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VACUOLAR UPTAKE OF THE PHYTOALEXIN MEDICARPIN BY THE GLUTATHIONE CONJUGATE PUMP

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Key Word Index—*Vigna radiata* syn *Phaseolus aureus* (mung bean); Leguminosae; isoflavonoid phytoalexins; glutathione *S*-transferase; vacuolar uptake.

Abstract—We have studied the uptake of [^{3}H]-medicarpin and its glutathione conjugate(s) into vacuolar membrane vesicles from etiolated hypocotyls of mung bean (*Vigna radiata*). Unconjugated medicarpin is taken up at a low rate in the presence or absence of MgATP. However, [^{3}H]-medicarpin-glutathione conjugate(s), prepared by incubation of medicarpin with a total maize glutathione *S*-transferase preparation, is taken up more than four-fold faster than medicarpin in the presence of MgATP, and this uptake is MgATP-dependent. Uptake of medicarpin-glutathione was not significantly inhibited by the ionophore gramicidin-D, but was strongly inhibited by vanadate and the alternative transport substrate *S*-(2,4-dinitrophenyl) glutathione. Our results demonstrate, in a model system, the potential utilization of the high affinity, high capacity, uncoupler-insensitive glutathione conjugate pump for the vacuolar transport of an isoflavonoid phytoalexin.

INTRODUCTION

Rapid and localized accumulation of isoflavonoid phytoalexins is a key feature of the disease resistance response of leguminous plants. Although considerable attention has been paid to the biochemistry and molecular biology of the isoflavonoid pathway [1], little is known about the subcellular site(s) of phytoalexin accumulation. This is an important consideration, because many phytoalexins are as toxic to the host plant as they are to its pathogens, and mechanisms must therefore exist for the sequestration of phytoalexins from the sensitive molecular machinery of the host.

In sorghum, red deoxyanthocyanidin phytoalexins accumulate in small membrane vesicles within the cytoplasm [2]. Constitutively expressed isoflavonoids have been shown to occur as vacuolar localized malonyl glycosides in tissues or cell cultures of several species including chickpea, soybean and alfalfa [3–7]. Elicitation or infection can lead to both *de novo* synthesis of isoflavonoid phytoalexins from distant precursors, and/or release of pre-existing isoflavone precursors from vacuolar stores [7–10]. The relative rates of synthesis and mobilization of isoflavonoid conjugates appear to be under complex feed-back and feed-forward control mechanisms, that are sensitive to the nature and extent of elicitation and to the rate of flux through the phenylpropanoid pathway [5, 8, 10–12]. Isoflavone glucosyltransferase and isoflavone glucoside malonyltransferase appear to be primarily cytoplasmic (operationally soluble) enzymes [8, 13], whereas isoflavone malonylglucoside malonylesterase is located in the vacuoles [8]. However, little is known of the mechanisms involved in vacuolar transport of isoflavonoid phytoalexins or their immediate isoflavone precursors.

Plants detoxify many phytotoxic foreign compounds by conjugation to the tripeptide glutathione (GSH), catalyzed by the enzyme glutathione *S*-transferase (GST) [14, 15]. The conjugates are then transported to and stored in the vacuole, and a MgATP-dependent glutathione *S*-conjugate 'export' pump (GS-X pump) active with such conjugates has been identified in plant vacuolar membranes [16–18]. Recently, it has been demonstrated that this transport system also functions in the vacuolar transport of endogenous natural products, such as the anthocyanin pigments. The *Bronze* 2 (*Bz2*) gene of maize encodes one of the final enzymes of anthocyanin synthesis, a GST that recognizes cyanidin 3-glucoside (C3G) [19]. In the absence of *BZ2*, anthocyanin is present in the cytosol, where it is oxidized to a brown pigment [19].

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When *BZ2* is present, GSH conjugates of C3G are synthesized, and purple anthocyanin accumulates in the vacuoles. Transport of anthocyanin-GS conjugate to the vacuole is inhibited by vanadate, a potent inhibitor of the GS-X pump [16–19], but is insensitive to bafilomycin, which inhibits the vacuolar H^+-ATPase. Two dicot mutations in anthocyanin sequestration, *an9* in Petunia (M. Alfenito, R. Buell, E. Souer, J. N. M. Mol, R. Koes and V. Walbot, unpublished results) and *fl3* in carnation (E. Larsen, M. Alfenito, W. Briggs and V. Walbot, unpublished results), are complemented following introduction of *BZ2* by particle bombardment, suggesting that GSTs may play a role in anthocyanin sequestration in both monocots and dicots.

Induction of GST transcripts and activities has been shown to accompany the production of isoflavonoid phytoalexins in elicitor-treated or infected plant cells [20, 21]; and xenobiotics induce both GST activity and the GS-X pump [18]. We have, therefore, investigated the possibility that the GS-X pump might also function in the transport of plant defence compounds into the vacuole. We here demonstrate that medicarpin, the major phytoalexin of alfalfa and several other legume species, is a substrate for glutathionation by total maize GSTs, and that the GSH conjugate(s) of medicarpin is actively transported into vacuolar membrane vesicles of mung bean (*Vigna radiata*), a model system for studies on vacuolar transport [17, 18], by the high affinity GS-X pump.

RESULTS AND DISCUSSIONS

[^{3}H]-Medicarpin-glutathione conjugate (medicarpin-GS) was synthesized by incubating [^{3}H]-medicarpin with affinity purified total maize GSTs in the presence of 20 mM GSH. Free [^{3}H]-medicarpin was mixed with 20 mM GSH and frozen immediately to avoid spontaneous conjugation, as a negative control. We then compared the rates of uptake of free medicarpin and medicarpin-GS into vacuolar membrane vesicles, purified from etiolated hypocotyls of mung bean, in the presence and absence of MgATP (Fig. 1). High level MgATP-dependent uptake of [^{3}H]-medicarpin was dependent on preincubation of this compound with GSH and GSTs. Free [^{3}H]-medicarpin incubated in the presence of GSH in the absence of GSTs was taken up at 16.7 ± 3.6 and 7.4 ± 1.3 nmol mg^{-1} 20 min^{-1} in the presence and absence of MgATP, respectively (Fig. 1). In contrast, [^{3}H]-medicarpin-GS was taken up at 81.0 ± 13.3 and 11.3 ± 0.4 nmol mg^{-1} 20 min^{-1} in the presence and absence of MgATP respectively (Fig. 1).

MgATP-dependent uptake of [^{3}H]-medicarpin-GS was strongly inhibited by vanadate and *S*-(2,4-dinitrophenyl) glutathione (DNP-GS), but was relatively insensitive to uncouplers. Whereas inclusion of vanadate (10 μM) or DNP-GS (100 μM) in the assay medium inhibited [^{3}H]-medicarpin uptake by more than 85%, addition of the ionophore gramicidin-D reduced uptake by only 17% (Table 1).

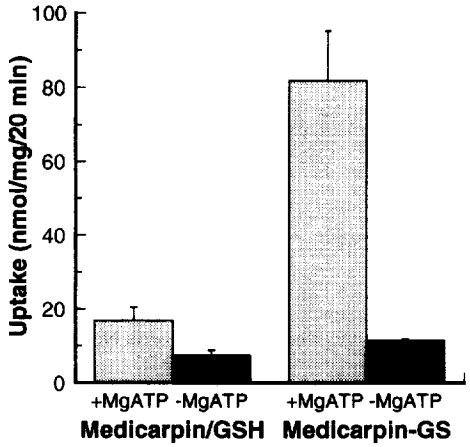

Fig. 1. MgATP-dependence of [^{3}H]-medicarpin uptake by vacuolar membrane vesicles of mung bean before (medicarpin/GSH) and after maize GST-mediated conjugation with GSH (medicarpin-GS). [^{3}H]-medicarpin or [^{3}H]-medicarpin-GS was added at a concentration of 65 μM. MgATP was either omitted (−MgATP) or added at a concentration of 3 mM (+MgATP). Values shown are means ± s.e. (n = 3).

MgATP-dependent, uncoupler-insensitive uptake increased as a simple Michaelian function of [^{3}H]-medicarpin-GS concentration (Fig. 2). Calculated K_m and V_{max} values for uptake were 21.5 ± 15.5 μM and 77.8 ± 23.3 nmol mg^{-1} 20 min^{-1}, respectively.

Direct involvement of the GS-X pump in the accumulation of [^{3}H]-medicarpin-GS by vacuolar membrane vesicles is therefore supported by three independent observations. Firstly, glutathionation of medicarpin selectively increases MgATP-dependent uptake. MgATP-independent uptake is marginally affected by glutathionation, but MgATP-dependent uptake is stimulated approximately six-fold, con-

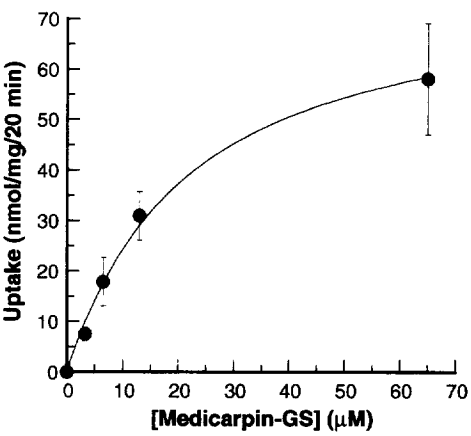

Fig. 2. Concentration-dependence of MgATP-dependent, uncoupler-insensitive uptake of [^{3}H]-medicarpin-GS into vacuolar membrane vesicles. Uptake was allowed to proceed for 20 min in standard uptake medium containing 3 mM MgATP and 5 μM gramicidin-D. The kinetic parameters were K_m 21.5 ± 15.5 μM and V_{max} 77.8 ± 23.3 nmol mg^{-1} 20 min^{-1}. Values shown are means ± s.e. (n = 3).

Table 1. Effects of different inhibitors on uptake of [^{3}H]-medicarpin-GS by vacuolar membrane vesicles. [^{3}H]-medicarpin-GS was added at a concentration of 65 μM. MgATP was either omitted (−MgATP) or added at a concentration of 3 mM (+MgATP). Gramicidin-D, vanadate and DNP-GS were added at concentrations of 5, 10 and 100 μM, respectively. Values outside parentheses are means ± s.e. (n = 3); values inside parentheses are rates of uptake expressed as percentage of control

Treatment	[^{3}H]-Medicarpin-GS uptake (nmol mg^{-1} 20 min^{-1})	
	+MgATP	−MgATP
Control	85.6 ± 13.3 (100)	16.7 ± 6.0 (100)
+Gramicidin-D	71.2 ± 3.0 (83.2)	13.2 ± 2.1 (79.0)
+Gramicidin-D + vanadate	12.9 ± 0.9 (15.1)	17.4 ± 0.5 (104.2)
+Gramicidin-D + DNP-GS	11.7 ± 2.8 (13.7)	5.6 ± 3.1 (33.5)

firming that medicarpin-GS is the transported species in these assays and that MgATP is the energy source. Secondly, uptake is directly energized by MgATP. The inability of uncoupler to inhibit [^{3}H]-medicarpin-GS uptake strongly implies that the H^+-electrochemical gradient that would otherwise be established by the vacuolar H^+-ATPase in the presence of MgATP does not drive uptake. Rather, the pronounced inhibition of MgATP-dependent uptake exerted by vanadate supports the hypothesis that GS-X pump-mediated uptake is strictly dependent on ATP hydrolysis and formation of a phosphoenzyme intermediate [16–18]. Finally, [^{3}H]-medicarpin-GS and the model GS-X pump substrate, DNP-GS, the transport of which has been previously analyzed in this system [17, 18], compete for uptake indicating that both are transported by the same uptake system.

The efficacy of medicarpin-GS as a substrate for the vacuolar GS-X pump is striking. Even though the K_m for medicarpin-GS uptake is undoubtedly an overestimate, because the yield from the conjugation reaction was not quantified, it is nevertheless two- to 25-fold lower than those estimated previously for DNP-GS (80 μM; [17]) and C3G-GS (46 μM) in this system, and glutathione-*S*-*N*-ethylmaleimide and metolachlor-GS uptake into barley vacuoles (500 μM and 40–60 μM, respectively [16]). Moreover, the capacity of the GS-X pump for medicarpin-GS uptake is high (V_{max} = 78 nmol mg^{-1} 20 min^{-1}) compared to that of DNP-GS (V_{max} = 12 nmol mg^{-1} 20 min^{-1}; [17]) and is comparable to that estimated for C3G-GS (Z.-S. Li, M. Alfenito, V. Walbot and P. A. Rea, unpublished results). Thus, although maize anthocyanin was the first natural substrate shown to be sequestered in the vacuole through the concerted actions of cytosolic GSTs and the vacuolar GS-X pump in plants [19], medicarpin, and presumably other isoflavonoid phytoalexins, are equally strong candidates for physiological substrates of this transport system. Although we have found no reports of the occurrence of medicarpin itself in mung bean, this plant does accumulate closely related isoflavone and pterocarpan phytoalexins [22].

Anthocyanins are permanently stored in the vacuole, perhaps as a result of subsequent modifications, such as malonylation and formation of complexes. In addition, plants store compounds in the vacuole that can subsequently be retrieved. For bulky organic compounds, little is known about the mechanism of this compartmentalization or how vacuolar compounds re-enter the cytoplasm. Isoflavonoid malonyl glycosides belong to the group of compounds that can be retrieved, a process that occurs during the early stages of infection/elicitation [8, 20]. Our results, demonstrating that the isoflavonoid medicarpin is a substrate for glutathionation and the preferential uptake of medicarpin-GS conjugate(s) compared to free medicarpin, provide a plausible explanation for the vacuolar compartmentalization of this cytotoxic compound. If it is generally true that glutathionation is a prerequisite for vacuolar compartmentalization, then many cytotoxic phytochemicals are likely to be substrates for cytoplasmic GSTs and the GS-X pump.

It must be cautioned that the results reported in this paper were obtained using model systems. Mung bean was chosen for the uptake studies because the GS-X pump in vacuolar membranes of this species has been well characterized [17, 18], and the kinetics of uptake of other flavonoid-derived compounds (anthocyanin glucosides) determined in this system (Z.-S. Li, M. Alfenito, V. Walbot and P. A. Rea, unpublished results). It remains to be determined whether alfalfa has a GS-X pump with similar characteristics, or whether alfalfa GSTs can readily act on medicarpin.

It is interesting to speculate that one function for the GST(s) induced following the hypersensitive response to avirulent fungal pathogens could be to facilitate the vacuolar storage of antimicrobial compounds in the healthy cells around the hypersensitive lesion. The vacuolar storage forms of isoflavonoids/pterocarpans are usually the corresponding malonyl glucosides [8, 21]. Because glutathionated anthocyanin glucoside is a substrate for the GS-X pump [19], the question remains as to whether medicarpin conjugates, in addition to the aglycone, are also substates for the vacuolar GS-X pump. Furthermore, our results raise a number of other important biochemical questions: do glycosylation and malonylation occur

in the cytoplasm, vacuole, or both? How is glutathione removed from the conjugates? What determines whether a vacuolar compound is permanently or temporarily sequestered? Answers to these questions, with respect to the isoflavonoid phytoalexins, will provide clues as to how the host plant integrates the balance between attack and defence during the determinative stages of pathogen infection.

EXPERIMENTAL

Reagents. GSH and 1-chloro-2,4-dinitrobenzene were purchased from Fluka; creatine kinase (type I from rabbit muscle, 150–250 U mg protein^{-1}), creatine phosphate and gramicidin-D were from Sigma; cellulose nitrate membrane filters were from Millipore.

Preparation of [^{3}H]*-medicarpin-GS.* [^{3}H]-Medicarpin was produced by base-catalyzed tritium exchange from tritiated H_2O using unlabelled medicarpin isolated from fenugreek (*Trigonella foenum-graecum*) seedlings exposed to 3 mM $CuCl_2$.

Purification of GST and conjugation reactions. Two-week-old maize B73N seedlings were grown under continuous light at 21°. Twenty-four hours before harvesting, the seedlings were exposed to a mild treatment with 2,4-dichlorophenoxyacetic acid and atrazine [14]. This stimulated maximal GST expression. Two grams of root and shoot tissue was ground to homogeneity in 50 ml of 500 mM Na-Pi pH 7.8 (Buffer A). The extract was centrifuged at 7000 *g* for 10 min at 4°. The supernatant was filtered through miracloth and mixed with 2 ml of *S*-hexylglutathione-conjugated agarose beads (Sigma). After a 5 min incubation at room temp, the beads were sedimented at 500 *g* at 4°. The supernatant was discarded. The beads were resuspended in 25 ml of prechilled Buffer A, and centrifuged again at 500 *g* at 4°. This step was repeated × 3. The beads were then resuspended in 2 ml of Buffer B (20 mM GSH, 500 mM Na-Pi pH 7.8) at room temp for 5 min. The beads were sedimented at 500 *g* at room temp. The supernatant was assayed for GST activity by the method of Mannervick and Gutjenberg [24].

[^{3}H]-Medicarpin (0.5 μCi, 4.15 μCi μmol^{-1}) was conjugated with GSH by incubating with 25 μl of total purified GSTs from maize for 3 hr at room temp in the dark. Control, unconjugated samples were prepd by mixing [^{3}H]-medicarpin (0.5 μCi) with cold Buffer B and immediately freezing the mixture in liquid N_2.

Synthesis of S-(2,4-*dinitrophenyl*) *glutathione* (*DNP-GS*). DNP-GS was synthesized from 1-chloro-2,4-dinitrobenzene and GSH by a modification of the enzymatic procedure in refs [17, 25].

Preparation of vacuolar membrane vesicles. Vacuolar membrane vesicles were purified from etiolated hypocotyls of *V. radiata* cv Berken (mung bean) as described [17, 18]. The advantages of using vacuolar membrane vesicles rather than intact vacuoles for uptake studies have been presented [17].

Measurement of uptake. Unless otherwise indicated, uptake of [^{3}H]-medicarpin or [^{3}H]-medicarpin-GS was measured at 25° in 200 μl reaction vols containing 3 mM ATP, 3 mM $MgSO_4$, 10 mM creatine phosphate, 16 U ml^{-1} creatine kinase^{-1}, 50 mM KCl, 0.1% (w/v) bovine serum albumin, 400 mM sorbitol and 25 mM Tris–Mes buffer, pH 8.0. Uptake was initiated by the addition of 12 μl membrane vesicles (30–40 μg protein) and brief mixing of the samples on a vortex mixer. Uptake was terminated by the addition of 1 ml ice-cold wash medium (400 mM sorbitol, 3 mM Tris–Mes, pH 8.0) and vacuum filtration of the suspension through prewetted HA cellulose nitrate filters (pore diameter 0.45 μm). The filters were rinsed twice with 1 ml ice-cold wash medium, air-dried and radioactivity was determined by liquid scintillation counting.

Protein estimation. The protein content of mung bean vacuolar membrane vesicle preps was estimated by a modification of the method in ref. [26].

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