

The Institute for Plant Nutrition and Soil Science  
of the Christian-Albrechts-Universität Kiel

**Alleviation of cadmium toxicity by silicon or sulfate supply in plants**

**Dissertation**

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Jiawen Wu, M. Sc.

born in Shaanxi, China

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Dean: Prof. Dr. Joachim Krieter

1. Examiner: Prof. Dr. Karl-Hermann Mühling

2. Examiner: PD Dr. Hans-Peter Mock

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## Table of Contents

Chapter 1 .....	1
1. General introduction .....	2
1.1 Cadmium.....	2
1.2 Silicon .....	5
1.3 Sulfur.....	9
1.4 Objectives .....	12
1.5 References.....	14
Chapter 2 .....	23
Silicon-enhanced oxalate exudation contributes to alleviation of .....	23
cadmium toxicity in wheat .....	23
Chapter 3 .....	33
Reduction of apoplastic fluid-Cd as affected by silicon results in decrease of Cd in wheat	33
Chapter 4 .....	72
Sulfate supply enhances cadmium tolerance in <i>Vicia faba</i> L. plants .....	72
Chapter 5 .....	99
Sulfate facilitates cadmium accumulation in leaves of <i>Vicia faba</i> L. at flowering stage.....	99
Chapter 6 .....	108
6. General discussion .....	109
6.1 Mitigation of Cd toxicity by Si in wheat .....	109
6.2 Enhancement of Cd tolerance by sulfate in faba bean .....	114
6.3 References.....	118
Chapter 7 .....	122
Summary.....	122

## **Chapter 1**

### **General introduction**

## 1. General introduction

### 1.1 Cadmium

Cadmium (Cd) is an atomic number 48 chemical element which belongs to period 5 in the periodic table. Its boiling point is 767 °C and melting point is 321 °C with a 112.4 standard atomic weight (Wuana and Okieimen 2011). The atomic weight of Cd is a mixture of eight different stable isotopes consisting of  $^{106}\text{Cd}$ , 1.25%;  $^{108}\text{Cd}$ , 0.89%;  $^{110}\text{Cd}$ , 12.49%;  $^{111}\text{Cd}$ , 12.80%;  $^{112}\text{Cd}$ , 24.13%;  $^{113}\text{Cd}$ , 12.22%;  $^{114}\text{Cd}$ , 28.73% and  $^{116}\text{Cd}$ , 7.49% with corresponding natural abundances (Meija et al. 2016). As a non-essential element, Cd is toxic for plants and thereby interrupting plant growth, photosynthesis, respiration and nutrient homeostasis and other metabolic processes (Nagajyoti et al. 2010; Wang et al. 2017). After taking up by plants, Cd will accumulate in different organs of plants including edible organs, which could result in Cd entry into human bodies via food chain (Hasan et al. 2009; Satarug et al. 2017). Gastrointestinal, pulmonary and dermal absorption of Cd will damage human health including kidney, lung and bones (Godt et al. 2006). Therefore, it is urgent to study strategies to reduce Cd toxicity in plants.

#### 1.1.1 Cadmium sources

Although Cd is a detrimental element, it is widely used in solders as an alloying metal, in electroplating as an anti-corrosion, in plastics as a stabilizer, in nickel (Ni)-Cd batteries, or in pigment. Thus, it is unavoidable to introduce Cd into air, water and soil (Cloquet et al. 2006). Generally, main sources of Cd are anthropogenic activities like mining, smelting and fossil fuel combustion and phosphate fertilizer application, and natural sources like weathering and erosion of Cd-containing rocks or volcanic activity (Bi et al. 2009; Pruvot et al. 2006). Hutton (1983) quantified the relative significance of different sources of Cd contributed to air, water and land respectively in the European Community. They found atmospheric Cd mostly originated from steel industry, zinc (Zn) production and volcanic action, while phosphate fertilizer manufacture and zinc production accounted for a large emission into aquatic systems. The largest contribution of Cd to land was waste disposal (Hutton 1983). Considering the long-term consumption of cereals and legumes by humans, JECFA (joint FAO/WHO Expert Committee on Food Additives) and CCFAC (Codex Committee on Food Additives and Contaminants) suggests a limit of 0.1 mg kg<sup>-1</sup> for Cd in crops (Commission;

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2002). European Community proposes a limit of  $0.2 \text{ mg kg}^{-1}$  for Cd in wheat grain (European Commission 2001), while FAO/WHO has a limit of maximum  $70 \text{ } \mu\text{g Cd day}^{-1} \text{ person}^{-1}$  (Ryan et al. 1982; Wagner 1993).

### **1.1.2 Cadmium uptake, translocation and distribution**

In Cd-polluted agricultural soils, plant roots contact with Cd directly. Bioavailability of Cd plays a vital role in determining Cd uptake by plants which includes forms of  $\text{Cd}^{2+}$ , Cd-inorganic compounds like  $\text{CdCl}^+$  and  $\text{CdOH}^+$ , and Cd-organic compounds like Cd-organic acids and Cd-humate. Once Cd entry onto the surface of roots, Cd will enter into plants through apoplastic, symplastic and coupled trans-cellular pathways (Fig. 1A). Apoplast of roots consist of cell walls and extracellular spaces, and Cd is absorbed through mass flow and diffusion in the apoplast (Vazquez et al. 2007; White 2012). Mass flow is driven by transpiration, while diffusion is determined by a multitude of factors such as diffusive layer thickness, transporter density, root exudates, and concentration gradient (Degryse et al. 2012; White 2012). Structurally, apoplastic absorption is interrupted by endodermis because Casparian bands and suberin lamellae in endodermis are low permeable to water and solutes (Barberon 2017), thus Cd will only be taken up through symplastic pathway or trans-cellular pathway (Fig. 1B). It is always difficult to distinguish symplastic pathway and trans-cellular pathway technically, thus trans-cellular pathway is also regarded as symplastic pathway. Cd is non-essential element for plants without specific transporter for Cd uptake, but Cd can share transporters with other essential nutrients (Clemens et al. 2002). For example, iron (Fe)-regulated transporter-like protein, IRT1 for Fe uptake (Vert et al. 2002), low-affinity cation transporter, LCT1 for calcium (Ca) uptake (Clemens et al. 1998) or natural resistance-associated macrophage protein, Nramp5 for manganese (Mn) locate in roots and have function of taking up Cd by roots in plants (Sasaki et al. 2012). Curie et al. (2009) also reported that Cd can be taken up in the form of Cd-chelator through yellow strip-1 like protein (YSL) as the same function on Fe uptake. After reaching into central cylinder in roots, Cd will be transported into shoots via xylem loading. Heavy metal P1B-ATPase family has been discovered to be responsible for Cd acropetal transport. For example, AtHMA2 and AtHMA4 (heavy metal P1B-ATPase 2 and 4) which are transporters for zinc (Zn) acropetal transport, have function of loading Cd into xylem. AtHMA3, heavy metal P<sub>1B</sub>-ATPase 3, has function of sequestering Cd into vacuoles (Romè et al. 2016). Cd transport from roots into shoots via

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xylem loading, and then Cd will be taken up by leaf cells or transport into grains via xylem-to-phloem pathway. Tanaka et al. (2007) showed that 90-100% of Cd in grains of rice (*Oryza sativa* L.) originated from phloem. Homolog of wheat low-affinity cation transporter 1, OsLCT1, a gene related to Cd transport in phloem has been identified in rice (Uraguchi et al. 2011).

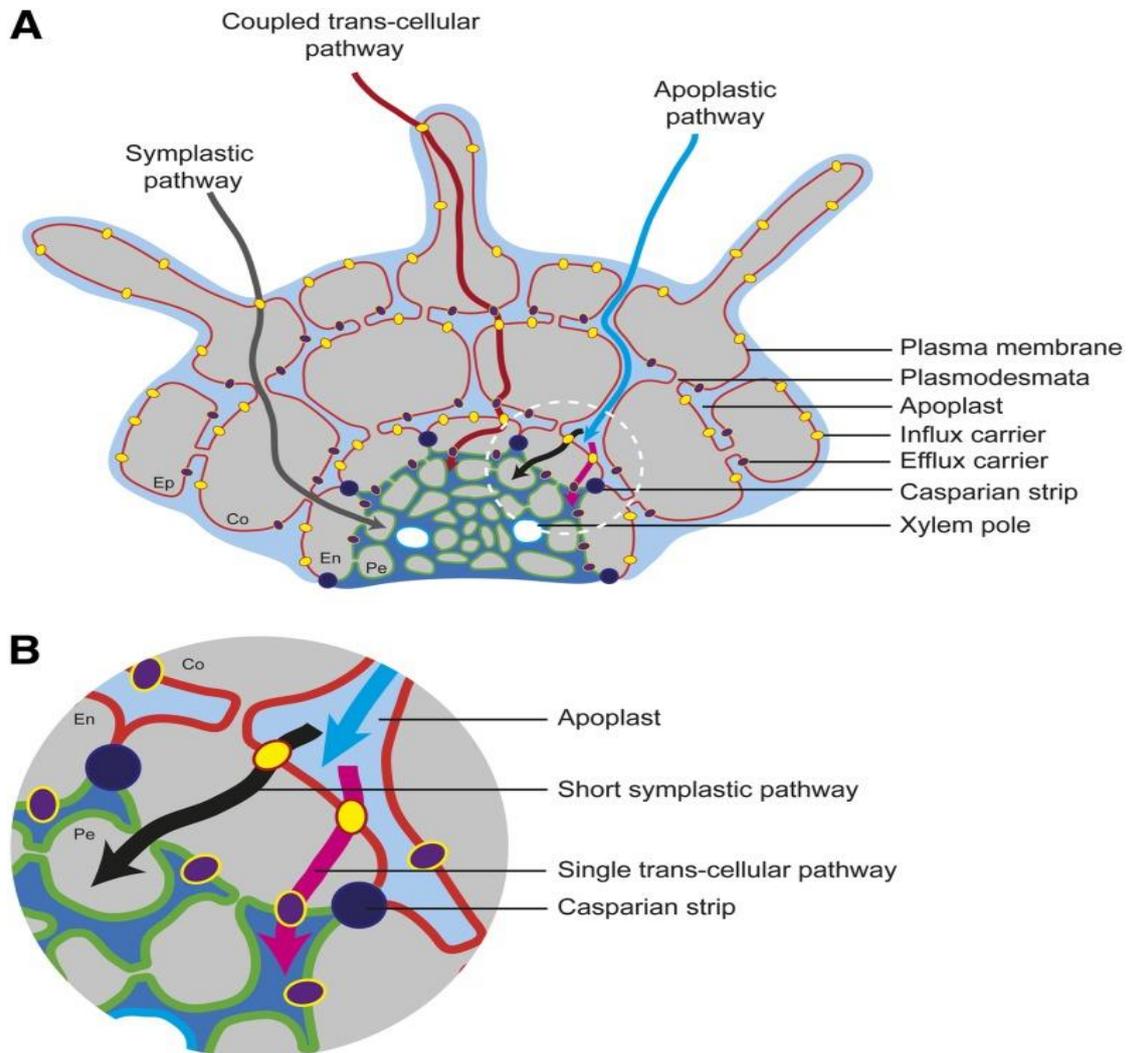


Fig. 1 Models of nutrients uptake by roots in plants. (A), Overview of apoplastic, symplastic and coupled trans-cellular pathways taking up nutrients by roots; (B), Magnification of white circled areas in Fig. 1A. Ep, epidermis; Co, cortex; En, endodermis; Pe, pericycle (Barberon and Geldner 2014).

### 1.1.3 Adverse effects of cadmium in plants

As a non-essential element, Cd is toxic to plant growth, even at very low concentration due to its high solubility and mobility (Gallego et al. 2012). Although Cd could replace the

function of Zn to keep a marine diatom (*Thalassiosira weissflogii*) growth when Zn is lacking, non-beneficial function of Cd is discovered in higher plants (Lane et al. 2005). It is well documented that Cd inhibits plant growth. As discussed in Section 1.1.2, Cd shares transporters with other essential nutrients, thus Cd could affect other mineral nutrients uptake and interrupt nutrient homeostasis (Sarwar et al. 2010). For example, Cd entry into leaves through calcium (Ca) channel disturbs plant-water relationship resulting in stomatal closure, and thereby decreasing transpiration rate (Gothberg et al. 2004; Perfus-Barbeoch et al. 2002). In addition, chemical similarity of Cd with Zn, Ca or Fe, Cd is easy to replace those cations from active sites such as enzymes or signaling components (Roth et al. 2006). When Cd accumulates in chloroplasts, photosynthesis apparatus will be damaged. Fagioni et al. (2009) showed that Cd reduced antenna proteins of PSI, while PSII proteins were less affected; and ATP-synthase complex and cytochrome b6/f were unchanged by Cd toxicity by analyzing proteomics of thylakoid membrane. Except those detrimental effects of Cd toxicity, Cd also induces production of reactive oxygen species (ROS) including singlet oxygen ( $^1\text{O}_2$ ), superoxide anion ( $\text{O}_2^{\cdot-}$ ), hydroxyl radical ( $\text{OH}^{\cdot}$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (Wu et al. 2013). As a non-redox element, although Cd does not involve in Fenton and Haber-Weiss reactions directly, Cd can still produce ROS indirectly. In general, three pathways are related to ROS production by Cd: (1) replacement of redox-active element such as Fe resulting in more ROS produced by more redox-active element; (2) increase of NADPH oxidase which leads to electron released from NADPH, and thereby generating  $\text{O}_2^{\cdot-}$  from  $\text{O}_2$  through using the electron; (3) binding Cd with  $-\text{SH}$  group such as glutathione (GSH) leading to reduced antioxidant capacity (Cuypers et al. 2010). When over-production of ROS by Cd, proteins, nucleic acids and lipids in plants will be damaged resulting in disruption of plant metabolism (Romero-Puertas et al. 2007).

## 1.2 Silicon

Silicon (Si) is the second most abundant element after oxygen in the earth's crust, ranging from 3.5 to 40 mg Si L<sup>-1</sup> in soils (Broadley et al. 2012). However, the majority of Si in soils is in the form of  $\text{SiO}_2$  containing 50-70% of Si, followed by the form of silicates or aluminum silicate (Ma and Yamaji 2006). Through weathering processes Si could be released into soil solution or surface water in the form of silicic acid ( $\text{H}_4\text{SiO}_4$ ) when pH is below 9.  $\text{H}_4\text{SiO}_4$ , an uncharged monosilicic acid, which is the only available form of Si for plants, ranging from

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0.1 to 0.6 mM in natural soil solution (Sommer et al. 2006). When concentration of  $H_4SiO_4$  is more than 2 mM, Si will be polymerized (Epstein 1999; Wu et al. 2013).

### 1.2.1 Silicon in plants

Epstein (1994) found a wide range of Si concentrations in shoots of different species ranging from 0.1% to 10% dry weight in tissues. According to the varieties of Si contents, plants are classified into high, intermediate and low Si accumulators (Epstein 1999). Regarding Si uptake by roots, active, passive or rejective models are correlated with Si accumulation in plants. More specifically, active uptake of Si represents that Si concentrations in shoots are higher than that derived from transpiration stream; passive uptake indicates that Si concentrations in shoots are equal to that derived from transpiration stream; and rejective uptake means that Si concentrations in shoots are lower than that derived from transpiration stream (Epstein 1994; Epstein 1999). Generally, monocots involved with active uptake of Si have higher Si accumulation than dicots involved with passive or rejective uptake of Si (Debona et al. 2017). Rice, a typical high Si-accumulator, deploys polar distribution of Si transporters consisting of low silicon 1 (OsLsi1) influx transporter and low silicon 2 (OsLsi2) efflux transporter located at distal and proximal sides of exodermis and endodermis respectively. Homologs of OsLsi1 have also been characterized in maize (*Zea mays* L.) (*ZmLsi1*, *ZmLsi6*), in barley (*Hordeum vulgare* L.) (*HvLsi1*, *HvLsi6*), in pumpkin (*Cucurbita moschata* L.) (*CmLsi1*), in wheat (*TaLsi1*), in cucumber (*Cucumis sativus* L.) (*CSiT-1*, *CSiT-2*) and in soybean (*Glycine max*) (*GmLsi1*) (Ma and Yamaji 2015).

### 1.2.2 Alleviation of cadmium toxicity by silicon

As discussed in section 1.1.3, agricultural soils are contaminated by Cd unavoidably due to mining, sewage sludge, phosphate fertilizer application, and thereby Cd will enter into plants (Sarwar et al. 2010). As a result, plants growth is damaged by Cd toxicity, even Cd accumulates into human bodies via food chain and threatens human health (European Commission 2001). It has been well documented that Si plays a pivotal role in alleviating Cd toxicity in plants (Adrees et al. 2015; Wu et al. 2013).

It is widely accepted that Si supply reverses retardation of plant growth induced by heavy metal stress including Cd toxicity (Rizwan et al. 2016a; Wu et al. 2013). For example, Si addition considerably increases dry weight of shoots and roots in winter wheat under Cd stress (Wu et al. 2016) and durum wheat (*Triticum turgidum* L.) (Rizwan et al. 2012). Similar

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beneficial effects of Si were also reported in other species such as rice (Ji et al. 2017), tomato (Wu et al. 2015), cucumber (Feng et al. 2010), maize (Vaculik et al. 2009), alfalfa (*Medicago sativa* L.) (Kabir et al. 2016) and pakchoi (*Brassica chinensis* L.) (Song et al. 2009) under Cd toxicity. In agreement with Cd stress, Si also reverses the decreased biomass induced by Cu toxicity (Keller et al. 2015), Al (aluminum) toxicity (Dorneles et al. 2016), Cr (chromium) toxicity (Huda et al. 2017), Zn toxicity (Song et al. 2011) and As (arsenic) toxicity (Sanglard et al. 2016). Nevertheless, Masarovic et al. (2012) showed that Si supply decreased dry weight of shoots and roots in sorghum (*Sorghum bicolor* L.) under Zn stress when compared with Zn treatment alone. Bokor et al. (2014) also showed that Si accelerated adverse physiological stress induced by Zn toxicity such as decrease of biomass in maize. Bokor et al. (2015) reasoned the negative effect of Si under Zn stress was that Si supply disrupted mineral homeostasis in plants. Taken together, Si supplementation alleviates heavy metal toxicity including Cd toxicity, induced plant growth retardation, but this positive effect of Si is lost under some certain unfavorable conditions such as under high level of Zn stress.

Si alleviates reduced biomass induced by Cd toxicity. Therefore, giving raise to a question, why could Si increase biomass of plants under Cd stress? Many studies have indicated that Si reduces Cd concentrations in plants such as in rice (Shi et al. 2005), in peanut (Shi et al. 2017), in maize (Liang et al. 2005) and in field bean (*Pisum sativum* L.) (Rahman et al. 2017) is the consequence of increasing biomass. In general, reduction of Cd as affected by Si in plants is positively related to enhance photosynthesis and antioxidant capacity due to less Cd toxicity in Si-supplied plants, and thus improving plant growth and increasing biomass of plants. In accordance with this theory, Malcovska et al. (2014) reported that Si addition increased chlorophyll concentrations of leaves and photosynthesis rate, and also alleviated oxidative stress in maize under Cd toxicity. Nwugo and Huerta (2008) also showed that Si recovered Cd-inhibited plant growth and photosynthesis, especially improved light use efficiency in low level-Cd stressed plants. Moreover, Silva et al. (2017) indicated that spectral emission and the Fr (red fluorescence)/Ffr (far red fluorescence) ratio responses to alleviation of Cd stress by Si were faster than chlorophyll concentrations and biomass changes, which were recommended to detect amelioration of Cd toxicity by Si as a marker. Regarding enhancement of antioxidant capacity in Cd-stressed plants by Si supply, it has been well reviewed by Kim et al. (2017). In plants, antioxidant system consists of enzymatic antioxidants such as superoxide dismutase, peroxidase, catalase, glutathione reductase, and

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ascorbate peroxidase, and non-enzymatic antioxidants such as glutathione, ascorbate and carotenoids (Cuypers et al. 2010). A multitude of studies have showed that Si supply mitigates Cd toxicity through improving of antioxidant capacity (Farooq et al. 2013; Shi et al. 2010; Wu et al. 2015), but whether Si involves in enhancement of antioxidant capacity in plants directly is still unclear.

Comparatively, Vaculik et al. (2009) reported that Si addition substantially increased biomass of maize plants under Cd toxicity, but Cd concentrations in shoots and roots were also increased by Si, and they explained the improved Cd concentrations in plants was attributed to longer distance of suberin lamellae deposition from root apex induced by Si under Cd stress since the delayed suberin development decreased apoplastic blocking capacity of Cd. Later, Vaculik et al. (2012) showed that Si affected Cd accumulation in maize was Cd concentration-dependent, and it was assumed that apoplastic barriers development in roots was only partially ascribed to different Cd uptake under low and high Cd conditions, and reduction of availability of Cd in shoots by sequestering Cd in cell walls was the reason for alleviation of Cd toxicity by Si. Based on those findings, it seems that Si-mediated reduction of Cd uptake reaching alleviation of Cd toxicity is not the only strategy to improve Cd-stressed plant growth. By compartmentalizing Cd into less active organelles such as cell walls or vacuoles without decreasing Cd accumulation might also be the mechanism of mitigation of Cd toxicity by Si in plants. For example, Ma et al. (2015) illustrated that Si-increased Cd accumulation in cell walls of rice existing in the form of [Si-hemicellulose matrix]Cd complexes by using suspension cells cultivation. Williams and Vlamis (1957) also indicated that Si ameliorated Mn toxicity without affecting Mn concentrations in leaves, but Mn was homogenously distributed in leaves of Si-supplied barley rather than concentrated Mn in leaves of non-Si-treated barley. Nevertheless, recent research reported different results that Si addition reduced Cd accumulation in cell walls of rice by suppressing expression of genes related to Cd uptake (Shao et al. 2017). They reasoned the different findings in rice as affected by Si under Cd stress is the way of different plant-level cultivation (Shao et al. 2017); suspension cell cultivation (Ma et al. 2015) and whole plant cultivation (Shao et al. 2017) respectively. Therefore, underlying mechanism regarding alleviation of Cd toxicity by Si is still needed to be discovered.

## 1.3 Sulfur

Sulfur (S) is one of macronutrients in plants ranging from 0.1% to 0.5% based on dry weight in plants (Hawkesford et al. 2012). As a constituent of methionine and cysteine, sulfur plays an important role in plant life cycle because those two amino acids are the precursors of S-containing coenzymes and secondary compounds (Droux 2004). For example, glutathione (GSH) comprises glutamate, cysteine and glycine, namely  $\gamma$ -L-glutamyl-L-cysteinylglycine, which accounts for more than 90% of water soluble thiol contents. Glycine coupled in GSH is replaced by alanine in some legume plants (Srivalli and Khanna-Chopra 2008).

### 1.3.1 Sulfur assimilation in plants

Although plants can absorb atmospheric sulfur dioxide ( $\text{SO}_2$ ) and utilize them by above-ground plants, major source of sulfur is still from root uptake in the form of sulfate (Hawkesford et al. 2012). As can be seen in Fig. 2, sulfate is taken up by plants via sulfate transporters, and then sulfate is activated by ATP via ATP sulfurylase (ATP-S). Afterwards, the product, 5'-adenylylsulfate (APS) is reduced to sulfite, and sulfite is further reduced to sulfide via sulfite reductase. Sulfide is incorporated with *O*-acetylserine to form cysteine via *O*-acetyl(thiol)lyase. Subsequently, cysteine is coupled with glycine and glutamate to synthesize glutathione (GSH) via formation of the intermediate  $\gamma$ -glutamylcysteine (Rausch and Wachter 2005). As the precursor of phytochelatin (PC), GSH is polymerized to produce PC via PC-synthase (Brunetti et al. 2011).

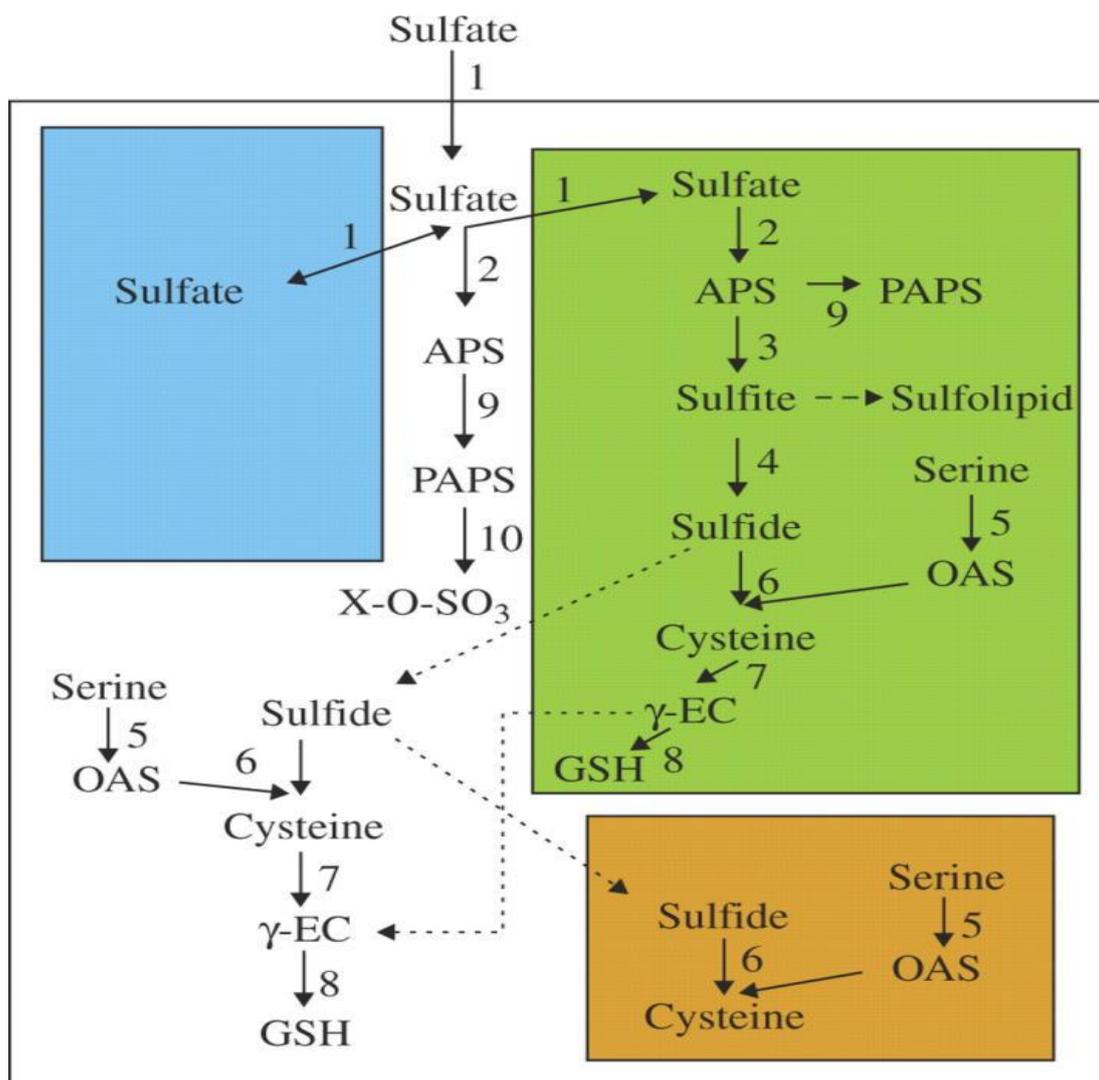


Fig. 2 Schematic illustration of sulfur assimilation in plants. 1, sulfate transporter; 2, ATP sulfurylase; 3, APS reductase; 4, sulfite reductase; 5, serine acetyltransferase; 6, *O*-acetylserine thiollyase; 7,  $\gamma$ -glutamylcysteine synthetase; 8, glutathione synthetase; 9, APS kinase; 10, sulfotransferase. Dashed lines represent multiple reaction steps; dotted lines indicate unconfirmed transport steps. Blue box represents vacuoles; green box indicates plastids; brown box shows mitochondria (Kopriva 2006).

### 1.3.2 Effects of sulfur on Cd stress in plants

Some studies have reported that metal exposure could affect sulfur assimilation in plants. Nussbaum et al. (1988) showed that APS reductase and ATP-sulfurylase (ATP-S) enzymes activities were increased by Cd toxicity in maize. Takahashi et al. (2000) also reported that after several hours Cd treatment, *Sultr1;1* and *Sultr2;1*, genes encoding sulfate transport, expression levels were up-regulated. Sulfur assimilation reduces inorganic sulfate into organic

cysteine (Cys) and GSH which are precursors of phytochelatins (PCs). PCs consist of ( $\gamma$ -Glu-Cys) $_n$ -Gly ( $n = 2-11$ ), and Cys-rich peptides in PCs are from GSH via phytochelatin synthase (PCS) in plants (Wojcik and Tukiendorf 2004). It is well documented that Cd stimulates production of PCs, and Cd-PC LMW (low molecular weight) complexes are formed rapidly in the cytosol, and then Cd-PC LMW is transported into the vacuole via ABC-type transporters and synthesized Cd-PC HMW (high molecular weight) complexes. Cd-PC HMW complexes are more stable than Cd-PC LMW complexes, and thereby Cd is detoxified through sequestering Cd into the vacuole (Yang and Chu 2011) (Fig. 3).

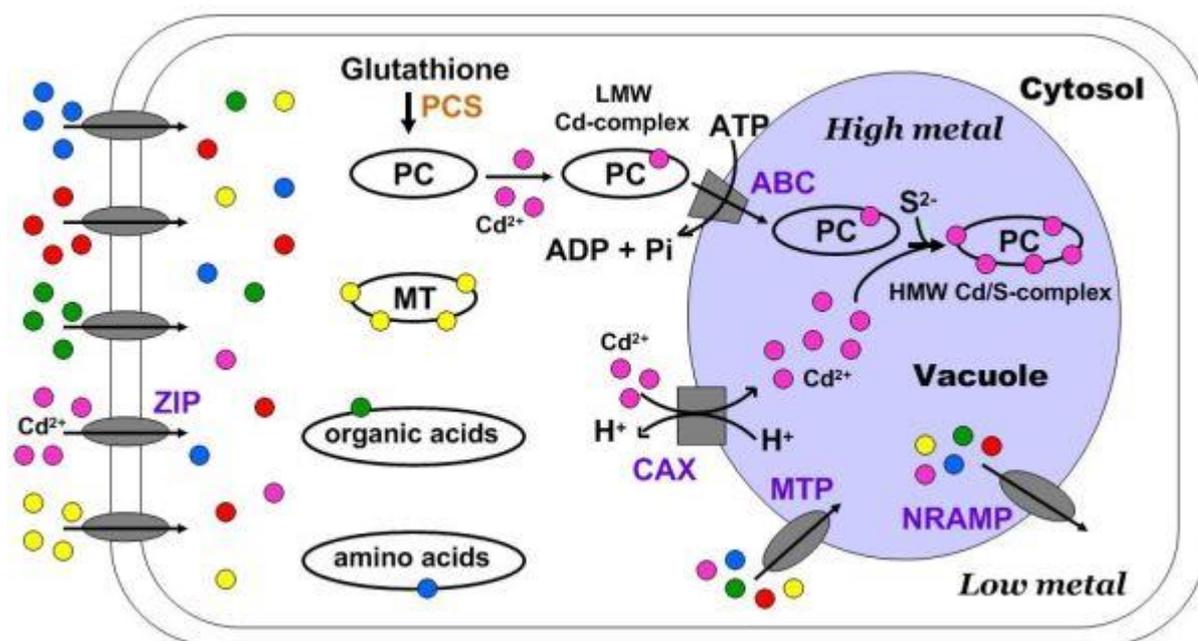


Fig. 3 Detoxification of Cd toxicity by phytochelatins (PCs) in cells. ZIP, zinc/iron-regulated transporters; MT, metallothionein; CAX, cation/proton exchanger; MTP, metal tolerance protein; NRAMP, natural resistance associated macrophage protein. LMW, low molecular weight; HMW, high molecular weight (Yang and Chu 2011).

However, in recent years, progressive processes have achieved to reduce sulfur compounds emission into environment and applying refined fertilizer without sulfur like urea and diammonium phosphate resulting in sulfur deficiency stress for plants (Astolfi et al. 2010). Thus, question is raised that how sulfur supply affects Cd toxicity in plants. To date, effects of sulfur on Cd accumulation in plants are conflicting. Hassan et al. (2005) found that sulfur decreased Cd concentrations in both roots and shoots of rice as a result of increased growth parameters by sulfur which performed as 'dilution' effect. Fan et al. (2010) also reported that

excessive sulfur reduced Cd accumulation in grains of brown rice at expense of losing rice yield. But, opposite results were reported by McLaughlin et al. (1998) showed that sulfur enhanced Cd contents in shoots of Swiss chard (*Beta vulgaris* L.) because of increased Cd availability in the growth medium, and López-Chuken and Young (2010) also reported that sulfate increased Cd accumulation in maize (*Zea mays* L.). However, studies on how sulfate supply affects subcellular accumulation of Cd and whether subcellular accumulation of essential nutrients contributes to Cd tolerance as affected by sulfate in plants are still lacking. On the other hand, several studies have indicated that exogenous application of sulfur alleviates Cd toxicity by improving antioxidant capacity in plants. For example, Anjum et al. (2008) reported that enhancement of AsA and GSH contents in mustard (*Brassica campestris* L.) by sulfur addition contributed to alleviation of Cd toxicity and thus improving plant growth. Liang et al. (2016) also reported that improvement of AsA-GSH cycle and promotion of PCs synthesis as affected by sulfur supply resulted in suppression of Cd toxicity due to repression of oxidative stress in pakchoi (*Brassica chinensis* L.). Similar function of sulfur on Cd toxicity regarding enhancement of antioxidant capacity were also found in other species, like wheat (Khan et al. 2015), rice (Hassan et al. 2005) and microalgae (*Chlamydomonas moewusii*) (Mera et al. 2016). Except well-known antioxidants, GSH and AsA, phenolic compounds are also known as antioxidants in plants. Diverse structures of phenolic compounds have the ability to donate electron agents or hydrogen atoms which could reduce the production of oxidant radical species (Martini et al. 2017; Sgherri et al. 2003). Thus, it is interesting to study whether phenolic compounds involve in alleviating oxidative stress as affected by sulfur supplementation.

## **1.4 Objectives**

Facing Cd pollution all over the world, strategies for alleviation of Cd toxicity in plants are urgent to be studied. Nutrients application is regarded as an eco-friendly and cost-effective strategy to counteract Cd stress. In this thesis, we focused on assessing effects of Si or sulfate on Cd toxicity in plants.

### **1.4.1 Effects of Si on Cd toxicity in wheat**

Wheat has been recognized as intermediate-Si accumulator. Although, some studies have showed that Si supply reduces Cd concentrations in wheat plants (Hussain et al. 2015; Rizwan et al. 2016b), the underlying mechanism is still unclear.

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To confirm whether Si supply decreases Cd accumulation is Cd concentration-dependent, 1, 5, 25  $\mu\text{M}$   $\text{CdCl}_2$  were applied in the presence or absence of Si in wheat plants hydroponically. Subcellular distribution of Cd as affected by Si was also determined. To test whether Si decreases Cd accumulation by modulating root exudation, exudation of low molecular weight organic acids under Cd with or without Si addition was studied (Chapter 2).

To evaluate how Si affects time-dependent Cd stress in wheat, apoplastic and symplastic Cd uptake, cell wall properties and suberin lamellae development were investigated in Si-supplied and non-Si-supplied plants under short- and long-term Cd exposures (Chapter 3).

#### **1.4.2 Effects of sulfur on Cd toxicity in faba bean (*Vicia faba* L.)**

It is well documented that sulfur application alleviates Cd toxicity by enhancing antioxidant capacity in plants (Anjum et al. 2008; Khan et al. 2015). Phenolic compounds are well studied antioxidants, and acetylated and non-acetylated kaempferol glycosides account for the majority of phenolic compounds in faba bean leaves (Neugart et al. 2015). To date, information on if sulfur mitigates oxidative stress induced by Cd through modulating phenolic compounds is still lacking. On the other hand, how excess supply sulfate affects Cd accumulation in plants is also unclear.

To fill the gap whether phenolic compounds perform as antioxidants to ameliorate Cd toxicity as affected by sulfate in faba bean, effects of sulfate on oxidative stress, Cd distribution and phenolic compounds were determined (Chapter 4).

To test how excess sulfate supply affects Cd accumulation at flowering stage, stable isotope of Cd was used to trace Cd from flower bud stage to flowering stage. To investigate whether mineral nutrients and S-containing compounds are related to Cd accumulation, correlations of Cd with nutrients, sulfate and non-protein thiol were also determined (Chapter 5).

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## **Chapter 2**

### **Silicon-enhanced oxalate exudation contributes to alleviation of cadmium toxicity in wheat**

Jiawen Wu, Christoph-Martin Geilfus, Britta Pitann, and  
Karl-Hermann Mühling

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## Silicon-enhanced oxalate exudation contributes to alleviation of cadmium toxicity in wheat



Jiawen Wu, Christoph-Martin Geilfus, Britta Pitann, Karl-Hermann Mühling\*

Institute of Plant Nutrition and Soil Science, Kiel University, Hermann-Rodewald-Str. 2, 24118, Kiel, Germany

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## ABSTRACT

Cadmium (Cd) contamination is a threat to agricultural plant production. Silicon (Si) supply to Cd-stressed plants is known to ameliorate growth by decreasing the concentrations of Cd in plant tissues. To elucidate the underlying mechanism in wheat, hydroponic experiments were conducted under Cd stressed conditions in the presence or absence of Si, with special emphasis on root morphology, root exudation of oxalate and Cd allocation. We found that Si supply decreased Cd accumulation in plant, which resulted in better plant growth. Further studies of subcellular distribution showed that Si did not accelerate Cd accumulation into cell walls or vacuoles. Since acropetal translocation of Cd was not affected regardless of Si addition as well, we reason the Si-related decrease in leaf Cd concentrations was based on exclusion at the root side. This effect can be traced back to oxalate exudation by roots. In this study, the ability to exude oxalate through the root apex in Cd-stressed plants was weakened, while Si addition recovered the ability, thus reducing Cd uptake by roots. To our knowledge, we showed the first time that Si decreases Cd uptake by the increase of root oxalate exudation as an avoidance mechanism. Meanwhile, we also found Si supply increased total number of root tips which might facilitate root oxalate exudation.

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## 1. Introduction

Increased concern about cadmium (Cd) entering the human food chain, especially by agricultural practice, was addressed in recent years. Of all heavy metals, Cd attracted a considerable interest due to its high solubility and mobility in agricultural soils (Bingham, 1979), which not only has implications on yield and quality of plant-based foods, but also it is potentially harmful to human health (Mortvedt, 1996). Consumption of Cd-loaded food is threatening human health, e.g., leading to renal dysfunction, bone demineralization, and cancer (Bernard, 2008; Godt et al., 2006). On the plant level, Cd toxicity, even at very low concentration, disturbs metabolic key processes such as photosynthesis, transpiration, respiration or nutrients homeostasis (Ali et al., 2014; Stohs and Bagchi, 1995). As a consequence, Cd toxicity induces plant growth retardation resulting in yield losses (Aery and Rana, 2003; Lux et al., 2011). Thus, it is urgent to decipher strategies that help the plant to reduce Cd uptake and its accumulation.

In this regard, silicon (Si) was reported to alleviate Cd stress (Adrees et al., 2015; Wu et al., 2013). It is widely accepted that Si-related mitigation of Cd toxicity is based on a decrease of Cd accumulation in plants such as rice (*Oryza sativa* L.) (Kim et al., 2014), durum wheat (*Triticum turgidum* L.) (Naeem et al., 2015), maize (*Zea mays* L.) (Cunha et al., 2008), tomato (*Solanum lycopersicum* L.) (Wu et al., 2015), peanut (*Arachis hypogaea* L.) (Shi et al., 2010), and cotton (*Gossypium hirsutum* L.) (Farooq et al., 2013). Taken together, previous studies showed that Si mainly decreased root Cd uptake and/or restricted root-to-shoot Cd translocation. Ma et al. (2015) have already demonstrated, by using suspension cells of rice, that negatively charged Si-hemicellulose is formed that is able to bind Cd cations which ultimately prevents the cellular uptake of Cd. For protoplasts, Greger et al. (2016) indicated that Si-caused decreases in Cd uptake in wheat are related to, e.g., down-regulation of *LCT1* (low affinity cation transporter 1) and *HMA2* (heavy metal ATPase 2), as well as up-regulation of *PCS1* (phytochelatin synthase 1) and *IRT1* (iron-regulated transporter 1).

Beside this, avoidance mechanism that is based on the root exudation of low molecular weight organic acids (LMWOA) contributes tremendously to decrease Cd uptake into plants (Clemens et al., 2002). For example, Pinto et al. (2008) showed that

\* Corresponding author.

E-mail address: [khmuehling@plantnutrition.uni-kiel.de](mailto:khmuehling@plantnutrition.uni-kiel.de) (K.-H. Mühling).

under Cd stress sorghum mainly exuded malate, while maize preferably released citrate to resist Cd toxicity. This is in line with findings of Zhu et al. (2011) who concluded that root oxalate exudation is the key factor to exclude Cd from entering roots in tomato. Taken together, it seems that root exudation is correlated to amelioration of Cd toxicity, but is species-dependent. Up to date, it is still unknown whether Si contributes to the reduction of Cd uptake that is based on the release of root exudation.

Thus, the aim of this study is to test whether root oxalate exudation facilitates Si-mediated amelioration of Cd toxicity in wheat (*Triticum aestivum* L.). According to our preliminary experimental data, the amount of root oxalate exudation is much higher than citrate and malate in wheat, thus we choose oxalate as a target in the present study. Besides, we also examined acropetal translocation and subcellular distribution of Cd as affected by Si.

## 2. Materials and methods

### 2.1. Plant material and cultivation

Seeds of wheat (*T. aestivum* L. cv. JB Asano) were surface sterilized and soaked in aerated deionized water for 6 h. Afterwards, seeds were sown into quartz sand and germinated for 10 d. Uniform wheat seedlings were selected and 4 plants per pot were transferred into 5 L nutrient solution including macronutrients (mM:  $\text{KH}_2\text{PO}_4$ , 0.2;  $\text{K}_2\text{SO}_4$ , 1.0;  $\text{Ca}(\text{NO}_3)_2$ , 2.0;  $\text{MgSO}_4$ , 0.5) and micronutrients ( $\mu\text{M}$ : Fe-EDTA, 200;  $\text{H}_3\text{BO}_3$ , 5;  $\text{MnSO}_4$ , 2;  $\text{ZnSO}_4$ , 0.5;  $\text{CuSO}_4$ , 0.3;  $(\text{NH}_4)_2\text{Mo}_7\text{O}_{24}$ , 0.01). To avoid osmotic stress, the strength of nutrient solution was gradually increased from 1/4 strength (for 3 d) to 1/2 strength (for 4 d) and to full strength at day 8. The pH was adjusted to  $6 \pm 0.1$  with HCl and NaOH and renewed every 3 d. Wheat seedlings were cultivated under controlled conditions in the greenhouse of Kiel University ( $54^\circ 20' 50.4''\text{N}$ ,  $10^\circ 06' 59.9''\text{E}$ ) at  $20/15^\circ\text{C}$  (day/night) and  $14\text{ h d}^{-1}$  photoperiod from 6:00 to 20:00 with  $250\ \mu\text{mol photon m}^{-2}\text{ s}^{-1}$  light intensity recorded by a light meter (Li-189, Lincoln, USA).

### 2.2. Experimental treatments

#### 2.2.1. Experiment 1: Cd allocation in plants as affected by Si

The first experiment is to analyze Cd concentrations in the shoot and root, and subcellular Cd distribution in different organs. The experimental design was as follows: after 10 d germination, uniform seedlings were pre-cultivated with or without Si addition for 7 d. Then seedlings were subjected to Cd treatments for 14 d.  $\text{Na}_2\text{SiO}_3$  and  $\text{CdCl}_2$ , served as the source of Si and Cd, respectively. Additional NaCl was supplemented to treatments without added Si to compensate the extra  $\text{Na}^+$  induced by  $\text{Na}_2\text{SiO}_3$ . In this experiment, two levels of Si (0 and 1 mM) and four levels of Cd (0, 1, 5, 25  $\mu\text{M}$ ) were conducted. This resulted in eight treatments: control, Si, Cd1, Si + Cd1, Cd5, Si + Cd5, Cd25, and Si + Cd25. After 14 d Cd treatment, plants were harvested. To remove adhering nutrients on plant material, plants were rinsed with deionized water at least three times. Plants were divided into shoots and roots and fresh weight was recorded.

For dry weight determination, shoots and roots were dried at  $65^\circ\text{C}$  until constant weight. Another set of fresh plants was divided into old leaves (the oldest 2 leaves), middle-aged leaves (the rest of leaves), young leaves (the youngest 2 leaves), sheaths + stems and roots, subsequently frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ .

#### 2.2.2. Experiment 2: effects of Si on root morphology and oxalate exudation under Cd stress

In this second experiment, plants cultivation was the same as described in experiment 1 (Section 2.2.1). After 7 d pre-cultivation, plants were stressed with  $5\ \mu\text{M}$  Cd with or without Si. This resulted

in four treatments: control, Si, Cd ( $5\ \mu\text{M}$  Cd), Si + Cd (1 mM Si and  $5\ \mu\text{M}$  Cd). After 14 d Cd treatment, one set of intact plants was taken for collection of root oxalate exudation while another set of plants was harvested for determination of root morphology.

#### 2.2.3. Experiment 3: effects of oxalate supply on Cd concentrations in plants

In order to test the effect of exogenous oxalate supply on Cd concentrations in wheat, a third experiment was implemented. Germination was the same as described above (see Section 2.1). After 10 d germination, seedlings were cultivated for 7 d with only nutrient solution as mentioned above (see Section 2.1). After 7 d cultivation, plants supplied with  $5\ \mu\text{M}$  Cd were subjected to different exogenous oxalate concentrations (0, 2.5, 5, 25  $\mu\text{M}$ ). The molar ratios of Cd to oxalate were 1:0, 1:0.5, 1:1, and 1:5. The pH of the solution was adjusted to  $6 \pm 0.1$  with HCl and NaOH. After 30 h Cd treatment, plants were divided into shoots and roots, and dried at  $65^\circ\text{C}$  until constant weight.

### 2.3. Translocation factor and root morphology

Cd translocation capacity from organ A to organ B was estimated by the translocation factor (TF). The Cd-TF was calculated according to Shi et al. (2010) as follows:

$$\text{TF}(\%)_{(A \text{ to } B)} = \text{Cd}_{(B)} / \text{Cd}_{(A)} * 100\%. \quad (1)$$

Root samples from experiment 2 were harvested and scanned by a high resolution scanner (V700 photo, Epson Perfection, Amsterdam, the Netherlands), then the pictures were analyzed by imaging software (WinRHIZO, Pro2009a) to obtain different root parameters, e.g., root surface area, total root length, total root tip amount and average root diameter.

### 2.4. Subcellular distribution of Cd

Tissues were separated into cell walls, organelle and soluble fractions based on their different settling after centrifugation according to the method of Weigel and Jäger (1980). Briefly, different plant tissues (old leaves, middle-aged leaves, young leaves and roots) were homogenized in grinding medium [0.25 M sucrose, 50 mM Tris-HCl buffer (pH 7.5), 1 mM dithioerythritol]. The homogenized solution was filtered through cheesecloth and the residue on the cheesecloth was washed at least three times with grinding medium. To obtain the total cell wall, the filtrate was centrifuged at 300g for 30 s. The gained pellet together with the washed plant residues was regarded as cell wall. The filtrate was then further centrifuged at 20,000g for 45 min, and the obtained pellet was regarded as organelle fraction, while the supernatant was taken as soluble fraction. All the steps were conducted at  $4^\circ\text{C}$ . Cd concentration in the soluble fraction was determined directly after dilution whereas solid materials of organelle and cell wall fractions were digested with pure  $\text{HNO}_3$  first and then determined after dilution (see Section 2.6 in details).

### 2.5. Collection of root exudates and oxalate analysis

As described in experiment 2 (see Section 2.2.2), after treatment, seedlings were removed from pots, and roots were rinsed with deionized water three times. To collect root exudates, plants were subsequently immersed into aluminum foil wrapped beakers containing 250 mL deionized water (one plant per beaker) and placed in the greenhouse under light conditions at  $20^\circ\text{C}$ . After 6 h, one drop 0.02% sodium azide was added into solution to avoid microbial degradation. Then, the solution was filtered through  $0.45\text{-}\mu\text{m}$  membrane filter and concentrated to 2 mL by using a

rotary evaporator (Büchi, R-114, Germany) at 40 °C with 0.1 kPa. The concentrated solution was filtered through a 0.22- $\mu\text{m}$  membrane filter again and then stored at  $-20\text{ }^\circ\text{C}$  until analysis of organic acids (Xie et al., 2013).

Oxalate was determined by ion chromatography (Dionex ICS-5000, Sunnyvale, USA). The mobile phase was 100 mM NaOH at a flow rate of  $1\text{ mL}\cdot\text{min}^{-1}$  and the total run time was 28 min. The injection volume was 10  $\mu\text{L}$ .

### 2.6. Determination of Cd concentration

Finely ground samples were digested with pure  $\text{HNO}_3$  at  $190\text{ }^\circ\text{C}$  for 45 min (10 min pre-heating, 15 min heating, 20 min cooling) in a microwave oven (Mars 6, CEM Corporation, USA) with the settings described in details by Jezeck et al. (2015a). Samples were diluted with 2%  $\text{HNO}_3$  and determined by inductively coupled plasma mass spectroscopy (ICP-MS; Agilent 7700, Agilent Technologies Inc., USA) as described by Jezeck et al. (2015b).

### 2.7. Statistical analysis

The statistical analysis was achieved using SPSS 16.0 software. Values were subjected to student's *t*-test or one-way ANOVA according to Duncan's Multiple Range Test at the 5% significance.

## 3. Results

### 3.1. Plant growth

After 14 d Cd treatment, all Cd stressed plants, especially old leaves showed leaf chlorosis, regardless of low ( $1\text{ }\mu\text{M}$  Cd), intermediate ( $5\text{ }\mu\text{M}$  Cd) or high ( $25\text{ }\mu\text{M}$  Cd) Cd level. However, Si supply obviously counteracted the Cd stressed symptoms.

As shown in Table 1, Si supply did not change plant biomass as compared with control. Also, low Cd supply had no significant effect on biomass as well. Under intermediate (Cd5) or high (Cd25) Cd stress, the biomass was significantly reduced in comparison with control, and Si addition significantly increased biomass compared with the corresponding Cd-stress except for root dry weight (Table 1).

### 3.2. Cd concentrations and contents in shoots and roots

In wheat seedlings, Cd concentrations in roots were about 14–20 times of that in shoots (Fig. 1A, C). The increase of Cd concentrations in nutrient solution from 1 to  $25\text{ }\mu\text{M}$  was followed by increasing Cd concentrations and contents in shoots and roots as well (Fig. 1). Compared with the corresponding Cd treatment, Si addition significantly decreased Cd concentrations in both shoots and roots regardless of low, intermediate or high Cd level (Fig. 1A,

C). The same trend was observed for Cd contents with the exception of high Cd stress; here, Si supply had no significant effects on Cd contents in shoots and roots (Fig. 1B, D).

### 3.3. Subcellular distribution of Cd

Under low Cd stress ( $1\text{ }\mu\text{M}$  Cd), Si supply significantly decreased total Cd concentrations in young leaves, middle-aged leaves, old leaves, and roots (Fig. 2A–D). Within the different leaves, Si addition significantly decreased Cd concentrations in cell walls, the organelle and soluble fractions of both young- and middle-aged leaves (Fig. 2A, B). In old leaves, Si supplement only decreased Cd concentrations in the soluble fractions, whereas Si did not change Cd concentrations in cell walls and the organelle fractions (Fig. 2C). In roots, Si supply remarkably reduced Cd concentrations in the organelle and soluble fractions, while it had no effect on Cd concentrations in cell walls. (Fig. 2D)

Under intermediate Cd stress (Cd5), in young leaves Si addition significantly decreased Cd concentrations in cell walls, the organelle and soluble fractions which was reflected by a decrease in total Cd concentration (Fig. 2E). In middle-aged leaves, Si supply did not affect Cd concentrations in cell walls, but obviously reduced Cd concentrations in both organelle- and soluble fractions resulting in a decrease of total Cd concentrations (Fig. 2F). In old leaves, decrease of Cd concentrations in soluble fractions by Si contributed to decreased total Cd concentrations when no different Cd concentrations were shown in cell walls and organelle fractions regardless of Si supply or not (Fig. 2G). In roots, Si addition significantly decreased total-, cell wall- and soluble fraction- Cd concentrations while Si did not significantly affect organelle fraction Cd concentrations (Fig. 2H).

Under high Cd stress ( $25\text{ }\mu\text{M}$  Cd), Si supplementation reduced organelle and soluble fractions, as well as total Cd concentrations, while Si addition had no effect on cell wall Cd concentrations in both young- and old leaves (Fig. 2I, K). In middle-aged leaves, Cd concentrations in cell walls, soluble fractions, even total leaves were not considerably changed with added Si, but organelle fraction Cd concentrations were decreased (Fig. 2J). In roots, Si addition did not significantly affect Cd concentrations in organelle fractions whereas it remarkably decreased total Cd concentrations including cell walls and soluble fractions Cd (Fig. 2L).

With regard to subcellular distribution proportion, soluble fraction of Cd (mainly consists of vacuolar compartment) accounted for the largest one, ranging from 59% to 80% in the leaf and root differently. Cell wall of Cd occupied 19–36% while organelle fraction had the minimum percentage with 1–5% in different organs (Table 2). Nevertheless, compared with corresponding Cd stress, Si addition had no significant impact on subcellular distribution percentage irrespective of low, intermediate or high Cd stress (Table 2).

### 3.4. Translocation factor

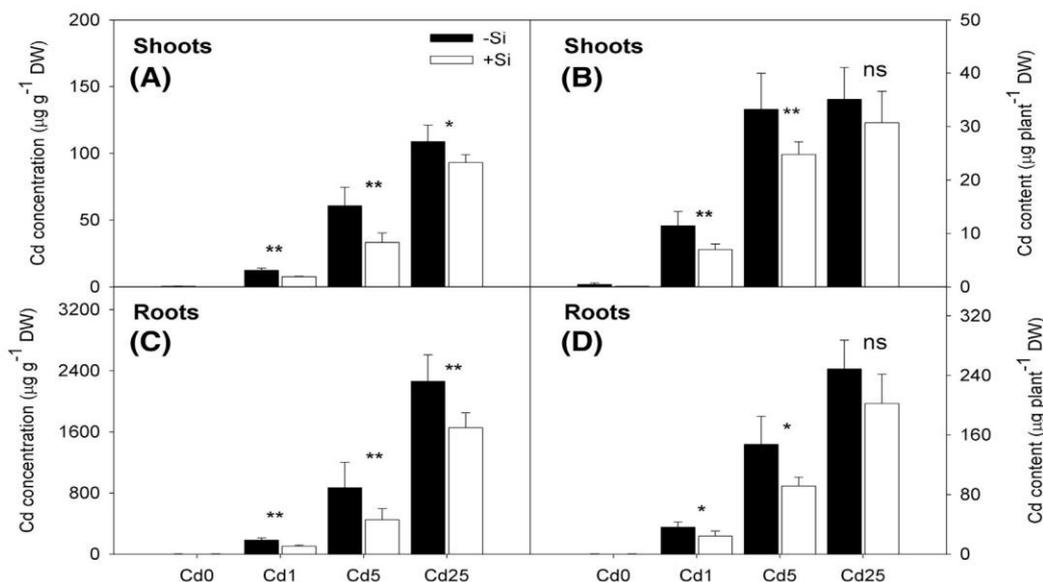
Cd translocation from roots to shoots was not affected by Si addition at the condition of low and intermediate Cd levels (Fig. 3A). At high Cd level, Si supplementation even facilitated Cd root-to-shoot translocation, which was correlated with enhanced Cd roots to sheaths + stems translocation by Si (Fig. 3B). At low Cd level, Si addition did not change TF of root-to-shoot and root-to-(sheath + stem) (Fig. 3A, B), but Si supply inhibited Cd translocation from sheaths + stems to middle-aged leaves (Fig. 3D). At the intermediate Cd level, TF values were not significantly affected by Si supply (Fig. 3). At high Cd level, Si addition accelerated Cd translocation from root-to-(sheath + stem) without affecting sheaths + stems to leaves translocation (Fig. 3B–E).

**Table 1**

Effects of Si on biomass in wheat under Cd stress.

Treatment	Fresh weight (g)		Dry weight (g)	
	Shoot	Root	Shoot	Root
Control	7.38 ± 1.01a	3.94 ± 0.73a	0.95 ± 0.13a	0.23 ± 0.03a
Si	6.71 ± 1.42a	3.57 ± 0.96a	0.92 ± 0.25ab	0.21 ± 0.07ab
Cd1	7.19 ± 0.89a	3.48 ± 1.08a	0.92 ± 0.10ab	0.20 ± 0.06ab
Si + Cd1	6.92 ± 0.92a	3.99 ± 0.94a	0.92 ± 0.11ab	0.24 ± 0.06a
Cd5	3.88 ± 0.59c	2.54 ± 0.43b	0.55 ± 0.06c	0.17 ± 0.02b
Si + Cd5	5.49 ± 0.91b	3.38 ± 0.84a	0.77 ± 0.16b	0.20 ± 0.03ab
Cd25	1.91 ± 0.21d	1.20 ± 0.30c	0.32 ± 0.03d	0.11 ± 0.02c
Si + Cd25	2.05 ± 0.32d	1.58 ± 0.22c	0.33 ± 0.05d	0.12 ± 0.02c

Different letters in the same column represent significant differences at  $P < 0.05$ . Values are means ± SD ( $n = 5$ ). Cd1,  $1\text{ }\mu\text{M}$  Cd; Si + Cd1, 1 mM Si with  $1\text{ }\mu\text{M}$  Cd; Cd5,  $5\text{ }\mu\text{M}$  Cd; Si + Cd5, 1 mM Si with  $5\text{ }\mu\text{M}$  Cd; Cd25,  $25\text{ }\mu\text{M}$  Cd; Si + Cd25, 1 mM Si with  $25\text{ }\mu\text{M}$  Cd.



**Fig. 1.** Effects of Si supply (1 mM Si) on Cd concentrations (A, C) and contents (B, D) in shoots and roots of wheat under low (1  $\mu\text{M}$ , Cd1), intermediate (5  $\mu\text{M}$ , Cd5) and high (25  $\mu\text{M}$ , Cd25) Cd stresses. Values are means  $\pm$  SD ( $n=5$ ). Asterisk "\*" represents significant difference at  $p < 0.05$  while "\*\*\*\*" indicates significant difference at  $p < 0.01$  according to student *t*-test.

### 3.5. Root morphology

With the treatment of 5  $\mu\text{M}$  Cd for 14 d, Si supply increased root biomass, but did not reach significant level in root dry weight (Table 1). Compared with control, Cd stress did not change root surface area, total root length and average root diameter, but significantly decreased total number of root tips. Si supplementation remarkably increased the total number of root tips, as well as root surface area under Cd stress, while it had no effect on total root length and average root diameter (Table 3).

### 3.6. Root oxalate exudation

In comparison to the non-stressed control, Cd toxicity markedly decreased oxalate exudation to a level that was below the detection limit. The addition of Si to Cd-stressed plants increased root oxalate exudation to 192  $\mu\text{g}$  per plant and 46 ng per root tip, respectively (Fig. 4).

### 3.7. Exogenous oxalate supply under Cd stress

Under conditions of short-term (30 h) exposure to Cd stress, exogenous oxalate addition did not change plant biomass in shoots and roots (Fig. 5A, B). Nevertheless, exogenous oxalate significantly decreased Cd concentrations in shoots and roots compared with non-oxalate addition under Cd stress. The higher the oxalate concentrations, the less Cd was in shoots or roots (Fig. 5C, D).

## 4. Discussion

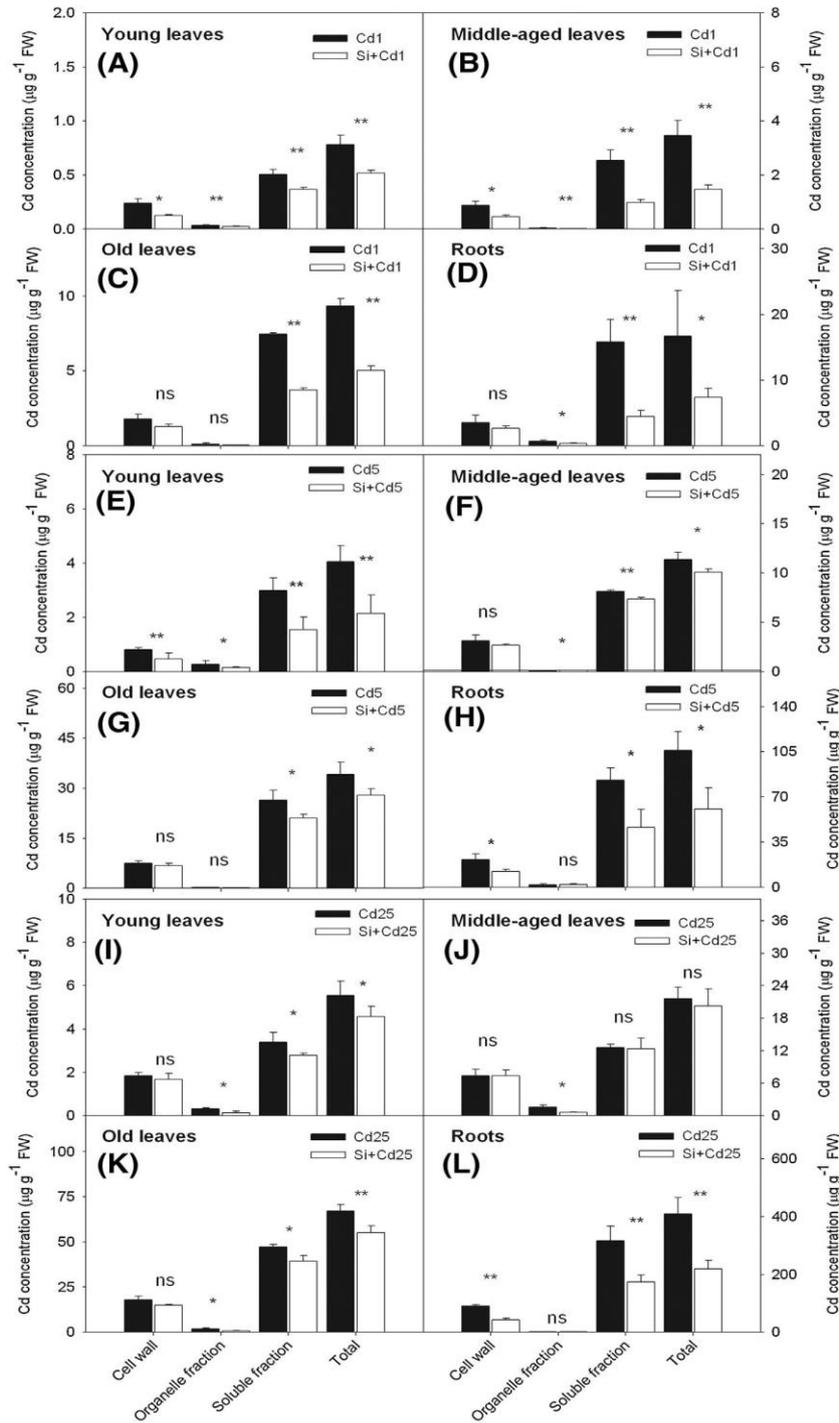
### 4.1. Effects of Si on biomass and Cd accumulation in plants

Cd accumulates in soils as a result of industrial pollution, phosphate fertilizer application, sewage sludge and wastewater usage etc. (Mortvedt, 1996). In agreement with previous studies (Kim et al., 2014; Adrees et al., 2015; Hussain et al., 2015), we here reported in bread wheat (*T. aestivum* L.) that Cd toxicity impaired

shoot and root biomass formation especially with high Cd stress, and Si ameliorated these adverse effects on growth (Table 1). We also found that Si addition significantly depressed Cd concentrations in both shoots and roots under all Cd doses (low, intermediate and high), and suppressed Cd contents as well (Fig. 1), which is consistent with previous studies in other plants (Farooq et al., 2013; Nwugo and Huerta, 2008). In this study, under high Cd stress, Si addition decreased Cd concentrations ( $\mu\text{g g}^{-1}$ ) in roots and shoots and did not affect Cd contents ( $\mu\text{g plant}^{-1}$ ) (Fig. 1D). This might be explained by a dilution effect of Si: Si addition, to some extent, increased biomass, though it did not reach significant level (Table 1). This phenomenon was previously observed by Rizwan et al. (2012) in durum wheat, which showed that amorphous Si supply significantly decreased Cd concentrations but did not change Cd contents in shoots.

### 4.2. Effects of Si on subcellular Cd distribution in plants

In general, plants employ two strategies to resist heavy metal toxicity including Cd: tolerance and avoidance (Clemens, 2006). The former is to compartment heavy metal into less metabolically active tissues such as cell wall or vacuole whereas the latter represents inhibiting heavy metal to enter into plants (Clemens, 2006; Clemens et al., 2013). Although it is well known that Si is able to ameliorate Cd stress in plants (Adrees et al., 2015; Wu et al., 2013), information on Si involved in tolerance and/or avoidance mechanism to mitigate Cd toxicity in wheat is still lacking. In this study, we reported the effect of Si-addition on the Cd concentrations in different subcellular fractions (soluble fractions, cell walls, organelle fractions) of organs (root and leaves), with leaves in different developmental stages (young, middle-aged and old leaves). The order of Cd concentrations in the different organs was: roots > old leaves > middle-aged leaves > young leaves. The order of Cd concentrations in the different subcellular fractions was: soluble fractions > cell walls > organelle fraction (Fig. 2). Regardless of Si addition or not, the proportions of Cd in different subcellular fractions were not affected (Table 2). Unlike in other plants, in

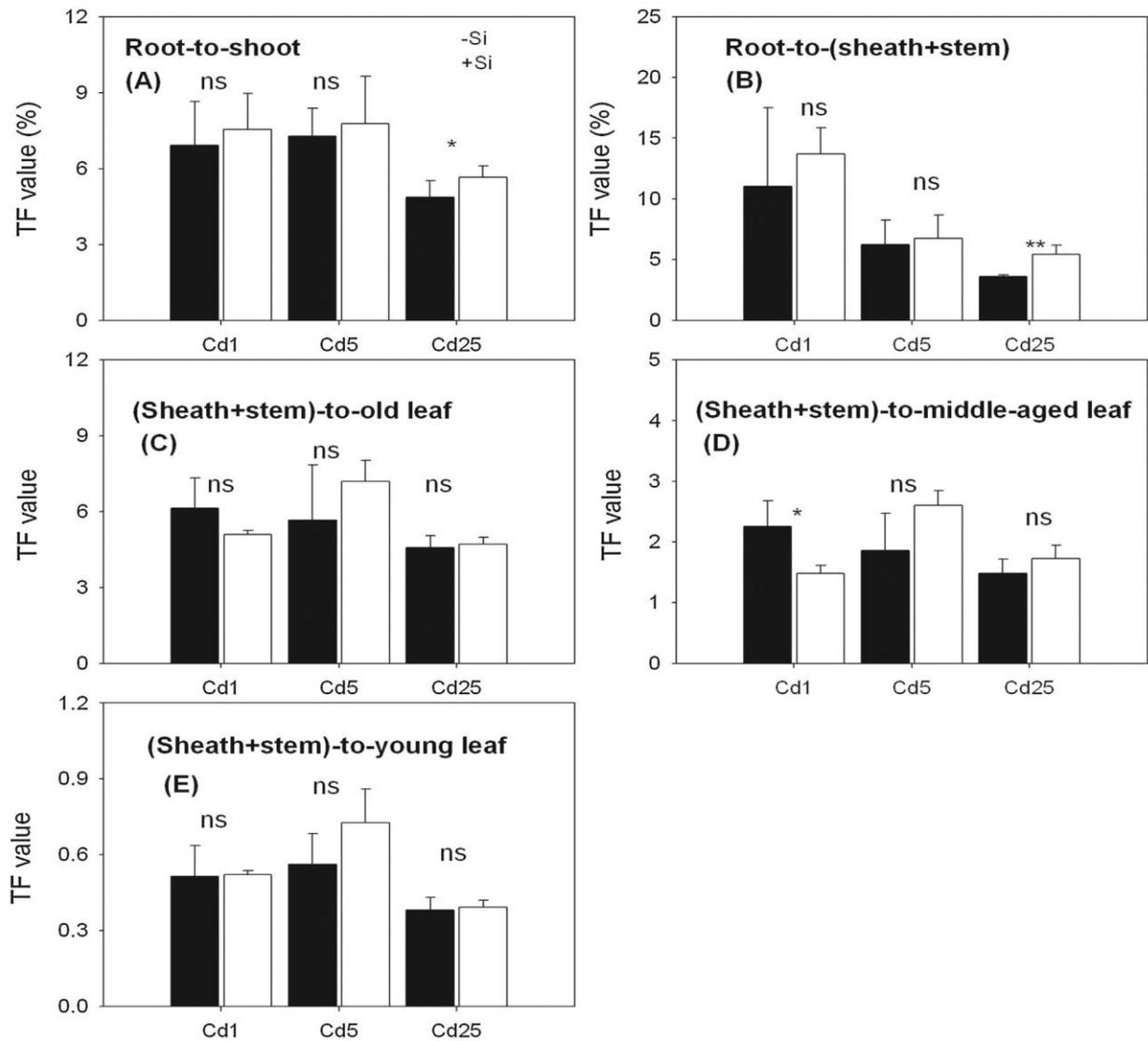


**Fig. 2.** Subcellular distribution of Cd in young-, middle-aged, old leaves and roots under low Cd (Cd1, 1  $\mu\text{M}$  Cd; A–D), intermediate Cd (Cd5, 5  $\mu\text{M}$  Cd; E–H) and high Cd (Cd25, 25  $\mu\text{M}$  Cd; I–L) stresses as affected by Si. Values are means  $\pm$  SD ( $n=4$ ). Asterisk "\*" represents significant difference at  $p < 0.05$  while "\*\*\*\*" indicates significant difference at  $p < 0.01$  according to student  $t$ -test.

**Table 2**  
Effects of Si on subcellular distribution proportions of Cd in wheat.

Treatment	Young leaves (%)			Middle-aged leaves (%)			Old leaves (%)			Roots (%)		
	SF	OF	CW	SF	OF	CW	SF	OF	CW	SF	OF	CW
Cd1	65±6	5±0	30±5	73±5	1±0	26±5	80±2	1±1	19±3	72±12	4±2	24±11
Si+Cd1	71±2	5±0	24±2	67±3	2±0	31±3	74±3	1±0	25±3	60±2	4±0	36±2
Cd5	73±1	3±0	24±2	72±4	1±0	27±4	77±1	1±0	22±0	78±2	2±1	20±3
Si+Cd5	70±3	5±1	25±3	73±1	1±0	26±1	75±2	1±0	24±2	76±4	3±1	21±4
Cd25	61±4	5±1	34±4	59±4	7±2	34±3	71±2	2±1	27±3	77±3	1±0	22±3
Si+Cd25	61±3	3±2	36±5	61±6	3±1	36±5	71±1	2±0	27±1	79±1	1±0	20±1

Values are means ± SD (n=4). Cd1, 1 μM Cd; Si+Cd1, 1 mM Si with 1 μM Cd; Cd5, 5 μM Cd; Si+Cd5, 1 mM Si with 5 μM Cd; Cd25, 25 μM Cd; Si+Cd25, 1 mM Si with 25 μM Cd. SF, soluble fraction; OF, organelle fraction; CW, cell wall. No significant difference is found between Cd and corresponding Si+Cd treatments according to student *t*-test at  $p < 0.05$  or  $p < 0.01$ .

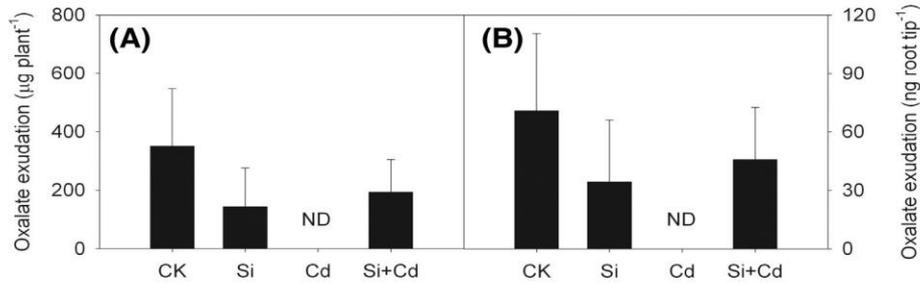


**Fig. 3.** Effects of Si on Cd translocation in wheat. Cd1, 1 μM Cd; Cd5, 5 μM Cd; Cd25, 25 μM Cd. Values are means ± SD (n=4). Asterisk "\*" represents significant difference at  $p < 0.05$  while "\*\*" indicates significant difference at  $p < 0.01$  according to *t*-test.

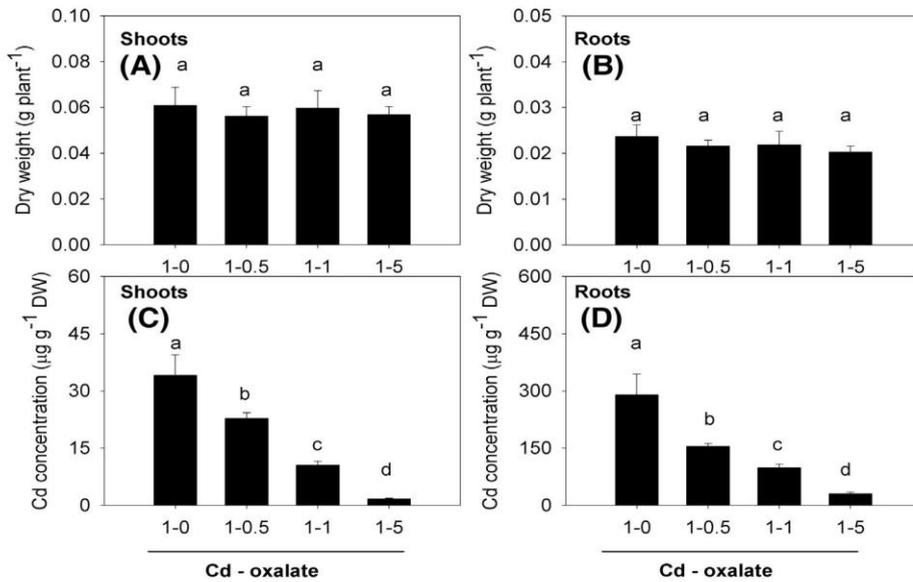
**Table 3**  
Effects of Si on root morphology under Cd stress grown hydroponically.

Treatment	Root surface area (cm <sup>2</sup> )	Total root length (cm)	Total root tip amount	Average root diameter (mm)
Control	1.45 ± 0.2ab	339 ± 123ab	4931 ± 1910a	0.066 ± 0.02a
Si	1.52 ± 0.6ab	366 ± 119a	4174 ± 1513a	0.056 ± 0.01a
Cd	1.05 ± 0.3b	236 ± 121b	2556 ± 1218b	0.101 ± 0.10a
Si + Cd	2.23 ± 1.6a	355 ± 139ab	4212 ± 1307a	0.062 ± 0.03a

Different letters in the same column represent significant differences at  $P < 0.05$ . Values are means ± SD (n = 10). Control, without Si and Cd; Si, 1 mM Si; Cd, 5 μM Cd; Si + Cd, 1 mM Si with 5 μM Cd.



**Fig. 4.** Effects of Si on oxalate exudation by roots under Cd stress in wheat. Values are means ± SD (n = 5). CK, control; Si, 1 mM Si; Cd, 5 μM Cd; Si + Cd, 1 mM Si with 5 μM Cd. A, oxalate exudation content per plant; B, oxalate exudation content per root tip. ND, not detected.



**Fig. 5.** Effects of exogenous oxalate on biomass (A, B) and Cd concentrations (C, D) under Cd stress in wheat. Values are means ± SD (n = 4). Bars with different letters mean significant differences at  $P < 0.05$ . 1–0, 1–0.5, 1–1 and 1–5 represent the molar ratios of Cd concentration to oxalate concentration in growth medium.

which Si facilitates Cd to accumulate in cell walls (Vaculík et al., 2012; Ye et al., 2012) or vacuoles (Ma et al., 2016) to enhance Cd tolerance, it seems that Si prefers to modulate avoidance mechanism to alleviate Cd toxicity in wheat rather than tolerance in *planta*.

#### 4.3. Effects of Si on Cd translocation

As reported in previous studies, Cd hyper-accumulators prefer to transport more Cd into shoots (Clemens et al., 2013) while Cd-

sensitive plants including wheat tend to accumulate Cd into roots (Berkelaar and Hale, 2000). Whether Si inhibits Cd translocation in wheat to alleviate Cd stress is also studied here. The translocation factor (TF) is used to evaluate the translocation capability of Cd from one organ to another organ (Lukačová et al., 2013; Nouri et al., 2009). We found at low and intermediate Cd levels, Si addition had no significant influence on TF values of root-to-shoot. At a high Cd level, Si supplementation facilitated root-to-shoot Cd translocation (Fig. 3), even though it was observed that Si addition decreased the Cd concentrations in the shoot. This means that other physiological

factors than the translocation of Cd must be the reason for the amelioration of Cd stress by Si. We assume it is likely that Si decreases the Cd uptake via effects exerted on the root rather than lowering the acropetal translocation of Cd.

#### 4.4. Effects of Si on Cd uptake regarding to root oxalate exudation

Since Si supply reduced Cd accumulation in wheat in this study without affecting tolerance mechanism (See Section 4.2) and Cd acropetal translocation (See Section 4.3), we speculated that an avoidance mechanism plays a pivotal role in Si-mediated reduction of Cd uptake. Root exudates, especially LMWOA, are suggested to be efficient ligands to alleviate Cd toxicity in plants as they can reduce the bioavailability of Cd (Cieřliński et al., 1998; Nigam et al., 2001; Pinto et al., 2008). Zhu et al. (2011) demonstrated that a Cd-resistant tomato cultivar accumulated less Cd as a result of increased oxalate exudation, by which Cd was excluded from entering the roots. Previous studies have shown Si-mediated alleviation of Cd toxicity is associated with decreased Cd uptake by roots (Hussain et al., 2015; Liu et al., 2013), but the underlying mechanism of Si-mediated decrease of Cd uptake with involvement of LMWOA is poorly understood. Because our preliminary experimental data showed the amount of oxalate exudation by roots is higher than citrate and malate in wheat, so we only studied oxalate exudation in the present study. We found, after 14 d of Cd treatment alone, oxalate exudation was reduced, but this effect was ameliorated by Si supply, leading to increased oxalate exudation (Fig. 4). As we know, this is new information that exogenous addition of Si improves the capacity to exude oxalate under conditions of Cd stress. Since oxalate exudation is a well-established mechanism for Cd-sensitive plants to exclude excessive uptake of Cd (Cieřliński et al., 1998), we reason that wheat is using the same mechanism to avoid Cd toxicity. Surprisingly, Si is promoting this effect. Based on this we reason that the application of Si has the potential to improve growth under conditions of Cd toxicity via effects exerted on the exudation of oxalate. However, it is not clear that Si restored oxalate exudation in the presence of Cd directly (like through up-regulation genes of oxalate production and exudation) or indirectly (like increase of root tip number as the reason that oxalate is mainly exuded by root apex).

In a follow-up experiment, different molar ratios of Cd to oxalate were added into the nutrient solution to study whether increasing oxalate concentrations in the root medium can reduce uptake of Cd in wheat. As a result, the exogenous supply of oxalate significantly inhibited root Cd uptake in a dose dependent manner, and Cd concentrations were decreased with increasing external oxalate (Fig. 5). As it is shown Cd-stress alone depressed the exudation of oxalate in wheat (Fig. 4) while simultaneous Si-application increased it, and combined with those results that exogenously supplied oxalate inhibited Cd uptake, we could conclude the increase of root oxalate exudation is favorable for keeping Cd from entering into wheat.

In addition, silicon supply considerably increased the root surface area and total number of root tips under Cd stress compared with non-Si-supplied plants (Table 3). Oxalate is mainly secreted from root apex via anion channels (Zhu et al., 2011). Accordingly, in this study, an increased total number of root tips may contribute to enhanced root oxalate exudation. In contrast, Berkelaar and Hale (2000) reported that larger root surface area and number of root tips is positively correlated with higher Cd accumulation. In this study, however, plants with a larger amount of root tips had less Cd accumulation over 14 d Cd treatment which further supports the higher root oxalate exudation is the consequence of lower Cd uptake and increased total number of root tips may be the reason of increased root oxalate exudation. On

the other hand, Cd stress alone without Si application declined oxalate exudation. This resulted in higher Cd accumulation (Fig. 1, 4; Table 3), which further indicates that enhanced root oxalate production is a promising mechanism to lower Cd uptake in wheat. In the present study, it seems that Cd-stressed plants promoted oxalate exudation is the second response as affected by Si indirectly as a result of increased root tip number. Nevertheless, whether Si executes molecular modulation to increase oxalate exudation under Cd stress directly still needs study.

In regard to avoidance mechanism, it is noteworthy that the phenomenon of metal detoxification by Si in culture medium exists which is well documented in aluminum (Al), such as formation of Al-Si complexes in culture solution or hydroxyaluminum silicates (HAS) in the apoplast of maize root apex (Wang et al., 2004). In this study, Cd<sup>2+</sup> activity percentages were not affected regardless of Si addition ranging from 71.54% (1 and 5 μM Cd application) to 71.51% (25 μM Cd application) in the growth medium calculated by Visual MINTEQ 3.1 software, showing no external amelioration mechanism exists here. However, whether Si (or silicic acid) reacts with Cd resulting in less phytotoxic Cd complexes (such as formation HAS under Al toxicity) in the root apoplast to detoxify Cd toxicity remains to be elucidated.

## 5. Conclusion

We demonstrated that Si supply achieved better plant growth is related to the decrease of total Cd concentrations in shoots and roots. Although Si decreased Cd accumulation in plants, the subcellular distribution pattern of Cd was not affected which indicates that Si did not facilitate Cd accumulation into cell walls or vacuoles. Since the translocation factor was not decreased by Si as well, we reason that the decreased leaf Cd concentrations were based on exclusion at the root side rather than the reduced acropetal translocation. The increase of root oxalate exudation as mediated by Si under Cd stress contributes to decreased Cd uptake by roots, and increased total root tips probably causes enhanced root oxalate exudation.

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## **Chapter 3**

### **Reduction of apoplastic fluid-Cd as affected by silicon results in decrease of Cd in wheat**

Jiawen Wu, Hans-Peter Mock, Ricardo F.H. Giehl, Britta Pitann, Michael Melzer, and Karl-Hermann Mühling

to be submitted

## **Reduction of apoplastic fluid-Cd as affected by silicon results in decrease of Cd in wheat**

Jiawen Wu<sup>a</sup>, Hans-Peter Mock<sup>b</sup>, Ricardo F. H. Giehl<sup>b</sup>, Britta Pitann<sup>a</sup>, Michael Melzer<sup>b</sup> and Karl-Hermann Mühling<sup>a\*</sup>

<sup>a</sup>Institute of Plant Nutrition and Soil Science, Kiel University, Hermann-Rodewald-Str. 2, 24118, Kiel, Germany

<sup>b</sup>Department of Physiology and Cell Biology, Leibniz Institute of Plant Genetics and Crop Plant Research, 06466, Gatersleben, Germany

\*Corresponding author,  
Prof. Dr. Karl-Hermann Mühling,  
Email: [khmuehling@plantnutrition.uni-kiel.de](mailto:khmuehling@plantnutrition.uni-kiel.de)

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## **Highlights**

Decrease of Cd concentration by Si under short-term exposure lays the foundation for higher Cd resistance of Si-supplied wheat plants under long-term Cd exposure.

## Abstract

Silicon (Si) alleviates cadmium (Cd) toxicity in plants, but knowledge on mitigation of short-term Cd stress by Si is still lacking. In this study, effects of Si on Cd toxicity under both short- and long-term exposures in wheat were investigated. Generally, Si considerably reduced Cd accumulation, which is ascribed to decreased root Cd absorption rather than acropetal Cd translocation. Under short-term stress, Si significantly decreased apoplastic and symplastic Cd in roots, although symplastic uptake transporters of Cd expression remained unaffected, reflected by unchanged expression of *Nramp5*, *LCT1*, *TM20* and *HMA2*. Since transpiration rate was not altered regardless of Si or Cd addition showing that apoplastic mass flow was unaffected, and no changes occurred in cell wall Cd absorption under short-term stress, we reason that decreased symplastic Cd is the consequence of limitation in apoplastic diffusion of Cd induced by Si. Compared with Cd treatment, Si supply delayed suberin lamellae deposition in roots under short-term Cd exposure. We assume that longer distance of suberin lamellae from root apex by Si+Cd treatment than Cd treatment resulted in larger apoplastic space, which could be the reason for decrease of apoplastic fluid-Cd concentration. Contrastingly, under long-term stress, Si did not affect apoplastic fluid-Cd, but Si decreased cell wall bound- and symplastic Cd. Nevertheless, cell wall properties and transcript levels of transporters related to Cd influx and transport were not affected by Si under Cd toxicity. After the long-term treatment, Si enhanced suberization of roots. The result that apoplastic fluid-Cd only accounted for 3.8% of Cd after 7 d Cd treatment implies that to less extent suberization reduced Cd accumulation through blocking apoplastic pathway. Taken together, we reason that lower Cd concentration under short-term treatment by Si supply contributes to less Cd toxicity in long-term Cd stressed-plants.

**Key words:** Apoplast, cadmium, diffusion, oxalate, silicon, suberin

## Introduction

Cadmium (Cd), a non-essential element, pollutes a large scale of agricultural soils worldwide, which mainly originates from industry, mining, sewage sludge, phosphorus fertilizers (Nagajyoti et al. 2010). When plants are cultivated in Cd-polluted soils, Cd will enter into plants leading to plant growth retardation, inhibition of photosynthesis and nutrient imbalances, resulting in negative effects on human health when entering the food-chain (Aery and Rana 2003; Lux et al. 2011). The characteristics of high solubility, mobility and toxicity of Cd render it urgent to figure out strategies to ameliorate Cd toxicity.

One of the most cost-effective and efficient methods for preventing adverse effects of Cd is to supply beneficial nutrients (Rizwan et al. 2016a). In this regard, silicon (Si) appears as a good candidate, because a broad set of studies have shown that Si is able to successfully mitigate various biotic and abiotic stresses including Cd toxicity (Adrees et al. 2015; Wu et al. 2013). A multitude of studies regarding alleviation of stresses by Si supply focus on rice (*Oryza sativa* L.) plants due to the high Si demand and accumulation. Generally, mechanisms of Si-mediated alleviation of Cd toxicity are as follows: (1) Si supplementation traps more Cd into cell walls and exists in the form of [hemicellulose-Si matrix]-Cd complexes in rice cell walls to detoxify Cd toxicity (Ma et al. 2015). Similar findings, that Si facilitated Cd accumulation into cell walls, resulting in reduction of the root-to-shoot Cd translocation, were also reported for cabbage (*Brassica campestris* L.) (Wu et al. 2016b) and *Kandelia obovata* (Ye et al. 2012); (2) suppression of gene expression related to Cd uptake and transport by Si addition (Kim et al. 2014; Shao et al. 2017); (3) Si-modulated metabolic activities enhance Cd tolerance, i.e., by improving photosynthesis (Nwugo and Huerta 2008), altering the levels of organic acids *in planta* (Wu et al. 2015) or readjusting the cell redox status (Farooq et al. 2016).

Although, Rizwan et al. (2016b) reported that in durum wheat (*Triticum turgidum* L.), an intermediate Si accumulator, Si supply reduced Cd accumulation, the co-precipitation of Si and Cd was not found either in the shoot or in the root, suggesting that, unlike rice, the formation of Si-Cd complexes is not the underlying mechanism for the Si-induced alleviation of Cd toxicity in wheat. In accordance with our previous studies which showed that Si supply attenuates Cd accumulation in bread wheat (*Triticum aestivum* L.) by suppressing Cd uptake by roots rather than acropetal translocation of Cd, and we further indicated that Si-improved oxalate exudation by roots contributes to reduced Cd uptake (Wu et al. 2016a). However, the

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underlying mechanism(s) of alleviating Cd toxicity by Si in wheat is still poorly understood.

Similar to other essential cations, Cd is taken up by roots through apoplastic and symplastic pathways (Clemens et al. 2002; White 2012). As a non-essential element for plants, Cd has no exclusive transporters for uptake and is taken up via non-specific cation transporters, such as for zinc, iron, or calcium (Romè et al. 2016). Nramp (natural resistance associated macrophage proteins) family, P-type heavy metal ATPases family, and ZIP (Zinc-regulated transporter/Iron-regulated transporter-like protein) family are all known to be involved in Cd uptake, sequestration and translocation (Clemens et al. 2002), for example, Nramp5 (Natural resistance-associated macrophage protein 5) (Ishimaru et al. 2012) and LCT1 (Low-affinity Cation Transporter 1) (Uraguchi et al. 2011), which are both localized in the root plasma membrane and responsible for Cd influx; TM20 (Transmembrane 20), which was reported to have efflux Cd activity and to hinder root Cd uptake in wheat (Kim et al. 2008); HMA3 (Heavy Metal ATPase 3), which is characterized to sequester Cd into the vacuole, and HMA2 (Heavy Metal ATPase 2), which is able to load Cd into xylem (Clemens et al. 2013).

In order to address how Si affects Cd absorption through apoplastic and symplastic pathways, in the present study, Si-mediated Cd absorption through apoplastic mass flow and diffusion as well as symplastic uptake were determined under both short- and long-term Cd treatments. To distinguish effects of Si on apoplastic fluid- or cell wall bound-Cd, cell wall properties such as adsorption, cation exchange capacity (CEC) and Cd in different components of cell walls were investigated. Additionally, suberin lamellae was determined under both short- and long-term Cd exposure to assess whether Si altered apoplastic diffusion barriers in roots is related to alleviation of Cd toxicity in plants. Expression of genes related to Cd uptake and transport were determined, including *Nramp5*, *LCT1*, *TM20*, *HMA3*, and *HMA2*. To compare with our previous studies, which indicated enhanced oxalate exudation prevents root Cd uptake under long-term Cd stress (Wu et al. 2016a), oxalate exudation was analyzed under short-term Cd stress, thus testing whether Si executes a direct function to mitigate Cd toxicity by promotion of root oxalate exudation.

## **Materials and methods**

### **Plant material and cultivation**

Seeds of wheat (*T. aestivum* L. cv. JB Asano) were surface sterilized and then thoroughly

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washed with deionized water, followed by soaking in aerated 0.5 mM CaCl<sub>2</sub> for 6 h. Subsequently, seeds were germinated until seedlings were about 9 cm and uniform seedlings were transferred to pots containing the following hydroponic solution: macronutrients [mM: K<sub>2</sub>SO<sub>4</sub>, 1.0; KH<sub>2</sub>PO<sub>4</sub>, 0.2; MgSO<sub>4</sub>, 0.5 and Ca(NO<sub>3</sub>)<sub>2</sub>, 2.0] and micronutrients [ $\mu$ M: Fe-EDTA, 200; ZnSO<sub>4</sub>, 0.5; CuSO<sub>4</sub>, 0.3; H<sub>3</sub>BO<sub>3</sub>, 5; MnSO<sub>4</sub>, 2 and (NH<sub>4</sub>)<sub>2</sub>Mo<sub>7</sub>O<sub>24</sub>, 0.01]. The strength was raised stepwise from one-fourth- to full-nutrient solution in 7 d to avoid osmotic stress. All plants were grown under controlled conditions in the greenhouse (day/night, 20/15 °C) with a photoperiod from 6:00 to 20:00.

### **Experimental treatments**

After 7 d of hydroponic cultivation with or without 1 mM Si (Na<sub>2</sub>SiO<sub>3</sub>) pre-treatment, wheat seedlings were stressed with 5  $\mu$ M Cd (CdCl<sub>2</sub>) in the presence or absence of Si addition, which resulted in four treatments: (1) Control, (2) Si, (3) Cd, and (4) Si+Cd. Treatments without Si application were supplied with NaCl to compensate for extra Na<sup>+</sup> ions supplied by Na<sub>2</sub>SiO<sub>3</sub>. The pH of solution was adjusted to 6  $\pm$  0.1 with NaOH and HCl. Both Cd speciation and Cd activity (Cd<sup>2+</sup> accounts for 71.5% of total Cd) were not affected by Si supply under our experimental conditions based on the calculation of Visual MINTEQ 3.1, a chemical equilibrium model. Plants were harvested at different time points after Cd exposure: 1 h, 6 h, 1 d (24 h), 7 d (168 h) or 14 d (336 h).

### ***In vitro* intact-roots cultivation**

After 7 d pre-cultivation, shoots of plants were decapitated and the roots were still remained in the culture solution. Subsequently, the culture solution was changed to the corresponding treatment solution (namely Control, Si, Cd, and Si+Cd treatment solution). After 24 h treatment, roots were washed with deionized water, dried, and acid-digested to measure Cd concentrations by ICP-MS (inductively coupled plasma mass spectroscopy; Agilent 7700, Agilent Technologies Inc., USA).

### **Cd accumulation and apoplastic fluid-, cell wall-bound and symplastic Cd in roots**

After germination, uniform seedlings were transferred to 5-L plastic pots (four plants per pot) for 7 d cultivation, and then plants were treated with or without Cd/Si. Four biological replicates were designed and one pot was regarded as one biological replicate. Finally, Cd stressed plants were divided into shoots and roots after 1 h, 6 h, 1 d, 7 d, and 14 d Cd exposure, respectively. The shoots and roots were dried at 65°C for 4 d and ground into powder with a grinding miller (Retsch, MM400, Germany) for Cd determination. The

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translocation factor (TF) of Cd was calculated as the percentage of Cd concentration of the shoot to the Cd concentration of the root (Shi et al. 2010).

After 1 h, 6 h, 1 d, 7 d, and 14 d Cd exposure with or without Si addition, roots of seedlings/plants were rinsed with deionized water and gently dried with tissue paper. Subsequently, the roots were desorbed based on the method of Redjala et al. (2011) and Yin et al. (2015). Briefly, two roots of individual treatments were desorbed in 200 mL 5 mM ice-cold  $\text{CaCl}_2$  for 20 min and the desorption solution was renewed every 10 min. Cd concentration in the pooling desorption solution was measured and regarded as Cd in root apoplastic fluid. Subsequently, the roots were frozen in liquid nitrogen for 2 min to destroy the membrane integrity, and then the roots were desorbed in newly changed 500 mL 5 mM  $\text{CaCl}_2$  at room temperature for another 40 min. The Cd concentration in the solution after 40 min desorption represents symplastic Cd, while the remaining Cd in the residues of roots is regarded as cell wall-bound Cd.

#### **Isolation and fractionation of cell wall, cell wall adsorption, and cation exchange capacity (CEC) of cell wall**

*Cell wall isolation:* Plants roots with or without Si treatment after 6 h and 7 d Cd stress were harvested, frozen in liquid nitrogen, and then stored in  $-80^\circ\text{C}$  for cell wall extraction. According to Zhong and Lauchli (1993), roots were ground into powder in liquid nitrogen and then incubated with ice-cold 75% ethanol for 20 min. The supernatant was discarded after centrifugation at 1000 g for 10 min. Subsequently, the pellets were washed with ice-cold acetone, mixed methanol and chloroform (1:1, v/v), and methanol, successively, and each washing step was repeated at least three times and incubated in an ice-water bath for 10 min. Eventually, the remaining pellets were vacuum frozen-dried and stored at  $-20^\circ\text{C}$  until further analysis.

*Cell wall adsorption:* Si- and non-Si treated plants without Cd treatment were harvested at different time points (6 h and 7 d) to measure the capacities of cell wall-Cd adsorption according to Zhu et al. (2012). About 20 mg dried-cell wall materials from each treatment were transferred to 2 mL Eppendorf tubes with 1.8 mL adsorption solution consisting of 5 or 50  $\mu\text{M}$   $\text{CdCl}_2$  in 0.5 mM  $\text{CaCl}_2$  at pH 4.5. The tubes were shaken overnight and then centrifuged at 12,000 g for 10 min. Cd concentration in the supernatant was determined and the difference of Cd concentration in the supernatant before and after Cd adsorption represents capability of Cd adsorption in the cell wall.

*CEC (Cation Exchange Capacity) of cell wall:* Based on the method of Dragišić Maksimović et al. (2012) with minor modifications, cell walls were incubated with 50 mM BaCl<sub>2</sub> at pH 5.0 in an ice-water bath for 2 h. Afterwards, the samples were centrifuged at 2000 g for 15 min and the pellets were repetitively incubated with fresh 50 mM BaCl<sub>2</sub> for another two times as described above. After incubation, the pellets were thoroughly washed with deionized water and frozen-dried. The Ba (barium) concentrations in the pellets were determined by ICP-MS after acid digestion. The amount of Ba in cell walls represents CEC of cell wall for the reason that Ba ion could replace other cations in cell walls. Thus, the unit of CEC was shown as cation charge equivalents substituted by Ba.

*Fractionation of cell wall:* Cd concentrations in different components of cell walls were determined by extracting cell wall components sequentially. Pectin was isolated by hot water twice in 1 h and the resulting pellets were regarded as without pectin (CW-P), followed by washing the CW-P pellets with 4% KOH containing 0.1% NaBH<sub>4</sub> in triplicate in 24 h. The resulting pellets were recognized to remove pectin and partial hemicellulose (CW-P-HC1). Subsequently, the CW-P-HC1 pellets were continued to be triply extracted by 24% KOH containing 0.1% NaBH<sub>4</sub> in 24 h. After centrifugation, the pellets were regarded to be without pectin and hemicellulose (CW-P-HC1-HC2) (Yang et al. 2011; Zhong and Läubli 1993). All the pellets from each step were acid-digested and measured for Cd concentrations by ICP-MS. As a result, we determined Cd concentrations in cell walls (CW-Cd), Cd concentrations in pectin components (P-CW) through subtracting CW-Cd with CW-P, Cd concentrations in hemicelluloses 1 components (HC1-Cd) through subtracting CW-P with CW-P-HC1, and Cd concentrations in hemicelluloses 2 components (HC2-Cd) through subtracting CW-P-HC1 with CW-P-HC1-HC2.

### **Transpiration rate**

After germination, uniform seedlings were transferred to 1 L pots (25 seedlings per pot). Four biological replicates were designed and one pot was regarded as one biological replicate. The nutrient solution was refreshed twice every day and pH was adjusted to  $6 \pm 0.1$  with NaOH and HCl.

Transpiration rate was gravimetrically measured and calculated based on the water loss of plants before and after treatment revised by subtracting water loss of evaporation. After 6 h, 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, and 7 d treatment, transpiration rates of plants in four treatments (Control, Si, Cd, and Si+Cd) were determined.

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**Determination of suberin lamellae deposition in roots**

After 6 h or 7 d treatment, 0-4.5 cm and 5-10 cm root segments from root apex were fixed with 4% (w/v) paraformaldehyde for 2 h under vacuum. After fixation, samples were washed twice with 1x PBS for 1 min and cleared with Clearsee solution for 5 weeks at room temperature. Clearsee solution consisted of 10% (w/v) xylitol powder, 15% (w/v) sodium deoxycholate and 25% (w/v) urea (Kurihara et al. 2015).

After clearing, root segments were incubated in 0.01% (w/v) Fluorol Yellow 088 dissolved in lactic acid at 70 °C for 30 min. Fluorol Yellow 088 should be freshly prepared. Subsequently, samples were rinsed with deionized water three times and 5 min for each time, followed by incubating into 0.5% (w/v) aniline blue for 30 min in the dark at room temperature. Afterwards, samples were rinsed with deionized water three times again and 10 min for each time. Confocal laser scanning microscopy (CLSM) equipped with standard GFP filter was used to observe suberin lamellae of the root segments (Lux et al. 2005).

**Oxalate exudation**

The method of root exudation collection and oxalate analysis are based on Xie et al. (2013) with small modifications. Briefly, five roots of plants were immersed in 250 mL 0.5 mM CaCl<sub>2</sub> for 6 h, and then solutions were filtered and concentrated for oxalate measurement. Oxalate concentrations were determined by ion chromatography (Dionex ICS-5000, Sunnyvale, USA). Oxalate exudation of roots was determined after plants treated with or without Cd in the presence or absence of Si for 6 h.

**Gene expression**

After 6 h or 7 d treatment, total RNA of roots was extracted using TRIzol (Invitrogen) according to manufacturer's instructions, followed by reversing RNA to cDNA by using a Verso cDNA synthesis kit (Thermo Fisher Scientific) including DNase I treatment. Quantitative RT-PCR (qRT-PCR) was conducted by using PowerUp™ SYBR™ green master mix (Applied Biosystems) with specific primers shown in Table 2. The qRT-PCR was run with conditions as follows: 10 min at 95°C; 40 cycles of 15 s at 95°C, 30 s at 59°C, 30 s at 72°C, and followed by establishment of melting curves with 10 s at 95°C, and continuous increment from 65°C to 95°C in a CFX96 Touch RT-PCR detection system (Bio-Rad). Four biological replicates and two technical replicates were performed for individual treatments. Transcript levels of genes were normalized with endogenous control (Actin) as used by Greger et al. (2016) and Kovács et al. (2014) in wheat according to the method of  $2^{-\Delta\Delta CT}$ .

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## Statistical analysis

All the values in this study were subjected to One-Way ANOVA according to Duncan's test or student *t*-test analysis at  $p < 0.05$  or  $p < 0.01$  by using SPSS software (SPSS Inc. 16.0).

## Results

### Effects of Si on biomass, Cd accumulation in plants, and apoplastic and symplastic distribution in roots

To test whether Si executes distinct mechanisms to alleviate Cd toxicity under short- and long-term Cd treatments, Cd accumulation as affected by Si addition from 1 h to 14 d was investigated in this study. As shown in Fig. 1, Cd stress did not affect dry weight of shoots in wheat after 6 h and 1 d Cd stress, but after 7 d and 14 d Cd stress, shoot dry weight was significantly decreased by Cd compared with corresponding control. When compared with Cd treatment alone, Si supply did not considerably alleviate Cd-stressed decrease of shoot dry weight after 7 d Cd treatment, whereas Si recovered shoot dry weight induced by Cd toxicity after 14 d Cd treatment (Fig. 1A). In regard to dry weight of roots, after 6 h, 1 d and 7 d Cd stress, no significant difference was found among treatments regardless of Si or Cd addition. However, after 14 d Cd toxicity, Cd considerably damaged plant growth shown by decreased root dry weight, and Si application mitigated the reduced dry weight of roots induced by Cd toxicity (Fig. 1B).

Further study on Cd concentration, it was observed that under less than 6 h Cd exposure, Si supplementation significantly decreased Cd concentrations in roots rather than in shoots. In contrast, Si supply reduced Cd concentrations in both roots and shoots after longer than 6 h (until 14 d in this study) Cd exposure (Fig. 2). Intriguingly, the translocation capacity of Cd was not affected by Si irrespective of short- or long-time Cd stress (Fig. 2C). Under less than 1 d Cd stress, Si supply decreased Cd concentrations in the apoplastic fluid and the symplast when compared with Cd treatment alone, whereas cell wall-bound Cd was not significantly affected by Si with the exception that Si reduced cell wall-bound Cd concentration after 1 d Cd exposure (Fig. 3). Under long-term Cd exposure (7 d and 14 d), the concentrations of cell wall-bound Cd and symplastic Cd in roots treated with Si were lower than that in the corresponding Cd treatments without Si. No significant difference was found in apoplastic fluid-Cd as affected by Si after long-term Cd exposure (Fig. 3).

As shown in Table 1, after 1 h Cd treatment, most Cd accumulated in the apoplastic fluid

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of roots which comprised 52.4% of total Cd, followed by symplastic Cd with 36.1%, and the resulting 11.5% Cd presented in the form of cell wall-bound Cd. In contrast, when plants were treated with Cd for longer than 1 d, the proportions of Cd distribution were reversed to: symplastic Cd (52.5–58.4%) > cell wall-bound Cd (36.7–37.8%) > apoplastic fluid-Cd (3.8–10.8%). On the other hand, when Si-supplied and non-supplied plants were compared after short-term Cd treatment (1 h and 6 h), it was found that Si addition significantly suppressed the percentages of Cd in apoplastic fluid and promoted the percentages of symplastic Cd with no effects on cell wall bound-Cd proportions (Table 1). Under long-term Cd treatment (> 1 d), Si supplementation had no impacts on Cd distribution in roots with the exception that Si sparingly increased the proportions of apoplastic fluid-Cd (Table 1).

### **Effects of Si on Cd toxicity under short-term treatment**

To study how Si modulates symplastic uptake of Cd, expression levels of genes related to Cd uptake, sequestration and translocation were investigated. Under short-term Cd exposure (6 h), transcript levels of all target genes were not affected by either Cd stress or Si addition when compared with control, with the exception of *HMA3* which was down-regulated by Si under Cd stress (Fig. 4A-E).

Regarding the apoplast, cell walls of roots cultured with and without Si were isolated for analyzing cell wall properties. Under short-term Cd stress (6 h), Cd treatment significantly increased root CEC of cell walls compared with control, and Si addition did not affect CEC irrespective of Cd stress (Fig. 5A). Detailed analysis of Cd in different components of cell walls, it was found that Si supply did not affect Cd accumulation in any components of cell walls, but Cd concentrations in pectin components were higher than that in hemicelluloses 1 and hemicelluloses 2 components (Fig. 5B). Study on Cd adsorption of cell walls cultured with or without Si showed that Si supply did not affect capacity of cell walls to absorb Cd in the incubation solution with either 5  $\mu\text{M}$  Cd or 50  $\mu\text{M}$  Cd (Fig. 5C).

*In vitro* Cd adsorption by intact roots and milled powdery roots was also investigated to test how Si affects Cd absorption. The results showed that Si-supplied plants had no effects on Cd adsorption by powdery roots in the incubation solution with either 5  $\mu\text{M}$  Cd or 50  $\mu\text{M}$  Cd, but it significantly decreased Cd adsorption by intact roots when compared with non-Si-supplied plants (Fig. 5D).

To assess whether Si supply affects apoplastic uptake of Cd through mass flow, we determined transpiration rate since transpiration provides force for apoplastic mass flow. We

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found that transpiration rates of plants were not affected regardless of Cd or Si supply (Fig. 5E). However, apoplastic diffusion barrier, suberin lamellae development was accelerated to be closer to root apex by Cd stress when compared with control after a short-term Cd treatment (6 h), but Si addition promoted longer distance of suberin to root apex under Cd toxicity (Fig. 6A, B). Yellow colour represented suberin deposition in root segments (5-10 mm from root apex) showed that suberin deposition of sole Cd treatment was closer to root apex than Si+Cd treatment (Fig. 6B).

Under short-term Cd treatment (6 h), root exudation of plants-treated with or without Si was collected to analyze oxalate concentrations. Regardless of Si or Cd addition, concentrations of oxalate exudation were not changed (Fig. 7A). As to Cd concentrations under Cd stress in the presence or absence of Si, Si supply had no effects on Cd concentrations under short-term Cd treatment in shoots, but Si addition significantly reduced Cd concentrations in roots (Fig. 7B).

#### **Effects of Si on Cd toxicity under long-term treatment**

By contrast with short-term treatment, after prolonged exposure to treatments (7 d), the expression levels of genes related to Cd uptake, sequestration and translocation were also determined. The expression levels of *Nramp5*, *LCT1* and *HMA3* were significantly up-regulated by Cd regardless of Si supply (Fig. 8A, B and D). Compared with control, the expression of *TM20* was down-regulated by Si. But Si up-regulated the expression level of *TM20* under Cd stress when compared with Cd stress alone (Fig. 8C). The expression level of *HMA2* was not affected regardless of Cd or Si (Fig. 8E).

Regarding the apoplast, under long-term Cd stress (7 d), CEC of cell walls was not affected by either Cd or Si supply (Fig. 9A). With regard to Cd in fractionated components, Cd concentrations in pectin components were much higher than that in hemicelluloses 1 and hemicelluloses 2 components. Si supplementation significantly decreased Cd concentrations in pectin components and total cell walls, and Si did not affect Cd concentrations in hemicelluloses 1 and hemicelluloses 2 components (Fig. 9B). After prolonged cultivation with Si, Cd adsorption of cell walls was not changed by Si in the incubation solution with either 5  $\mu\text{M}$  Cd or 50  $\mu\text{M}$  Cd (Fig. 9C).

Under long-term treatment (7 d), Cd stress did not affect suberin deposition when compared with control. However, Si addition considerably shortened the distance of suberin lamellae deposition to root apex regardless of Cd addition (Fig. 10C). Root segments of 25-30

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mm from root apex clearly showed that yellow deposition (suberin lamellae) was closer to root apex in Si and Si+Cd-treated plants when compared with control and Cd-treated plants (Fig. 10D).

## **Discussion**

### **Effects of Si on Cd absorption by roots and accumulation in plants**

Beneficial effect of Si on improving biomass of plants under Cd toxicity has been well documented (Guntzer et al. 2012; Wu et al. 2013). However, when we investigated effects of Si on dry weight of wheat plants after 6 h to 7 d Cd treatment here, and we found that Si only increased dry weight of plants after long-term Cd stress (Fig. 1). We also found that Si supply considerably decreased Cd concentrations in shoots of wheat after long-term Cd stress (Fig. 2A), which is in accordance with previous studies, e.g. in rice (Nwugo and Huerta 2008), pakchoi (*Brassica chinensis* L.) (Song et al. 2009), durum wheat (Rizwan et al. 2016b), and cabbage (Wu et al. 2016b). In cabbage (Wu et al. 2016b) and pakchoi (Song et al. 2009) plants, they reason that reduction of shoot Cd accumulation by Si supply is associated with repressed acropetal Cd translocation, because Si-supplied plants preferred to sequester more Cd in cell walls of roots instead of transporting Cd into shoots. In rice, Ma et al. (2015) also showed that Si-enhanced Cd in cell walls combining with Si and hemicelluloses components by using suspension cells, whereas Shao et al. (2017) reported that Si decreased Cd in cell walls of rice. In the present study, a time-course Cd exposure experiment showed that Si addition significantly decreased Cd concentrations in both shoots and roots without affecting the translocation factor in wheat (Fig. 2), suggesting that restrained Cd absorption by roots is the reason for decreased Cd accumulation rather than reduced acropetal Cd translocation in Si-treated wheat plants.

Question gives raise for how Si reduces Cd absorption by roots in wheat. To answer this question, apoplastic and symplastic uptake of Cd as affected by Si was investigated here. Under short-term Cd treatment Si supplementation significantly suppressed Cd concentrations in the apoplastic fluid and symplast without affecting cell wall-bound Cd, whereas under long-term Cd treatment, Si addition remarkably decreased cell wall-bound Cd and symplastic Cd without affecting apoplastic fluid-Cd (Fig. 3). These results indicate that Si mediated distinct mechanisms to ameliorate Cd toxicity under short- and long-term Cd exposure.

### **Alleviation of Cd toxicity by Si under short-term Cd stress**

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Previous studies on amelioration of Cd toxicity by Si in plants focused on several days or even several months' effects of Si (Farooq et al. 2016; Zhang et al. 2008), thus information on short-term effects of Si on Cd stress is limited. Ma et al. (2016) reported that Si decreased Cd toxicity after 12 h stress through modifying cell walls and increasing more Cd in cell walls of rice. In the present study, we did not find any effects of Si on cell wall properties after 6 h Cd stress (Fig. 5), but we found Si significantly decreased Cd in apoplastic fluid and the symplast (Fig. 3). We reason that using single cells and longer Cd stress time as short-term treatment by Ma et al. (2016) resulted in different findings from ours. To distinguish Si decreased symplastic Cd through down-regulation of gene expression related to Cd uptake and transport or because reduced Cd in apoplastic fluid, thereby decreasing symplastic uptake of Cd, we determined expression levels of *Nramp5* (Ishimaru et al. 2012) and *LCT1* (Uraguchi et al. 2011) (function of Cd influx), *TM20* (Kim et al. 2008) (function of Cd efflux), *HMA3* (Kim et al. 2014) (function of sequestering Cd into vacuoles) and *HMA2* (Clemens et al. 2013) (function of loading Cd into xylem). Si addition had no significant effects on expression levels of *Nramp5*, *LCT1*, *TM20* and *HMA2* (Fig. 4), suggesting that Si decreased symplastic Cd is due to decreased Cd in apoplastic fluid rather than down-regulation of transporters related to Cd uptake. Based on those results, we conclude that Si reduced apoplastic fluid-Cd is the consequence of decreased Cd concentrations under short-term Cd exposure.

Apoplastic absorption is driven by diffusion and mass flow in roots. Mass flow is supported by transpiration, while diffusion depends on diffusive layer thickness, concentration gradient, cell wall structure or root exudates, among others (Degryse et al. 2012; White 2012). Transpiration rates were not affected by Si under Cd toxicity in this study (Fig. 5E), which implies that Si-decreased Cd absorption is not through effects on mass flow. Combined the results of unchanged cell wall properties, unchanged expression of genes related to Cd uptake and unchanged mass flow absorption, we conclude that Si decreased apoplastic diffusion of Cd resulting in decreased Cd concentrations in roots under short-term treatment. Interestingly, a short-term Cd absorption experiment with excised intact roots, in which transpiration of shoots were eliminated, showed that Si supply substantially decreased Cd absorption by roots (Fig. 5D), further reinforcing that mass flow was not the reason for mitigation of Cd by Si. However, another Cd absorption experiment with milled powdery roots showed no effects of Si on Cd adsorption (Fig. 5D), implying that Si-supplied plants deployed some barriers to restrain Cd entry into roots. As apoplastic diffusion barriers, suberin lamellae development

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was determined here, and we found that Si was able to lengthen the distance of suberin deposition from root apex under Cd stress compared with Cd treatment alone (Fig. 6), which indicates that Si addition increased larger apoplastic diffusion space longitudinally. It is generally considered that acceleration of suberin lamellae reduces Cd uptake by roots (Lux et al. 2011). However, our results showed that Si delayed suberin lamellae deposition decreased Cd. Vaculík et al. (2009) also found that Si delayed suberin lamellae development from root apex under Cd toxicity, but they showed Si increased Cd in roots and shoots which is not in agreement with the majority of studies that Si decreased Cd accumulation in plants. Considering that Cd mostly accumulated in the apoplast under short-term Cd exposure and gradually decreased as prolonged Cd treatment time (Table 1), we reason that Si increased apoplastic space is the result of decreased apoplastic fluid-Cd like the ‘dilution’ effect under short-term Cd exposure. To confirm if this ‘dilution’ effect of Si also affects other mineral nutrients, Fe and Zn in the apoplastic fluid, the cell wall and symplastic fluid as affected by Si were also determined. We found that Fe and Zn in the apoplastic fluid and the percentages of apoplastic Fe and Zn distribution were not affected by Si under Cd stress regardless of Cd exposure time (Fig. S1 and Table S1). Because plants were pre-cultivated for 7 d with non-Cd nutrient solution before Cd treatment, thus apoplastic fluid-Fe and -Zn were already steady after 7 d pre-cultivation. Those results indicated that Si-decreased apoplastic fluid-Cd through increasing apoplastic space could only be effective under short-term Cd stress. It is well documented that suberin lamellae development varied with environmental conditions (Enstone and Peterson 2005). Suberin lamellae development are differently affected by different nutrient deficiency, for example deficiencies of Mn, Zn, Fe nutrients delay development of suberin lamellae and deficiencies of S and K or salt stress enhance suberin lamellae development, which could happen in several hours (Barberon et al. 2016). The plasticity of suberin lamellae deposition is negatively regulated by stress hormone ethylene and positively regulated by abscisic acid (ABA) in *Arabidopsis* (Barberon et al. 2016). However, further studies are needed to decipher whether Si-regulated stress hormone such as ethylene or ABA for alleviation of Cd toxicity through modulating plasticity of suberin development in wheat.

In addition, it is widely accepted that tolerance and avoidance are two strategies for plants to resist Cd toxicity (Clemens 2006). In our previous study, we showed that Si stimulated an avoidance rather than tolerance mechanism in wheat to mitigate Cd toxicity by promoting root

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oxalate exudation after long-term Cd treatment (Wu et al. 2016a). However, it remained unclear whether Si also modulates a similar avoidance mechanism under short-term Cd exposure (6 h). In the present study, we found that Si addition had no significant effects on oxalate exudation by roots under short-term Cd stress (Fig. 7A), but significantly decreased Cd concentration in roots (Fig. 7B). Thus, we suggest that oxalate exudation involved in suppression of root Cd uptake, but Si did not regulate this process under short-term Cd stress, implying enhancement of oxalate exudation is not the direct mechanism for alleviation of Cd toxicity.

### **Alleviation of Cd toxicity by Si under long-term Cd stress**

By contrast with short-term Cd exposure, Si significantly reduced Cd in cell walls (Fig. 3B), which is consistent with result reported by Shao et al. (2017). They reason that reduced Cd by Si supply in rice is associated with down-regulation of *OsHMA2* and *OsNramp5*. However, we found non-changed expression of *HMA2* by Si (Fig. 8E), which confirmed that effects of Si on acropetal Cd translocation is not the reason for mitigation of Cd toxicity as shown by non-changed translocation factor (Fig. 2C). Kim et al. (2014) also indicated that Si reduced Cd by decreasing *OsHMA2* and *OsHMA3* expression in rice. But, Cui et al. (2017) found that silica nanoparticles up-regulated expression of *OsHMA3* in cells of rice. By using protoplast of wheat cells, Greger et al. (2016) showed that silicate down-regulated *LCT1* and up-regulated *PCSI* expression. In the present study, Cd up-regulated expression of *Nramp5*, *LCT1*, and *HMA3*, but Si did not affect the up-regulated expression of those genes induced by Cd toxicity (Fig. 8A, B and D). Based on those findings, it seems that down-regulation of transporter related to Cd uptake and transporter is not responsible for decreasing Cd accumulation in wheat plants. Interestingly, compared with Cd stress alone, Si up-regulated the expression of *TM20* (Fig. 8C) which was reported to have the function of Cd efflux from roots, thus reducing the Cd accumulation in roots of wheat (Kim et al. 2008). However, the detailed function of *TaTM20* is still needed to be discovered. When we further analyzed cell wall properties, we found that although CEC of cell wall and Cd adsorption of cell wall were not affected by Si supply, Cd in pectin components was considerably decreased in Si-supplied plants (Fig. 9). Ma et al. (2015) reported most of Cd in hemicelluloses components of cell walls, and Si enhanced Cd accumulation in hemicelluloses components in rice cells. The different findings could be explained by different plants, and roots and single cells used. Therefore, to answer how Si decreases Cd in cell walls of wheat under long-term Cd exposure,

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we also determined suberin lamellae development. Si supply enhanced suberin lamellae development regardless of Cd toxicity under long-term treatment (Fig. 10). This is in agreement with previous findings that Fleck et al. (2011) indicated that Si supply accelerated suberization of the exodermis and endodermis in rice roots, but the underlying mechanism is still unclear. Wheat plants are non-exodermis species (Taleisnik et al. 1999), so we just found that Si addition accelerated endodermal suberization of roots in wheat (Fig. 10). The suberization of roots by Si repressed apoplastic pathway which could result in reduction of Cd. However, after long-term Cd exposure, changeable apoplastic Cd showed by Cd in apoplastic fluid only accounted for a minor percentage of total Cd, suggesting that blocking apoplastic absorption after long-term Cd exposure could not play a dominant role in decreasing Cd. However, in our previous published paper, we found that Si-enhanced oxalate exudation contributes to reduction of Cd after long-term Cd stress in wheat (Wu et al. 2016a). Therefore, we conclude that Si decreased Cd concentrations under short-term Cd exposure lays a foundation for higher Cd resistance of Si-supplied wheat plants under long-term Cd exposure.

## **Conclusion**

Reduction of Cd accumulation as affected by Si is attributed to reduce root Cd absorption rather than acropetal Cd translocation regardless of Cd exposure time. Si-decreased apoplastic fluid-Cd plays a crucial role in decrease of Cd accumulation under short-term Cd stress. The increase of apoplastic space longitudinally through delaying suberin lamellae development as affected by Si contributes to the decreased apoplastic fluid-Cd performed as ‘dilution’ effect. Under long-term Cd exposure, Si enhanced suberization of roots which could repress apoplastic pathway of absorption, but apoplastic pathway accounted for a minor percentage of Cd adsorption. Although, Si did not change expression of *Nramp5*, *LCT1*, *HMA2* and *HMA3*, Si up-regulated expression of *TM20* which involves in Cd efflux from roots under long-term Cd exposure. Overall, Si supply decreases Cd in roots through regulating suberin lamellae development under short-term Cd exposure, which is correlated with less Cd toxicity in Si-supplied plants under long-term Cd exposure. Therefore, Si-supplied plants have higher resistance than non-Si-supplied plants under long-term Cd treatment.

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Table 1 Effects of Si on time-dependent subcellular Cd distribution in roots of wheat.

Percentage (%)	Treatment	1 h	6 h	1 d	7 d	14 d
Apoplastic fluid-Cd	Cd	52.4±4**	34.5±3**	10.8±2**	3.8±0*	5.2±1*
	Si+Cd	34.4±2	14.4±3	6.0±1	5.2±1	7.8±2
CW bound-Cd	Cd	11.5±4 <sup>ns</sup>	25.9±2 <sup>ns</sup>	36.7±2 <sup>ns</sup>	37.8±2 <sup>ns</sup>	37.6±4 <sup>ns</sup>
	Si+Cd	15.6±1	31.9±3	38.8±10	32.9±3	32.6±5
Symplastic Cd	Cd	36.1±5**	39.7±4**	52.5±1 <sup>ns</sup>	58.4±2 <sup>ns</sup>	57.2±4 <sup>ns</sup>
	Si+Cd	50.0±2	53.8±5	55.2±10	61.8±4	59.6±3

Values are means ± SD (n=4). According to *t*-test, asterisk '\*\*' represents significance between Cd and Si+Cd treatments at  $p < 0.01$  while asterisk '\*' shows significance at  $p < 0.05$ ; 'ns', no significance; 'CW', cell wall.

Table 2 Sequences of specific-primers used for qRT-PCR.

Gene	Accession number	Primer sequences	Product size (bp)
<i>Nramp5</i>	KX165386.1	Forward: 5'-TCTGGGTGATTCTGATTGGC-3' Reverse: 5'-GGCTTTGGATACTCGGTCTT-3'	118
<i>LCT1</i>	AF015523.2	Forward: 5'-ACCATACCAGGACGGTGAGC-3' Reverse: 5'-CGGCGAAACGGAAGAACG-3'	121
<i>TM20</i>	DQ323065.1	Forward: 5'-CCGATCCTCTTGCACAATA-3' Reverse: 5'-ATGGACAGCATGAAGCTCAC-3'	77
<i>HMA3</i>	KF683298.1	Forward: 5'-CCGTCTCTCAGTCCCGTATTG-3' Reverse: 5'-GGAGCCACCTGAAGGAAGAC-3'	156
<i>HMA2</i>	JF489141.1	Forward: 5'-GGGCATCCGCTTATTTGG-3' Reverse: 5'-TTCCACTGCCTTTCTCCCTC-3'	100
<i>Actin</i>	GQ339780.1	Forward: 5'-GTGGACGCACAACAGGTA-3' Reverse: 5'-AGCGAGGTCAAGACGAAG-3'	110

**Figure Legends:**

Fig.1 Effects of Si on time-dependent (6 h to 14 d) dry weight in shoots (A), in roots (B) of wheat. Values are means  $\pm$  SD (n = 4). Different letters represent significant difference at  $p < 0.05$  among four treatments determined by Duncan's test.

Fig.2 Effects of Si on time-dependent (1 h to 14 d) Cd concentrations in shoots (A), in roots (B) and root-to-shoot Cd translocation (C) of wheat. Values are means  $\pm$  SD (n = 4). According to *t*-test, double asterisks '\*\*' represent significance between Cd and Si+Cd treatments at  $p < 0.01$ , while single asterisk '\*' shows significance at  $p < 0.05$ ; 'ns', no significance.

Fig. 3 Effects of Si on time-dependent (1 h to 14 d) apoplastic fluid-Cd (A), cell wall bound-Cd (B) and symplastic Cd (C) in roots. Values are means  $\pm$  SD (n = 4). Statistical analysis is the same as described in Fig. 2.

Fig. 4 Effects of Si on expression of different genes related to Cd uptake and transport under short-term Cd exposure. Values are means  $\pm$  SD (n = 4). Different letters indicate significance at  $p < 0.05$  determined by Duncan's test.

Fig. 5 Effects of Si on CEC (cation exchange capacity) of cell walls (A), Cd concentrations in different components of cell walls (B) and Cd adsorption by cell walls (C) under short-term Cd exposure, and *in vitro* Cd adsorption experiments with intact roots or milled powdery roots in the presence or absence of Si (D), and time-course transpiration rates as affected by Si under Cd stress (E). Values are means  $\pm$  SD (n = 4). Different letters indicate significance at  $p < 0.05$  determined by Duncan's test in Fig. 5A, B and E. According to *t*-test, double asterisks '\*\*' represent significance at  $p < 0.01$ , while single asterisk '\*' shows significance at  $p < 0.05$  in Fig. 5C and D; 'ns', no significance; CW, cell wall; P, pectin; HC1; hemicelluloses 1; HC2, hemicelluloses 2.

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Fig. 6 Effects of Si on suberin lamellae development under short-term Cd exposure. Values are means  $\pm$  SD ( $n = 4$ ). Different letters indicate significance at  $p < 0.05$  determined by Duncan's test. Yellow colour shows the deposition of suberin lamellae in Fig. 6B.

Fig. 7 Effects of Si on root oxalate exudation (A) and Cd concentrations in shoots (B) under short-term Cd treatment. Values are means  $\pm$  SD ( $n = 4$ ). Different letters indicate significance at  $p < 0.05$  determined by Duncan's test in Fig. 7A. Double asterisks '\*\*' represent significance at  $p < 0.01$ , and 'ns' shows no significance determined by  $t$ -test in Fig. 7B.

Fig. 8 Effects of Si on expression of different genes related to Cd uptake and transport under long-term Cd exposure. Values are means  $\pm$  SD ( $n = 4$ ). Different letters indicate significance at  $p < 0.05$  determined by Duncan's test.

Fig. 9 Effects of Si on CEC (cation exchange capacity) of cell walls (A), Cd concentrations in different components of cell walls (B) and Cd adsorption by cell walls (C) under long-term Cd exposure. Values are means  $\pm$  SD ( $n = 4$ ). Different letters indicate significance at  $p < 0.05$  determined by Duncan's test in Fig. 9A, B. The 'ns' represents no significance determined by  $t$ -test in Fig. 9C.

Fig. 10 Effects of Si on suberin lamellae development under long-term Cd exposure. Values are means  $\pm$  SD ( $n = 4$ ). Different letters indicate significance at  $p < 0.05$  determined by Duncan's test. Yellow colour shows the deposition of suberin lamellae in Fig. 10B.

Fig. S1 Effects of Si on time-dependent (0 h to 14 d) apoplastic fluid-Fe and Zn (A, D), cell wall bound-Fe and Zn (B, E) and symplastic Fe and Zn (C, F) in roots. Values are means  $\pm$  SD ( $n = 4$ ). According to  $t$ -test, double asterisks '\*\*' represent significance between Cd and Si+Cd treatments at  $p < 0.01$ , while single asterisk '\*' shows significance at  $p < 0.05$ ; 'ns', no significance.

Fig. 1

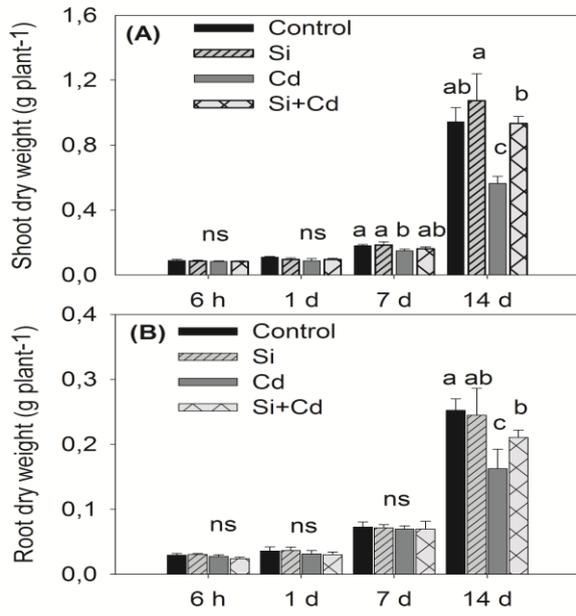


Fig. 2

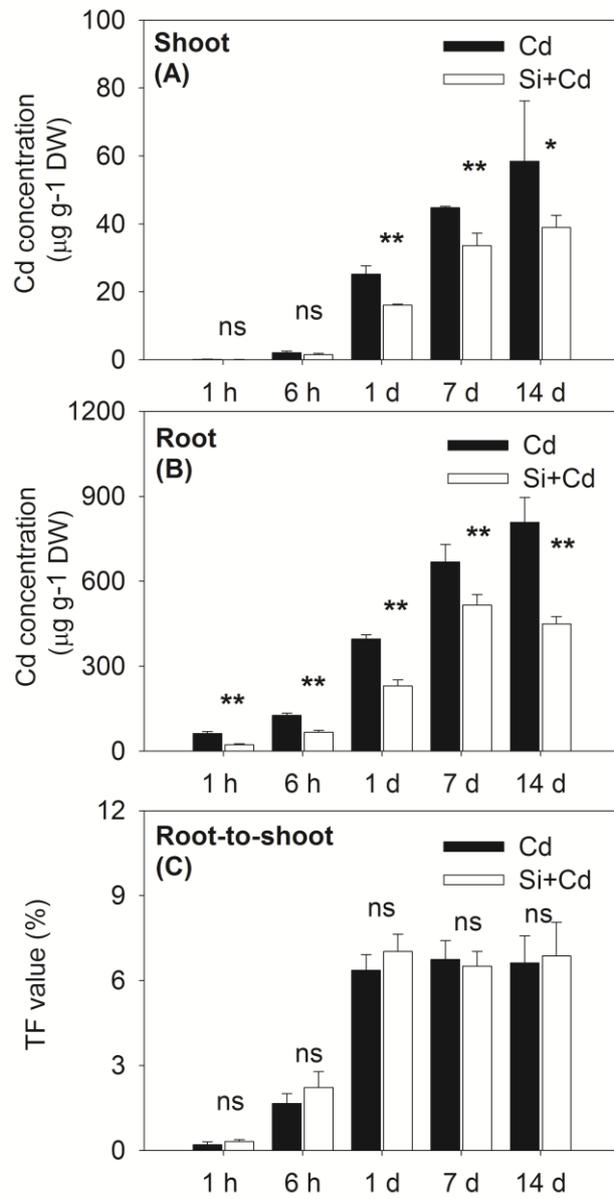


Fig. 3

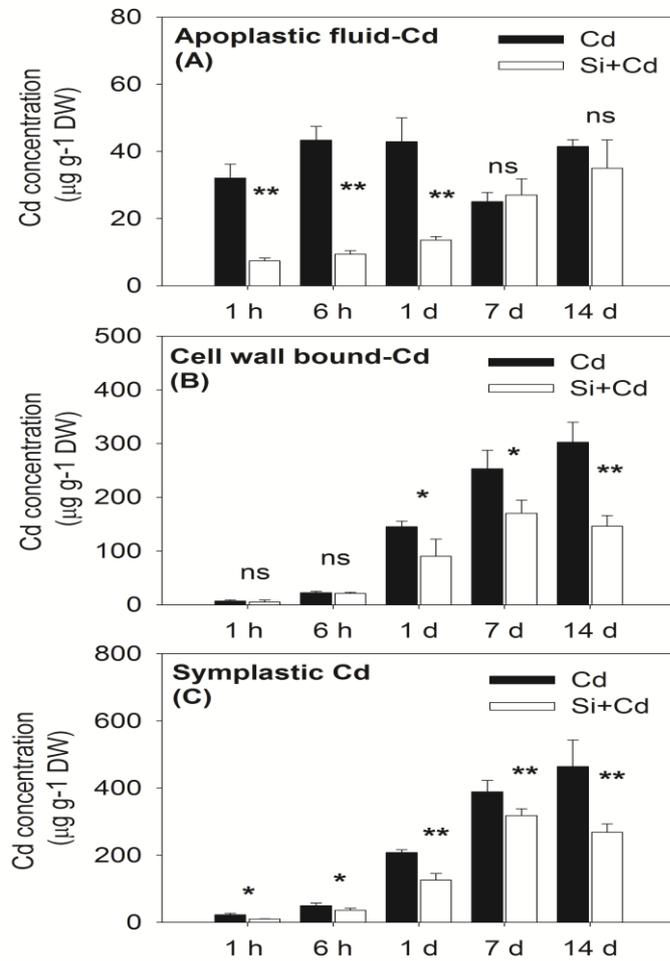


Fig. 4

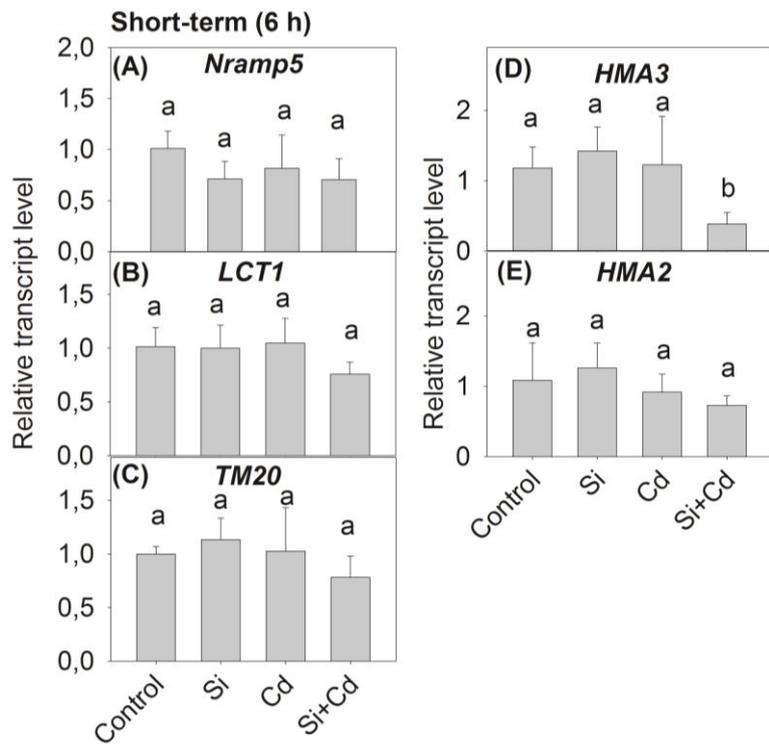


Fig. 5

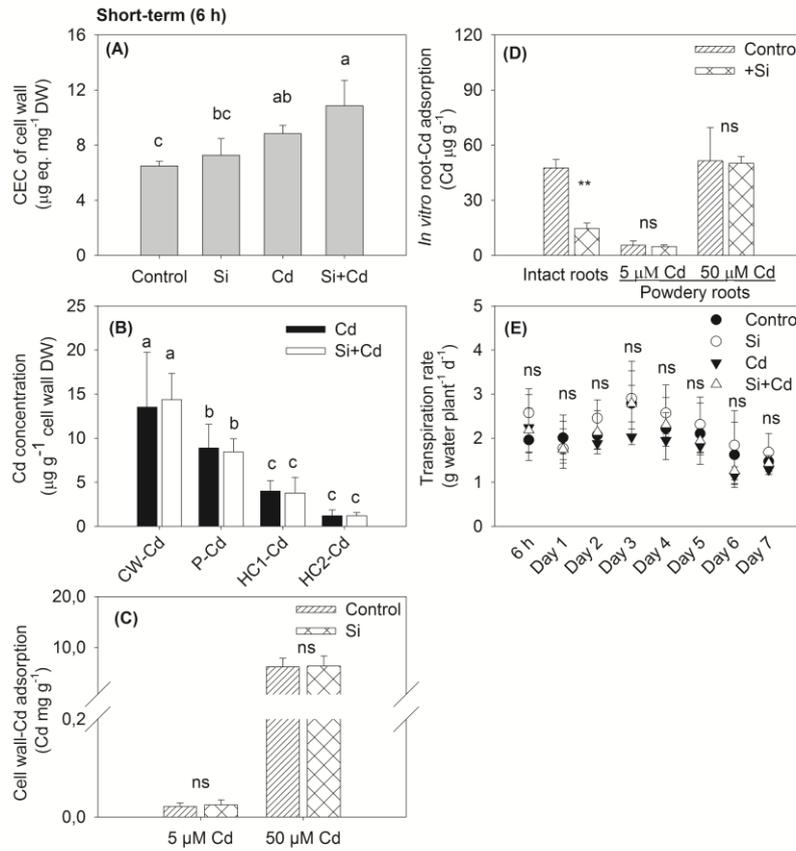


Fig. 6

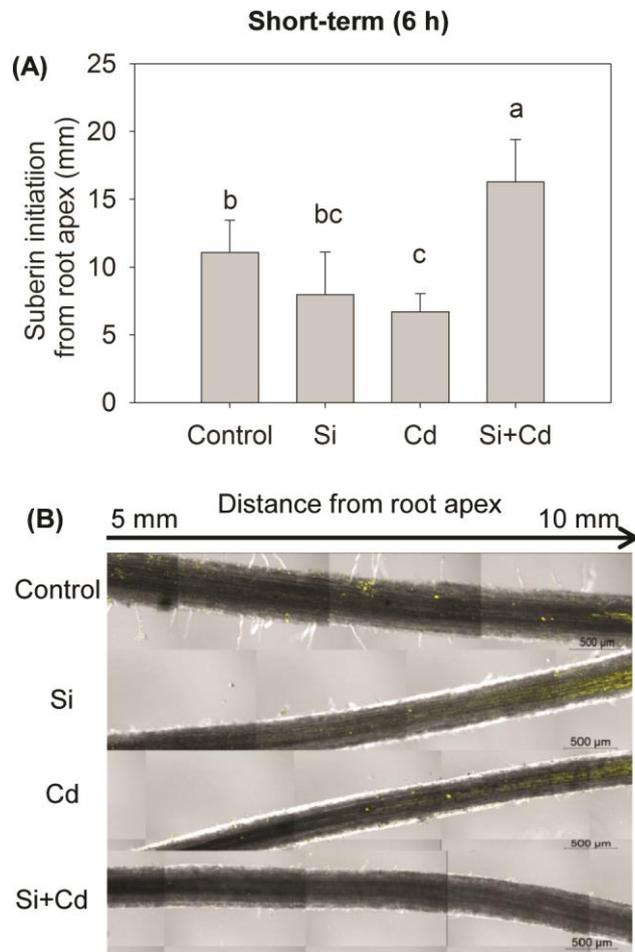


Fig. 7

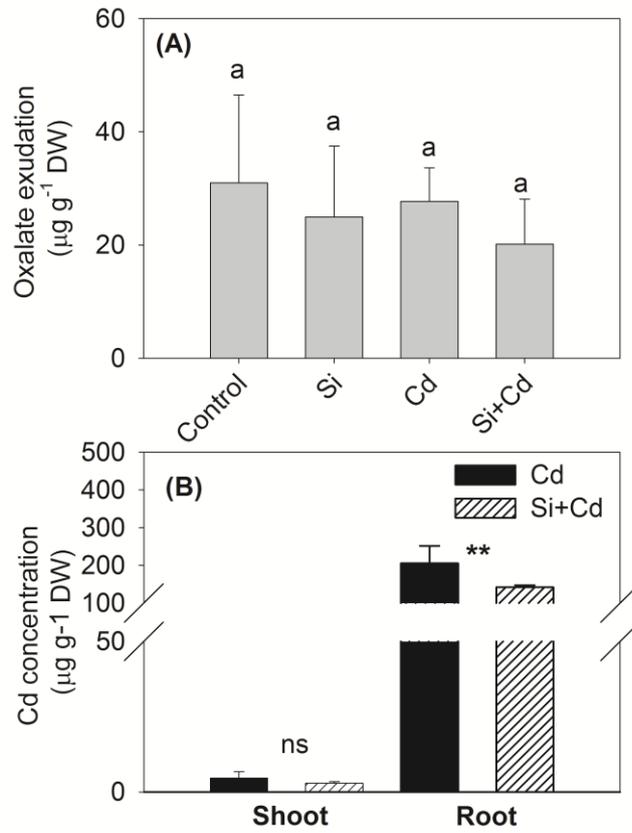


Fig. 8

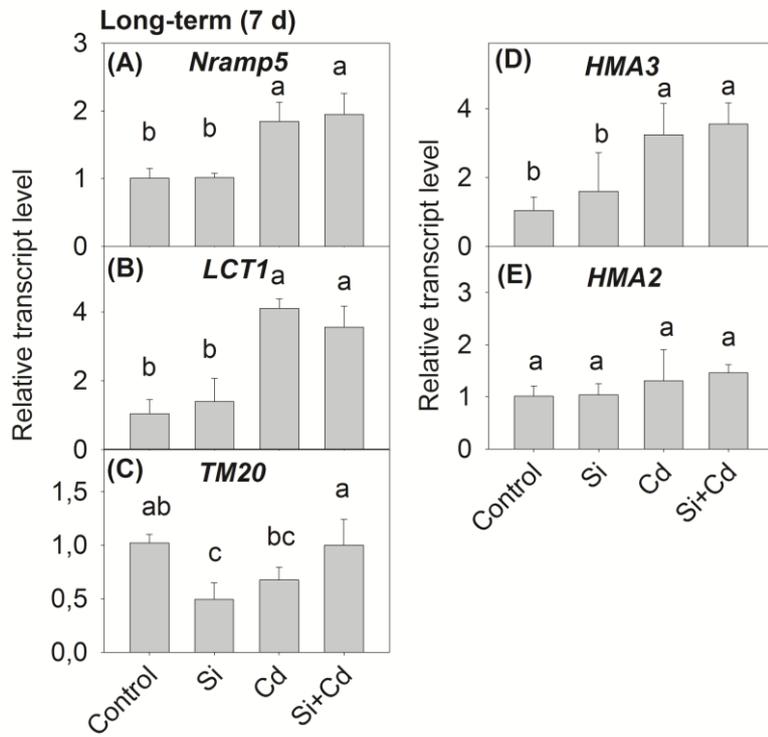


Fig. 9

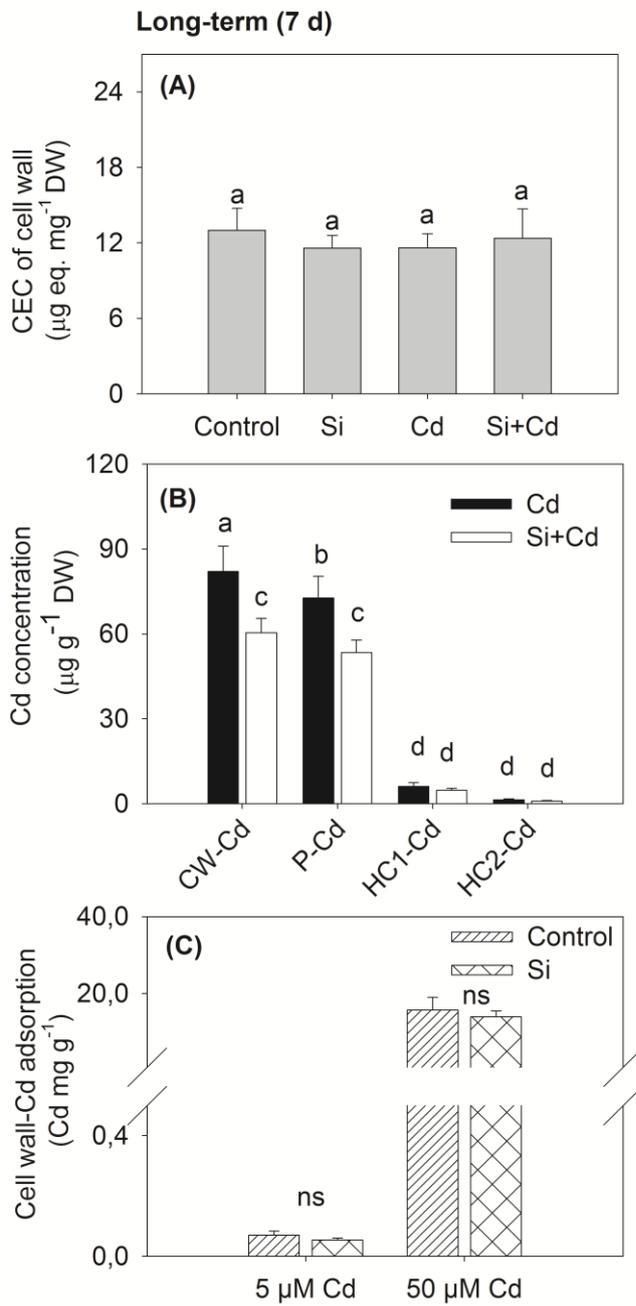


Fig. 10

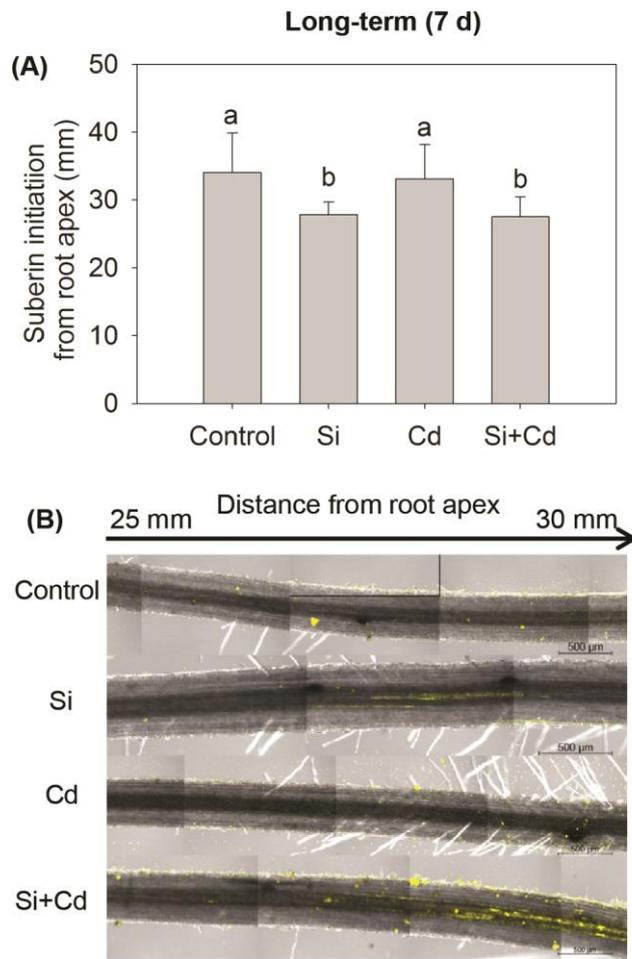


Fig. S1

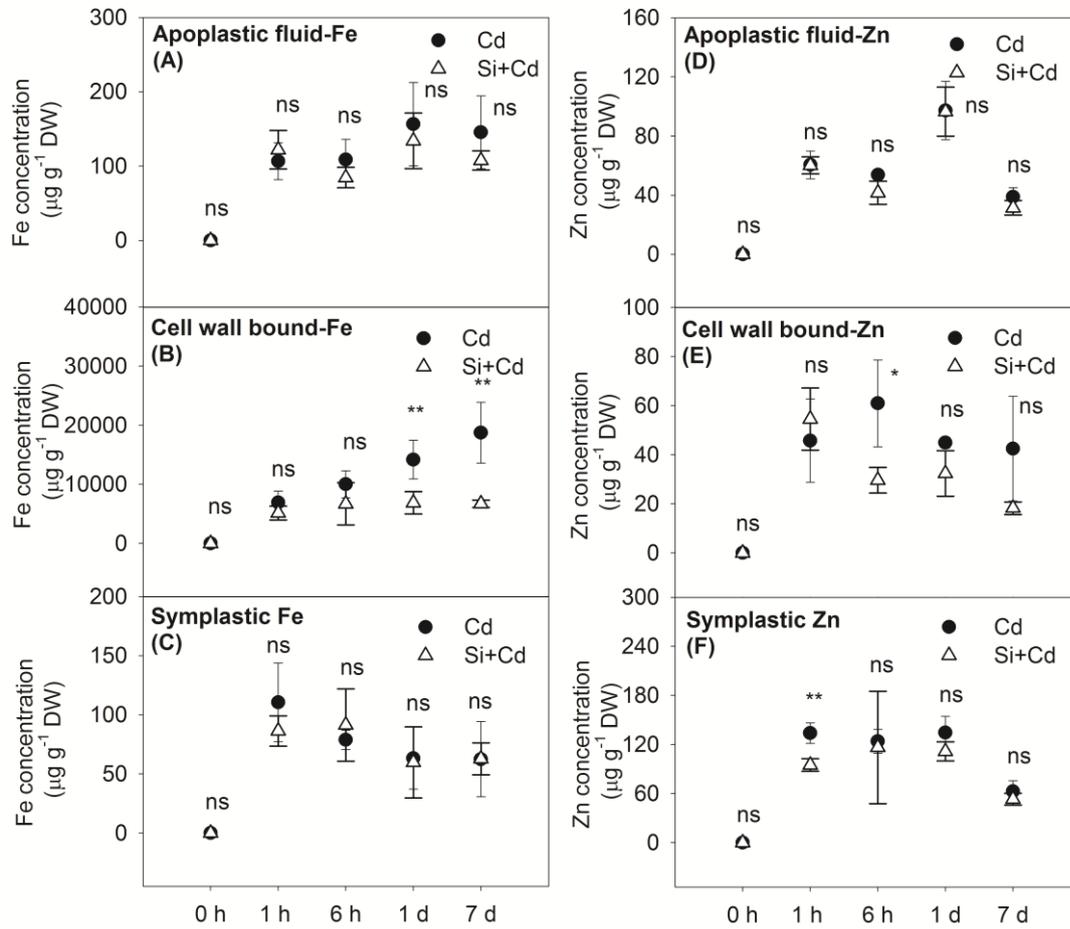


Table S1 Effects of Si on time-dependent subcellular Fe and Zn distribution in roots of wheat.

	Percentage (%)	Treatment	1 h	6 h	24 h	7 d
<b>Fe</b>	Apoplastic fluid-Fe	Cd	1.7±0.1	1.1±0.4	1.3±0.7	0.8±0.3
		Si+Cd	2.4±0.8	1.4±0.7	2.1±1.1	1.6±0.3
	Cell wall bound-Fe	Cd	96.3±1.5	98.1±0.5	98.2±0.8	98.8±0.4
		Si+Cd	95.9±1.3	96.8±1.9	97.0±1.4	97.6±0.5
	Symplastic Fe	Cd	2.0±0.7	0.8±0.2	0.5±0.2	0.4±0.1
		Si+Cd	1.7±0.6	1.7±0.3	0.9±0.5	0.9±0.3
<b>Zn</b>	Apoplastic fluid-Zn	Cd	25.3±2.2	23.4±1.6	34.4±2.2	27.0±3.5
		Si+Cd	28.8±3.3	24.2±8.0	39.9±4.8	30.5±1.2
	Cell wall bound-Zn	Cd	16.0±3.3	20.9±3.6	17.3±1.4	29.1±1.3
		Si+Cd	25.8±4.5	17.0±5.9	13.5±3.7	17.8±1.6
	Symplastic Zn	Cd	58.7±5.0	55.7±5.3	48.3±1.1	43.9±9.9
		Si+Cd	45.4±1.4	58.8±1.2	46.6±5.8	51.7±1.8

Values are means ± SD (n=4). According to *t*-test, no significant difference was found between Cd and Si+Cd treatments.

## **Chapter 4**

### **Sulfate supply enhances cadmium tolerance in *Vicia faba* L. plants**

Jiawen Wu, Hans-Peter Mock, and Karl-Hermann Mühling

to be submitted

## Sulfate supply enhances cadmium tolerance in *Vicia faba* L. plants

Jiawen Wu<sup>1</sup>, Hans-Peter Mock<sup>2</sup>, Karl-Hermann Mühling<sup>1\*</sup>

<sup>1</sup> Institute of Plant Nutrition and Soil Science, Kiel University, Hermann-Rodewald-Str. 2, 24118, Kiel, Germany

<sup>2</sup> Department of Physiology and Cell Biology, Applied Biochemistry, Leibniz Institute of Plant Genetics and Crop Plant Research, 06466, Gatersleben, Germany

\*To whom correspondence should be addressed,

Prof. Dr. Karl-Hermann Mühling,

Email: [khmuehling@plantnutrition.uni-kiel.de](mailto:khmuehling@plantnutrition.uni-kiel.de)

## Abstract

Sulfur deficiency has become one of unfavorable conditions in agriculture due to progressive program of reducing sulfur emission into environment in recent decades. In addition, cadmium (Cd) pollution is increasingly widespread as the development of industrialization, mining, smelting, sewage sludge, and phosphate fertilizer application. However, how surplus sulfate supply affects Cd toxicity is still unclear. Experiments with deficient, sufficient and excess sulfate levels under Cd stress were conducted in faba bean hydroponically. Sufficient and excess sulfate supply significantly increased biomass of Cd-stressed plants when compared with deficient sulfate supply. Intriguingly, sulfate application also increased Cd concentrations in leaves. Based on increased Cd concentrations without damaging plant growth by sulfate application, we conclude that sulfate supply enhances Cd tolerance in plants. Sulfate application increased  $\text{CdSO}_4^0$  proportion in the growth medium which is partially related to increase Cd in faba bean because  $\text{CdSO}_4^0$  facilitates Cd diffusion in plants. Further study on Cd localization, we found that increased Cd preferred to accumulate in epidermis of leaves which might contribute to enhancement of Cd tolerance. Detrimental effects on plasma membrane integrity shown by Evan's blue, lipid peroxidation shown by MDA, and  $\text{H}_2\text{O}_2$  contents induced by Cd toxicity were alleviated by surplus sulfate supply compared with deficient sulfate supply. To test whether phenolic compounds involve in sulfate-enhanced Cd tolerance in faba bean since phenolic compounds play a role in scavenging ROS in plants, we determined effects of sulfate on phenolic compounds under Cd stress. As a result, phenolic compounds were not significantly affected by sulfate application. However, Cd toxicity induced tendency of reducing two flavonoid compounds, robinin and kaempferol-3-O-acetyl-rhamnogalactoside-7-O-rhamnoside. To further assess how those two compounds affect Cd toxicity, foliar application of robinin, and kaempferol-3-O-rhamnoside, the precursor of kaempferol-3-O-acetyl-rhamnogalactoside-7-O-rhamnoside, were conducted in Cd stressed-plants. Foliar application of those two flavonoid compounds substantially depressed Cd concentrations in leaves of faba bean. Taken together, sulfate accelerates Cd accumulation in leaves of faba bean and compartmentalizes Cd in epidermis of leaves, thus sulfate supply enhances Cd tolerance. Although sulfate mitigates oxidative stress induced by Cd toxicity, phenolic compounds as antioxidants are not involved in scavenging ROS based on unchanged phenolic compounds as affected by sulfate supply.

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**Keywords:** Cd, localization, oxidative, phenolic, sulfur.

## 1. Introduction

Due to industrialization, mining, smelting, sewage sludge and phosphate fertilizer application, cadmium (Cd)-polluted soils are widespread (Tudoreanu and Phillips 2004). Cd is readily taken up by plant roots, and then accumulated in plants (Wu et al. 2013). As a non-essential element, Cd has no specific transporter for Cd uptake, but Cd can be taken up and transported by sharing transporters with essential elements such as manganese (Mn), zinc (Zn), calcium (Ca) or iron (Fe) (Clemens et al. 2002). Thus, in Cd-polluted soils, it is unavoidable that Cd will enter into plants. Although Cd is non-redox bivalent element and does not participate in Haber-Weiss and Fenton reactions directly, it could still produce ROS (reactive oxygen species) including  $O_2^{\cdot-}$  (superoxide anion),  $H_2O_2$  (hydrogen peroxide),  $OH^{\cdot}$  (hydroxyl radical) and  $^1O_2$  (singlet oxygen) indirectly (Wu et al. 2015). In general, three pathways contribute to production of ROS by Cd, (1) Cd replaces redox-active elements like Fe from proteins, which results in more redox-active elements and thereby producing more ROS; (2) induction of NADPH oxidase by Cd, which could generate  $O_2^{\cdot-}$  from  $O_2$  through using electron derived from NADPH; (3) Cd could bind with  $-SH$  group such as GSH (glutathione) and reduces the capacity of scavenging ROS (Cuypers et al. 2010). As a result, Cd-induced ROS could react with proteins, lipids and nucleic acids, resulting in lipid peroxidation, membrane damage, enzyme inactivation, and disrupt cellular metabolism (Romero-Puertas et al. 2007).

Detoxification of Cd in plants by chelating Cd with compounds and storing in vacuoles has been well documented, especially phytochelatins (PCs) compounds (Brunetti et al. 2011). As the precursor of PCs, GSH is produced from sulfate assimilation, which also plays a role as an antioxidant. Combined with the situation that sulfur deficiency in arable soils induced by refined fertilizer production and reduction of sulfur emission into environment (Hirai et al. 2004), how sulfate supply affects Cd toxicity in plants is increasingly attracting attention. Anjum et al. (2008) showed that sulfur ameliorated Cd toxicity in mustard (*Brassica campestris* L.) by increasing AsA (ascorbate) and GSH contents. Khan et al. (2015) also reported that sulfur addition alleviated oxidative stress induced by Cd toxicity through increasing proline and GSH production in wheat (*Triticum aestivum* L.). Except GSH and AsA, phenolic compounds are also well known as antioxidants (Sgherri et al. 2003). Due to structure of phenolic compounds, they play roles in denoting electron agents or hydrogen

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atoms which prevents formation of oxidant radical species (Martini et al. 2017). To date, whether sulfur supply alleviates oxidative stress by modulating phenolic compounds under Cd stress is still unclear.

Faba bean (*Vicia faba* L.) seeds containing high proteins and minerals consumed by human beings and animals (Crépon et al. 2010). It has been studied that the majority of phenolic compounds in leaves of faba bean are acetylated and non-acetylated kaempferol glycosides (Neugart et al. 2015). It will be interesting to elucidate whether those phenolic compounds involve in alleviation of Cd toxicity as influenced by sulfur nutrition. Therefore, phenolic compounds as affected by deficient, sufficient and excess sulfate supply under Cd toxicity in faba bean were determined here. To assess oxidative stress, plasma membrane integrity shown by Evan's blue, lipid peroxidation shown by MDA and H<sub>2</sub>O<sub>2</sub> in plants under Cd stress as affected by different levels of sulfate were investigated. With the help of Cd staining dye, effects of sulfate on Cd localization was also determined.

## 2. Materials and methods

### 2.1 Plant cultivation and treatment

Faba bean (*V. faba* L. cv. Fuego) seeds were surface sterilized and then soaked in aerated 0.5 mM CaCl<sub>2</sub> for 12 h. Subsequently, seeds were germinated in quartz sands for 10 d. After germination, uniform seedlings were transferred for hydroponics with three different sulfate levels (S1, deficient sulfate; S2, sufficient sulfate; and S3, excess sulfate). Compositions of nutrient solution with sufficient sulfate (S2) were macronutrients (mM) [Ca(NO<sub>3</sub>)<sub>2</sub> 2.0; KH<sub>2</sub>PO<sub>4</sub>, 0.1; K<sub>2</sub>HPO<sub>4</sub>, 0.1; K<sub>2</sub>SO<sub>4</sub>, 1.0; KCl, 0.2; MgSO<sub>4</sub>, 0.5] and micronutrients (μM) [Fe-EDTA, 60; H<sub>3</sub>BO<sub>3</sub>, 10; CuSO<sub>4</sub>, 0.2; MnSO<sub>4</sub>, 2; ZnSO<sub>4</sub>, 0.5; NiSO<sub>4</sub>, 0.01; (NH<sub>4</sub>)<sub>2</sub>MoO<sub>7</sub>O<sub>24</sub>, 0.05]. When K<sub>2</sub>SO<sub>4</sub> and MgSO<sub>4</sub> were replaced by KCl and MgCl<sub>2</sub> on the basis of S2 nutrient solution, the resulting solution was regarded as S1 nutrient solution. Extra 5 mM sulfate in the form of Na<sub>2</sub>SO<sub>4</sub> added into S2 nutrient solution resulted in S3 nutrient solution. Sodium ion (Na<sup>+</sup>) induced by Na<sub>2</sub>SO<sub>4</sub> were compensated by NaCl in S1 and S2 nutrient solution. The pH of nutrient solution was adjusted to 6.0 ± 0.1 with 0.1 M NaOH and 0.1 M HCl. The strength of nutrient solution was stepwise-changed from one-quarter to full-strength in 7 d to avoid osmotic stress. After 7 d cultivation, plants with three sulfate levels were stressed with or without 50 μM Cd in the form of CdCl<sub>2</sub> respectively, which resulted in six treatments

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including S1, S2, S3, S1+Cd, S2+Cd and S3+Cd. Proportions of different Cd speciation as affected by three different sulfate levels were calculated by Visual MINTEQ 3.1 (a chemical equilibrium model) (Mera et al. 2016) respectively. The nutrient solution was renewed every 3 d, and plants were grown in the greenhouse with a photoperiod from 8:00 to 22:00 and temperature (24/18 °C; day/night). After 7 d Cd stress, plants were harvested for analysis.

## 2.2 Determination of Cd

After Cd treatment, plants were separated into shoots and roots, and dried in an oven until reaching constant weight, and then dry weight of shoots and roots was measured. Afterwards, the dried materials were grinded into powder by a miller (MM400, Retsch, Germany). The powdery materials were digested with ultra-pure HNO<sub>3</sub> (Sigma-Aldrich) in a microwave oven (Mars 6, CEM Corporation, USA) for 45 min at 190 °C consisting of 10 min pre-heating, 15 min heating and 20 min cooling. The digested solution was diluted into 100 mL volumetric flask with 18.2 MΩ.cm deionized H<sub>2</sub>O. Before measuring Cd by ICP-MS (inductively coupled plasma mass spectroscopy; Agilent 7700, Agilent Technologies Inc., USA), samples were diluted again with 2% (v/v) HNO<sub>3</sub>. Cd content, namely total Cd amount in plants, was calculated as follows,

$$\text{Cd content } (\mu\text{g plant}^{-1}) = \text{Cd concentration } (\mu\text{g g}^{-1}) * \text{dry weight } (\text{g plant}^{-1}) \quad \text{Equation 1}$$

## 2.3 Localization of Cd

Fresh root segments (0.5-1.0 cm from root apex) and leaves (0.5\*0.5 cm<sup>2</sup>) were harvested and fixed in FAA [formaldehyde (10 mL)-acetic acid (7 mL)-ethanol (2 mL)] solution at 4 °C overnight. Subsequently, samples were dehydrated in a series of successive acetone concentrations from 25% (v/v), 50% (v/v), and 75% (v/v) to 100% (v/v). Every step was incubated for 20 min and the step with 100% acetone was repeated three times. Technovit<sup>®</sup> 7100 kit was used for embedding samples. The following steps including pre-infiltration, infiltration, and polymerization were conducted according to manufacturers' instruction. The embedded samples were cut into 25 μm thickness sections by a rotary microtome (RMT-20, Radical Scientific Equipments pvt. Ltd, India).

The sections of roots and leaves were stained with Leadmium Green AM dye (a specific dye for localizing Cd) for 30 min at room temperature in the dark. The Leadmium Green AM solution was freshly prepared according to manufacturers' instruction. Briefly, 50 μg Leadmium Green was dissolved into 50 μL DMSO as stock solution, and the working

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solution was diluted with 0.85% (w/v) NaCl according to a ratio of 1:10. After staining, the sections were washed with 0.85% (w/v) NaCl for several times, and then fluorescence was observed by using a fluorescence microscope (DMI6000B, Leica, Microsystems, Wetzlar, Germany) equipped with a DFC-360FX camera at 495 nm excitation and 523-530 nm emission. Images were processed by an image processing package, Fiji software (<https://imagej.net/Fiji/Downloads>).

#### 2.4 Assay of Evan's blue

Fresh root tips (0-2.5 cm from root apex) and third oldest leaves were washed with deionized H<sub>2</sub>O, and gently blotted with tissue paper. Afterwards, root samples were stained with 0.025% (w/v) Evan's blue in 100  $\mu$ M CaCl<sub>2</sub> (pH 5.6) for 15 min, and then washed with 100  $\mu$ M CaCl<sub>2</sub> (pH 5.6) until Evan's blue on the surface of samples was washed out. Leaf samples were stained with 0.25% (w/v) Evan's blue in 100  $\mu$ M CaCl<sub>2</sub> (pH 5.6) for 5 h, and then leaves were boiled in 95% (v/v) ethanol for 5 min to remove the chlorophyll. Subsequently, the stained samples were homogenized with 1% (w/v) SDS to release Evan's blue. Homogenate was centrifuged at 12,000 g for 10 min, and the OD<sub>600</sub> (optical density at 600 nm) value of supernatant was determined by a spectrophotometer (Thermo Scientific, Helios Gamma, England) (Baker and Mock 1994; Yamamoto et al. 2001).

#### 2.5 Measurement of MDA and H<sub>2</sub>O<sub>2</sub>

Malondialdehyde (MDA) contents were determined based on TBA (thiobarbituric acid) test which could detect MDA as the product of lipid peroxidation. Briefly, 0.2 g fresh materials were homogenized in 2 mL 0.1% (w/v) TCA (trichloroacetic acid) in an ice bath, and the homogenate was centrifuged at 12,000 g for 10 min at 4 °C. Aliquot of 1 mL supernatant was mixed with 1 mL 0.1% (w/v) TBA [dissolved in 20% (w/v) TCA], followed by incubating at 100 °C for 30 min, and then put in an ice bath to stop the reaction. Subsequently, the samples were centrifuged at 10,000 g for 5 min at 4 °C, and then OD values were determined at 532 nm and 600 nm by a spectrophotometer. Finally, MDA amount was calculated according to extinction coefficient 155 mM<sup>-1</sup> cm<sup>-1</sup> (Velikova et al. 2000).

For the measurement of H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide), extraction was the same as described for MDA. After 0.1% (w/v) TCA extraction and centrifugation, 0.5 mL supernatant was added into 0.5 mL 10 mM potassium phosphate buffer (pH 7.0), followed by adding 1 mL 1 M KI, and incubated for 15 min. The absorbance of supernatant was measured at 390 nm by a

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spectrophotometer. The H<sub>2</sub>O<sub>2</sub> contents were calculated based on the standard curve of H<sub>2</sub>O<sub>2</sub> (Velikova et al. 2000).

#### 2.6 Phenolic compound determination

After Cd treatment, one set of plants were vacuum frozen-dried, grinded into powder and stored at -20 °C for determining phenolic compounds. According to a ratio of 10 mg samples to 400 µL 80% (v/v) methanol, around 10 mg samples were mixed with 80% (v/v) methanol and then homogenized 45 s at 5000 rpm twice by using FastPrep homogenizer with zirconium dioxide beads. Subsequently, samples were centrifuged at 14,000 rpm for 10 min at 4 °C. Supernatant was transferred to new Eppendorf tubes, while the pellets were re-extracted with 100% (v/v) methanol again based on a ratio of 10 mg samples to 400 µL 100% (v/v) methanol. Afterwards, the supernatant from two times' extraction was combined, and mixed with solvent A according to the mixture of 80% sample with 20% solvent A. The mixture was centrifuged at 14,000 rpm for 5 min at 4 °C, and then 150 µL supernatant was taken for running HPLC (high-performance liquid chromatography). The program was set up according to method of Petridis et al. (2016). Briefly, program started with 100% solvent A [0.1% (v/v) formic acid in water] and 0% solvent B [0.1% (v/v) formic acid in acetonitrile], and before 10 min ending set with 60% solvent A and 40% solvent B. The temperature of column was kept at 35°C (BEH phenyl column, 1.7 µm, 100 x 2.1 mm). Detection of absorbance was determined at 280 nm.

UPLC-PDA-ESI-MS (ultra-performance liquid chromatography-photodiode array electrospray ionisation-mass spectrometry) was used to identify phenolic compounds. UPLC program was set up the same as that for HPLC. Both positive and negative ion modes were conducted in MS experiments with a mass range from 50 to 1000 *m/z*. All the parameters set up were the same as described by Brauch et al. (2018).

#### 2.7 Foliar application of flavonoids under Cd stress

Faba bean seedlings were cultivated with sufficient sulfate nutrition as described above (section 2.1). After 7 d cultivation, plants were treated with 50 µM Cd in the form of CdCl<sub>2</sub>. Meanwhile, foliar application of 5 µM robinin (R) dissolved in 2% (v/v) methanol and 5 µM kaempferol-3-O-rhamnoside (K) dissolved in 2% (v/v) methanol were performed in Cd-stressed plants daily, and 2% (v/v) methanol was also supplied to leaves of Cd-stressed plants as control, which resulted in three treatments Cd, Cd+R and Cd+K. To reach homogenous distribution of solutions on leaves surface, 0.1% (v/v) Silwet L-77 was added into foliar

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application solutions as a wetting agent. Three biological replicates were designed for every treatment, and three plants in one 5-L pot combined together as one biological replicate. After 9 d Cd stress, plants were separated into shoots and roots, and dried until reaching constant weight. Afterwards, the dried materials were ground into powder, and digested for measuring Cd concentration as described above (section 2. 2).

### **3. Statistic analysis**

All the values in this study were means  $\pm$  SD from three or four biological replicates. Experiment was repeated three times for determining Cd concentrations in roots and in shoots independently, and data presented here was from one of the independent experiments. Significant differences were determined by Duncan's test and One-way ANOVA at the level of 0.05 by using SPSS (SPSS Inc. 16.0).

### **4. Results and discussion**

#### 4.1 Effect of sulfate on biomass under Cd stress

Sulfur deficiency has become the problem for plant production, because progress regarding reduction of sulfur emissions into the atmosphere has been achieved in recent decades (Hirai et al. 2004). Sulfur deficiency leads to shortage of amino acids such as cysteine and methionine resulting in inhibition of protein synthesis and decrease of chlorophyll concentrations in leaves. As a result, sulfur deficiency decreases plant growth, crop yield and quality (Hawkesford 2000). In consistent with the deleterious effects of deficient sulfur, our results also showed that S1 (deficient sulfur) significantly reduced shoot dry weight of faba bean plants when compared with S2 (sufficient sulfur) and S3 (excess sulfur) (Fig. 1). On the other hand, S-containing metabolite GSH is the precursor of PCs, and PCs play a pivotal role in detoxifying Cd by sequestering Cd into vacuoles in form of PCs-Cd complexes (Uraguchi et al. 2017). We hypothesize that, under Cd toxicity, exogenous sulfur supply could recover stressed-plant growth induced by sulfur deficiency, and thereby enhancing Cd tolerance. In the present study, we confirmed the hypothesis that exogenous sulfur supply significantly increased plant biomass compared with deficient sulfur supply under Cd toxicity (Fig. 1).

#### 4.2 Effect of sulfate on Cd accumulation and distribution

In regard to Cd concentration, sufficient and excess sulfur addition substantially promoted Cd concentrations in leaves of faba bean when compared with deficient sulfur (Fig. 2 A), but Cd concentrations in roots were unaffected regardless of sulfur supply (Fig. 2B). Further investigation of Cd amounts in plants, we found Cd contents of leaves in sufficient and excess sulfur-treated plants were 4.7 and 4.5 times of that in deficient sulfur-treated plants (Fig. 2C), while Cd contents of roots were not significantly changed compared with deficient sulfur treatment (Fig. 2D). Based on those results, we conclude that an increased Cd accumulation in leaves by sulfur supply was related to both increased biomass and Cd concentration, whereas sulfur supply did not affect Cd accumulation in roots. Comparatively, Zhang et al. (2014) reported that surplus sulfur reduced Cd concentrations in roots but increased shoot Cd in rice (*Oryza sativa* L.) plants. Liang et al. (2016) showed different effects of sulfate that in Cd tolerant cultivar, sulfur supply decreased Cd concentrations in leaves with increasing Cd concentrations in roots, whereas in Cd sensitive cultivar, sulfur addition did not affect Cd in leaves with increasing Cd in roots of pakchoi (*Brassica chinensis* L.). In maize (*Zea mays* L.), López-Chuken and Young (2010) found that sulfur application enhanced Cd accumulation in shoots. Llugany et al. (2013) reported that in Cd non-hyperaccumulators, surplus sulfur increased Cd concentrations in roots and decreased Cd in shoots of Cd sensitive ecotype, but sulfur did not affect Cd concentrations in Cd tolerant ecotype; in Cd hyperaccumulators, sulfur application enhanced Cd concentrations in shoots of *Noccaea caerulescens* without affecting Cd in roots, while sulfur supply had no effects on Cd concentrations in both roots and shoots of *Noccaea praecox*. Altogether, effect of sulfur on Cd accumulation is species-specific dependent.

In the present study, when we used a chemical equilibrium model (Visual MINTEQ 3.1) to calculate Cd ions in nutrient solution, we found that percentages of  $\text{CdSO}_4^0$  were increased as sulfate concentrations increased in the growth medium, which were 0.1%, 5.5% and 22.6% in deficient sulfate-supplied, sufficient sulfate-supplied and excess sulfate-supplied nutrient solution respectively (Table 1).  $\text{CdSO}_4^0$  complexes in nutrient solution plays a crucial role in promoting Cd uptake by maize (López-Chuken and Young 2010). Mera et al. (2016) also reported that exogenous application of sulfate in the growth medium enhanced the percentage of  $\text{CdSO}_4^0$  which affected Cd tolerance of microalgae (*Chlamydomonas moewusii*). Therefore, we reason that surplus sulfate increased Cd in the form of  $\text{CdSO}_4^0$ , to some extent, contributes to increased Cd accumulation in faba bean plants in this study. To assess how sulfur affects

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Cd distribution in leaves and roots, Cd specific dye was used to visualize Cd in the cross-section of leaves and roots. Signals of Cd in sufficient and excess sulfate-supplied leaves were stronger than that in deficient sulfate-treated leaves (Fig. 3A-C), which is in accordance with Cd concentrations in leaves (Fig. 2A). However, more Cd was accumulated in upper and low epidermis of leaves (Fig. 3A-C) in cross-section of leaves, implying that increased Cd by surplus sulfate in leaves prefers to accumulate in epidermis. With regard to Cd localization in roots, Cd staining showed that Cd was accumulated in epidermis and endodermis of deficient sulfate treated-roots (Fig. 3D), while Cd was uniformly distributed in root cross-section of sufficient and excess sulfate treated-plants (Fig. 3E, F). Combined with that unchanged Cd concentrations in roots regardless of sulfate supply (Fig. 2B), it seems that retention of Cd in epidermis and endodermis in roots of deficient sulfate supplied-plants is the partial reason for restraining Cd translocation from roots into leaves resulting in low Cd concentrations in leaves compared with sufficient and excess sulfate-supplied plants (Fig. 2A and Fig. 3).

#### 4.3 Effects of sulfate on oxidative stress and phenolic compounds under Cd toxicity

It is well documented that Cd toxicity leads to oxidative stress in plants (Cuypers et al. 2010). In agreement with this detrimental effect of Cd, we also found that Cd-induced oxidative stress in faba bean. Plasma membrane integrity shown by Evan's blue, lipid peroxidation shown by MDA, and H<sub>2</sub>O<sub>2</sub> were damaged or elicited by Cd stress (Fig. 4, 5). Although sulfur deficiency decreased biomass of plants, no oxidative stress was investigated here (Fig. 4, 5). However, the heaviest oxidative stress was showed in deficient sulfate supplied-plants under Cd toxicity, while the oxidative stress was alleviated in sufficient and excess sulfate supplied-plants compared with deficient sulfate supplied-plants under Cd stress (Fig. 4, 5). Previous studies have well documented that sulfur reduced oxidative stress induced by Cd toxicity through modulating AsA-GSH in both Cd hyperaccumulator (Indian mustard, *Brassica juncea* L) (Bashir et al. 2015) and Cd non-hyperaccumulator (rice, *O. sativa* L.) (Jung et al. 2017).

Except AsA and GSH, phenolic compounds are also another important group of antioxidants which have been well known that play roles in scavenging ROS (Abou Samra et al. 2011). Márquez-García et al. (2012) showed that Cd increased total phenolic compounds especially rutin, cinnamic acid derivatives and epicatechin, which correlated with enhanced antioxidant capacity in *Erica andevalensis*. Manquián-Cerda et al. (2016) also indicated that

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Cd-induced chlorogenic acid in blueberry (*Vaccinium corymbosum* L.) resulted in Cd tolerance. Nevertheless, information on whether phenolic compounds involve in detoxification of Cd toxicity as affected by sulfate is still lacking. To test whether sulfate modulates phenolic compounds to alleviate Cd stress in faba bean, we determined compositions of phenolic compounds as affected by different sulfate levels under Cd toxicity. Majority of phenolic compounds were detected in leaves, whereas phenolic compounds in roots were below the detectable threshold. Eight abundances of peaks were found in leaves of faba bean regardless of sulfate or Cd treatment (Fig. 6). According to peak area, phenolic compounds were not remarkably affected by sulfate or Cd treatment, but areas of peak 3 and peak 7 showed decreased trends by Cd toxicity irrespective of sulfate levels (Fig. S1). Peak 3 and peak 7 were identified to be robinin (also known as kaempferol-3-O-robinoside-7-O-rhamnoside) and kaempferol-3-O-acteyl-rhamnogalactoside-7-O-rhamnoside respectively by using UPLC-PAD-ESI-MS<sup>n</sup> method. To further study functions of those two flavonoids on Cd toxicity, foliar application of robinin and kaempferol-3-O-rhamnoside in Cd-stressed faba bean was investigated. The chemical, kaempferol-3-O-acteyl-rhamnogalactoside-7-O-rhamnoside, was commercially unavailable, thus the precursor of this compound, kaempferol-3-O-rhamnoside was supplied. We found that dry weight of shoots and roots were unaffected by foliar supply of robinin and kaempferol-3-O-rhamnoside under Cd stress when compared with Cd treatment alone (Fig. 7A, B). However, foliar application of either robinin or kaempferol-3-O-rhamnoside significantly decreased Cd concentrations in leaves of faba bean (Fig. 7C), whereas application of those two flavonoids did not affect Cd concentrations in roots (Fig. 7D).

Due to the diversity of structures, phenolic compounds have multiply functions in plants (Ben Farhat et al. 2013). To date, it is still limited information on biological functions of robinin or kaempferol-3-O-rhamnoside in plants. Yin et al. (2014) indicated that kaempferol-3-O-rhamnoside-7-O-rhamnoside acted as an endogenous inhibitor of polar auxin transport in shoots of *Arabidopsis*. In the present study, foliar supply of kaempferol-3-O-rhamnoside or robinin (kaempferol-3-O-robinoside-7-O-rhamnoside) reduced Cd concentrations in leaves (Fig. 7C). Further studies are needed to elucidate whether kaempferol-3-O-rhamnoside or kaempferol-3-O-robinoside-7-O-rhamnoside plays a competitive role in depressing the synthesis of kaempferol-3-O-rhamnoside-7-O-rhamnoside, and thereby alleviating Cd toxicity by increasing polar auxin transport.

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## 5. Conclusion

Sulfate application significantly increased biomass of faba bean under Cd stress when compared with deficient sulfate supply. Intriguingly, sufficient and excess sulfate supply also increased Cd concentrations in leaves of faba bean. Since  $\text{CdSO}_4^0$  facilitates Cd diffusion in plants, we assume the increased  $\text{CdSO}_4^0$  percentage in the growth medium by surplus sulfate might be related to enhance Cd accumulation in leaves. The increase of Cd concentrations in leaves without damaging plant growth implies that sulfate supply enhanced Cd tolerance in plants. Localization of Cd showed that sulfate accelerated Cd accumulation in epidermis of leaves, which thus contributes to enhanced Cd tolerance. Regarding oxidative stress, sulfate supply recovered plasma membrane integrity, decreased MDA and  $\text{H}_2\text{O}_2$  contents induced by Cd toxicity. Phenolic compounds as antioxidants were not significantly affected by sulfate supply, suggesting phenolic compounds did not involve in scavenging ROS induced by Cd toxicity as affected by sulfate. However, Cd treatment induced trends of decreasing robinin and kaempherol-3-O-acteyl-rhamnogalactoside-7-O-rhamnoside in plants. Foliar application of robinin and kaempherol-3-O-rhamnoside remarkably decreased Cd concentrations in leaves, showing that those two flavonoids play roles in alleviation of Cd toxicity in faba bean.

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Table 1 Effects of exogenous sulfate on Cd speciation in the growth medium

Treatments	Speciation name (% of total concentration)						
	Cd <sup>2+</sup>	CdCl <sup>+</sup>	CdCl <sub>2</sub> <sup>0</sup>	CdSO <sub>4</sub> <sup>0</sup>	Cd(SO <sub>4</sub> ) <sub>2</sub> <sup>2-</sup>	CdNO <sub>3</sub> <sup>+</sup>	CdHPO <sub>4</sub> <sup>0</sup>
S1+Cd	58.4	38.1	1.5	<b>0.1</b>	none	0.4	1.5
S2+Cd	59.5	32.0	1.0	<b>5.5</b>	0.1	0.4	1.5
S3+Cd	72.6	1.1	none	<b>22.6</b>	1.3	0.5	1.9

Percentages of different Cd speciation were calculated by Visual MINTEQ 3.1 (a chemical equilibrium model). S1+Cd, deficient sulfate with Cd treatment; S2+Cd, sufficient sulfate with Cd treatment; S3+Cd, excess sulfate with Cd treatment.

### Figure captions

Fig.1 Dry weight of shoots (A) and roots (B) as affected by three different levels of sulfate under Cd stress. Values are means  $\pm$  SD (n = 4). Different letters above bars represent significant difference at the level of 0.05 determined by Duncan' test. S1, deficient sulfate; S2, sufficient sulfate; S3, excess sulfate; S1+Cd, deficient sulfate with Cd treatment; S2+Cd, sufficient sulfate with Cd treatment; S3+Cd, excess sulfate with Cd treatment.

Fig. 2 Cd concentrations (A, B) and Cd contents (C, D) in leaves and roots of faba bean as affected by three different levels of sulfate under Cd stress. Values are means  $\pm$  SD (n = 4). Different letters above bars represent significant difference at the level of 0.05 determined by Duncan' test. S1+Cd, deficient sulfate with Cd treatment; S2+Cd, sufficient sulfate with Cd treatment; S3+Cd, excess sulfate with Cd treatment.

Fig. 3 Localization of Cd in cross-section of leaves (A-C) and roots (D-F) as affected by three different levels of sulfate under Cd stress. UE, upper epidermis; LE, low epidermis; EN, endodermis; EP, epidermis.

Fig. 4 Effects of sulfate on plasma membrane integrity of leaves (A) and roots (B) shown by Evan's blue under Cd stress. Values are means  $\pm$  SD (n = 4). Different letters above bars represent significant difference at the level of 0.05 determined by Duncan' test. S1, deficient sulfate; S2, sufficient sulfate; S3, excess sulfate; S1+Cd, deficient sulfate with Cd treatment; S2+Cd, sufficient sulfate with Cd treatment; S3+Cd, excess sulfate with Cd treatment.

Fig. 5 Effects of sulfate on MDA (malondialdehyde) (A, B) and H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide) (C, D) of leaves (A, C) and roots (B, D) under Cd stress. Values are means  $\pm$  SD (n = 4). Different letters above bars represent significant difference at the level of 0.05 determined by Duncan' test. S1, deficient sulfate; S2, sufficient sulfate; S3, excess sulfate; S1+Cd, deficient sulfate with Cd treatment; S2+Cd, sufficient sulfate with Cd treatment; S3+Cd, excess sulfate with Cd treatment.

Fig. 6 Chromatogram of phenolic compounds derived from leaves of faba bean.

Fig. 7 Effects of foliar application of kaempherol-3-O-rhaminose (K) and robinin (R) on dry weight and Cd concentrations in Cd-stressed faba bean. Values are means  $\pm$  SD (n = 3). Different letters above bars represent significant difference at the level of 0.05 determined by Duncan' test.

**Supplementary figure caption**

Fig. S1 Peak areas of eight abundant peaks as affected by three different levels of sulfate under Cd stress. Values are means  $\pm$  SD (n = 4). S1, deficient sulfate; S2, sufficient sulfate; S3, excess sulfate; S1+Cd, deficient sulfate with Cd treatment; S2+Cd, sufficient sulfate with Cd treatment; S3+Cd, excess sulfate with Cd treatment.

Fig. 1

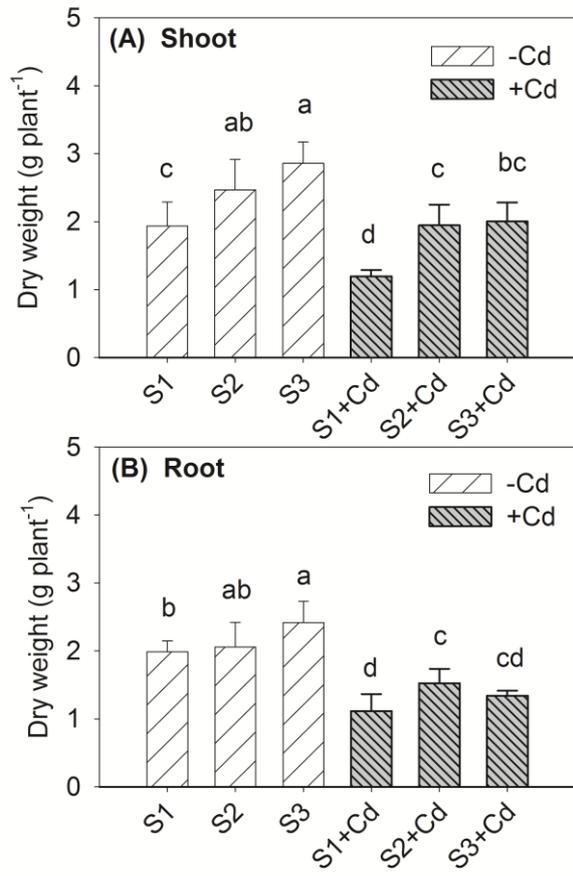


Fig. 2

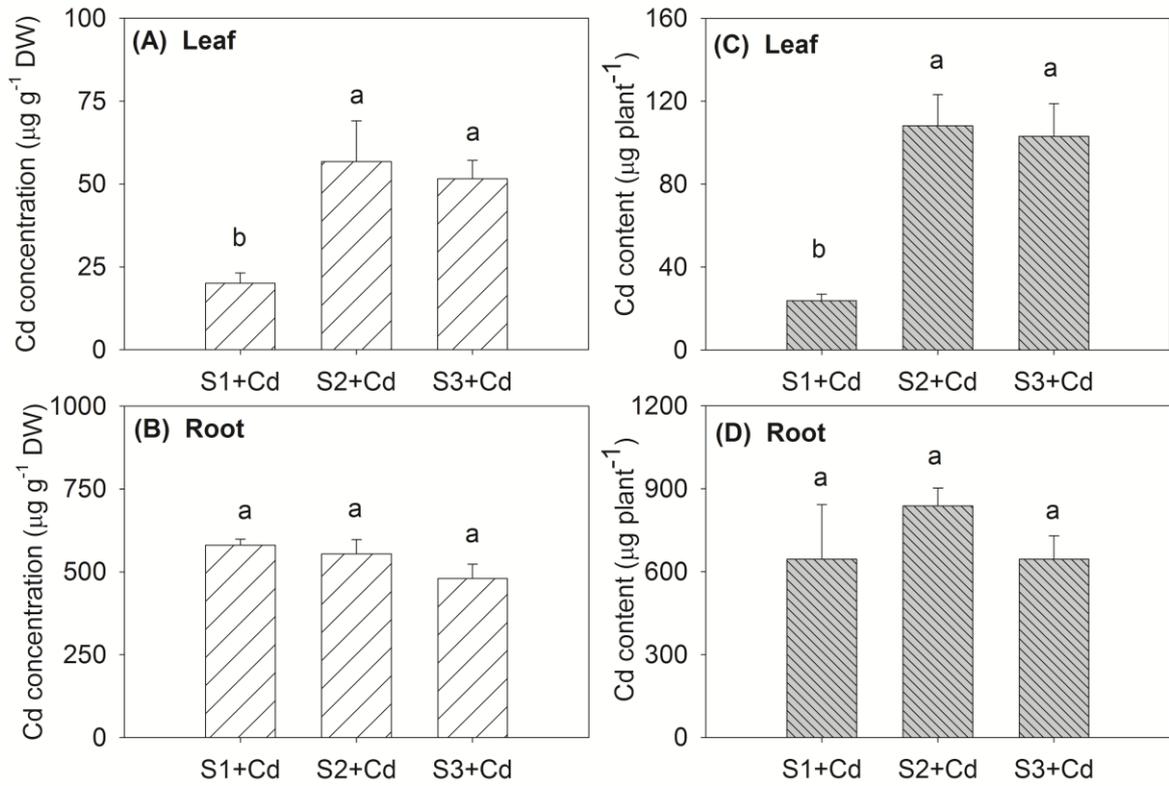


Fig. 3

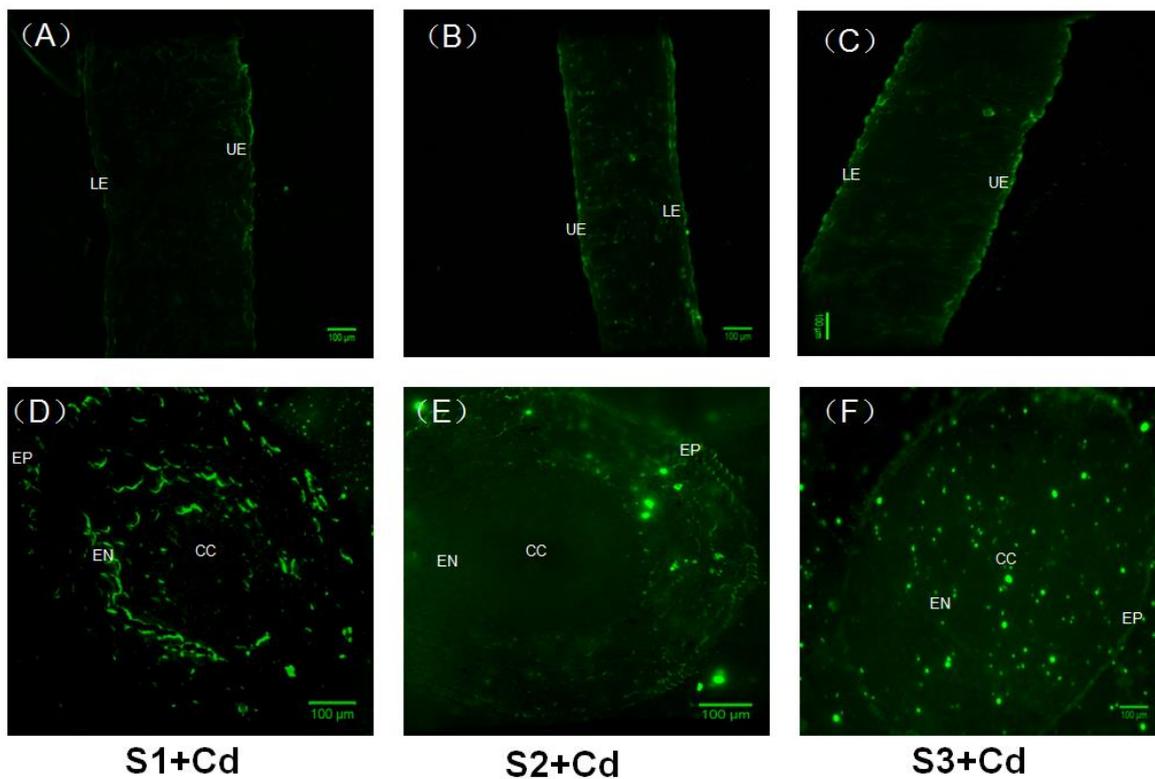


Fig. 4

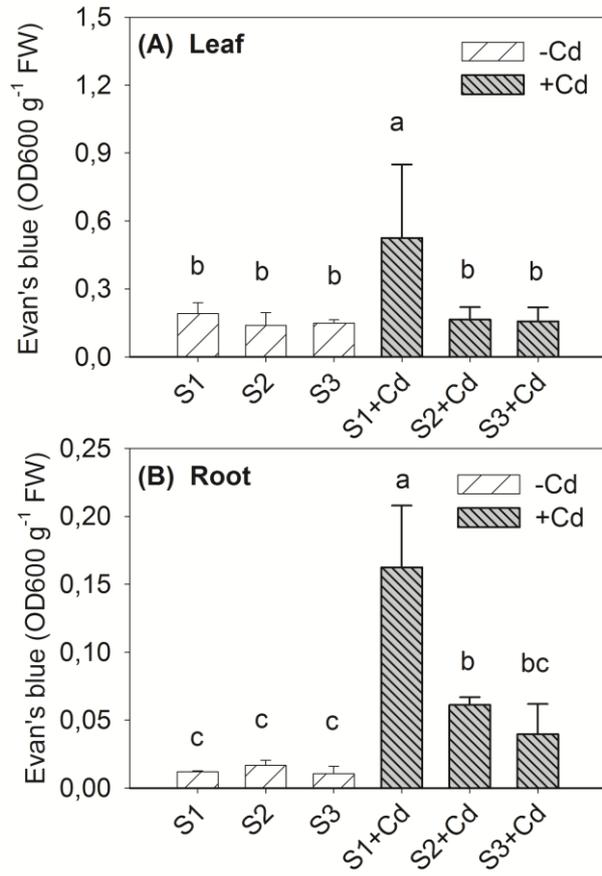


Fig. 5

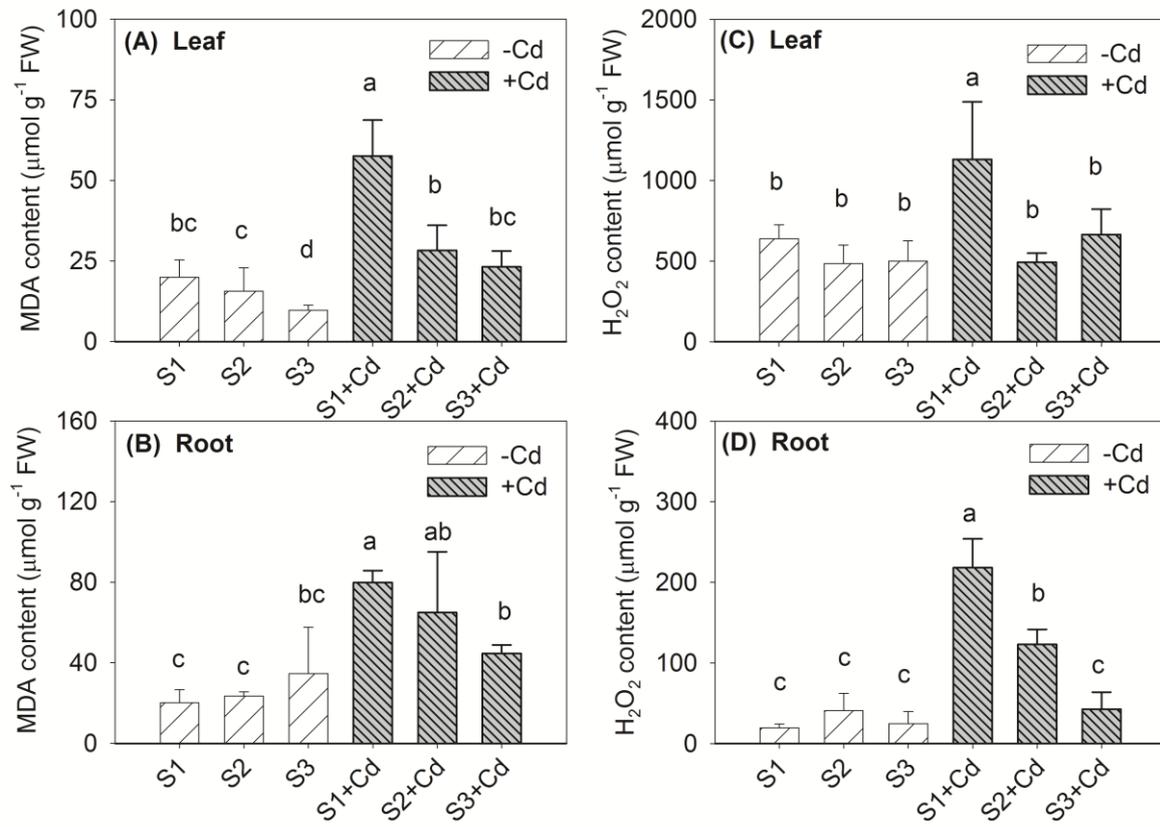


Fig. 6

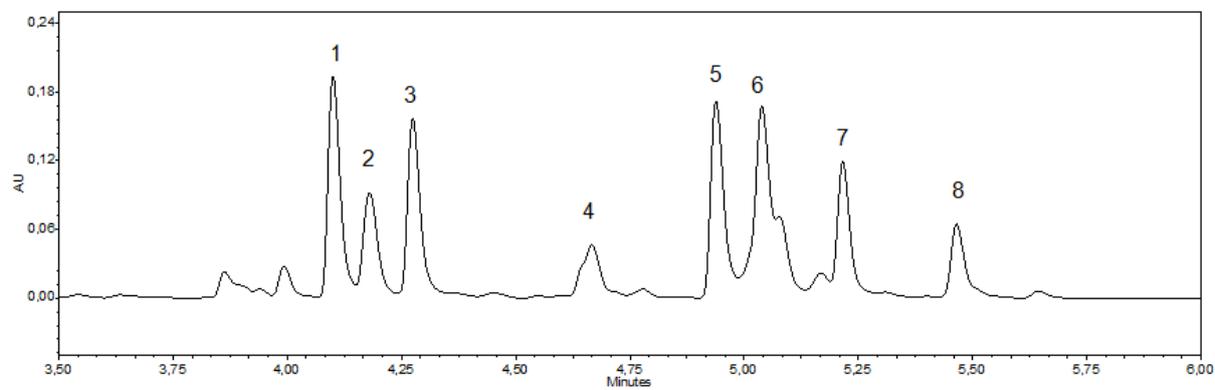


Fig. 7

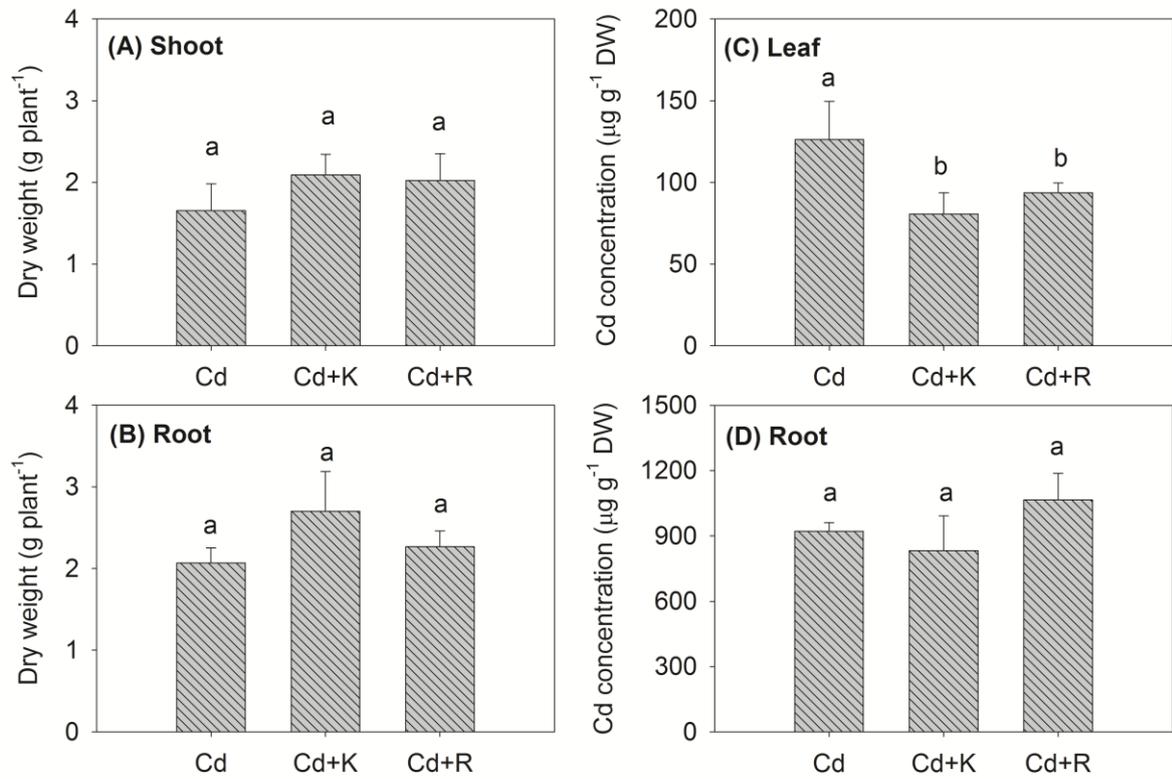
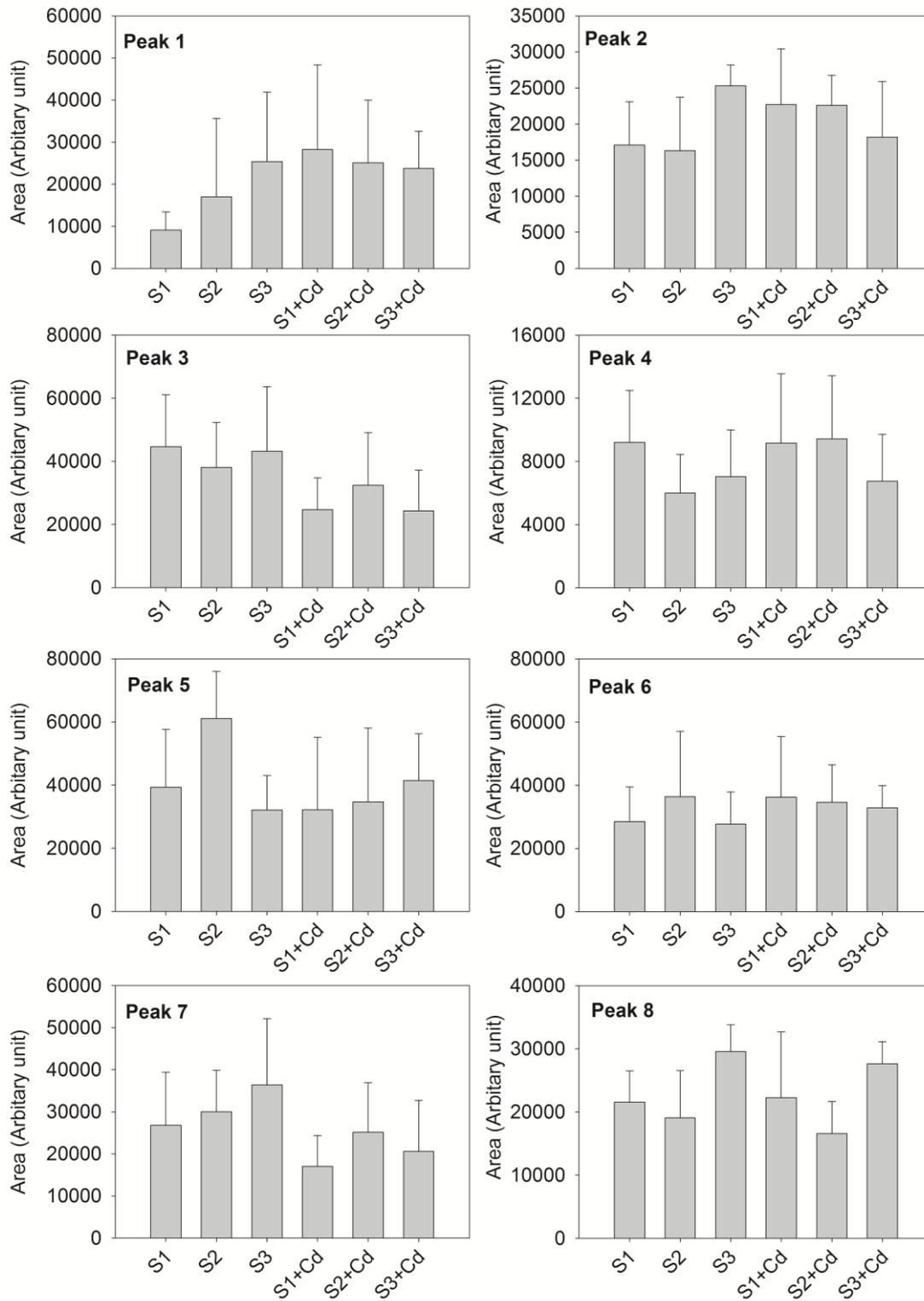


Fig. S1



## Chapter 5

### **Sulfate facilitates cadmium accumulation in leaves of *Vicia faba* L. at flowering stage**

Jiawen Wu, Amit Sagervanshi, and Karl-Hermann Mühling

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## Sulfate facilitates cadmium accumulation in leaves of *Vicia faba* L. at flowering stage

Jiawen Wu\*, Amit Sagervanshi, Karl Hermann Mühling

Institute of Plant Nutrition and Soil Science, Kiel University, Hermann-Rodewald-Str. 2, 24118 Kiel, Germany

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### ABSTRACT

Cadmium (Cd) is a toxic element, and is prevalent all over the world because of industrialization, mining, sewage sludge, or pesticide supply. Sulfur deficiency is also a frequent problem faced in agriculture. To date, information relating to effects of sulfate on Cd toxicity is still limited. To elucidate how sulfate affects Cd accumulation in faba bean, subcellular accumulation of Cd in leaves consisting of apoplastic washing fluid, symplastic fluid and the cell wall under deficient, sufficient and excess sulfate treatments were investigated in the present study. By using stable isotope of Cd ( $^{106}\text{Cd}$ ), we also traced Cd accumulation in young leaves at flowering stage from early and newly uptake of Cd in the same plants as affected by sulfate. We found that excess sulfate supply significantly increased newly uptake of Cd without affecting early uptake of Cd when compared with sufficient sulfate treatment, which resulted in enhanced total Cd in leaves by excess sulfate. Since newly uptake of Cd in leaves was from root uptake directly, we conclude that excess sulfate supply enhanced Cd originated from root uptake directly rather than re-translocation from old leaves, which is related to increased Cd accumulation in young leaves of faba bean. Subcellular analysis showed that the enhanced Cd by excess sulfate addition was a consequence of enhanced Cd in cell walls, while Cd accumulation in the apoplastic washing fluid and symplastic fluid were unchanged. The increased Cd by excess sulfate supply might be related to increased proportion of Cd speciation  $\text{CdSO}_4^0$  in the growth medium because of faster diffusion of  $\text{CdSO}_4^0$  than  $\text{Cd}^{2+}$ . To test whether macronutrients, micronutrients, sulfate and non-protein thiol involved in cell wall-Cd accumulation as affected by sulfate, correlations of subcellular Cd with subcellular macronutrients, micronutrients, sulfate, and non-protein thiol were analyzed. We found that cell wall-Cd was negatively correlated with K and Ca concentrations, whereas cell wall-Cd was positively correlated with Cu and symplastic non-protein thiol concentrations. However, when compared with sufficient sulfate, excess sulfate decreased K concentration and increased symplastic non-protein thiol concentration without changing Ca and Cu concentrations. Based on those results, reduction of K concentration and enhancement of symplastic non-protein thiol concentration by sulfate supply might be a reason for increase of cell wall-Cd concentration. Taken together, increased Cd in cell walls of leaves by sulfate supply contributes to enhance Cd accumulation.

### 1. Introduction

In recent decades, cadmium (Cd), as a heavy metal, is increasingly widespread all over the world because of industry development, mining, sewage sludge and phosphate fertilizer supply (Bingham, 1979; Wu et al., 2016). As a result, agricultural soils are polluted by Cd, which leads to Cd accumulation in plants and in human bodies via food chain (Aery and Rana, 2003). Thus, it is urgent to explore strategies to mitigate Cd toxicity.

It is well-known that Cd could combine with phytochelatins (PCs) and stored in the vacuoles in the form of Cd-PCs complexes as the detoxification mechanism in plants, and sulfur assimilation provides

precursors for synthesis of PCs. (Clemens, 2006; Uraguchi et al., 2017). Progressive process has achieved to reduce sulfur compounds emission into environment and refined fertilizer application without sulfur like urea and di-ammonium phosphate, which results in sulfur deficiency stress for plants (Astolfi et al., 2010). Some studies have shown that Cd toxicity facilitates sulfate uptake by plants through up-regulation of sulfate transporters expressions, e.g. SULTR1;2 (Nocito et al., 2002; Yamaguchi et al., 2016). However, limited information regarding effects of excess sulfur on Cd toxicity in plants is available.

Anjum et al. (2008) reported that sulfur reduced Cd toxicity and enhanced plant growth through improving ascorbate (AsA) and glutathione (GSH) contents in mustard (*Brassica campestris* L.). Liang et al.

\* Corresponding author.

E-mail addresses: [jwwu@plantnutrition.uni-kiel.de](mailto:jwwu@plantnutrition.uni-kiel.de) (J. Wu), [asagervanshi@plantnutrition.uni-kiel.de](mailto:asagervanshi@plantnutrition.uni-kiel.de) (A. Sagervanshi), [khmuehling@plantnutrition.uni-kiel.de](mailto:khmuehling@plantnutrition.uni-kiel.de) (K.H. Mühling).

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(2016) also found that sulfate alleviated Cd-induced oxidative stress and growth inhibition through up-regulation of PCs synthesis and AsA-GSH cycle in pakchoi (*Brassica chinensis* L.). Similar ameliorative roles of sulfur in Cd stress were reported in rice (*Oryza sativa* L.) (Hassan et al., 2005), in wheat (*Triticum aestivum* L.) (Khan et al., 2015) and in microalgae (*Chlamydomonas moewusii*) (Mera et al., 2016a) as well. However, studies on how sulfate supply affects subcellular Cd and whether subcellular essential nutrients contribute to Cd tolerance as affected by sulfate in plants are still lacking. In addition, results on sulfur-mediated uptake and accumulation of Cd in different plants are controversial. For example, Hassan et al. (2005) reported that sulfur decreased Cd concentrations in both roots and shoots of rice as a result of increased growth parameters by sulfur. Fan et al. (2010) also indicated that excessive sulfur reduced Cd accumulation in brown rice with the loss of rice yield. But, McLaughlin et al. (1998) showed that sulfur enhanced Cd contents in shoots of Swiss chard (*Beta vulgaris* L.) because of increased Cd availability in the growth medium. López-Chuken and Young (2010) also reported that sulfate increased Cd accumulation in maize (*Zea mays* L.).

In the present study, experiments with deficient, sufficient and excess sulfate treatments were conducted to investigate how sulfur affects Cd accumulation in faba bean (*Vicia faba* L.). As a transition from vegetative stage to reproductive stage, flowering stage is a special growth stage. Wei et al. (2006) reported that Cd concentrations in leaves at flowering stage was up to 85.8% of that at mature stage in *Solanum nigrum* L., and Wang et al. (2017) also showed that flowering stage was optimal for predicting Cd accumulation in pepper (*Capsicum annuum* L.) fruits. Therefore, how sulfate affects Cd accumulation at flowering stage was studied here, and we compared early and newly uptake of Cd in young leaves in the same plants by using stable isotope of Cd ( $^{106}\text{Cd}$ ) as a tracer. Cd in apoplastic washing fluid, cell wall and symplastic fluid as affected by sulfate supply were analyzed. To test whether sulfate affects Cd accumulation by modulating macronutrients such as potassium (K), calcium (Ca) and magnesium (Mg), and micronutrients such as manganese (Mn), iron (Fe), copper (Cu) and zinc (Zn), sulfate, and non-protein thiol, we determined nutrients, sulfate and non-protein thiol concentrations and also analyzed correlations of Cd with those parameters.

## 2. Materials and methods

### 2.1. Plant cultivation and treatment

Seeds of faba bean (*Vicia faba* L. cv. Fuego) were surface sterilized with 2% sodium perchlorate for 5 min, and then washed with deionized water thoroughly. Subsequently, seeds were soaked in 0.5 mM  $\text{CaCl}_2$  with aeration for 12 h. After 12 d germination in the quartz sand, uniform seedlings were transferred to 10-L black pots (three seedlings per pot) with nutrient solution. Four biological replicates were designed for all the treatments, and one individual pot was regarded as one biological replicate. Three different sulfur (S) levels were added in the form of sulfate: deficient sulfur (S1), 2.71  $\mu\text{M}$  sulfate; sufficient sulfur (S2), 1502.71  $\mu\text{M}$  sulfate; excess sulfur (S3), 6502.71  $\mu\text{M}$  sulfate. For S2 treatment, compositions of nutrients were macronutrients (mM) containing  $\text{Ca}(\text{NO}_3)_2$ , 2.0;  $\text{K}_2\text{SO}_4$ , 1.0;  $\text{KH}_2\text{PO}_4$ , 0.1;  $\text{K}_2\text{HPO}_4$ , 0.1; KCl, 0.2;  $\text{MgSO}_4$ , 0.5 and micronutrients ( $\mu\text{M}$ ) containing Fe-EDTA, 60;  $\text{H}_3\text{BO}_3$ , 10;  $\text{ZnSO}_4$ , 0.5;  $\text{CuSO}_4$ , 0.2;  $\text{MnSO}_4$ , 2;  $\text{NiSO}_4$ , 0.01;  $(\text{NH}_4)_2\text{Mo}_7\text{O}_{24}$ , 0.05. For S1 treatment,  $\text{MgSO}_4$  and  $\text{K}_2\text{SO}_4$  were replaced by  $\text{MgCl}_2$  and KCl respectively when compared with S2 treatment. For S3 treatment, extra 5 mM  $\text{Na}_2\text{SO}_4$  were added when compared with S2 treatment. The Na ions induced by extra  $\text{Na}_2\text{SO}_4$  addition were compensated to S1 and S2 treatments in the form of NaCl. It is well documented that  $\text{Cl}^-$  (chloride) in the growth medium more than 20 mM might have Cl toxicity in Cl sensitive species, while the Cl concentration could be four to five times higher in Cl tolerant species without affecting plant growth (Broadley et al., 2012). In the present study, Cl concentrations in the

growth medium of all the treatments were lower than 20 mM. Accordingly, Cl toxicity was negligible here.

At flower bud stage, plants were carefully transferred to 1-L containers (one plant per container as one biological replicate, four biological replicates per treatment) consisting of corresponding nutrient solution with or without 20  $\mu\text{M}$   $\text{CdCl}_2$ . After 3 d 20  $\mu\text{M}$   $\text{CdCl}_2$  treatment, plants were stressed with 20  $\mu\text{M}$   $^{106}\text{CdCl}_2$  for 1 d again. After Cd treatment, fresh weight of shoots and roots from one set of Cd-stressed plants was measured, and then samples were dried and used for Cd measurement. Top five fully-expanded leaves from another set of Cd-stressed plants were harvested for apoplastic and symplastic analysis. The proportions of different Cd speciation in the growth medium were calculated by a chemical equilibrium model, Visual MINTEQ 3.1 (Mera et al., 2016b). Plants were cultured in the greenhouse at Kiel University with a photoperiod from 8:00–22:00 and temperature (24/18 °C; day/night). The pH of nutrient solution was adjusted to  $6.0 \pm 0.2$  with NaOH and HCl. Before Cd treatment, the solution was changed every week, while it was changed daily after Cd stress.

### 2.2. Isolation of apoplastic washing fluid and symplastic fluid

After Cd treatment, top five fully-expanded leaves were selected for apoplastic washing fluid (AWF) and symplastic fluid isolation based on infiltration-centrifugation technique (Mühling and Sattelmacher, 1995; Shahzad et al., 2013). Briefly, leaves were washed with deionized water, and then intact leaves were gently placed into 60 mL plastic syringes which were filled with 40 mL deionized water. By pulling the plungers of syringes, the leaves were infiltrated with an estimated 20 kPa. Afterwards, leaves were blotted with tissue paper and centrifuged at 71 g for 5 min at 4 °C, and the collected fluid was AWF. After centrifugation, the leaves were frozen in liquid nitrogen for several seconds, and then thawed at room temperature. Subsequently, the thawed leaves were centrifuged at 327 g for 5 min, and the collected fluid was regarded as symplastic fluid. Both AWF and symplastic fluid were stored at  $-80$  °C.

The residues of leaves were followed by extracting cell walls based on the method of Zhong and Läuchli (1993). After symplastic fluid isolation, the leaves residues were incubated with 75% ethanol at 4 °C for 20 min, and then washed with ice-cold acetone, methanol and chloroform mixture (1:1, v/v), and methanol sequentially. Afterwards, the pellets were vacuum frozen-dried and stored at  $-20$  °C.

### 2.3. Nutrients determination

Samples of AWF and symplastic fluid derived from top five fully-expanded leaves were diluted with 2%  $\text{HNO}_3$ , while cell walls samples were digested with ultra-pure grade  $\text{HNO}_3$  by a microwave oven (Mars 6, CEM Corporation, USA) first and then diluted with 2%  $\text{HNO}_3$ .

Macronutrients like K, Ca and Mg were determined by AAS (Atomic Absorption Spectrometry, AAS 5EA, Thermo Electron S, Jena, Germany), and K, Ca and Mg standard solutions (Merck, Darmstadt, Germany) were independently used for making corresponding standard curves. Micronutrients like Mn, Fe, Cu and Zn were determined by ICP-MS (inductively coupled plasma mass spectroscopy, Agilent 7700, Agilent Technologies Inc., USA), and multi-element ICP-standard-solution (ROTI<sup>®</sup>STAR, Roth, Germany) was used for establishing standard curves.

### 2.4. Determination of Cd and calculation

After Cd treatment, samples of top five fully-expanded leaves, shoots and roots were treated with  $\text{HNO}_3$  as described for mineral nutrients analysis. The final ratio of stable isotope between  $^{106}\text{Cd}$  and  $^{114}\text{Cd}$  was determined by ICP-MS, while the total Cd was measured by AAS.

The total amount of Cd ( $\text{Cd}^{\text{T}}$ ) consists of early uptake of Cd ( $\text{Cd}^{\text{E}}$ )

and newly uptake of Cd ( $Cd^N$ ), shown as follows,

$$Cd^T = Cd^E + Cd^N \quad (1)$$

The ratios of  $^{106}Cd$  and  $^{114}Cd$  in samples are calculated as follows,

$$R_{(106/114)} = \frac{Cd^E \times f_{106-nat} + (Cd^T - Cd^E) \times f_{106-enr}}{Cd^E \times f_{114-nat} + (Cd^T - Cd^E) \times f_{114-enr}} \quad (2)$$

After combining like terms by mathematic analysis, the Eq. (2) is transformed into,

$$Cd^E = \frac{Cd^T \times [f_{106-enr} - R_{(106/114)} \times f_{114-enr}]}{R_{(106/114)} \times (f_{114-nat} - f_{114-enr}) + f_{106-enr} - f_{106-nat}} \quad (3)$$

In the equations,  $R_{(106/114)}$  represents the ratio of  $^{106}Cd$  and  $^{114}Cd$  in samples which was measured by ICP-MS. The  $f_{106-nat}$  and  $f_{106-enr}$  are abundances of  $^{106}Cd$  in nature and in the enriched  $^{106}Cd$  stable isotope respectively, while  $f_{114-nat}$  and  $f_{114-enr}$  are abundances of  $^{114}Cd$  in nature and in the enriched  $^{114}Cd$  stable isotope respectively. The stable isotope of Cd, enriched  $^{106}Cd$ , was purchased from Neonest AB, Solna, Sweden, which is water-soluble. The abundances of enriched  $^{106}Cd$  are following:  $^{106}Cd$ , 74.1%;  $^{108}Cd$ , 7.3%;  $^{110}Cd$ , 4.2%;  $^{111}Cd$ , 3.5%;  $^{112}Cd$ , 3.3%;  $^{113}Cd$ , 3.1%;  $^{114}Cd$ , 2.7%;  $^{116}Cd$ , 1.8%, while natural abundances of Cd are  $^{106}Cd$ , 1.25%;  $^{108}Cd$ , 0.89%;  $^{110}Cd$ , 12.49%;  $^{111}Cd$ , 12.80%;  $^{112}Cd$ , 24.13%;  $^{113}Cd$ , 12.22%;  $^{114}Cd$ , 28.73%;  $^{116}Cd$ , 7.49%.

Consequently, early uptake of Cd ( $Cd^E$ ) was calculated based on Eq. (3), and then newly uptake of Cd ( $Cd^N$ ) could also be calculated according to Eq. (1), namely,

$$Cd^N = Cd^T - Cd^E \quad (4)$$

### 2.5. Sulfate and non-protein thiol measurement

Sulfate concentrations in the AWF and symplastic fluid derived from top five fully-expanded leaves were determined by turbidimetric method according to Kolmert et al. (2000). Simply, when barium added into solution containing sulfate, it resulted in solute turbid. Thus, sulfate could be measured by a spectrophotometer at 420 nm. Standards of sulfate were used for determination of sulfate contents.

In regard to non-protein thiol measurement, aliquots of 1 mL AWF and symplastic fluid derived from top five fully-expanded leaves were mixed with 0.8 mL deionized water and 0.2 mL 50% (w/v) trichloroacetic acid. After 15 min reaction, the samples were centrifuged at 10,000 g for 10 min. Subsequently, 0.5 mL supernatant was added into 1 mL 0.4 M Tris-HCl (pH 8.9), and then mixed with 0.025 mL 0.01 M DTNB [5, 5'-Dithiobis-(2-nitrobenzoic acid)]. Within 5 min, the absorbance was recorded at 412 nm (Nagalakshmi and Prasad, 2001).

### 2.6. Statistic analysis

All values in the present study were subjected to one-way ANOVA analysis by using SPSS software (SPSS Inc. 16.0). Significant differences were determined according to Duncan's test at the level of 0.05. Pearson's correlation coefficients were calculated by using SPSS software (SPSS Inc. 16.0). In the present study, the units of Cd and nutrients concentrations in cell walls were indicated as  $\mu g g^{-1}$  FW. It is noteworthy that the fresh weight of cell walls was converted from dry weight of cell walls based on unchanged ratio of cell wall biomass to whole leaf biomass regardless of fresh weight or dry weight.

## 3. Results

### 3.1. Effects of sulfate on biomass and Cd concentrations in shoots and roots

Deficient sulfate significantly decreased the shoot fresh weight (Fig. 1A), whereas the fresh weight of the root was not affected (Fig. 1B)

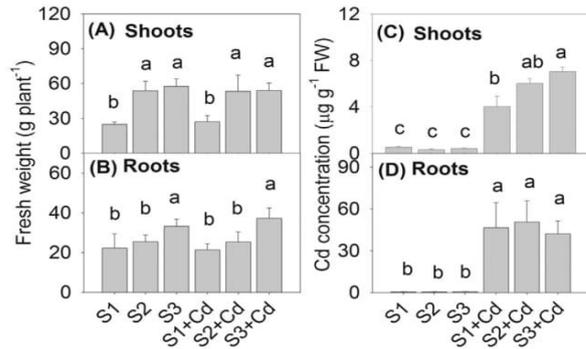


Fig. 1. Effects of sulfate on fresh weight (A, B) and Cd concentrations (C, D) in shoots and roots under Cd stress. Values are means  $\pm$  SD (n = 4). Different letters indicate significance at  $p < 0.05$  determined by Duncan's test and one-way ANOVA. S1, deficient sulfate; S2, sufficient sulfate; S3, excess sulfate; S1 + Cd, deficient sulfate with 20  $\mu M$  CdCl<sub>2</sub>; S2 + Cd, sufficient sulfate with 20  $\mu M$  CdCl<sub>2</sub>; S3 + Cd, excess sulfate with 20  $\mu M$  CdCl<sub>2</sub>.

when compared with sufficient sulfate addition regardless of Cd stress. Excess sulfate supply had no impact on the shoot biomass (Fig. 1A), but it increased the root biomass in contrast to sufficient sulfate irrespective of Cd toxicity (Fig. 1B).

Sulfate application did not affect Cd concentrations in both shoots and roots of non-Cd-stressed plants (Fig. 1C, D). However, excess sulfate significantly increased Cd concentrations in shoots when compared with deficient sulfate treatment under Cd toxicity (Fig. 1C). In Cd-stressed plants, Cd concentrations in roots were not changed by sulfate supply (Fig. 1D).

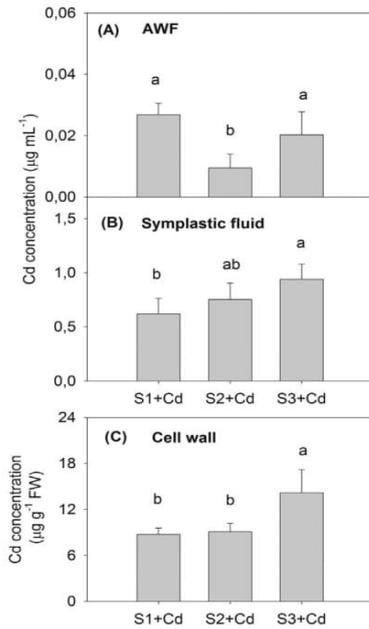
### 3.2. Effects of sulfate on subcellular Cd accumulation

Compared with sufficient sulfate supply, deficient sulfate remarkably increased Cd concentrations in the AWF without changing Cd concentrations in symplastic fluid and cell walls (Fig. 2). Excess sulfate supply also promoted Cd concentrations in the AWF (Fig. 2A) and had no significant effect on Cd concentrations in symplastic fluid (Fig. 2B), but it increased Cd concentrations in cell walls (Fig. 2C).

Regarding Cd contents in leaves, total Cd accumulation was increased with increasing exogenous sulfate addition. Compared with deficient sulfate, sufficient and excess sulfate supply did not affect Cd accumulation in the AWF and symplastic fluid, but they enhanced Cd accumulation in cell walls which contributed to the increase of total Cd contents (Table 1). More specifically, Cd accumulation in the AWF, symplastic fluid and cell walls from early uptake of Cd were not affected regardless of sulfate supplementation, resulting in unchanged total Cd accumulation from early uptake of Cd by sulfate (Table 1). Comparatively, excess sulfate substantially increased Cd accumulation from newly uptake of Cd in leaves, especially increased subcellular Cd accumulation in cell walls at flowering stage (Table 1).

When compared contributions of early and newly uptake of Cd to total Cd accumulation as affected by sulfate, as can be seen in Table 1, newly uptake of Cd accounted for 66% of total Cd in excess sulfate-treated plants. Nevertheless, newly uptake of Cd only occupied 34% of total Cd in plants under deficient sulfate treatment. In detail, the proportions of Cd distribution in cell walls from early uptake of Cd were 66% and 26% in deficient sulfate and excess sulfate-treated plants respectively, while the percentages were 34% and 74% in deficient and excess sulfate-treated plants from newly uptake of Cd (Table 1).

In addition, in the growth medium, sulfate addition remarkably changed Cd speciation percentages (Table 2), especially CdCl<sup>+</sup> and CdSO<sub>4</sub><sup>0</sup>. In deficient and sufficient sulfate-treated solution, the percentages of CdCl<sup>+</sup> to total Cd was 35.1% and 32.6% respectively, while



**Fig. 2.** Effects of sulfate on Cd concentrations in the AWF (apoplastic washing fluid, A), symplastic fluid (B) and cell wall (C) of faba bean leaves. Values are means  $\pm$  SD (n = 4). Different letters indicate significance at  $p < 0.05$  determined by Duncan's test and one-way ANOVA. S1 + Cd, deficient sulfate with 20  $\mu$ M CdCl<sub>2</sub>; S2 + Cd, sufficient sulfate with 20  $\mu$ M CdCl<sub>2</sub>; S3 + Cd, excess sulfate with 20  $\mu$ M CdCl<sub>2</sub>.

it was only 1.1% in excess sulfate-treated solution. Regarding CdSO<sub>4</sub><sup>0</sup>, the percentages were 0.1%, 5.6%, 27.0% for deficient, sufficient and excess sulfate treatments, respectively (Table 2).

3.3. Effects of sulfate on nutrients concentrations

In comparison with sufficient sulfate, deficient sulfate increased concentrations of K, Ca and Mg in leaves under Cd stress (Fig. 3D, H, L). More specifically, Ca and Mg concentrations in the AWF, symplastic

**Table 2**  
Speciation of Cd as affected by sulfate in the growth medium.

Treatments	Speciation name (% of total concentration)						
	Cd <sup>2+</sup>	CdCl <sup>+</sup>	CdCl <sub>2</sub> <sup>0</sup>	CdSO <sub>4</sub> <sup>0</sup>	Cd(SO <sub>4</sub> ) <sub>2</sub> <sup>2-</sup>	CdNO <sub>3</sub> <sup>+</sup>	CdHPO <sub>4</sub> <sup>0</sup>
S1 + Cd	61.6	35.1	1.1	0.1	–	0.4	1.7
S2 + Cd	58.7	32.6	1.0	5.6	0.1	0.4	1.6
S3 + Cd	67.6	1.1	–	27.0	2.1	0.5	1.7

Proportions of different Cd speciation were calculated by Visual MINTEQ. 3.1 (a chemical equilibrium model). Here '–' represents none. S1 + Cd, deficient sulfate with 20  $\mu$ M CdCl<sub>2</sub>; S2 + Cd, sufficient sulfate with 20  $\mu$ M CdCl<sub>2</sub>; S3 + Cd, excess sulfate with 20  $\mu$ M CdCl<sub>2</sub>.

fluid and cell walls were all increased (Fig. 3E-L). For K, only K concentrations in the AWF were increased and K in the symplastic fluid and cell walls were unaffected (Fig. 3A-C). By contrast, excess sulfate did not affect total concentrations of Ca and Mg compared with sufficient sulfate supply (Fig. 3H, L). Concentrations of Ca and Mg in the AWF, symplastic fluid and cell walls were not changed either (Fig. 3E-L). However, excess sulfate significantly declined total concentrations of K in leaves in contrast to sufficient sulfate (Fig. 3D). Specifically, excess sulfate decreased K in symplastic fluid, but it did not affect K concentrations in the AWF and cell walls (Fig. 3A-C).

In regard to micronutrients, deficient sulfate addition showed higher total concentrations of Mn, Fe and Zn than sufficient sulfate under Cd toxicity (Fig. 4D, H, P). Deficient sulfate increased Mn and Zn concentrations in the AWF, symplastic fluid and cell walls when compared with sufficient sulfate (Fig. 4), while deficient sulfate only increased Fe concentrations in the AWF (Fig. 4E). Deficient sulfate increased Cu concentrations in the AWF without affecting Cu concentrations in symplastic fluid and cell walls (Fig. 4I-K) by contrast with sufficient sulfate. Excess sulfate supply did not affect Mn, Fe, Cu, and Zn concentrations when compared with sufficient sulfate (Fig. 4)

3.4. Effects of sulfate on sulfate and non-protein thiol concentrations under Cd stress

As can be seen in Fig. 5, sulfate concentrations of AWF in excess sulfate-treated plants were higher than that in deficient and sufficient sulfate-treated plants (Fig. 5A). However, sulfate concentrations in symplastic fluid were not changed regardless of sulfate levels under Cd stress (Fig. 5B). Non-protein thiol concentrations in symplastic fluid of

**Table 1**  
Effects of sulfate on Cd contents and distribution in young leaves of faba bean under Cd stress.

	Treatments	AWF ( $\mu$ g leaves <sup>-1</sup> ) Distribution (%)	Symplastic fluid ( $\mu$ g leaves <sup>-1</sup> ) Distribution (%)	Cell wall ( $\mu$ g leaves <sup>-1</sup> ) Distribution (%)	Total Cd amount ( $\mu$ g leaves <sup>-1</sup> ) Distribution (%)
Early uptake of Cd ( <sup>65</sup> Cd)	S1 + Cd	0.019 $\pm$ 0.00a (67%)	0.805 $\pm$ 0.14a (70%)	1.119 $\pm$ 0.31a (66%)	2.082 $\pm$ 0.16a (66%)
	S2 + Cd	0.009 $\pm$ 0.00a (31%)	0.790 $\pm$ 0.29a (53%)	1.588 $\pm$ 0.29a (52%)	2.208 $\pm$ 0.11a (49%)
	S3 + Cd	0.018 $\pm$ 0.01a (58%)	0.776 $\pm$ 0.29a (58%)	1.339 $\pm$ 0.37a (26%)	2.473 $\pm$ 0.59a (34%)
Newly uptake of Cd ( <sup>109</sup> Cd)	S1 + Cd	0.009 $\pm$ 0.00a (33%)	0.350 $\pm$ 0.11b (30%)	0.566 $\pm$ 0.19c (34%)	1.074 $\pm$ 0.24c (34%)
	S2 + Cd	0.018 $\pm$ 0.01a (69%)	0.715 $\pm$ 0.19a (47%)	1.484 $\pm$ 0.10b (48%)	2.292 $\pm$ 0.24b (51%)
	S3 + Cd	0.013 $\pm$ 0.00a (42%)	0.567 $\pm$ 0.21ab (42%)	3.832 $\pm$ 0.94a (74%)	4.820 $\pm$ 1.03a (66%)
Total Cd amount	S1 + Cd	0.028 $\pm$ 0.01a (100%)	1.156 $\pm$ 0.24a (100%)	1.685 $\pm$ 0.48c (100%)	3.156 $\pm$ 0.39c (100%)
	S2 + Cd	0.027 $\pm$ 0.01a (100%)	1.505 $\pm$ 0.48a (100%)	3.072 $\pm$ 0.39b (100%)	4.501 $\pm$ 0.34b (100%)
	S3 + Cd	0.032 $\pm$ 0.02a (100%)	1.344 $\pm$ 0.40a (100%)	5.172 $\pm$ 0.73a (100%)	7.293 $\pm$ 1.35a (100%)

Values are means  $\pm$  SD (n = 4). Different letters indicate significance at  $p < 0.05$  determined by Duncan's test and one-way ANOVA. AWF, apoplastic washing fluid; S1 + Cd, deficient sulfate with 20  $\mu$ M CdCl<sub>2</sub>; S2 + Cd, sufficient sulfate with 20  $\mu$ M CdCl<sub>2</sub>; S3 + Cd, excess sulfate with 20  $\mu$ M CdCl<sub>2</sub>.

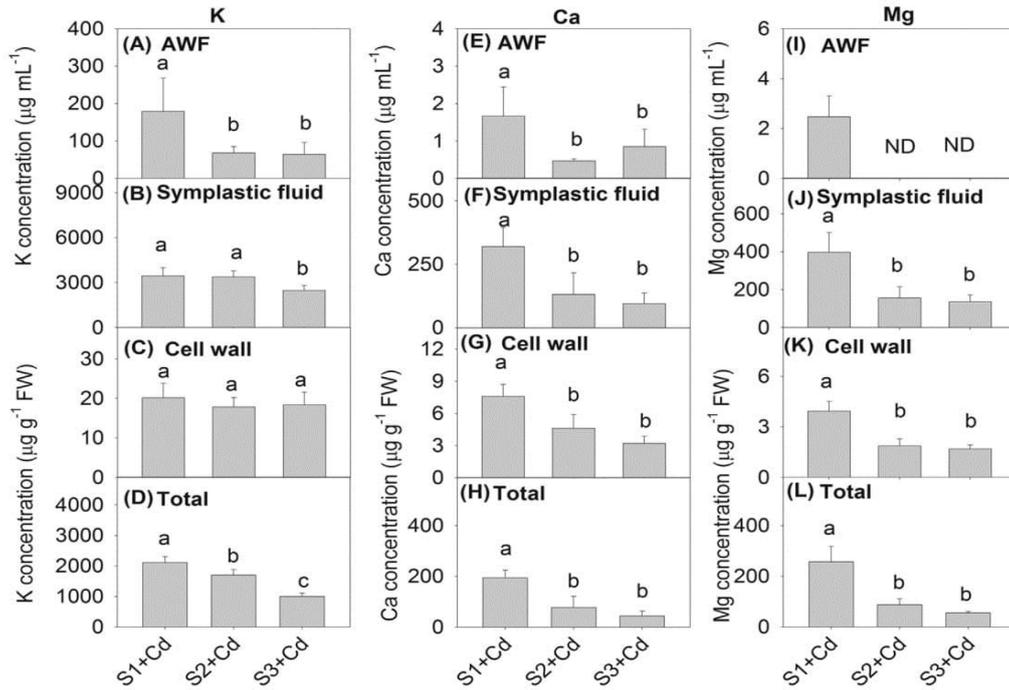


Fig. 3. Effects of sulfate on subcellular concentrations of macronutrients under Cd stress. K (potassium, A-D), Ca (calcium, E-H), and Mg (magnesium, I-L). Values are means  $\pm$  SD ( $n = 4$ ). Different letters indicate significance at  $p < 0.05$  determined by Duncan's test and one-way ANOVA. AWF, apoplasmic washing fluid; S1 + Cd, deficient sulfate with 20  $\mu\text{M}$  CdCl<sub>2</sub>; S2 + Cd, sufficient sulfate with 20  $\mu\text{M}$  CdCl<sub>2</sub>; S3 + Cd, excess sulfate with 20  $\mu\text{M}$  CdCl<sub>2</sub>.

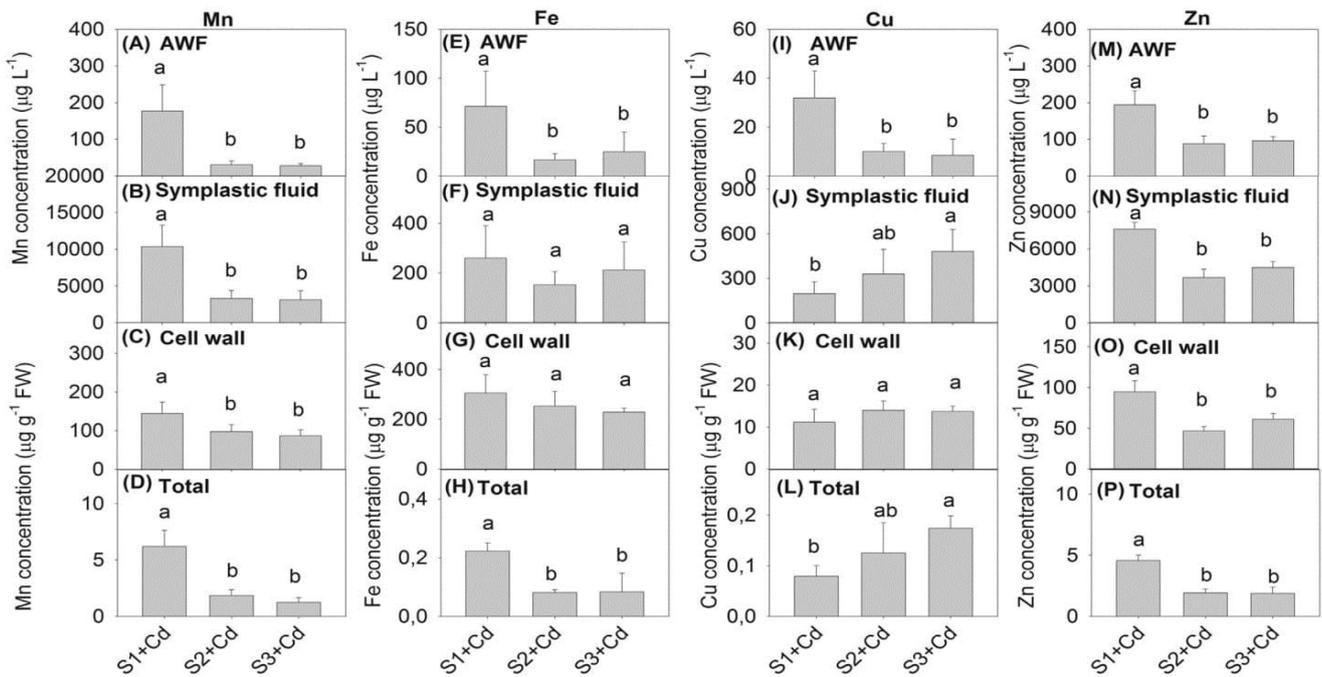


Fig. 4. Effects of sulfate on subcellular concentrations of micronutrients under Cd stress. Mn (manganese, A-D), Fe (iron, E-H), Cu (copper, I-L), and Zn (zinc, M-P). Values are means  $\pm$  SD ( $n = 4$ ). Different letters indicate significance at  $p < 0.05$  determined by Duncan's test and one-way ANOVA. AWF, apoplasmic washing fluid; S1 + Cd, deficient sulfate with 20  $\mu\text{M}$  CdCl<sub>2</sub>; S2 + Cd, sufficient sulfate with 20  $\mu\text{M}$  CdCl<sub>2</sub>; S3 + Cd, excess sulfate with 20  $\mu\text{M}$  CdCl<sub>2</sub>.

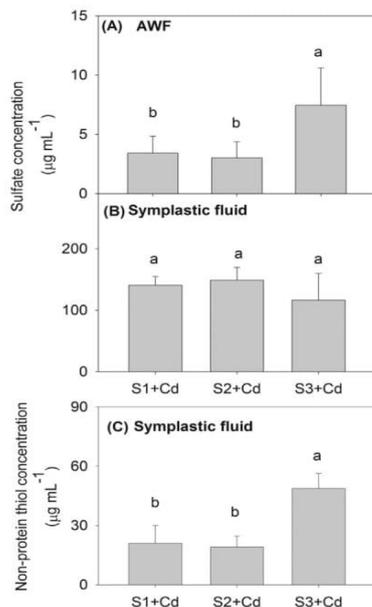


Fig. 5. Sulfate (A, B) and non-protein thiol (C) concentrations as affected by different sulfate treatments under Cd stress. Values are means  $\pm$  SD ( $n = 4$ ). Different letters indicate significance at  $p < 0.05$  determined by Duncan's test and one-way ANOVA. AWF, apoplastic washing fluid; S1 + Cd, deficient sulfate with  $20 \mu\text{M CdCl}_2$ ; S2 + Cd, sufficient sulfate with  $20 \mu\text{M CdCl}_2$ ; S3 + Cd, excess sulfate with  $20 \mu\text{M CdCl}_2$ .

excess sulfate-treated plants were higher than that in deficient and sufficient sulfate-treated plants under Cd stress (Fig. 5C), while non-protein thiol concentrations in the AWF were below experimental detection.

#### 4. Discussion

##### 4.1. Effects of sulfate on biomass and Cd accumulation in faba bean

Sulfur deficiency could lead to chlorosis of leaves, retardation of plant growth, and loss of yields (Hawkesford, 2000). In the present study, we found the same stress that deficient sulfur decreased fresh weight of shoots regardless of Cd toxicity in faba bean (Fig. 1A), and excess sulfate did not affect shoot fresh weight compared with sufficient sulfate (Fig. 1A). When excess sulfate enters into plants, plants deploy strategy to store them without changing plant growth (Rennenberg, 1984). As reported by Kowalska (2005), plant growth and leaf area were unaffected regardless of excessive sulfate levels, although sulfate contents in plants were increased as sulfate in nutrient solution increased. As deficient sulfate significantly reduced biomass of shoots regardless of Cd stress (Fig. 1A), we reason that all the changes of deficient sulfate-treated plants under Cd stress were induced by stunted plant growth. Therefore, we focused on effects of excess sulfate on Cd toxicity by contrast with sufficient sulfate supply.

Excess sulfate did not change biomass of shoots in comparison with sufficient sulfate (Fig. 1A) under Cd toxicity, but it increased Cd concentrations in the apoplast of leaves (Fig. 2A, C). As a result, total Cd contents in leaves of excess sulfate-treated plants were higher than that in sufficient sulfate-treated plants (Table 1), indicating that excess sulfate increased Cd concentrations is the reason for increase of Cd accumulation rather than increased biomass. Stable isotope of Cd ( $^{106}\text{Cd}$ ) was used to trace Cd from early and newly uptake in the same plants. The similar method was also used to study Zn uptake, translocation and remobilization in rice by supplying stable isotope of Zn (Wu

et al., 2010). Intriguingly, the results showed that the majority of Cd in young leaves was from newly uptake of Cd at flowering stage in excess sulfate-treated plants (66%) while it was a minor percentage in deficient-stressed plants (34%), and promoted newly uptake of Cd by excess sulfate was mainly accumulated in cell walls (Table 1). Based on those results, we conclude that excess sulfate facilitates accumulation of newly uptake of Cd in cell walls of leaves in faba bean. Koren et al. (2013) reported that Cd ligand environment, to a large extent, modified subcellular distribution pattern of Cd in leaves of *Noccaea (Thlaspi) praecox*:  $\text{CdSO}_4$ -stressed plants preferentially accumulated Cd in the apoplast while  $\text{CdCl}_2$ -stressed plants preferred to accumulate Cd in the vacuoles. The case that in our study, excess sulfate remarkably promoted Cd speciation  $\text{CdSO}_4^0$  to 27.0% from 5.6% in sufficient sulfate treatment in the growth medium (Table 2), which could explain why excess sulfate facilitated Cd accumulation in cell walls. Surprisingly, excess sulfate only facilitated newly uptake of Cd in cell walls at flowering stage, but did not affect early uptake of Cd when compared with sufficient sulfate treatment (Table 1). However, the underlying mechanism is unknown. We speculate that Cd in the form of  $\text{CdSO}_4^0$  in the growth medium was not absorbed by plants at flower bud stage, while at late flowering stage,  $\text{CdSO}_4^0$  begun to be absorbed by plants. The diffusion of  $\text{CdSO}_4^0$  is faster than  $\text{Cd}^{2+}$ , and  $\text{CdSO}_4^0$  prefers to accumulate in cell walls as reported by Koren et al. (2013). Mera et al. (2016b) also showed that in microalgae, the increase of sulfate resulted in the enhancement of  $\text{CdSO}_4^0$  proportion, but they found antagonistic effect of sulfate on Cd when sulfate concentration was below  $14.2 \text{ mg L}^{-1}$ , while synergistic effect of sulfate on Cd when sulfate concentration exceeded  $14.2 \text{ mg L}^{-1}$ . We assume that the capacity of microalgae cell walls was not enough to sequester increased  $\text{CdSO}_4^0$  by sulfate, thus synergistic effect of sulfate on Cd was investigated when sulfate concentration exceeded  $14.2 \text{ mg L}^{-1}$ . On the other hand, in the present study, excess sulfate only enhanced newly uptake of Cd without affecting early uptake of Cd. Since newly uptake of Cd originated from root uptake directly, we reason that the enhanced Cd in young leaves by excess sulfate was from root uptake directly rather than re-translocation from old leaves. In regard to Cd in roots, Cd concentrations were not affected regardless of sulfate levels (Fig. 1D), which further implies that excess sulfate accelerated Cd accumulation in leaves without retaining Cd in roots. Overall, sulfate increases Cd accumulation in cell walls by newly uptake of Cd at flowering stage which results in increased total Cd accumulation in leaves. To assess whether sulfate supply increases Cd in cell walls related to nutrients and S-containing compounds, we determined correlations of Cd in cell walls with nutrients, sulfate and non-protein thiol. Cd concentrations were negligible in non-Cd-stressed plants (Fig. 1C, D), thus we just determined nutrients, sulfate and non-protein thiol concentrations in Cd-stressed plants.

##### 4.2. Effects of sulfate on nutrients under Cd stress

To date, no biological function of Cd is discovered in plants. Nevertheless, Cd is able to enter into plants via transporters of essential nutrients, such as Ca, Zn, Fe, or Mn (Clemens et al., 2002; Wu et al., 2013). Because of the competition of Cd with other essential nutrients, mineral homeostasis is disrupted by Cd toxicity in plants (Sebastian and Prasad, 2016; Stein et al., 2017). Some studies also showed that exogenous application of Zn (Koleli et al., 2004), or Mg (Kudo et al., 2015) could reduce Cd accumulation in plants. To elucidate whether sulfate increases cell wall-Cd involving in nutrients effects, we analyzed relationships of Cd in cell walls with nutrients concentrations under different sulfate treatments. We found Cd in cell walls was negatively correlated with K and Ca concentrations, and positively correlated with Cu (Table S1-2).

In plants, Ca is a component of cell wall (Jarvis, 1982). Antagonistic effect of Ca on Cd could reduce Cd accumulation showed by Huang et al. (2017) that exogenous application of Ca decreased Cd concentrations in plants. However, compared with sufficient sulfate, excess

sulfate did not affect Ca concentrations in leaves (Fig. 3E-H), but excess sulfate significantly decreased K concentrations under Cd stress (Fig. 4D). It seems that reduction of K by sulfate is related to increase of Cd. It is well known that K is the most abundant inorganic cation in the symplast of plants (Leigh and Jones, 1984), which is also shown in our study (Fig. 3). K plays a multitude of roles in plants such as involvement of photosynthesis, enzyme activation, protein synthesis, osmoregulation including stomata movement and cell extension (Hawkesford et al., 2012). Regarding the relationship of K and Cd toxicity, Liu et al. (2012) reported that exogenous supply of K declined Cd concentrations in rice seedlings, and Ahmad et al. (2016) also confirmed this beneficial function of K on decreasing Cd in chickpea (*Cicer arietinum* L.). Even Liu et al. (2013) showed that deficient K protected rice plants from Cd toxicity through stimulation of antioxidant enzymes activities. Based on those information, it seems that excess sulfate enhanced Cd accumulation in leaves of faba bean through decreasing K concentration is reasonable (Fig. 3 and Table 1), and we speculate that K plays a regulatory role in Cd toxicity in excess sulfate-treated plants. Nevertheless, it is still unclear how K affects cell wall-Cd in plants. We hypothesize that stimulating antioxidant defense capacity (Liu et al., 2013) and/or inducing osmotic stress proteins by decreased K (Alahari and Apte, 2004) contribute to Cd resistance. Limited information is available about effects of Cu on Cd accumulation. It is unknown why cell wall-Cd was positively correlated with Cu concentration. However, Cu concentrations in the AWF, symplastic fluid and cell walls were unchanged under Cd stress as affected by excess sulfate (Fig. 4I-L), indicating that sulfate did not modulate this synergetic effects of Cu on Cd in this study.

#### 4.3. Sulfate and non-protein thiol concentrations under Cd stress as affected by sulfate supply

Küpper et al. (2004) indicated that young leaves combined more S-containing compounds (e.g. phytochelatins; PCs) than mature leaves. As reported earlier, sulfur supply enhances Cd tolerance by improving GSH, non-protein thiol and PCs, and sequestering thiol-Cd containing compounds like PCs-Cd into vacuoles to detoxify Cd toxicity (Anjum et al., 2008; Zhang et al., 2013). In the present study, excess sulfate increased symplastic non-protein thiol concentrations (Fig. 5C), but symplastic Cd concentrations were not affected (Fig. 2B), which indicates that sulfate supply increases -SH group and then storage Cd with -SH group such as PCs-Cd compounds in vacuoles is not the case here. Regarding whether sulfate supply increases Cd in cell walls correlating with S-containing compound, we found that Cd in cell walls was positively correlated with symplastic non-protein thiol concentrations (Table S3). To date, limited information is available on how symplastic non-protein thiol affects Cd in cell walls. We assume that enhanced non-protein thiol involves in regulatory function such as antioxidant capacity, and thereby affecting Cd accumulation in plants indirectly.

#### 5. Conclusion

Excess sulfate accelerates Cd accumulation in leaves of faba bean at flowering stage when compared with sufficient sulfate. The enhanced Cd by sulfate supply was from newly uptake of Cd rather than early uptake of Cd, which suggests that the enhanced Cd originates from root uptake directly rather than re-translocation from old leaves. The enhanced Cd by sulfate supply prefers to accumulate in cell walls resulting in higher Cd tolerance in faba bean. Enhancement of Cd in cell walls is correlated with decreased K concentration and increased symplastic non-protein thiol concentration. The underlying mechanisms of antagonistic effect of K and synergetic effect of symplastic non-protein thiol on Cd in cell walls are still unclear. Higher percentage of  $\text{CdSO}_4^0$  in the growth medium as a result of sulfate supply might also contribute to increase Cd in leaves because  $\text{CdSO}_4^0$  diffusion is faster than  $\text{Cd}^{2+}$ .

#### Acknowledgments

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#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ecoenv.2018.03.042>.

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## **Chapter 6**

### **General discussion**

## 6. General discussion

Cd pollution is increasingly serious because of mining, industry development, sewage sludge and phosphate fertilizer application (Clemens et al. 2013). Cd toxicity damages photosynthesis, respiration, nutrient homeostasis, and thereby hampering plant growth. Moreover, once Cd enters into edible organs of plants, Cd will threaten human health via food chain (Mortvedt 1996). Reducing Cd toxicity is becoming urgent, and one of the eco-friendly and cost-effective strategies is to apply nutrients to antagonize Cd stress.

### 6.1 Mitigation of Cd toxicity by Si in wheat

Si has been well documented as one beneficial element which alleviates biotic and abiotic stresses including Cd stress in plants (Debona et al. 2017; Kim et al. 2017; Wu et al. 2013).

#### 6.1.1 Beneficial effects of Si on biomass and Cd accumulation in wheat

Zhang et al. (2008) reported that Si addition increased shoot biomass by 125%–171% and root biomass by 100%–106% under 2  $\mu\text{M}$  Cd treatment at four different growth stages in rice. Farooq et al. (2016) showed that Si supply recovered biomass of roots and shoots in rice after Cd stress. Similar ameliorative effect of Si was also found in other species, such as pea (Rahman et al. 2017), maize (Akhtar et al. 2017), strawberry (Treder and Cieslinski 2005), cucumber (Wu et al. 2015), durum wheat (*Triticum turgidum* L.) (Rizwan et al. 2016). In the present study, Cd concentration-dependent experiments were performed to study how Si addition affects Cd toxicity in wheat. In accordance with published studies, we also found Si executed beneficial effect on biomass in intermedium Cd-stressed wheat plants. After 14 d Cd treatment, low Cd (1  $\mu\text{M}$ ) stress had no impact on biomass in wheat plants, and Si had no effect on biomass in low Cd-stressed plants either; Si addition significantly increased biomass in wheat suffered from intermedium Cd (5  $\mu\text{M}$ ) treatment when compared with Cd treatment alone; Si supply did not affect biomass under high Cd (25  $\mu\text{M}$ ) treatment (Chapter 2, Table 1). However, Si considerably decreased Cd concentrations in shoots regardless of Cd concentrations in the growth medium (Chapter 2, Fig. 1). Hussain et al. (2015) also showed that Si supply at the boot stage reduced Cd concentrations in grains of wheat irrespective of Cd levels. Reduction of Cd in above-ground plants is generally attributed to Cd uptake and translocation in plants. Zhang et al. (2008) reported Si reduced Cd translocation from roots to shoots in rice, which was also found in tomato (Wu et al. 2015). Vaculík et al. (2009) showed Si addition facilitated root-to-shoot Cd translocation in maize and Vatehová et al. (2012) also

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observed same function of Si in *Brassica juncea* and *Brassica napus*. Shi et al. (2010) even showed acropetal translocation of Cd was cultivar-dependent in peanut: in Cd sensitive cultivar, Si decreased acropetal translocation of Cd; in Cd tolerant cultivar, Si had no effect on acropetal translocation of Cd. It indicates that Si effect on root-to-shoot Cd translocation is species and cultivar-dependent. In our work, translocation factors (TF) of root-to-shoot Cd were not affected by Si addition under low and intermedium Cd exposures, and TF value was even increased under high Cd exposure as affected by Si (Chapter 2, Fig. 3). Those results illustrated that Si-mediated repression of Cd in wheat is not related to acropetal translocation of Cd, thus it should be reduction of Cd uptake by roots. To confirm whether time-dependent Cd stress is also affected by Si addition, experiments after 1 h, 6 h, 1 d, 7 d and 14 d Cd treatment in the presence or absence of Si were conducted. Cd concentrations in shoots and roots were decreased by Si, but acropetal translocation of Cd was unchanged by Si supply regardless of Cd time exposure (Chapter 3, Fig. 2), confirming that Si-decreased Cd in wheat is ascribed to reduction of Cd entry in roots rather than hampering acropetal translocation of Cd. Therefore, we focused on effects of Si on Cd entry in roots of wheat in the following-up experiments. Intriguingly, under short-term Cd treatment (< 6 h), Si remarkably decreased Cd concentrations in apoplastic fluid-Cd and in the symplast of roots without changing cell wall-bound Cd, while under long-term Cd treatment, Si considerably reduced Cd concentrations in cell walls and the symplast of roots without affecting apoplastic fluid-Cd (Chapter 3, Fig. 3). It seems that Si executes distinct mechanisms to alleviate Cd toxicity in wheat.

### **6.1.2 Effects of Si on Cd toxicity under short-term treatment**

Like other nutrients, Cd entry into roots also has apoplastic and symplastic pathways (Barberon and Geldner 2014). Radial transport of Cd from epidermis, cortex cells of roots to endodermis through apoplastic and symplastic pathways (Redjala et al. 2009). In this study, the majority of Cd accumulated in the apoplastic fluid of roots under sole Cd treatment, and Si decreased the distribution of apoplastic fluid-Cd under short-term Cd exposure (Chapter 3, Table 1), showing that Cd absorption via the apoplastic pathway plays a crucial role and Si modulated the apoplastic pathway under short-term Cd exposure. Si supply significantly decreased Cd concentrations in the apoplastic fluid and in the symplast of roots without affecting cell wall-bound Cd under short-term Cd exposure (Chapter 3, Fig. 3), which is consistent with findings reported by Ye et al. (2012) in *Kandelia obovate*. They reasoned that Si-restricted apoplastic transport of Cd which contributed to alleviation of Cd toxicity by

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increasing cell wall-bound Cd. However, in our study, Si addition did not affect CEC of cell wall (Chapter 3, Fig. 5A) and Cd accumulation in different fractions of cell walls in roots under short-term Cd stress (Chapter 3, Fig. 5B). Furthermore, no cell wall-Cd adsorption differences were found in Si-treated plants compared with non-treated plants (Chapter 3, Fig. 5C), implying that Si-reinforced cell wall bound-Cd is not the ameliorative reason for Cd toxicity in wheat.

The apoplast consists of apoplastic fluid and cell wall, and apoplastic absorption is driven by diffusion and mass flow in roots. Mass flow is supported by transpiration, while diffusion depends on diffusive layer thickness, concentration gradient, cell wall structure or root exudates, among others (Degryse et al. 2012; White 2012). Since cell wall properties were not affected by Si under short-term Cd treatment, we reason that Si-decreased apoplastic fluid-Cd results in amelioration of Cd stress. Furthermore, transpiration rates were not affected by Si under Cd toxicity (Chapter 3, Fig. 5E), which implies that Si-decreased Cd absorption is not through effects on the pathway of mass flow. A short-term excised intact-root Cd absorption experiment, eliminating the shoot effects (Chapter 3, Fig. 5D), showed that Si supply substantially decreased Cd absorption by roots, which further confirmed that mass flow was not the reason for the mitigation of Cd by Si, indicating that Si-decreased apoplastic diffusion of Cd in roots. Combined with the results that Cd adsorption of powdery root of Si-supplied plants was not affected and Cd adsorption of intact roots in Si-added plants was decreased (Chapter 3, Fig. 5), we assume that Si-supplied plants deployed some apoplastic diffusion barriers to restrain Cd entry into roots. When we further investigated suberin lamellae deposition, we found Si remarkably lengthened the distance of suberin deposition from root apex under Cd stress compared with Cd treatment alone (Chapter 3, Fig. 6), which implies that Si addition increased longitudinal apoplastic space under Cd toxicity. Under short-term Cd stress, the majority of Cd accumulated in the apoplast and Cd concentration in the apoplastic fluid trended to be steady as Cd exposure time increased (Chapter 3, Fig. 3A and Table 1). Considering that the same Cd concentrations in the growth medium regardless of Si addition and non-changed root growth between Cd and Si+Cd treatments (Chapter 3, Fig. 1B), and thereby Cd entry into root surface should be the same for all Cd-treated plants, we hypothesize that Si increased apoplastic space is the result of decreased apoplastic fluid-Cd like the ‘dilution’ effect under short-term Cd exposure. To confirm if this ‘dilution’ effect of Si also affects other mineral nutrients, divalent ions such as Fe and Zn in the apoplastic fluid,

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the cell wall and symplastic fluid as affected by Si were also determined. We found that Fe and Zn in the apoplastic fluid and the percentages of subcellular Fe and Zn distribution were not affected by Si under Cd stress regardless of Cd exposure time (Chapter 3, Fig. S1 and Table S1). Because plants were pre-cultivated for 7 d with non-Cd nutrient solution before Cd treatment, we reason apoplastic fluid-Fe and -Zn were already steady after 7 d pre-cultivation, which indicated that Si-decreased apoplastic fluid-Cd through increasing apoplastic space could only be under short-term Cd stress.

With regard to symplastic uptake of Cd, Cd shares transporters with other essential divalent elements for the reason that plants deploy no specific transport system for a non-essential element such as Cd (Clemens et al. 2002). In this study, we chose *Nramp5* (Ishimaru et al. 2012) and *LCT1* (Uraguchi et al. 2011) (function of Cd influx), *TM20* (Kim et al. 2008) (function of Cd efflux), *HMA3* (Kim et al. 2014) (function of sequestering Cd into vacuoles) and *HMA2* (Clemens et al. 2013) (function of loading Cd into xylem) to study whether Si regulates the symplastic Cd uptake pathway, sequestration or acropetal transport of Cd at transcriptional level. Under short-term Cd exposure, Si addition had no significant effects on expression levels of *Nramp5*, *LCT1*, *TM20* and *HMA2* (Chapter 3, Fig. 4), suggesting that Si application sparingly affected symplastic Cd uptake and acropetal Cd transport. Based on those results, we conclude that Si mainly reduced apoplastic fluid-Cd through enhancing apoplastic space, which contributes to decrease of Cd accumulation under short-term Cd stress.

### **6.1.3 Effects of Si on Cd toxicity under long-term treatment**

In contrast to short-term Cd stress, Si-reduced Cd accumulation is also attributed to the suppression of root Cd absorption rather than acropetal Cd translocation under long-term Cd stress (Chapter 3, Fig. 2). At the transcriptional level, non-changed expression of *HMA2*, which is involved in Cd xylem loading, as affected by Si (Chapter 3, Fig. 8E) confirmed that effects of Si on acropetal Cd translocation is not the reason for the mitigation of Cd toxicity. As to apoplastic Cd absorption by roots, Si had no impacts on apoplastic fluid-Cd concentration under long-term Cd stress (Chapter 3, Fig. 3A). The explanation could be that apoplastic fluid did not play a pivotal role in Cd resistance after the long-term Cd stress because apoplastic fluid-Cd proportions dropped from 52.4% under short-term Cd treatment to 3.8% under long-term Cd treatment (Chapter 3, Table 1). Regarding cell wall properties, Si supply reduced total cell wall bound-Cd because of decreased pectin bound-Cd without

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affecting CEC of cell wall (Chapter 3, Fig. 9B) and cell wall-Cd adsorption (Chapter 3, Fig. 9C), implying that Si-repressed Cd in cell walls is related to reduce Cd absorption from bulk solution or promotion of Cd transport into symplast rather than alteration of cell wall properties. However, no differences were found in bulk solution neither the speciation of Cd nor Cd activity (free Cd ion accounts for 71.5%) as affected by Si supply as calculated by Visual MINTEQ 3.1. Referring to symplastic uptake, Cd stimulated the expression of *Nramp5*, *LCT1*, and *HMA3*, but Si did not affect the up-regulated expression of those genes induced by Cd toxicity (Chapter 3, Fig. 9), indicating that Si did not affect symplastic Cd uptake and sequestration in roots. Comparatively, Kim et al. (2014) indicated that Si-reduced Cd in rice by decreasing *OsHMA2* and *OsHMA3* expression, while Shao et al. (2017) also reported that Si repressed expression of *OsHMA2* and *OsNramp5* resulting in reduction of Cd accumulation in rice. Greger et al. (2016) showed that silicate down-regulated *LCT1* and up-regulated *PCSI* determined by semi-quantitative RT-PCR which was related into decrease of Cd in protoplast of wheat cells. It is noteworthy that Cd can only share transporters with essential nutrients, if transporters related to Cd uptake and transport were down-regulated, other essential elements will be decreased as well. In the present study, Si did not affect gene expression of *Nramp5*, *LCT1* and *HMA3* which involve in Cd uptake and transport (Chapter 3, Fig. 8), but Si decreased Cd accumulations in plants after long-term Cd exposure (Chapter 3, Fig. 2-3), implying that symplastic uptake of Cd was not affected by Si addition. Altogether, Si reduces cell wall bound- and symplastic Cd without affecting cell wall properties and symplastic uptake capacity of Cd under long-term Cd treatment (Chapter 3, Fig. 9). We reason the decreased Cd is a consequence of reduced Cd entry into roots. Fleck et al. (2011) indicated that Si supply accelerated suberization of the exodermis and endodermis in rice roots and the underlying mechanism is still unclear. Nevertheless, in wheat, as a non-exodermis species (Taleisnik et al. 1999), we also found that Si addition accelerated suberin lamellae development, namely Si shortened the distance of suberin deposition from root apex (Chapter 3, Fig. 10), which resulted in repression of apoplastic bypass flow of Cd and thus decreased Cd in plants. Vaculík et al. (2009) reported that Si increased Cd concentrations in both shoots and roots of maize by lengthening distance of suberin lamellae development from root apex previously, but the results were not in agreement with the most of studies that Si addition decreased Cd accumulation in plants. Also, percentages of apoplastic fluid-Cd only occupied 3.8% and 5.1% for Cd and Si+Cd treatments respectively after 7 d Cd stress

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(Chapter 3, Table 1), suggesting apoplastic uptake of Cd was not dominant after long-term Cd exposure. Thus, we assume that acceleration of suberization by Si addition only partially contributes to decrease Cd under long-term Cd stress.

Interestingly, compared with Cd stress alone, Si up-regulated the expression of *TM20* (Chapter 3, Fig. 8C) which was reported to have the function of Cd efflux from roots, thus reducing the Cd accumulation in roots of wheat. However, the detailed function of *TaTM20* is still needed to be discovered. To test whether avoidance involves in mitigation of Cd toxicity as affected by Si, we determined low molecular organic acids exudation by roots. We found that Si-enhanced oxalate exudation contributes to reduction of Cd under long-term Cd stress (Chapter 2, Fig. 4-5), whereas Si supply had no effects on oxalate exudation by roots under short-term Cd stress (Chapter 3, Fig. 7). Overall, Si increased efflux of Cd and exudation of oxalate are related to reduce absorption of Cd under long-term Cd exposure. However, we assume that Si-decreased Cd under short-term Cd stress lays the foundation for alleviation of Cd toxicity under long-term Cd stress. Because Si-decreased Cd in short-term Cd stressed-plants resulting in less Cd accumulation, thus long-term Cd stressed-plants with Si addition have higher resistance to Cd stress.

## **6.2 Enhancement of Cd tolerance by sulfate in faba bean**

It is well-documented that phytochelatins (PCs) play a pivotal role in detoxifying Cd toxicity, which could combined with Cd ions and then sequester PCs-Cd complexes in vacuoles to reduce Cd toxicity (Clemens 2006). As the precursor of PCs, glutathione (GSH) is produced from sulfur assimilation (Brunetti et al. 2011). In arable soils, we are also facing the problem of sulfur deficiency because of progressive process for reducing sulfur compounds emission into environment (Astolfi et al. 2010). Therefore, the question is raised that whether excess sulfate application will alleviate Cd toxicity. To answer this question, we studied effects of deficient, sufficient and excess sulfate supply on Cd stress in faba bean (*V. faba* L.) here.

Deficient sulfur induced decrease of S-containing amino acids and chlorophyll synthesis, thereby reducing plant growth (Hawkesford 2000). In agreement with this, our results showed that deficient sulfate treatment significantly decreased biomass of shoots at both seedling stage and flowering stage by contrast with sufficient sulfate supply (Chapter 4, Fig. 1). Cd concentrations were also decreased in deficient supplied-plants when compared with

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sufficient and excess sulfate-supplied plants (Chapter 4, Fig. 2). The better plant growth with higher Cd concentrations in sufficient and excess sulfate-supplied plants than deficient supplied-plants indicates that sulfate application enhances Cd tolerance in plants. Localization of Cd showed that excess sulfate facilitated Cd accumulation in epidermis of leaves (Chapter 4, Fig. 3) which might be related to enhancement of Cd tolerance by sulfate. We reason that more Cd accumulation in epidermis of leaves could reduce Cd in mesophyll cells, thus resulting in higher photosynthesis rate. In consistence with adverse oxidative stress as induced by Cd toxicity in plants (Cuypers et al. 2010), we also found that Cd stress damaged plasma membrane integrity, increased MDA and H<sub>2</sub>O<sub>2</sub> concentrations in plants, but excess sulfate remarkably alleviated those detrimental effects when compared with deficient sulfate treatment (Chapter 4, Fig. 4, 5). It is well known that sulfate supply mitigates Cd toxicity by increasing GSH concentrations because enhanced GSH increases AsA-GSH cycle which is efficient for scavenging ROS (Bashir et al. 2015; Jung et al. 2017). Phenolic compounds have also been known as antioxidants (Martini et al. 2017), but it is still unclear whether phenolic compounds involve in alleviation of Cd toxicity as affected by sulfate. In this study, we found that phenolic compounds were not significantly affected regardless of Cd or sulfate treatment, but peak 3 and peak 7 phenolic compounds had the tendency of decline by Cd toxicity (Chapter 4, Fig. 6 and S1). By using UPLC-PDA-ESI-MS, we identified the two phenolic compounds are kaempferol-3-O-robinoside-7-O-rhamnoside (robinin) and kaempferol-3-O-acteyl-rhamnogalactoside-7-O-rhamnoside respectively. To further investigate how those two compounds affect Cd toxicity in faba bean, foliar application was conducted in Cd-stressed plants. The commercial chemical, kaempferol-3-O-acteyl-rhamnogalactoside-7-O-rhamnoside, is unavailable, so the precursor of this compound, kaempferol-3-O-rhamnoside was used for foliar application. Interestingly, foliar supply of robinin or kaempferol-3-O-rhamnoside significantly decreased Cd concentrations in leaves (Chapter 4, Fig. 7), suggesting that certain phenolic compounds involved in mitigation of Cd toxicity, whereas sulfate did not involve in this beneficial effect.

Flowering stage is important for yield and quality of seeds. Wei et al. (2006) found that Cd concentrations in leaves at flowering stage was much higher than that mature stage in *Solanum nigrum* L. Wang et al. (2017) also reported that the best stage for predicting Cd accumulations in pepper (*Capsicum annuum* L.) fruits was flowering stage. Therefore, we studied how sulfate affects Cd accumulation in leaves of faba bean at flowering stage. By

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using stable isotope  $^{106}\text{Cd}$ , we traced Cd accumulation in leaves from early uptake of Cd and newly uptake of Cd at flowering stage. We found that sulfate supply substantially increased Cd accumulation in cell walls of leaves resulting in increased Cd in leaves, but sulfate only increased Cd in cell walls from newly uptake of Cd (Chapter 5, Table 1). Koren et al. (2013) reported that different ligands of Cd affected Cd distribution at subcellular level in *Noccaea (Thlaspi) praecox*;  $\text{CdSO}_4$  facilitated Cd accumulate in cell walls of leaves, and the form of  $\text{CdCl}_2$  facilitated Cd accumulate in vacuoles of leaves. When we calculated speciation of Cd in the growth medium as affected by sulfate, it is interesting to find that sulfate supply obviously increased percentage of  $\text{CdSO}_4^0$  (Chapter 5, Table 2), indicating that increased  $\text{CdSO}_4^0$  might be related to increase Cd in cell walls as affected by sulfate supply. López-Chuken and Young (2010) also showed that sulfate increased  $\text{CdSO}_4^0$  proportion in the growth medium. However, at seedling stage, although  $\text{CdSO}_4^0$  proportion in the growth medium was increased as sulfate concentration in the growth medium increased (Chapter 4, Table 1) and Cd concentrations in leaves were increased in sufficient sulfate-treated plants compared with deficient sulfate-treated plants, Cd concentrations in sufficient sulfate-treated plants were not further increased when compared with Cd in excess sulfate-treated plants (Chapter 4, Fig. 2). Based on those results, we conclude that to some extent sulfate increased  $\text{CdSO}_4^0$  proportion in the growth medium contributes to increase Cd in shoots, but effect of increasing Cd as  $\text{CdSO}_4^0$  increases will disappear when sulfate concentration in the growth medium is higher than a certain value. Although diffusion of  $\text{CdSO}_4^0$  is faster than  $\text{Cd}^{2+}$ , absorption of  $\text{CdSO}_4^0$  by plants is limited. As Ferri et al. (2017) demonstrated that one critical value of sulfate concentration in the growth medium existed for sulfate uptake, when external sulfate concentration exceeded this critical value, sulfate uptake will not be increased.

As a toxic element, Cd has no specific transporters, but Cd entry into plant through sharing transporters with Fe, Zn, Mn or Ca (Clemens et al. 2013), thus Cd toxicity could induce nutrition imbalance in plants (Sarwar et al. 2010). To test whether mineral nutrients are associated with enhancement of Cd in cell walls by sulfate, we determined macronutrients and micronutrients concentrations in the AWF, symplastic fluid and cell walls. We found negative relationships of K and Ca with cell wall-Cd, and positive relationship of Cu with cell wall-Cd (Chapter 5, Table S1-2). However, compared with sufficient sulfate, excess sulfate did not affect Ca and Cu concentrations and decreased K concentrations (Chapter 5, Fig. 3-4), showing that decrease of K by sulfate involved in increase of Cd in cell walls. It is still

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unclear why K is correlated with Cd in cell walls. Siddiqui et al. (2012) showed that exogenous application of K decreased Cd concentrations and enhanced antioxidant system in faba bean. K supply decreased Cd concentrations was also reported in rice (Liu et al. 2012) and chickpea (*Cicer arietinum* L.) (Ahmad et al. 2016). Based on those findings, it seems it is reasonable that sulfate-decreased K concentration resulted in increase of Cd concentration.

To assess correlations of sulfate and non-protein thiol concentrations with Cd concentrations in cell walls, we determined sulfate and non-protein thiol concentrations in the AWF and symplastic fluid. We found that non-protein thiol concentration in symplastic fluid was positively correlated with Cd in cell walls (Chapter 5, Table S1). Excess sulfate significantly increased non-protein thiol concentration in symplastic fluid compared with sufficient sulfate, suggesting that non-protein thiol concentration enhanced by sulfate is one reason for increasing Cd in cell walls. Although non-protein thiol concentration in symplastic fluid was increased by excess sulfate supply compared with sufficient sulfate supply, symplastic Cd concentration was not affected (Chapter 5, Fig. 2 and Fig. 5), which implies that excess sulfate did not accelerate Cd in vacuoles with increasing symplastic non-protein thiol concentration. We assume that increase of non-protein thiol by sulfate under Cd stress plays a regulatory role in enhancement of Cd tolerance such as improvement of antioxidant capacity.

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## **Chapter 7**

## **Summary**

## 7. Summary

Cadmium (Cd) pollution in agricultural soils is increasingly serious due to industry development, mining, sewage sludge and phosphate fertilizer. Plants growing there take up and accumulate Cd, which will affect plant growth and even threat human health after consuming Cd-containing edible organs of plants. In this thesis, effects of silicon (Si) and sulfate on Cd toxicity were studied.

It is well documented that Si alleviates biotic and abiotic stresses including Cd toxicity, but the underlying mechanisms are still poorly understood. Wheat hydroponics experiments under short- and long-term Cd treatment in the presence and absence of Si showed that exogenous Si supply significantly decreased Cd in roots and shoots. However, the root-to-shoot Cd translocation was not affected regardless of Si supply. We found that under short-term treatment, Si considerably decreased Cd in apoplastic fluid. Si neither affected gene expression levels of symplastic transporters related to Cd uptake and transport nor cell wall properties including capacity of adsorbing Cd. *In vitro* experiments of roots showed that Si decreased Cd in intact roots, but had no effects on milled powdery roots, suggesting that Si induced apoplastic barriers resulting in decrease of Cd in the apoplastic fluid. Regarding apoplastic barriers, Si delayed suberin lamellae development under short-term Cd exposure. Combined with most of Cd in apoplastic fluid, we reason that delayed suberin lamellae development by Si enlarged longitudinal apoplastic space, thereby decreasing Cd concentrations in apoplastic fluid as a `dilution` effect. Under long-term Cd exposure, Si decreased Cd in cell walls. Gene expression related to Cd influx and transport was unaffected, but Cd efflux transcript was up-regulated by Si. In addition, oxalate exudation which is able to reduce Cd accumulation in plants was also enhanced by Si under long-term Cd exposure. Taken together, Si-supplied plants have low Cd accumulation and less Cd phytotoxicity under short-term exposure which deploys higher Cd resistance for plants under long-term exposure.

Products of sulfate assimilation, glutathion (GSH) and phytochelatin (PC) are chelators of Cd, thereby detoxifying Cd. To date, it is still unclear whether excess sulfate supply will alleviate Cd toxicity. Therefore, effects of deficient, sufficient and excess sulfate on Cd toxicity were studied in faba bean. Sulfate supply reversed detrimental effects of Cd on biomass and oxidative stress, but also increased Cd concentrations in leaves, suggesting that sulfate enhances Cd tolerance in faba bean. We found that sulfate accelerated Cd in cell walls of leaves, which were negatively correlated with K concentration, and positively correlated

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with symplastic non-protein thiol concentration. In the growth medium, sulfate supply remarkably increased percentage of  $\text{CdSO}_4^0$ . Since diffusion of  $\text{CdSO}_4^0$  is faster than  $\text{Cd}^{2+}$ , we reason that promotion of  $\text{CdSO}_4^0$  is also partially related to enhancement of Cd in leaves.

## Zusammenfassung

Landwirtschaftliche Nutzflächen werden aufgrund von Industrie, Bergbau, Klärschlamm und Phosphatdünger zunehmend mit Cadmium (Cd) verunreinigt. Pflanzen nehmen Cd auf und akkumulieren es, was das Pflanzenwachstum beeinträchtigt sowie die menschliche Gesundheit nach dem Verzehr von Cd-haltigen Nutzpflanzen schädigt. Daher wurden in dieser Studie die Effekte von Silizium (Si) und Sulfat auf die Cd Toxizität untersucht.

Es ist bekannt, dass Si biotische und abiotische Stressfaktoren, einschließlich Cd-Toxizität, reduziert. Die zugrunde liegenden Mechanismen sind jedoch kaum verstanden. Weizen, im hydroponischen System angezogen, zeigte unter Kurz- und Langzeit Cd-Behandlung in Gegenwart und Abwesenheit von Si, dass die exogene Si-Zugabe, die Cd-Konzentration in Wurzeln und Sprossen signifikant verringerte. Die Cd-Translokation von Wurzel zu Spross wurde jedoch nicht beeinflusst. Unter Kurzzeitbehandlung wurde die Cd-Konzentration im Apoplast durch exogene Si-Zufuhr verringert. Si beeinträchtigt weder die Genexpression von Cd-spezifischen Symplast-Transportern, noch änderten sich Zellwand-spezifische Eigenschaften wie die Kapazität Cd zu absorbieren. *In vitro* Experimente zeigten, dass Si die Cd-Konzentration in intakten, aber nicht in pulverisierten Wurzeln verringerte. Weiterhin, führt Si-Zugabe zu einer verzögerten Ausbildung der Suberinlamellen, was darauf hindeutet, dass Si über die Veränderung apoplastischer Barrieren eine niedrige Cd-Konzentration im Apoplast induziert. Die verzögerte Suberinlamellen-Entwicklung vergrößert den longitudinalen apoplastischen Raum und führt somit zu dem sogenannten „dilution“ Effekt. Nach Langzeit Cd-Behandlung, verringerte Si den Cd Gehalt in den Zellwänden. Während die Genexpression von Transkripten für den Cd-Influx und Cd-Transport nicht verändert waren, zeigte ein Cd-Efflux Transkript erhöhte Genexpression bei Anwesenheit von Si. Weiterhin war die Exsudation von Oxalat erhöht, was die Cd Akkumulation in Pflanzen reduziert. Zusammenfassend konnte gezeigt werden, dass die Zugabe von Si, die Cd Akkumulation und Toxizität in Pflanzen nach Kurzzeitbehandlung verringert, was zu einer erhöhten Cd Toleranz unter Langzeitbedingungen beiträgt.

Glutathion (GSH) und Phytochelatin (PC), Produkte der Sulfat-Assimilierung, sind Cd-Chelatoren und können Cd detoxifizieren. Es ist jedoch noch immer unklar, ob eine übermäßige Sulfat Zugabe die Cd Toxizität reduziert. Daher wurden die Auswirkungen von unzureichender, ausreichender und übermäßiger Sulfat-Zugabe, auf die Cd Toxizität in Faba Bohnen untersucht. Sulfat Zugabe wirkte schädlichen Cd-induzierten Effekten wie reduzierter

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Biomasse oder oxidativen Stress entgegen, und führte zu einer erhöhten Cd Konzentration in Blättern, was darauf hindeutet, dass Sulfat die Cd Toleranz in Faba Bohnen erhöhte.

Sulfate induzierte eine hohe Cd-Konzentration in den Zellwänden der Blätter, was negativ mit der K-Konzentration und positiv mit der Konzentration an symplastischen nicht-proteinartigen Thiolen korrelierte. In Wachstumsmedium erhöhte die Sulfat Zugabe die  $\text{CdSO}_4^0$  Konzentration. Da  $\text{CdSO}_4^0$  schneller diffundiert als  $\text{Cd}^{2+}$  wurde angenommen, dass  $\text{CdSO}_4^0$  die erhöhte Cd Konzentration in Blättern mit beeinflusst.

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## Curriculum vitae

### **Personal Information**

Name: Jiawen Wu

Gender: Female

Date of birth: 29<sup>th</sup> July 1989

Email: [wujiawende@126.com](mailto:wujiawende@126.com)

Nationality: Chinese

### **Education**

#### **August 2017 – March 2018**

Visiting scientist, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) (Germany)

#### **October 2014 – April 2018**

Ph.D study, Institute of Plant Nutrition, Kiel University (Germany)

#### **September 2011 – June 2014**

M. S. College of Horticulture, Northwest A&F University (China)

#### **September 2007 – June 2011**

B. S. College of Horticulture, Northwest A&F University (China)

### **Publications**

**Wu, J.W.**, Geilfus C.M., Pitann, B. and Mühling, K.H. 2016. Silicon-enhanced oxalate exudation contributes to alleviation of cadmium toxicity in wheat. *Environmental and Experimental Botany*. 131, 10-18

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