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Effects of phosphorus supplied in soil on subcellular distribution and chemical forms of cadmium in two Chinese flowering cabbage (*Brassica parachinensis* L.) cultivars differing in cadmium accumulation

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ABSTRACT

Differences in the subcellular distribution and chemical speciation of Cd between two Chinese flowering cabbage (*Brassica parachinensis* L.) cultivars, Lubao70 (LB70, low-Cd cultivar) and ChixinNO.4 (CX4, high-Cd cultivar) were investigated under various soil Cd and P treatments. Subcellular fractionation of Cd-containing tissues showed that a higher proportion of Cd was bound to the cell wall fraction of LB70 than that of CX4, indicating that Cd compartment functioned better in LB70. Compared to CX4, LB70 had lower proportions of Cd in inorganic form and water-soluble form, but higher proportions of Cd in proteins/pectates integrated form, implying that the low Cd accumulation in LB70 is associated with the low *in vivo* mobility of Cd. In both cultivars, shoot and root Cd concentration and translocation of Cd from the roots to the shoots obviously decreased with increasing soil P level. It was found that phosphorus (P) played important roles in Cd uptake and translocation via the processes involved in bonding Cd to the cell wall fraction and forming Cd-phosphate complexes. It is suggested that use of low-Cd cultivars in conjunction with P supply is a much useful way to reduce the pollution risk of Cd in the food chain.

1. Introduction

In recent years, the contamination of agricultural soil with cadmium (Cd) has become a significant environmental problem in large areas of the globe (Nicholson et al., 2003; Murtaza et al., 2008; Wong et al., 2002) with Cd becoming a threat to human health when it passes up the food chain (Satarug et al., 2003). Various soil clean-up techniques have been proposed and have proved effective in some areas (Mulligan et al., 2001). However, employing these on polluted farmland in many developing countries is problematic because of the time they take and/or the high costs of remediation (Salt et al., 1995a; Ebbs et al., 1997). Furthermore, farmers cannot afford to leave agricultural soils fallow during treatments due to the high demand for their food products.

The selection of crop cultivars with a genetic tendency to accumulate low Cd concentrations in their edible parts is currently proposed as a practical solution, reducing the risk of soil Cd entering the human food chain without land lying fallow (McLaughlin et al., 1994; Wang et al., 2009). This cultivar selection strategy is based on the fact that significant differences exist between cultivars of the same species in the uptake and subsequent distribution of trace elements (Grant et al., 2008). Indeed, wide variations in the accumulation of Cd have been documented among cultivars of not only many staple crops (McLaughlin et al., 1994; Clarke et al., 2002), but also in several leafy vegetables (Wang et al., 2009; Liu et al., 2010). Scientists recognize that the development of a cultivar selection strategy requires a thorough understanding of underlying Cd accumulation processes at the genetic, molecular, biochemical, physiological and agronomic levels (Tanhuanpää et al., 2007; Grant et al., 2008; Wang et al., 2009). Metal transporters and chromosome ploidy level have been reported to play important roles on the Cd accumulation of plants (Elizabeth and Guerinot, 2006; Kováčik et al., 2010a,b). Except for these heritable factors, substantial studies found that plant nutrients could also influence the Cd accumulation of plants through affecting the activity and bioavailability of Cd in the soil–plant environment. Hence, the interactions of Cd with some key nutrient elements in soils or in soil–plant systems have been studied (Bolan et al., 2003; Yu and Zhou, 2009; Sarwar et al., 2010). Phosphorus (P) makes up about 0.2% of plant dry weight and is a macronutrient which frequently limits plant growth (Schachtman et al., 1998). It is a component of key

Abbreviations: AP, available P; DTPA, digestion and diethylene triamine pentaacetic acid; FAAS, flame atomic absorption spectrophotometry; TF, translocation factor.

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molecules such as nucleic acids, phospholipids and ATP; it also plays a role in controlling key enzymic reactions and thus influences the regulation of metabolic pathways (Theodorou and Plaxton, 1993). Moreover, the addition of P-based materials to soils could influence the bioavailability of heavy metals such as Pb, Cd and Zn (Zwonitzer et al., 2003; Bolan et al., 2003; Yu and Zhou, 2009). However, there was no well-documented evidence to identify the intrinsic mechanism for the role of P nutrition in developing plant tolerance against Cd (Sarwar et al., 2010).

The Chinese flowering cabbage (*Brassica parachinensis* L.) is an important leafy vegetable of Southeast Asia, which is cultivated and consumed during the majority of the year, as well as being abundantly exported to Europe, America, Australia and other regions. Previously, we have found it to be vulnerable to Cd contamination from the soil, with genotype-dependent differences of up to four fold between shoot Cd concentrations among 31 cultivars. It has been reported that the biological activity of Cd in plants is associated with its subcellular distribution and its chemical forms, both of which can greatly affect the amount of free Cd ions in plant cells and so potentially influence the migration and accumulation of Cd throughout the plant (Wang et al., 2009; Zhang et al., 2009). While inter-species variation in the subcellular distribution and chemical speciation of Cd has been investigated in some detail (Wang et al., 2008; Zhang et al., 2009), intra-species differences have received much less attention, and there is little information available on the influence of P on either of these parameters in plants.

We therefore sought to determine the subcellular distribution and chemical forms of Cd in both low- and high-Cd accumulating Chinese flowering cabbage cultivars and to study the influence of P on these variables.

2. Materials and methods

2.1. Plant material

Two cultivars (with the same ploidy level), cv. Lubao70 (LB70) and cv. Chixin-NO.4 (CX4), were selected from 31 Chinese flowering cabbage cultivars used in our previous studies. When grown in soil containing cadmium (Cd) concentrations varying from 0.114 to 1.127 mg kg^{-1}, the shoot Cd concentrations of LB70 were consistently lower (by at least 65%) than those of CX4. Therefore, LB70 and CX4 were identified as low-and high-Cd cultivars, respectively.

2.2. Soil preparation and experiment design

The original soil used in this study was collected from an experimental garden in the suburbs of Qingyuan city (111°55′E, 23°30′N), Guangdong province, China. It was air-dried and then ground with a wooden roller and passed through a sieve (5 mm mesh) for pot experiments. Prior to its use in experiments, the main parameters of the soil were measured using routine analytical methods for testing soil agricultural chemistry (Lu, 2000). The soil pH was 5.63; its organic matter content, total N, total P, available P and available K were 1.38%, 1.42 g kg^{-1}, 0.26 g kg^{-1}, 5.5 mg kg^{-1}, and 39.1 mg kg^{-1}, respectively. Total and extractable concentrations of Cd in the soil were determined by flame atomic absorption spectrophotometry (FAAS, Hitachi Z-5300, Japan), followed by mixed-acid (HNO_3–$HClO_4$–HF) digestion and diethylene triamine pentaacetic acid (DTPA) extraction (Amacher, 2001), respectively. The concentrations of total Cd and DTPA-extractable Cd were 0.189 and 0.095 mg kg^{-1}, respectively.

Two Cd treatments, Cd_1 (Cd = 0.529 mg kg^{-1} DW soil) and Cd_2 (Cd = 1.182 mg kg^{-1} DW soil), were jointly applied with three available P (AP) treatments, P_1 (AP = 13.7 mg kg^{-1} DW soil), P_2 (AP = 56.2 mg kg^{-1} DW soil) and P_3 (AP = 108.5 mg kg^{-1} DW soil). The P_1, P_2 and P_3 treatments were achieved by adding KH_2PO_4 to original soil at 175, 877 and 1974 mg kg^{-1} DW soil, respectively. The Cd_1 and Cd_2 treatments were obtained by mixing the original soil with a heavily Cd-contaminated soil (without other heavy metal contaminations) in ratios of 163:1 and 62:1, respectively. The range of Cd concentrations in the resultant soils was representative of most reported cases of Cd contamination of agricultural soils in China (Wang et al., 2001).

A pot experiment was conducted in a greenhouse at Sun Yat-sen University, Guangdong province, China. It consisted of a randomized complete block design with three replicate pots per treatment. Each pot (22 cm in diameter and 18 cm in height), was filled with 2.5 kg prepared soil, and then N (800 mg pot^{-1} $CO(NH_2)_2$) and K fertilizer (500 mg pot^{-1} K_2SO_4) were applied prior to a 2 week equilibration period. In October 2009, 20 seeds were sown in each pot and 2 weeks after germination, seedlings were thinned to four per pot. The plants were regularly watered to maintain moderate soil moisture. Harvesting of both shoots and roots was carried out after a 40 day growth period. Plant materials were divided into three portions in order to investigate the Cd subcellular distribution, examine the chemical forms of Cd and determine the total Cd concentrations of samples, respectively. Each portion was weighed and then frozen in liquid N_2 until use.

2.3. Separation of tissue fractionations

Cells were separated into three fractions (the cell wall fraction, the soluble fraction, and the organelle-containing fraction) according to the method reported by Wang et al. (2009). Frozen materials were homogenized in cooled extraction buffer (50 mM Tris–HCl, 250 mM sucrose and 1.0 mM $C_4H_{10}O_2S_2$, pH 7.5) with a chilled mortar and a pestle. The homogenate was sieved through nylon cloth (80 μm), and liquid was squeezed from the residue. The residue was washed twice with homogenization buffer; as it contained mainly cell walls and cell wall debris it was designated as the 'cell wall fraction'. The filtrate was centrifuged (20 000*g*, 45 min). The supernatant and pellet were designated the 'soluble fraction' (including vacuoles) and the 'organelle containing fraction' (excluding vacuoles), respectively. All of the above steps were performed at 4 °C. The subcellular fractions were dried at 70 °C to constant weight and then digested at 145 °C for 24 h with an oxidizing mixture of acids (HNO_3–$HClO_4$, 4:1, v/v). Cadmium concentrations in the digests were determined by FAAS (Hitachi Z-5300, Japan). The detection limit of Cd analysis was 0.5 μg L^{-1}. The precision of the analytical procedures for plant material was assessed using a Certified Reference Material (CRM) (GBW-07603) provided by the National Research Center for CRM, China. Total Cd concentrations in shoot and root samples were determined with the same method following acid digestion with HNO_3–$HClO_4$ (4:1, v/v). The Cd concentrations of all subcellular fractions were based on the fresh weights of samples before separation. To verify the accuracy of the experimental results, the percentage recovery of Cd was calculated by dividing the sum of the Cd concentrations of the three fractions by the total Cd concentration of the shoots and roots.

2.4. Extraction of Cd in different chemical forms

Cadmium associated with various chemical forms (F1–F5) was successively extracted by designated solutions in the following order (Wu et al., 2005):

(1) 80% ethanol, extracting inorganic Cd giving priority to nitrate, chloride, and aminophenol cadmium, F1;
(2) distilled water (d-H_2O), extracting water-soluble Cd with organic acids and $Cd(H_2PO_4)_2$, F2;
(3) 1 M NaCl, extracting pectate- and protein-integrated Cd, F3;
(4) 2% HAC, extracting insoluble $CdHPO_4$, $Cd_3(PO_4)_2$, and other Cd-phosphate complexes, F4;
(5) cadmium in residues, F5.

Frozen plant material was cut into small pieces of 1–2 mm^3, mixed with 37.5 ml of the appropriate extraction solution and incubated at 30 °C (18 h), then the extraction solution was separated and the residual material was re-extracted with additional extraction solution (37.5 ml) under the same conditions for another 2 h; the resulting solution was combined with the extract previously obtained. This procedure was repeated twice. The combined extracts (150 ml) were evaporated to constant mass and digested with an oxidizing mixture of acids (HNO_3–$HClO_4$, 4:1, v/v). The residual plant material was extracted with the next extraction solution in the sequence, using the procedure described above. The Cd concentration of the plant material remaining after all of the extractions had been conducted was determined by digesting it with HNO_3–$HClO_4$ (4:1, v/v). The concentrations of Cd associated with different chemical forms were determined by FAAS (Hitachi Z-5300, Japan). The percentage recovery of Cd was calculated by dividing the sum of the Cd concentrations of all of the chemical forms by the total Cd present in shoots and roots.

2.5. Data analysis and statistical method

To identify the characteristics of Cd transportation from roots to shoots, the translocation factor (TF) (Hart et al., 1998) was calculated using the following equation:

$$TF = C_{shoot}/C_{root}$$

where C_{shoot} and C_{root} are the Cd concentrations (based on fresh weight) in the shoots and roots of each cultivar, respectively. SPSS 16.0 for Windows was used for statistical analyses. Two-way ANOVA (generalized linear models) was performed to evaluate the effects of soil Cd and P on plant Cd accumulation. The significance of the observed differences was determined using least significant difference (LSD) tests.