Anthocyanin accumulation and expression analysis of biosynthesis-related genes during chili pepper fruit development

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Abstract

Chili pepper (Capsicum annuum L.) cv. Árbol and Uvilla fruits differing in anthocyanin contents were analyzed to characterize the accumulation patterns. The maximum accumulation of the aglycon delphinidin occurred 20 days post-anthesis (DPA) with higher content in Uvilla than in Árbol fruits. Regarding the cDNA library, 9 186 cDNA clones were selected. The clones with high homology to genes concerning anthocyanin biosynthesis, such as encoding chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3’,5’-hydroxylase (F3’5’H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), UDP Glc-flavonoid 3-O-glucosyl transferase (UFGT), and also those possibly involved in anthocyanin transport into the vacuoles, an anthocyanin permease (ANP) and a glutathione S-transferase (GST) were used for gene expression analysis. In general, the expression of all investigated genes was developmentally regulated in both Árbol and Uvilla. CHS and CHI transcripts were expressed at the maximal level at 10 DPA and then consistently declined throughout fruit development. F3’5’H, DFR, UFGT and GST expression exhibited a positive correlation with anthocyanin accumulation and the highest transcript levels were detected prior to or by the time of maximum anthocyanin accumulation depending on the chili pepper type. Pericarp fruit tissues from cv. Tampiqueño 74, an anthocyanin non-accumulator, also showed CHS, CHI, F3H, ANS, and ANP expression at some developmental stages.

Additional key words: anthocyanin transport, Capsicum annuum, cDNA library, pericarp.

Introduction

Anthocyanins are pigments widespread in nature that are responsible for fruit, stem, leaf, flower, tuber, and root colors (orange, red, purple, and blue) in many plant species (Holton and Cornish 1995, Wu and Prior 1998, Grotewold 2006). These compounds have several biological functions including attracting pollinators and preventing photo-oxidative damage (Zhang et al. 2012). The protection conferred against UV damage is related to their antioxidant capacity which has been extensively studied in many plants. Anthocyanins have also shown to display important nutraceutical properties that prevent heart disease and cancer, which may also be linked to their antioxidant capacity (e.g. Reddy et al. 2005, Lamy et al. 2007). Some chili pepper fruits synthesize and accumulate delphinidin, specifically delphinidin-3-trans-coumaroylrutinoside-5-glucoside (nasunin, 89 %) and delphinidin-3-cis coumaroylrutinoside-5-glucoside (4.6 %), as the main anthocyanins (Borovsky et al. 2004, Sadilova et al. 2006, Lighthourn et al. 2008).

The anthocyanin biosynthetic pathway has been thoroughly studied in maize, Arabidopsis, snapdragon, and petunia. Multiple researchers have detected no major differences in the number and type of participating anthocyanin structural genes among these species (Reif...
et al. 1985, Britsch et al. 1993, Jez et al. 2000). Instead, regulation of anthocyanin biosynthesis seems to be the foremost distinction among maize, snapdragon, and petunia (Quattrocchio et al. 1993). Both regulatory and structural genes are indispensable for anthocyanin biosynthesis. Regulatory genes are responsible for the activation and/or repression of structural genes. Several transcription factors, including basic Helix-Loop-Helix (bHLH), MYB and WD40 repeat, regulate different steps of the anthocyanin biosynthetic pathway (Quattrocchio et al. 1993, De Vetten et al. 1997, Spelt et al. 2000, Mathews et al. 2003, Preston et al. 2004). Studies on anthocyanin biosynthesis in chili pepper are very scarce. The A locus responsible for the presence of anthocyanins in the foliage, flower, and immature fruits of chili pepper has been mapped (Ben Chaim et al. 2003). Mapping of anthocyanin-related genes in the Solanaceae, including chili pepper, has also been reported (De Jong et al. 2004). The participation of a MYB transcription factor in fruit anthocyanin regulation has been investigated (Borovsky et al. 2004). The chili pepper Myb factor transcripts were only detected in anthocyanin-pigmented fruits. Partial expression analysis of some of the anthocyanin-biosynthetic genes (CHS, CHI, DFR and ANS) in green and violet chili pepper fruits was also reported in that study (Borovsky et al. 2004). According to these authors CHS and CHI gene expression was constitutive. However, more recently, expression studies in non-anthocyanin- and anthocyanin-pigmented chili pepper leaves, flowers, and fruits revealed a positive correlation between the presence of these pigments and the accumulation of transcripts of three biosynthetic genes (CHS, DFR, and ANS) and also those of Myc, Myb, transcription factors but not of Wd40 (Stommel et al. 2009). Therefore, there is still controversy on the regulation of the expression of structural genes in chili pepper and also incomplete data on the expression of additional anthocyanin-biosynthetic genes in Capsicum.

In this study, the accumulation pattern of anthocyanins in two chili pepper types was investigated. A cDNA library from pericarp tissues was constructed, and expressed sequence tags (ESTs) with high homology to anthocyanin-related genes were isolated and used for a comparative expression analysis of all anthocyanin-biosynthetic genes and two genes possibly involved in anthocyanin trafficking into the vacuole in chili pepper fruits with different anthocyanin contents.

Materials and methods

Seeds of chili pepper (Capsicum annum L.) cv. Árbol (partially anthocyanin-pigmented fruits), cv. Uvilla (totally black anthocyanin-pigmented fruits), and cv. Tampiqueño 74 (T74; anthocyanin non-pigmented fruits) were germinated in 100 cm³ plastic pots after three weeks of germination. A soil mixture constituted by three parts of Sunshine Mix 3 (SunGro Horticulture, Bellevue, WA, USA), one part of Vermiculite (SunGro Horticulture), one part of Perlite (Perlit de la Laguna, Durango, Mexico), one part of sludge, and two parts of forest soil was used for germination and development of plants. Plants were grown in a greenhouse and fertilized every two weeks with a FerVialFol (Agroquímicos Rivas, Celaya, Mexico) solution (N:P:K 30:20:10) containing macro and micronutrients.

Chili pepper fruits were harvested at 10, 20, 30, 40, 50, and 60 d post-anthesis (DPA). Pericarp was directly dissected from fruits, immediately immersed into liquid nitrogen, and kept at -80 °C. Pericarp tissue was used for both anthocyanin extraction (three samples; six fruits per sample and per plant; three different plants), and for total RNA isolation (three to four samples; four fruits per sample and per plant; three different plants). All anthocyanin analyses were carried out while protecting the samples from direct light. Anthocyanins from pigmented pericarp tissue (1 g fresh mass; samples stored at -80 °C) from Árbol, Uvilla and T74 were extracted with 10 cm³ of acidified methanol, following a previously reported protocol (Borovsky et al. 2004). Briefly, anthocyanin-methanolic extracts were centrifuged at 12 000 g for 10 min at room temperature and the supernatant was concentrated to 0.5 cm³. To release the anthocyanidins (aglycons), the anthocyanin extracts were centrifuged at 12 000 g for 10 min at room temperature, and supernatants were concentrated to 0.5 cm³, hydrolyzed by boiling in a 2 M HCl solution (1 h), and purified with an equal volume of ethyl acetate. Samples were further heated at 80 °C for 3 min, extracted with an equal volume of isooamyl alcohol, dried at 35 °C, and finally dissolved in 1 cm³ of methanol:HCl (100:0.01; v/v). Delphinidin separation was carried out in a LC-1445 HPLC (GBC, Dandenong, Victoria, Australia) apparatus equipped with a LC1150 pump, a UV-VIS detector LC1205K, and a C18 column (Vydac 201SP54, Hesperia, CA, USA). Detection of delphinidin was carried out at 530 nm and the retention time of a commercial standard was used for delphinidin quantification (Chromadex, Laguna Hills, CA, USA). For cDNA library preparation, total RNA was isolated from pericarp tissues of 20 DPA Uvilla fruits using the PureLink Micro-to-midi Total RNA kit (Invitrogen, Carlsbad, CA, USA). RNA integrity was verified using 1 % (m/v) ethidium bromide-stained agarose gel and a Bioanalyzer 2100 with RNA 6000 Nano Assay (Agilent Technologies, Stockport, UK). mRNA (1.5 μg) was isolated from 200 μg of total RNA using the Dynabeads mRNA direct kit (Dynal-Biotech, Oslo, Norway). The cDNA library was generated using the CloneMiner cDNA library construction kit according to the manufacturer’s protocol (Invitrogen). Sequencing reactions were carried out either by BigDye v 3.1
ANTHOCYANIN ACCUMULATION DURING FRUIT DEVELOPMENT

Results and discussion

To investigate the anthocyanin accumulation pattern in fruits, the content of these pigments was measured in delphinidin equivalents during fruit development (Table 1). It was observed that Uvila accumulated more delphinidin than Árbol and no anthocyanin accumulation was detected in T74 (Table 1). Uvila synthesized important amounts of anthocyanins starting at 5 DPA (data not shown), reached the highest content at 10, 20, and 30 DPA, then the content significantly declined (40 to 60 DPA). Anthocyanins were undetectable at 10 DPA in Árbol but attained the highest anthocyanin accumulation in the pigmented pericarp areas at 20 DPA. Albeit, this anthocyanin accumulation was lower compared with the maximum value observed in Uvilla fruits. Anthocyanin amount gradually decreased after 30 DPA in Árbol. Anthocyanin contents in Uvila and Árbol fruits are similar to those found by Sadilova et al. (2006) [312.5 mg kg⁻¹(f.m) for violet pepper fruits], and by Lightbourn et al. (2008) [90 and 208 mg kg⁻¹(f.m.) for violet and black chili pepper fruits, respectively]. Total anthocyanins in chili pepper fruits were lower than those in bilberry and black currant (Nyman and Kumpulainen 2001). This might be due to the fact that chili pepper fruits accumulate anthocyanins only in the outer layer of mesocarp cells, whereas above mentioned berries accumulate these pigments in the entire fruit.

Genes encoding anthocyanin biosynthetic enzymes or regulatory proteins have been isolated using cDNA libraries (Deluc et al. 2008, Griesser et al. 2008). Since Uvila fruits showed the highest anthocyanin content at 20 DPA, pericarp tissues from fruits at this stage were used to generate the cDNA library. In this study, cDNA fragments were identified by homology with the structural genes encoding CHS, CHI, F3'H, DFR, ANS, UFGT, and GST transcript levels were related to the actin transcript level and expressed as relative densitometric units (DU).
Table 1. Delphinidin content [mg kg⁻¹(f.m.)] in pericarp tissues of chili peppers fruits at different developmental stages. Means ± SD (n = 3). No delphinidin content was observed in cv. T74.

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60 DPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Árbol</td>
<td>0.0 ± 0</td>
<td>250.5 ± 20.5</td>
<td>139.5 ± 55.0</td>
<td>95.0 ± 25.6</td>
<td>20.3 ± 12.1</td>
<td>13.1 ± 7.8</td>
</tr>
<tr>
<td>Uvilla</td>
<td>309.9 ± 13.5</td>
<td>336.7 ± 50.6</td>
<td>305.3 ± 35.5</td>
<td>96.1 ± 1.5</td>
<td>56.3 ± 7.1</td>
<td>76.6 ± 8.7</td>
</tr>
</tbody>
</table>

Fig. 1. Semi-quantitative RT-PCR analysis of anthocyanin-related genes in Árbol, Uvilla and Tampiqueño 74 chili pepper fruits at different developmental stages. Actin mRNA was used as a loading control. DPA - days post-anthesis.

Table 2. Analysis of the transcription of anthocyanin-related genes using semi quantitative RT-PCR in chili pepper fruits at different developmental stages. Transcript level for each gene was related to the actin transcript level and expressed as relative densitometric units (DU). Means ± SD (n = 3 - 4). 0.0a = deviation values lower than 10⁻³.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>DPA</th>
<th>CHS</th>
<th>CHI</th>
<th>F3H</th>
<th>F3’5’H</th>
<th>DFR</th>
<th>ANS</th>
<th>UFGT</th>
<th>ANP</th>
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<td>2.02±0.12</td>
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<td>0.98±0.11</td>
<td>0.59±0.11</td>
<td>0.02±0.02</td>
<td>0.44±0.02</td>
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<td>1.31±0.09</td>
<td>1.90±0.12</td>
<td>1.82±0.09</td>
<td>0.57±0.03</td>
<td>1.70±0.07</td>
<td>1.46±0.09</td>
<td>5.42±0.13</td>
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<tr>
<td></td>
<td>30</td>
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<td>0.05±0.05</td>
<td>1.32±0.09</td>
<td>1.60±0.01</td>
<td>0.64±0.05</td>
<td>1.40±0.05</td>
<td>0.89±0.01</td>
<td>0.23±0.05</td>
<td>1.81±0.11</td>
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<td>0.01±0.0⁰</td>
<td>0.96±0.0⁰</td>
<td>0.38±0.01</td>
<td>0.02±0.0⁴</td>
<td>0.00</td>
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<td>0.71±0.07</td>
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<td>2.62±0.11</td>
<td>2.52±0.12</td>
<td>0.72±0.03</td>
<td>2.29±0.09</td>
<td>1.83±0.07</td>
<td>1.56±0.07</td>
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<td>0.98±0.09</td>
<td>0.84±0.05</td>
<td>0.11±0.0⁴</td>
<td>0.90±0.03</td>
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<td>0.01±0.0⁰</td>
<td>0.42±0.07</td>
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<td>1.21±0.04</td>
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<td>60</td>
<td>0.02±0.0²</td>
<td>0.03±0.0⁰</td>
<td>0.72±0.01</td>
<td>0.01±0.0⁴</td>
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<td>1.37±0.05</td>
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<tr>
<td>T74</td>
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<td>0.71±0.06</td>
<td>0.84±0.07</td>
<td>0.00</td>
<td>0.00</td>
<td>0.20±0.01</td>
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<td>0.19±0.05</td>
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<td>0.92±0.06</td>
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<td>0.62±0.02</td>
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to each gene of interest, those sequences exhibiting the highest homology to the respective gene from another species were selected for expression analysis. The sequence size of these selected cDNAs ranged from...
Reverse transcription (RT)-PCR assays were utilized to study the expression of anthocyanin biosynthesis-related genes. Pericarp tissues from Árbol and Uvilla chili pepper fruits at six developmental stages were used for RT-PCR analysis (Fig. 1, Table 2). Additionally, pericarp tissues from fruits of T74 (anthocyanin non-accumulating fruits) at different developmental stages were used as negative controls. In general, the expression of all studied genes was developmentally regulated in Árbol and Uvilla. Higher transcript levels of CHS, F3H, F3'5'H, DFR, UFGT, and ANP were observed in Uvilla than in Árbol fruits. Several additional differences in expression pattern were detected in the two chili pepper types. All genes, except ANS, were expressed at their highest levels in 10 DPA Uvilla fruits. CHS transcripts were detected at the highest level in 10 DPA Árbol and Uvilla fruits and subsequently decreased after 20 DPA; however, higher expression of this gene was recorded at 10 DPA in Uvilla fruits compared with Árbol fruits. CHS was also expressed in almost all the developmental stages of the T74 fruits. CHI was expressed at the highest level at 10 DPA and then declined throughout fruit development in both pigmented chili peppers until the transcripts were almost undetectable at 40 - 60 DPA (Fig. 1, Table 2). CHI transcripts were detected only at 10 DPA in T74 fruits.

F3H transcripts were present during all stages of fruit development with maximum at 20 - 30 DPA and 10 DPA in Árbol and Uvilla, respectively, and a further decay with maturation was observed in both fruit types. This gene was also expressed almost at the same extent in all the developmental stages of the T74 fruits. In the case of F3'5'H in Árbol fruits, transcripts were detected at 10 DPA, peaked at 20 DPA, then decreased slightly at 30 - 40 DPA, and were undetectable at 50 and 60 DPA. In Uvilla fruits, the highest F3'5'H transcripts were observed at 10 DPA, diminished from 20 to 40 DPA, and no expression was recorded at 50 and 60 DPA. T74 fruits did not exhibit expression of this gene at any stage. In Árbol fruits, DFR and UFGT transcripts were almost undetectable at 10 DPA, peaked at 20 DPA, then gradually decreased, and no expression at the late ripening stages was recorded. In contrast, in Uvilla fruits the highest level of DFR and UFGT was detected at 10 DPA, a gradual reduction in transcripts was observed from 20 to 40 DPA, and no expression was found at 50 or 60 DPA. Fruits of T74 did not show detectable DFR and UFGT transcripts at any developmental stage. ANS expression in Árbol fruits increased from 10 to 30 DPA and then decreased, and stayed almost constant from 40 to 60 DPA, whereas in Uvilla pepper the transcripts increased from 20 to 60 DPA. ANS was irregularly expressed in fruits of T74 at all developmental stages.

Although the ANP and GST genes are not directly related to the anthocyanin biosynthetic pathway, both have been proposed to participate in anthocyanin vacuolar sequestration (Marrs et al. 1995, Mueller et al. 2000, Mathews et al. 2003). The highest ANP expression occurred by the time of maximum anthocyanin accumulation (20 DPA in Árbol and 10 DPA in Uvilla) (Tables 1 and 2, Fig. 1). In later fruit ripening stages (50 and 60 DPA), ANP transcripts were not detected. T74 fruits did express this gene from 10 to 40 DPA but not at 50 - 60 DPA. GST was expressed in Árbol and Uvilla but not in T74.

Anthocyanin accumulation in different organs and plant tissues usually correlates with content and activity of enzymes involved in the biosynthetic pathway. This relationship has been reported in several fruits including bilberry, apple, orange, and grape, and flowers like morning glory and petunia (Jaakola et al 2002, Lo Piero et al. 2005, Castellarin et al. 2006, Morita et al. 2006, Espley et al. 2007, Ahmed et al. 2009). In the present work, a positive correlation between anthocyanin accumulation and transcription of some but not all of the analyzed genes was observed for both Árbol and Uvilla fruits (Tables 1 and 2, Fig. 1). For example, the expression of CHS and CHI was developmentally regulated in Árbol and Uvilla, and although the transcript levels displayed a positive correlation with anthocyanin accumulation, these two genes were also expressed in fruits from the anthocyanin non-pigmented T74 suggesting that they are not exclusive or specific for anthocyanin biosynthesis. Borovsky et al. (2004) previously reported that the expression of CHS (assayed by Northern blot using a petunia CHS probe) and CHI (determined by RT-PCR employing primers designed from a CHI gene of petunia and a tomato EST for a putative CHI) genes in fruits of purple and green genotypes was not correlated with the presence or accumulation of anthocyanins since the corresponding transcripts were detected in fruits at different ripening stages and also in petals and leaves of both genotypes. Furthermore, while CHS expression was developmentally regulated, that of CHI was constitutively expressed in fruits, petals, and leaves of both genotypes (Borovsky et al. 2004). On the other hand, according to Stommel et al. (2009) CHS expression was positively correlated with the presence of anthocyanins in the leaves, flowers, and fruits of the purple-black (anthocyanin producer) chili pepper line 06C59 but was not expressed in tissues of the anthocyanin non-producer line 06C19-2.

In the present work, F3H expression also seemed to be developmentally regulated but it was detected at different levels in each developmental stage of Árbol and Uvilla fruits as well as in T74 (Fig. 1, Table 2). These results for CHS, CHI, and F3H gene expression could be explained by the fact that these genes are involved in other flavonoid biosynthesis pathways like that of flavonols. F3’5’H, DFR, UFGT, and GST were also transcriptionally regulated during fruit development and showed maximum transcription when anthocyanins accumulated to either the highest concentration (Árbol) or just prior to this stage (Uvilla) but they were not detected in fruits of T74 indicating that their expression was exclusive or specific just for anthocyanin-pigmented fruits (Tables 1 and 2, Fig. 1).
expression in purple chili pepper fruits previously reported by Borovsky et al. (2004) or in black fruits (Stommel et al. 2009) also showed a positive correlation between anthocyanin accumulation and the transcript levels. No previous information on the expression of F3’5’H or UFGT has been reported for chili pepper; nevertheless, the results of the present work suggest that all these genes are indeed related to anthocyanin accumulation in both chili pepper types. In this study, it was also concluded that there was no a positive correlation between the expression of ANS and the presence of anthocyanins since the transcripts were detected in all stages of fruit development in T74 (Fig. 1, Table 2). Conversely, Borovsky et al. (2004) reported that ANS transcripts were exclusively detected when anthocyanin was accumulated. Higher expression of ANS was also detected in line 06C59 than line 06C19-2 (Stommel et al. 2009). ANP transcripts were recorded in fruits of Árbol, Uvilla and T74, suggesting that ANP function is not exclusively related to anthocyanin transport into the vacuole. Finally, GST was expressed at the early and middle (10, 20, 30, and 40 DPA) but not at the late stages (50 and 60 DPA) of fruit development in Árbol and Uvilla, and no detectable transcript levels were observed in T74 fruits indicating a positive correlation with anthocyanin accumulation. Recently, a strong correlation was recorded between GST expression and the accumulation of anthocyanin in Vitis vinifera cell cultures (Conn et al. 2008). Furthermore, Arabidopsis TT19 mutants defective in a GST have shown reduced anthocyanin transport into vacuole (Kitamura et al. 2004). GST expression has not been previously reported in chili pepper fruits. In this work, a cDNA clone with high homology to GST-AN9 from petunia (Mueller et al. 2000) and to GST TT19 from Arabidopsis (Kitamura et al. 2004) was used for expression analysis. In our study, this gene also exhibited a positive correlation with anthocyanin accumulation (Tables 1 and 2, Fig. 1) suggesting its participation in the compartmentalization of these pigments in the vacuole.

In conclusion, delphinidin was accumulated in Árbol and Uvilla chili pepper fruits to different levels but not in T74 fruits. The expression of all the studied genes was developmentally regulated in Árbol and Uvilla. F3’5’H, DFR, UFGT, and GST expression showed a positive correlation with anthocyanin accumulation whereas CHS, CHI, F3H, and ANP were expressed in anthocyanin-pigmented as well as non-pigmented chili pepper fruits.

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