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Genetic background of Anthocyanin build-up in pepper

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List of Abbreviations:

SiRNA- Small interfering RNA

DNA- Deoxyribo Nucliec Acid

MSAP- Methylation-Sensitive Amplification Polymorphism

AFLP- Amplified Fragment Length Polymorphism

FLS- flavonol synthase

LAR - leucoanthocyanidin reductase

ANR- anthocyanidin reductase

FAS- Flower Anthocyanidin Synthase

GF- Green Fruit

LF- Lilac Fruit

PCR- Polymorphic Chain Reaction

FAO- Food and Agriculture Organization of the United Nations

UV- Ultraviolet

TBE- Tris Borate EDTA

ABSTRACT

Pepper (*Capsicum spp*.), a member of the *Solanaceae* family, is a widely eaten vegetable and spice crop worldwide. The leaves, flowers, and fruits all have hues of red, blue, and purple. The pepper's purple hue is due to the presence of anthocyanin, delphinidin-3-p-coumaroylruntinoside-5-glucoside. It enhances the health of both people and plants. Even though it has a lot of nutritional and commercial value, unneeded anthocyanin buildup results in purple and black blotches on the fruit, Our aim was to find out the differences in DNA methylation patterns between the purple and green sections of the same pepper pod.

In this study we use the pepper pod genotype which has two distinct color sectors: green and purple, which are associated with different levels of pigmentation. The hypothesis was that the DNA methylation patterns would differ between the two-color sectors, reflecting the regulatory mechanisms underlying pigmentation variation in fruit development. To test this hypothesis, the DNA was isolated separately from the green sector and from the purple sector of the same pepper pod. The DNA was then subjected to a technique called (MSAP), which uses methylation-sensitive restriction enzymes to detect methylation differences in the DNA. The restriction enzymes used were *Hpa*II and *Msp*I, which are isoschizomers that recognize the same sequence (CCGG) but have different sensitivity to methylation. *Hpa*II can only cut the sequence when both cytosines are unmethylated, while *Msp*I can cut the sequence regardless of the methylation status of the cytosines. By comparing the fragments obtained with enzymes, it is possible to infer the methylation status of the CCGG sites in the DNA.

The MSAP technique also involves the use of 20 primer combinations that anneal to the restriction sites and amplify the fragments by selective polymerase chain reaction (PCR). The primers have selective nucleotides at their 3’ ends, which increase the specificity and resolution of the technique. The primer combinations that resulted in clearly detectable fragments were evaluated; these were mainly primers containing 4 selective nucleotides. In many cases, the use of primers with 3-3 selective nucleotides resulted in smears fragments on the gel for reliable evaluation.

The MSAP analysis revealed that out of the 36 primer combinations tested, 20 yielded an evaluable pattern, and their amplified fragments resulted in 187 distinct patterns. Of these 187, 121 were patterns that were either monomorphic or not indicative of methylation differences

between the green and purple sectors. However, the patterns obtained with the 20 primer combinations differed in 11 cases in the green and purple sectors, in that both *Hpa*II and *Msp*I could cleave in the purple sector, indicating that the section was not methylated, whereas in the green sector only *Hpa*II digested the sample, indicating that the section was methylated. This suggests that there are significant differences in DNA methylation patterns between the green and purple sectors of the same pepper pod, and that these differences may be involved in the regulation of pigmentation variation in fruit development.

1. INTRODUCTION AND OBJECTIVES

Anthocyanins, a class of water-soluble pigments, are widely distributed in the plant kingdom. They are part of the flavonoid family, characterized by an anthocyanidin core combined with sugars and acyl compounds. Depending on the pH, they can display colors ranging from red to purple to blue. These compounds are present in various plant tissues, including leaves, stems, roots, flowers, and fruits, serving as secondary metabolites. Anthocyanins have multiple functions, such as attracting pollinators and aiding in seed dispersal, as well as providing protection against environmental stresses. Growing evidence suggests that anthocyanins have beneficial effects on health and could contribute to the prevention of chronic and degenerative diseases, making them an intriguing focus for both breeders and researchers.

Pepper, scientifically known as *Capsicum annuum* L., holds significant agricultural importance globally. It serves as a rich reservoir of essential nutrients such as vitamins, carotenoids, and flavonoids. Bell pepper fruits exhibit a wide range of pigments, including chlorophyll and carotenoids, resulting in various colors such as green, yellow, red, white, and purple etc. Interestingly, among the cultivated pepper varieties, there are several with purple-colored fruits, which could potentially serve as a valuable source of anthocyanins. The purple hue in peppers results from a sole anthocyanin compound, specifically delphinidin-3-p-coumaroyl runtinoside-5-glucoside, which is a flavonoid. However, it's worth noting that this purple pigmentation is only present in immature fruits and fades as they ripen. Unfortunately, unripe purple bell pepper fruits are not well-received due to their bitter taste and lack of sweetness. Up to this point, there are not commercially available sweet and fully mature purple bell peppers. Additionally, the sporadic purple patches on the fruit surface, triggered by environmental factors, negatively affect their visual appeal.

Molecular markers are specific DNA sequences linked to positions within the genome. They enable the identification of variations or polymorphisms within species or among individuals of a species in specific DNA regions. These epigenetic changes, pertain to alterations in the genome, including histone modifications, DNA methylation, and siRNA, without impacting the actual DNA sequence. Of all the various epigenetic mechanisms, DNA methylation, particularly involving cytosine methylation, has been the most extensively. researched in the context of plants. Methylation Sensitive Amplified Polymorphism (MSAP) represents a modification of the Amplified Fragment Length Polymorphism (AFLP) method.

It involves digesting genomic DNA with methylation-sensitive restriction endonucleases and subsequently amplifying the digested fragments, where a methyl group is added to a cytosine residue, resulting in C5-methylcytosine. DNA methylation is a type of epigenetic modification that affects chromatin accessibility and structure, along with histone modifications and non-histone proteins. DNA methylation also plays a role in regulating gene expression, transposon silencing, chromosome interactions, and trait inheritance. Some examples of plant traits that are influenced by DNA methylation are anthocyanin and carotenoid production, which are pigment biosynthesis processes that show differential gene expression during fruit ripening.

Objective:

In this study we are investigating differential methylation patterns in color-segmented sectors of a single genotype fruit, we have analyzed whether there are significant differences in DNA methylation patterns between the green and purple sectors of the same pepper pod. By examining these color-specific methylation profiles, we hope to gain insights into the regulatory mechanisms underlying pigmentation variation in fruit development.

2. LITREATURE REVIEW

2.1 Peppers (Capsicum annuum L.)

The *Solanaceae* family, specifically the *Solaneae* tribe and the *Solanoicleae* subfamily, is home to the *Capsicum* genus. The majority of the 2000 species and 75 genera that make up the *Solanaceae* family are tropical in origin (Chamroy, 2023). With maize, beans, and squash, chili (*capsicum*) is one of the oldest crops grown in the Americas. Along with roughly twenty-five wild and semi-cultivated variants, there are five primary species of chilies that are grown commercially: *Capsicum chinense*, *Capsicum annuum*, *Capsicum pubescens*, *Capsicum baccatum*, and *Capsicum frutescens*. Peppers (*Capsicum annuum* L.) are grown in subtropical climates all over the world, including Mexico. They can be classified as sweet or spicy (Anaya-Esparza et al., 2021).

The Food and Agriculture Organization of the United Nations (FAO) stated that the world's pepper production exceeded 39 million tons in 2020 (FAO, 2020; Wang et al., 2023). The importance of pepper (*Capsicum annuum* L.) in the field of horticulture arises from its numerous culinary uses and its role as an ornamental plant. Its value lies in its abundant presence of flavonoids, carotenoids, and vitamins, rendering it a valuable source of these beneficial compounds (Tang et al., 2020). Within the pepper family, purple peppers are unusual and unique in color due to the formation and accumulation of anthocyanins (Ou et al., 2013). Due to their high anthocyanin content, purple peppers can withstand high heat and drought well. Anthocyanin concentrations are usually higher in the fruit's outer skin (Meng et al., 2022). Furthermore, certain pepper types yield visually beautiful purple fruits due to the development of anthocyanins during fruit growth (Liu et al., 2022).

2.1.1 Potential benefits of the purple pepper

Peppers offer a range of nutraceutical advantages owing to their phytochemical composition, which can be affected by factors such as genotype, ripeness stage, and various environmental, cultivation, and post-harvest conditions (Hernández-Pérez et al., 2020). Along with that, plant have a visible trait called purpleness that is present from the early seedling stage and is inherited continuously throughout the plant's life cycle (Huang et al., 2021). The exposure to light triggers the production of anthocyanins in purple peppers. Different lengths of exposure to light result in different expressions of several structural genes and transcription factors associated with the formation of anthocyanins, which in turn cause variations in the amounts of anthocyanin

accumulation (Zhou et al., 2022). About 21 days following pollination, pepper fruit reaches its maximum size, and its characteristic purple hue is seen during the whole growing season (Tang et al., 2020; Zhou et al., 2022). Capsaicin is gaining importance within the pharmaceutical sector because of its impact on the nervous system, substantial reduction in total cholesterol levels in the bloodstream, heart, and aorta, and its effectiveness in pain relief (Figure 1) (Adaszek et al., 2019; Hernández-Pérez et al., 2020).

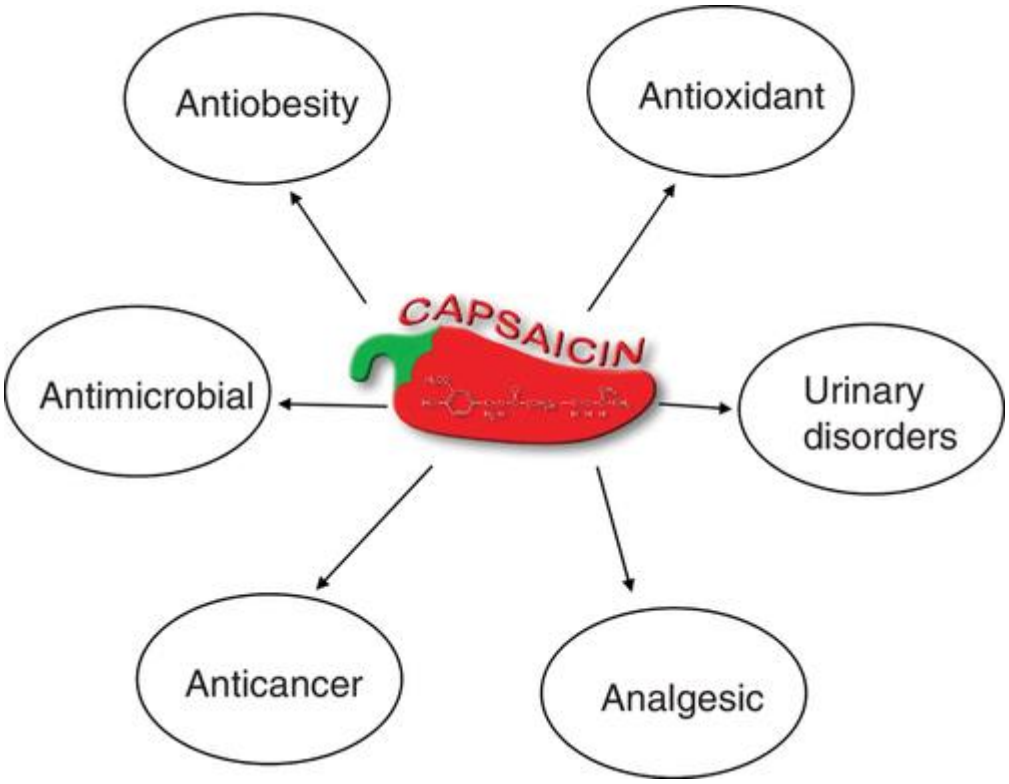


Figure 1: *Possible advantages associated with the utilization of capsaicin for human well-being (Hernández-Pérez et al., 2020).*

A pepper fruit's visual attractiveness, flavor, chemical composition, and nutritional value are all considered while evaluating its quality (Wang et al., 2023). Despite their potential relevance, a number of extremely nutritious and noteworthy but lesser-known solanaceous vegetables—such as Thai brinjal, husk tomato/tomatillo, bird's eye chili, and tree tomato/tamarilo—are not commercially farmed (Chamroy, 2023). More people are adopting healthy eating habits, with an increasing emphasis on including functional foods high in flavonoids and anthocyanins in

diets. Purple fruits and vegetables are now valued commercially as a result of this increased interest (Kovács et al., 2022).

Understanding the genetics of the pepper genome has advanced significantly in recent years. Significant progress was made in 2014 when the pepper genome was successfully sequenced and assembled. The whole genomes of *C. annuum* 'Zunla 1' and the wild species 'Chiltepin' were sequenced as part of this project (Wang et al., 2023). Using techniques from genetics, genetic engineering, and molecular biology, advances have been made in the investigation of the regulation of anthocyanin synthesis and metabolism (Meng et al., 2022).

2.1.2 Medicinal and dietary applications of purple pepper

Various germplasm resources exist for peppers, with purple peppers being an uncommon variety. In contrast to the typical green peppers, purple peppers display purple cotyledons, stems, leaves, flowers, and fruits, and they boast elevated anthocyanin levels. Purple peppers exhibit robust resilience to environmental stressors, including high temperatures and drought, and they also possess certain resistance traits against pests (Huang et al., 2021; YiHu et al., 2011). As per the findings, it has been observed that the vitamin C content in purple bell pepper cultivars falls below that of green, red, and orange bell peppers, which typically range between 62 and 162 mg per 100 grams (M.-H. Choi et al., 2023). In Figure 2, Pepper and eggplant exhibited the greatest overall antioxidant activity. Considering their complementary nutritional compositions, a consistent consumption of all three vegetables together would contribute to over 20% of the Dietary Reference Intake for several of the examined phytochemicals (Rosa-Martínez et al., 2021).

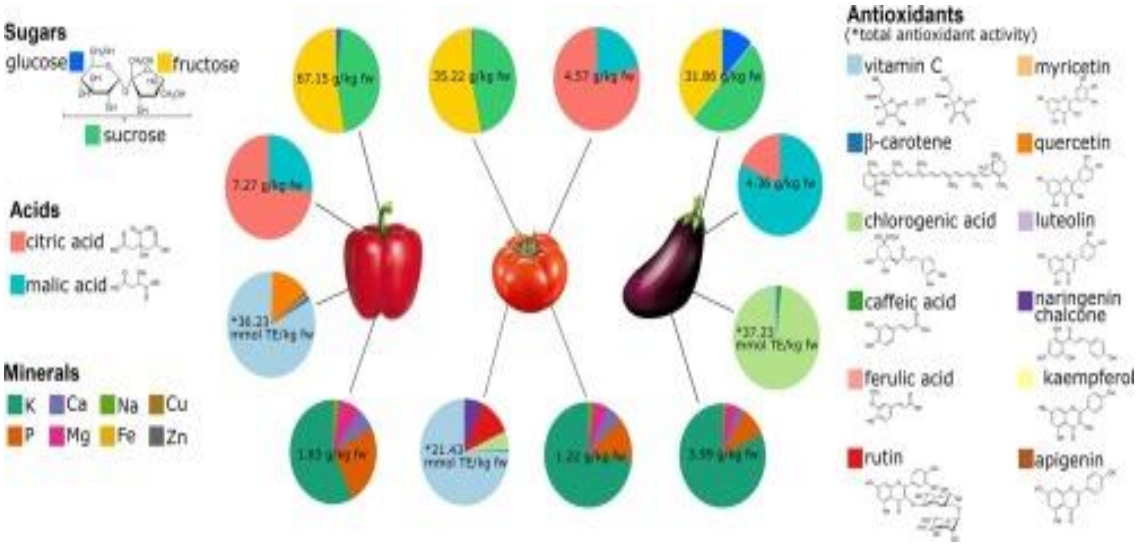


Figure 2: *Figure showing the comparative concentration of various sugars, acids, minerals, and antioxidants between three individuals of Solanaceae members (Pepper, tomato, egg-plant)(Rosa-Martínez et al., 2021).*

Furthermore, scientific research indicates that bioactive compounds derived from bell peppers offer properties such as anti-inflammatory, antidiabetic, antimicrobial, and immunomodulatory effects, among various other benefits (Anaya-Esparza et al., 2021; Carvalho et al., 2015). Indigenous communities often explore the medicinal properties of herbs and spices, utilizing them for their potential health advantages and incorporating them into therapeutic traditions. The Mediterranean diet is renowned for its exceptional nutritional richness, as well as its exquisite taste and flavor. The spices incorporated into this diet are celebrated for their anti-diabetic, anti-inflammatory, anti-hyperlipidemic, and anti-hypertensive attributes (Carvalho et al., 2015).

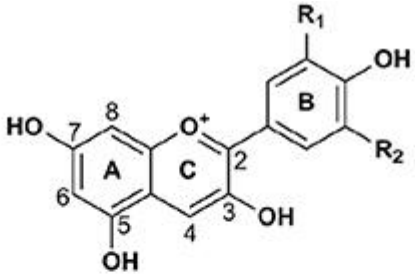
2.2 Anthocyanin

2.2.1 Biological function of Anthocyanins

Natural pigments called anthocyanins are edible, water-soluble, and have important medicinal and nutritional qualities (Li et al., 2022). Anthocyanins are common flavonoid compounds that are present in plants and are distinguished by their unique C6-C3-C6 molecular structure (Fig:3) (Belwal et al., 2020). One important class of flavonoids that makes up a large group of secondary metabolites in plants are anthocyanins. These glycosylated polyphenolic chemicals are

found in flowers, seeds, fruits, and other plant tissues (Figure 3). They exhibit a wide range of colors, from orange, red, and purple to blue (Liu et al., 2018).

These water-soluble flavonoid compounds are primarily located within cell vacuoles, their hue, a characteristic related to color, is significantly influenced by the intravacuolar conditions. In the natural environment, over 600 distinct anthocyanins have been documented (Smeriglio et al., 2016). Because anthocyanins are strong antioxidants, they protect plants from a variety of biotic and abiotic stressors (Ahmed et al., 2015). Anthocyanin pigments are the main component of the color-driven attraction approach, which leads to their antioxidant efficacy (Benvenuti et al., 2016). Analogously with other natural colors, anthocyanins can degrade during extraction, purification, manufacturing, and storage. The stability of anthocyanins can be affected by various factors, including temperature, pH levels, and the chemical makeup of the pigment (Le et al., 2019).



Anthocyanidin	R_1	R_2	Pepper	Eggplant	Tomato	Potato
Pelargonidin (Pg)	H	H				X
Cyanidin (Cy)	OH	H				X
Delphinidin (Dp)	OH	OH	X	X	X	X
Peonidin (Pn)	OCH_3	H				X
Petunidin (Pt)	OH	OCH_3			X	X
Malvidin (Mv)	OCH_3	OCH_3			X	X

Figure 3: *The typical chemical framework of anthocyanins and the six most prevalent anthocyanins found in solanaceous vegetables, represented as "x." (Liu et al., 2018).*

Anthocyanins are flavonoids with di- or tri-hydroxy B-ring substitutions and a flavylium cation, which allows them to absorb visible light within the 500–550 nm range due to their conjugated double bonds. The broad array of colors exhibited by anthocyanins is determined by the degree of hydroxylation and the type and quantity of substituted groups they possess. Anthocyanin

aglycones, also referred to as anthocyanidins, usually feature hydroxy substitutions at either penta-(3, 5, 7, 3′, 4′) or hexa-(3, 5, 7, 3′, 4′, 5′) positions (Figure 4). (Cavalcanti et al., 2011; Landi et al., 2015)

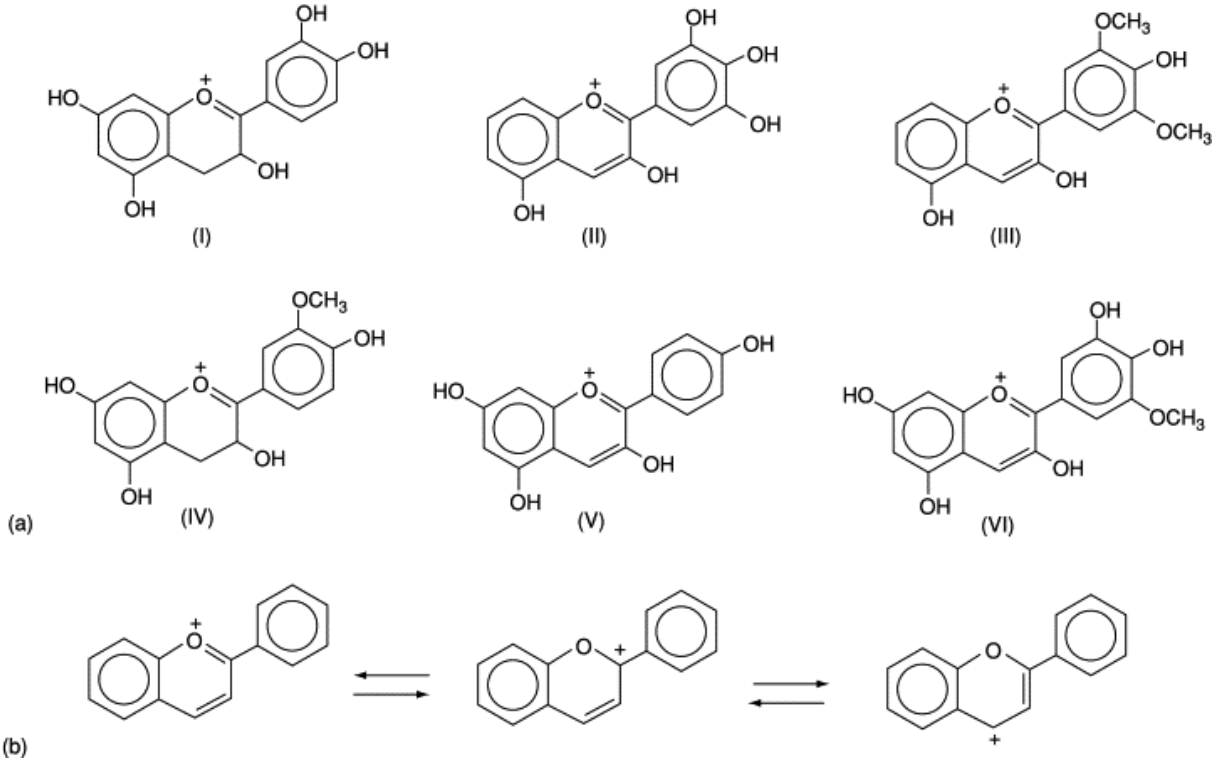


Figure 4: *The structures presented here depict six of the commonly encountered anthocyanidins. All anthocyanidins are characterized by the presence of hydroxyl groups at the 3rd, 5th, and 7th positions. However, it's worth noting that each structure may feature unique hydroxyl or methoxyl groups on the so-called B ring. (I) cyanidin, (II) delphinidin, (III) malvidin, (IV) peonidin, (V) pelargonidin, and (VI) petunidin (Horbowicz et al., 2008).*

2.2.2 Antioxidant activity of anthocyanins in plants

Anthocyanins, being the most extensively oxidized flavonoids, play a crucial role in preserving the typical cellular redox equilibrium by offsetting free radicals with the aid of their hydroxyl groups, making them effective antioxidants in the plant kingdom (Pang et al., 2023). Anthocyanins, the primary group of flavonoids in plants, are pigments marked by a flavylium cation (AH+) structure that acts as an acid. This specific configuration is intricately associated with their antioxidant properties (Tena et al., 2020). After anthocyanins are dissolved in a liquid solution, various bioassays can be employed to assess their antioxidant potential (Figure 5). Typically, two distinct mechanisms are employed to elucidate how anthocyanins function as

antioxidants: hydrogen atom donation (HAT) and single-electron transfer (SET) (Carvalho et al., 2015; Tena et al., 2020).

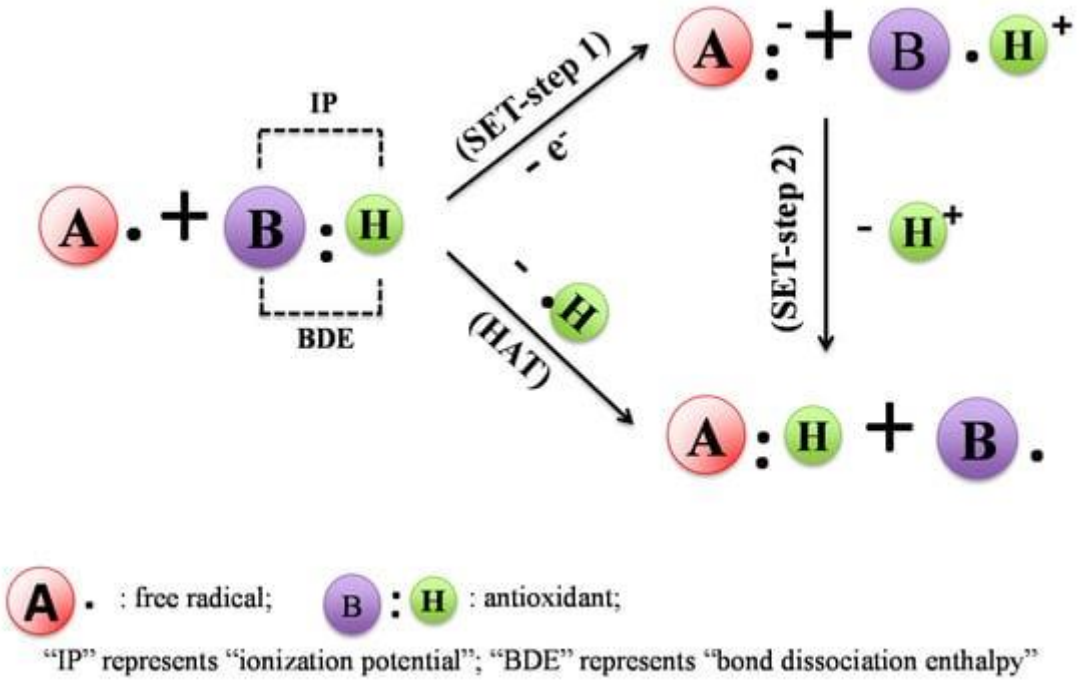


Figure 5: *Mechanisms of how antioxidants counteract free radicals involve single-electron transfer (SET) and hydrogen atom abstraction (HAT). Within the SET mechanism, the ionization potential (IP) of the antioxidant plays a pivotal role in assessing its antioxidant effectiveness. In the case of the HAT mechanism, the key parameter for evaluating the antioxidant's action is the bond dissociation enthalpy (BDE)(Tena et al., 2020).*

Peppers contain a wealth of antioxidants, encompassing chlorophyll, carotenoids, tocopherols, and capsaicinoids. Moreover, they generate both neutral and acidic phenolic antioxidants, which hold significance in the context of plant defense mechanisms (Padilha et al., 2015). The presence of antioxidants can be affected by various environmental stress factors, both related to living organisms (biotically) and non-living factors (abiotically) (Kopta et al., 2020; Sahid et al., 2022). In plant situations marked by stress, the antioxidant activity tends to elevate, as it represents a mechanism for plants to defend themselves against challenging environmental conditions (Mahmood et al., 2021).

2.2.3 Biosynthetic pathway of anthocyanin

The process of flavonoid biosynthesis is considered one of the central metabolic pathways under current investigation. In purple pepper, the primary anthocyanin is delphinidin-3-trans-coumaroylrutinoside-5-glucoside (Zhou et al., 2022). A substantial body of evidence suggests that, among various environmental factors, light stands out as a prominent regulatory element in the anthocyanin biosynthesis pathway (Ma et al., 2021).

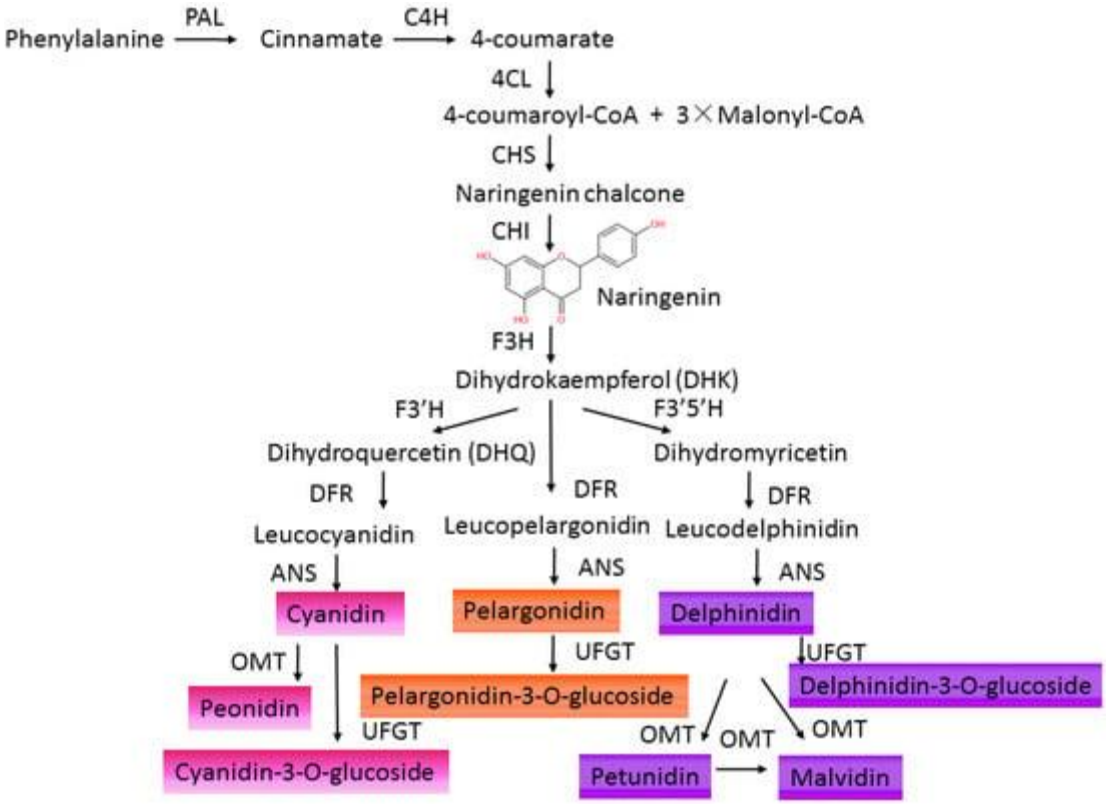


Figure 6: *The pathway of anthocyanin biosynthesis (Ma et al., 2021).*

It can be deduced that the light-induced signalling pathway triggers factors that activate the transcription of genes responsible for anthocyanin biosynthesis, ultimately leading to anthocyanin production as a means of enhancing stress tolerance (Ma et al., 2021; Takos et al., 2006). The pathway gives rise to the production of anthocyanins, with additional offshoots for the synthesis of flavonols (through the involvement of flavonol synthase [FLS]) and the production of CTs (involving leucoanthocyanidin reductase [LAR] and anthocyanidin reductase [ANR] (Figure 6). This pathway's genetic and biochemical aspects have been examined and defined in petunia (*Petunia hybrida*), maize (*Zea mays*), snapdragon (*Antirrhinum majus*), and

Arabidopsis (Sun et al., 2015). Numerous plant species have a highly conserved anthocyanin biosynthesis pathway. Two unique routes lead to the biosynthesis of anthocyanin: the flavonoid pathway and the phenylpropanoid pathway, which both begin with phenylalanine (Jung et al., 2019; Tanaka & Ohmiya, 2008). Multiple transcription factors control the expression of the genes that produce anthocyanins, which is how anthocyanin biosynthesis is regulated at the transcriptional level (Albert et al., 2011).

2.3 Methylation-Sensitive Amplified Polymorphism (MSAP) Technique:

In eukaryotes, cytosine residues in nuclear DNA are often methylated. On the other hand, methylated cytosines in higher plants are spread between the sequences ^{5m}CG and ^{5m}CNG, namely CTG, CAG, and CCG, but in mammals they are a subset of those found as CG dinucleotides. The discovery that 5-methylcytosine accounts for 2.5–11.6% of cytosine residues in mammals but 20–40% in angiosperms reflects this extra specificity (Kovařík et al., 1997; Portis et al., 2004). Animal studies, which indicate that methylation of 5CG dinucleotides has both epigenetic and mutagenesis impacts on numerous cellular functions, provide a large portion of our knowledge regarding DNA methylation in higher species (Rawoof et al., 2020). Since the promoter regions of quiet genes have been discovered to be more methylated than sequences that are actively transcribed, heavy cytosine methylation has also been revealed to have a significant role in gene expression in higher plants. Significant variations in cytosine methylation levels have been seen in rice, tomato, and maize tissue types; these variations can be attributed to the control of gene expression throughout development and differentiation. It is yet unclear how methylation controls the expression of genes (Y. Choi et al., 2002; Law & Jacobsen, 2010; Portis et al., 2004; Zhang & Zhu, 2012).

The techniques for identifying DNA methylation have evolved throughout time. Unmethylated cytosine has been detected by using bisulfites to convert it into thymine (Portis et al., 2004). Amplified fragment length polymorphism (AFLP) has been modified and used recently to measure the amount of cytosine methylation in the genomes of rice, cotton, apple, and Arabidopsis; to assess the pattern and extent of cytosine methylation in micropropagated banana, oil palm, potato, grapevine, and Arabidopsis plants (Cervera et al., 2002); to examine the DNA methylation status of strawberry and apple shoot tips during cryopreservation; to identify fingerprint clones in grapevine; and to identify modifications in the DNA methylation pattern of

winter wheat lines following vernalization treatments (Imazio et al., 2002; Sherman & Talbert, 2002). Utilising the isoschizomers *Hpa*II and *Msp*I, which vary in how sensitive they are to methylation of their recognition sequences, forms the basis of the approach. The methylation status of the internal or exterior cytosine residues influences the activity of both enzymes, which are capable of recognising the tetranucleotide sequence 5′-CCGG. Whereas *Msp*I cleaves hemi- or fully methylated C^{5m}CGG but not ^{5m}CCGG, *Hpa*II is inert when one or both of the two cytosines are completely methylated (both strands methylated) but cleaves the hemi-methylated sequence (just one strand methylated) (McClelland et al., 1994; Portis et al., 2004).

2.3.1 Advancements in DNA methylation techniques.

Methods involve employing restriction enzymes that vary in their responsiveness to methylation, such as the pairing of *Msp*I and *Hpa*II isoschizomers (Figure 7). These enzymes play a role in the Methylation Sensitive Amplified Polymorphism (MSAP) technique and differentially cleave their recognition site 5'-CCGG-3' according to disparities in cytosine residue methylation (Tanaka et al., 2014). In recent times, MSAP analyses have gained significance as a valuable tool for addressing inquiries within the growing field of "ecological epigenetics," which investigates epigenetic phenomena within an ecological framework (Rey et al., 2020). MSAP has been employed for the evaluation of DNA methylation diversity and epigenetic composition within natural plant populations. It has also been applied to identify connections between environmental conditions, observable characteristics, and methylation patterns at specific genetic loci (Akimoto et al., 2007; Alonso et al., 2016). MSAP represents an adaptation of the AFLP technique where multiple endonuclease isoschizomers that target the same restriction site but respond differently to DNA methylation are simultaneously utilized as 'methylation-sensitive cutters' in conjunction with the same 'unbiased cutter.' Subsequently, the patterns of generated bands from these cutters are compared (Alonso et al., 2016). The cutting efficiency of enzymes is significantly influenced by the methylation status of both the outer and inner cytosine residues within the 5'-CCGG-3' sequences they recognize. Therefore, it is possible to ascertain the methylation status of particular bands by considering the cutting ability of each enzyme at the restriction site. MSAP-based assessments can be conducted on various species, irrespective of their genome size and the availability of a reference genome (Chwialkowska et al., 2017).

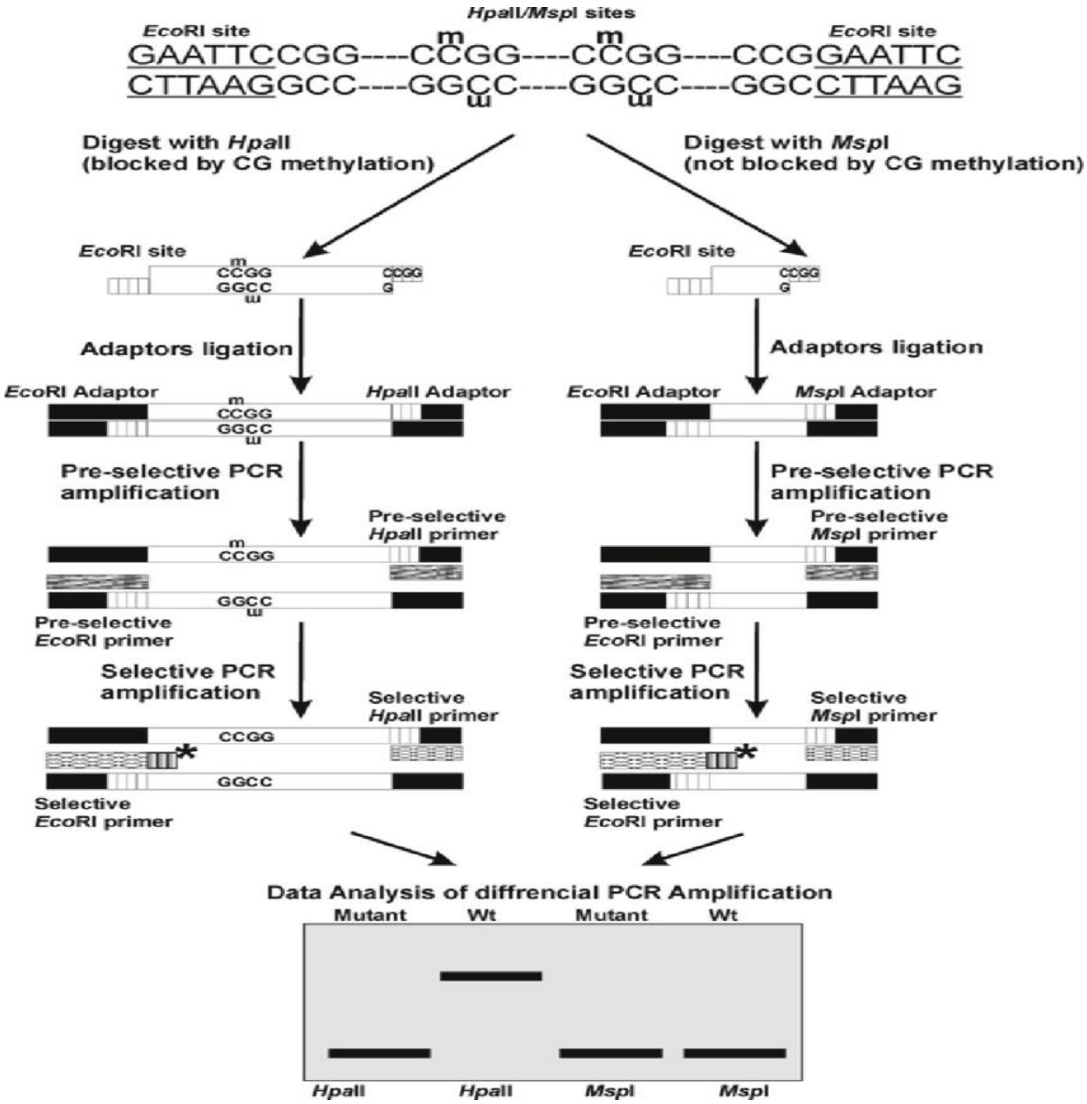


Figure 7: *Illustration of the MSAP procedure: Initially, DNA is subjected to digestion with methylation-sensitive (HpaII) and methylation-insensitive (MspI) endonucleases, followed by the ligation of the resulting DNA fragments to particular adaptors. Subsequently, these ligated DNA fragments are employed as templates in an initial PCR reaction utilizing specific primers. The resulting PCR products are then utilized as DNA templates in a subsequent selective PCR reaction, employing three selective nucleotides as fluorescently labeled primers (indicated by asterisks).*

2.3.2 Comparing High-Resolution DNA Methylation Techniques:

Bisulfite sequencing stands out as a primary method for examining DNA methylation due to its exceptional precision, as it yields results at a single-nucleotide level. In this process, genomic DNA is subjected to treatment with sodium bisulfite, inducing the deamination of unmethylated cytosines, causing their transformation into uracil while preserving the stability of methylated

cytosines. Subsequently, the bisulfite-treated DNA undergoes amplification through PCR using specific primers, and uracil residues are substituted with thymine (González-Benito et al., 2020a). The amplified fragments are then sequenced, facilitating the identification of methylated cytosines. This method is also suitable for comprehensive, genome-wide investigations. Nevertheless, it is worth noting that bisulfite treatment, while providing high-resolution outcomes, has significant limitations, including its substantial cost, time-intensive nature, and labor-intensive aspects, particularly when applied to genome-wide analyses of DNA methylation (Fulneček & Kovařík, 2014; González-Benito et al., 2020a).

Alternative approaches rely on employing distinctively methylation-sensitive restriction enzymes, like the pairing of isoschizomers. These particular restriction enzymes are integral components of the Methylation Sensitive Amplified Polymorphism (MSAP) technique, as they differentially cleave the 5'-CCGG-3' recognition site in response to variances in cytosine residue methylation (González-Benito et al., 2020a). A key benefit of this method in the realm of plant conservation is that MSAP enables investigations into non-model systems, even when their genomes have not been sequenced. This is because the amplification of restriction fragments is not contingent on the presence of genome sequence data (Fulneček & Kovařík, 2014).

In conclusion of comparison DNA methylation is one of the best-studied epigenetic changes, plays a vital role in controlling eukaryotic growth and development processes involving gene expression regulation (Jiang et al., 2020). In both development and disease, cytosine methylation in DNA is crucial for maintaining genomic stability, imprinting, and complexity. Furthermore, transposon silencing and gene regulation depend on DNA methylation (W.-F. Li et al., 2019)

2.3.3 Application of MSAP in studying epigenetic differences in pepper:

Over recent years, numerous research investigations have concentrated on exploring how epigenetic mechanisms might contribute to the adaptive responses of species to evolving environmental conditions, both in the short and long run. Epigenetic alterations encompass modifications to the genome, such as adjustments to histones, DNA methylation, and siRNA activity, without exerting any influence on the underlying DNA sequence (González-Benito et al., 2020). Among the various epigenetic mechanisms studied in plants, DNA methylation stands out as one of the most extensively researched. It has been proposed that, unlike other epigenetic processes, methylation could play a pivotal role in maintaining a consistent gene expression pattern

as cells undergo mitotic division (Abid et al., 2017). Epigenetic alterations can be genetically triggered, for instance, through gene-body methylation influenced by single-nucleotide polymorphisms.

Epigenetics serves as a crucial element in our comprehension of natural selection, inheritance, and potentially other evolutionary mechanisms. Nevertheless, a challenge in linking observable traits with particular epigenetic changes lies in the fact that both epigenetic and phenotypic variations can exhibit variability within natural systems (Akhter et al., 2021; Xia et al., 2016). The removal of DNA methylation from an epigenetic mark can occur through the action of DNA glycosylase/lyase or when methylated sites are inadequately preserved (Kohli & Zhang, 2013). Epigenetic and post-translational modifications can exert control over the process of anthocyanin biosynthesis (Cai et al., 2019; Khan & Abbas, 2023). However, there is a lack of available information regarding the relationship between heterosis and epigenetic factors like DNA methylation in pepper (Xu et al., 2015).

2.3.4 Genomic Insights into Capsicum Fruit Pigmentation

Anthocyanin regulation and biosynthesis genes' expression levels are regulated in part by DNA methylation and histone modification. In citrus, the Ruby2-Ruby1 gene cluster's functions were categorized by the methylation levels of the promoters. Histone H3K9 demethylase JMJ25 altered the promoter of the repressor gene MYB182 in poplar, which had a negative effect on the production of anthocyanins.(S. Li et al., 2022). Transposon insertions in the pepper and radish promoter and intron that increased the expression of the MYB gene (Ohno et al., 2020). As of right now, chromosome 8 has fine-mapped the gene Flower Anthocyanidin Synthase (FAS), which codes for an anthocyanidin synthase and indicates the presence of eggplant flower anthocyanin. Similarly, green fruit was caused by a mutation in Ca3GT, which was traced to chromosome 10 in pepper (Liu et al., 2020). The primary cause of pepper's purple fruit color may be delphinium chloride. The green pepper line's fruits had a larger cyanidin 3-O-galactoside concentration than the purple pepper L29 fruits, suggesting that the green pepper fruit also partially accumulated anthocyanins (Meng et al., 2022).

Research focused on enhancing yield, resistance, and various traits in pepper has experienced a significant surge. The ongoing enhancements in the pepper genome sequence and the creation of genetic maps are poised to drive substantial progress in the study of pepper fruit color. This

development has opened up fresh prospects for investigating the mechanisms underlying fruit color formation in pepper.(Shu et al., 2023; Wang et al., 2023; Wu et al., 2023).

3. MATERIAL AND METHODS

Figure 8 : *The pepper pod genotype used in the experiment.*

The plant used was one of the genotypes of an F2 generation deriving from the cross of 'Kaldom' and 'Black Pearl'. The 'Black Pearl' contains a retrotransposon insertion in its MYB gene which governs the anthocyanin biosynthesis. As a result of this insertion, the pepper is accumulating elevated amounts of anthocyanins. The DNA was isolated separately from the purple and from the green sector as indicated in Figure 8.

3.1 DNA isolation

Genomic DNA was isolated according to the E.Z.N.A. ® SP Plant DNA Kit protocol from a single genotype which showed sectoral colouration in its berries. The DNA was isolated separately from the green sector and from the lilac sector as well, according to the following protocol:

1. Grind fresh/frozen tissue samples in liquid nitrogen.
2. Transfer 50 mg ground tissue to a nuclease-free 1.5 mL microcentrifuge tube.
3. Add 400 µL SP1 Buffer and 5 µL RNase A. Vortex at maximum speed to mix thoroughly.
4. Incubate at 65°C for 10 minutes. Mix samples twice during incubation by inverting the tube.
5. Add 140 µL SP2 Buffer. Vortex to mix thoroughly.
6. Let sit on ice for 5 minutes.
7. Centrifuge at maximum speed (≥10,000 x g) for 10 minutes.
8. Insert a Homogenizer Mini Column into a 2 mL Collection Tube.
9. Carefully transfer the supernatant to the Homogenizer Mini Column. Do not disturb or transfer any of the insoluble pellet.
10. Immediately centrifuge at maximum speed for 2 minutes.

11. Transfer cleared lysate to a 1.5 mL microcentrifuge tube. Measure the volume of the lysate.
12. Add 1.5 volumes SP3 Buffer. Vortex immediately to obtain a homogenous mixture.
13. Insert a HiBind® DNA Mini Column into a 2 mL Collection Tube.
14. Transfer 650 µL sample to the HiBind® DNA Mini Column.
15. Centrifuge at maximum speed for 1 minute.
16. Discard filtrate and reuse the collection tube.
17. Repeat Steps 14-16 until all of the sample has been transferred to the column.
18. Transfer the HiBind® DNA Mini Column to a new 2 mL Collection Tube.
19. Add 650 µL SPW Buffer.
20. Centrifuge at maximum speed for 1 minute.
21. Discard filtrate and reuse the collection tube.
22. Repeat Steps 19-21 for a second SPW Buffer wash step.
23. Centrifuge the empty HiBind® DNA Mini Column at maximum speed for 2 minutes to dry the column.
24. Transfer the HiBind® DNA Mini Column into a clean 1.5 mL microcentrifuge tube.
25. Add 50-100 µL Elution Buffer heated to 65°C.
26. Let sit at room temperature for 3-5 minutes.
27. Centrifuge at maximum speed for 1 minute.
28. Repeat Steps 25-27 for a second elution step.
29. Store eluted DNA at -20°C

Concentration and purity of the DNA was determined by using the NanoDrop 1000 spectrophotometer. And then by agarose gel electrophoresis.

3.2 Methylation Sensitive Amplified Polymorphism (MSAP)

Methylation-sensitive restriction enzymes were used as "frequent cutter" enzymes, the procedure called for using the isoschizomers. The *Hpa*II/*Msp*I adapter was created, whereas the primer and adapter for the "rare cutter" enzyme *Eco*RI were the same as those employed in conventional AFLP analysis. The list of MSAP primers and adapters used are listed in table 1.

Table 1: *MSAP primer and adapter used.*

Adapter / primer name	Sequence 5'-3'
*Eco*RI – adapter – F	CTCGTAGACTGCGTACC
*Eco*RI – adapter – R	AATTGGTACGCAGTC
*Hpa*II / *Msp*I – adapter F	GATCATGAGTCCTGCT
*Hpa*II / *Msp*I – adapter R	CGAGCAGGACTCATGA
*Eco*RI – Pre-selective	GACTGCGTACCAATTCA
*Hpa*II / *Msp*I - Pre-selective	ATCATGAGTCCTGCTCGG
*Hpa*II / *Msp*I - TCAA	ATCATGAGTCCTGCTCGGTCAA
*Hpa*II / *Msp*I - TCAC	ATCATGAGTCCTGCTCGGTCAC
*Hpa*II / *Msp*I – GCT	ATCATGAGTCCTGCTCGGGCT
*Hpa*II / *Msp*I - CAG	ATCATGAGTCCTGCTCGGCAG
*Eco*RI – ACG	GACTGCGTACCAATTCACG
*Eco*RI – AAG	GACTGCGTACCAATTCAAG
*Eco*RI – ACA	GACTGCGTACCAATTCACA
*Eco*RI – AAC	GACTGCGTACCAATTCAAC
*Eco*RI – ACC	GACTGCGTACCAATTCACC
*Eco*RI – AAT	GACTGCGTACCAATTCAAT
*Eco*RI – ATC	GACTGCGTACCAATTCATC
*Eco*RI – AGC	GACTGCGTACCAATTCAGC
*Eco*RI - AGG	GACTGCGTACCAATTCAGG

To detect MSAP two sets of digestion reactions were carried out simultaneously. For the first reaction, the digestion 250 µg DNA was used (Table 2).

Table 2: *Digestion components.*

Template DNA	250 µg
Tango buffer	2 µl
HpaII or *MspI*	0.5 µl
water	up to 17.5 µl

Samples were incubated overnight at 37 ºC and then were inactivated at 65 ºC for 20 minutes in case of the *HpaII* digested samples and 80 ºC for 20 minutes in the case of the *MspI* digested set of samples. A second set of digestion was carried out using the *Eco*RI enzyme (Table 3).

Table 3: *EcoRI mediated digestion components.*

Previously digested template DNA	17.5 µl
Tango buffer	2 µl
*Eco*RI	0.5 µl

Samples were incubated overnight at 37 ºC and then were inactivated at 65 ºC for 20 minutes.

The ligation reaction was carried out using the following protocol to ligate the adaptor sequences to the digested DNA (Table 4)

Table 4: *Adapter ligation components.*

Digested DNA	10 µl
10x buffer	4 µl
*Eco*RI adapter Forward	0.5 µl
*Eco*RI adapter Reverse	0.5 µl
*Msp*I/*Hpa*II adapter Forward	0.5 µl
*Msp*I/*Hpa*II adapter Reverse	0.5 µl
T4 ligase	1 µl
water	3 µl

Overnight ligated products were used for the pre-selective PCR.

3.2.1 Pre-selective PCR conditions were the following:

Table 5: *Components for Pre-selective PCR.*

Adapter ligated template	4 µl
*Msp*I/*Hpa*II primer	1.5 µl
*Eco*RI primer	1.5 µl
Phusion Master Mix	10 µl
water	3 µl

2 min precycle at 98 °C; 30 cycles: denaturation at 98 °C for 10 s, annealing at 56 °C for 30 s, polymerization at 72 °C for 30 s.

3.3 Agarose Gel electrophoresis

After PCR amplification in pre-selective, agarose gel electrophoresis was used to analyze the success of the PCR amplifications, for which 1% TBE agarose gel was applied (1 g SeaKem® LE agarose powder to 100 ml 0.5X TBE buffer) The electrophoretic contidions were 90 V for 20 minutes.

From the pre-selective PCR 50x dilution were made and used as a template for further selective PCRs. For the reactions we used Dream Taq™ polymerase, the conditions were set according to the manufacturer's protocol and according to the annealing temperature of the primers.

3.4 Selective PCR conditions used.

For the selective PCR the following primers were applied (Table 5). The PCR protocol was set to the following:

2 min pre-cycle at 94 °C; 30 cycles: denaturation at 94 °C for 10 s, annealing at 65 °C for 30 s, polymerization at 72 °C for 1 min.
Amplicons were separated on 2% TBE agarose gel containing 2 g agarose and 100 ml TBE buffer.

3.5 Silver staining

For the silver staining 6% polyacrylamide gel was used. The gel was prepared from a ready stock solution by mixing 30 ml of the 6% stock acrylamide gel, 30 µl 25% TEMED and 30 µl freshly made 25% APS. The mixture was then poured in between the open slit of the gel cassette and was solidified under UV light for 36 minutes. Automated Laser Fluorometer was used to separate the fragments.

The method of the staining was the following:

1. Remove the gel from the cassette.

2. Wash the gel with distilled water.
3. Fix the gel by pouring 200 ml distilled water + 3 ml 65% HNO_3 solution on it, shake for 5 minutes.
4. Wash the gel with distilled water 2 times.
5. Pour the Developer Solution (12 g Na_2CO_3 + 200 ml ultrapure water complemented with 30% 70 μl formaldehyde and shake for 10-15 minutes.
6. Wash the gel with distilled water 2 times.
7. Fix the gel again by shaking it in 200 ml ultrapure water + 62.5 ml glacial acetic acid for 5 minutes.
8. Wash the gel with distilled water 2 times.
9. Let the gel soak in 200 ml ultrapure water + 69 ml 87% glycerin for 10-15 minutes.

4. RESULTS AND DISCUSSION

4.1 Results:

The objective of the study was to study the methylation pattern of the same genotype exhibiting sectorial colouration in its berry. The aim is to identify the differences between the anthocyanin-pigmented and non-pigmented sectors using the methylation-sensitive amplified polymorphism (MSAP) technique.

4.2 Methylation Sensitive Amplified Polymorphism (MSAP) Analysis:

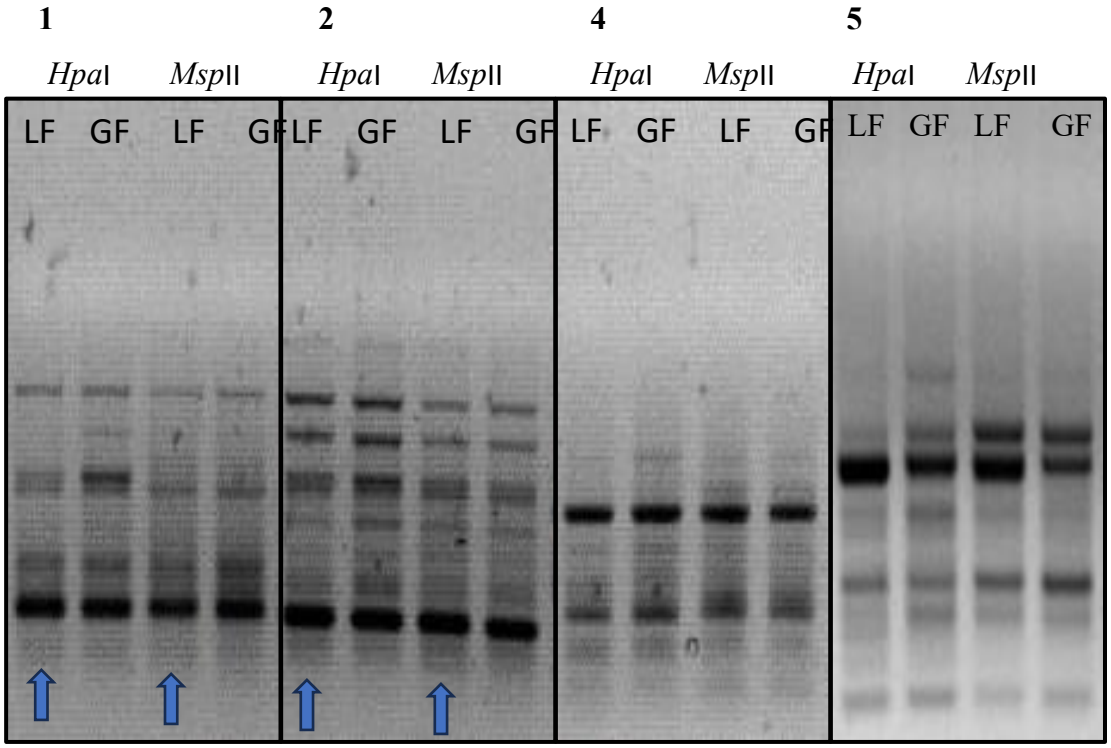
Preliminary tests were conducted using the pre-amplification products obtained from sample pepper, in order to define the conditions that would yield distinct amplified fragments on the sequencing gel. We tested different combinations of selective primers, with three selective nucleotides at the *Eco*RI end and three selective nucleotides at the *Hpa*II–*Msp*I end (from *Msp/Hpa* TCAA/TCAC/GCT/CAG). Therefore, from three to 4 selective nucleotides were tested. Only some of the primer combinations produced scorable bands and were used in this study; other combinations resulted in a smear or yielded too many amplified fragments to make accurate scoring possible.

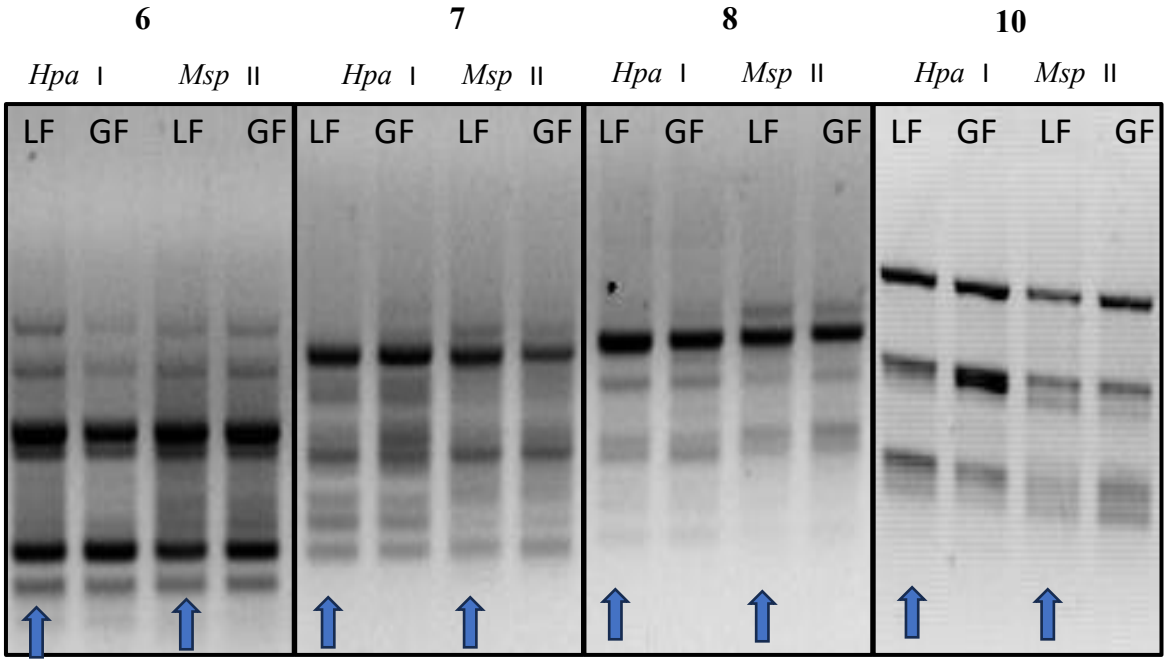
DNA methylation profiles were originated for genomic DNA extracted from same pepper. A total of (1020) fragments were amplified by 20 primer combinations. For each primer combination, PCR amplification originated from 1 to 36 (primer combination *Msp/Hpa-Eco*) fragments. With each MSAP primer combination (Table 6) we analyzed 4 lanes corresponding to two sources of genomic DNA (sample from LF (Lilac/ Purple Fruit) and GF (Green Fruit) of the same pod of pepper), each restricted with *Msp/Hpa-Eco*. Comparison of the MSAP patterns revealed two main kinds of polymorphism: (i) Bands always appearing after digestion with some *Msp/Hpa-Eco* primer combination these bands were the result of DNA methylation at the 5′-CCGG sites but did not highlight changes in methylation. (ii) Bands showing polymorphism, these bands resulted from changes in DNA methylation status Changes in DNA methylation status were detected by comparison of MSAP patterns between LF and GF

Table 6: *Primer Combinations used.*

No. Of combinations		
1	*Msp/Hpa* TCAA	*Eco* ACG
2	*Msp/Hpa*TCAA	*Eco* AAG
3	*Msp/Hpa*TCAA	*Eco* AAC
5	*Msp/Hpa*TCAA	*Eco* ACC
6	*Msp/Hpa*TCAA	*Eco* AAT
7	*Msp/Hpa*TCAA	*Eco* ATC
8	*Msp/Hpa*TCAA	*Eco* AGC
10	*Msp/Hpa*TCAC	*Eco* ACG
11	*Msp/Hpa*TCAC	*Eco* AAG
12	*Msp/Hpa*TCAC	*Eco* ACA
13	*Msp/Hpa*TCAC	*Eco* AAC
14	*Msp/Hpa*TCAC	*Eco* ACC
15	*Msp/Hpa*TCAC	*Eco* AAT
16	*Msp/Hpa*TCAC	*Eco* ATC
23	*Msp/Hpa*GCT	*Eco* ACC
32	*Msp/Hpa*CAG	*Eco* ACC
33	*Msp/Hpa*CAG	*Eco* AAT
34	*Msp/Hpa*CAG	*Eco* ATC
35	*Msp/Hpa*CAG	*Eco* AGC
36	*Msp/Hpa*CAG	*Eco* AGG

On each gel photos presented below, the order of the samples is the follows: 1st sample digested with *Msp*I, 1st sample digested with *Hpa*II, 2nd sample digested with *Msp*I, 2nd sample digested with *Hpa*II and so on. Methylation pattern changes within and/or in between the samples, brackets indicate the fragments characteristic of that given genotype and wished to cut and clone different bands.


1
2
4
5
HpaI
MspII
LF
GF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7
8
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Hpa I
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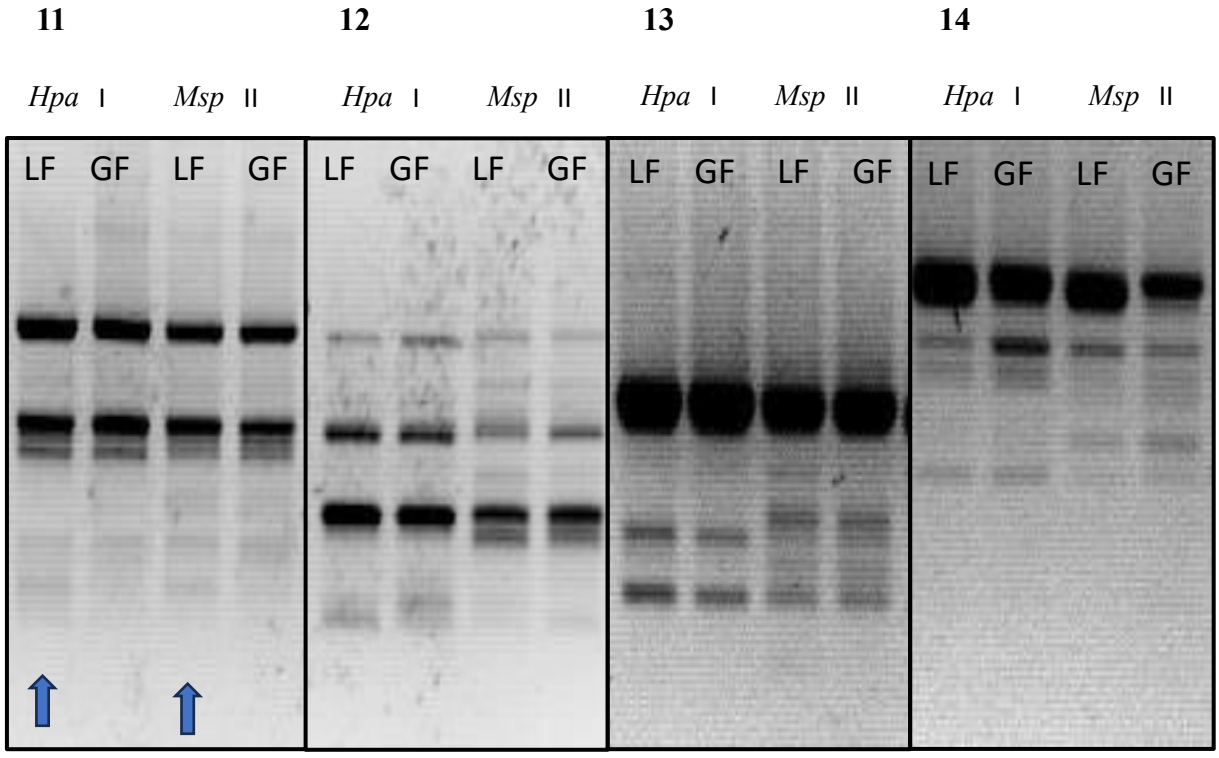
LF
GF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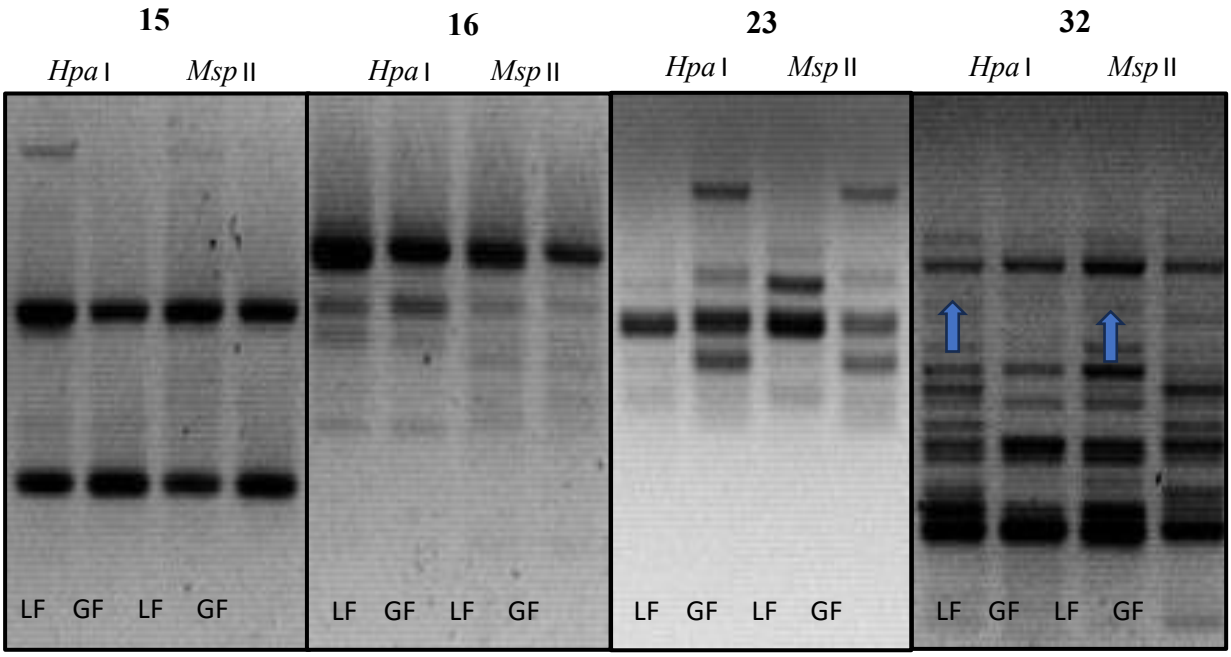
12
13
14
Hpa I
Msp II
LF GF LF GF


15
16
23
32
Hpa I
Msp II
LF GF LF GF

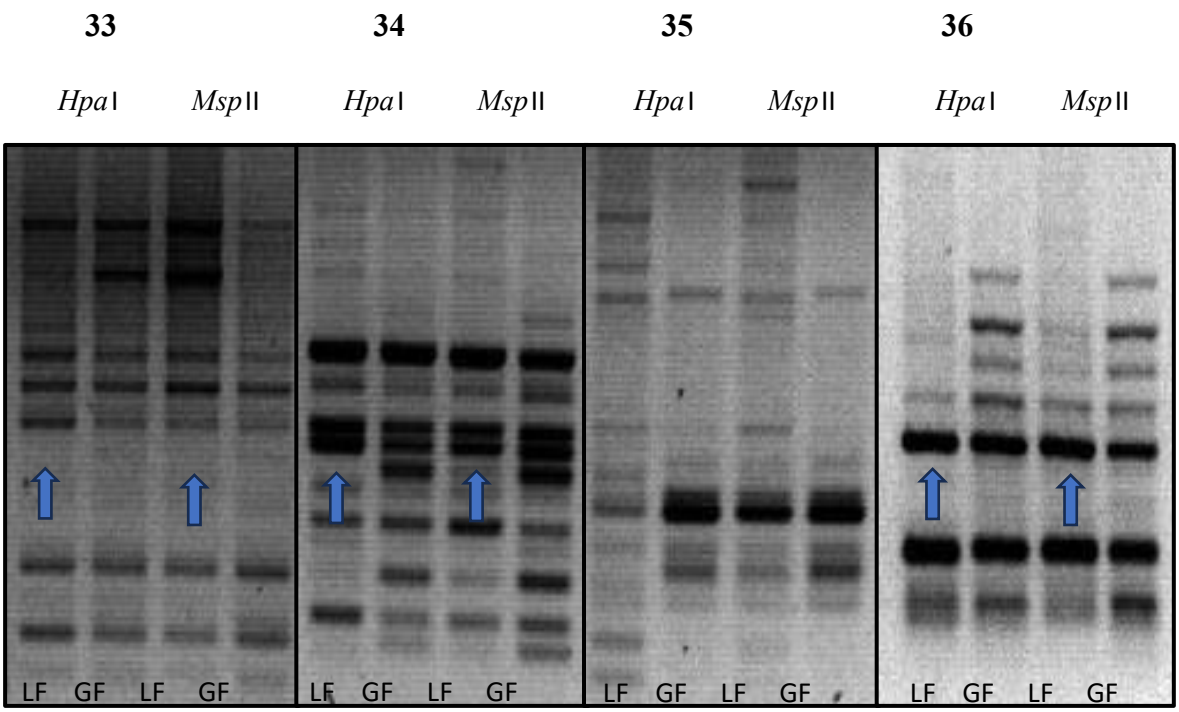


Figure 9: *The gel photos shows that the 20 primer combinations produced different patterns in the green (GF) and purple (LF) sectors, arrows indicating that the region was not methylated in the purple sector as both HpaI and MspII were able to cleave fragments. {based on (Portis et al., 2004)}.*

Table 7: *Number of bands observed after amplification with the 20 MSAP primer combinations used.*

Primer combinations	Total number Of Bands	Purple sector (L.F)	Green sector (G.F)
1-*Msp/Hpa*TCAA-*Eco*ACG	47	6	12
2-*Msp/Hpa*TCAA-*Eco*AAG	50	4	10
3-*Msp/Hpa*TCAA-*Eco*AAC	52	8	12
5-*Msp/Hpa*TCAA-*Eco*ACC	58	4	13
6-*Msp/Hpa*TCAA-*Eco*AAT	42	4	10
7-*Msp/Hpa*TCAA-*Eco*ATC	51	6	12
8-*Msp/Hpa*TCAA-*Eco*AGC	48	5	14

10-*Msp/Hpa*TCAC-*Eco*ACG	55	8	14
11-*Msp/Hpa*TCAC-*Eco*AAG	53	6	12
12-*Msp/Hpa*TCAC-*Eco*ACA	58	6	10
13-*Msp/Hpa*TCAC-*Eco*AAC	52	4	9
14-*Msp/Hpa*TCAC-*Eco*ACC	47	6	14
15-*Msp/Hpa*TCAC-*Eco*AAT	54	7	12
16-*Msp/Hpa*TCAC-*Eco*ATC	49	8	13
23-*Msp/Hpa*GCT-*Eco*ACC	56	6	16
32-*Msp/Hpa*CAG-*Eco*ACC	47	8	12
33-*Msp/Hpa*CAG-*Eco*AAT	45	8	14
34-*Msp/Hpa*CAG-*Eco*ATC	51	5	9
35-*Msp/Hpa*CAG-*Eco*AGC	56	8	10
36-*Msp/Hpa*CAG-*Eco*AGG	49	6	12
Total	1020	193	240

Despite the fact that the polyacrylamide gel allows a higher resolution due to its 6% concentration, resulting in a more accurate evaluation, the evaluation of our samples was performed on the basis of the 2% agarose gel photographs. The primer combinations that resulted in clearly detectable fragments were evaluated; these were mainly primers containing 4 selective nucleotides. In many cases, the use of primers with 3-3 selective nucleotides resulted in so-called 'smears' or too many fragments on the gel for reliable evaluation.

To investigate the overall methylation pattern of the samples, DNA was isolated from different, well distinguishable coloured sectors of a single crop (genotype 49). Of the 36 primer combinations, 20 yielded an evaluable pattern, and their amplified fragments resulted in 187 distinct patterns. Of these 187, 121 were patterns that were either monomorphic or not indicative of methylation differences between different colored sectors. The different pattern differences between green and purple sectors are summarised in the following table (Table 7).

Table 8: *Changes in patterns of cytosine methylation between the purple and the green sectors revealed by MSAP.*

Type	Purple		Difference	Green		Difference between the methylation pattern between the purple and green sectors
	H	M		H	M	
I	+	-	I1	+	+	6
			I2	-	-	1
			I3	-	+	3
II	-	+	II1	+	+	7
			II2	-	-	8
			II3	+	-	2
III	+	+	III1	-	-	8
			III2	+	-	11
			III3	-	+	2
IV	-	-	IV1	+	+	7
			IV2	+	-	4
			IV3	-	+	7

The 'H' and 'M' columns in the table indicate digestion by restriction endonucleases *HpaI* and *MspII*, and the '+' and '-' symbols indicate the presence or absence of fragments. The 4 methylation patterns occurring in purple fruits were designated by Roman numerals (I, II, III, IV), while deviations from these were further grouped into categories 1, 2, 3. In the last column, we indicated the number of pattern deviations detected within each category. The highest number of variations was detected within category III. In this category, the patterns obtained with the 20 primer combinations differed in 11 cases in the green and purple sectors, in that both *HpaI* and *MspII* could cleave in the purple sector, indicating that the section was not methylated, whereas in the green sector only *HpaI* digested the sample, indicating that the section was methylated (Table 8).

5. CONCLUSIONS AND SUGGESTIONS

Our specific focus was to compare the DNA methylation patterns between the green and purple sectors within the same fruit. By unravelling these sector-specific epigenetic signatures, we aspire to shed light on the underlying mechanisms governing color variation.

The DNA was extracted from the given pepper cultivars and PCR was performed for the cultivars using 20 MSAP primer combinations. The evaluation of primer combinations is critical for successful DNA methylation studies. In our investigation, we observed that primers containing 4 selective nucleotides consistently produced clear and detectable fragments. However, the use of primers with only 3-3 selective nucleotides often resulted in undesirable "smears" or an excessive number of fragments on the gel. These smears can hinder reliable evaluation and interpretation of methylation patterns. Therefore, researchers should prioritize primer designs that incorporate 4 selective nucleotides to enhance specificity and sensitivity in detecting methylation differences.

To delve deeper into the overall methylation landscape, we isolated DNA from distinct colored sectors within a single crop genotype (genotype 49). Among the 36 primer combinations tested, 20 yielded patterns that were evaluable. These amplified fragments generated a remarkable 187 distinct patterns. However, upon closer examination, we found that 121 of these patterns were either monomorphic (unchanging) or did not reveal significant methylation differences between the various colored sectors.

The most intriguing findings emerged from the remaining 66 patterns, where differential methylation was evident between green and purple sectors. Specifically, in 11 cases, the patterns obtained with the 20 primer combinations exhibited distinct behavior in the green versus purple sectors. Notably:

- **Purple Sector**: Both *Hpa*I and *Msp*II could cleave the DNA, indicating an absence of methylation.
- **Green Sector**: Only *Hpa*I digestion occurred, suggesting methylation at specific sites.

These differential responses highlight the dynamic interplay between DNA methylation and color expression. The green-to-purple transition appears to involve intricate epigenetic modifications, with *Hpa*I and *Msp*II acting as sensitive indicators. Further exploration of these patterns

may unravel key regulatory elements governing fruit coloration. Our study underscores the importance of fine-tuning primer selection and provides a foundation for future investigations into the fascinating world of plant epigenetics.

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DECLARATIONS

MATE Organizational and Operational Regulations

III. Requirements for Students
III.1. Study and Examination Regulations Appendix 6.13: The MATE Uniform Thesis / thesis / final thesis / portfolio guidelines

Annex 4.2: Declaration of public access and authenticity of the thesis/thesis/dissertation/portfolio

DECLARATION

the public access and authenticity of the thesis/dissertation/portfolio[1]

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Year of publication: __2023_____

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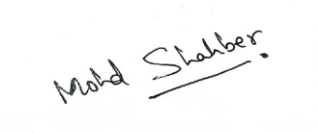

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