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Phytochelatins are only partially correlated with Cd-stress in two species of *Brassica*

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Abstract

Synthesis of phytochelatins (PCs) is triggered in response to cadmium (Cd) and Cd-PC complexes are non-toxic. However, the relationship between PCs and Cd-tolerance is not clear. *Brassica napus* and *Brassica juncea* were compared to determine if the level of Cd-induced stress (as measured by accumulation of Cd, biomass, chlorophyll content and photosystem II efficiency) is related to the accumulation of PCs. While both species contained similar concentrations of Cd, *B. juncea* was more Cd-tolerant over the lifetime of the plant. After short-term exposure (4–7 days) to Cd, concentrations of chlorophyll declined in both species but maximum photosystem II efficiency was unaffected. Both species produced PCs in response to the lowest Cd treatment. With 50–200 μM Cd in solution, roots of *B. juncea* produced about four times more PCs than did *B. napus* but PCs in the leaves of *B. juncea* were about half that of *B. napus.* Concentrations of PCs did not increase with concentrations of Cd in the tissues and the only stress correlated with PC accumulation was reduced chlorophyll content. Differential tolerance cannot be explained in terms of differential Cd-induced stress, differential Cd or concentrations of PCs in the leaves; some other mechanism(s) offers *B. juncea* increased protection from Cd-toxicity.

Keywords: *Brassica juncea*; *Brassica napus*; Cadmium; Chlorophyll; Fluorescence; Phytochelatins; Stress

1. Introduction

Phytochelatins (PCs) have the general structure (γ-Glu-Cys)$_n$-Gly, where $n = 2$–11 [1]. Metal ions form non-toxic complexes with PCs through interactions with the thiol group of Cys. Phytochelatins are synthesized from glutathione (GSH) by an enzyme, γ-glutamylcysteine dipeptidyl transpeptidase, commonly referred to as PC synthase [2]. This enzyme is activated by a number of metal ions; however, PC complexes formed in vivo have been associated only with Cd, As or Cu (reviewed in Ref. [3]). Cytosolic Cd-PC complexes acquire S^{2-}, perhaps at the tonoplast, and form high molecular weight complexes [4] that move into the vacuole [5]. Due to acidic conditions in the vacuole, the complex dissociates and Cd^{2+} becomes chelated by vacuolar organic acids such as citrate, oxalate and malate [6]. It is not clear whether apo-PCs are degraded in the vacuole or whether they return to the cytoplasm and shuttle other metal ions to the vacuole.

While PCs play a role in detoxification and compartmentalization of Cd, the contribution of PCs to Cd-tolerance is not clear. The most powerful evidence in support of PCs comes from studies involving *Arabidopsis* mutants. In a series of Cd-sensitive mutants that varied in their ability to accumulate PCs, the amount of PCs positively correlated with tolerance to Cd [7,8]. In addition, cultured cells of azuki beans (*Vigna angularis*) that were Cd-sensitive lacked PC synthase activity [9]. Overexpression of the PC synthase gene [10] and the glutathione reductase gene [11] from *Escherichia coli* in *Brassica juncea* resulted in increased biosynthesis of PCs and glutathione, respectively, and increased Cd-tolerance. One might not always expect concentrations of PCs to be higher in metal-tolerant plants as compared to metal-sensitive relatives; it may depend on how much Cd is taken up by the plants. For example, in *Silene vulgaris*, the concentrations of PCs produced by Cu-sensitive and Cu-tolerant populations did not differ when the plants were experiencing equal Cu-induced stress (although it took more Cu to stress the tolerant population) [12]. With 10 μM Cu in solution, the Cu-sensitive plants had a higher concentration of PCs, and experienced more Cu-induced stress, than did the Cu-tolerant plants. A similar pattern was found in

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cell cultures of *Cucumis sativus*; at any given concentration of Cd in solution, the concentrations of both PCs and Cd were highest in cells of the sensitive line [13]. This relationship breaks down under severe metal-toxicity; above a certain threshold of toxicity, the concentrations of PCs decline in both sensitive and tolerant plants presumably due to irreparable damage to the cell [12].

Other evidence, however, questions the relationship between PCs and Cd-tolerance. In three of five transgenic lines of *Arabidopsis* treated with Cd, overexpression of PC synthase resulted in an approximate doubling of PC synthesis and an approximate 75% reduction in root length as compared to wild type *Arabidopsis* [14]. This PC-induced hypersensitivity to Cd was thought to arise from direct toxicity of the PC molecule, and could be corrected by addition of GSH to the medium [14]. In natural ecotypes of *S. vulgaris*, Cd-sensitive plants produced more PCs than did Cd-tolerant plants across a range of concentrations of Cd [15,16]. In Cd-tolerant *Thlaspi caerulescens*, the amounts of PCs were less than those of a Cd-sensitive relative, *T. arvense*, despite having accumulated more Cd [17]. It has been shown that PCs are not an effective mechanism of Cd-tolerance under conditions of chronic exposure [18,19] possibly due to the corresponding high demands for sulphate reduction.

Clearly, the presence of PCs alone is not enough to confer Cd-tolerance. A better understanding of the protective role of PCs may arise from evaluating Cd-induced production of PCs in tandem with Cd-induced stress responses. Given the relationship between Cd-induced PC synthesis and Cd-induced stress, one must examine PC production over a range of Cd-induced stresses, especially when investigating its relative role in Cd-sensitive and Cd-tolerant plants [12].

Plant stress has been measured using a number of variables including delayed or stunted growth, chlorosis, increased rates of respiration, reduced rates of photosynthesis and reduced photosynthetic efficiency. Variables such as biomass and reproductive success represent integrated plant responses to the stressor whereas measures of photosynthetic efficiency represent a more immediate (direct or indirect) stress response [20]. Cadmium, among other stressors, has been shown to reduce photosystem II (PSII) maximum photochemical efficiency [21,22], which can be quantified through measures of chlorophyll fluorescence yield. Specifically, the ratio of F_v/F_m (variable fluorescence/maximal fluorescence) is a direct measure of the efficiency of PSII photochemistry [20]. Under stress-free conditions, F_v/F_m is fairly constant ($\sim$0.8); most light energy is converted to chemical energy, which results in a flow of electrons from PSII to PSI [23]. When under stress, a greater proportion of light energy is not quenched by photochemistry through electron transfer; it is dissipated as heat or light (fluorescence) and F_v/F_m declines.

In this study, long-term (lifetime) responses of *B. juncea* and *B. napus* to Cd will be compared to obtain a relative measure of their respective levels of Cd-tolerance. The responses of both species to short term (up to 1 week) exposure to Cd will be compared to determine if the accumulation of PCs is related to the level of Cd-induced stress. Stress will be measured in terms of biomass, accumulation of Cd, chlorophyll content and PSII efficiency. If PCs are related to Cd-tolerance, the more Cd-tolerant species will have the highest concentration of PCs and will show fewer symptoms of Cd-stress. Alternatively, if PC production is simply a reflection of internal [Cd], then the two species should not differ in the concentration of PCs at any level of Cd-induced stress.

2. Materials and methods

2.1. Plant material

Seeds of *B. juncea* (L.) Czern. (AC-Vulcan 95-ISOL) and *B. napus* L. (AC-Excel 95), supplied by Dr. G. Rakov at Agriculture and Agri-Food Canada, Saskatoon, germinated on moistened filter paper. Two-day-old seedlings were transferred into pots filled with industrial sand (99.8% quartz; 1–2 mm diameter grains) and placed in a greenhouse. Pots were watered once daily with 1/2 strength Hoagland's nutrient solution [24] set to pH 5.6. Twelve-day-old plants were transferred into 1.4 l mason jars filled with 1/2 strength Hoagland's nutrient solution (pH 5.6) in a growth chamber set to 20 °C and illuminated for 16 h per day (180 ± 15 μmol m^{-2} s^{-2}). After addition of $CdCl_2$, three replicates of each treatment were maintained daily at pH 5.6 to ensure Cd in solution was available to the plants.

2.2. Long-term exposure to Cd

Twenty two-day-old plants were treated with 0–400 μM $CdCl_2$ added to fresh nutrient solution. Solutions were replaced weekly. Each plant was harvested when its last seed pod was fully dried. As leaves dropped from each plant, they were collected and stored at 60 °C until the remainder of the plant was harvested. All tissues were dried to constant weight at 60 °C. The dry weights of roots, shoots (stem plus leaves) and seeds (total mass per plant) were measured.

2.3. Short-term exposure to Cd

Twenty two-day old plants were treated with 0–200 μM $CdCl_2$ added to fresh nutrient solution. Plants were harvested 4 or 7 days post Cd-treatment depending on the type of analysis done.

2.3.1. Biomass and Cd-accumulation

After 4 days of exposure to Cd, the roots were washed for 30 min in 1 mM $CaSO_4$; cation exchange between Ca^{2+} and Cd^{2+} removes Cd that may have been adsorbed to the root surface. Weights of roots and shoots, which had been dried at 60 °C to constant weight, were recorded. Samples were acid-digested using a modified EPA SW-846 test method 3051 [25]. Dried plant tissue was cut into fine pieces (1–2 mm) and 0.10–0.12 g of each sample was placed in a test tube with 1.0 ml of Omni-Trace® nitric acid (EM Science) and heated to 85–90 °C. Reagent (nitric acid and distilled water) blanks were processed to ensure Cd was not added during sample preparation. Tomato leaves (NIST Standard #1573a) were also processed to quantify recovery of Cd during the extraction procedure. Once the