benefits in humans. He and Giusti (2010) found that these compounds exhibit potent antioxidant properties, which help reduce oxidative stress and potentially lower the risk of cardiovascular diseases and certain cancers. Tsuda et al. (2012) emphasize that anthocyanin-rich diets contribute to cellular health by neutralizing reactive oxygen species. This dual significance—contributing to both plant and human health—underscores the importance of understanding anthocyanin biosynthesis in crops like peppers, where pigment content is linked to both visual and nutritional quality.

Peppers (*Capsicum spp*.) hold significant interest in research due to their agricultural, nutritional, and economic importance worldwide. Stintzing and Carle (2007) found that the pigmentation of peppers, influenced by the accumulation of anthocyanins and other carotenoids, is a key factor in determining both their market appeal and nutritional quality. Howard et al. (2000) noted that anthocyanins in peppers contribute to a rich color profile and serve as indicators of antioxidant capacity, making them valuable targets in plant breeding programs aimed at enhancing health-related compounds in crops. This interest aligns with consumer demand for foods with functional health benefits, as anthocyanin-rich peppers can offer potential protective effects against oxidative stress and related health conditions.

Peppers are also a model species for studying pigmentation processes, as they exhibit a wide variation in anthocyanin content across different cultivars and environmental conditions.

Lightbourn et al. (2007) demonstrated that anthocyanin concentration varies widely among pepper cultivars, affecting both color and antioxidant capacity. Liu et al. (2018) observed that environmental factors such as light and temperature modulate anthocyanin biosynthesis in peppers, influencing gene expression and leading to variation in pigment accumulation. Studying these factors in peppers can thus inform breeding strategies aimed at optimizing pigment content and stability under diverse growing conditions, which has practical applications for both crop improvement and nutritional enhancement.

Conserved DNA-Derived Polymorphism (CDDP) markers are valuable tools for studying the genetic regulation of anthocyanin biosynthesis in crops like peppers, where pigment production is influenced by both genetic and environmental factors said Ford et al. (1998). These markers help track polymorphisms in key regulatory genes, such as *MYB* and *ERF*, which control anthocyanin pathways. Allan and Espley (2018) showed that *MYB* transcription factors, responsive to environmental stimuli, play a direct role in activating these pathways. Additionally, Deng et al. (2022) demonstrated that *ERF5* enhances anthocyanin accumulation by interacting with *MYBA* and *F3H* genes, suggesting that CDDP markers could clarify similar regulatory networks in peppers. This combined approach of CDDP markers and molecular analysis offers a promising method for advancing genetic insights and improving crop pigment traits as mentioned by Boss et al. (1996).

This study primarily addresses s anthocyanin biosynthesis in peppers and precisely, it seeks to determine how effectively CDDP markers can track polymorphisms in genes from the MYB and ERF transcription factor families, both of which are crucial in regulating pigment accumulation. The MYB family is known to directly influence anthocyanin biosynthetic genes, responding to environmental cues such as light and temperature, while the *ERF* family plays a complementary role, especially in stress responses, enhancing anthocyanin production under various conditions (Allan & Espley, 2018; Jia et al., 2018).By investigating this aspect, this research aims to provide actionable insights into enhancing anthocyanin stability in peppers, which could inform targeted breeding strategies to improve both the nutritional quality and commercial appeal of pepper crops.

2. LITERATURE REVIEW

2.1. Overview of Anthocyanin Biosynthesis

2.1.1. *Capsicum spp*

Capsicum species, commonly referred to as peppers, are a key genus in the Solanaceae family, encompassing several economically important species such as *Capsicum annuum*, *C. frutescens*, *C. chinense*, *C. baccatum*, and *C. pubescens*. These species vary widely in size, shape, color, and pungency level, traits that have been exploited through both traditional and modern breeding techniques. Botanically, these plants are characterized by their woody stems, simple leaves, and typically star-shaped flowers, leading to fruits that vary from small, round structures to long, fleshy pods depending on the species and cultivar (Bosland and Votava, 2012).

2.1.1.1. Technological Advancements in *Capsicum* Cultivation

Advancements in agricultural technology have greatly impacted the cultivation and breeding of *Capsicum* species. Modern techniques such as hydroponics and controlled environment agriculture (CEA) are being increasingly used to optimize conditions for anthocyanin production in peppers. These methods allow for precise control of factors such as light, temperature, and nutrients, which are crucial for inducing and maximizing anthocyanin synthesis in pepper fruits (Kumar et al., 2015). Additionally, the use of genetic engineering and molecular breeding techniques has enabled the development of *Capsicum* varieties with improved traits such as increased disease resistance, enhanced nutritional content, and greater yield stability (Barry et al., 2008).

2.1.1.2. Nutritional and Medicinal Value of *Capsicum*

Peppers are not only valued for their culinary uses but also for their nutritional and medicinal properties. They are rich sources of vitamins A, C, and E, and contain significant levels of antioxidants, which contribute to health benefits such as anti-inflammatory properties and enhanced immune function. The anthocyanins present in purple and dark red peppers are particularly noted for their antioxidant capacity, which can help in preventing chronic diseases such as heart disease and cancer (Menichetti et al., 2016). Furthermore, the capsaicin in hot peppers has been extensively studied for its pain-relieving properties, showcasing the diverse medicinal applications of the genus (Derry et al., 2015).

2.1.1.3. Economic Relevance of *Capsicum spp*

Capsicum species are of significant economic value globally, with their fruits used in various forms such as fresh, dried, and powdered for culinary spices. The global market for peppers is expanding, with significant production centers in China, Mexico, Turkey, Indonesia, and India. Beyond culinary uses, *Capsicum* extracts are also widely used in the pharmaceutical and cosmetic industries due to their bioactive compounds like capsaicin and pigments such as anthocyanins, which have antioxidant properties (Smith et al., 2016). This economic importance underlines the need for continued agricultural innovation and breeding focused on enhancing desirable traits such as fruit color and nutritional content.

2.1.1.4. Pepper Features and Their Influence on Anthocyanin Biosynthesis

The morphology of pepper fruits is diverse, ranging from small, round, berry-like structures in some wild species to larger, lobed or elongated fruits in cultivated varieties. The color of peppers is particularly variable, with fruits exhibiting colors that span green, yellow, orange, red, and purple, the latter being indicative of significant anthocyanin accumulation. Anthocyanin biosynthesis in peppers is influenced by both genetic and environmental factors. Genetically, several alleles have been identified that regulate anthocyanin production, with expression patterns often tightly controlled by developmental cues and light exposure (Zhang and Winkel-Shirley, 2003). Environmental conditions such as light intensity, temperature, and water stress significantly affect anthocyanin levels in *Capsicum* fruits. Studies have shown that cooler temperatures and moderate light levels can enhance anthocyanin synthesis in some pepper varieties, likely through the activation of specific transcription factors within the anthocyanin biosynthetic pathway (Lois, 2010).

2.1.1.5. Comparative Analysis with Other Solanaceous Crops

Anthocyanin biosynthesis in peppers shares many similarities with other solanaceous crops, such as tomatoes and eggplants, but also exhibits unique regulatory characteristics. Studies comparing these crops have revealed that while the basic biosynthetic pathways are conserved, the control points and intensity of expression of the biosynthetic genes can vary significantly (Ballester et al., 2010). This comparative analysis helps in understanding the evolutionary adaptations and can guide breeding strategies to enhance desirable traits in peppers.

2.1.1.6. Nutritional and Commercial Significance of Anthocyanins in Peppers

The increased interest in anthocyanins is also due to their potential health benefits, including anti-inflammatory and anticarcinogenic properties, as well as their ability to improve visual health. In peppers, high anthocyanin content not only enhances the visual and nutritional appeal of the fruits but also adds value to the crops from a commercial perspective, potentially leading to higher market prices (Butelli et al., 2008).

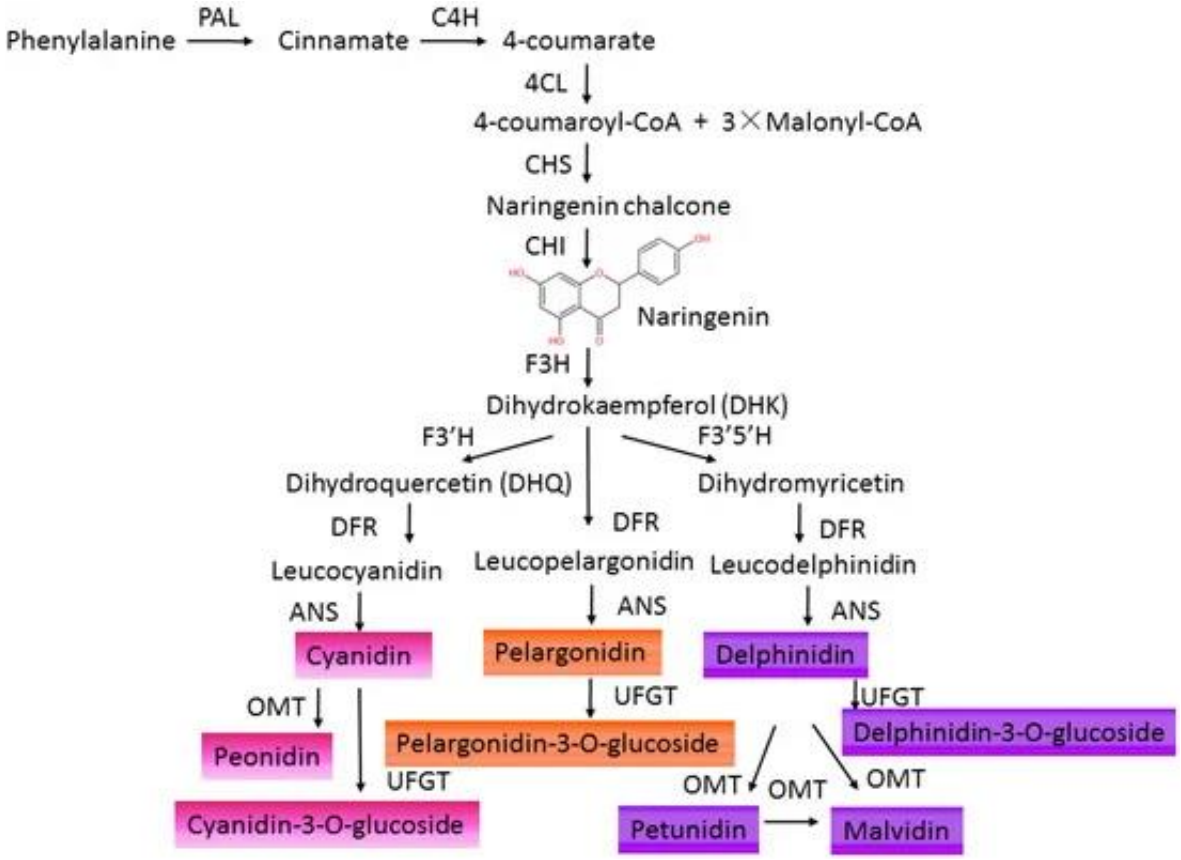
2.1.2. Anthocyanins: General Properties and Biosynthetic Pathway

Anthocyanins are a group of water-soluble pigments that belong to the flavonoid class of phytochemicals, known for their roles in the coloration of fruits, flowers, and leaves. The primary function of anthocyanins in plants is to attract pollinators and seed dispersers, as well as to provide protection against various biotic and abiotic stresses, such as UV radiation and extreme temperatures. The basic biosynthetic pathway of anthocyanins begins with the phenylpropanoid pathway where the amino acid phenylalanine is converted into flavonoids, which are then specifically modified into anthocyanins (Tanaka et al., 2008).

2.1.2.1. Key Enzymes and Steps in the Anthocyanin Pathway

The anthocyanin biosynthetic pathway (Figure 1) involves several key enzymes including phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavonoid 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose: flavonoid glucosyltransferase (UFGT). Regulation of these enzymes occurs at both the transcriptional and post-transcriptional levels (Grotewold, 2006).

Figure 1: The pathway of anthocyanin biosynthesis. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 30 hydroxylase; F3′5′H, flavonoid 3050hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, UDP-galactose flavonoid 3-O-galactosyltransferase; OMT, O-methyl transferase.
(Source: Ma et al., 2021)



2.1.2.2. Genetic Regulation of Anthocyanin Biosynthesis

In-depth studies have elucidated the genetic regulation of anthocyanin biosynthesis, highlighting the role of transcription factors from the MYB, bHLH, and WD40 families. These transcription factors form a complex known as the MBW complex, which directly activates the transcription of anthocyanin biosynthetic genes. In peppers, specific MYB and bHLH transcription factors have been identified that are essential for the regulation of anthocyanin production. Research has shown variability in anthocyanin content among different pepper species, which can be attributed to differences in the expression levels and activity of these transcription factors (Borovsky et al., 2004; Stommel and Bosland, 2006). For instance, the *Capana10g002666* gene has been associated with intense purple pigmentation in certain pepper varieties (Liu et al., 2016).

Also, in the studies conducted on mulberry fruits, it was demonstrated that ERF5 interacts with *F3H* and *MYBA* of the flavonoid pathway, each crucial in anthocyanin synthesis. In the process, ERF5 was discovered to bind directly to the promoter regions of *MYBA* and *F3H*, hence enhancing the transcriptional activities of such genes. The interaction thus suggests that ERF5 is a key

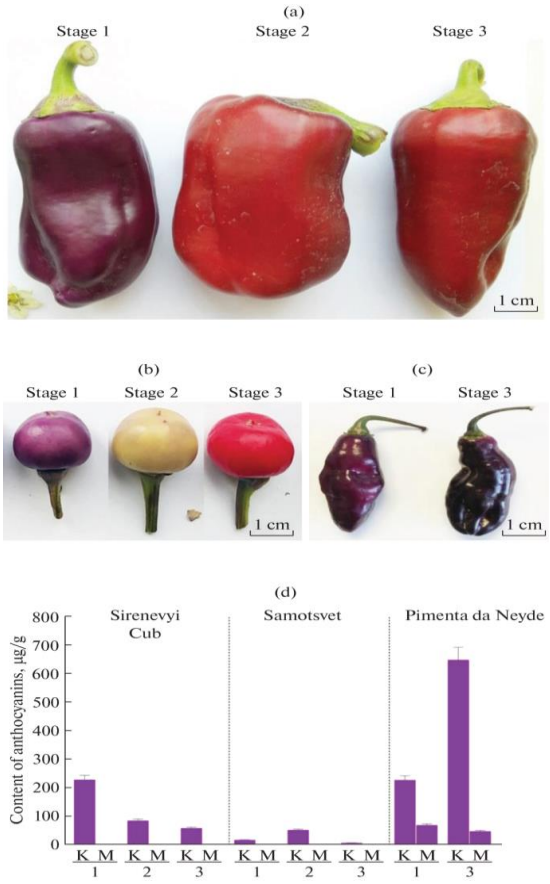
mediator in the effects of ethylene on anthocyanin accumulation in particular tissues and under certain conditions-for example, during fruit ripening or under certain environmental stimuli. This has been confirmed by bioassays such as EMSA (Electrophoretic Mobility Shift Assay) and other luciferase-based bioassays (Deng et al., 2022).

In addition to transcription factors, microRNAs (miRNAs) play a vital role in the genetic regulation of anthocyanin biosynthesis by modulating gene expression at the post-transcriptional level. He et al. (2016) found that miRNAs like *miR828* and *miR858* are significant regulators in this pathway. Specifically, *miR828* targets MYB repressors, lifting inhibitory controls on anthocyanin production, particularly in response to environmental cues like light and temperature. Yang et al. (2019) further observed that *miR858* provides regulatory balance by modulating both MYB activators and repressors, enabling plants to fine-tune anthocyanin accumulation based on developmental and environmental conditions. Together, these miRNAs ensure that anthocyanin synthesis aligns with optimal growth conditions.

2.1.2.3. Molecular Mechanisms of Anthocyanin Accumulation

Anthocyanin levels in plants vary significantly, shaped by genetic and environmental factors. In peppers, this variation is pronounced, with cultivars displaying a spectrum of color intensities and patterns (Figure 2) due to differing expression of anthocyanin biosynthetic genes, regulated by a network of transcription factors. Developmental stages also impact anthocyanin accumulation, in certain species increasing as the fruit matures such as grapes where the ripening leads to intensifies pigmentation, but this is not the case for peppers. These regulatory genes respond to environmental cues, resulting in substantial differences in anthocyanin content within the same species under varying conditions (Matus et al., 2009).

Figure 1: Comparative Analysis of Anthocyanin Content in Different Stages of Pepper Maturation.
(Source: Filyushin et al., 2023).



2.1.2.3.1. Agronomic Factors

The cultivation practices including soil type, nutrient management, and irrigation practices markedly influence anthocyanin production. Nutrients such as nitrogen, phosphorus, and potassium need to be optimally balanced as excessive nitrogen, for example, can dilute anthocyanin concentrations by promoting excessive vegetative growth. Soil pH and organic matter content also affect the availability of these nutrients and hence the plant's physiological response including anthocyanin synthesis (Kumar et al., 2015). Post-harvest factors such as storage temperature and light exposure can further modify anthocyanin levels, making these critical considerations for maintaining the nutritional quality and visual appeal of peppers during marketing (Barry et al., 2008).

2.1.2.4. Anthocyanin Biosynthesis and Plant Stress Responses

Anthocyanins are not only important for coloration but also play significant roles in plant defense mechanisms. They have been found to have antioxidative properties, which help plants combat

oxidative stress caused by various environmental stresses, including drought, salinity, and exposure to heavy metals. In peppers, anthocyanins can contribute to increased resistance against pathogen attack by acting as antimicrobial agents (Chalker-Scott, 1999).

Hormones such as abscisic acid (ABA), jasmonic acid (JA), and ethylene significantly influence anthocyanin biosynthesis, especially under stress conditions. Jia et al. (2018) demonstrated that ABA, often activated during drought or salinity stress, enhances MYB transcription factor activity, thereby upregulating anthocyanin biosynthetic genes. Shan et al. (2020) found that JA, released in response to mechanical damage or herbivory, works synergistically with bHLH transcription factors to activate *MYB* genes, which promotes anthocyanin production as a defense mechanism. Ethylene, associated with fruit ripening, has also been shown to enhance anthocyanin accumulation; Chervin et al. (2019) observed that ethylene treatment in fruit crops like tomatoes and grapes accelerates pigmentation by promoting *MYB* expression during ripening. This hormone-driven regulation allows plants to dynamically adjust anthocyanin levels in response to environmental stresses and developmental cues, thereby optimizing pigment stability and stress tolerance.

2.2. Technological Advances in Anthocyanin Research

2.2.1. Recent Genetic Studies and Advances

Advancements in genetic engineering and molecular biology techniques have opened new avenues for enhancing anthocyanin production in peppers. The CRISPR/Cas9 system has been successfully employed to modify key genes in the anthocyanin pathway, resulting in altered pigment profiles (Zhang et al., 2020).

This technology has been used to alter anthocyanin-related transcription factors both in tomato (*Solanum lycopersicum*) and grapevine (*Vitis vinifera*), proving its versatility in changing pigment biosynthesis. CRISPR-Cas9 was employed in targeting SlMYB, a repressor of anthocyanin biosynthesis in tomatoes, thereby enhancing the pigmentation of fruits in the process (Li et al., 2018). Meanwhile, in the case of grapevine, *VvMYBA* and *VvMYB5a* transcription factors were knocked down by this method, resulting in a decrease in anthocyanin levels and changing the color of the grape skin (Ren et al., 2021).

2.2.2. Genetic Engineering and Breeding for Enhanced Anthocyanin Content

With the advancing tools in genetic engineering, efforts have been made to enhance anthocyanin content in crops for their nutritional and aesthetic values. In peppers, conventional breeding techniques have been supplemented by molecular breeding approaches to select high-anthocyanin-producing variants. Genetic engineering, such as overexpression of specific MYB transcription factors, has shown promise in increasing anthocyanin levels effectively and specifically in pepper fruits (Giovannoni, 2007). For instance, when *MYB* expression was induced by the CaMV 35S promoter rather than by other promoters, an increase in anthocyanin accumulation was noted in various carrot organs (Fan et al., 2020; Xu et al., 2020).

As of right now, the anthocyanidin biosynthesis of a number of plants, such as tobacco (*Nicotiana tabacum*), apple (*Malus domestica*), radish (*Raphanus sativus*), freesia (*Freesia hybrida*), gerbera (*Gerbera hybrida*), rose (*Rosa hybrida*), and others, has been shown to exhibit expression of *MYBs* (Shen et al., 2019; Fan et al., 2020; Li T. et al., 2020; Li Y. et al., 2020, Li et al., 2021; Zhong et al., 2020).

2.2.3. Genetic variation and pigmentation with molecular markers

The conserved DNA-derived polymorphism (CDDP) marker is a molecular tool that has proven effective in distinguishing genetic variations among samples, particularly those that exhibit differences in pigmentation. For example, In *Elaeagnus macrophylla*, an endangered species, CDDP markers were used to analyze genetic diversity across populations, revealing structural genetic variation tied to color differences and contributing to conservation and breeding strategies aimed at maintaining biodiversity and phenotypic stability (Wang et al., 2020). In *Rosa rugosa*, CDDP markers helped researchers investigate genetic diversity in relation to anthocyanin levels, providing a clear link between genetic markers and pigmentation in rose petals, thus enhancing understanding of the genetic regulation of color traits (Saïdi et al., 2017).

In pepper *(Capsicum annuum)*, AFLP markers were used to assess genetic diversity related to anthocyanin pigmentation. DNA from different pepper varieties was digested and amplified, revealing polymorphisms that correlated with color differences. AFLP provided a genome-wide perspective on anthocyanin-associated traits, helping distinguish pepper cultivars by pigmentation (Rao et al., 2003).

In apple *(Malus domestica)*, SNP markers helped identify regions linked to red coloration in apple skin due to anthocyanin accumulation. Through genome-wide association studies (GWAS), researchers pinpointed SNPs near MYB transcription factors that regulate anthocyanin biosynthesis. This high-precision mapping provided insight into genetic variations influencing pigmentation, showing SNP markers' effectiveness in tracking color-related traits (Chagné et al., 2013).

In Chinese cabbage *(Brassica rapa)*, HRM analysis detected variations in anthocyanin genes, focusing on pigmentation in cabbage leaves. Differences in DNA melting curves revealed sequence variations linked to anthocyanin levels. HRM's sensitivity to small genetic changes made it ideal for identifying anthocyanin-related variants in distinct cabbage phenotypes (Wang et al., 2014).

CDDP markers work by targeting conserved sequences in DNA, such as gene-specific regions or conserved domains, which can be amplified using specific primers (Collard & Mackill, 2009). This method is advantageous in assessing genetic diversity and differentiation, offering a high level of specificity and reproducibility in comparison to other marker systems like RAPD or AFLP (Gupta & Varshney, 2000). Due to these features, CDDP markers have become valuable in studying traits linked to pigmentation, including those involved in anthocyanin biosynthesis.

2.2.3.1. CDDP and Anthocyanin

CDDP markers are highly effective in analyzing genetic variation within anthocyanin biosynthetic pathways, allowing researchers to differentiate plant varieties based on their anthocyanin levels and resulting coloration (Joshi et al., 2015; Zhang et al., 2018). This ability is particularly valuable for plant breeding programs, where the selection of specific pigmentation traits is essential for developing cultivars with targeted color characteristics. Additionally, CDDP markers help identify key genetic loci that control anthocyanin production, providing insights into the genetic mechanisms underlying color variation. This understanding not only sheds light on the evolutionary pathways of pigment synthesis but also enhances the commercial and aesthetic appeal of both horticultural and agricultural crops (Zhang et al., 2018). By linking genetic differences to visible traits, CDDP markers effectively integrate molecular research with practical applications in plant breeding and cultivar development.

In the same direction, studies on lily flowers showed that MYB transcription factors specifically, those belonging to the R2R3-MYB family are essential for triggering genes involved in late anthocyanin biosynthesis, such as *ANS*, frequently in conjunction with other regulators like ERF (Wang et al., 2023).

3. MATERIAL AND METHODS

3.1.**Plant Material**

Table 1: List of Plant Samples Categorized by Color at Economic Maturity Stage.

(Sourece: own work)

Number	Sample code	Color
1	12943-1	Green
2	12943-2	Purple
3	12943-3	Purple
4	12943-4	Green
5	12941-1	Green
6	12941-2	Purple
7	12941-3	Purple
8	12941-4	Purple
9	12935-4	Purple
10	12935-5	Purple
11	12933-1	Purple
12	12933-2	Purple
13	12933-3	Purple
14	12932-1	Purple
15	12932-3	Purple
16	12998-1	Purple
17	12927-2	Green
18	12927-3	Green
19	12924-1	Green
20	12949-3	Purple
21	12949-4	Purple
22	12999-1	Purple
23	12999-3	Purple

Plant material was provided by Gábor Csilléry. For the studies we used 23 near isogenic lines which are either purple (18) at economic ripeness and turn red at biological maturity, or green (5) at economical ripeness and turn red at biological maturity (Table 1).

3.2. DNA Isolation And nanodrop measuring

The process for DNA extraction from fresh tissue samples based on E.Z.N.A. Plant DNA Kit by Omega Bio-tek is outlined as follows: leaves were used as primary tissues, grounded in liquid nitrogen.

Next, 50 mg of the ground tissue is transferred into a nuclease-free 1.5 mL microcentrifuge tube. To this, 400 µL of SP1 Buffer and 5 µL of RNase A are added, and the mixture is vortexed at maximum speed to ensure thorough mixing. The sample is then incubated at 65°C for 30 minutes. After incubation, 140 µL of SP2 Buffer is added, followed by vortexing and resting on ice for 5 minutes. The sample is centrifuged at maximum speed (≥10,000 x g) for 10 minutes.

Following centrifugation, a Homogenizer Mini Column is placed into a 2 mL Collection Tube, and the sample is centrifuged at maximum speed for 2 minutes. The cleared lysate is then transferred into a new 1.5 mL microcentrifuge tube. To this, 1.5 volumes of SP3 Buffer diluted with 100% ethanol are added, followed by vortexing. A HiBind® DNA Mini Column is inserted into a 2 mL Collection Tube, and 650 µL of the sample is transferred to the column, then centrifuged at maximum speed for 1 minute. This step is repeated until all of the sample has passed through the column.

Next, 650 µL of SPW Wash Buffer, diluted with 100% ethanol, is added, and the mixture is centrifuged at maximum speed for 1 minute. A second wash with the SPW Wash Buffer is performed. The empty HiBind® DNA Mini Column is then centrifuged at maximum speed for 2 minutes to ensure it is thoroughly dried. The column is transferred into a clean 1.5 mL microcentrifuge tube, and 50 µL of pre-heated Elution Buffer (at 65°C) is added twice. The sample is left to rest at room temperature for 3-5 minutes before being centrifuged at maximum speed for 1 minute each time. This elution step is repeated once more to ensure optimal DNA recovery. Finally, the eluted DNA is stored at -20°C until further use.

Concentration was measured by the Nanodrop 1000 Thermo Fisher Scientific spectrophotometer.

3.3. Primers

For the studies four different primers (Table 2) were applied, these primers targeted two transcription factor families, the ERF and the MYB.

Table 2: Primers.
(Source: Collard and Mackill, 2009)

ERF	ERF1	CACTACCGCGGSCTSCG	17
	ERF2	GCSGAGATCCGSGACCC	17
MYB	MYB1	GGCAAGGGCTGCCGC	15
	MYB2	GGCAAGGGCTGCCGG	15

3.4. PCR analysis

The PCR reactions were conducted using a DNA concentration of 20 ng/µl for each sample. The PCR mixture included 6 µl of sterile H_2O, 1 µl of 10x buffer, 0.25 µl of dNTPs from a 2 mM stock, 0.2 µl of 25 mM $MgCl_2$, 1.5 µl of primers at a concentration of 100 pM, 0.05 µl of Dream Taq polymerase (5 U/µl), and 1 µl of the DNA sample. Reaction conditions were as follows:

The PCR protocol began with an initial denaturation step at 94°C for 2 minutes to separate the DNA strands. This was followed by 30 cycles of amplification, each consisting of denaturation at 94°C for 30 seconds, primer annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute. After the cycling steps, a final extension was performed at 72°C for 5 minutes to ensure complete synthesis of all DNA fragments. The reaction was then held at 4°C to preserve the PCR products and were conserved at -20°C.

3.5. Gel electrophoresis

To separate DNA, gel electrophoresis was utilized. The process involves applying an electric current, which causes the charged molecules to migrate through the gel. A 1% agarose gel was prepared by dissolving 1.5 grams of agarose in 150 ml of TBE solution and heating it in a microwave. After allowing it to cool, 5 µl of ethidium bromide (EtBr, 10 µg/µl) was added. The electrophoresis was carried out using a 0.5x TBE buffer at a voltage of 90 volts for 20-30 minutes. The DNA bands were then visualized under a UV transilluminator, and images of the gel were captured for analysis.

3.6. Statistical methods

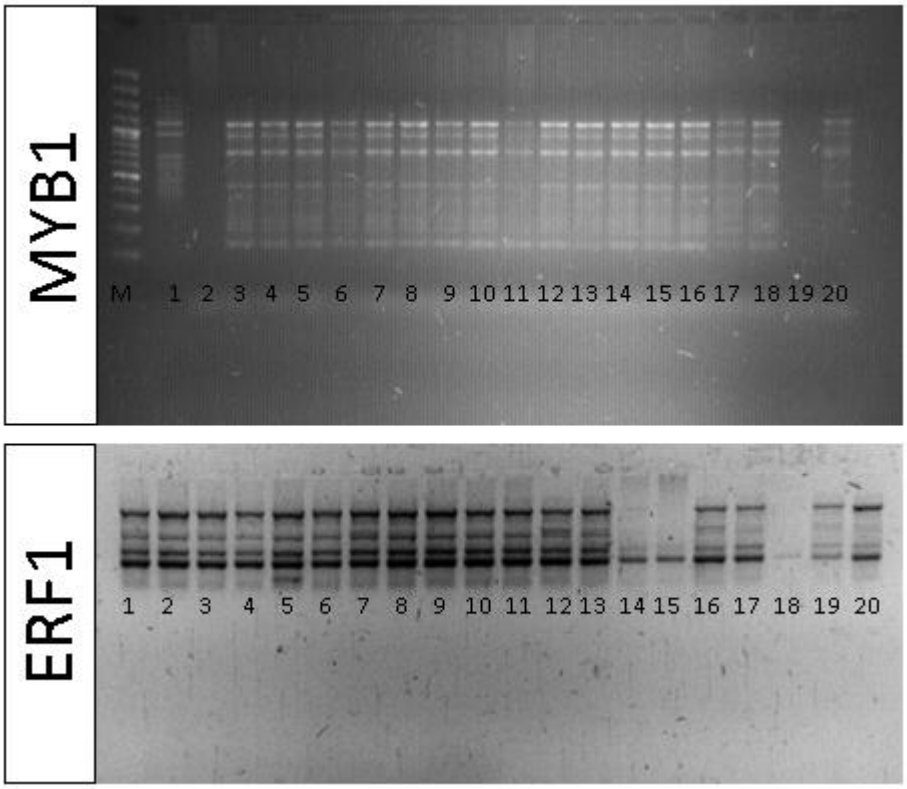
The Jaccard index and the principal component analysis (PCA) were calculated using the PAST 4.03 software (Hammer et al., 2001). The polymorphic information content (PIC), effective multiplex ratio, expected heterozygosity index (H), marker index, and discriminative power were calculated using the iMEC: Online Marker Efficiency Calculator program, developed by Amiryousefi et al. (2018). This program allows for various calculations based on previous research. It is an R software-based analytical website accessible online. The expected heterozygosity was determined based on the research of Liu et al. (1978), the polymorphic information content (PIC) according to Botstein et al. (1980), and the average heterozygosity, marker index, and discriminative power were based on the work of Tessier et al. (1999). Higher values of these indices indicate a higher level of polymorphism among the genotypes.

4. DISCUSSION

DNA amplification using the CDDP markers (*MYB1*, *ERF1*,) yielded distinct band patterns, as shown in the gel electrophoresis images (Figure 3). The bands indicate successful amplification of target regions, with white bands in the upper image representing fragments generated by the *MYB1* marker, while black bands in lower image represent fragments from the *ERF1* marker. The presence or absence of specific bands highlight polymorphisms between the different plant lines, which could be linked to the genetic differences in color expression at economic and biological maturity stages.

Figure 2: Fragments generated by CDDP marker MYB1 and ERF1(M:GeneRuler 100 bp Plus Ladder)

(Source: own work)



4.1. Marker Efficiency and Polymorphism Analysis

The marker data derived using iMEC (Table 3) indicates different levels of polymorphic information content (PIC), effective multiplex ratio (EMR), marker index (MI), discriminative power (DP), and resolving power (RP) across the four primers. The PIC values ranged from 0,253 (*MYB1*) to 0,308 (*ERF2*), suggesting moderate polymorphism within the analyzed lines. Higher PIC values indicate greater genetic diversity among the samples, with *ERF2* being the most informative marker for this study. The EMR values indicate the number of effective alleles detected, with *MYB2* having the highest EMR (7,434), suggesting that it is the most effective marker in amplifying diverse alleles.

Table 3: Marker data based on iMEC
(Source: own work)

	PIC	**EMR**	**MI**	**DP**	**RP**
MYB1	0,253	2,957	0,012	0,456	0,261
MYB2	0,255	7,434	0,012	0,448	1,043
ERF1	0,278	4,826	0,011	0,354	0,870
ERF2	0,308	6,217	0,008	0,212	0,435

4.2. Comparison of Genetic Similarity using Jaccard Index

The Jaccard index table (Table 4) presents pairwise genetic similarity among the analyzed lines. Higher values indicate greater similarity between samples. Notably, lines such as 12943-2 and 12943-3, both purple, exhibit a high similarity index, indicating genetic closeness. Conversely, the green breeding lines (12941-1 and 12927-2) show distinct clustering patterns, although there is a great similarity between the samples belonging to both colors, green and purple (between 12943-1 and 12943-2, 12943-1 and 12943-3 and between 12943-4 and 12941-2).

Table 4: Jaccard index

(Source: own work)

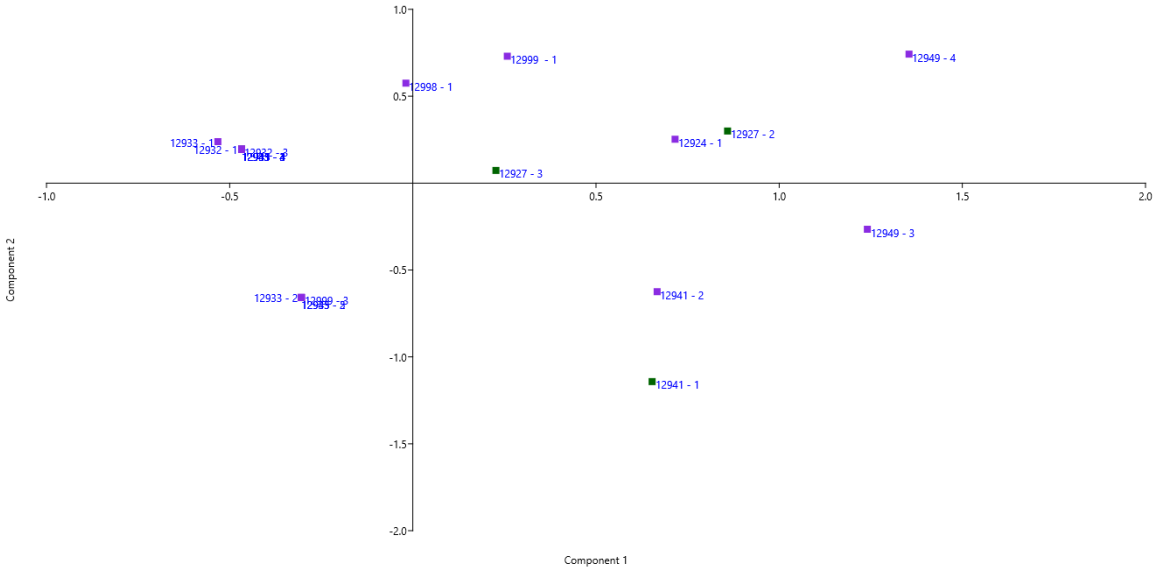
	12943 - 1	12943 - 2	12943 - 3	12943 - 4	12941 - 1	12941 - 2	12941 - 3	12941 - 4	12935 - 4	12935 - 5	12933 - 1	12933 - 2	12933 - 3	12932 - 1	12932 - 3	12998 - 1	12927 - 2	12927 - 3	12924 - 1	12949 - 3	12949 - 4	12999 - 1	12999 - 3
12943 - 1	1																						
12943 - 2	0,952381	1																					
12943 - 3	1	0,952381	1																				
12943 - 4	1	0,952381	1	1																			
12941 - 1	0,818182	0,857143	0,818182	0,818182	1																		
12941 - 2	0,869565	0,909091	0,869565	0,869565	0,863636	1																	
12941 - 3	1	0,952381	1	1	0,818182	0,869565	1																
12941 - 4	1	0,952381	1	1	0,818182	0,869565	1	1															
12935 - 4	1	0,952381	1	1	0,818182	0,869565	1	1	1														
12935 - 5	0,952381	1	0,952381	0,952381	0,857143	0,909091	0,952381	0,952381	0,952381	1													
12933 - 1	0,954545	0,909091	0,954545	0,954545	0,782609	0,833333	0,954545	0,954545	0,954545	0,909091	1												
12933 - 2	0,952381	1	0,952381	0,952381	0,857143	0,909091	0,952381	0,952381	0,952381	1	0,909091	1											
12933 - 3	1	0,952381	1	1	0,818182	0,869565	1	1	1	0,952381	0,954545	0,952381	1										
12932 - 1	1	0,952381	1	1	0,818182	0,869565	1	1	1	0,952381	0,954545	0,952381	1	1									
12932 - 3	1	0,952381	1	1	0,818182	0,869565	1	1	1	0,952381	0,954545	0,952381	1	1	1								
12998 - 1	0,954545	0,909091	0,954545	0,954545	0,782609	0,833333	0,954545	0,954545	0,954545	0,909091	0,913043	0,909091	0,954545	0,954545	0,954545	1							
12927 - 2	0,869565	0,826087	0,869565	0,869565	0,863636	0,833333	0,869565	0,869565	0,869565	0,826087	0,833333	0,826087	0,869565	0,869565	0,869565	0,913043	1						
12927 - 3	0,954545	0,909091	0,954545	0,954545	0,863636	0,913043	0,954545	0,954545	0,954545	0,909091	0,913043	0,909091	0,954545	0,954545	0,954545	0,913043	0,913043	1					
12924 - 1	0,875	0,833333	0,875	0,875	0,791667	0,84	0,875	0,875	0,875	0,833333	0,84	0,833333	0,875	0,875	0,875	0,84	0,84	0,916667	1				
12949 - 3	0,833333	0,869565	0,833333	0,833333	0,826087	0,875	0,833333	0,833333	0,833333	0,869565	0,8	0,869565	0,833333	0,833333	0,833333	0,875	0,875	0,875	0,88	1			
12949 - 4	0,84	0,8	0,84	0,84	0,76	0,88	0,84	0,84	0,84	0,8	0,807692	0,8	0,84	0,84	0,84	0,88	0,88	0,88	0,884615	0,92	1		
12999 - 1	0,913043	0,869565	0,913043	0,913043	0,75	0,875	0,913043	0,913043	0,913043	0,869565	0,875	0,869565	0,913043	0,913043	0,913043	0,956522	0,875	0,875	0,807692	0,84	0,92	1	
12999 - 3	0,952381	1	0,952381	0,952381	0,857143	0,909091	0,952381	0,952381	0,952381	1	0,909091	1	0,952381	0,952381	0,952381	0,909091	0,826087	0,909091	0,833333	0,869565	0,8	0,869565	1

4.3. Principal Component Analysis (PCA)

The PCA plot (Figure 4) shows the distribution of the breeding lines on their genetic markers and their respective colors. It appears that the findings show no complete separation of clusters composed solely of purple or green lines, with some overlap among certain samples (12943-1 and 12943-2), (12943-1 and 12943-3). It appears that the genetic marker was unable to distinguish between the green and purple samples, indicating no significant genetic differentiation between the two-color groups.

Figure 3: PCA by sample color

(Source: own work)



5. CONCLUSIONS AND PROPOSALS

CDDP markers such as *ERF2* and *MYB2* show moderate effectiveness in assessing genetic diversity among *Capsicum* near-isogenic lines with different fruit colors. *ERF2* had the highest PIC value, indicating it as the most informative marker, while MYB2 demonstrated the highest EMR, effectively amplifying diverse alleles.

Genetic variation among samples with the help of CDDP markers but they did not fully account for the observed phenotypic differences in fruit color between purple and green lines. Jaccard Index values indicated genetic similarity among color-specific lines (purple lines 12943-2 and 12943-3), but overall clustering patterns showed overlap between color groups.

PCA distribution indicated overlapping clusters, with no complete separation of purple and green lines. This suggests that the genetic markers used in this study were insufficient for distinct genetic differentiation by color.

Observed polymorphisms (Banding patterns from *MYB1* and *ERF1*) highlighting some genetic traits related to color. However, these markers alone may not be adequate to establish genetic links to color traits conclusively.

The approach of using CDDP markers on *Capsicum* follows their effective application in *Paeonia*, where CDDP markers targeted genes within the flavonoid pathway (such as *CHS, ANS, DFR, CHI, F3H, and F3'H*) directly linked to anthocyanin accumulation and color expression. This application highlighted the regulatory role of MYB transcription factors in anthocyanin pathways, with genetic diversity among cultivars correlating with pigmentation outcomes (Wang et al., 2014).

In future studies, integrating AFLP and SNP markers could prove beneficial, as these have shown efficacy in genetic studies of color traits in *Capsicum annuum* and *Malus domestica* (Rao et al., 2003; Chagné et al., 2013). Combining CDDP markers with these markers could provide a genome-wide view, increasing the likelihood of identifying color-associated genetic variations.

Implementation of HRM analysis to identify minor sequence variations in anthocyanin pathway genes might perform well demonstrated in *Brassica rapa*, HRM's sensitivity can detect slight genetic changes related to pigmentation. This could aid in tracking variations across *Capsicum* lines with high precision (Wang et al., 2014).

CRISPR-Cas9 gene editing to manipulation anthocyanin-related transcription factors, such as *MYB* and *ERF* families might validate their role in pigmentation effects. Studies on *Solanum lycopersicum* and *Vitis vinifera* have successfully used CRISPR for this purpose, supporting its potential applicability in *Capsicum* research (Wang et al., 2014).

6. SUMMARY

Our investigation about the genetic diversity focused on 23 near-isogenic lines (NILs) of a plant species of *Capsicum spp*, focusing on lines that display variations in fruit color at different stages of maturity. The study compares lines that exhibit a transition from green to red and from purple to red as they progress from economic to biological maturity. Using Conserved DNA-Derived Polymorphism (CDDP) markers targeting MYB and ERF transcription factor families, this research aims to understand the genetic factors influencing color development in these plants.

DNA was extracted from plant samples, followed by polymerase chain reaction (PCR) amplification using four specific primers (*MYB1*, *MYB2*, *ERF1*, *ERF2*). The amplified DNA fragments were analyzed through gel electrophoresis, and the genetic diversity was further evaluated using various genetic indices, including polymorphic information content (PIC), effective multiplex ratio (EMR), and discriminative power (DP). Additionally, the genetic similarities among the lines were assessed using the Jaccard index, and a Principal Component Analysis (PCA) was conducted to visualize the genetic relationships.

The results revealed moderate genetic diversity among the breeding lines, with the *ERF2* marker displaying the highest PIC value, suggesting it is the most informative for this study. The PCA shows clear groupings of the breeding lines based on their genetic profiles and color phenotypes. The genetic analysis highlighted distinct clustering of green and purple lines, yet some samples are found among a clustering of different coloration.

While these markers revealed moderate genetic variation, they did not fully account for the observed differences in fruit coloration between NILs.

In conclusion, this work contributes to foundational knowledge of genetic markers and transcription factors related to fruit color variation in Capsicum. It highlights the need for further research, particularly with other markers and this might be groundwork for targeted breeding approaches.

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LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

4CL – 4-Coumarate CoA Ligase

ABA – Abscisic Acid

AFLP – Amplified Fragment Length Polymorphism

ANS – Anthocyanidin Synthase

bHLH – Basic Helix-Loop-Helix (transcription factor family)

CaMV 35S – Cauliflower Mosaic Virus 35S Promoter (a widely used promoter in genetic engineering)

C4H – Cinnamate 4-Hydroxylase

CDDP – Conserved DNA-Derived Polymorphism

CHI – Chalcone Isomerase

CHS – Chalcone Synthase

CRISPR/Cas9 – Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR-associated protein 9

DP – Discriminative Power

DFR – Dihydroflavonol 4-Reductase

EMR – Effective Multiplex Ratio

EMS – Ethyl Methane Sulfonate

EMSA – Electrophoretic Mobility Shift Assay

ERF – Ethylene Response Factor

EtBr – Ethidium Bromide

F3H – Flavanone 3-Hydroxylase

F3′H – Flavonoid 3′-Hydroxylase

F3′5′H – Flavonoid 3′,5′-Hydroxylase

FLS – Flavonol Synthase

GWAS – Genome-Wide Association Studies

JA – Jasmonic Acid

MBW Complex – MYB-bHLH-WD40 complex, a regulatory complex involved in activating anthocyanin biosynthetic genes

MI – Marker Index

miRNA – MicroRNA

MYB – (Refers to a family of transcription factors named after MYeloBlastosis oncogene, commonly involved in regulating gene expression in plants)

NILs – Near-Isogenic Lines

OMT – O-Methyl Transferase

PAL – Phenylalanine Ammonia-Lyase

PCA – Principal Component Analysis

PCR – Polymerase Chain Reaction

PIC – Polymorphic Information Content

RAPD – Random Amplified Polymorphic DNA

RP – Resolving Power

ROS – Reactive Oxygen Species

SlMYB – *Solanum lycopersicum* MYB (a MYB transcription factor gene in tomatoes)

SNP – Single Nucleotide Polymorphism

TBE – Tris-Borate-EDTA (a buffer used in gel electrophoresis)

UFGT – UDP-Glucose: Flavonoid 3-O-Glucosyltransferase

VvMYBA – *Vitis vinifera* MYBA (a MYB transcription factor gene in grapevine)

VvMYB5a – *Vitis vinifera* MYB5a (a MYB transcription factor gene in grapevine)

WD40 – A family of proteins involved in various cellular processes, including gene expression

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DECLARATION

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