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Phenylalanine biosynthesis and its relationship to accumulation of capsaicinoids during *Capsicum chinense* fruit development

L.A. CASTRO-CONCHA, F.M. BAAS-ESPINOLA, W.R. ANCONA-ESCALANTE, F.A. VÁZQUEZ-FLOTA, and M.L. MIRANDA-HAM*

Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Mérida, 97200, Yucatán, México

Abstract

Activities of phenylalanine (Phe) biosynthetic enzymes chorismate mutase (CM) and arogenate dehydratase (ADT) and of phenylalanine ammonia lyase [PAL, an enzyme that directs Phe towards capsaicinoid (CAP) synthesis] were analyzed during *Capsicum chinense* Jacq. (habanero pepper) fruit development. A maximum CM activity coincided with a maximum CAP accumulation. However, ADT exhibited two activity peaks, one during the early phase (10 - 17 days post-anthesis, DPA) and another during the late phase (35 - 37 DPA); only the latter coincided with CAP. Interestingly, PAL activity was inversely related to CAP accumulation; lower activities coincided with a maximum CAP content. These results suggest the operation of a control mechanism that coordinated Phe synthesis and its channeling towards CAP synthesis during the course of fruit development.

Additional key words: arogenate dehydratase, chorismate mutase, fruit placenta, habanero pepper, phenylalanine ammonia lyase.

Introduction

The burning sensation that is typical of hot peppers is caused by capsaicinoids (CAP), a group of arylamides, containing a vanilloid moiety and an acyl side chain, that are derived from phenylalanine (Phe) and a branched amino acid (valine or leucine), respectively (Stewart *et al*. 2005, Aza-González *et al*. 2011). Most of the genes involved in the CAP biosynthetic pathway have been identified (Vázquez-Flota *et al*. 2007). However, very little is known about primary amino acid metabolism in pepper placentas. The placenta is the tissue holding the seed inside the fruits, and in *Capsicum*, it is the only tissue displaying CAP biosynthetic capacity (Ruiz-Lau *et al*. 2010). A positive correlation was found between nitrate content and CAP accumulation in the placentas of pepper plants grown under various nitrogen doses (Monforte-González *et al*. 2010). In addition, increased ammonia assimilation precedes CAP accumulation in response to elicitation with salicylic acid and methyl jasmonate in isolated placentas (Ancona-Escalante *et al*. 2013). Furthermore, several pepper Phe-overproducing cell lines growing *in vitro* show an increased accumulation of CAP and their intermediates suggesting that a higher Phe supply might be channeled to CAP synthesis (Salgado-Garciglia and Ochoa-Alejo 1990, Ochoa-Alejo and Salgado-Garciglia 1992). Phenylalanine is formed *via* the shikimate pathway from chorismate through the sequential action of chorismate mutase (CM; EC 5.4.99.5), prephenate aminotransferase, and arogenate dehydratase (ADT; EC 4.2.1.91), which yield prephenate, arogenate, and Phe, respectively (Cho *et al*. 2007). For CAP synthesis to occur, firstly Phe must be deaminated by phenylalanine ammonia lyase (PAL; EC 4.3.1.24). In pepper, this enzyme is coded by a three-gene family (Curry *et al*. 1999), the members of which are differentially regulated (Kim and Hwang 2014, Kim *et al*. 2014).

Nonetheless, CAP accumulation in pepper pods varies according to developmental stage, apparently as result of the balance between synthesis and oxidation (Contreras-Padilla and Yahia 1998, Stewart *et al*. 2005). However, it remains unknown whether this developmentally associated pattern also involves the availability of an

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Abbreviations: ADT - arogenate dehydratase; CAP - capsaicinoids; CM - chorismate mutase; DPA - days post-anthesis; f.m. - fresh mass; PAL - phenylalanine ammonia lyase; Phe - phenylalanine.

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*Author for correspondence; fax: (+999) 9813900, e-mail: mirham@cicy.mx

internal Phe supply. To aid in elucidating this point, we examined Phe biosynthesis enzymes and PAL, which channels Phe to CAP formation. Enzyme and CAP data were jointly analyzed, and the accumulation of some CAP synthesis intermediates was examined throughout a complete developmental cycle of *C. chinense* pods.

Materials and methods

Habanero pepper (*Capsicum chinense* Jacq.) cv. Chak k'an-iik pods were collected between 10 and 40 days post-anthesis (DPA) and assigned developmental stages from 1 to 7 as shown in Fig. 1. Each phase corresponds to approximately 5 DPA. The habanero pepper plants were cultivated in a greenhouse at the Centro de Investigación Científica de Yucatán (Mérida, México; 21° 02' 38'' N, 89° 38' 22'' W, 8 m above sea level). The plants were grown in a mixture of soil, *Perlite*, *Vermiculite*, peat moss, and coconut fiber and watered to a full field capacity every other day. The plants were fertilized once a week with *MaxiGro*® and *MaxiBloom*® (*General Hydroponics*, Sevastopol, CA, USA). After collection and arrival to the laboratory, the pods were washed with a commercial soap and thoroughly rinsed under running tap water. The pods were then dissected into placentas and pericarps (colored parts), and the separated tissues were frozen in liquid nitrogen and stored at -80 °C for further analyses.

Activities of CM, ADT, and PAL were determined in protein extracts obtained by homogenizing ground frozen tissues in extraction buffers in a polytron (*Model T10*, *IKA*, USA) at the full speed. For CM and PAL, the buffer contained 20 mM Tris-HCl, pH 7.8, 10 mM 2-mercapto-ethanol, and 5 % (m/v) polyvinylpolypyrrolidone (PVPP); for ADT, 50 mM Tris-HCl pH 8.5, 5 mM 2-mercaptoethanol, 5 % PVPP, 1 μM leupeptin, and 1 μM pepstatin. The homogenate was centrifuged at 24 400 *g* and 4 °C for 15 min. Activity of CM was determined in a reaction mixture containing 0.15 mM barium chorismate, 20 mM Tris-HCl, pH 7.8, and the protein extract in a final volume of 300 mm^3. The mixture was incubated at 37 °C for 20 min and the reaction was stopped by adding 400 mm^3 of 1 M HCl and incubating for further 10 min. The mixture was then neutralized with 3.2 cm^3 of 1 M NaOH and the amount of phenylpyruvate formed was quantified at 320 nm (Cotton and Gibson 1965) using a spectrophotometer (*Genesys*^{TM}, *Thermo-Electron* Corp, USA). An ADT assay reaction mixture contained 50 mM Tris-HCl, pH 7.5, 2.5 mM arogenate, 0.25 mM tyrosine, 1 μM leupeptin, 1 μM pepstatin, and the protein extract in a final volume of 200 mm^3 (Fisher and Jensen 1987). The mixture was incubated at 32 °C for 20 min before adding an internal standard (0.833 mM glycine) and 100 mm^3 of ortho-phthalaldehyde (54 mg dissolved in 1 cm^3 of ethanol, 9 cm^3 of 0.4 M sodium borate, pH 9.4, and 200 mm^3 of 2-mercapto-ethanol). The reaction product (Phe) was derivatized at room temperature for 90 s and then injected to an HPLC system (*Agilent Technologies*, Germany) equipped with a fluorescence detector. A mobile phase was water-ethanol (2:3; v/v); a flux and run time were 1 cm^3 min^{-1} and 7 min, respectively. The derivatized products were detected at 360 nm (excitation) and 455 nm (emission). Arogenate used in this assay was enzymatically prepared from prephenate (Connelly and Siehl 1987) utilizing a partially purified prephenate aminotransferase from *C. chinense* leaves (EC 2.6.1.78; Ripper and Matringe 2002). Activity of PAL was determined in the desalted protein extracts as described by Ochoa-Alejo and Gómez-Peralta (1993). Briefly, a reaction mixture including 10 mM L-Phe, 50 mM Tris-HCl, pH 8.8, and the protein extract in a final volume of 2 cm^3 was prepared. The mixture was incubated at 37 °C for 1 h, and the reaction was stopped by addition of 500 mm^3 of 6 M HCl; the products were then extracted in 10 cm^3 of diethyl ether. After solvent evaporation, the residue was dissolved in 50 μM NaOH, and *trans*-cinnamic acid that had formed was quantified at 290 nm. The total protein in the extracts was measured according to Peterson (1977).

The total soluble amino acids were quantified as ninhydrin derivatives (Cocking and Yemm 1954). Capsaicinoids (capsaicin and dihydrocapsaicin), ferulic acid, and vanillin were determined by HPLC (Collins *et al*. 1995). Briefly, the metabolites were extracted with acetonitrile and separated using an HPLC system equipped with a diode array detector. The filtered samples were injected (20 mm^3) to a reverse phase column (*ZORBAX Octadecyl Silane* C18, particle size 5 μm, 4.6 × 150 mm). The run conditions were as follows: isocratic mobile phase 30A:70B [A - methanol-water (10:90), B - 100 % methanol]; flux 1 cm^3 min^{-1}; running time 10 min; room temperature. Capsaicin, dihydrocapsaicin, ferulic acid, and vanillin were identified and quantified using commercial standards as references.

All data were subjected to analysis of variance (*ANOVA*)

Results

Capsaicinoids (capsaicin + dihydrocapsaicin) are exclusively synthesized and accumulated in the placenta. In habanero peppers, the developmental pattern of CAP accumulation has been described for the complete fruit (Contreras-Padilla and Yahia 1998). In this study, CAP content in placental tissue was analyzed throughout the

developmental cycle, from an early stage (stage 1; approximately 20 mm long; 10 - 15 DPA) to almost fully ripe pods (stage 7; approximately 40 - 50 mm long; 36 - 40 DPA). Peppers at stage 1 were defined as those with enough placental tissue to be manually excised from the pods. Stages 2, 3, 4, 5, 6, and 7 corresponded to 16 - 20, 21 - 25, 26 - 30, 31 - 35, 36 - 40, and 41 - 45 DPA (Fig. 1). Pod expansion occurred from stage 1 to stage 4, whereas ripening (defined as turning color from green to orange) began at stage 5 (Fig. 1).

Capsaicinoids reached a high content in placental tissue from very early phases [72 μmol g^{-1}(f.m.); stage 2] reaching a maximum prior to ripening [132 μmol g^{-1}(f.m.); stage 5] and decreasing slightly thereafter (Fig. 2). These results contrast with the accumulation pattern determined in whole pods (Contreras-Padilla and Yahia 1998) where a maximum content was reached much later, at approximately

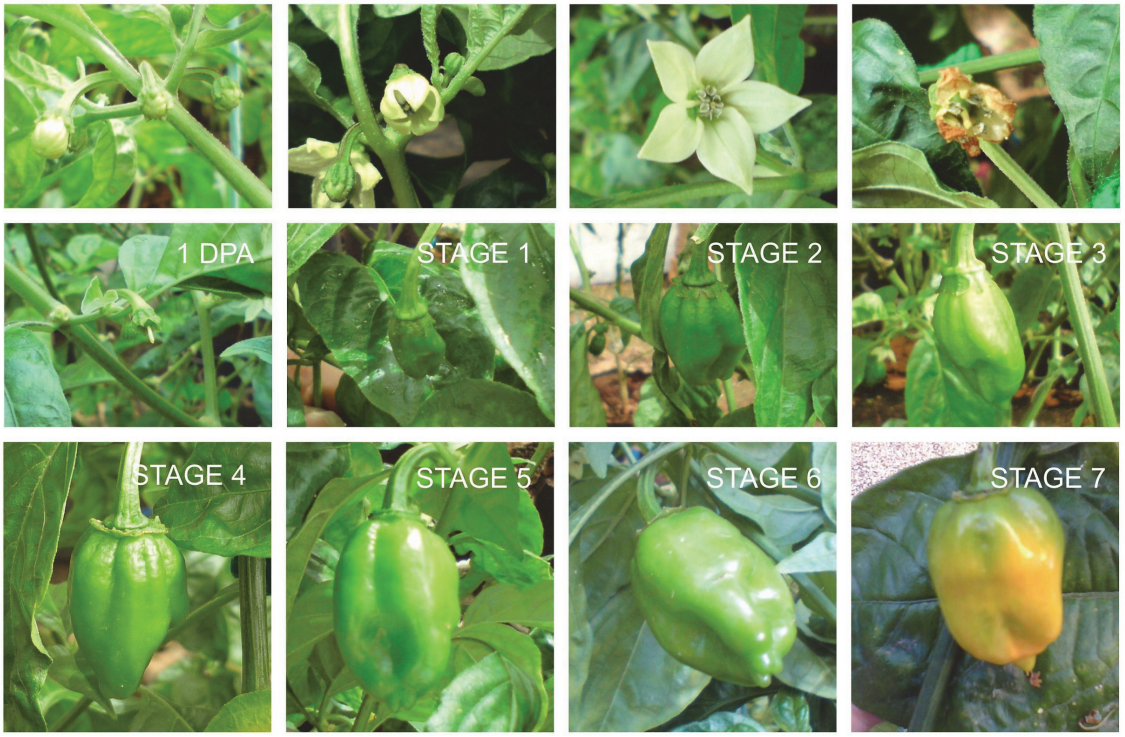

Fig. 1. The developmental cycle of *Capsicum chinense* pods.

50 DPA. This might indicate that the placentas achieved a full biosynthetic CAP capacity from an early developmental stage unlike whole pods; thus, the biosynthetic capacity can be better estimated by analyzing the isolated tissues in which CAP are synthesized and accumulated (Monforte-González *et al*. 2010).

Ferulic acid and vanillin, two CAP synthesis intermediates, were detected throughout most of the pod developmental stages; however, the content of these intermediates was much lower than that of CAP [between 0.3 and 2.3 nmol g^{-1}(f.m.) for ferulic acid and between 0.6 and 4 nmol g^{-1}(f.m.) for vanillin, Fig. 3]. No vanillylamine could be detected. Both intermediates were present during the first stages of development, when important quantities of these compounds are being channeled, not only during pod growth but also during initial synthesis and accumulation of CAP. Vanillin was detected up to stage 3, and thereafter became undetectable possibly due to its rapid conversion into vanillylamine and CAP.

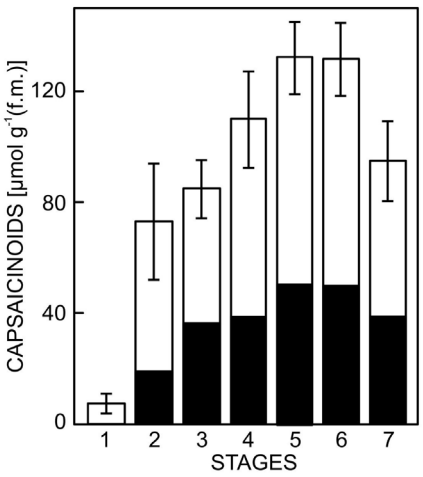

Fig. 2. The capsaicinoid [capsaicin (*white bars*) + dihydrocapsaicin (*black bars*)] content in the pod placental tissue of *Capsicum chinense* at various developmental stages. Means of three independent experiments with three replicates ± SDs.

The content of Phe (Fig. 4) remained very low at the beginning of fruit development [approximately 2.5 nmol g^{-1}(f.m.)] but increased significantly at stage 4 [approximately to 22 nmol g^{-1}(f.m.)] prior to a maximum CAP accumulation at stage 5; thereafter, the Phe content decreased steadily to 13 nmol g^{-1}(f.m.) at stage 7.

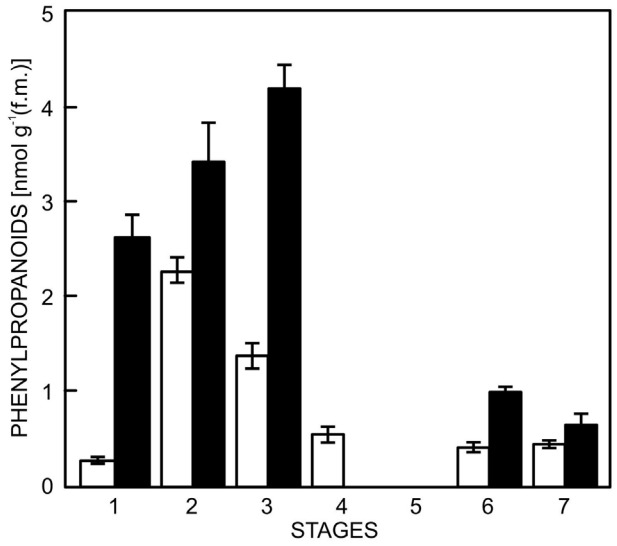

Fig. 3. The content of two intermediaries in the phenylpropanoid pathway, ferulic acid (*white bars*) and vanillin (*black bars*), in the placental tissues of *Capsicum chinense* at various developmental stages. Means of three independent experiments with three replicates ± SDs.

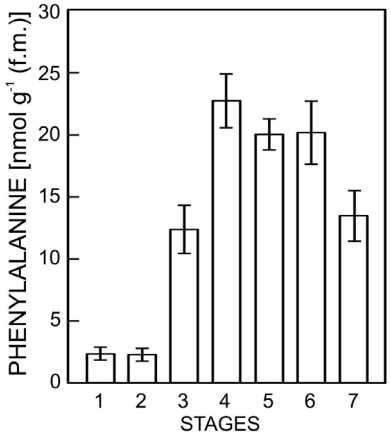

Fig. 4. The phenylalanine content in the placental tissues of *Capsicum chinense* at various developmental stages. Means of three independent experiments with three replicates ± SDs.

To determine whether the pepper placental tissue itself has a potential to supply Phe required for CAP synthesis, activities of CM and ADT, two key enzymes involved in its synthesis, were analyzed in this tissue. Activities of CM and ADT were present throughout the analyzed period (Fig. 5*A*,*B*). In this way, CM activity, which is the first committed step in synthesis of Phe and tyrosine from chorismate, was maintained between 0.09 and 0.14 nmol mg^{-1}(protein) min^{-1}, and the highest values occurred at later developmental stages (Fig. 5*A*). In contrast, ADT activity, which is involved in the last reaction of Phe synthesis, exhibited two peaks of maximum activity; one at early stages (stages 1 and 2) and second at a later stage (stage 6) (Fig. 5*B*). For both enzymes, the activity values were similar to values obtained in previous studies in other tissues and species such as *Arabidopsis thaliana*, spinach, and tomato leaves; and petunia petals (Cho *et al*. 2007, Lancien *et al*. 2007, Maeda *et al*. 2010).

Phenylalanine ammonia lyase directs Phe into a secondary metabolism, deaminating this amino acid to produce cinnamic acid, which can be converted to many phenolic compounds including CAP. In the habanero

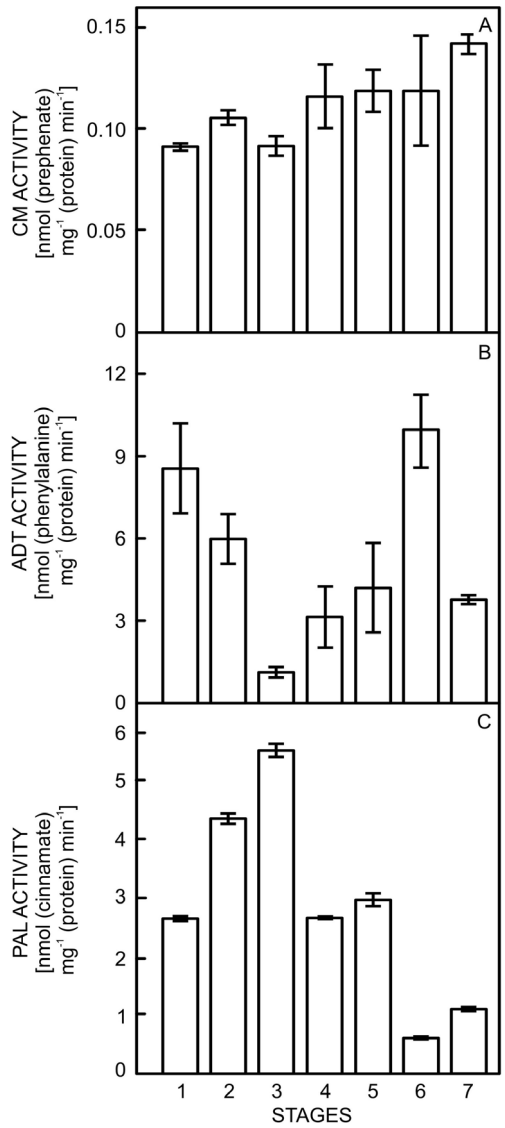

Fig. 5. The activities of chorismate mutase (CM) (*A*), arogenate dehydratase (ADT) (*B*), and phenylalanine ammonia lyase (PAL) (*C*) in the placental tissues of *Capsicum chinense* at various developmental stages. Means of three independent experiments with three replicates ± SDs.

pepper placentas, PAL activity doubled from stages 1 to 3 [up to 5.5 nmol mg^{-1}(protein) min^{-1}], prior to a maximum CAP accumulation; thereafter, PAL activity decreased to its initial value (stages 4 - 5) and then to even lower values [approximately 1 nmol mg^{-1}(protein) min^{-1}; Fig. 5*C*].

Discussion

We measured CM and ADT activities since these best reflect the biosynthetic potential of placental tissue. Both these enzymes forming Phe, as well as PAL, are under both transcriptional regulation and post-transcriptional regulation (Herrmann and Weaver 1999, Shi *et al*. 2013). Accumulation of CAP in the habanero pepper placentas was monitored throughout fruit development. This tissue is the sole site of CAP synthesis, and the metabolites are accumulated in epidermal blisters (Stewart *et al*. 2005). High amounts of CAP were detected from the early stages, and moderate changes were detected during fruit expansion and ripening (Fig. 2). Even when biosynthetic intermediates (such as ferulic acid and vanillin) were detected at very low amounts (two magnitude orders lower than CAP), some interesting features were noted throughout the developmental process (Fig. 3). Ferulic acid is the last CAP intermediate that exhibits a propanoid lateral chain attached to the phenolic ring. Therefore, in addition to its participation in CAP formation, ferulic acid and all previous phenylpropanoids can be diverted towards the synthesis of many other compounds such as flavonoids and lignins (Boerjan *et al*. 2003). Vanillin results from shortening a lateral side chain of ferulic acid and can be considered the first committed step towards CAP. Both vanillin and ferulic acid were detected in placenta during the first stages of fruit development, preceded by a peak in ADT activity and coinciding with the highest PAL activity. Moreover, both compounds accumulated early, prior to the development of the maximum CAP content (Fig. 3).

In contrast, the content of Phe (Fig. 4), from which these are derived, increased prior to CAP accumulation (stage 4), and then decreased steadily although activities of CM and ADT, two enzymes involved in its synthesis, were present throughout the entire developmental period (Fig. 5*A*,*B*). Interestingly, PAL, which uses Phe as substrate, was also active throughout the developmental cycle although a low activity of this enzyme coincided with a maximum CAP accumulation (Fig. 5*C*). Inhibition of PAL and other biosynthetic enzymes by CAP has previously been observed (Kim *et al*. 2009).

Taken together, these data suggest that in habanero peppers, the placental tissue can produce Phe throughout fruit development; however, this amino acid is readily used in synthesis of not only CAP but also other metabolites such as lignin, coumarins, benzoic acid, flavonoids, and some proteins (Núñez-Palenius and Ochoa-Alejo 2005). Moreover, a mechanism controlling flux from Phe into secondary metabolites is also implied because Phe accumulation in this tissue can shut down its synthesis or deviate chorismate to synthesis of tyrosine (Zulak *et al.* 2007). In this context, we note that proteins controlling the orchestration of fruit development and secondary metabolism routes, such as anthocyanin synthesis, have been described in *Arabidopsis thaliana* (Gou *et al*. 2011). Furthermore, the reprogramming of primary and secondary metabolism in response to internal pools of nitrogen has been observed in *Arabidopsis* (Scheible *et al*. 2004).

Recently, it has been revealed that in pepper fruits, two periods of significant transcriptomic changes occur. One was detected during early developmental phases (between 10 and 20 DPA), and the other occurred at the switch from the ripe to senescent stage (Martínez-López *et al*. 2014). Interestingly, the latter presented a higher specialized gene expression than previous phases. In this study, we analyzed the enzymatic activities involved in Phe synthesis (CM and ADT) and in routing Phe towards secondary metabolism (PAL). Following this approach, we showed the importance of coordination between pathways that supply starting compounds and those involved in formation of secondary metabolites in the placental tissues of habanero pepper.

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