

Molecular Biology of Chili Pepper Anthocyanin Biosynthesis

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Dedicated to Dr. Estela Sánchez de Jiménez for her invaluable contributions to plant biochemistry

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Abstract. Chili pepper (*Capsicum* spp.) is an important horticultural crop worldwide. Chili pepper fruits from different *Capsicum* species have been highly consumed in Mexico since pre-Columbian times. Some *Capsicum* species synthesize and accumulate anthocyanins in different tissues and organs. Although the anthocyanin biosynthetic pathway has been established for different plant species, very few studies on anthocyanin chemistry, biochemistry and molecular biology of these pigments produced by chili peppers have been reported. In this review we describe the information on the type of anthocyanins synthesized and accumulated in chili pepper, and also on the molecular biology of the biosynthetic pathway. Additionally, we discuss the applications of current knowledge for the genetic manipulation, through genetic engineering, of this trait, and also the future anthocyanin-related research areas in *Capsicum*.

Key words: *Capsicum*, chili pepper, anthocyanins.

Resumen. El chile (*Capsicum* spp.) es uno de los cultivos hortícolas más importantes a nivel mundial. Los frutos de diferentes especies de *Capsicum* han sido utilizados para su consumo en México desde tiempos precolombinos. Algunas especies de *Capsicum* sintetizan y acumulan antocianinas en diferentes tejidos y órganos. A pesar de que la ruta de biosíntesis de las antocianinas se ha establecido para diversas especies vegetales, se han publicado relativamente pocos estudios para el caso de *Capsicum* acerca de la química, bioquímica y biología molecular de estos pigmentos. En la presente revisión describimos la información existente sobre las antocianinas que se sintetizan y se acumulan en las plantas de chile, y también la referente a la biología molecular de la biosíntesis de esos pigmentos. Además, se discuten las aplicaciones del conocimiento actual para la manipulación por ingeniería genética de estas características, así como las futuras áreas de investigación de las antocianinas en *Capsicum*.

Palabras clave: *Capsicum*, chile, antocianinas.

Introduction

Anthocyanins are pigments widespread in nature and responsible for the red, blue and purple colors in many plant tissues including fruits, stems, leaves, flowers and roots [1]. Anthocyanins are derived from cyanidin by: a) addition or subtraction of hydroxyl groups, b) hydroxyl methylation degree, and c) carbohydrate moiety, number and position. As exceptions, few anthocyanins are glycosylated at C-3 hydroxyl (Fig. 1) [2]. Anthocyanins are accumulated at different levels in plants (Table 1) and have several biological functions, such as attracting pollinators and preventing plant photo-oxidative damage. These properties are related to their antioxidant capacity, which has been extensively studied in many plants. On the other hand, anthocyanins have important nutraceutical applications for human health. For example, several studies have shown that anthocyanins exhibit antioxidant and anti-inflammatory

activities [3], and they even enhance the inhibition of tumor cell growth [4].

Anthocyanins have been characterized in different plants, including eggplant, petunia, potato and berries, among others [5-7]. Currently, anthocyanin profile may be determined by several physicochemical methods; for instance, using HPLC and MS [1]. The anthocyanin profile is quite diverse, depending mostly on the plant species; e.g. in berries, numerous kinds of anthocyanidins are found, whereas in eggplant delphinidin is the only anthocyanin present in the fruits [6]. Similarly, the anthocyanin profile in different fruits including berries, apples, peaches and plums, and their main aglycons (antho-

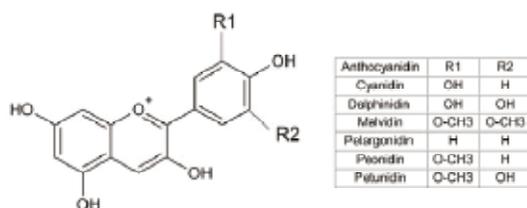


Fig. 1. Chemical structure of some anthocyanidins.

Table 1. Anthocyanin accumulation levels in fruits from different plant species.

Plant species	µg/g fresh weight	Reference
Chili pepper (<i>C. annum</i> L., Solanaceae)	320	[10]
Eggplant skin (<i>S. melongena</i> L., Solanaceae)	450	[10]
Grape peel (berry skin, <i>Vitis vinifera</i> L. cv. Merlot)	20,000	[26]
Bilberry (<i>Vaccinium myrtillus</i>)	3960	[28]
Strawberry (<i>Fragaria ananassa</i> cv. Jonsok)	238	[7]

cyanidin type), sugar moieties and acylated groups have been analyzed [1]. This investigation showed that cyanidin was the only aglycon found in all fruits, whereas delphinidin, petunidin and malvidin were exclusively observed in berries. Glucose and rutinose were the most common moiety sugars of anthocyanins from all the previously mentioned fruits. Glucose was found in all fruits, except in apple, and rutinose was merely absent in apple, grape and blackberry, whereas acylated groups were mainly found in berry fruits.

In the particular case of *Capsicum* spp., there are chili peppers from different species showing pigmentation due to the presence of anthocyanins. This pigmentation is found in flowers, fruits and foliage (Fig. 2). Purple is the most frequent color observed in chili peppers, although it is possible to find some other colors, such as black and magenta. There are few reports describing the presence of anthocyanins in chili pepper fruits [8-11]. For instance, it was reported that delphinidin was present in an Israeli-cultivar fruit of *C. annuum* [8]. Likewise, in a German chili pepper (*C. annuum* L.) it was found that the main anthocyanins were delphinidin-3-*trans*-coumaroylrutinoside-5-glucoside (nasunin 89%) and delphinidin-3-*cis* coumaroylrutinoside-5-glucoside (4.6%) [10]. The same anthocyanin profile was found in other chili pepper plant [*Capsicum annuum* L. genotypes G05C69-12 (black fruit/black foliage) and G05C74-12 (violet fruit/green foliage)], in which full-size immature fruit and fully expanded leaves were analyzed [9]. It is very important to notice that delphinidin is the only anthocyanidin found, so far, in chili peppers.

Information on the biochemistry and molecular biology of anthocyanins in *Capsicum* species is very scarce. Here we

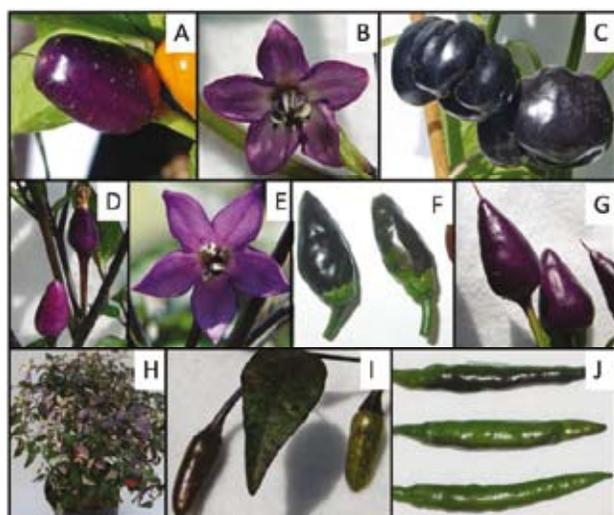


Fig. 2. Chili pepper plants accumulating anthocyanins. A-B) *Capsicum annuum* L. accession 387; C) *Capsicum annuum* L. accession 1109; D-E) *Capsicum eximium* L. accession 1546; F) *Capsicum annuum* L. 'Uvilla'; G) Bolivian rainbow pepper (Pepper Joe Seeds); H-I) Fluorescent purple pepper (Pepper Joe Seeds); *Capsicum annuum* L. árbol type. Pepper Joe Seeds (www.pepperjoe.com). Accessions depicted in this figure were supplied by IPK Gatersleben, Genebank Department, Foundation Leibniz, Institute of Plant Genetics and Crop Plant Research (IPK); 06466-Gatersleben, Germany.

present a general overview of anthocyanin biosynthesis, with a particular emphasis on the current advances in the biochemistry and molecular biology of these pigments in chili peppers.

Molecular aspects of biosynthetic pathway of anthocyanins

Anthocyanin-biosynthesis pathway genes were first described in maize (*Zea mays*), petunia (*Petunia hybrida*) and snapdragon (*Antirrhinum majus*) [12] (Fig. 3). Despite anthocyanin biosynthetic pathway is common in plants accumulating these pigments, there are diverse anthocyanin-pigmentation patterns in plants. This might be due to the fact that some structural genes are not expressed, their regulation patterns are different, and/or some others genes encode enzymes with different substrate specificity. Petunia plants do not show pelargonidin accumulation because their dihydroflavonol reductase (DFR) enzyme does not use dihydrokaempferol as substrate, since it only reduces dihydromyricetin and less efficiently dihydroquercetin, which are precursors for delphinidin and cyanidin biosynthesis, respectively [13]. Similarly, Rose flowers are not able to accumulate delphinidin, and this is attributed to their deficiency of *F3'5'h* (flavonoid-3',5'-hydroxylase) gene. Interestingly, when the *viola F3'5'h* gene was over-expressed by genetic engineering in some Rose cultivars, this resulted in delphinidin accumulation, and consequently the blue color in flowers showed up [14].

Despite that the anthocyanin biosynthetic pathway, including enzymes, regulation and genes involved is well known in the above-mentioned species, in other anthocyanin-accumulating plants like the solanaceous *Solanum tuberosum*, *Solanum melongena*, *Physalis ixocarpa* and *Capsicum* spp. the identities,

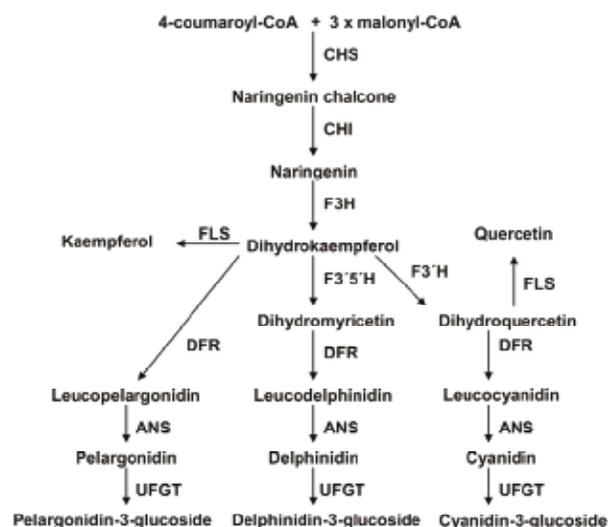


Fig. 3. Anthocyanin biosynthesis pathway in plants. CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone 3-hydroxylase; F3'H: flavonoid 3'-hydroxylase; F3'5'H: flavonoid-3',5'-hydroxylase; DFR: dihydroflavonol 4-reductase; ANS: anthocyanidin synthase; UFGT: UDP-Glc-flavonoid 3-O-glucosyl transferase; and FLS: flavonol synthase (Modified from [12]).

level and expression patterns of anthocyanin genes, as well as their tissue-specificity expression, is barely known. Regarding anthocyanin production in *Capsicum* spp., it has been proposed that locus *A* (incompletely dominant gene) is responsible for the purple color of the foliage, flower and immature fruits of chili pepper [15]. On the other hand, it has been established that the gene *c* controls the anther-filament color [16]. Recently, crosses of several chili pepper varieties bearing a different anthocyanin-accumulation pattern, have been used as an approach for mapping the genes that control anthocyanin biosynthesis [17]. Results from this study showed that the genes that control anthocyanin accumulation are located in chromosome 10. Likewise, loci *A* and *Fc* (locus for the purple anther filament in pepper) have been mapped in the same position of chromosome 10, indicating that both loci were allelic. Moreover, color mutations in tomato and potato were mapped at the corresponding locus *A* of chili pepper, suggesting that anthocyanin expression in tomato, potato and pepper is controlled by orthologous genes [18]. On the other hand, the anthocyanin-related genes *Chs* (chalcone synthase), *Chi* (chalcone isomerase), *F3h* (flavanone 3-hydroxylase), *F3'h* (flavonoid 3'-hydroxylase), *F3'5'h*, *Dfr*, *Ans* (anthocyanidin synthase), *Ufgt* (UDP-Glc-flavonoid 3-O-glucosyl transferase), *Rt* (encoding an anthocyanidin-3-glucoside rhamnosyltransferase), *An1*, *An2*, *An11* (MYB domain transcriptional regulatory genes) and *Jafl3* (encoding a bHLH transcription factor) from *Petunia* have been localized in the tomato genetic map [19]. In such study, *An2* gene, which encodes a MYB transcription factor, was located in chromosome 10, in a similar region in which locus *A* was mapped in chili pepper. These results suggest that both the tomato region in chromosome 10 and the chili pepper locus *A* are homologous. Similarly, using conserved sequence regions from *An2* and an EST homologous from tomato, a cDNA fragment isolated from chili pepper was found to co-segregate with locus *A* [8]. When *A* sequence was compared to GenBank database, a 61% amino acid identity to AN2 (petunia) and 56% to AN1 (tomato MYB transcription factor) were observed. When transcript levels from locus *A* were evaluated by northern assays in two chili pepper fruits, e.g. pigmented-fruit var. 5226 and non-pigmented-fruit PI 159234, they were detected only in the anthocyanin-pigmented chili pepper fruits from var. 5226 at all developmental stages, except in the mature period when the fruits turned red, but not purple. The *A* transcripts were not detected in the anthocyanin non-pigmented PI 159234 fruits at any developmental stage. In addition, *Chs*, *Chi*, *Dfr* and *Ans* expression was analyzed by northern blot analysis using several molecular probes derived from solanaceous gene sequences. It was observed that *Chs* transcripts were detected in both pigmented and non-pigmented fruits, with a maximal expression level during the first developmental stages and hardly noticeable at the ripe fruit stage. Similarly, *Chi* transcripts were constitutively expressed in pigmented and non-pigmented fruits. On the contrary, *Dfr* and *Ans* transcripts were only detected in the anthocyanin-pigmented fruits, starting their accretion at the young fruit stage and reaching the utmost accumulation at the mature unripe fruit.

Later on, the expression of MYC (bHLH), MYB(*A*) and WD40 transcription factors genes was analyzed in *C. annuum* leaves from temperature and light stressed plants [20]. It was observed that under high light conditions ($435 \mu\text{mol s}^{-1} \text{m}^{-2}$) the temperature only affected *Myb(A)* gene expression, whereas under low light conditions ($215 \mu\text{mol s}^{-1} \text{m}^{-2}$) temperature had no effect on the expression of the regulatory genes suggesting that the expression of transcription factor genes is dependent on light and temperature interactions. Also, it was reported that *Chs*, *Dfr* and *Ans* expression was higher in mature leaves than in the younger ones, and this expression was positively correlated with anthocyanin accumulation [20].

In other study, the expression of the regulatory genes *Myc*, *Myb(A)* and *Wd40* was analyzed in flowers, fruits and leaves from chili pepper line 06C19-2 (non-anthocyanin pigmented; with white flowers, green fruit and green foliage) and line 06C59 (anthocyanin pigmented; with purple flowers, black fruit and black leaves) showing different anthocyanin-accumulation patterns [11]. The *Wd40* profile expression in fruits was similar in both chili pepper lines, whereas the expression of *Myc* and *Myb(A)* genes was positively correlated with fruit and flower anthocyanin accumulation. Interestingly, the positive correlation for *Myc* and *Myb(A)* expression and anthocyanin accumulation, was not observed in leaves from both chili pepper lines. *Chs*, *Dfr* and *Ans* expression was higher in anthocyanin-pigmented flowers, fruits and leaves than in non-pigmented plant counterparts.

In summary, the expression of some anthocyanin-related genes, such as *Chs*, *Chi*, *Dfr* and *Ans*, has been studied in chili pepper; nonetheless, a group of anthocyanin-linked genes, such as *F3h*, *F3'h*, *F3'5'h*, *Ufgt* have not been plenty analyzed. Moreover, these last anthocyanin genes might be necessary for delphinidin biosynthesis. Fortunately, the identity and function of each regulatory gene type, such as *Myc* (bHLH), *Myb* and *Wd40*, are known in chili pepper [21].

Regulation of the anthocyanin biosynthetic pathway

Regulation of anthocyanin biosynthesis in numerous plants involves a complex network of transcription factors that interact among themselves [21]. In other plants, it has been observed that regulation of anthocyanin biosynthesis entails some transcription factor types, such as basic helix-loop-helix (bHLH), MYB and WD40 repeats. In *Petunia* spp., AN1 (bHLH) directly activates *Dfr* counterpart, whereas *An1* gene expression depends on AN2 and AN4 (MYB domain proteins), which activate transcription in petals and anthers, respectively [22, 23]. On the other hand, *An2* and *An4* activities are regulated by AN11 (WD40) protein, which is expressed independently from *An1* and *An2* genes. Moreover, the *An2* over-expression in the *an11* mutant restores *Dfr* promoter activity [24].

Regulation of anthocyanin-structural genes by the complex network of transcription factors is different depending on the plant species [21, 22]. For example, in maize, the expression of *R* and *C1* regulatory-gene families transcriptionally activate all structural genes of the anthocyanin pathway [21]. In con-

trast, in snapdragon and *Petunia*, it seems that the anthocyanin biosynthetic pathway is controlled at two levels: mutations of *Petunia* regulatory genes indicate that the first genes, such as *Chs*, *Chi* and *F3h*, are independently expressed from regulatory genes, while the late genes, such as *Dfr* and *Ans*, are dependent on the regulatory gene expression [22].

Regulation of the anthocyanin pathway in stems, foliage, flowers and immature fruits of an anthocyanin-accumulator chili pepper (*C. annuum* inbred line 5226) has been reported [8]. In this case, it has been proposed that *Petunia* and *Capsicum* anthocyanin pathway regulation seems to be quite comparable, since petunia-anther pigmentation was not controlled by *An2*, but by *An4*, whereas in chili pepper *A* (MYB) regulated the anthocyanin-pigmentation in all plant tissues, except in anthers. Likewise, it was revealed by northern assays using probes from solanaceous gene sequences, that the first genes of the anthocyanin pathway, such as *Chs* and *Chi*, were expressed in both pigmented (5226) and non-pigmented (PI159234) fruits, whereas genes encoding for the last enzymes, such as *Dfr* and *Ans*, were only expressed in pigmented fruits, following the *A* pattern expression [8]. The precedent results, suggest again a precise regulation of chili pepper anthocyanin pathway, similarly as in *Petunia*, where the first genes of the anthocyanin pathway are constitutively expressed, and the genes for the last steps are dependent on regulatory gene expression. However, other studies have shown that *Chs*, *Dfr* and *Ans* expression was higher in mature leaves than in younger ones [20]; also, *Chs*, *Dfr* and *Ans* expression was higher in pigmented flowers, fruits and leaves, compared to non-pigmented ones [11].

As it was previously mentioned, *MYC* and *MYB* (*A*) expression in chili pepper has been positively correlated with anthocyanin accumulation in fruits and flowers, but not in leaves [11]. These results demonstrated that *Capsicum* anthocyanin biosynthesis is differentially regulated depending on the tissues. On the contrary, similarly as in *Petunia*, *Wd40* expression was not correlated with other structural or regulatory genes of the anthocyanin pathway, and even it was expressed in non-anthocyanin accumulating tissues [24].

Anthocyanin accumulation usually positively correlates with transcript accumulation of genes encoding enzymes involved in the biosynthetic pathway. This correspondence has been reported for several fruits such as bilberry, apple and grape, and flowers such as morning glory and petunia, where maximal anthocyanin accumulation showed a positive correlation with *Chs*, *F3'h*, *F3'5'h*, *Dfr*, *Ans* and *Ufgt* expression [25-28]. In chili pepper, this positive correlation was observed for *Chs*, *Dfr*, *Ans*, *MYC* and *MYB* (*A*), whereas *Chi* was expressed constitutively in anthocyanin-varieties [38], or the degree of anthocyanin accumulation levels can differ substantially [39]. It was reported that those non-anthocyanin-pigmented varieties usually have mutations in structural and/or regulatory genes [19, 30], and/or anthocyanin-biosynthesis repressor genes are also participating [40, 41]. In chili pepper plants, anthocyanin accumulation is quite diverse. It is possible to find anthocyanin pigmentation in different plant parts, such as leaves, petals, sepals, stems, anther filaments, anthers and fruits, among others

(Fig. 2). Besides being found in several plant parts, anthocyanin accumulation can be observed at different degrees of pigmentation in the same plant structure, depending on the developmental stage or chili pepper variety [8]. All these facts indicate that chili pepper anthocyanin biosynthesis and accumulation is a highly regulated and complex process. Future work will be conducted to have a more detailed view of the anthocyanin-related structural genes expression and regulation in chili pepper since until now only few genes have been investigated (*Chs*, *Chi*, *Dfr*, *Myb*, *Myc*, *Wd40*), but their regulation or their role in regulation in different organs or tissues has not been clearly established. Furthermore, no knowledge on the regulation by environmental factors has been generated. We are currently carrying out expression experiments of structural genes like *F3h*, *F3'h*, *F3'5'h*, *Ufgt*, and also genes possibly involved in the sequestration of anthocyanins into the vacuole [anthocyanin permease (*Anp*) and glutathione s-transferase (*Gst*)]. Virus induced gene silencing experiments are also underway in our laboratory to assign the regulatory function of *Myb*, *Myc* and *Wd40* on the anthocyanin-related genes in chili pepper tissues. The basic knowledge derived from all these studies might allow us to manipulate this pathway by genetic engineering in order to improve the nutraceutical value of chili pepper fruits in the future.

Applications

Up to our knowledge, the only published research where the available anthocyanin biosynthesis information was applied to obtain a biotechnological product was recently reported [14]. In this case, a *Rosa hybrid*, plant that does not produce violet-blue flowers, due to the absence of delphinidin-based anthocyanins, was genetically manipulated to down-regulate the endogenous *Rose Dfr* gene and to over-express the *Iris x hollandica Dfr* and *Viola* spp. *f3'5'h* genes in order to obtain a novel violet-colored flower due to delphinidin accumulation.

So far, the best alternative to increase anthocyanin accumulation is by over-expressing *Myb*-type regulatory genes using homologous and heterologous constructs, since they are able to activate structural genes such as *Dfr* and *Ufgt* [42, 43]. *Myb* genes are also competent to repress transcription, since when they are mutated a substantial increase in anthocyanin accumulation occurs [40]. Therefore, this approach may be used in those plants that possess the anthocyanin-structural genes, but due to loss of function mutations or another genetic phenomenon they are not able to accumulate anthocyanins.

Since genetic transformation methods in chili pepper, either by *Agrobacterium*-mediated or by another type of protocol, are not completely reliable and predictable [44, 45], *Myb* over-expression is not an easy task in *Capsicum*. As a consequence, Virus Induced Gene Silencing (VIGS) has been used in order to effectively suppress various anthocyanin biosynthesis-related genes, such as in *Petunia* [46], or capsaicinoid biosynthesis-related genes in chili pepper [47]. Therefore, VIGS can be applied successfully to chili pepper in order to elucidate gene function, by loss-of-function, or to induce a significant increase

Table 2. Environmental and chemical factors affecting anthocyanin accumulation in different plants.

Factors		Anthocyanin accumulation		Plant	Reference
		Decrease	Increase		
Temperature	High	X		Chrysanthemum	[51]
				Grape	[52]
	Low		X	Grape	[53]
High-temperature, low-light		X		<i>Arabidopsis</i>	[54]
UV light			X	Red turnip	[55]
P and N limitation			X	<i>Arabidopsis</i>	[56]
Sucrose			X	<i>Arabidopsis</i>	[57]
Methyl jasmonate			X	Apple	[58]
Abscisic acid			X	Grape	[59]
Ethylene			X	Apple	[60]

in anthocyanin accumulation by repressing negative regulators of this biosynthetic pathway.

Several reports have described the nutraceutical properties of anthocyanins, because these compounds have shown antioxidant, antimicrobial, anti-inflammatory and anti-carcinogenic properties [48]. Usually, anthocyanin-accumulating plant extracts are used to carry out those studies; however, anthocyanin-accumulating transgenic plants have not commonly been used. An exception is the report described very recently [49], in which *Delila* and *Roseal* genes from snapdragon were expressed in tomato to generate purple tomatoes, which over-accumulated anthocyanins (2.83 mg/g fresh weight) at comparable levels to those found in blackberries and blueberries. In a test, cancer-susceptible mice were fed with a purple-tomato rich diet, and they showed a mean extension of life span of 145 to 180 days.

As it is described in Table 2, anthocyanin accumulation is affected by several chemical and physical stresses; therefore, this is an open area to explore and elucidate the plausible role of anthocyanins in the tolerance to different kind of stresses. For instance, the anthocyanin content in maize showed a positive correlation when the plants were exposed to linear increases of selenium; therefore, changes in anthocyanin levels could be used as an indicator of selenium presence [50].

As it can be realized, anthocyanins are pigments widely distributed among plants, including *Capsicum*. However, there is a lack of information regarding anthocyanin biosynthesis control and regulation in *Capsicum*. Therefore, it is imperative to continue studying this interesting pigment pathway as a fundamental step to utilize, in the near future, the generated information for the genetic manipulation of this trait in *Capsicum* species.

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