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The crucial role of roots in increased cadmium-tolerance and Cd-accumulation in the pea mutant *SGECd*^{t}

A.A. BELIMOV[1]*, N.V. MALKOV[1], J.V. PUHALSKY[1], V.E. TSYGANOV[1], K.B. BODYAGINA[1,2], V.I. SAFRONOVA[1], K.-J. DIETZ[3], and I.A. TIKHONOVICH[1,2]

All-Russia Research Institute for Agricultural Microbiology, Pushkin, 196608, St.-Petersburg, Russia[1]
Saint-Petersburg State University, 199034, St.-Petersburg, Russia[2]
Bielefeld University, D-33501, Bielefeld, Germany[3]

Abstract

Elucidation of mechanisms underlying plant tolerance to cadmium, a widespread toxic soil pollutant, and accumulation of Cd in plants are urgent tasks. For this purposes, the pea (*Pisum sativum* L.) mutant *SGECd*^{t} (obtained by treatment of the laboratory pea line SGE with ethylmethane sulfonate) was reciprocally grafted with the parental line SGE, and four scion/rootstock combinations were obtained: SGE/SGE, *SGECd*^{t}/*SGECd*^{t}, SGE/*SGECd*^{t}, and *SGECd*^{t}/SGE. They were grown in hydroponics in the presence of 1 μM $CdCl_2$ for 30 d. The SGE and *SGECd*^{t} scions on the *SGECd*^{t} rootstock had a higher root and shoot biomass and an elevated root and shoot Cd content compared with the grafts having SGE rootstock. Only the grafts with the SGE rootstock showed chlorosis and roots demonstrating symptoms of Cd toxicity. The content of nutrient elements in roots (Fe, K, Mg, Mn, Na, P, and Zn) was higher in the grafts having the *SGECd*^{t} rootstock, and three elements, namely Ca, Fe, and Mn, were efficiently transported by the *SGECd*^{t} root to the shoot of these grafts. The content of other measured elements (K, Mg, Na, P, and Zn) was similar in the root and shoot in all the grafts. Then, the non-grafted plants were grown in the presence of Cd and subjected to deficit or excess concentrations of Ca, Fe, or Mn. Exclusion of these elements from the nutrient solution retained or increased differences between SGE and *SGECd*^{t} in growth response to Cd toxicity, whereas excess of Ca, Fe, or Mn decreased or eliminated such differences. The obtained results assign a principal role of roots to realizing the increased Cd-tolerance and Cd-accumulation in the *SGECd*^{t} mutant. Efficient translocation of Ca, Fe, and Mn from roots to shoots appeared to counteract Cd toxicity, although Cd was actively taken up by roots and accumulated in shoots.

Additional key words: calcium, grafting, heavy metals, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc.

Introduction

Cadmium (Cd) is a widespread soil pollutant and often accumulates in plants including agricultural crops. It has a high cellular toxicity, which can be deleterious not only to plants but also to animals and humans. Plants evolved a number of biochemical mechanisms to minimize Cd penetration and accumulation, in particular in the symplast, and to cope with Cd inside tissues (Sanita di Toppi and Gabrielli 1999, Dong *et al.* 2007, Hasan *et al.* 2009, Verkleij *et al.* 2009, Kulaeva and Tsyganov 2011, Lin and Aarts 2012). These and other studies identified a root, which is in the closest contact to soil-borne Cd, as a plant organ being most vulnerable to Cd toxicity. Efficient subcellular compartmentation and loading into the xylem for transport to the shoot are essential features of metal tolerance in whole plants.

Mechanisms of Cd tolerance and safe accumulation were revealed by using plant mutants having alterations in related traits and by exploiting a natural variation of tolerance. The role of phytochelatins in Cd detoxification was unraveled with Cd-sensitive *Arabidopsis thaliana* mutants, such as *cad2*, deficient in γ-glutamylcysteine synthetase and thus in phytochelatin synthesis (Howden

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Abbreviation: GSH - glutathione.
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* Corresponding author; e-mail: belimov@rambler.ru

et al. 1995, Cobbett *et al.* 1998). Likewise, mutants were instruments in identifying metal-specific transport mechanisms or metal-binding proteins involved in tolerance to Cd, Cu, Zn, and Hg, such as the metal sensitive *A. thaliana* mutant *cup1-1* (Van Vliet *et al.* 1995). Counteracting the negative effect of Cd on nutrient uptake by *A. thaliana* roots also contributes to Cd tolerance of the *cad1* mutant (Larson *et al.* 2002). The Cd-tolerant *A. thaliana* mutant *cdr3-1D* is characterized by an increased expression of the *AtPDR8*/*AtPDR12* transporter, excluding Cd from roots, and by an increased glutathione (GSH) content (Wang *et al.* 2011). The *A. thaliana* mutant *MRC-22* demonstrated that the root tip senses Cd and modulates the direction of root growth and root patterning (Watanabe *et al.* 2010). The significance of phytochelatins, GSH, and antioxidant enzymes in the response to Cd toxicity was also shown in the Cd-tolerant mutant *cadH-5* of *Oryza sativa* (Shen *et al.* 2012). The translocation of Cd from roots to shoots by Zn and Mn transporters was demonstrated using the Cd-sensitive mutant of *O. sativa* (He *et al.* 2009). These examples show the fundamental role of mutants and their analysis in dissecting mechanisms contributing to heavy metal tolerance. Most studies used *A. thaliana* as a genetic model, or addressed the question of hyperaccumulators which exhibit the particular trait of heavy metal tolerance (*e.g.*, Yang *et al.* 2005, Pollard *et al.* 2014). Others studied Cd tolerance in crop species in order to understand the mechanism of Cd accumulation and to develop strategies to modify it in edible parts (*e.g.*, Shen *et al.* 2012).

The pea mutant *SGECd*^{t} having a single recessive mutation was obtained by treatment of the pea line SGE with ethylmethane sulfonate, and it shows an increased Cd tolerance and Cd accumulation (Tsyganov *et al.* 2007) but a decreased tolerance to and accumulation of Hg (Belimov *et al.* 2015, 2016). The mutant locus *cdt* is localized on the VI linkage group of the pea genetic map, but the sequence of the corresponding gene has not been identified yet (Kulaeva and Tsyganov 2013, Tsyganov *et al.* 2013). Moreover, *SGECd*^{t} displays higher root sap flow rates both in the presence and in the absence of a toxic Cd concentration mitigating often encountered negative effects of Cd on plant water status and transport of water and nutrient elements from roots to shoots (Belimov *et al.* 2015). Recently, we also reported physiological differences between the *SGECd*^{t} and the wild type SGE when exposed to other metal ions, *e.g.*, an increased shoot biomass of the mutant in the presence of 400 µM Zn, an increased root and shoot biomasses in the presence of 100 µM Fe, and an elevated root Mn or Zn content in the presence of 400 µM Mn or Zn, compared to SGE plants (Belimov *et al.* 2016). These multiple alterations suggest that the mutation in the *SGECd*^{t} exerts a pleiotropic effect and is involved in regulatory mechanisms which interfere with a short and long distance transports of nutrient elements and water from roots to shoots.

The present report aimed to identify a plant organ, root or shoot, of the *SGECd*^{t} mutant which mediates the increased tolerance to Cd and causes Cd accumulation. Such knowledge is essential for subsequent addressing molecular mechanisms underlying the response of plants to Cd toxicity.

Materials and methods

Seeds of the wild-type pea (*Pisum sativum* L.) line SGE and its Cd-tolerant mutant *SGECd*^{t} were surface sterilized and scarified by treatment with 98 % (m/v) H_2SO_4 for 30 min, rinsed with sterile water, and germinated on filter paper in Petri dishes at 25 °C in the dark for three days. Seedlings were transferred to plastic pots (four pots with 10 seeds per genotype) containing 800 cm^3 of a nutrient solution [µM]: KH_2PO_4, 400; KNO_3, 1200; $Ca(NO_3)_2$, 60; $MgSO_4$, 250; KCl, 250; $CaCl_2$, 60; Fe-tartrate, 10; H_3BO_3, 2; $MnSO_4$, 4; $ZnSO_4$, 3; NaCl, 6; Na_2MoO_4, 0.06; $CoCl_2$, 0,06; $CuCl_2$, 0.06; $NiCl_2$, 0,06; pH 5.5. Seedlings were cultivated in growth chambers in the dark (for increasing shoot elongation) for 2 d and under an irradiance of 400 µmol m^{-2} s^{-1}, a 12-h photoperiod, a minimum/maximum temperature of 18/23 °C, and a relative humidity of 60 % for further 2 d. Scions were cut 1 cm above cotyledons, joined the wedge with rootstocks, and fixed by means of plasticine. Two pots with 10 grafted plants were prepared for each reciprocal graft: the scion SGE/rootstock SGE; scion *SGECd*^{t}/rootstock *SGECd*^{t}; scion *SGECd*^{t}/rootstock SGE; and scion SGE/rootstock *SGECd*^{t}. The pots were covered with transparent plastic bags to maintain high air humidity and incubated for 10 d as described above. During this period, abortive grafts were removed and four successful uniform grafts were left in each pot for further cultivation. Then, the bags were removed and the nutrient solution was changed and supplemented with 1 µM $CdCl_2$. Preliminary experiments had established this Cd concentration as appropriate to ensure survival of the grafted plants with a significant effect on plant growth, and the grafts also showed no differences in plant growth in the absence of Cd. The plants were cultivated for 30 d with the change of the Cd-supplemented solution every third day. Then, the shoots were cut and the roots were soaked in a 1 mM Pb-citrate solution (pH 11) for 10 min and washed in deionized water for desorption of apoplastically bound elements. Then, the plant tissue was dried at room temperature, weighed and stored paper bags for elemental analysis.

To determine the role of nutrient elements (Ca, Fe, and Mn) in differentiation of the Cd-treated SGE and *SGECd*^{t}, the seeds of both pea genotypes were surface sterilized, scarified, and germinated as described above. The plants (two pots with four plants for each treatment) were cultivated in the same manner as described above with differences listed below. To obtain Ca, Fe, or Mn deficient conditions, $Ca(NO_3)_2$ and $CaCl_2$, Fe-tartrate or $MnSO_4$ were eliminated from the solution. To obtain excess concentrations of these nutrients, the full solution was supplemented with 120 µM $CaCl_2$, 10 or 100 µM Fe-tartrate, and 4 or 400 µM $MnSO_4$. Addition of 120 µM Ca and 10 µM Fe or 4 µM Mn doubled the concentrations of these elements in the full solution resulting in 240 µM Ca, 20 µM Fe, or 8 µM Mn, respectively. Treatments with 100 µM Fe or 400 µM Mn, being sufficient for a growth inhibition of both genotypes (Belimov *et al.* 2016), had final concentrations of 110 µM Fe or 408 µM Mn. The solution was supplemented with 2 µM $CdCl_2$ in all treatments except of the untreated control. The nutrient solutions were changed at five and nine days after planting. Plants were cultivated for 12 d after planting in the growth chamber as described above. The plants were dried at room temperature, weighed, and the percentage of *SGECd^t* biomass to SGE biomass was calculated for roots and shoots.

The roots and shoots of individual plants were ground with a household mill *MKM6000* (*BOSCH*, Berlin, Germany) and digested in a mixture of concentrated HNO_3 and 38 % (v/v) H_2O_2 at 70 °C using a digester *DigiBlock* (*LabTech*, Sorisole, Italy). Content of elements (Ca, Cd, Fe, K, Mg, Mn, Na, P, S, and Zn) in the digested plant samples were determined using an inductively coupled plasma emission spectrometer ICPE-9000 (*Shimadzu*, Kyoto, Japan) following manufacturer's instructions.

Statistical analysis of the data was performed using the software *STATISTICA v. 10* (*StatSoft Inc.*, Tulsa, USA). The Fisher LSD test (one way *ANOVA*) was used to evaluate differences between means.

Results

Both the grafts with *SGECd^t* rootstocks had higher root and shoot biomasses in the presence of Cd compared with the grafts having SGE rootstocks (Fig. 1*A,B*). Cadmium content in the roots and shoots of grafts with *SGECd^t* rootstocks was also higher than in those with the SGE rootstocks (Fig. 1*C,D*). Only the grafts with the SGE rootstocks showed chlorosis of shoots demonstrating symptoms of Cd toxicity (Fig. 2).

In order to address nutrient homeostasis in the grafted plants as a possible cause for toxicity development, the content of nutrient elements were quantified in the roots. Amounts of macronutrients K, P, and Mg, as well as of micronutrients Fe, Mn, and Zn, and also of Na were higher in the grafts having the *SGECd^t* rootstocks (Fig. 3) than in the other grafts. Several elements (K, Mg, Na, P, and Zn) showed a similar content in the roots and shoots in all the plants. However Ca, Fe, and Mn content was higher in the shoots of the grafts having the *SGECd^t* rootstocks suggesting that these elements, independently of the scion, were more actively transported by the *SGECd^t* roots to any grafted shoot. The efficient translocation of these elements likely played a role in Cd tolerance of the grafts.

To test this hypothesis, both the genotypes were grown in the presence of Cd and subjected to deficient or excessive concentrations of Ca, Fe, and Mn. Strongly reduced Ca content in the nutrient solution had no effect on genotypic differences in shoot biomass (Fig. 4*A*) and increased a difference in root biomass (Fig. 4*B*). On the contrary, high Ca concentrations decreased the extent of the genotypic differences (Fig. 4*A,B*) as indicated by the biomass ratio between both the genotypes (Fig. 4*C*). Significant genotypic differences in shoot and root biomasses were observed after exclusion of Fe or in the presence of 20 µM Fe in the nutrient solution, however, the supplementation with 110 µM Fe eliminated differences between the Cd-treated genotypes (Fig. 4*A,B*). In the Mn-free nutrient solution, the root and shoot biomasses of the Cd-treated grafs with the *SGECd^t* scions were higher than those with the SGE scions. Interestingly, additional 8 µM Mn eliminated the genotypic difference in root biomass. The biomass of both the Cd-treated genotypes in the presence of 404 µM Mn was similar.

Discussion

The results of the experiment with reciprocally grafted plants clearly assign a crucial role of roots in increasing Cd-tolerance and Cd-accumulation of the *SGECd^t* mutant. Only the grafts with the mutant rootstock manifested the mutant phenotype. This physiological trait concerns the ability to grow and respond to toxic Cd (Fig. 1, 2) as firstly reported by Tsyganov *et al.* (2007) in *Pisum sativum*. It is likely that an increased Cd tolerance of the *SGECd^t* roots of this species contributes to an efficient nodulation in the presence of Cd (Tsyganov *et al.* 2005), but the observed effect on nodule formation was not related to the expression of genes encoding glutathione and phytochelatins (Kulayeva and Tsyganov 2015). The findings are also in line with a previous report, which revealed an enhanced water uptake and water transport from roots to shoots in an *SGECd^t* mutant, both in the absence of Cd or the presence of Cd in the nutrient solution. A modified root function in the *SGECd^t* mutant is evident from an increased root xylem exudation (Belimov *et al.* 2015). It was concluded that this trait not only alleviates a disturbance in *SGECd^t* water status caused by Cd toxicity but also facilitates uptake and translocation of Cd into the shoot *via* an enhanced water flow. Indeed, several studies reported negative effects of

Cd on plant water relations (Poschenrieder and Barcelo 1999, Perfus-Barbeoch *et al.* 2002, Nedjimi *et al.* 2009) and a decreased accumulation of heavy metals under water-limited conditions (Leferve *et al.* 2009, Disante *et al.* 2014). Those data and the results from this study indicate a close relationship between plant water status and tolerance to and uptake of toxic metals.

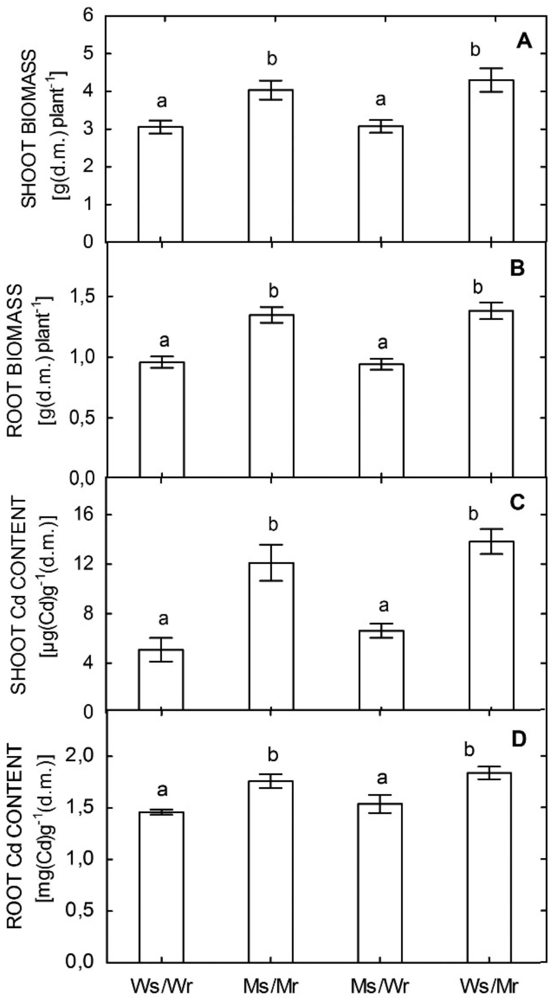

Fig. 1. Shoot (*A*) and root (*B*) biomasses and Cd content in shoots (*C*) and roots (*D*) of grafted pea plants grown in the presence of 1 µM $CdCl_2$. Reciprocal grafts: scion SGE/rootstock SGE (Ws/Wr); scion $SGECd^t$/rootstock $SGECd^t$ (Ms/Mr); scion $SGECd^t$/rootstock SGE (Ms/Wr); scion SGE/rootstock $SGECd^t$ (Ws/Mr). Means ± SEs, $n = 8$. Different letters indicate significant differences between treatments (LSD test, $P < 0.05$).

Based on previous results, it was proposed that the Cd-treated $SGECd^t$ mutant is able to incorporate essential nutrient elements more efficiently. Uptake and accumulation concerned in particular Ca, Fe, Mg, Mn, and S in roots and B, Ca, Mg, Mn, and Zn in shoots. It was suggested that maintenance of nutrient homeostasis in the $SGECd^t$ mutant counteracts Cd-induced inhibition of nutrient uptake (Tsyganov *et al.* 2007, Belimov *et al.* 2016). Toxic Cd concentrations are known to significantly inhibit uptake of mineral elements by roots and thereby affect the ability of plants to maintain nutrient homeostasis. Thus, an efficient nutrient management is considered as an important mechanism of tolerance to toxic metals (Zornoza *et al.* 2002, Yang *et al.* 2004, Rodriguez-Serrano *et al.* 2009, Nazar *et al.* 2012). This study expands these results by demonstrating an improved availability of several nutrients in the roots of the Cd-treated grafts with a rootstock of the Cd tolerant $SGECd^t$ mutant. The results indicate the importance of the root for nutrient uptake in the presence of Cd.

At the same time, the grafting experiment shows that the $SGECd^t$ roots efficiently directed three elements, namely Ca, Fe, and Mn, to a long distance transport to the shoot (Fig. 3). A positive role of Ca in counteracting toxic effects caused by Cd was repeatedly reported. Particularly, Ca-deficient *Typha latifolia* increases Cd accumulation due to Cd-induced expression of the Ca^{2+}-channel gene *TlTPC1* encoding a putative membrane transporter (Rodriguez-Hernandez *et al.* 2015). An increased shoot Cd content accompanied by growth inhibition has been observed in Ca-deficient rice plants, probably as a result of reduced GSH content and expression of genes related to stress defense (Cho *et al.* 2012). Addition of excess Ca to the Cd-supplemented nutrient solution stimulates growth *via* protection of photosystem II in *Phaseolus coccineus* leaves (Drazkiewicz and Baszynski 2008), expression of phytochelatin synthase in *Latuca sativa* roots (He *et al.* 2005), and a decrease in Cd content of maize roots (Sterckeman *et al.* 2011). There is also evidence that addition of Fe to a nutrient solution decreases uptake and transport of toxic Cd from roots to shoots in soybean (Cataldo *et al.* 1983) and increases leaf biomass and chlorophyll content in Cd-treated lettuce plants (Thys *et al.* 1991). Several reports demonstrated alleviation of Cd toxicity by treatments of plants with Mn due to a decrease in Cd content in maize roots (Palove-Balang *et al.* 2006), rice leaves (Huang *et al.* 2017), and roots and leaves of two closely related Mn-hyper-accumulating species *Phytolacca americana* (Peng *et al.* 2008) and *P. acinosa* (Liu *et al.* 2013). The beneficial effect of Mn on Cd-treated rice seedlings is associated with decreases in Cd content, free proline content, lipid peroxidation, water loss, and activity of ascorbate peroxidase and glutathione peroxidase and Mn also prevents inhibition of K and Mg uptake by roots probably due to a competition between Mn and Cd (Rahman *et al.* 2016). Our present experiments show that the genotypic differences between the SGE and $SGECd^t$ in their growth response to Cd significantly depended on Ca, Fe, or Mn concentrations in the nutrient solution. The growth advantage of the $SGECd^t$ was maintained or even enhanced under the shortage of these elements, but it decreased or was even eliminated in the excess concentrations (Fig. 4). The results provide new information on the important role of these nutrient elements and the effect of their efficient transportation to the mutant shoot for counteracting Cd toxicity. It should be mentioned that in plants, large families of ion transporters are involved in a local transport of Ca, Fe, and Mn, as well as in long-distance transport (Pittman 2005, Sharma *et al*. 2016). In addition, an increased transport of different nutrient elements and also of Cd by the *SGECd^t* mutant is accompanied by a greater xylem flow induced by root-pressure, which is possibly linked to the function of aquaporins (Belimov *et al.* 2015). We propose that in the *SGECd^t* mutant root, key genes involved in regulation of uptake of water and

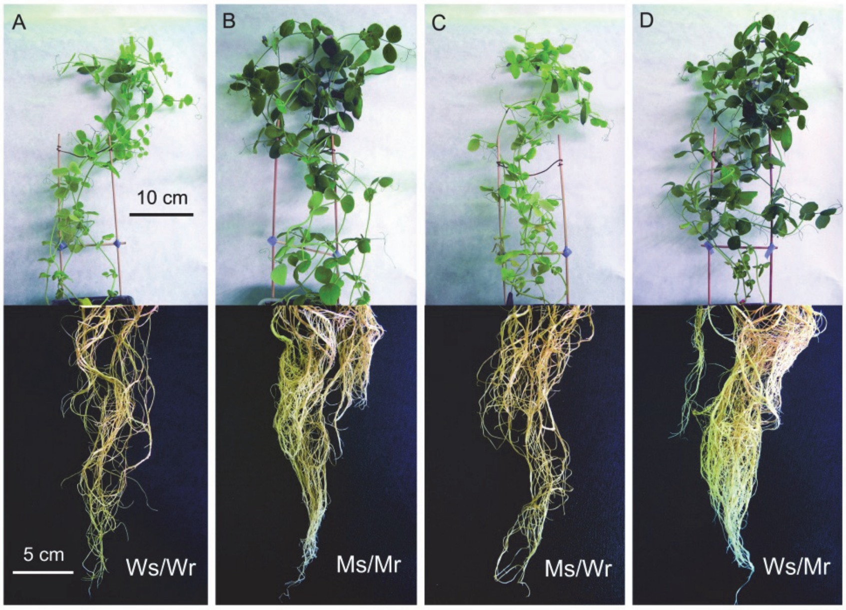

Fig. 2. Images of grafted pea plants grown in the presence of 1 μM $CdCl_2$. Reciprocal grafts: *A* - scion SGE/rootstock SGE (Ws/Wr); *B* - scion *SGECd^t*/rootstock *SGECd^t* (Ms/Mr); *C* - scion *SGECd^t*/rootstock SGE (Ms/Wr); *D* - scion SGE/rootstock *SGECd^t* (Ws/Mr).

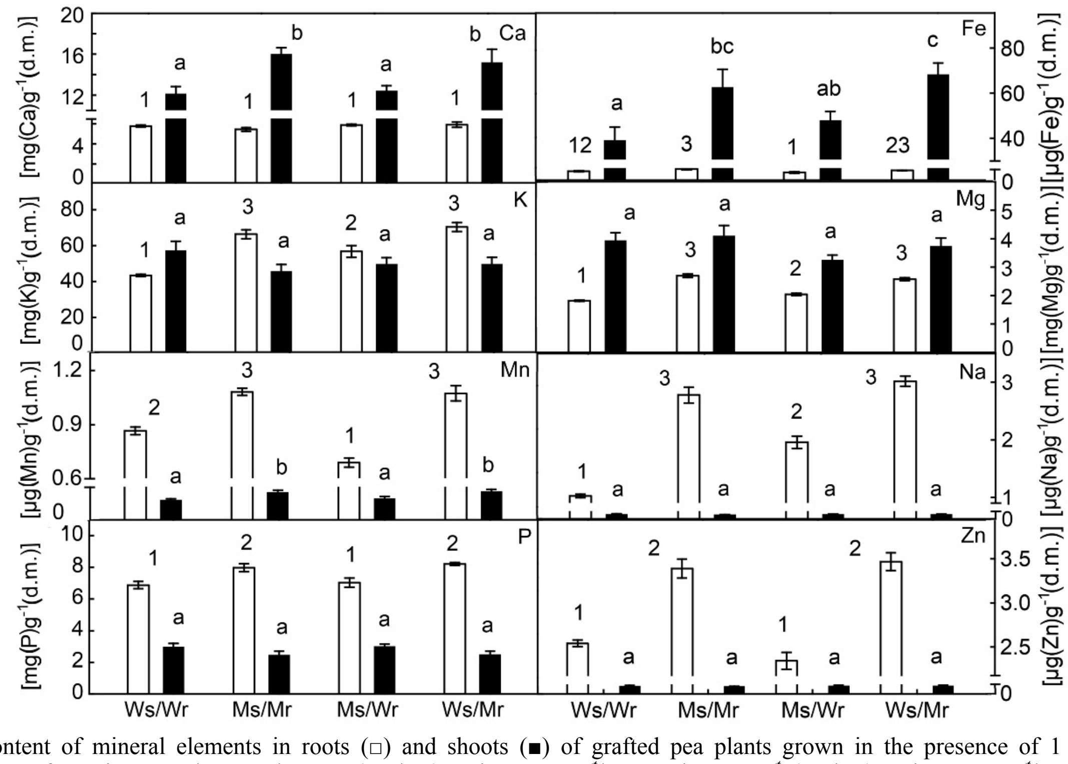

Fig. 3. Content of mineral elements in roots (□) and shoots (■) of grafted pea plants grown in the presence of 1 μM $CdCl_2$. Reciprocal grafts: scion SGE/rootstock SGE (Ws/Wr); scion *SGECd^t*/rootstock *SGECd^t* (Ms/Mr); scion *SGECd^t*/rootstock SGE (Ms/Wr); scion SGE/rootstock *SGECd^t* (Ws/Mr). Means ± SEs, *n* = 5. Different numbers or letters show significant differences between treatments of roots and shoots, respectively (LSD test, *P* < 0.05).

transport of nutrient elements were affected and that this modification was responsible for supplying the shoot with nutrients (particularly Ca, Fe, and Mn) enabling active shoot growth and photosynthesis. This, in turn, could ensure normal growth and function of the root *via* supplying the root with photoassimilates. This hypothesis could be addressed by transcriptome profiling, and experiments in this direction have been recently started.

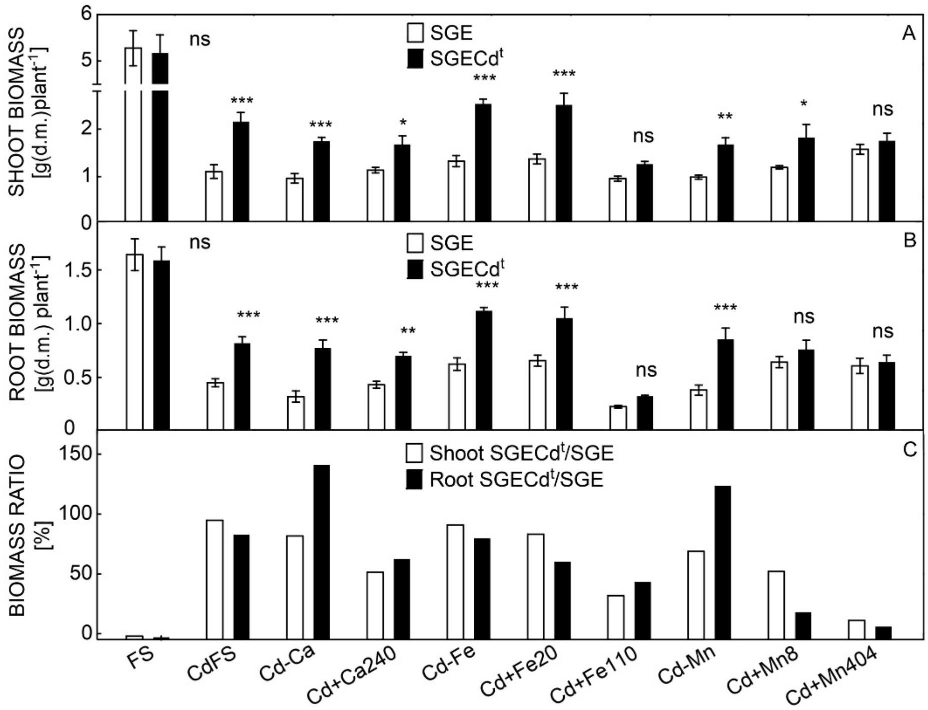

Fig. 4. Shoot (*A*) and root (*B*) biomasses and the percentage of biomass of *SGECd*^{t} relative to SGE for roots and shoots (*C*) of non-grafted plants grown under different nutritional conditions. Plants of all treatments, except the Cd-untreated control in the full solution (FS), were grown in the presence of 2 µM $CdCl_2$. Treatments: CdFS - Cd-supplemented full solution containing 120 µM Ca, 10 µM Fe, and 4 µM Mn; Cd-Ca - Ca deficient solution; Cd+Ca240 - full solution supplemented with 120 µM $CaCl_2$; Cd-Fe - Fe deficient solution; Cd+Fe20 - full solution supplemented with 10 µM Fe-tartrate; Cd+Fe110 - full solution supplemented with 100 µM Fe-tartrate; Cd-Mn - Mn deficient solution; Cd+Mn8 - full solution supplemented with 4 µM $MnCl_2$; Cd+Mn404 - full solution supplemented with 400 µM $MnCl_2$. Means ± SEs, $n = 8$. *Asterisks* show significant differences between genotypes (LSD test, *,**,*** - $P < 0.05$, 0.01, and 0.001, respectively, ns - nonsignificant differences).

Taken together, the grafting experiments with reciprocal scion/rootstock combinations reveal that tolerance to Cd as well as the uptake and translocation of Cd from roots to shoots of the *SGECd*^{t} mutant is controlled by the roots. This finding emphasizes the major role of roots as important organs for determining accumulation of toxic heavy metals in plants. It is proposed that the mutation in *SGECd*^{t} was related to a regulatory gene that modulated activity of several gene(s) and related molecular transporters. The affected genes were localized and functioned in roots, and they were involved in regulation of uptake and transport to the shoot not only of Cd but also of water and mineral elements, in particular Ca, Fe, and Mn. Improved availability of essential nutrients alleviated the Cd stress in the mutants despite the active Cd uptake by roots and accumulation of it in shoots.

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