Characteristics and regulation of anthocyanin biosynthesis in pepper-review

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Abstract: Pepper is an important horticultural crop due to its culinary as well as ornamental applications. Some *Capsicum* varieties build up anthocyanins in their different organs. The biosynthesis of these pigments – beside genetic determinism – depends on diverse factors such as the environment, developmental stage and type of tissue. Though anthocyanin biosynthetic pathway has been first described in the 1800s and from then on it has been well established even in species belonging to *Solenaceae*, information on the pathway is scarce in case of *Capsicum* spp. This review comprises the current knowledge on the biochemistry and molecular biology of the anthocyanin biosynthetic pathway.

Keywords: anthocyanin, biosynthetic pathway, *Capsicum*

Anthocyanins

Anthocyanins comprise a major branch of the phenylpropanoid pathway, thus they are found ubiquitously in nature. These pigments are responsible for a range of colours – from red to purple according to pH - in various fruits, vegetables and flowers and to a lesser extent in mosses. They colour different organs and tissues at their different stages of development and their accumulation is environmentally dependent. Besides colouring these water soluble pigments serve a wide variety of functions, e.g.; since they contribute to other organoleptic or nutritional qualities of plants such as flavour, moreover, they exhibit pharmaceutical applications to human health, anthocyanins are termed as nutraceutical compounds. As for their medical applications, studies showed that these secondary metabolites induce apoptosis, promote DNA repair and they also play a role in the protection against oxidative stress and in the inhibition of tumor cell proliferation (Winkel-Shirley, 2001a; Wang and Stoner, 2008; Welch et al., 2008; Kocic et al., 2011; Benvenuti et al., 2016). Furthermore polyphenols also possess cardio-protective effects besides their anti-inflammatory, analgesic, bactericidal, fungicidal, spasmolytic properties and antioxidant qualities (Cisowska et al., 2011; Wallace 2011; Yousuf et al., 2016). In the case of plants, anythocyanins support them in preventing ultra violet and oxidative light stresses and in attracting insect pollinators, thus they contribute to seed dispersal as well. They could also play a role in male fertility in the case of some plant species, signalling during nodulation, in auxin transport, and in defense mechanisms against antimicrobial agents, and in feeding deterrents (Holton and Cornish, 1995; Winkel-Shirley, 2002; Falcone Ferreyra Mí et al., 2012; Kumar and Pandey, 2013).

Anthocyanins emerge from a diverse family of aromatic molecules, called flavonoids. Flavonoids comprise six major subgroups in higher plants beside anthocyanins; chalcones, flavones, flavonols, flavandiols, condensed tannins (or proanthocyanidins) and aurones (Winkel-Shirley, 2001a). Specialized forms of flavonoids are also synthesized by some plant species, e.g.: isoflavonoids are synthesized almost exclusively by some leguminous plants and polymerized forms of phlobaphenes are synthesized by some maize, gloxinia and sorghum varieties (Winkel-Shirley, 2001a; Falcone Ferreyra Mí et al., 2012). To date, more than 9,000 flavonoid compounds have been found in different plants, therefore forming one of the largest families of natural products (Wang et al., 2011).

As of *Capsicum annuum* L., its fruit colour is mainly due to the mixture of different carotenoids

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Figure 1. Mutants showing different nodal colouration

and chlorophylls, thus its fruit rarely contains anthocyanins. Although there are good examples for the co-occurence of anthocyanins and carotenoids in the fruits of *Solanaceae* e.g. the tamarillo and tomato (Sadilova et al., 2006). There are various species of *Capsicum spp*. which show anthocyanin pigmentation not only in their fruits but in the flowers and foliage as well (Aza-González et al., 2012), however flower organs are rarely coloured (Chaim et al., 2003). Although, in a mutant collection comprising of almost 400 mutations maintained by Gábor Csilléry (*Figure 1*) different vegetative and generative organs show anthocyanin colouration (*Figure 2*) (Csilléry, 2016b, a).

Ripe fruit colour varies from yellow to red and it can even be brown as a combination of red and green colour. The colour of unripe pepper fruit however can vary from ivory to nearly black including yellow, different shades of green, lilac and purple (Anderson, 2006). Foliar pigmentation together with the lengthy maturation period with its purple and black

Figure 2. Mutants showing different level of anther colouration

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colours provides ornamental interest of pepper as well as culinary applications (Lightbourn et al., 2008). Different analytical experiments showed that the main and only anthocyanidin found in the fruit, foliage and in the flower of pepper is delphinidin-3-p-coumaroyl-runtinoside-5-glucoside (Sadilova et al., 2006; Aza-González and Ochoa-Alejo, 2012).

Genetic background of anthocyanin biosynthesis

Genetic background of anthocyanin pigmentation has been studied extensively since the early work of Mendel on pea (*Pisum sativum* L.) flower colour in the 1800s. By then anthocyanin biosynthesis pathway has been well established in the case of maize (*Zea mays)*, petunia (*Petunia x hybrida)*, snapdragon (*Antirrhinum majus*) and lately Arabidopsis (*Arabidopsis thaliana)* is also used as a model plant (Holton and Cornish, 1995; Winkel-Shirley, 2001b; Shi and Xie, 2014). Pigmentation pattern is diverse despite of the biosynthetic pathway in the listed plants share many common reactions. The discrepancies can either be explained by the different regulation of structural genes, or that some structural genes are not expressed in plants. In addition, some genes encode enzymes with different substrate specificity, for e.g. a major difference is that petunia does not produce pelargonidin pigments, while snapdragon and maize are incapable of synthesizing delphinidin. This can be explained by the substrate specificity, since petunia dihydroflavonol reductase enzyme does not use dihydrokaempferol as substrate (Holton and Cornish, 1995).

Locus *A* is responsible for the anthocyanin biosynthesis in the immature fruit, flower and in the foliage in *Capsicum spp* (*Figure 4)*. This *A* locus is incompletely dominant and is responsible for the violet and black colour in the flower, foliage and immature fruit. Another gene; *MoA* (Deshpande, 1933; Daskalov and Poulos, 1994), in the presence of *A* intensifies the purple colouration of the tissues. Number of genes (*al-1, al-2, al-3, al-4*, *al-5,* and *al-6, al-7* in *Capsicum chinense* furthermore, *al-8* in *C. chacoense*) are responsible for the development of anthocyanin-less tissues (*Figure 4)* (Csillery, 1980; Csillery, 1983).

Purple colouration can also occur in the style and in the filament even in the abscence of *A*, in this case, purpe colour is determined by *Asf*, whilst *As* in the abscence of *A* or *Asf* will colour the style purple (*Figure 4)* (Odland, 1960; Lippert et al., 1966).

Anther is the only tissue where the anthocyanin pigmentation is not controlled by *A*, locus *Fc* is responsible for the purple pigmentation of anther filament (Borovsky et al., 2004). Mapping of genes related to anthocyanin biosynthesis has been reported in the family of *Solanaceae* family including pepper. In the case of pepper, genes controlling anthocyanin biosynthesis have been mapped to chromosome 10. Both *A* and *Fc* were mapped in the same position of chromosome 10, suggesting that they are allelic (Chaim et al., 2003).

Genomes of species belonging to *Solanaceae* – especially tomato (*Solanum lycopersicum*), petunia (*Petunia x hybrida*), potato (*Solanum tuberosum*), eggplant (*Solanum melongena*) and pepper (*Capsicum annuum* L.*)* - share extensive co-linearity of gene order although there are several chromosomal rearrangements in their genomes, meaning that if a gene position has been determined in one of the species, its position may coincide in the others (De Jong et al., 2004).

Such orthologous loci are the *A* on pepper chromosome 10 in a region that coincides with the chromosomal region of potato *F* and *I* – controlling flower and tuber skin colour - *fap10.1* and other major genes for anthocyanin present in eggplant and tomato *ag* (*anthocyanin gainer*), or *Aft* (*Anthocyanin fruit)* a dominant mutation introgressed from *S. chilense*.

Further mapping of genes showed that petunia *An2* is located in the same site on chromosome 10 where *A* was mapped in pepper (Borovsky et al., 2004; De Jong et al., 2004; Paran and van der Knaap, 2007; Lightbourn et al., 2008).

Regulatory and structural elements of the pathway

Regulatory as well as structural genes are necessary for the anthocyanin biosytnhesis (Aza-Gonzalez et al., 2013). Enzymes of the biosynthetic pathway have already been characterized and plenty of genes coding for these enzymes have been cloned and they showed high sequence similarity among species (Stommel et al., 2009).

Enzymes involved in pepper anthocyanin biosynthesis are; phenylalanine ammonia-lyase (*PAL*), cinnamate 4-hydroxylase (*C4H*), 4-coumarate: CoA ligase (*4CL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), flavonoid 3′,5′-hydroxylase (*F3′5′H*), dihydroflavonols 4-reductase (*DFR*), anthocyanin synthase (Quattrocchio et al., 1993), and UDP-glucose flavonoid 3-glucosyltransferase (*UFGT*). Additional enzymes which are not directly linked to anthocyanin biosythetic pathway are the anthocyanin permease (*ANP*) and gluthaione S-transferase (Thorup et al., 2000) both act in sequestration of anthocyanin in the vacuole. Additionally 3/5-O-glycosyltransferases (*3GT/5GT*), rhamnosyl transferase (*RT*) and O-methyltransferase (*OMT*) or anthocyanin methyltransferase can further modify flavonoids to produce anthocyanins (*Figure 4)* (Holton and Cornish, 1995; Borovsky et al., 2004; Stommel et al., 2009; Aza-Gonzalez et al., 2013; Aguilar-Barragán and Ochoa-Alejo, 2014).

The first enzyme of the anthocyanin biosynthetic pathway is *CHS* that forms tetrahydroxychalcone by using malonly-CoA and 4-coumaroyl CoA as substrate. Then, tetrahydroxychalcone will be isomerized by *CHI* to naringenin, which will be converted to dihydrokaempferol by *F3H. F3'5'H* will then hydroxylate the dihydrokaepmferol to form colouress dihydroflavonols. These will be converted to coloured anthocyanins by *DFR, ANS* and *UFGT.*

Enzymes of anthocyanin biosynthesis pathway can be grouped based on either being coded by early or late structural genes. Early structural genes (EBG) of the pathway are the *CHI, F3H, F3'5'H* the late genes (LBG) are the *DFR, ANS, UFGT* and *RT* (*Figure 4)* (Zhang et al., 2015). Just as in the case of the Petunia, the early genes of the pathway in pepper are expressed independently of regulatory genes whereas late genes are *A*-dependent (Quattrocchio et al., 1993). Genomic comparison of *A* revealed that there is no sequence difference in the coding region in green, - or purple fruited peppers, thus colour differences are due to the variations in the promoter regions (Borovsky et al., 2004).

In the case of pepper the regulation of the pathway is controlled by three types of transcription factor (TF) families: bHLH MYC, R2R3-MYB and WD40 repeat proteins (Stommel et al., 2009). Although in Arabidopsis, at least six transcription factors belonging to MYB, bHLH, WD40, WRKY, zinc finger, and MADS box proteins are involved in the pathway (Terrier et al., 2009).

Most plant MYB proteins are characterized by two imperfect repeats; R2 and R3 making R2R3-MYB the largest TFs gene family in plants. There are two other subfamilies of MYB TFs, the MYB 1R and MYB 3R (Li et al., 2011). The smallest subfamily is the 4R MYB group comprises of R1 R2 repeats (Dubos et al., 2010). These subfamilies differ in the number of their imperfect repeats of the conserved MYB DNA-binding motif.

The repeats encode three α-helices of 50 to 53 amino acids, and the second and the third helices form a helix-turn-helix (HTH) structure when they bind to the DNA (Hichri et al., 2011; Aguilar-Barragán and Ochoa-Alejo, 2014). R2R3-MYB TFs are involved

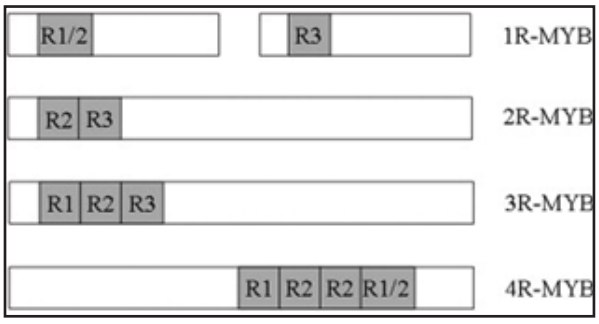

Figure 3. Imperfect repeats of MYB transcription factor

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in diverse physiological and biochemical processes including the regulation of secondary metabolism, control of cell morphogenesis, regulation of meristem formation, floral and seed development and the control of the cell cycle. Some of them are also involved in various defense and stress responses and in light and signal transduction pathways (Du et al., 2009; Berenschot and Quecini, 2014). Anthocyanin-related R2R3-MYB proteins are encoded by *colorless1 (C1)* - for seed pigmentation - and *Pl* - for the pigmentation of other plant tissues –multigene families were first described in maize (Gonzalez et al., 2008; Stommel et al., 2009). MYB proteins together with its MYC partners act both as the activators of the structural genes, and MYB alone activates the gene coding for the bHLH transcription factor (Liu et al., 2015).

Another widespread transcription factor is the basic helix-loop-helix (bHLH) protein also known as MYC. These proteins contain a conserved bHLH domain in their C-terminus region, which consists of a DNA-binding basic region followed by two helices (Zhao et al., 2013; Aguilar-Barragán and Ochoa-Alejo, 2014). Plant MYC proteins were also first described in maize where these R-like proteins interact with C1 MYB proteins and by binding to the promoters of the biosynthesis genes they activate anthocyanin synthesis in maize (Stommel et al., 2009).

WD40 or WDR (WD repeat) proteins are characterized by a peptide motif of 44–60 amino acids, typically delimited by a core region that contains the glycine-histidine (GH) dipeptide on the N-terminal side (11–24 residues from the N-terminus) and the tryptophan-aspartate dipeptide at the C-terminus (Stommel et al., 2009; Hichri et al., 2011). The WD motif is tandemly repeated 4 to 16 times thus forming the WDR protein, which then acts as a platform to facilitate diverse protein-protein interactions (Stommel et al., 2009; Aguilar-Barragán and Ochoa-Alejo, 2014).

At least one member of each of the transcription factor families is required to control tissue, - and developmental stage specific expression of anthocyanin structural genes (Li et al., 2011). Different varieties of species have been investigated and showed that the anthocyanin pathway is activated by similar MYB, MYC and WD40 proteins, suggesting that their function is conserved (Quattrocchio et al., 2006).

As discussed anthocyanin pigmentation in *Capsicum annuum* is influenced by an incompletely dominant gene, *A* which encodes Myb_a transcription factor, which is absent from genotypes that do not accumulate anthocyanins (Lightbourn et al., 2007).

If the *A* locus is present, TFs form a complex (MBW) which then interacts with the promoters of the structural genes of the anthocyanin biosynthetic pathway in order to modulate their expression. Before or after its assembly of the WD40 and MYC, the bHLH protein binds to a specific amino acid sequence: (DE) Lx2(RK)x3Lx6Lx3R on helices 1 and 2 of the R3 repeat of MYB. When it is coupled, the MYB will bind to the recognition element of the consensus sequence AACCTA of the anthocyanin biosynthesis structural gene promoter and MYC will be bound to the E-box of the promoter containing consensus sequence CAGCTG (Lightbourn et al., 2007).

As mentioned above structural genes can be grouped whether they are early, or late biosynthetic genes. Interaction of MBW complexes lead to an increase of the expression of the LBGs; *DFR, ANS, UFGT* and *RT* which are *A*-dependent. Separation pattern of the early and late genes varies according to plant species, for example: anthocyanin biosynthesis of Arabidopsis is regulated at *F3´H*, while the pathway of petunia is regulated at the *DFR* step (Quattrocchio et al., 1993; Gonzalez et al., 2008).

Different expression studies have been carried out that rectify the regulation of *A*-dependent genes. For example, a study investigated a purple fruited and a green fruited genotype. In the purple fruited anthocyanins became visible 10 days post-anthesis, reached their maximum level around day 20 and they disappeared upon ripening. Anthocyanins could not be detected

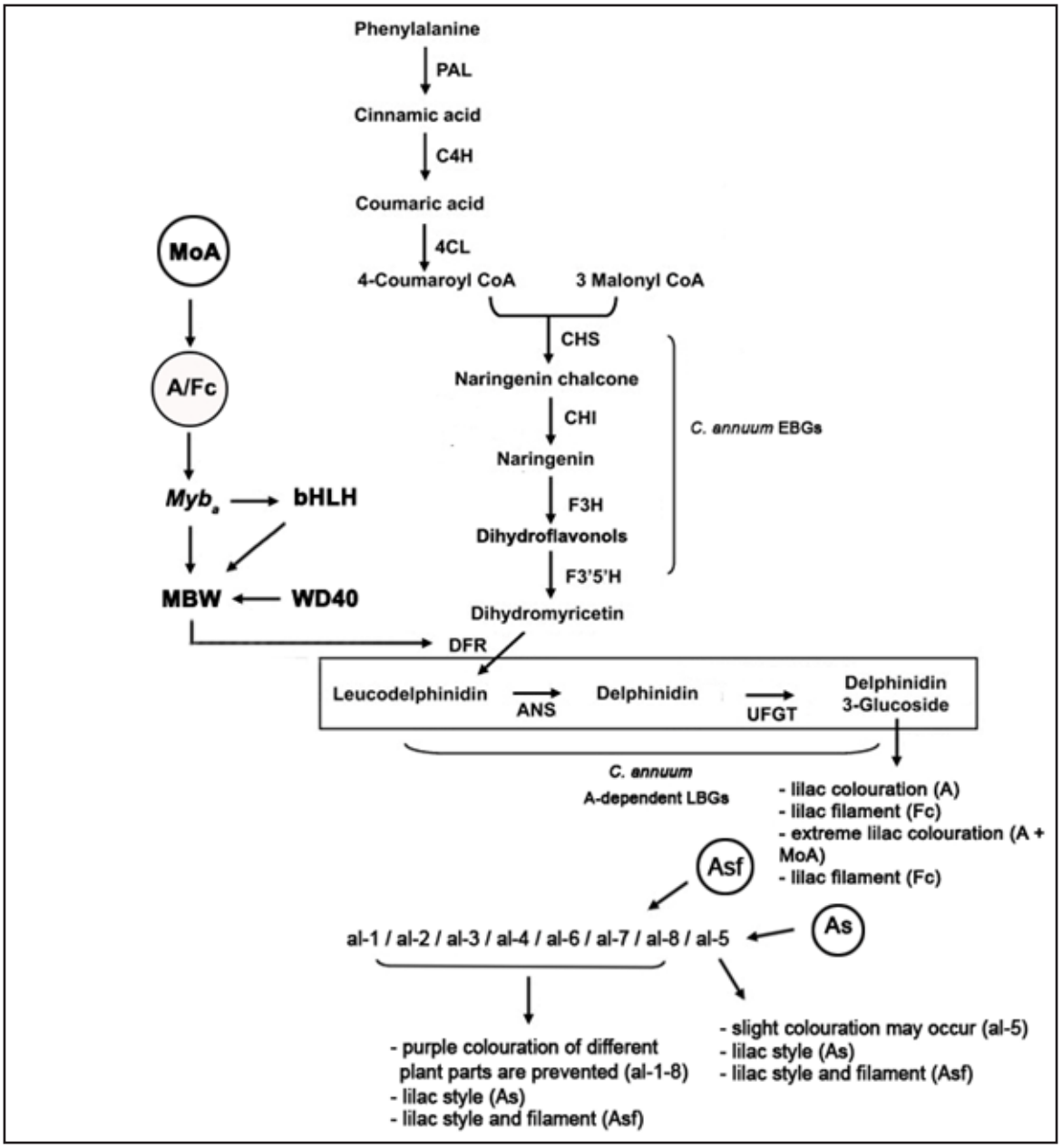

Figure 3. Simplified anthocyanin biosynthetic pathway

at any stage of fruit development in the green fruited plants. Northern analysis showed that in the purple fruited plant transcript accumulation was detected at all stages of fruit development, except for the ripe fruit and in flower petals and leaves, while no transcripts could be detected in the green fruited genotype, which can be explained by the lack of expression of *A.* PCR amplification of the *A* gene showed that the length of the PCR products from both genotypes are indistinguishable. Expression analysis of different structural genes of the pathway showed results that the early genes (*CHI, CHS)* were present in all tissues of both genotypes. On the other hand, late genes, such as *DFR* and *ANS* could only be detected in the purple fruited and not in the green fruited genotype (Borovsky et al., 2004).

Regulatory and structural genes were also examined in order to investigate how they influence tissue-specific expression. Two related genotypes of common pedigree were selected; one with purple flowers and black fruit and foliage, the other with white flowers and green fruit and foliage. Though the second genotype lacked anthocyanin in its fruit, flower or foliage,

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it contained some in its internodes, meaning that the lack of anthocyanin in its other tissues could be a result of differential tissue-specific gene expression. Expression *of CHS, DFR, ANS* from the biosynthetic genes and *Myc, Myb*$_a$ and *Wd* genes were analyzed in the flower, fruit, and foliar tissues of both anthocyanin-pigmented and non pigmented genotypes. Transcript levels of structural genes were higher in anthocyanin pigmented tissues than in the non pigmented genotype. *Wd* transcript levels did not differ significantly between the genotypes. *Myb*$_a$ and *Myc* transcript levels – consistent with the higher levels of structural gene transcripts – were higher in the floral and fruit tissues of the anthocyanin-pigmented genotype. However, in leaf tissue there was no significant difference in either of the regulatory transcript levels between the two genotypes. The *Myb*$_a$ and *Myc* transcript levels of the leaf tissue were significantly lower in the case of the anthocyanin-pigmented genotype than in the flower or in the fruit tissue. This suggests that other mechanisms contribute to the anthocyanin regulation of the foliage (Stommel et al., 2009).

Factors affecting purple colouration

Post-transcriptional gene silencing by miRNAs could explain the differential expression. These microRNAs are endogenous single-stranded approximately 20-24 nucleotide long RNAs that are associated with the RNA-induced silencing complex (RISC). They play important regulatory roles in both animals and plants by targeting mRNAs for cleavage or translational repression. They are likely to influence the output of many protein-coding genes. The majority of these genes codes for transcriptional factors. Beside regulation of gene expression, they also play role in plant development, signal transduction, protein degradation, response to environmental stress and pathogen invasion, and regulate their own biogenesis (Bartel, 2004; Zhang et al., 2006; Li et al., 2007). Different studies suggest that miRNAs could be involved in the regulation of the anthocyanin biosynthetic pathway. In Arabidopsis, increased miRNA156 activity enhanced the accumulation of anthocyanins, whereas reduced miRNA156 activity resulted in high levels of flavonols. It was also suggested that one of the miRNA156 targets SQUAMOSA PROMOTER BINDING PROTEIN LIKE 9 (SPL), negatively regulates anthocyanin accumulation by directly preventing expression of biosynthetic genes through the destabilization of a MBW transcriptional complex. Studies of Litchi (*Litchi chinensis* Sonn.) and Chinese radish (*Raphanus sativus* L.) indicated the same, i.e. several target genes for the miRNAs encode TFs involved in anthocyanin biosynthesis, including MYB, bHLH, WD40 repeat, SPL, auxin response factor, ethylene insensitive 3, WRKY and MADS-box proteins (Gou et al., 2011; Liu et al., 2016; Sun et al., 2017). A study targeting tomato miRNAs brought into light that miRNA858 regulates anthocyanin biosynthesis by modulating the expression of two R2R3-MYB transcription factors (Jia et al., 2015). Virus induced gene silencing (VIGS) vector systems have been developed from both RNA and DNA plant viral sources to specifically silence target genes in plants (Lange et al., 2013). This VIGS technique has been employed successfully in silencing of the R2R3-MYB transcription factor in *Capsicum spp.* (Kim et al., 2017). Silencing of the MYB also altered MYC and WD40 transcript levels in the *CaMYB* silenced leaves.

Expression of flavonoid pathway genes were also altered in the silenced plants (Zhang et al., 2015). In another study similar results were found when Tobacco rattle virus (TRV) constructs were used for VIGS in *Capsicum eximium.* Chili pepper fruits were transformed with TRV2-MYB and TRV2-WD40 constructs. Compared to control, these plants demonstrated reduced accumulation of anthocyanins in their fruits. This reduction both included the structural and the TF genes. Plants transformed with TVR2-MYB constructs exhibited decreased expression of *CHS, CHI, F3'5'H, DFR* and *3GT* genes, whereas there was no decrease in the level of *F3H.* Chilies infected with the TRV2-WD40 construct displayed reduction in *CHS, F3H , F3'5'H, DFR* and *3GT* but not in *CHI* in their fruit (Aguilar-Barragán and Ochoa-Alejo, 2014).

Even environmental conditions have an impact on the degree of anthocyanin pigmentation. Numerous articles describe the effect of light on anthocyanin biosynthesis (Mancinelli, 1985; Takos et al., 2006; Cominelli et al., 2008; Albert et al., 2009; Nakatsuka et al., 2009). Temperature is another well known factor which effects anthocyanin accumulation. Although a study demonstrated that anthocyanin content of leaves from *C. annuum* was not influenced by temperature whether the plants were grown under either low or high light conditions. High light positively influenced *CHS, DFR* and *ANS* expression both at low and high temperature. As of regulatory genes, they had a constant level of expression under all circumstances, except that low temperature – high light condition triggered a higher Myb_a expression (Lightbourn et al., 2007). However, both structural and regulatory gene transcript levels increased under low-temperature treatment of *Zea mays* L. seedlings (Christie et al., 1994).

Nutrient deficiency can also trigger anthocyanin colouration. The most affecting one is phosphorus (P) availability, a distinctive symptom of a plant suffering from P shortage is anthocyanin pigmentation (Jiang et al., 2007), although nitrogen deficiency can also lead to anthocyanin accumulation. Boron, magnesium, sulphur and zinc deficiencies were too reported to enhance anthocyanin accumulation (Chalker-Scott, 2002). Nutrient shortage generates similar responses to oxidative stress response as a result of high light conditions, thus the photo-protective role of these molecules could also be relevant for stress caused by nutrient deficiency (Henry et al., 2012). Nitrogen starvation affects the photosynthesis of plants via the interruption of the photosynthetic membrane due to starch accumulation, resulting in an increased light sensitivity. To prevent oxidative damage, the plant will produce an elevated amount of anthocyanins and flavonols which serve as photo-protective pigments (Stewart et al., 2001). A relationship is hypothesised between nutritional deficiencies, anthocyanin build-up and water stress. Foliar pigmentation caused by P stress is speculated to play role in the osmoregulation of water stress induced by low P levels (Chalker-Scott, 2002). Different environmental conditions such as flooding or cold soil conditions can both result in a decreased P uptake (Steyn et al., 2002) due to the relative shortage of P, thus leading to anthocyanin pigmentation. Symptoms caused by this relative shortage are transient and mainly affect young seedlings. Upon warmer soil and aerial conditions the colouration will gradually disappear (http1).

Another argument supporting the research of anthocyanin content of different pepper varieties is from the breeder's point of view. Thanks to the ever detailed genetic map of *C. annuum* linkage groups of different economically important genes have been described (Prince et al., 1993; Yi et al., 2006; Lee et al., 2009; Wu et al., 2009; Cheng et al., 2016). For example: *anthocyaninless* gene could possibly be linked to the L^3 gene of TMV resistance (Csilléry and Ruskó, 1980; Zatykó and Moór, 1998), linkage of *A* to a major quantitative trait locus for fruit shape index *fs10.1* – or locus *O* that is responsible for the round shaped fruit - has been described both in pepper and in potato (Peterson, 1959; Chaim et al., 2003)). The *anthocyaninless* gene is also used as a marker in producing hybrid lines, where anthocyaninless, male-sterile plants are used as female crossing partners (Csillery et al., 1986). Investigation of further linkages between different economically important genes and the use of molecular markers could help the breeders in the selection of desirable trait combinations in the early cotyledonous stage.

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