Doctoral (PhD) thesis

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Comparative analytical and genetic study of anthocyanin accumulating pepper genotypes during ripening

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1. Background and objectives

Peppers (*Capsicum annuum* L.), members of the *Solanaceae* family, are among the most extensively cultivated vegetables. Peppers grown for fresh consumption hold significant importance not only within Hungary but also in neighbouring nations. Their market value is influenced by several factors, including shape, flavour, and pungency. Furthermore, colour constitutes a vital aspect of their value. As pepper fruits mature, their colours can vary due to the pigments accumulated within them. This characteristic makes the *Capsicum* family an excellent model for studying the biosynthetic pathways of secondary metabolites crucial to fruit coloration.

Capsicum species encompass numerous phytonutrient compounds with advantageous effects on human health, possessing a significant degree of antioxidant capacity. These compounds include the pigments responsible for the fruits' coloration, such as chlorophylls, carotenoids, and anthocyanins. Among these, the beneficial impacts of anthocyanins have been acknowledged and demonstrated for several decades. Their contributions encompass areas such as protection against oxidative stress, antioxidant capacity, antimicrobial activity, anti-inflammatory roles, cardiovascular protection, defence against neurodegenerative diseases, and more.

Current trends indicate an increasing consumer awareness of the functional food market. This refers to the pursuit of foods that provide health benefits in addition to savouriness. Sweet peppers excel in meeting these consumer demands, boasting one of the highest vitamin C contents and carotenoid accumulations among fruits and vegetables. Furthermore, they exhibit a high total polyphenol content compared to the potato family. When these already favourable nutritional attributes are coupled with the additive effects of anthocyanins, peppers rightfully earn the classification of a superfood.

The cultivation of peppers in our country boasts a history spanning centuries. Collections curated by breeders like Gábor Csilléry harbour a wealth of mutants showcasing varying degrees of anthocyanin build-up. Analysing the nutritional data and conducting genetic testing on these mutants can serve as a robust foundation for the advancement of pepper breeding as a means to produce functional food.

At the outset of this research, the following objectives were established:

- To explore the regulatory mechanisms governing the anthocyanin biosynthetic pathway within both generative and vegetative tissues of various pepper species, cultivars, and breeding lines, each exhibiting diverse levels of anthocyanin build-up during the ripening process.
- To conduct an in-depth analysis of the interplay between transcription factors responsible for orchestrating anthocyanin biosynthesis regulation and the structural genes inherent to the biosynthetic pathway.
- To delve into the nutritional attributes of anthocyanin-rich pepper genotypes throughout the ripening stages, with particular focus on the polyphenolic constituents present in the fruits.
- To investigate the antimicrobial properties inherent to anthocyanin-rich peppers.
- To understand the complex interaction between genotype, environment, and phenophase, collectively influencing the process of anthocyanin biosynthesis in peppers.

2. Material and method

2.1. Plant material

The experiments were conducted using five generations of anthocyanin containing mutant lines from either *C. chinense* or *C. annuum*, along with reciprocal hybrid plant material from *C. annuum* × *C. chinense* or *C. chinense* × *C. annuum* species. These plant materials were provided by PepGen Ltd. In addition to commercially available extreme purple varieties such as *C. annuum* 'Cancun', 'Royal Black', 'Azteco', and 'Black Pearl', an additional variety named 'Zulu' was also provided by PepGen Ltd. As controls *C. annuum* 'Kaldom', 'Soroksári', 'Fehérözön', and *C. chinense* 'Bhut Jolokia' were applied. The mapping population used in my research originated from a cross between *C. annuum* 'Kaldom' and *C. annuum* 'Black Pearl'.

2.2. Genotyping of the anthocyanin containing breeding lines

To genotype the anthocyanin-breeding lines, I developed degenerate primers targeting conserved regions of MYBa, a gene associated with anthocyanin production, as well as two other R2R3-MYB transcription factors believed to regulate anthocyanin biosynthesis. Following the sequencing of these transcription factors' coding and promoter regions, I designed a tetra-primer amplification refractory mutation system (tARMS) for SNP detection. I employed gene-specific primers to identify retrotransposon insertions that amplify anthocyanin accumulation in extreme purple peppers. Additionally, genotyping was performed using techniques such as iPBS, CAPS, SSR, MSAP, and AFLP

2.3. Effect of the LINE-1 retrotransposon on the anthocyanin biosynthesis

Regarding the effect of the LINE-1 retrotransposon insertion on anthocyanin biosynthesis, I first identified varieties containing this insertion, characterized by elevated anthocyanin accumulation in both vegetative and generative organs. Utilizing 'Kaldom' as the mother plant, I performed castration and subsequently pollinated it with pollen sourced from 'Black Pearl' individuals. The resulting F_1 generations were segregated into two groups and cultivated under distinct light conditions. I investigated the impacts of light exposure and retrotransposon insertion on both vegetative and generative organs using analytical methods and gene expression analyses. Following self-pollination of the F_1 plants, I recorded phenotypic data for 196 F_2 plants across a ten-week period, assessing ten different tissues using a four-degree scale. Among the F_2 plants, 103 were selected for

genotyping. Furthermore, I conducted an examination of gene expression patterns within the generative organs of the F_2 plants.

2.4.CaMYBa virus induced gene silencing (VIGS)

For VIGS, I used pTRV-1, pTRV-2 (Tobacco Rattle Virus) vectors and pTRV-PDS construct as control. After ligation of the prepared insert and vector, I first transformed *E. coli* (DH5α) and then *A. tumefaciens* (GV3101) with the recombinant construct by heat shock method after $CaCl_2$ treatment. After transformation of *A. tumefaciens*, I infiltrated 3-3 leaves of four leaf staged young *N. benthamiana* plants. Following the appearance of 'photobleaching' caused by the PDS construct, leaves showing symptoms were crushed in a mortar and rubbed into leaves of pepper (*C. annuum* 'Black Pearl') previously sprayed with carborundum using a glass rod. Following gene silencing, gene expression assays of the plants were performed.

2.5.Variation in phytonutrients during ripening

Fruits were collected at four distinct stages of maturity: two economically mature stages at 20 and 30 days after fruit set (GS1 and GS2), two economically mature stages at 40 days after fruit set, the sooty stage (T), and the full biological maturity stage at 60 days after fruit set (B). At the time of collection, I determined the average colour, pH and soluble solids content of the fruits. I also measured the Total Monomer Anthocyanin (TMA), Total Polyphenol (TPC), Total Flavonoid (TFC), Total Carotenoid (TC) content, Ferric Reducing Ability of Plasma (FRAP) and three enzymatic antioxidants: the activity of catalase, peroxidase and superoxide dismutase. I also investigated gene expression changes in the anthocyanin biosynthesis of fruits during the same ripening stages.

2.6. Antimicrobial activity of pepper extracts

The antimicrobial activity of the pepper extracts was determined by agar diffusion method according to the slightly modified CLSI (Clinical and Laboratory Standards Institute) protocol M02. I used four Gram-positive (*Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 10876, *Enterococcus faecalis* ATCC 15433, *Staphylococcus aureus* ATCC 29213) and three Gram-negative (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 10145) bacteria. For the plant extracts, solutions of 100 mg/ml were prepared, after pipetting into the wells, the dishes were incubated at 35±1 °C for 24 h and the diameter of the zones of inhibition was measured to the nearest millimetre.

3. Results and discussion

The experiments focus on three R2R3-MYB transcription factors. Of these, only the *CaMYBa* or *CaAN2* (hereafter *Ca10g11650*) gene was shown to regulate anthocyanin biosynthesis in both vegetative and generative tissues of pepper at the beginning of my research. In addition to this gene, two other R2R3-MYB transcription factors, *Ca10g11690* and *Ca10g11710,* which are thought to regulate anthocyanin biosynthesis, are also located on chromosome 10.

Degenerate primers were designed for the conserved regions of the three transcription factors to investigate whether the different colour variations are caused by the presence or absence of these genes. I was able to detect *Ca10g11690* and *Ca10g11710* genes in addition to the *Ca10g11650* gene, independent of the colour of vegetative and generative tissues and the species or cultivar under study. The SNPs either detected or described in the literature were not completely certain to be phenotypically related. Since sequence polymorphisms did not lead to consistent results, plants were tested with SSR, iPBS, AFLP and MSAP in addition to the tARMS marker system, however, none of the marker systems alone was able to distinguish plants by fruit colour, even though one SRR was only 18925 base pairs 5' from the *A* locus, and this marker could not clearly indicate differences between samples.

To investigate the role of the *Ca10g11650* gene in anthocyanin biosynthesis, a segregating population was developed. Among the crossing partners, the paternal 'Black Pearl' variety carries a LINE- 1 retrotransposon insertion in its *Ca10g11650* gene, which amplifies anthocyanin biosynthesis through a gain-of-function mutation. The F_1 generation was divided into two groups, group Hybrid-1 was exposed to an average of 2304 W/m^2 of global radiation per day during the months from germination to sample collection, and group Hybrid-2 was exposed to 3200 W/m^2 per day during the same period. Among the groups of hybrids, higher gene expression was measured for samples kept under a grow lamp, with *Ca10g11690* and *Ca10g11710* also being expressed most strongly in group 2. The phytonutrients measured for the Hybrid-2 group were higher than those of the Hybrid-1 group both at the GS2 phase and at full biological maturity. For this group, even higher values for TFC than 'Black Pearl' were measured in both maturation phases, while for FRAP a higher, although not significantly different, value was detected in the GS2 phase.

The phenotypic distribution of F_2 individuals suggests that anthocyanin build-up in the population is influenced by one locus. The phenotypic data suggest

that enhanced anthocyanin biosynthesis - due to LINE-1 retrotransposon insertion - was more pronounced in generative organs. Comparing phenotypic and genotypic data, 10 plants were scored in which peppers produced conflicting results. Gene expression patterns of the generative organs through enhanced anthocyanin biosynthesis of F_2 individuals grouped by genotype and phenotype were investigated. For anthers, filament and style, *Ca10g11650* expression was detected in both paternal and heterozygous individuals. However, the expression of *Ca10g11690* and *Ca10g11710* genes was only detectable in the style and filament, but not in the anther.

Silencing of *Ca10g11650*, the *MYBa* gene, did not cause the complete loss of anthocyanins; however, it affected both the early and late structural genes in the biosynthetic pathway, as well as genes encoding the transcription factors bHLH and WD40. Among the early structural genes, the expression of *CHI* was significantly decreased in both flower and leaf in the gene silenced plants, *F3H* was not decreased in flower, while the expression of *CHS* in either tissue showed no significant difference compared to the control plant. Despite several reports discusses the effect of the *MYBa* gene on late structural genes, this effect was not significant in *ANS* in any tissue. In gene silenced plants, however, there was a significant decrease in the expression of *DFR* and *UFGT* in both flower and leaves. The expression of *bHLH* was decreased, but not significantly, and the expression of *WD40* was barely decreased in flowers and not significantly in leaves.

The presence of anthocyanins in *C. annuum* anthocyanin-bearing breeding lines is transient, and they degrade during maturation. Thus, TMA is detected only in the early phenophases, except in *C. chinense* 'Pim. Ney.' and '11270', where anthocyanin content was measurable in all phenophases. In two cases, the presence of anthocyanins was also detectable in white peppers, although no phenotypic changes were observed in the fruits. In the case of TPC, on average higher values were measured on dry weight basis at the economically mature stages, and a decreasing trend was observed during the ripening stage. The highest value was observed in the 'Pim. Ney.', whose values were significantly different from those of the other genotypes. At commercial maturity, I expected higher values for purple-fruited mutants due to increased anthocyanin accumulation, whereas I measured 1.5-2 times higher values for white-fruited peppers at the GS1 stage. Most genotypes showed high antioxidant capacity values at GS1, followed by a decrease at the turning stage and a slight increase at the biological ripening stage. The highest antioxidant capacity was observed in 'Pim. Ney.' genotype,

however, it was the only spicy genotype, so the capsaicin content may have contributed to the higher antioxidant capacity values. As ripening progressed, the amount of flavonoids in the fruits decreased, but there was a significant increase in carotenoid content, with an eightfold increase in the case of the cultivar 'Soroksári'. Examining the correlation between phytonutrient composition and antioxidant capacity of the fruits, FRAP and TPC values showed a strong positive correlation (r = 0.906), and TMA showed a strong positive correlation with both FRAP (r = 0.849) and TPC (r = 0.848), indicating that the presence of anthocyanins may be related to an increased antioxidant capacity during ripening. However, this only determined the trend of each phytonutrient, not its quantity, as both antioxidant capacity and TPC were higher in non-purple genotypes than in purple genotypes, thus not confirming the hypothesis that higher anthocyanin content is a detectable contributor to either TPC or FRAP. The results show that TMA, TPC and FRAP were influenced by genotype, while the most influential factor on TFC was the phenophase.

Gene expression studies were performed in the same four phenophases and used for a correlation analysis to determine the relationship between anthocyanin content and the genes tested. There is no significant correlation between the anthocyanin content and between *CHS* expression, neither between the *CHS* expression and the three R2R3-MYB transcription factors. In contrast, the expression of *CHI* is strongly correlated with anthocyanin content ($r=0.589^{**}$) and with the expression of the *Ca10g1170* MYB transcription factor ($r=0.978^{**}$), while at a lower significance level it is also correlated with the expression of the *Ca10g11650* gene ($r=0.487^{*}$). *F3H* also shows a strong correlation at a high significance level with all transcription factors tested and with anthocyanin content. The expression of *ANS* and *UFGT* showed a strong correlation with TMA, while this was not detected for *F3'5'H, DFR* and *GST*. However, a positive correlation was detected between *DFR* and two MYB transcription factors tested at a lower significance level. In contrast, *ANS* was also strongly correlated with the expression of all three transcription factors at higher significance levels. Regarding the R2R3-MYB transcription factors, the expression of all three genes tested showed a strong positive correlation with the anthocyanin content of the fruits.

The potential use of anthocyanin contianing breeding lines as natural antioxidants and possible ’super foods’ having antimicrobial effects was tested. Based on the agar diffusion experiments, the *C. chinenese* peppers had on average the highest antimicrobial activity against the bacteria tested. Overall, the extracts

were most effective against *E. faecalis*, the most resistant bacterium being *B. cereus*. In relation to the antimicrobial activity of the peppers, the phytonutrient make-up of the berries at two stages of maturity for *C. annuum* peppers and at full biological maturity for *C. chinenese* varieties, with particular attention to polyphenolic compounds was tested. Total polyphenol content and total flavonoid content had no significant effect on inhibition zones. In contrast, total monomeric anthocyanin content had a significant effect on the size of the inhibition zones. TMA had the greatest effect on *E. coli* and *E. faecalis* bacteria. The genotype effect was significant for all species tested except *P. aeruginosa* and *B. subtilis*. The combined effect of maturation phase and genotype was not significant only for *E. coli*.

4. Conclusions and Recommendations

After examining the sequence polymorphisms of the genes encoding the R2R3-MYB transcription factors *Ca10g11650, Ca10g11690* and *Ca10g11710*, it is likely that the detected polymorphisms are due to different species or variety usage, as I could not link any of the differences examined with complete certainty to the different phenotypes. Based on the literature, there is no agreement on the effect of *CaMYBa* on structural genes of the anthocyanin biosynthetic pathway, nor on how the expression of structural genes and the anthocyanin content of the fruits are related. As in the case of gene silencing, I could not detect a statistically verifiable correlation between *Ca10g11650* and *CHS* expression in the fruit, but the trend observed in the tissues examined in both experiments suggests that *Ca10g11650* does not directly regulate *CHS* but presumably affects upstream genes in the pathway. It is therefore appropriate to extend the studies to other genes of the general phenylpropanoid pathway. In contrast, *CHI* and *F3H* are closely associated with *Ca10g11650* expression, based on the pooled data. In this light, the results obtained in vegetative and generative tissues suggest that *Ca10g11650* not only regulates the late genes of the biosynthetic pathway, but also affects the early genes *CHI* and *F3H.*

Regarding the phytonutrients, there is a strong positive correlation between anthocyanin content and antioxidant capacity of the samples, thus it can be stated that anthocyanins contribute statistically verifiably to antioxidant capacity in the studied varieties and breeding lines, and therefore the consumption of anthocyanin-producing varieties should become part of a functional diet. Although stress-induced anthocyanin build-up is an undesirable trait in some varieties, the effect of anthocyanins against *E. coli* and *E. faecalis* has been demonstrated, so it is worth considering these plants as functional food. When only one factor was changed between the growing conditions of peppers deriving from the same cross having the same genetic background, not only were there differences in the expression of genes involved in anthocyanin biosynthesis, but also TMA, TPC, TFC and FRAP values were higher in F_1 individuals receiving more light, so this should be taken into account in the cultivation technology when shading.

5. New scientific results

1. It was demonstrated that the sequence polymorphisms described so far amongst purple and anthocyaninless varieties are highly species and cultivar dependent and not generalized within *Capsicum* species.
2. Among the four phytonutrients tested during the four phenophases, TMA, TPC and FRAP were mainly influenced by genotype, whereas TFC was mainly influenced by the phenophase tested.
3. Using tissue-specific expression studies, it was demonstrated that in addition to the *Ca10g11650* gene, *Ca10g11690* and *Ca10g11710* also play a role in anthocyanin biosynthesis in both vegetative and generative tissues. Combining the gene expression data measured at 4 phenophases of ripening and gene silencing of *Ca10g11650*, it was demonstrated that the *Ca10g11650* gene affects not only the late genes of the anthocyanin biosynthetic pathway, but also the early genes *CHI* and *F3H*.
4. It was shown that while *CHI* and *F3H* expression are correlated with the anthocyanin content of the tissues studied, this correlation is only trend-like for *CHS*.
5. It was demonstrated that anthocyanins have a detectable role in both the total antioxidant capacity and in the antimicrobial activity of the fruits, especially against a Gram-positive (*E. faecalis*) and a Gram-negative (*E. coli*) bacterial species.
6. It was shown that F_1 individuals from the same cross with the same genetic background differed not only in anthocyanin biosynthetic activity when exposed to different amounts of light, but also in the amount of other polyphenolic components and ultimately in the total antioxidant capacity of the fruit.
7. By studying a segregating population of *C. annuum* 'Black Pearl' crossed with 'Kaldom', I have shown that anthocyanin biosynthesis is determined by a single gene. However, by analysing the generative tissues of F_2 individuals from the same cross, I have shown that *Ca10g11650* is involved in the colouration of fruit, petal, style and filament, as well as *Ca10g11690* and *Ca10g11710*. However, in the case of the anthers, only *Ca10g11650* was found to play a role.

6. The author's publication activity

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