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Differential subcellular distribution and chemical forms of cadmium and copper in *Brassica napus*

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ABSTRACT

Metal subcellular fractions and chemical profile highly reflect their level of toxicity to plants. Cadmium and Cu, two different but potentially toxic metals, were compared in the present study for their subcellular distribution and chemical forms in two *Brassica napus* cultivars (Zheda 622 and ZS 758). Five-week-old seedlings were hydroponically exposed to metal stress and analyzed after 15 days of treatment. In both cultivars, Cd was less retained at cell wall, thus major part of Cd accumulated in the soluble fraction. By contrast, handsome amount of Cu was sequestrated in both cell wall and vacuole containing fraction. Across sensitive organelles, Cu preferentially accumulated in chloroplasts, while Cd was equally distributed in chloroplasts and mitochondria; the two metals intruded nucleus at lesser degree. Further, Cd and Cu differentially interacted with various cellular ligands, and the extent of interaction was higher in the tolerant cultivar ZS 758. Copper was remarkably sequestrated by phosphates, and secondarily by peptide-ligands; inversely, the role of phosphates was secondary in Cd complexation, which was mainly achieved by peptide-ligands. Additional amount of Cu was aggregated with oxalates, but oxalate-bound Cd was scarcely detected. Current results have demonstrated varied toxicological and detoxification pathways of Cd and Cu in *B. napus*, suggesting that the efficiency of different alleviation strategies could vary against Cd and Cu toxicity to plants.

1. Introduction

Increasing metal accumulation in soil, mainly originated from growing industrial activity, intensive use of fertilizers and improper disposal of wastes, has become a major environmental issue in the modern era (Adriano, 2001; Kabata-Pendias, 2011). Cadmium (Cd) and copper (Cu) are among elements of most concern as they can reach high level in soil (van Assche and Clijsters, 1990) with respect to their frequent occurrence and high contents in common sources of soil contamination i.e. metal mining wastes, sewage sludge, phosphate fertilizers (Kikuchi et al., 2007; Nagajyoti et al., 2010). Moreover, both metals are critical for crop productivity, being prone to inhibit plant growth at relatively low concentration (Ali et al., 2014a,b; de Vos et al., 1993). For many crops including oilseed rape (*Brassica napus* L.), the critical concentration of Cu toxicity is believed to be around 20 mg/kg of leaf dry weight (Davis and Becker, 1978; Marschner, 1995). Likewise, Cd concentrations above 5–10 mg/kg leaf dry weight are considered as toxic to most cultivated plants (White and Brown, 2010). In *Brassica napus* however, no significant biomass reduction could be detected up to leaf concentration in the range of 60 mg Cd/kg of dry weight (Selvam and Wong, 2009), corroborating the view that this species is one of the most tolerant to Cd (Yadav and Srivastava, 2014).

Phytotoxicity effects of Cd and Cu are generally related to their potential interference in a number of vital cellular processes such as photosynthesis, respiration, and metabolism of essential elements (Andresen and Kupper, 2013; Lin and Wu, 1994; Ravet and Pilon, 2013; Wu and Zhang, 2002). In addition, both elements can cause oxidative stress, altering the functionality of membranes through lipid peroxidation (Gill and Tuteja, 2010). Structural and ultrastructural damages to cell organelles are also recurrent manifestations of Cd and Cu toxicity (Ali et al., 2013; Peng et al., 2005); more seriously, the accumulation of these metals in nucleus can cause severe DNA damages, likely to interfere with plant

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development and crop productivity (Moura et al., 2012).

Although posing comparable treats to plant development, Cd and Cu present different chemical behavior and biological relevance (Cuypers et al., 2012). Copper is a redox-active and an essential metal for plant functioning in normal conditions (Yruela, 2005), whereas Cd lacks redox properties, and no report exist as to its beneficial role in higher plants, except for some Cd-hyper accumulating populations like *Thlaspi caerulescens* (Verbruggen et al., 2009). Therefore, Cd and Cu are likely to present some specificity in their toxicological and detoxification pathways in plant. Hence, it is interesting to gain insight into the suspected differential metabolism of Cd and Cu in plant, in the prospect of making progress in applied technologies so as to exploit the full potential of plant species against metal stress. A number of previous studies have been conducted in this regard, and a differential behavior of Cd and Cu has been evidenced in relation to plant cellular redox imbalance and oxidative stress (Cuypers et al., 2011), to anti-oxidative response (Stoiber et al., 2010), to mineral nutrients composition (Mwamba et al., 2016), to phenolic metabolism (Kováčik et al., 2009), and to the kinetics of MAPK (mitogen-activated protein kinase) activation (Jonak et al., 2004). Similarly, it has been observed a varied accumulation pattern of Cd and Cu in *B. napus*, evoking distinct uptake pathways (Mwamba et al., 2016). However, no report exists to compare their distribution at subcellular level and their pattern of interaction with cellular ligands, which directly determine the level of metal intra-cellular activity, thus the extent of potential toxicity to plant (van Assche and Clijsters, 1990). Therefore, a compared analysis of Cd and Cu subcellular distribution and chemical forms is worth attention, in order to gain further insight into the differential metabolism of these metals in plant.

The subcellular distribution and chemical forms of cu have been rarely examined in cultivated plants, although some reports exist in non-agricultural plants (Xu et al., 2013). By contrast, Cd has received tremendous attention in this regard, and has been investigated for its subcellular distribution and chemical forms in various cultivated plants including maize and pea (Lozano-Rodriguez et al., 1997), bean (Velazquez et al., 1992), lettuce (Ramos et al., 2002) and tobacco (Vogeli-Lange and Wagner, 1990). Obtained results proved however contrasting; for instance, alleviation of Cd toxicity in maize and lettuce was mainly associated with metal immobilization at cell wall, while vacuolar sequestration played the major role for Cd detoxification in bean, barley and tobacco. These variations may be suggestive of species-dependent behavior, thus driving the interest to examine the subcellular distribution and chemical forms of Cd and Cu in oilseed rape (*Brassica napus* L.), as this species has received little attention in this regard, despite its importance as a worldwide source of edible oil (Zhou, 2016) and a candidate plant for metal contaminated soils (Yadav and Srivastava, 2014). Hence, the present study was conducted to characterize the subcellular distribution and chemical forms of Cd and Cu in *B. napus*, suggesting that the two metals could present some specificity considering their distinct functional and chemical behavior. In current investigation, two *B. napus* cultivars differing in metal tolerance were used so as to associate the pattern of metal subcellular distribution and chemical forms with plant tolerance.

2. Materials and methods

2.1. Plant material and experimental conditions

Seeds of two leading oilseed rape (*B. napus* L.) cultivars (cvs. Zheda 622 and ZS 758), previously identified as differing in both Cd and Cu tolerance (Mwamba et al., 2016) were obtained from the College of Agriculture and Biotechnology, Zhejiang University (China). Mature seeds were germinated and grown in peat soil under ambient conditions (20–24 °C temperature and 55–60% relative humidity) in a green house. Four-week-old seedlings were selected for morphological uniformity and transferred to a modified Hoagland nutrient solution (3 plants per 5-L plastic pot) prepared (in μM) as follows: 4000 Ca $(NO_3)_2 \cdot 4H_2O$, 4000 $(NH_4)_2SO_4$, 4000 K_2SO_4, 4000 KNO_3, 1300 KH_2PO_4, 1000 $MgSO_4 \cdot 7H_2O$, 50 Fe-EDTA, 10 H_3BO_3, 5 $MnSO_4 \cdot H_2O$, 5 $ZnSO_4 \cdot 7H_2O$, 1 $CuSO_4 \cdot 5H_2O$, 0.5 $Na_2MoO_4 \cdot 2H_2O$. Seedlings were first allowed to acclimatize in free-metal solution for one week, then Cd as $CdCl_2.2 \cdot 5H_2O$ and Cu as $CuCl_2 \cdot 2H_2O$ were added to make desired concentrations: (1) control; (2) 50 μM Cd; (3) 50 μM Cu; (4) 200 μM Cd; and (5) 200 μM Cu. Throughout the experiment, the nutrient medium was continuously aerated through air pump and renewed every 4 days. The pH of the solution was adjusted on daily basis at 5.7 ± 0.1 with 1 M NaOH or HCl. The experiment was laid out in a completely randomized design and treatments were replicated thrice. After 15-d metal treatments, root and leaf samples (top-most or second fully expanded) were harvested and used fresh or kept frozen in liquid N_2 with respect to laboratory assay.

2.2. Plant tissues fractionation and metal analysis

Root and leaf (second fully developed) samples were separated into five different subcellular fractions (cell wall, chloroplast, nuclei, mitochondria and soluble fraction) by the gradient centrifugation technique as described by Wu et al. (2013) with some modifications. Fresh samples were weighted and homogenized in pre-cooled (4 °C) extraction solution containing 250 mM sucrose, 50 mM Tris–HCl (pH 7.5), and 1.0 mM dithioerythritol (DTE), then filtered through a 80 μm nylon cloth. The residue on the nylon was further washed with the extraction solution and designated as cell wall fraction. The filtrate was subjected to a differential centrifugation at $1500 \times g$ for 10 min ($2500 \times g$ for 20 min if root sample), $5000 \times g$ for 20 min, then $15{,}000 \times g$ for 35 min, and the obtained pellets were classified respectively as plastid fraction, nucleus fraction and mitochondria fraction; the resultant supernatant was referred to as soluble fraction mostly containing vacuole. All steps were performed at 4 °C. The obtained cell fractions were successively oven-dried at 70 °C to constant weight, ashed at 500 °C for 12 h, incubated in concentrated nitric acid until complete dissolution, then filtered prior metal analysis. Cadmium and Cu contents in different fractions were determined by graphite furnace atomic absorption spectrophotometry (Analyst 800, Perkin-Elmer, USA).

2.3. Analysis of metal chemical forms

Fractions of Cd and Cu related to different chemical forms were extracted sequentially following the protocol of Fu et al. (2011) with minor modifications. Five different extraction solutions designated to extract distinct chemical forms of metals were used in this order: (i) 80% ethanol, extracting inorganic metals including nitrate, chloride, and aminophenol Cd or Cu (Fi), (ii) deionized water, extracting water soluble metals of organic acid complexes (Fii), (iii) 1 M NaCl, extracting pectate and protein-integrated metals (Fiii), (iv) 2% acetic acid, extracting phosphate-sequestrated metals (Fiv), and (v) 0.6 M HCl, extracting oxalate-bound metals (Fv). Samples (roots and second fully developed leaves) were weighted and homogenized in 80% ethanol at a ratio of 1:100 (W:V); the homogenate was shaken for 22 h at 25 °C then centrifuged at $5000 \times g$ for 10 min. The resulting supernatant was pooled in a tube and the pellets re-suspended twice in the extraction solvent, and each time shaken for 2 h at 25 °C and centrifuged at $5000 \times g$ for 10 min. Supernatants of the three centrifugations were then pooled in a common tube. The pellets underwent the same steps