



# Net cadmium flux and gene expression in relation to differences in cadmium accumulation and translocation in four apple rootstocks



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## ABSTRACT

In order to investigate the variations in Cd uptake, translocation and tolerance among four apple rootstocks, seedlings were exposed to either 0 or 50  $\mu\text{M}$   $\text{CdCl}_2$  for 50 days. Net  $\text{Cd}^{2+}$  influxes in the rhizosphere were greatest at 600  $\mu\text{m}$  in root meristematic zone of all apple rootstocks. There were large variations in net  $\text{Cd}^{2+}$  influxes among different apple rootstocks and the lowest  $\text{Cd}^{2+}$  influxes into roots and leaf petiole vascular bundles were observed in *Malus baccata* (Mb). Net  $\text{Cd}^{2+}$  influxes into roots of all apple rootstocks were significantly decreased after the treatment with metabolic inhibitors and channel blockers, such as vanadate, verapamil and *N*-ethylmaleimide. Moreover, net  $\text{Cd}^{2+}$  influxes into roots were also affected by exogenous  $\text{Fe}^{3+}$  concentrations. Cd exposure caused decline in photosynthesis and biomass increment in all apple rootstocks except in *M. baccata* (Mb). Among four apple rootstocks, *M. baccata* (Mb) displayed the lowest Cd concentrations and amounts in tissues, and the smallest bio-concentration factor (BCF) and translocation factor ( $T_f$ ) under Cd exposure. Gene expression levels regarding Cd uptake, transport and detoxification were in line with the tendencies of Cd absorption, transport and tolerance, respectively. *M. baccata* (Mb) had lower transcript levels of genes related to Cd uptake and translocation but stronger gene expression for Cd detoxification. On the basis of this experiment, it is concluded that *M. baccata* (Mb) is superior to other three apple rootstocks to be used as rootstock in Cd contaminated soil.

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## 1. Introduction

Cadmium (Cd), a highly toxic heavy metal, is accumulated in orchard soils due to irrigation with wastewater discharge from mining operations and industrial emissions, the applications of metal-based pesticides, fungicides and fertilizers (Fang and Zhu, 2014; Li et al., 2014). When absorbed excessively by plants, Cd may negatively affect many important physiological processes such as diminishing net photosynthesis, decreasing chlorophyll synthesis, inducing oxidative stress, reducing enzyme activity and nutrient concentrations, inhibiting growth, resulting in lower crop productivity (Benavides et al., 2005; Clemens et al., 2013; Luo et al., 2013b; Zhang et al., 2002). Furthermore, Cd can be translocated efficiently from plant roots to leaves or fruits and therefore end up in foodstuff, which is responsible for human disease including cardiovascular disease, chronic anemia, lung cancers and nervous

system damage (Das et al., 1997; Hall, 2002; Nawrot et al., 2006). According to recent soil survey, about 19.4% of the farmland in China has been contaminated by heavy metals, especially Cd (Zhou et al., 2015). Previous studies have been carried out to investigate Cd accumulation in apple orchards and fruits, and shown that Cd in apple and other four fruits including pear, grape, peach-shaped plum and orange exceeded the safety standards (Fang and Zhu, 2014; Wang et al., 2015). The increasingly accumulation of Cd in the orchard soil not only limits fruit cultivation, but also poses a potential risk to human health. Therefore, how to prevent Cd toxicity to fruit trees and reduce Cd accumulation in fruits has become an important issue.

Previous studies have demonstrated that significant difference in the Cd uptake and distribution existed among plant species or even among cultivars within species (He et al., 2013b; Yu et al., 2006). Therefore, the selection and breeding of cash crops with low-Cd accumulation in edible parts will be a feasible and useful approach to reduce Cd accumulation. In recent years, notable progress has been made on the different mechanisms related to Cd absorption and translocation of from roots to shoots among species

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or cultivars, such as Chinese cabbage (Liu et al., 2010), rice (Liu et al., 2014), wheat (Farrell et al., 2005), oilseed rape (Wu et al., 2015), maize (Kurz et al., 1999) and peanut (Su et al., 2012). However, little information is available on the mechanisms underlying Cd uptake, accumulation and detoxification in apple trees. Grafting experiments indicated that rootstocks determine the Cd content in the aerial parts of plants and affect plants Cd tolerance (Savvas et al., 2013; Xin et al., 2013). For apple trees, rootstocks constitute their roots and are widely used for vegetative propagation. Hence, studies on the variation in Cd<sup>2+</sup> uptake, translocation and accumulation in different apple rootstocks are important and needed, by which high Cd tolerance with low Cd accumulation species could be selected to avoid Cd accumulation in human body.

It is well documented that Cd entry into the root cells is the first key process for accumulation and translocation (Farrell et al., 2005; He et al., 2011). Using the noninvasive micro-test technique (NMT), previous studies have demonstrated the dynamics of Cd<sup>2+</sup> fluxes in the rhizosphere of herbaceous or woody plants (He et al., 2011; Li et al., 2012; Sun et al., 2013a,b). Under the same Cd exposure conditions, significant differences in net Cd<sup>2+</sup> influxes among two oilseed rape cultivars were observed (Wu et al., 2015). Sun et al. (2013b) also found that net Cd<sup>2+</sup> influxes were higher in hyper-accumulator ecotypes of *Sedum alfredii* than those in non-accumulator ecotypes. A large variation in the net Cd<sup>2+</sup> fluxes at the root surface among cultivars of the same wheat species resulted in differences in plant Cd accumulation (Farrell et al., 2005). Generally, low grain Cd accumulators exhibited lower Cd<sup>2+</sup> fluxes than the high grain Cd accumulators (Farrell et al., 2005). Up to now, there has been little information on the dynamics of Cd<sup>2+</sup> fluxes in the rhizosphere in relation to differences in Cd accumulation among different apple roots. The net Cd<sup>2+</sup> fluxes in poplar roots is coupled with the plasma membrane (PM) H<sup>+</sup>-ATPases, and the inhibition of PM H<sup>+</sup>-ATPases decreased net Cd<sup>2+</sup> influxes (Ma et al., 2014). In the roots of *Suaeda salsa*, a halophyte plant, pre-treatment with Ca<sup>2+</sup> channel and thiol blockers suppressed the net Cd<sup>2+</sup> influxes, indicating Cd entry into root cells via Ca<sup>2+</sup> channel and Cd transport in the plants was regulated by thiol compounds (Li et al., 2012). In apple roots, whether and how PM H<sup>+</sup>-ATPases, Ca<sup>2+</sup> channel and thiol compounds would affect the net Cd<sup>2+</sup> fluxes remain uncharacterized. The influence of exogenous iron (Fe) on Cd uptake and accumulation have been explored in many plants. Gao et al. (2011b) found that the net Cd<sup>2+</sup> influxes decreased notably in roots of *Malus xiaojinensis* at high Fe level compared to Fe deficiency, resulting low accumulation in roots and shoots. The same results were observed in rice (Nakanishi et al., 2006) and pea (Cohen et al., 1998). However, no information is available on how exogenous Fe affects the net Cd<sup>2+</sup> fluxes in different apple rootstocks.

In recent years, significant progress has been made in elucidating the molecular basis of Cd<sup>2+</sup> uptake into plant cells. PM H<sup>+</sup>-ATPases which provide the proton motive force for ion translocation across the plasma membrane play a pivotal role in Cd<sup>2+</sup> uptake. A study in *M. xiaojinensis* found that the release of H<sup>+</sup> by PM H<sup>+</sup>-ATPases affected rhizosphere pH and contributed to Cd absorption (Gao et al., 2011a). In  $\gamma$ -ECS overexpressing poplar roots, Cd exposure led to the up-regulation of *HA2.1* and *AHA10.1* associated with higher Cd uptake, compared to the wild types (He et al., 2015). However, up to now, little is known about the function and regulation of genes encoding PM H<sup>+</sup>-ATPases in different apple roots exposed to Cd. As a nonessential element, Cd enters the plant cell by transporters for nutrient cations (e.g. Ca<sup>2+</sup>, Fe<sup>2+</sup> and Zn<sup>2+</sup>), often leading to reduction in these essential elements (Rodríguez-Serrano et al., 2009). Natural resistance associated macrophage protein (NRAMP) which has high affinity for Fe<sup>2+</sup> and Zn<sup>2+</sup> have been confirmed to transport Cd directly (Thomine et al., 2000).

Once inside the root cells, Cd can either be transported and sequestered in vacuoles by tonoplast localized transporters, such as ATP-binding cassette transporter (ABCC) (Park et al., 2012), or detoxified by metal-binding chelators, such as nicotianamine (NA) and metallothionein (MT), which are synthesized under Cd stress conditions and play a role in metal tolerance (Cobbett and Goldsbrough, 2002; Lin and Aarts, 2012). Vacuolar sequestered Cd could be remobilized again via tonoplast localized metal efflux transporters, such as NRAMPs (Lin and Aarts, 2012), and further loaded into the xylem by heavy metal ATPases (HMAs) for root-to-shoot transport (Grispen et al., 2011). Previous studies have demonstrated the role of ferric reduction oxidase2-like (*FRO2*-like), *NRAMP1/3*, *NAS1*, *MT2* and *HMA4* in Cd uptake, transport and detoxification (Gao et al., 2011a; Grispen et al., 2011; He et al., 2015; Ma et al., 2014; Oomen et al., 2009). However, it is still unclear about the function of these genes associated with variations in Cd accumulation and detoxification in different apple roots.

China, the largest producer of apple fruit worldwide, occupies about 48.4% of the total world amounts (FAOSTAT, 2012). The safety production of apple is a major public concern in recent years (Grembecka and Szefer, 2013; Wang et al., 2015). To date, few evaluations have been made on Cd uptake and accumulation in different apple rootstocks. In this study, four apple rootstocks were used to investigate Cd uptake and plant-internal partitioning in different tissues. The hypothesis is that variation in Cd uptake and accumulation exists among different apple rootstocks. To examine this hypothesis, the spatial and temporal characteristics of net Cd<sup>2+</sup> fluxes in relation to PM H<sup>+</sup>-ATPases, Ca<sup>2+</sup> channel and SH-binding ligands at the root surface, Cd concentrations in different tissues, total Cd amounts, bio-concentration factor (BCF), translocation factor (*T<sub>f</sub>*) and key genes involved in Cd uptake and translocation were analyzed.

## 2. Materials and methods

### 2.1. Plant materials and Cd exposure

The experiments were conducted at Shenyang Agricultural University, Shenyang, China (41°49'N, 123°34'E). Seedlings of *Malus baccata* Borkh. (Mb), *Malus hupehensis* Rehd. (Mh), *Malus micromalus* 'qingzhoulinqin' (Mm) and *Malus robusta* Rehd. (Mr) were used in the present study. Seeds were stratified at 0–4 °C for 40 days. After germination, seeds were planted in nursery seedling plate filled with plug seedling substrate. They were then placed in a greenhouse under natural light and temperature conditions (day: night temperature, 26 °C: 18 °C; relative air humidity, 50–60%). After 40 days, seedlings with similar size (6–7 leaves, about 8 cm tall) were selected and transferred to plastic pot (20 × 20 × 18 cm) filled with 4 kg sand. One seedling was planted in each pot. Seedlings were cultivated for 2 weeks before Cd exposure. Fifty milliliters full-strength modified Hoagland solution (Fe<sup>3+</sup>: 25 μM, pH 6.0) were slowly added to the pots in every 2 days. Distilled water (100 ml) was added every evening. Thirty-six plants with similar height and growth performance for four apple rootstocks were selected and divided into two groups, with 18 plants in each group: one group for the cadmium treatment (50 μM CdCl<sub>2</sub>) and the other for the control (0 μM CdCl<sub>2</sub>). Plants were treated with Cd being supplied daily with the nutrient solution for 50 days. The apex of each seedling was marked prior to the Cd treatment in order to distinguish tissues formed during the Cd treatment. Six plants for each species were harvested before Cd exposure and root dry weight were recorded in order to calculate root biomass increment formed during the Cd treatment. Plants were grown for 50 days in the absence or presence of Cd being supplied daily with the nutrient solution before harvest.

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