MgATP-Dependent Transport of Phytochelatins Across the Tonoplast of Oat Roots[1]

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In Cd-exposed oat (*Avena sativa*) roots Cd was found to be associated primarily with the phytochelatin (γ-glutamylcysteinyl)$_3$-glutamic acid [(γEC)$_3$G], with a peptide to Cd ratio of 1:3 (cysteine to Cd ratio of 1:1), even though both (γEC)$_2$G and (γEC)$_3$G were present in the roots. Phytochelatins are known to accumulate in the vacuoles of plant cells on exposure to Cd, but the mechanism is not clear. Here we present evidence for the transport of the phytochelatins (γEC)$_3$G and (γEC)$_2$G as well as the Cd complex Cd-(γEC)$_3$G across the tonoplast of oat roots. Transport of (γEC)$_3$G had a K_m for MgATP of 0.18 mM and a V_{max} of 0.7 to 1 nmol mg^{-1} protein min^{-1}. Transport of (γEC)$_3$G was also energized by MgGTP and to a lesser extent MgUTP and was highly sensitive to orthovanadate, with a 50%-inhibitory concentration of 0.9 μM. The Cd complex Cd-(γEC)$_3$G and (γEC)$_2$G were also transported in a MgATP-dependent, vanadate-sensitive manner. Therefore, this process is a candidate for the transport of both phytochelatins, and Cd as its peptide complex, from the cytoplasm into the vacuole.

Cd is a potentially toxic, nonessential metal that is accumulated from soil by plants. At moderate to high levels of Cd exposure a significant proportion of intracellular Cd is bound as a heterogeneous Cd-phytochelatin sulfide complex (for recent reviews, see Rauser, 1990; Steffens, 1990; Wagner, 1994). Evidence obtained using the inhibitor buthionine sulfoximine (see reviews by Rauser, 1990; Steffens, 1990) suggests that production of the Cd-phytochelatin complex is necessary for Cd detoxification within plants. The vacuole is the major site for accumulation of both Cd and phytochelatins (Krotz et al., 1989; Vögeli-Lange and Wagner, 1990). Recent identification of a Cd/H$^+$ antiport mechanism at the tonoplast provides one way for Cd to accumulate in the vacuole (Salt and Wagner, 1993). A Cd-sensitive mutant of the fission yeast *Schizosaccharomyces pombe*, capable of synthesizing phytochelatins but unable to form large amounts of Cd-phytochelatin sulfide complex, was shown to have a mutation within the *hmt1* gene encoding an ATP-binding cassette type of transport protein associated with the vacuolar membrane (Ortiz et al., 1992).

In contrast to those from wild type, vacuolar membrane vesicles from this mutant were unable to transport either phytochelatins or Cd-phytochelatin complex (Ortiz et al., 1994). This evidence strongly suggests that Cd detoxification in *S. pombe*, and by extension in plants, involves both the synthesis of phytochelatins and their compartmentalization within the vacuole.

To better understand the role of phytochelatins in Cd detoxification within plants, we have undertaken an investigation of the transport processes involved in their accumulation in the vacuole.

MATERIALS AND METHODS

Growth of Oats and *Schizosaccharomyces pombe*

Oat seeds (*Avena sativa*, purchased from Southern States Cooperative, Lexington, KY) (185 g) were surface sterilized for 1 min in 0.25% sodium hypochlorite, rinsed four times in 4 L of water, and spread evenly onto moist cheesecloth covering a wire mesh attached to a plastic frame (43.5 × 54.6 × 4.5 cm). The loaded frame was placed in a plastic tray (46 × 56 × 10 cm) containing 12 L of 0.5 mM CaSO$_4$ and 10 μM CdSO$_4$ when required. The solution was aerated using two 44-cm-long aquarium diffusers. Roots were harvested after 4 d of growth in the dark at 22°C. Freshly harvested roots were used for isolating tonoplast-enriched vesicles; otherwise, the roots were frozen in liquid N$_2$ and stored at −70°C until used for analyses of Cd-binding complex or phytochelatins.

The glutamate-requiring fission yeast *Schizosaccharomyces pombe* (Glu$^-$ATCC 38390) was obtained from the American Type Culture Collection. The yeast was maintained on master agar (2%, w/v) plates made with 30 g of Glc and 5 g of yeast extract per liter of distilled water (YED). Liquid cultures were prepared with defined Difco (Detroit, MI) yeast carbon base (YCB) medium supplemented with 0.5 mM glutamate, [^{14}C]glutamate (0.125–0.5 μCi/mL), 38 mM (NH$_4$)$_2$SO$_4$, and 0.5 mM CdSO$_4$ unless stated otherwise. The YCB and glutamate solutions were sterilized by filtration through 0.2-μm sterile filters; all other solutions were autoclaved. Flasks of liquid cultures were shaken on a gyratory shaker at 175 rpm and 30 to 32°C. Liquid inocu-

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Abbreviations: AMP-PNP, 5'-adenylylimidodiphosphate; BTP, bis-tris-propane or 1,3-bis[tris(hydroxymethyl)methylamino] propane; ΔpH, pH gradient; (γEC)$_3$G, (γ-glutamylcysteinyl)$_3$glutamic acid; V_o, void volume; V_t, total volume.

lum was prepared by transferring *S. pombe* from YED master agar plates into 125-mL Erlenmeyer flasks containing 20 mL of YCB and 5 mM glutamate without [^{14}C]glutamate and CdSO$_4$. Flasks were incubated overnight and a 10-mL inoculum was transferred to a 1-L flask containing 200 mL of YCB, 0.5 mM glutamate, 38 mM (NH$_4$)$_2$SO$_4$, 0.5 mM CdSO$_4$, and [^{14}C]glutamate (0.125–0.5 μCi/mL). Initial cell densities were approximately 2.7 × 10^7 cells/mL. Flasks were incubated for 48 h with shaking to yield approximately 1.2 × 10^{12} cells. Cells were harvested by centrifugation at 12,500g for 10 min at 4°C and either frozen with liquid N$_2$ and stored at −70°C or used for the purification of Cd-binding complex.

Purification of Oat Root Cd-Binding Complex

Ten grams of frozen oat roots were ground in liquid N$_2$ using a pestle and mortar. Chilled 50 mM Hepes, pH 8.0, was added to the frozen powder (tissue to buffer ratio of 1) and the tissue was ground until thawed. The crude root extract was centrifuged twice at 48,000g for 15 min at 4°C and the supernatant was either frozen in liquid N$_2$ and stored at −70°C or applied directly to either a Sephacryl S-100 gel filtration column (1.6 × 98 cm) or a Q-Sepharose Fast Flow ion-exchange column (0.6 × 0.25 cm). For gel filtration the column was equilibrated with 10 mM Hepes, pH 8.0, containing 300 mM KCl and calibrated with blue dextran and acetone to give a V_o and V_t of 80 and 172.5 mL, respectively. The linear flow rate was 25 cm/h. Crude extract (4.5 mL) was applied to the column and 2.5-mL fractions were collected and analyzed for Cd by atomic absorption spectrophotometry. Peak Cd-containing fractions were pooled and analyzed for thiols by HPLC. Total acid-soluble thiols in roots were determined by HPLC of root homogenates prepared in 0.1 M HCl (tissue to buffer ratio of 1). For ion exchange the supernatant was passed through a Q-Sepharose Fast Flow ion-exchange column that had been equilibrated with 10 mM Tris-HCl, pH 8.6. After the column was washed with 10 mL of 10 mM Tris-HCl, pH 8.6, the Cd-binding complex was eluted with approximately 1 mL of 10 mM Hepes, pH 8.0, 1 M KCl. The complex was either frozen in liquid N$_2$ and stored at −70°C or used immediately for phytochelatin purification.

Purification of Cd-Binding Complex from *S. pombe*

S. pombe cells from a 200-mL culture were suspended in 10 mL of buffer containing 50 mM Tris-HCl, pH 8.6, 1 mM PMSF, and 1% Tween (v/v) (buffer A). The cell suspension was vortexed for 5 min in a Pyrex culture tube (15 × 2.2 cm) containing 50% (v/v) acid-washed glass beads (0.25–0.3 mm diameter) and cooled on ice for 30 s, a procedure that was adapted from that of Moreno et al. (1991). This procedure, repeated six times, resulted in approximately 75% cell breakage as estimated by light microscopy. The crude extract was decanted and the glass beads were washed with 10 mL of buffer A. The wash was added to the

initial extract and the suspension was centrifuged at 48,000g for 15 min at 4°C. The complex was isolated using a Q-Sepharose column as described above for oat root Cd-binding complex. The complex was either frozen in liquid N$_2$ and stored at −70°C or used immediately for phytochelatin purification.

Purification of Thionitrobenzoate Derivatives of Phytochelatins for MS

Cd-binding complexes from *S. pombe* or oat roots were dissociated, on ice, by acidification with 6 M HCl to below pH 2, giving a final HCl concentration of 80 mM, EDTA was added (5 mM final concentration) to chelate free Cd, and the solution was incubated for 10 min followed by centrifugation in a microcentrifuge for 10 min at 4°C. The supernatant was neutralized with 2 M Hepes to approximately pH 7.0, fresh DTT was added (5 mM final concentration) to liberate free thiol groups, and the solution was incubated at 25°C for 20 min, then reacidified with 6 M HCl, incubated on ice for 10 min, and centrifuged for 10 min at 4°C in a microcentrifuge. An aliquot was loaded onto a Nucleosil C$_{18}$ reversed-phase HPLC column equilibrated with 0.1% TFA (v/v) at 37°C and phytochelatins were eluted over 30 min with a linear gradient of 0 to 15% acetonitrile in 0.1% TFA. Phytochelatins were detected using Ellman's reagent (Grill et al., 1985) and the thionitrobenzoate-derivatized phytochelatins were collected. Derivatized phytochelatins were then lyophilized and repurified by HPLC (Meuwly et al., 1994) and the structure of this purified material was determined using tandem MS (Meuwly et al., 1994). To check the integrity of these thionitrobenzoate-derivatized phytochelatins, samples were reduced with DTT to remove the thionitrobenzoate group and analyzed using C$_{18}$ reversed-phase HPLC and detection with Ellman's reagent. This clearly demonstrated that the derivatized phytochelatins used for MS were the same as those initially observed by HPLC.

Purification of ^{14}C-Labeled Phytochelatins for Transport Studies

[^{14}C]Cd-binding complex isolated from *S. pombe* exposed to [^{14}C]glutamate was dissociated and reduced, and aliquots (20–30 μL) were loaded onto a Nucleosil C$_{18}$ reversed-phase HPLC column as described above. The A_{220} of the column effluent was recorded first followed by continuous addition of Ellman's reagent and detection of thiols by A_{405} (Rauser, 1991). In this way the phytochelatins of interest could be identified in the UV recording. Larger aliquots were then chromatographed under the same conditions and the desired peptides were collected based on their A_{220} without the addition of Ellman's reagent. Phytochelatins were lyophilized and used immediately or stored in a desiccator. Stored peptides were reduced with fresh 5 mM DTT for 20 min, rechromatographed as before, and lyophilized before immediate use. The specific activity of purified [^{14}C](γEC)$_3$G varied between 0.07 and 0.73 mCi/mmol depending on the amount of [^{14}C]glutamate

used in the original culture. Just prior to use, the phytochelatins were quantified by HPLC and detected with Ellman's reagent to determine their oxidation state. Concentrations of phytochelatins presented are based on these data. The content of free thiol groups in phytochelatins were measured after reduction with DTT and again after C_{18} reversed-phase HPLC and lyophilization, and recoveries ranged from 70 to 100%.

Two forms of synthetic Cd-phytochelatin complex were prepared, Cd-[^{14}C](γEC)$_3$G and [^{109}Cd]Cd-(γEC)$_3$G. In the first case CdSO$_4$ was added to [^{14}C](γEC)$_3$G; in the second case CdSO$_4$ containing ^{109}Cd (0.2–0.6 mCi/mmol) was added to (γEC)$_3$G, all in a peptide to Cd ratio of 1:1.5 (Cys to Cd ratio of 1:0.5). The reaction occurred in 1 mL of transport buffer (buffer A) described below. Cd not complexed with (γEC)$_3$G was removed using a Sephadex G-10 column (5 × 1.5 cm) equilibrated with buffer A. Cd-(γEC)$_3$G complex was recovered in transport buffer in the void volume (83% of ^{109}Cd and 90% of [^{14}C](γEC)$_3$G applied). The synthetic Cd-(γEC)$_3$G complex had a peptide to Cd ratio of 1:1.5 based on the specific activities and recovery efficiencies of ^{109}Cd and [^{14}C](γEC)$_3$G. In oat roots (γEC)$_3$G is the only phytochelatin associated with Cd, in a peptide to Cd ratio of 1:3 (Cys to Cd ratio of 1:1); however, synthetic Cd-(γEC)$_3$G with a peptide to Cd ratio of 1:3 was insoluble. The higher loading capacity of the in vivo oat root Cd-binding complex for Cd is probably due to the presence of sulfide (W.E. Rauser, unpublished data). Because of this solubility problem, synthetic Cd-(γEC)$_3$G was produced with a peptide to Cd ratio of 1:1.5 (half of that in vivo).

Preparation of Sealed Tonoplast-Enriched Vesicles

The procedure was as described previously by Salt and Wagner (1993) except that the final concentration of protein in tonoplast-enriched vesicles was 5 to 10 mg/mL.

MgATP-Dependent Phytochelatin Transport

Transport of phytochelatins was monitored using their [^{14}C]glutamate-labeled analogs. Lyophilized phytochelatins were dissolved in nitrogen-purged buffer containing 25 mM Hepes-BTP at pH 7.0, 175 mM mannitol, 28 mM HCl adjusted with BTP to pH 7.0 (to stimulate the V-type ATPase and to provide the Cl counter anion) and 0.2 mM NaN$_3$ (buffer A). MgATP-dependent transport of (γEC)$_3$G was measured in the presence of 29 to 281 μM (γEC)$_3$G, 2 mM Tris-ATP, and tonoplast-enriched vesicles (0.1–0.3 mg/mL). Reactions were initiated by the addition of 300 mM MgSO$_4$ to a final concentration of 3 mM, to demonstrate MgATP dependence. The sample was incubated at 25°C, and 100-μL aliquots were assayed using the direct filtration method described below. Assays using [^{14}C](γEC)$_2$G as substrate contained 474 μM [^{14}C](γEC)$_2$G; this high concentration was used because of the low specific activity of this material. All additions were made in 2.5 mM Hepes-BTP at pH 7.0.

MgATP-Dependent Cd-(γEC)$_3$G Complex Transport

Transport of Cd-(γEC)$_3$G complex was monitored using either [^{109}Cd]Cd-(γEC)$_3$G or Cd-[^{14}C](γEC)$_3$G. MgATP-dependent Cd-(γEC)$_3$G complex transport was measured as for (γEC)$_3$G transport.

Kinetics of MgATP-Dependent (γEC)$_3$G Peptide Transport

The kinetics of MgATP-dependent (γEC)$_3$G transport were measured using [^{14}C](γEC)$_3$G. Vesicles were added to buffer A containing either 55 or 102 μM [^{14}C](γEC)$_3$G (0.06 and 0.08 μCi/mL) and various concentrations of ATP, to give a protein concentration of 0.28 or 0.43 mg/mL. The reaction was initiated by the addition of 300 mM MgSO$_4$ to all samples (including 0 mM ATP) to a final concentration of 3 mM. The samples were incubated at 25°C, and 200-μL aliquots removed after 5 min for assay using the direct filtration method. All additions were made in 2.5 mM Hepes-BTP at pH 7.0.

Proton Transport Activity

A ΔpH was generated in vesicles (acid inside) using the V-type ATPase (MgATP-dependent). Generation of ΔpH was monitored directly by measuring accumulation of [^{14}C]methylamine using the direct filtration assay. Vesicles were added to buffer A containing 2 mM Tris-ATP and 100 μM [^{14}C]methylamine (1 μCi/mL), to give a protein concentration of 50 to 100 μg/mL. The reaction was initiated by the addition of 300 mM MgSO$_4$ to a final concentration of 3 mM. The sample was incubated at 25°C, and 100-μL aliquots were assayed using the direct filtration method. All additions were made in 2.5 mM Hepes-BTP at pH 7.0.

Direct Filtration Assay

The procedure was essentially that of Churchill and Sze (1983). Samples were filtered through a 0.45-μm filter (Millipore HATF type HA, Millipore Corp., Bedford, MA) that had been pre-wetted with 1 mL of cold washing buffer containing 250 mM mannitol, 2.5 mM Hepes-BTP (pH 7.2), and 0.1% BSA. After filtration of the sample, the filter was rinsed quickly with 4 mL of cold washing buffer. Filtration was achieved using an applied vacuum of approximately 40 cm of Hg. For the measurement of [^{14}C]methylamine and [^{14}C]phytochelatins, filters were dried before counting in Beckman ReadySafe cocktail with a liquid scintillation spectrometer. The majority of samples were counted to give a 2-σ percentage of 2. For ^{109}Cd measurements filters were dried and analyzed by γ counting.

Loading of Vesicles with Potassium

Vesicles from the dextran cushion were recovered and diluted to 30 mL with buffer containing 2.5 mM Hepes-BTP at pH 7.2, 250 mM mannitol, 10 mM potassium gluconate, 0.1% BSA, 0.1 mM PMSF, and 0.1 mM DTT. After incubation at 10°C for 1 h, vesicles were sedimented at 90,000g for 30

min and the pellet was resuspended in the same buffer to a concentration of 5 to 10 mg/mL.

Protein Concentration Determination

Protein concentration was estimated using a modification of the method of Bensadoun and Weinstein (1976) as described by Salt and Wagner (1993).

Reagents

Chemicals were purchased from Sigma, including [^{14}C]methylamine (9.8 mCi/mmol) and [^{14}C]glutamate (229–240 mCi/mmol). Bicinchoninic acid protein assay reagent was obtained from Pierce (Rockford, IL) and ^{109}Cd (2.29 mCi/mg) was obtained from DuPont, NEN Products.

Data Analysis

All curve fitting was done using the Marquardt-Levenberg nonlinear regression algorithm on a personnel computer-based program as described by Brooks (1992).

RESULTS

Characterization of Phytochelatins Used for Transport Studies

S. pombe produced phytochelatins in higher abundance than oat roots and allowed the use of a glu^- strain for labeling with [^{14}C]glutamate; therefore, S. pombe cells were used as the source of phytochelatins. The phytochelatins (γEC)$_3$G and (γEC)$_2$G were purified from the Cd-binding complex of S. pombe by acidification, reduction with excess DTT, and separation by C$_{18}$ reversed-phase HPLC,
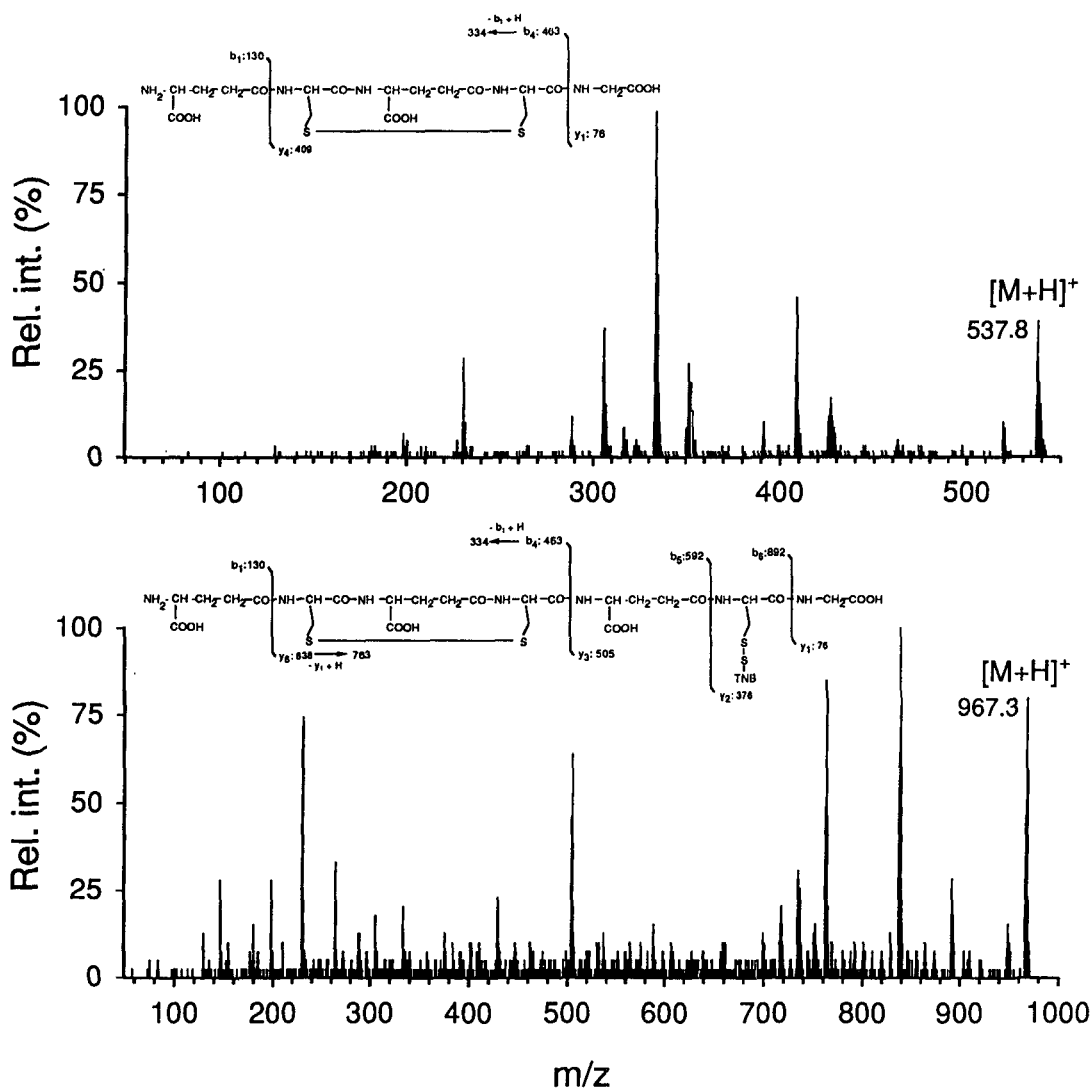

Figure 1. Identification of (γEC)$_2$G and (γEC)$_3$G using MS-MS analysis. Tandem MS spectra of the thionitrobenzoate-derivatized phytochelatins, (γEC)$_2$G and (γEC)$_3$G, isolated from Cd-exposed S. pombe cells. Insets, Major fragment ions observed in the MS-MS spectra. Rel. int., Relative intensity.

and their sequences were determined using tandem MS of their thionitrobenzoate derivatives (Fig. 1). Prior to MS, thionitrobenzoate-derivatized phytochelatins were observed to undergo spontaneous molecular rearrangement, forming disulfides with adjacent derivatized Cys residues with the release of thionitrobenzoate. This artifactual disulfide bridge formation in no way reflects the oxidation state of the phytochelatins used in transport studies. Phytochelatins used in transport studies were collected after reduction with DTT and C_{18} reversed-phase HPLC in a reduced, underivatized form and assayed just prior to use to quantify the number of free thiol groups. All phytochelatin concentrations and specific activities presented are based on these data. Free thiol groups were also measured after reduction with DTT and again after C_{18} reversed-phase HPLC and lyophilization, and recoveries ranged from 70 to 100%, suggesting that the majority of the phytochelatins used in transport studies were in their reduced form.

Transport of $(\gamma EC)_3G$ into Oat Root Tonoplast Vesicles

Cd-binding complex was isolated using a Sephacryl gel filtration column from oat roots that had been exposed to Cd. The complex contained only the phytochelatin $(\gamma EC)_3G$ even though both $(\gamma EC)_2G$ and $(\gamma EC)_3G$ were present in the root. $(\gamma EC)_3G$ and Cd were present in the complex in a ratio of 1:3. Because the in vivo Cd-binding complex of oat roots contained only $(\gamma EC)_3G$, we decided to investigate $(\gamma EC)_3G$ uptake into isolated tonoplast vesicles. In the presence of 2 mM MgATP and 47 μM $(\gamma EC)_3G$, tonoplast vesicles accumulated $(\gamma EC)_3G$ at an initial rate of 0.7 nmol mg^{-1} protein min^{-1} over the first 10 min (Fig. 2). By 70 min the uptake rate tended toward a minimum and reached a total accumulation of 18 nmol mg^{-1} protein. On addition of 0.02%
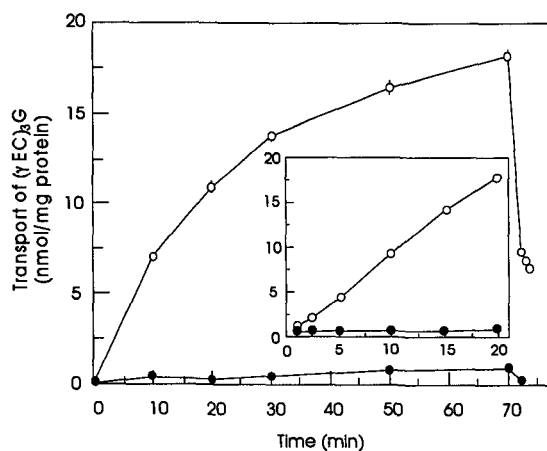

Figure 2. Time course of $(\gamma EC)_3G$ transport into oat root tonoplast vesicles. $(\gamma EC)_3G$ transport was detected using [^{14}C]$(\gamma EC)_3G$ as described in "Materials and Methods." Transport activity was measured in the presence of 47 μM $(\gamma EC)_3G$, 2 mM ATP and in the presence (●) or absence (○) of 3 mM MgSO$_4$. After 70 min, Triton X-100 was added to a final concentration of 0.02%. Inset, Transport of $(\gamma EC)_3G$, supplied at 30 μM, over the first 20 min.

Table I. *Transport of Cd-$(\gamma EC)_3G$ complex*

Assays were performed in the presence of 2 mM MgATP and Cd-$(\gamma EC)_3G$, and transport was monitored by uptake of either [^{109}Cd]Cd-$(\gamma EC)_3G$ or Cd-[^{14}C]$(\gamma EC)_3G$, at a concentration of 134 μM and with a peptide to Cd ratio of 1:1.5. All data are presented with the appropriate control in the absence of Mg subtracted and are presented as the mean ± SD of two independent experiments with three replicate samples per experiment.

Addition	Cd-$(\gamma EC)_3G$ Transport Substrate	
	[^{109}Cd]Cd-$(\gamma EC)_3G$	Cd-[^{14}C]$(\gamma EC)_3G$
	nmol mg^{-1} protein (20 min)$^{-1}$	
Control	16.9 ± 2.0	5.1 ± 1.2
Na$_3$VO$_4$ (50 μM)	8.4 ± 1.1	0.2 ± 0.2
NH$_4$Cl (5 mM)	5.8 ± 1.0	3.8 ± 0.9

Triton X-100 the accumulated $(\gamma EC)_3G$ was released. In the absence of Mg total accumulation of $(\gamma EC)_3G$ after 70 min was reduced by 95%. An independent experiment using different vesicle and phytochelatin preparations performed over a narrower time range (Fig. 2, inset) with 30 μM $(\gamma EC)_3G$ showed $(\gamma EC)_3G$ uptake over the first 15 min to be linear and equal to 0.93 nmol mg^{-1} protein min^{-1}. Transport assays done at $(\gamma EC)_3G$ concentrations of 29, 30, 47, 65, 72, 174, and 281 μM gave uptake rates of 1.05, 0.93, 0.7, 1.14, 1.31, 0.77, and 1.07 nmol mg^{-1} protein min^{-1}, respectively, giving a mean uptake rate of 1 ± 0.2 nmol mg^{-1} protein min^{-1}. This clearly demonstrates that even at the lowest $(\gamma EC)_3G$ concentration used, 29 μM, $(\gamma EC)_3G$ was present at a saturating concentration. The mean rate of $(\gamma EC)_3G$ transport should therefore approximate its V_{max}.

To determine the effect of the oxidation state of phytochelatins on their uptake, experiments were performed in the presence and absence of 3.5-fold excess DTT. In the presence of excess DTT the uptake of $(\gamma EC)_3G$ after 20 min was reduced by 20%. It is difficult to determine in this experiment whether the DTT was affecting the oxidation state of the transporter or the phytochelatin; however, it does demonstrate that the majority of transported phytochelatins were unaffected by DTT.

The phytochelatin $(\gamma EC)_2G$ was also observed to be transported into tonoplast vesicles in a MgATP-dependent, vanadate-sensitive manner. In the presence of 2 mM MgATP, uptake was 2.9 ± 1.8 nmol mg^{-1} protein (20 min)$^{-1}$; this was reduced by 97% in the presence of 50 μM vanadate (data not shown).

Transport of Cd-$(\gamma EC)_3G$ Complex into Oat Root Tonoplast Vesicles

Both Cd and $(\gamma EC)_3G$ were transported into tonoplast vesicles when present as a Cd-$(\gamma EC)_3G$ complex (Table I). In the presence of Cd, transport of $(\gamma EC)_3G$ had properties very similar to those observed for $(\gamma EC)_3G$ in the absence of Cd (Table II), being totally inhibited by vanadate and slightly inhibited by NH$_4$Cl. Transport of Cd in the presence of $(\gamma EC)_3G$ was likely due to both transport via the Cd/H antiport (Salt and Wagner, 1993), driven by the V-type ATPase, and transport of Cd as a Cd-$(\gamma EC)_3G$ com-

Table II. *Characteristics of* (γEC)$_3$G *peptide transport and* CH$_3$NH$_2$ *uptake into tonoplast vesicles*

Assays were performed in the presence of 2 mM MgATP unless otherwise stated. Transport of (γEC)$_3$G and CH$_3$NH$_2$ was monitored using [^{14}C](γEC)$_3$G and [^{14}C]CH$_3$NH$_2$, respectively. Where present, potassium was as 10 mM potassium gluconate in both the vesicles and the assay mixture. Data are presented as mean ± SD of one to four independent experiments with three replicate samples per experiment, where n represents the number of values used in the mean. All values have their control in the absence of Mg subtracted. Gramicidin D, valinomycin, oligomycin, verapamil, and quinidine were all added in ethanol, giving a final ethanol concentration of 1%.

Function	Chemical	Rate of (γEC)$_3$G Transport	n	Inhibition	Rate of CH$_3$NH$_2$ Uptake	n	Inhibition
		nmol mg^{-1} protein (20 min)$^{-1}$		%	nmol mg^{-1} protein (10 min)$^{-1}$		%
Energy source (nucleoside triphosphate)	MgATP (2 mM)	22.7 ± 2.5	12	0	15.7 ± 1.6	6	0
	MgATP (2 mM) + ethanol (1%)	21.3 ± 1.4	6	6	9.2 ± 3.0	9	41
	MgGTP (2 mM)	15.3 ± 1.7	9	33	4.1 ± 1.6	9	74
	MgUTP (2 mM)	12.2 ± 1.6	6	47	1.0 ± 1.0	6	93
	MgAMP-PNP (2 mM)	0	3	100	0.3 ± 0.2	3	98
Uncouplers	NH$_4$Cl (1 mM)	22.1 ± 0.9	6	4	6.5 ± 0.7	6	58
	NH$_4$Cl (5 mM)	20.5 ± 0.7	3	10	0	3	100
	Gramicidin D (10 μg/mL)	15.0 ± 0.3	3	33	0	3	100
	K/valinomycin (0.5 μM)	15.7 ± 0.5	3	26	1.3 ± 0.7	3	86
Inhibitors	Na$_3$VO$_4$ (50 μM)	1.0	3	96	13.7 ± 1.0	3	13
	Oligomycin (10 μg/mL)	0.7 ± 0.2	3	97	3.1 ± 0.3	3	67
	Oligomycin (1 μg/mL)	10.4 ± 0.3	3	51	5.8 ± 0.5	3	37
	Oligomycin (0.1 μg/mL)	18.0 ± 0.9	3	15	6.5 ± 1.0	3	29
	Verapamil (25 μM)	19.8 ± 0.6	3	7	7.1 ± 0.8	3	23
	Quinidine (25 μM)	21.9 ± 0.3	3	−3	7.1 ± 0.2	3	22
	Ethanol (1%) NaN$_3$ absent	18.3 ± 0.7	3	14	9.8 ± 1.3	3	−7

plex. Cd transport via the Cd/H antiport is insensitive to vanadate and sensitive to NH$_4$Cl. Therefore, we were able to estimate Cd transport via the Cd/H antiport to be approximately 50% of the total Cd transported into tonoplast vesicles in the presence of (γEC)$_3$G. The remaining vanadate-sensitive (8.5 nmol mg^{-1} protein (20 min)$^{-1}$) and NH$_4$Cl-insensitive (5.8 nmol mg^{-1} protein (20 min)$^{-1}$) Cd transport is novel and probably represents Cd transported as a Cd-(γEC)$_3$G complex. This is supported by the fact that (γEC)$_3$G and Cd were transported with ratios of 1:1.7 and 1:1.5 (peptide:Cd), measured as vanadate-sensitive or NH$_4$Cl-insensitive transport, respectively. These ratios are very close to the 1:1.5 (peptide:Cd) ratio with which the synthetic Cd-(γEC)$_3$G complex was produced, supporting the conclusion that the Cd and (γEC)$_3$G are transported as a complex.

MgATP Dependence of (γEC)$_3$G Transport

Based on initial rates (0–5 min), transport of (γEC)$_3$G increased with increasing concentrations of MgATP, producing Michaelis-Menten-type kinetics. A plot of initial velocity against substrate concentration at a (γEC)$_3$G concentration of 102 μM (Fig. 3) gave a K_m for MgATP of 0.18 ± 0.03 mM and a V_{max} of 0.68 ± 0.04 nmol mg^{-1} protein min^{-1}. The uptake rate of (γEC)$_3$G in the absence of ATP and presence of 3 mM Mg was zero (Fig. 3), demonstrating the MgATP dependence of (γEC)$_3$G uptake. A repeat experiment with different vesicle and phytochelatin preparations at 55 μM (γEC)$_3$G gave a K_m of 0.19 ± 0.07 mM and a V_{max} of 0.9 ± 0.09 nmol mg^{-1} protein min^{-1}. These V_{max} rates are in agreement with the mean uptake rate of 1 ± 0.2 nmol/mg protein observed over a 10-fold range of (γEC)$_3$G concentrations and the uptake rates of 0.7 and 0.93 nmol

mg^{-1} protein min^{-1} presented in Figure 2. This is further evidence that all experiments presented were done at saturating (γEC)$_3$G concentrations.

Energy Source for (γEC)$_3$G Transport

Transport of (γEC)$_3$G was stimulated in the presence of MgATP, MgGTP, and MgUTP (Table II), with the effective-

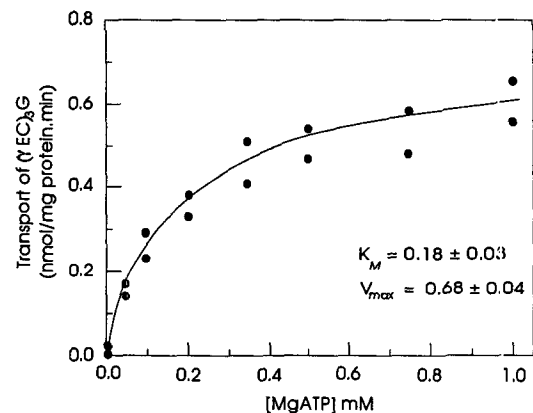**Figure 3.** Kinetics and MgATP dependence of (γEC)$_3$G transport into oat root tonoplast vesicles. (γEC)$_3$G transport was detected using [^{14}C](γEC)$_3$G as described in "Materials and Methods." (γEC)$_3$G was provided at a concentration of 102 μM. Data, with control values in the absence of MgSO$_4$ being subtracted, were plotted against [MgATP] to give Michaelis-Menten-type kinetics. Nonlinear regression using the Marquardt-Levenberg algorithm, as described in "Materials and Methods," gave a K_m for MgATP of 0.18 ± 0.03 mM and a V_{max} of 0.68 ± 0.04 nmol mg^{-1} protein min^{-1} using 16 data points representative of two experiments.

ness of the triphosphate nucleosides decreasing in the order $MgATP > MgGTP > MgUTP$. The rate of $(\gamma EC)_3G$ transport in the presence of MgGTP or MgUTP was 66 and 53% that with MgATP; this compares to 26 and 7%, respectively, for methylamine accumulation driven by the V-type ATPase. Neither transport of $(\gamma EC)_3G$ or accumulation of methylamine was detected in the presence of MgAMP-PNP (Table II), a nonhydrolyzable analog of ATP. The addition of NH_4Cl dissipated the ΔpH generated by the V-type ATPase, as monitored by methylamine; however, this treatment had no effect on the transport of $(\gamma EC)_3G$ (Table II). Gramicidin D, a proton ionophore, also dissipated the ΔpH and caused a 33% decrease in $(\gamma EC)_3G$ transport (Table II). The dissipation of the membrane potential generated by the V-type ATPase, using K and valinomycin, caused a 26% reduction in $(\gamma EC)_3G$ transport (Table II).

Inhibitors of $(\gamma EC)_3G$ Transport

The phosphate transition state analog sodium orthovanadate inhibited $(\gamma EC)_3G$ transport by 96% at 50 μM, whereas methylamine accumulation was affected only slightly (Table II). Sodium orthovanadate inhibited transport of $(\gamma EC)_3G$, giving a 50%-inhibitory concentration of 0.9 $\pm$ 0.02 μM (Fig. 4). Oligomycin, an inhibitor of mitochondrial ATPase and also of ATP-dependent daunomycin transport in canalicular membrane vesicles (Kamimoto et al., 1989), inhibited both $(\gamma EC)_3G$ transport and methylamine accumulation (Table II). Verapamil and quinidine,
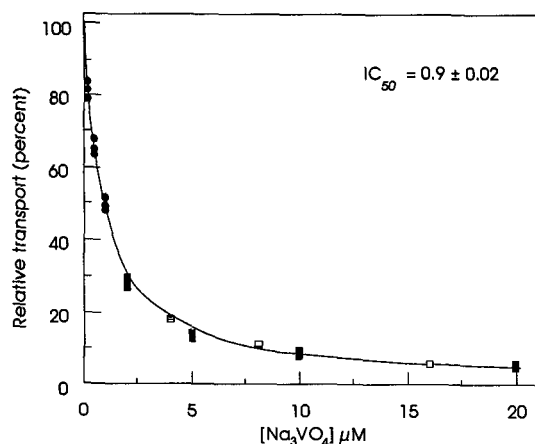

Figure 4. Sodium orthovanadate inhibition of $(\gamma EC)_3G$ transport into oat root tonoplast vesicles. $(\gamma EC)_3G$ transport was detected using [^{14}C]$(\gamma EC)_3G$ as described in "Materials and Methods." Transport activity was measured in the presence of 2 mM ATP and 3 mM $MgSO_4$ with control values in the absence of $MgSO_4$ being subtracted. The curve is a composite of three separate experiments with data plotted as a percentage of their respective control values in the absence of sodium orthovanadate. Transport rates in the absence of sodium orthovanadate for each experiment were 24.5 $\pm$ 0.5 (●), 37.7 $\pm$ 1.3 (■), and 34.2 $\pm$ 1.1 (□) nmol mg^{-1} protein (20 min)$^{-1}$. Nonlinear regression using the Marquardt-Levenberg algorithm, as described in "Materials and Methods," gave a 50%-inhibitory concentration (IC_{50}) value for sodium orthovanadate of 0.9 $\pm$ 0.02 μM using 30 data points.

inhibitors of multiple drug resistance transport proteins (for reviews, see Endicott and Ling, 1989; Gottesman and Pastan, 1993), had no effect on $(\gamma EC)_3G$ transport (Table II). In the absence of sodium azide, $(\gamma EC)_3G$ transport was only marginally inhibited and methylamine accumulation was increased only slightly (Table II).

DISCUSSION

Since $(\gamma EC)_3G$ is the only phytochelatin involved in Cd binding in oat roots, our investigations centered on its transport into isolated tonoplast vesicles. In the presence of MgATP $(\gamma EC)_3G$ was accumulated into tonoplast vesicles (Fig. 2). Using a vesicle volume of 10 μL/mg protein (Sze and Churchill, 1981) it can be calculated that $(\gamma EC)_3G$ was accumulated to a concentration 38-fold above that in solution. This suggests that $(\gamma EC)_3G$ was being actively transported against a concentration gradient. This transport was shown to be dependent on MgATP, because in the absence of either Mg (Fig. 2) or ATP (Fig. 3) transport was essentially zero. Also, in the presence of the nonhydrolyzable ATP analog AMP-PNP transport was zero (Table II). Taken together, these data suggest that transport of $(\gamma EC)_3G$ was energized by the hydrolysis of MgATP. Transport experiments done over a wide range of $(\gamma EC)_3G$ concentrations all showed very similar initial uptake rates, suggesting that all experiments were performed at saturating $(\gamma EC)_3G$ concentrations. This was also supported by kinetic experiments at 55 and 102 μM $(\gamma EC)_3G$, which gave similar V_{max}.

To confirm that we were measuring [^{14}]$(\gamma EC)_3G$ uptake into vesicles and not some radiolabeled contaminant, competition experiments were performed between labeled and unlabeled $(\gamma EC)_3G$. At a ratio of unlabeled to labeled $(\gamma EC)_3G$ of 4.3 $\times$ 10^{-4} (nmol/cpm), 5.6 $\times$ 10^4 cpm mg^{-1} protein (20 min)$^{-1}$ of labeled $(\gamma EC)_3G$ was accumulated into tonoplast vesicles. As the ratio of unlabeled to labeled $(\gamma EC)_3G$ was increased to 6.3 and then 11.5 $\times$ 10^{-4}, the amount of labeled $(\gamma EC)_3G$ accumulated in vesicles decreased to 3.5 $\times$ 10^4 and 1.5 $\times$ 10^4, respectively. This suggests that unlabeled $(\gamma EC)_3G$ can effectively compete with labeled $(\gamma EC)_3G$ for transport and supports our contention that we were measuring [^{14}C]$(\gamma EC)_3G$ in our transport assays.

Four observations suggest that transport of $(\gamma EC)_3G$ is coupled to the direct hydrolysis of ATP rather than being driven by the electrochemical potential (ΔpH + membrane potential) generated by the V-type ATPase:

1. The dissipation of ΔpH with NH_4Cl or gramicidin D or the dissipation of the membrane potential with K/valinomycin had little or no effect on the transport of $(\gamma EC)_3G$. We note here that gramicidin D and valinomycin are both small peptides that might interact directly with $(\gamma EC)_3G$ transport, causing slight inhibition. There is evidence that both gramicidin D and valinomycin are substrates for the P-glycoproteins (known to mediate multiple drug resistance in mammalian cells; for review, see Juranka et al., 1989; Gottesman and Pastan, 1993).

2. Transport of $(\gamma EC)_3G$ was sensitive to vanadate (Table II; Fig. 4), whereas the V-type ATPase, measured using

either methylamine uptake (Table II) (Churchill and Sze, 1983) or ATP hydrolysis (Salt and Wagner, 1993), was insensitive to vanadate.

3. Transport of $(\gamma EC)_3G$ was partially energized by both GTP and UTP (Table II), whereas the V-type ATPase, measured using methylamine uptake, utilized GTP poorly and UTP minimally (Table II) (Churchill and Sze, 1983).

4. Transport of $(\gamma EC)_3G$ had a K_m for MgATP of 0.17 to 0.19 mM, whereas the V-type ATPase of oat roots has a K_m of 0.25 to 0.35 mM (Wang and Sze, 1985; Randle and Sze, 1986; Salt and Wagner, 1993).

Cd transport in the presence of $(\gamma EC)_3G$ can be divided into ΔpH-dependent and -independent components, based on sensitivity to NH_4Cl (Table I). ΔpH-dependent Cd transport was insensitive to vanadate, which suggests that this Cd was being transported via the Cd/H antiport, which has been shown to be insensitive to 50 μM vanadate (13.8 versus 12.1 nmol Cd mg^{-1} protein [20 min]$^{-1}$) and sensitive to agents that dissipate ΔpH, including carbonyl cyanide p-(trifluoromethoxy)phenyl-hydrazone (22.9 versus 3.8) (Salt and Wagner, 1993) in oat root tonoplast vesicles. Therefore, Cd accumulated in a ΔpH-independent manner was probably due to a novel Cd transport system unrelated to the ΔpH-dependent Cd/H antiport. This is further supported by the sensitivity of this accumulation to vanadate (Table I). It can be calculated that this ΔpH-independent Cd transport system can accumulate Cd into tonoplast vesicles to a concentration 3.5-fold that in solution. This suggests that Cd is being actively accumulated against a concentration gradient.

The most likely explanation for this novel Cd transport is that in the presence of $(\gamma EC)_3G$, Cd is transported as a Cd-$(\gamma EC)_3G$ complex in a ΔpH-independent, vanadate-sensitive manner. This is supported by the fact that $(\gamma EC)_3G$ and Cd were transported with a ratio of approximately 1:1.5 (peptide:Cd) (measured as either vanadate-sensitive of NH_4Cl-insensitive transport); this was the ratio of the original synthetic Cd-$(\gamma EC)_3G$ complex. Cd was transported by this ΔpH-independent, vanadate-sensitive transport system with a rate of approximately 7 nmol Cd mg^{-1} protein (20 min^{-1}), which compares favorably with a rate of 14 nmol Cd mg^{-1} protein (20 min)$^{-1}$ for Cd transported via the Cd/H antiport reported by Salt and Wagner (1993). The source of the free Cd transported via the Cd/H antiport (Table I) is not known; however, a number of possibilities exist, including contamination by free Cd from the desalting step during purification of the synthetic Cd-$(\gamma EC)_3G$ complex and dissociation of the synthetic Cd-$(\gamma EC)_3G$ complex, either spontaneously or due to some interaction with the vesicles. The presence of free Cd in these experiments is puzzling; however, it does not alter the major finding that in the presence of $(\gamma EC)_3G$, Cd is transported in a novel ΔpH-independent, vanadate-sensitive manner.

Phytochelatins are only produced in significant amounts on exposure to Cd and various other metals. However, tonoplast vesicles prepared from roots of oat seedlings grown in the presence or absence of Cd had the same ability to accumulate, over 20 min, either Cd-$(\gamma EC)_3G$ or $(\gamma EC)_3G$ (data not shown). This observation does not preclude the possibility that there are changes in the initial rates of transport, an issue that requires further work. It also remains to be determined what the in vivo substrate is for the phytochelatin transport system described here. However, it would appear that in vitro $(\gamma EC)_2G$, $(\gamma EC)_3G$, and the Cd-PC complex Cd-$(\gamma EC)_3G$ are all transported.

CONCLUSION

We have described the MgATP-dependent, vanadate-sensitive transport of $(\gamma EC)_3G$ into isolated tonoplast vesicles from oat roots. Both Cd-$(\gamma EC)_3G$ and $(\gamma EC)_2G$ appear to be substrates for this transport system. This provides further support for the idea that the vacuole plays a central role in the sequestration and detoxification of Cd (as a Cd, sulfide-phytochelatin complex) in plant tissues exposed to moderate to high levels of Cd. Due to the properties of this transport system, we suggest that it may belong to the superfamily of ABC-type transporters.

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