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RESEARCH NOTE

Induction of capsaicinoid accumulation in placental tissues of *Capsicum chinense* Jacq. requires primary ammonia assimilation

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Abstract The activities of primary ammonia assimilation enzymes were analyzed in isolated placentas of habanero peppers (*Capsicum chinense* Jacq.). The placentas were cultured in vitro and exposed to conditions promoting capsaicinoid accumulation, such as treatments with salicylic acid (SA) and methyl jasmonate (MeJa). Although exposure to both inducers resulted in increased accumulation of capsaicinoids, the induction by SA was more pronounced. Glutamine synthetase (GS) activity, which incorporates ammonia into glutamine, increased more than six fold under such conditions, suggesting GS participation in fulfilling the demand for amino acids required to support the increase in capsaicinoid synthesis. Glutamate dehydrogenase (GDH), which has been involved in nitrogen assimilation in non-photosynthetic tissues such as placentas, was apparently not involved; its activity decreased in tissues exposed to the inducers. Thus, under the conditions tested, the activation of secondary metabolism required activation of basal nitrogen metabolism.

Keywords Solanaceae · Pepper · Elicitors · Salicylic acid · Methyl jasmonate

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Introduction

The spicy flavor of peppers is due to the presence of a group of compounds collectively known as capsaicinoids. Capsaicinoids are acid amides derived from the condensation of vanillylamine, a phenolic core derived from phenylalanine, and branched chain fatty acids of 9- to 11-carbon length, which are derived either from valine or leucine (Stewart et al. 2007). Capsaicinoids are present only in the *Capsicum* genus and confined to the epidermal cells of the placental tissue (Zamski et al. 1987). The biosynthetic capacity of pepper placentas was established in tissue culture when placental tissue from *C. frutescens* was maintained in a medium containing mineral salts, vitamins and sucrose (Lindsey and Yeoman 1984). Moreover, isolated placentas were fully capable of metabolizing labeled phenylalanine into capsaicin (Lindsey 1985). Nevertheless, the origin of the initial amino acids has not been established.

In *Capsicum*, the synthesis of capsaicinoids is affected by several environmental factors, such as water availability (Ruiz-Lau et al. 2010) and light and nutrients (Lindsey 1985). Nitrogen availability has a critical role in capsaicin accumulation (Johnson and Decoteau 1996), and this response is directly related to nitrate accumulation in the placenta (Monforte-González et al. 2010). Nitrogen availability (both its chemical form and quantity) also modifies amino acid synthesis, and these effects occur mainly through primary assimilation of nitrogen into glutamate (Hirel and Lea 2004). Glutamate is synthesized through the sequential action of glutamine synthetase (GS) and glutamate synthase (GOGAT), although glutamate dehydrogenase (GDH) may also play an important role under certain conditions (Miranda-Ham and Loyola-Vargas 1988). In this way, nitrogen's effects on capsaicin accumulation

could result from an increase in amino acid availability, which in turn could be related to an increase in nitrogen assimilation. This relationship between capsaicin content and primary nitrogen assimilation should be reflected in the activity of the enzymes involved in glutamate formation. We analyzed the activity of these enzymes in isolated placental tissue from habanero peppers (*Capsicum chinense* Jacq.), in which capsaicinoid accumulation was activated by exposure to salicylic acid and methyl jasmonate. Both of these compounds have elicited positive effects on the synthesis of secondary metabolites in several species, including *Fagopyrum esculentum* (Hu et al. 2011) and *Hypericum perforatum* L. (Gadzovska et al. 2012). The activities of the enzymes involved in primary ammonia assimilation (*i.e.,* GS, GOGAT and GDH) were then evaluated.

Materials and methods

Establishment of primary in vitro placental cultures

Unripened fruits of habanero pepper (*C. chinense* Jacq.) var. Chak k'an-iik, cultivated in a greenhouse at the Centro de Investigación Científica de Yucatán, were collected and washed with commercial soap and thoroughly rinsed in running tap water. Green pods (length 45 mm × diameter 25 mm) were collected approximately 25 days post anthesis. The fruits were surface sterilized with 80 % ethanol followed by 50 % commercial bleach (3 % sodium hypochlorite) and rinsed several times with sterile distilled water. Placental tissue was excised and, in order to prevent its disaggregation, submerged in 2.5 % (w/v) sodium alginate for 30 s, followed by a 2 h incubation in cold 1 % $CaCl_2$ with stirring. Before transfer to the culture media, the explants were rinsed with sterile distilled water to remove excess calcium. Alginate-covered placentas were maintained in 250 ml Erlenmeyer flasks containing 40 ml of MS medium supplemented with 30 g L^{-1} sucrose without growth regulators. The flasks were maintained at 25 °C, with continuous light and gentle shaking (80 RPM) for 12 h prior to the treatments. Three entire placentas (approx. 3 g FW) were kept in each flask.

Induction of capsaicinoid accumulation

Capsaicinoid accumulation was induced by exposing primary placental cultures to salicylic acid (SA) and methyl jasmonate (MeJa) (Gutiérrez-Carbajal et al. 2010). Stock solutions of SA in distilled water and MeJa in ethanol were filter sterilized before use. Dose–response curves from 5 to 500 µM were established for both inducers. Each inducer was added individually to the cultured placentas, and samples of both tissue and culture media were taken at 0, 24 and 36 h after the initiation of the experiments. After harvest, the tissues were blotted on paper to remove liquid excess, frozen in liquid nitrogen and stored at −80 °C until analysis. Culture media were lyophilized and kept at −20 °C until analysis.

Nitrogen assimilating enzyme activity

GS, NADH-GOGAT and both aminative (NADH−) and deaminative (NAD^+-dependent) GDH activities were analyzed as described in Miranda-Ham and Loyola-Vargas (1988). GDH and GOGAT activities were defined as nmoles of cofactor reduced or oxidized per min. GS activity was expressed as µmoles of γ-glutamyl hydroxamate formed per min. Protein content was determined according to Peterson (1977).

Metabolite determination in placental tissues

Ammonia and total amino acids pools were determined according to Streuli and Averell (1970) and Saneoka et al. (1995), respectively. Capsaicinoids were quantified in the placental tissue as well as in the culture medium by HPLC according to Collins et al. (1995).

Data analysis

All of the data were subjected to analysis of variance (ANOVA), and mean comparisons were made using Tukey's multiple-range test at a 5 % level of probability.

Results

Effect of salicylic acid and methyl jasmonate on capsaicinoid content

Treatments with both inducers of secondary metabolism (SA and MeJa) had positive effects on capsaicinoid accumulation. However, a more pronounced response was noted when SA was used as the inducer (Fig. 1a). Maximal response compared with controls (200 %) was observed in tissues exposed for 24 h to 500 µM SA, and no further increase was observed after a longer exposure (Fig. 1a). Although the placentas exposed to MeJa also showed an increase in capsaicinoid accumulation, the response was not as marked as with SA (Fig. 1b). A similar differential response to these inducers was observed in *C. chinense* suspension cultures (Gutiérrez-Carbajal et al. 2010; Altúzar-Molina et al. 2011): a 500 % increase in capsaicinoid content was detected after the cell suspensions were treated with 500 µM SA, and no accumulation was observed after the addition of MeJa. Comparable results were also detected in *Hiperycum hirsutum* and *H. maculatum* shoot

cultures in which both 50 and 200 μM SA concentrations favored hypericin production (Coste et al. 2011).

Capsaicinoid accumulation in the placental tissues of *C. chinense* cultured in vitro was 45 % and 1,000–5,000 % greater than observed in cell suspension cultures of *C. frutescens* and *C. chinense* respectively (Lindsey and Yeoman 1984; Gutiérrez-Carbajal et al. 2010). This result suggests that capsaicin biosynthesis requires specific tissue organization that is lost with dedifferentiation (Stewart et al. 2007). Isolated placental tissue was able to interact with the culture medium: the capsaicinoid content in the samples increased nearly 40 % over the initial values, even without any induction treatment (see controls in Fig. 1). Ammonia and soluble amino acid pools were maintained throughout the culture period, at about 40 and 25 μmoles g^{-1} FW, respectively. Thus, placental tissue remained metabolically active in the in vitro culture conditions assayed (data not shown). In contrast, placentas from detached habanero pepper pods, which were stored under the same environmental conditions as the cultured tissues, showed capsaicinoid levels approximately 30 % lower than those in the controls within the first 24 h after harvest (from 1,880 to 1,300 nmoles).

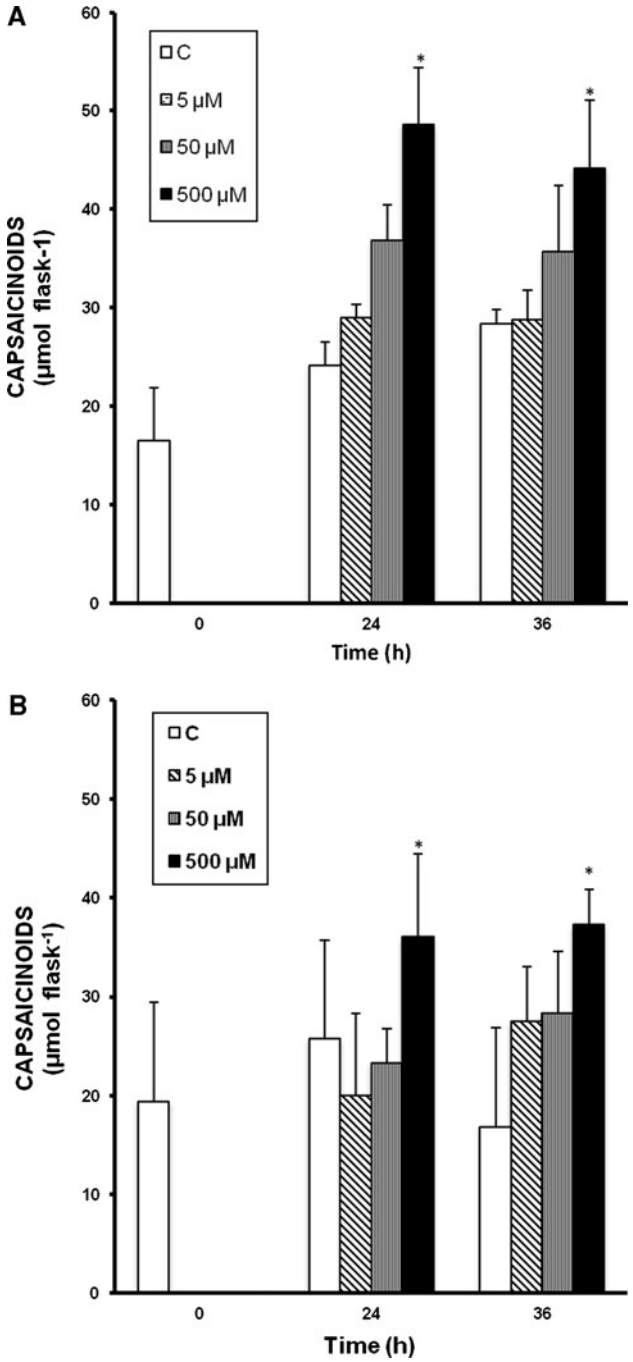

Fig. 1 Effect of **a** SA and **b** MeJa on the total amount of capsaicinoids per flask (pepper placentas + culture media). Each flask contained 3 g FW of placental tissue and 40 mL medium. Data correspond to the mean of three independent experiments with three replicates ±1 SD. *$P < 0.05$ Tukey's test was applied to compare treatments with controls

Ammonia assimilation enzyme activities in placentas treated with salicylic acid

Since capsaicinoid accumulation in habanero pepper pods has been correlated with nitrate content in placentas (Monforte-González et al. 2010), we analyzed the activities of the enzymes involved in primary nitrogen metabolism in the in vitro cultured placentas exposed to conditions that promoted maximal capsaicin accumulation: 500 μM SA or MeJa for 24 h. Under those conditions, GS activity increased 600 % in placentas exposed to 500 μM SA in comparison to unexposed controls (Fig. 2a). GOGAT activity increased as well, but to a lesser extent (Fig. 2b). Therefore, SA promoted increases both in capsaicinoid levels and in primary nitrogen assimilation. GDH has been shown to be involved in amino acid catabolism (NAD^+-dependent activity). However, under certain conditions, it can also catalyze the NADH-dependent amination of 2-oxoglutarate to form glutamate, particularly in non-photosynthetic tissues (Melo-Oliveira et al. 1996) such as placentas. Interestingly, both NADH− and NAD^+-GDH activities decreased to a third of those found in the controls (Fig. 2c, d), suggesting a minor role for these enzymes in supplying nitrogen for SA-induced capsaicinoid accumulation.

Ammonia assimilation enzyme activities in placentas treated with methyl jasmonate

In MeJa-exposed tissues (500 μM), which exhibited lower capsaicinoid induction compared to SA exposure, GS and GOGAT activities followed a trend similar to that observed in the SA treated tissues, albeit at lower levels, GS increased only 200 % (Fig. 3a, b), and the NAD^+- and

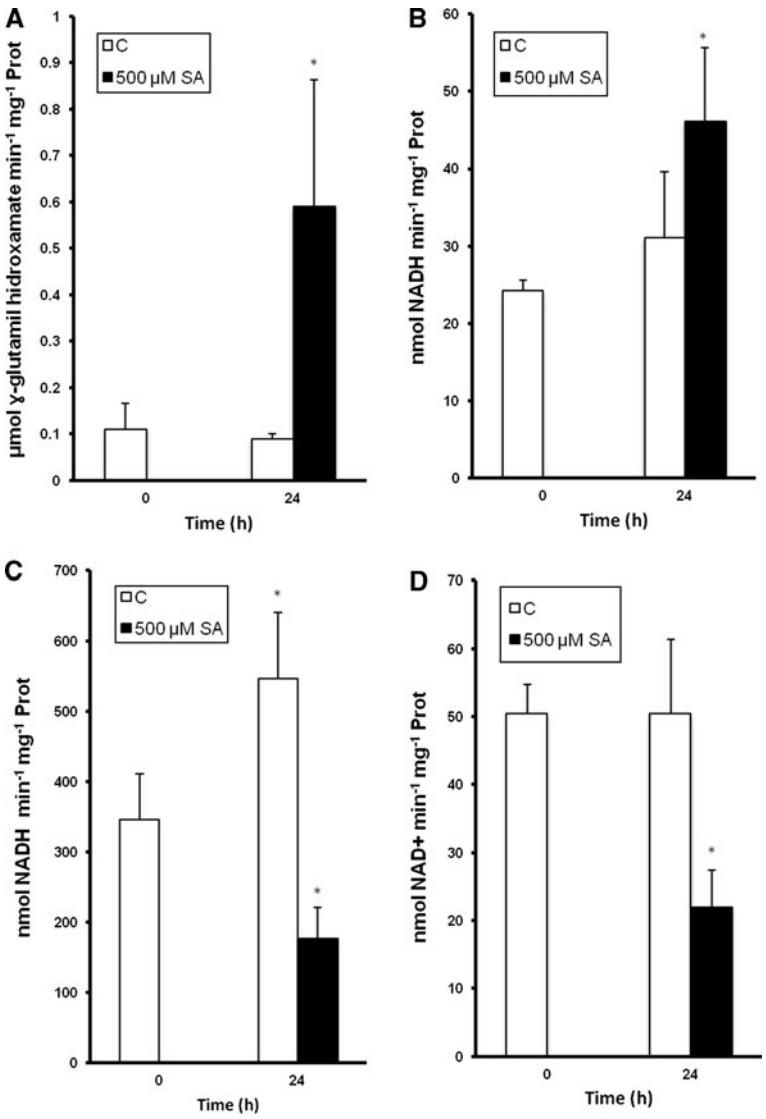

Fig. 2 Effect of 500 μM SA treatment on the activities of ammonia assimilation enzymes: **a** GS, **b** NADH-GOGAT, **c** NADH-GDH and **d** NAD^+-GDH. Data correspond to the mean of three independent experiments with three replicates ±1 SD. * $P < 0.05$ Tukey's test was applied to compare treatments with control

NADH-dependent GDH activities in the placental tissues were comparable to those in the controls (Fig. 3c, d).

Discussion

The data presented here suggest that the increase in capsaicinoid synthesis produced by inducer treatments requires an increase in primary ammonia assimilation. Available glutamine produced by GS may be required for reactions that employ this amino acid as an amine donor for the synthesis of those directly related to capsaicinoid synthesis, such as phenylalanine (Hirel and Lea 2004).

Placental tissues hold the seeds in place in fruits and are derived from the structures supporting ovules in the gynoecium. The tissue has therefore been generally regarded as structural in nature, and only a few studies on the primary metabolic activity of placental tissue have been conducted. Gene expression in placenta has been correlated with parthenogenesis and seed fertility (Testa et al. 2002). Recently, the transcript profiles of different pepper tissues (*C. annuum* cvs. Serrano and Anaheim) were made available (Góngora-Castillo et al. 2012). Placental tissue displays a less complex transcript profile than other tissues, suggesting relatively low metabolic activity. Our results, however, show that this tissue can support nitrogen assimilation into amino acids. Furthermore, enzymes involved in this process can adjust their function to accommodate different metabolic circumstances that may require a surplus of amino acids to use as building blocks, such as capsaicin production. Elicitor effects on GS activity were most striking (Figs. 2a, 3a). Glutamine synthetase is subjected to complex regulatory mechanisms, both at the transcriptional and post-transcriptional levels (Hirel and Lea 2004). In this study, we found that transcript levels corresponding to GS remained relatively constant,

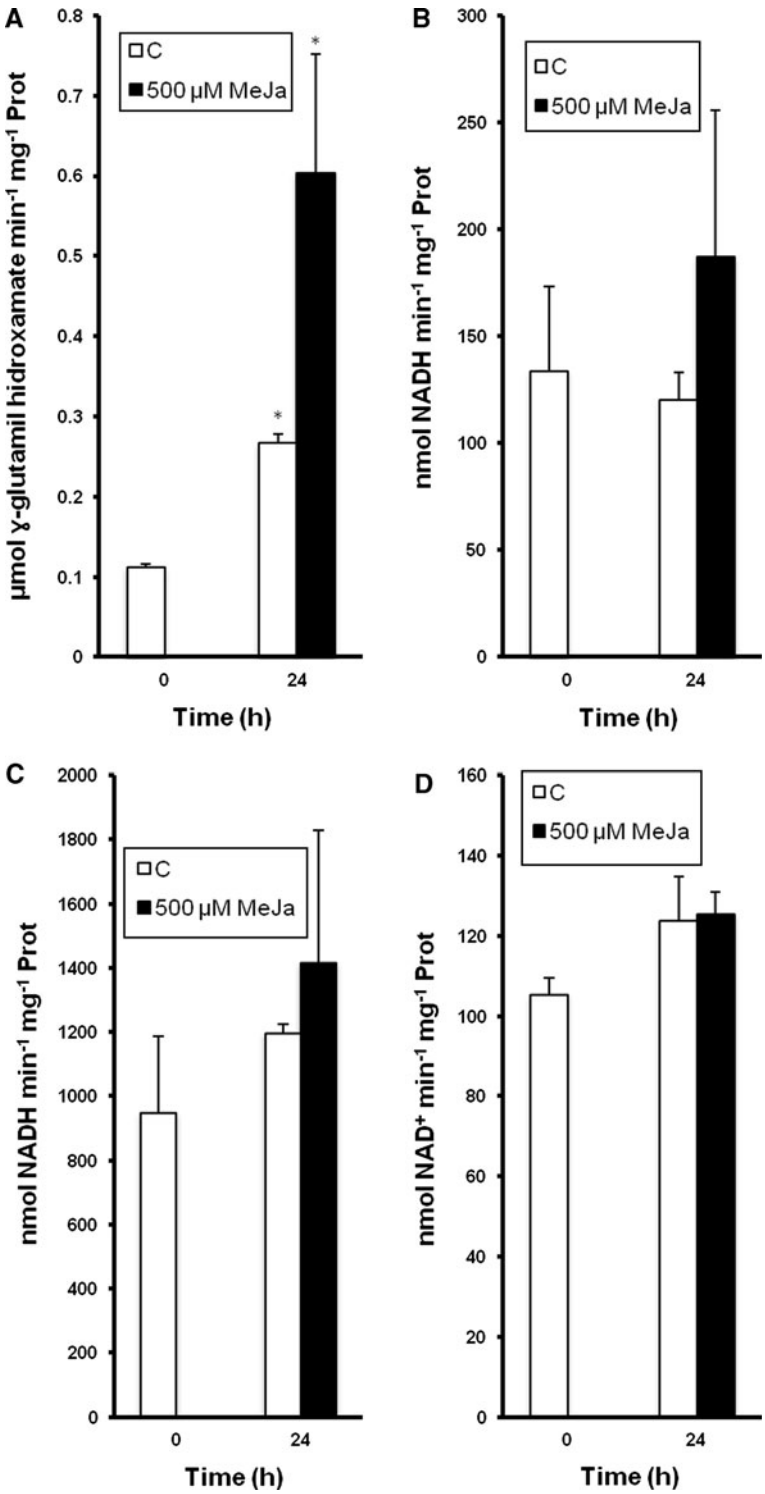

Fig. 3 Effect of 500 μM MeJa treatment on the activities of ammonia assimilation enzymes: **a** GS, **b** NADH-GOGAT, **c** NADH-GDH and **d** NAD^+-GDH. Data correspond to the mean of three independent experiments with three replicates ±1 SD. * $P < 0.05$ Tukey's test was applied to compare treatments with control

regardless of the observed increases in enzyme activity and capsaicin accumulation (data not shown).

Increases in GS activity in response to SA treatments have been recorded in tea plants (*Camellia sinensis*; Rana et al. 2008), even though the possibility of a correlation with purine alkaloids was not tested, the increase indicates that elicitors positively modified nitrogen assimilation. These effects were also observed in *C. chinense* placental tissues, in which an increase in GS activity coincided with higher capsaicinoid levels. This result suggests that a glutamine surplus could be channeled to secondary metabolism reactions (Figs. 2, 3). Nitrogen has a critical effect on the production of secondary metabolites, and common regulatory points between aromatic amino acid

biosynthesis and monoterpenoid alkaloids have been found in *Catharanthus roseus* (Van der Fits and Memelink 2000).

Total capsaicinoid content increased in the placental tissue of *C. chinense* when it was cultured in MS medium (see controls in Fig. 1), which contains 60 mM nitrogen (40 mM NO_3 and 20 mM NH_4). Accordingly, the activity of the ammonia assimilation enzymes increased even in the controls, which were not exposed to elicitors (Figs. 2, 3). These enzymes could act to maintain a steady flow of glutamate and glutamine for the synthesis of other amino acids, including phenylalanine, leucine and valine, which are the precursors of capsaicinoids (Figs. 2, 3). Ammonia and soluble amino acid pools were maintained throughout the culture period, and the induction treatments did not modify their levels. From the data obtained under these conditions, it is possible to conclude that the increased demand for nitrogen to produce capsaicinoids in the placentas was satisfied by the increased activity of glutamine synthetase, along with that of GOGAT. GDH did not seem to be involved in this process.

Our data suggest that the activation of the secondary metabolism in placental tissues requires the activation of basal nitrogen metabolism under the conditions tested. As far as we know, this is the first report of a detailed study in *C. chinense* that describes the role of enzymes involved in ammonium assimilation in relation to capsaicinoid accumulation.

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