Plant Science 176 (2009) 367–374

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

The origin of copper-induced medicarpin accumulation and its secretion from roots of young fenugreek seedlings are regulated by copper concentration

Dimitroula Tsiri [a], Ioanna Chinou [b], Maria Halabalaki [b], Kosmas Haralampidis [a], Caroline Ganis-Spyropoulos [a,*]

[a] Department of Botany, Faculty of Biology, University of Athens, Panepistimiopolis, Athens 157 81, Greece
[b] Department of Pharmacognosy and Chemistry of Natural Products, School of Pharmacy, University of Athens, Athens 157 84, Greece

ARTICLE INFO

Article history:
Received 17 October 2008
Received in revised form 25 November 2008
Accepted 2 December 2008
Available online 9 December 2008

Keywords:
Cell cultures
Copper elicitation
Fenugreek
Medicarpin
Medicarpin exudation
Trigonella foenum-graecum L.

ABSTRACT

Isoflavonoid pterocarpans, like medicarpin (M), are produced by leguminous plants in response to biotic or abiotic elicitation from either their glycosidic conjugate pools or by de novo synthesis. In an attempt to clarify M origin in response to copper elicitation, intact *Trigonella foenum-graecum* (fenugreek) seedlings and cell suspension cultures were treated with $CuCl_2$, and the accumulated isoflavonoid aglycones and their glycosides were determined by High Performance Liquid Chromatography (HPLC). Our results show that copper induces the de novo synthesis of M in a concentration dependent manner. In roots, where copper accumulated in high amounts, only part of M was formed de novo, while another part was formed at the expense of its malonyl glucoside (MGM). In contrast, when tissue copper concentration was low, like in shoots, or in roots treated with low $CuCl_2$, M was formed only by de novo synthesis. The increase of phenylalanine ammonia lyase activity (PAL) as well as the accumulation of chalcone synthase (*CHS*) and vestitone reductase (*VR*) specific transcripts are consistent with the de novo synthesis of M induced by copper. The non-linear negative correlation of the studied copper concentrations to the amount of M excreted in the seedling growth medium suggests the existence of an M secretion process which is regulated by copper concentration. The possible involvement of an ATP-dependent transporter in the copper-induced M excretion is discussed.

1. Introduction

Isoflavonoids are particularly prevalent in the Papilonoideae subfamily of the Leguminosae. Isoflavonoid phytoalexins are important plant resistance factors that are induced by biotic and abiotic elicitors. Different legume species produce different classes of isoflavonoid phytoalexins, like the pterocarpan medicarpin (M) in *Medicago sativa* [1] and *M. truncatula* [2,3], and maackian and formononetin (F) in *Trifolium repens* [4].

Isoflavonoids accumulate constitutively usually in the forms of glucosides and malonyl glucosides, primarily in plant roots but also in shoots [1,3,5,6]. In contrast, accumulation of their free forms in most cases is induced upon microbial or insect attack or upon abiotic elicitation, like UV light and heavy metals [1]. The metabolic relationship between pre-existing conjugates and free isoflavonoids produced in response to abiotic elicitors, like $CuCl_2$, has been studied in leguminous plants. In roots from elicited seedlings, their glycosidic conjugates served as metabolic pools, which released by hydrolysis the corresponding free aglycones [4,7]. However, whole alfalfa seedlings responded to copper (1 or 3 mM $CuCl_2$) elicitation by synthesizing M de novo [8]. Pterocarpan biosynthesis has been studied extensively, the enzymes leading to M biosynthesis are well characterized, and most of the corresponding genes have been cloned [1]. Phenylalanine ammonia lyase (PAL) is the enzyme which introduces phenylalanine to the phenylpropanoid pathway, chalcone synthase (CHS) is involved in the first step committed to flavonoid biosynthesis, while vestitone reductase (VR) catalyzes the penultimate step of M biosynthesis (Fig. 1). The expression of *PAL*, *CHS* and *VR* has been verified in healthy roots or cell suspension cultures of *Medicago* spp. [9–11], but *VR* is absent from shoots or leaves. Upon biotic and abiotic elicitation their activities increased in parallel to M synthesis [9,11–16].

The difference in the origin of copper-induced accumulation of M between tissues of the same or related species prompted the systematic investigation on M accumulation in response to copper

* Corresponding author. Fax: +30 2107274653.
E-mail address: cgspyro@biol.uoa.gr (C. Ganis-Spyropoulos).

Abbreviations: CHS, chalcone synthase; cDNA, complementary DNA; RP-HPLC, reverse phase high performance liquid chromatography; M, medicarpin; MGM, medicarpin-3-*O*-glucoside-6″-malonate; PAL, phenylalanine ammonia lyase; mRNA, messenger RNA; RT-PCR, reverse transcription-polymerase chain reaction; VR, vestitone reductase.

0168-9452/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2008.12.001

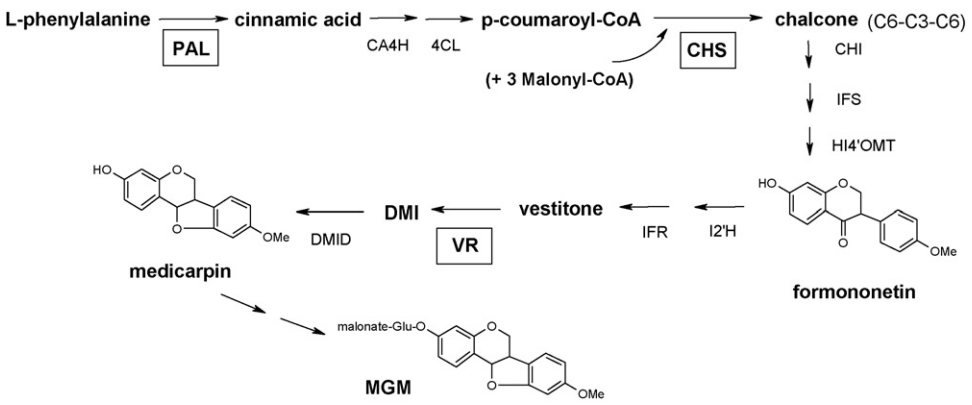

Fig. 1. Outline of the biosynthetic pathway leading to medicarpin. Enzyme and compound abbreviations used: PAL, phenylalanine ammonia lyase; CA4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate: coenzymeA ligase; CHS, chalcone synthase; IFS, isoflavone synthase; HI4′OMT, 2-hydroxyisoflavone 4′-*O*-methyltransferase; I2′H, isoflavone 2′-hydroxylase; IFR, isoflavone reductase; VR, vestitone reductase; DMID, 7,2′-dihydroxy-4′-methoxy-isoflavanol dehydratase. DMI, 7,2′-dihydroxyisoflavanol; MGM, medicarpin-3′-*O*-glucoside-6-*O*-malonate. Multiple arrows indicate two or more reactions.

elicitation. In view of the fact that *Trigonella foenum-graecum* L. (fenugreek) whole seedlings produced M in response to abiotic elicitation [17], we conducted a systematic investigation on M metabolism in roots, shoots and the growth medium of fenugreek seedlings and in fenugreek cell suspension cultures in response to copper elicitation. M accumulation was correlated with PAL activity and the expression of *CHS* and *VR* genes.

2. Materials and methods

2.1. Plant material and cell cultures

Trigonella foenum-graecum L. (fenugreek) seeds were purchased from a local market. Surface sterilized seeds were sown on moistened filter paper under sterile conditions and maintained at 25 ± 1 °C with a 16 h period of light (5.3 W m^{-2}) in a growth chamber. All experiments with seedlings and cell cultures were performed at the same temperature and light conditions.

Cell suspension cultures were initiated from callus cultures that were developed from roots of 4-day-old fenugreek seedlings [18]. For their initiation about 2 g of calli were transferred into 250 ml flasks containing 50 ml of culture medium containing 1.5 mg L^{-1} benzylaminopurine (BAP), 1.0 mg L^{-1} napthalene acetic acid (NAA) and 3% sucrose in Murashige and Skoog (MS) medium [19] pH 5.8. Cultures were maintained on an orbital shaker (120 rpm) and were subcultured initially 4 weeks after initiation of the liquid culture. The following subcultures were performed at 2 weeks intervals transferring 25 ml of the culture into 25 ml of fresh medium. Cultures were used for experiments between 4 and 7 subcultures after initiation from callus.

2.2. Elicitation and inhibition experiments

Four days after subculture, suspension cultures were elicited with copper by adding sterile $CuCl_2$ at a final concentration of 0.5 mM. An equal amount of water was added to control cultures. Cells were harvested by vacuum filtration, frozen in liquid N_2 and stored at −80 °C until analysis.

Six-day-old seedlings were treated hydroponically with $CuCl_2$ of different concentrations (0, 0.01, 0.05, 0.1, 0.5 and 1 mM). Seedlings (12 per vial) were placed into sterile vials, containing 120 ml of sterile water or $CuCl_2$, so that only their roots were immersed in the growth solution. At least 4 vials per treatment were used. After the stated period, growth media and seedlings were collected, their roots were washed with distilled water and blow dried, and the excised roots and shoots (200–300 mg/sample) were weighted and stored at −80 °C until analysis. For the inhibition assays, 5-day-old seedlings were transferred into vials containing 120 ml sterile de-ionized water (control), filter-sterilized potassium cyanide (100, 250 or 500 μM) (KCN, Sigma–Aldrich) or sodium orthovanadate (100, 250 or 500 μM) (OV, Sigma). After 24 h of incubation, $CuCl_2$ was added to the media to a final concentration of 0.01 mM and seedlings were incubated for another 24 h. All experiments were performed at least in triplicates.

2.3. Copper analysis

The copper content of shoots and roots of fenugreek seedlings treated with 0.1 mM $CuCl_2$ was determined by atomic absorption spectroscopy (Spectra AA 20 plus, VARIAN) after microwave wet digestion of the dried material in supra-pure concentrated nitric acid (70–71%). Before wet digestion roots were washed thoroughly with 10% HNO_3 to exchange surface-bound copper.

2.4. Isoflavonoid extraction, analysis and identification

Plant material was extracted with 80% methanol (1:10 w/v) with pestle and mortar. After centrifugation (4000 rpm, 10 min) the collected supernatants were filtered through nylon membrane filters (0.45 μm Titan2, SUN-SRi) and subjected to RP-HPLC analysis. Cells from cell cultures were extracted with 80% methanol (1:10 w/v) by ultrasonication for 30 min. The extracts were centrifuged (4000 rpm, 15 min) and the filtered supernatants were used for HPLC analysis. Cell suspension culture or seedling growth media were adjusted to pH 2.5 and were extracted twice with an equal volume of ethyl acetate. The organic fractions were pooled, evaporated to dryness under reduced pressure at 40 °C using rotoevaporator system (Buechi Rotavapor R-114), and the residues were redissolved in 1 ml methanol, filtered and subjected to analysis. All extracts were analyzed by HPLC using a quarternary gradient pump (PU-2089, Jasco, Japan) connected to a multi-wavelength detector (MD-2015, Jasco, Japan) under the following chromatographic conditions: 20 μl of sample were injected onto a Lichrosorb RP 18, 5.0 μm, 250 × 4 mm column; elution profile: 0–25 min, 55% A in B, 25–30 min 100% A, 30–35 min 100% A, 35–40 min step return to 100% B, 40–45 min re-equalibration with 100% B. [solvents: (A) acetonitrile and (B) water with 0.1% acetic acid]; flow rate: 1.5 ml min^{-1}; detection: at 283 nm. Isoflavonoid quantification was based on a calibration curve, plotting peak area as monitored at 283 nm against known concentrations of medicarpin.