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Brassica juncea Produces a Phytochelatin-Cadmium-Sulfide Complex[1]

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ABSTRACT

Phytochelatins (PCs) are enzymically synthesized peptides produced in higher plants and some fungi upon exposure to heavy metals. We have examined PC production in the Se-tolerant wild mustard *Brassica juncea* and found that it produces two types of PC-Cd complexes with the same characteristics as those from fission yeast *Schizosaccharomyces pombe*, including a high molecular weight PC-Cd-sulfide form.

Heavy metals, a group of elements including Cd, Cu, Zn, Bi, Ni, Hg, and Pb, are toxic to all organisms at varying concentrations. In response to those elements, animal cells and some fungi, including *Saccharomyces cerevisiae* and *Neurospora crassa*, synthesize metal-chelating proteins called metallothioneins (11, 13). In contrast, the fission yeast *Schizosaccharomyces pombe*, algae, and higher plants synthesize enzymically produced peptides as a result of exposure to these metals. These metal-binding peptides have been called PC,[2] cadystins, Cd-peptides, $\gamma(EC)_n$-G, and γ-glutamyl peptides; they will be referred to here, as in recent reviews (24, 28, 30), as PCs. PCs were first discovered in the fission yeast as components of metal-containing complexes from Cd-induced cells (19, 20); it was demonstrated that previous reports of metallothionein-like proteins from plants could be attributed to PCs (9) and that PCs were produced by all higher plants tested (7). PCs have also been shown to exist in the algae *Chlorella fusca* (6) and the yeast *Candida glabrata*, which produces both PCs and a metallothionein (17, 18).

PC synthesis is induced by a variety of metals, including Cd, Cu, Zn, Pb, Hg, Ni, Bi, Ag, and Au; in addition, multiatomic anions including SeO_4^{-2}, SeO_3^{-2}, and AsO_4^{-3} also cause PC synthesis (10). PCs have been shown to bind Cd and Cu directly (19, 25) and are believed to bind Pb and Hg by competition with Cd (1). The PCs produced by *S. pombe* have a structure identical to those of plants, consisting of repeating units of γ-glutamylcysteine followed by a single C-terminal glycine, with the number of repeating units ranging from 2 to 11 (9, 14, 15). The number of repeating units varies with the conditions of Cd exposure (12, 16). PCs are synthesized enzymically by PC synthase, a 25-kD protein that removes a γ-glutamylcysteine moiety from one molecule of GSH and couples it to another GSH. PC synthase was purified from a variety of plant species and was found to be produced constitutively in the absence of metal induction (8).

In addition to the PC-Cd complex isolated from *S. pombe* by Murasugi *et al.* (19), the same authors later described a second complex from the same organism, characterized by a higher apparent mol wt (approximately 10,000 M_r), which was composed of PCs, Cd, and acid-labile S^{-2} (21). This complex, referred to as the HMW PC-Cd complex, was essential for metal tolerance; several mutant strains deficient in production of the HMW complex were hypersensitive to Cd (22). The requirement for this additional form may be due to its altered properties relative to the LMW PC-Cd complex, including increased acid stability and an enhanced affinity for metals (25, 27). PC-Cd-S^{-2} complexes from *Candida glabrata* have been shown to have electronic and optical properties different from bulk CdS (3, 4). The HMW complex has not been a common feature in extracts from metal-induced plant cells, although Steffens *et al.* (5, 31) reported acid-labile S^{-2} and sulfite in PC-metal complexes isolated from tomato cells, and Verkleij *et al.* (33) have reported increased levels of S^{-2} in PC-Cd complexes from metal-resistant isolates of *Silene vulgaris*. Recently, however, a HMW PC-Cd complex from tomato has been reported (26).

Brassica juncea Czern L., a wild mustard, has been shown to tolerate high concentrations of Se and to accumulate high levels of this toxic element (2). High rates of sulfur uptake and assimilation have been reported in this organism and have been implicated in Se uptake. Using a wild mustard isolated from metal- and sulfate-rich soil, we have investigated whether the response of *B. juncea* to Cd is accompanied by incorporation of acid-labile S^{-2} into a HMW PC-Cd-S^{-2} complex.

MATERIALS AND METHODS

Plant Germination and Growth

Brassica juncea Czern L. and tomato (*Lycopersicon esculentum* cv Rutgers) seeds were surface sterilized with 0.5% sodium hypochlorite/1% SDS, washed with sterile H_2O, then

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[2] Abbreviations: PC, phytochelatin; HMW, high mol wt; LMW, low mol wt; S^{-2}, sulfide.

placed on 70-mL sterile Murashige and Skoog salts medium (Gibco) (containing 3% sucrose, 1 μg/mL nicotinic acid, 10 μg/mL thiamine HCl, 10 μg/mL pyridoxine HCl, 100 μg/mL myoinositol, and 1% agar) for germination. Plants were grown in a Nor-Lake illuminated growth chamber at 2700 lux (16 h/d) and induced with 3 mL sterile $CdCl_2$ added to the surface of the agar medium for 7 d subsequent to exposure of the third set of true leaves, 8 to 10 d after germination.

S. pombe Strains and Growth

S. pombe strain Sp223 (h^{-s} *ade6.216 ura4.294 leu1.32*) was grown in yeast glucose medium (5 g yeast extract [Difco], 20 g glucose/L) at 30°C and induced with 200 μM $CdCl_2$ at A_{595} = 0.4. After 30 h of growth, the cells were harvested by centrifugation and washed twice with 50 mM Tris-HCl, pH 8.0, then stored at −20°C.

Preparation of Cell-Free Extracts

Plants were removed from growth medium, carefully washed with H_2O to remove traces of agar, and frozen in liquid N_2. The tissue was ground to a fine powder and extracted with 50 mM Tris-HCl, pH 8.0, 1 mM *p*PMSF on ice. The extracts were cleared by centrifugation and stored at −80°C until analysis.

S. pombe cells were resuspended in a minimal volume of 50 mM Tris-HCl, pH 8.0, then disrupted by vortexing with glass beads. The extracts were cleared by centrifugation and stored at −80°C until analysis.

Protein Determination

Protein was determined by dye binding assay with dye concentrate purchased from Bio-Rad Laboratories. BSA served as a standard.

Gel Filtration Assay

Plant extracts containing 4 mg of protein (2 mg for *S. pombe*) were combined with 60 μL 80% glycerol and 10 μL 1 M DTT, then brought to 1 mL with 50 mM Tris-HCl, pH 7.8. For experiments involving radioactive tracer, 2.5 μL 0.1 mCi/mL $^{109}CdCl_2$ (NEN, 0.4 Ci/mmol) was added to this sample, followed by incubation on ice for 20 min. The sample was then loaded on a 1.8 × 90 cm column of Sephadex G-50 equilibrated with 50 mM Tris-HCl, pH 7.8. The column was developed with 50 mL/h of this buffer and 2-mL fractions were collected. ^{109}Cd profiles were determined by liquid scintillation counting with Beckman Ready Micro cocktail.

PC Purification and Composition Analysis

Cell-free extracts were applied to a DEAE-Sephadex column equilibrated with 50 mM Tris-HCl, pH 7.8. After washing with 2 bed volumes of buffer, the column was developed with a linear gradient of 0.1 to 0.5 M KCl. PC-containing fractions, as determined by a trial run with ^{109}Cd as a tracer, were combined and concentrated by vacuum centrifugation. The samples were then separated on the G-50 column described above and HMW- and LMW-containing fractions were pooled and concentrated. Each concentrated sample was desalted by extensive dialysis against 0.5 mM Tris-HCl, pH 8.0, 1 μM $CdCl_2$ using a Spectrum Molecularporous membrane with a mol wt cut-off of 500. Each sample was then lyophilized to dryness in preparation for amino acid composition analysis.

Amino acid composition analysis was performed by AAA Laboratories (Redmond, WA). Each sample was subjected to complete acid hydrolysis, derivatization, and separation by ion-exchange HPLC. Cysteine was determined as cysteic acid after performic acid oxidation.

S^{-2} Assay

S^{-2} in pooled G-50 column fractions was measured by the method of Rabinowitz (23). Lyophilized samples were brought to 0.7 mL with H_2O. One-half milliliter 1 M ZnOAc followed by 0.1 mL 6% (w/v) NaOH were added to each, after which all samples were vortexed for 10 s. One-quarter milliliter of *N,N'*-dimethyl-*p*-phenylene diamine HCl (0.1% in 6 N HCl) was added, the samples were swirled until clear, and 0.1 mL $FeCl_3$ (0.31% in 0.6 N HCl) was added followed by vigorous vortexing. After 30 min at room temperature, the A_{670} was measured in a Beckman DU-62 spectrophotometer. Na_2S served as the standard.

RESULTS

B. juncea Produces a HMW Cd-Containing Complex

B. juncea is known to have a relatively high rate of sulfur assimilation; in fact, it is this characteristic that is believed partly responsible for this species' high Se uptake (2). To determine if this vigorous sulfur uptake and assimilation was reflected by incorporation of acid-labile sulfide ion into HMW complexes with PCs and Cd, an extract from *B. juncea* induced by 100 μM $CdCl_2$ was assayed by gel filtration (Fig. 1A). Extracts of other Cd-induced species were studied for comparison: *S. pombe*, as the organism in which synthesis of the HMW PC-Cd complex has been most thoroughly studied, and *L. esculentum*, as a species reported to include S^{-2} and sulfite in PC-Cd complexes produced by tissue culture cells (2, 31).

In contrast to *L. esculentum*, which under these induction conditions produced no PCs detectable by gel filtration (data not shown), *B. juncea* produced two Cd-containing peaks that correspond in mobility to the LMW and HMW complexes observed in extracts from *S. pombe* (Fig. 1B). The level of Cd in the extract from *B. juncea* was approximately twice that in the *L. esculentum* extract (data not shown), implying that differences in levels of observed PC synthesis are not due solely to differences in ^{109}Cd specific activity. The concentrations of $CdCl_2$ used in these experiments resulted in no visible toxic effects on the seedlings. When *L. esculentum* was induced with 500 μM $CdCl_2$, PC production was increased, but no HMW complex was observed (Fig. 1C). This observation does not conflict with reports of S^{-2} generation from PCs from *L. esculentum*, because LMW PCs from *S. pombe* have also been shown to contain acid-labile S^{-2} in amounts lower than those in HMW PCs (25).

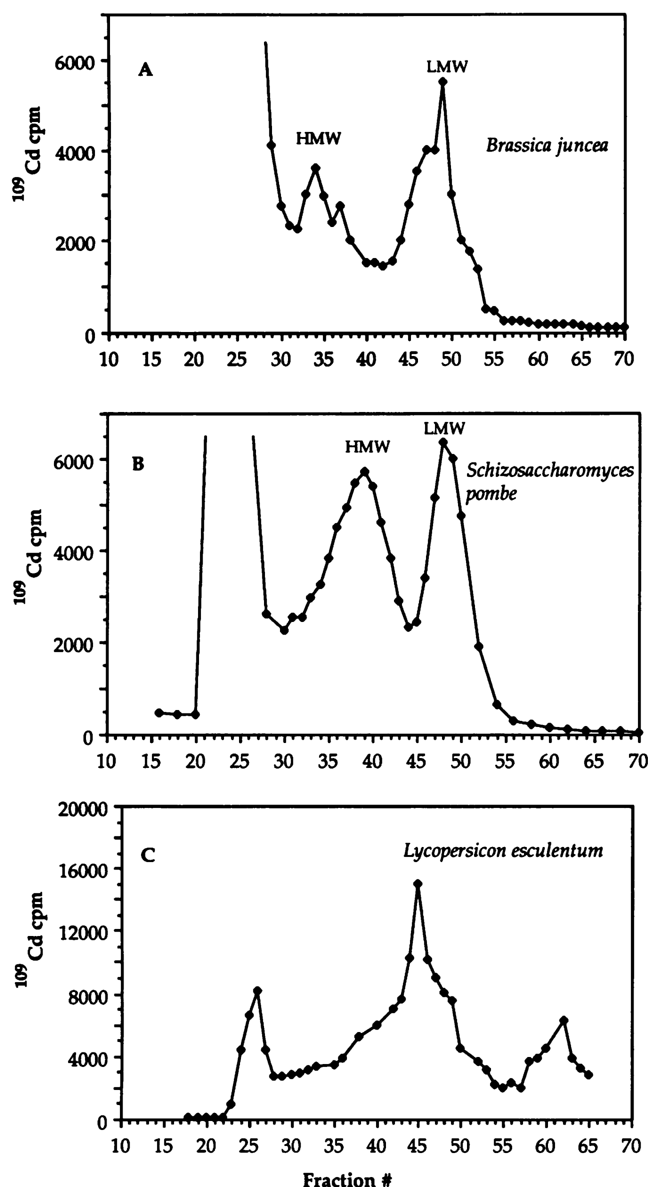

Figure 1. Cell-free extracts from $CdCl_2$-induced *B. juncea* (induced with 100 μM $CdCl_2$) (A), *S. pombe* (induced with 200 μM $CdCl_2$) (B), and *L. esculentum* (induced with 500 μM $CdCl_2$) (C) were labeled with 0.25 μCi ^{109}Cd and subjected to gel filtration chromatography.

Analysis of Cd, S^{-2}, and Amino Acids in the HMW Cd Complex

One salient difference between the LMW and HMW PC-Cd complexes found in *S. pombe* is the presence of acid-labile S^{-2} in the HMW complex at near-stoichiometric levels with the Cd (3, 4, 25). To determine if the HMW and LMW Cd-containing peaks in the gel filtration analysis of the *B. juncea* extract bore the same relationship with each other as do those from *S. pombe,* the S^{-2} content of each of the peaks was determined. Crude extracts were subjected to gel filtration as above (without inclusion of ^{109}Cd) and the appropriate fractions were pooled, lyophilized, and assayed for S^{-2} content (Table I). Samples from *S. pombe* were treated in the same manner to determine how the composition of HMW PCs, known from analysis of purified samples (25), would be reflected in this crude preparation. The pooled HMW fractions from *S. pombe* resulted in a S^{-2}/Cd ratio of 2.0, a value two- to threefold higher than those reported elsewhere (3, 4, 25). We attribute this difference to contaminating substances in the crude sample; the values are best used as a comparison between the HMW and LMW complexes. The HMW fractions from *B. juncea* yielded a S^{-2}/Cd ratio of 1.0, and the ratios from the pooled LMW fractions are lower in both cases by a factor of 2.5, indicating that the differences in mobility correlate with changes in complex composition.

As verification that the S^{-2}-containing complex from *B. juncea* included PCs, the complex was partially purified by ion-exchange chromatography and gel filtration, then subjected to amino acid composition analysis (Table II). As expected for PCs, the main amino acid components, cysteine, glutamate, and glycine, are present in ratios similar to those obtained from the *S. pombe* PCs treated in an identical manner, indicating that PCs are the main peptide components of the partially purified Cd- and S^{-2}-containing complex. Previous reports of highly purified PCs from *S. pombe* yielded an average peptide length for the HMW complex of $n = 3.8$ (25). The relatively low average size of the peptides in our samples is probably a result of contaminating glycine-containing peptides in the preparation. In addition, values for glutamate and aspartate are artificially higher due to the acid hydrolysis procedure that results in deamination of glutamine and asparagine.

DISCUSSION

The presence of a S^{-2}-containing HMW complex in extracts of *B. juncea* is significant in that only recently has a plant

Table I. *S^{-2} and Cadmium Contents of HMW and LMW PC Pools from S. pombe and B. juncea*

Pooled gel filtration column fractions containing HMW and LMW PC-Cd complexes from Cd-induced *S. pombe* and *B. juncea* were assayed for total Cd and for acid-labile S^{-2}.

Organism	HMW			LMW		
	S^{-2}	Cd	S^{-2}/Cd	S^{-2}	Cd	S^{-2}/Cd
	nmol			*nmol*		
S. pombe	33.5	16.4	2.0	9.5	12.0	0.8
B. juncea	8.2	7.9	1.0	3.9	9.3	0.42

Table II. *Amino Acid Composition of HMW PCs from S. pombe and B. juncea*

HMW PCs isolated from *S. pombe* and *B. juncea* as described in "Materials and Methods" were subjected to total acid hydrolysis and amino acid composition analysis. Amino acid content is described either by percentage or normalized to glycine content.

Amino Acid	*S. pombe*		*B. juncea*	
	%	*versus* Gly	%	*versus* Gly
Ala	2.8	0.18	3.5	0.23
Arg	1.4	0.09	0	0
Asp	5.8	0.39	7.3	0.49
Cys	25	1.8	25	1.8
Glu	34	2.3	31	2.1
Gly	15	1	15	1
His	0.4	0.03	0.5	0.03
Ile	1.5	0.1	2.3	0.15
Leu	2.2	0.15	3.0	0.2
Lys	1.9	0.13	2.4	0.16
Pro	1.6	0.11	1.5	0.1
Phe	0.6	0.04	0	0
Ser	2.9	0.19	3.3	0.22
Thr	1.7	0.11	2.1	0.14
Val	2.6	0.17	2.4	0.16

been reported to contain this type of PC complex (26). *S. pombe* and *C. glabrata* both incorporate S^{-2} into PC-Cd complexes and, in *S. pombe*, formation of these HMW complexes has been demonstrated to be essential for wild-type levels of metal tolerance. Verkleij *et al.* (33) observed a correlation between levels of PC-associated S^{-2} and Cd tolerance in *Silene vulgaris* plants, but the apparent mobility of the single PC-Cd complex in gel filtration remained unchanged. Formation of this distinct HMW PC complex in *B. juncea* may indicate that some of the genetic determinants responsible for formation of the HMW complex in fission yeast have functional analogs in this plant. The characteristics of *B. juncea* that might contribute to the formation of this HMW complex are unknown. The high levels of sulfur uptake by this plant may play an important role, because Cd exposure and the resulting burst of PC synthesis have been reported to deplete GSH faster than biosynthesis can replenish it (29, 32). Possibly, an increased flux of sulfur through the assimilatory sulfate reduction pathway might enable both a higher rate of PC synthesis and production of S^{-2} for incorporation into PC-Cd-S^{-2} complexes. Production of these complexes, with their demonstrated advantages in stability over the LMW complex, could contribute to higher metal tolerance by more effective sequestration.

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