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High specificity in response of pea mutant SGECd^t to toxic metals: Growth and element composition

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ABSTRACT

The present report aimed to better understand the mechanisms of plant co-tolerance to various toxic metals, and relationships between metal tolerance and metal accumulation. The pea (*Pisum sativum* L.) line SGE and its mutant SGECd^t, having increased tolerance to and accumulation of Cd, but decreased tolerance to and accumulation of Hg, were cultivated in hydroponics at a range of toxic concentrations of heavy metals (Cd, Co, Cr, Hg, La, Ni, Pb and Zn), as well as Al, Fe, Mn, NaCl and H^+ ions. The SGECd^t mutant showed increased tolerance to Co (increased root biomass at 12 and 25 μM Co and shoot biomass at 25, 50 and 100 μM Co), but similar root and shoot Co contents as SGE. No significant differences between SGE and SGECd^t in biomass response to other metals and low pH were detected. However at particular metal concentrations, SGECd^t tended to (Student's *t* test, $P < 0.05$) have increased: (i) shoot biomass (34%) in the presence of 400 μM Zn; (ii) root and shoot biomass (32%) in the presence of 100 μM Fe; (iii) root Mn or Zn contents (65% or 8%, respectively) in the presence 400 μM Mn or Zn, compared to SGE plants. No genotypic differences in the content of other toxic metals were observed, except for the previously reported increased Cd content and decreased Hg content in SGECd^t. Generally, metal toxicity decreased macro- and micro-element (nutrient) concentrations in plants, however opposite effects were also observed particularly on Hg-treated plants. SGECd^t had increased root Ca, Fe, Mg, Mn and S content and shoot B, Ca, Mg, Mn, Na and Zn content in Cd-treated plants. In the presence of toxic Hg the mutant contained less root and shoot Ca, K, Mg and S, but had increased root Co, Cr and Cu contents. Genotypic differences in individual nutrient elements were also observed following Ag, Al, La, Mn, Ni or Zn treatment. Taken together, the results indicate high specificity in phenotypic responses of SGECd^t exposed to toxic metals and that the mutation might affect some regulatory genes, which could modulate nutrient (particularly Ca) homeostasis and regulation of ion transporters.

1. Introduction

Heavy metals are widespread soil pollutants that can inhibit plant growth and accumulate in agricultural products. A number of elements such as Cd, Co, Cr, Cu, Hg, Ni, Pb, and Zn belong to the group of heavy metals, and physical and chemical properties of these elements vary greatly (Housecroft and Sharpe, 2008). Plants developed several mechanisms for decreasing or avoiding toxicity of heavy metals and preventing their excessive uptake by roots and translocation to reproductive organs. Although some interactions between plants and toxic metals are common for all or most heavy metals, some mechanisms are specific or prevalent for a particular metal (Patra and Sharma, 2000; Hall, 2002; Clemens, 2006; Dong et al., 2007; Nagajyoti et al., 2010; Verkleij et al., 2009; Lin and Aarts, 2012). Usually reports on this topic are devoted to a single metal, and most usually Cd (summarized by Sanita di Toppi and Gabrielli, 1999; Dong et al., 2007; Hasan et al., 2009), thereby missing important information about responses of the studied plant species or genotypes to other elements.

Mutagenesis is a powerful tool to understand the mechanisms of accumulation of, and tolerance to, toxic metals by plants. The important role of phytochelatins in Cd detoxification was shown using Cd-sensitive mutants *cad1* and *cad2* of *Arabidopsis thaliana* deficient in phytochelatin synthase (Howden et al., 1995) and γ-glutamylcysteine synthetase (Cobbett et al., 1998), respectively.

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Decreased P, K, Mg, S and Fe concentrations in the Cd-treated *cad1* mutant suggested that maintaining nutrient homeostasis contributes to Cd tolerance (Larsson et al., 2002). Experiments with a Cd-sensitive mutant of rice demonstrated that translocation of Cd from root to shoot may be mediated by Zn and Mn transporters exacerbating Cd toxicity in shoot (He et al., 2009). The Cd-tolerant *A. thaliana* mutant MRC-32 contained more Cd but exhibited pleiotropic phenotype with slow growth rate and alterations in leaf development, indicating that the mutation occurred at a regulatory gene controlling expression of several other genes (Watanabe et al., 2010). Another *A. thaliana* mutant MRC-22 had a Cd-phobic root response, suggesting mis-regulation of Cd sensing in the root zone (Watanabe et al., 2010). The *A. thaliana* mutant *cup1-1* with increased sensitivity and accumulation of Cd and Cu was similar to WT in its response to Hg, suggesting that different, metal-specific mechanisms were involved (Van Vliet et al., 1995).

Several mutants having altered metal relations were isolated and described in pea. Two pea mutants A79-397 (Gottschalk, 1987) and E107 (Welch and LaRue, 1990) were characterized by elevated exudation rate of Fe(III)-reducing substances to the surrounding medium resulting in increased sensitivity to Fe toxicity, which excessively enhanced Fe uptake and caused necrotic spots on the leaves. In addition, the E107 mutant excessively accumulated aluminum and manifested symptoms typical of Al toxicity (Guinel and LaRue, 1993). A chemically induced mutant SGECd^t was isolated from a laboratory pea line SGE and characterized by increased Cd tolerance and Cd accumulation (Tsyganov et al., 2007). In the presence of toxic Cd (4 μM), the mutant SGECd^t had lower contents of non-protein thiols and free proline, lower activities of catalase and peroxidase than wild-type (WT) plants, and maintained plant nutrient uptake, suggesting a Cd-insensitive phenotype. Under optimal conditions the SGECd^t mutant had 35% more oxidized glutathione in the roots than SGE plants. However it was concluded that mutation was not linked to glutathione and/or phytochelatin biosynthesis, since only marginal genotypic differences in their concentrations were found in Cd-treated plants (Tsyganov et al., 2007). A crucial role of the root in the increased Cd-tolerance and Cd-accumulation of SGECd^t was shown using reciprocally grafted plants (Malkov et al., 2007). SGECd^t had better water uptake by Cd-treated plants and higher root sap flow rate in both the presence or absence of toxic Cd (Belimov et al., 2015), suggesting that root water transport might be involved in mechanisms of increased tolerance and accumulation of Cd (Belimov et al., 2015). In contrast, Hg treatment of SGECd^t revealed decreased Hg-tolerance and foliar Hg-accumulation but had more negative effects on plant water relations compared to SGE plants (Belimov et al., 2015).

The present report aimed to characterize genotypic specificity of SGECd^t mutant in tolerance to and uptake of different heavy metals, as well as Al, Fe, Mn, NaCl and H^+ ions, to better understand mechanisms of metal co-tolerance, relationships between metal tolerance and accumulation, and effects of toxic metals on plant nutrient uptake.

2. Materials and methods

2.1. Plant growth conditions

The wild-type pea (*Pisum sativum* L.) line SGE and its Cd-tolerant mutant SGECd^t (see Introduction section for details) were used. Seeds were surface sterilized and scarified by treatment with 98% H_2SO_4 for 30 min, rinsed carefully with tap water and

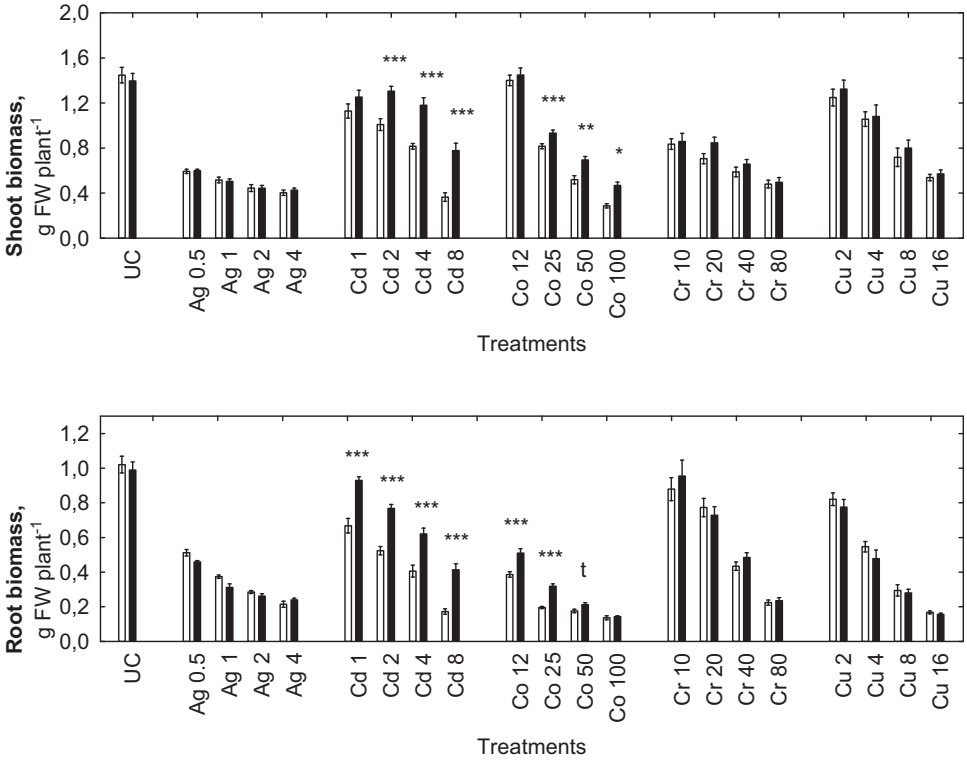

Fig. 1. Growth response of pea plants to toxic concentrations of Ag, Cd, Co, Cr and Cu.
Pea genotypes: wild type SGE (□), mutant SGECdt (■).
The axis of abscissa listed elements and their concentration in μM.
UC stands for untreated control in the complete nutrient solution
Asterisks show significant difference between pea genotypes at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) as determined by Fisher's LSD test (two way ANOVA; $n \geq 10$).
t shows significant difference between pea genotypes at $P < 0.05$ as determined by Student's test ($n = 15$) for a given treatment.

germinated on filter paper in Petri dishes for three days at 25 °C in the dark. Seedlings were transferred to plastic pots (one pot with 5 seeds per genotype and treatment) containing 800 mL of 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, 12; H_3BO_3, 2; $MnSO_4$, 1; $ZnSO_4$, 3; NaCl, 6; Na_2MoO_4, 0.06; $CoCl_2$, 0,06; $CuCl_2$, 0.06; $NiCl_2$, 0,06; pH = 5.5. The next day after planting (DAP) the nutrient solution was supplemented with different concentrations of metal salts such as: $AgNO_3$ (0.5, 1, 2, 4 μM), $AlCl_3$ (25, 50, 100, 200 μM), $CdCl_2$ (1, 2, 4, 8 μM), $CoCl_2$ (12, 25, 50, 100 μM), CrO_3 (10, 20, 40, 80 μM), $CuCl_2$ (2, 4, 8, 16 μM), $FeCl_3$ (25, 50, 100, 200 μM), $HgCl_2$ (0.5, 1, 2, 4 μM), $LaCl_3$ (60, 120, 240, 480 μM), $MnCl_2$ (200, 400, 800, 1600 μM), NaCl (12, 25, 50, 100 mM), $NiCl_2$ (10, 20, 40, 80 μM), $PbCl_2$ (15, 30, 60, 120 μM) or $ZnCl_2$ (50, 100, 200, 400 μM). When plants were cultivated in the presence of $AlCl_3$, $FeCl_3$ or $MnCl_2$, the nutrient solution was acidified up to pH = 4.5 via addition of 1 M HCl, because toxicity of these metals is shown in acid conditions. The effect of pH on plant growth was also estimated via addition of 1 M HCl to the nutrient solution and pH adjusted to 4.0, 3.5 or 3.0. Potassium phosphate was eliminated and concentration of KCl increased up to 650 μM, when plants were cultivated in the presence of $PbCl_2$, since lead precipitated as $Pb_3(PO_4)_2$. Preliminary experiments established a range of metal concentrations exerting slight to strong growth inhibition. In all experiments the nutrient solution was changed, and where necessary the supplements were added, at 5 and 9 DAP. Plants were cultivated for 12 days in growth chamber with 400 μmol m^{-2} s^{-1}, 12 h photoperiod with minima/maxima temperatures of 18 °C/23 °C respectively. Then root and shoot fresh weight (FW) of individual plants was determined. The plants were dried at room temperature and stored for elemental analysis. Experiments for each pea genotype and each treatment were repeated at least two times and included untreated controls.

2.2. Determination of metal concentrations

Plants treated with metal concentrations exerting significant (usually not maximal) root and shoot growth inhibition were chosen for elemental analysis. Shoots were cut and the roots intended for elemental analysis (except those treated with $PbCl_2$) were soaked in 1 mM Pb-citrate solution (pH = 11) for 10 min and washed in deionized water for desorption of apoplastically bound metals. In the same manner 1 mM $LaCl_3$ was used for desorption of Pb. Then roots and shoots of individual plants were dried, ground and digested in a mixture of concentrated HNO_3 and 38% H_2O_2 at 70 °C using DigiBlock (LabTech, Italy). Concentrations of elements in digested plant samples were determined using an inductively coupled plasma emission spectrometer ICPE-9000 (Shimadzu, Japan).

2.3. Statistical analysis

Statistical analysis of the data was performed using the software STATISTICA version 7.0 (StatSoft Inc., USA). Fisher's LSD test (two way ANOVA), Student's *t*-test, Wilcoxon matched pairs test and the confidence intervals were used to evaluate differences between means.

3. Results

The SGECd^t mutant had more root biomass at all Cd concentrations and shoot biomass at 2, 4 and 8 μM Cd compared

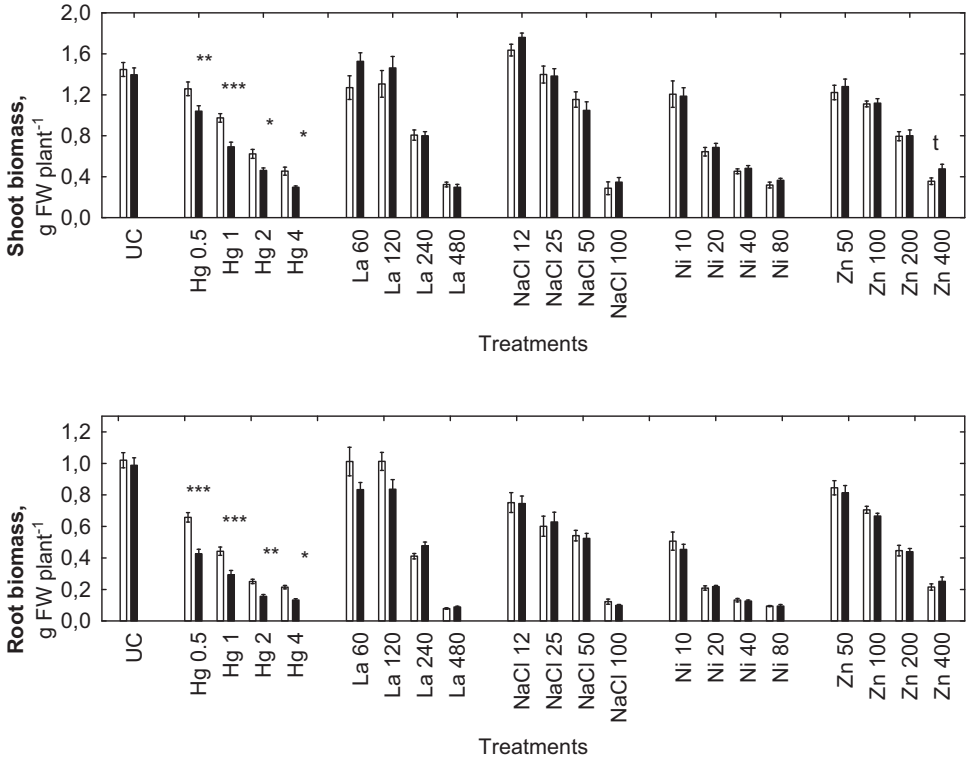

Fig. 2. Growth response of pea plants to toxic concentrations of Hg, La, NaCl, Ni and Zn.
Pea genotypes: wild type SGE (□), mutant SGECdt (■).
The axis of abscissa listed elements and their concentration in μM, except that NaCl is given in mM.
UC stands for untreated control in the complete nutrient solution
Asterisks show significant difference between pea genotypes at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) as determined by Fisher's LSD test (two way ANOVA; $n \geq 10$).
t shows significant difference between pea genotypes at $P < 0.05$ as determined by Student's test ($n = 10$) for a given treatment.

with WT (Fig. 1), confirming previous data (Tsyganov et al., 2007; Belimov et al., 2015). As in previous results (Belimov et al., 2015), root and shoot biomass of SGECd^t exposed to all Hg concentrations tested was lower that of WT (Fig. 2). This suggested that growth conditions in the present set of experiments were suitable to differentiate genotypic responses to toxic metals.

Treatments with Co revealed that SGECd^t had more root and shoot biomass than WT (Fig. 1). Genotypic differences were significant in root biomass at 12 and 25 μM Co, in shoot biomass at 25, 50 and 100 μM Co, and a tendency (*t*-test, $P = 0.029$, n = 15) for increased root biomass of SGECd^t compared to WT by 20% was also detected at 50 μM Co (Fig. 1).

Root and shoot biomass of SGECd^t and WT did not differ in response to a range of toxic concentrations of Ag, Cr, Cu (Fig. 1), La, NaCl, Ni, Zn (Fig. 2) and Al, Fe, Mn and Pb (Fig. 3). Both genotypes grew similarly at low pH values (Fig. 3). Among these treatments the following tendencies in genotypic differences expressed as a bigger biomass of SGECd^t were: (i) shoot biomass increased by 34% (*t*-test, $P = 0.046$, n = 10) in the presence of 400 μM Zn (Fig. 2); (ii) root (*t*-test, $P = 0.015$, n = 15) and shoot (*t*-test, $P = 0.036$, n = 15) biomass increased by 32% in the presence of 100 μM Fe (Fig. 3).

Cd-treatment increased root and shoot Cd content of the SGECd^t mutant by 2.3-fold and 1.9-fold respectively. Hg-treatment of the SGECd^t mutant decreased shoot Hg content by 37%, compared with WT (Table 1). Treatments with Mn or 400 μM Zn increased content of these metals in SGECd^t roots by 65% and 8%, respectively. No genotypic differences in the content of other toxic metals were observed on the treated plants (Table 1). Total root and shoot Co accumulation of SGECd^t was higher than of WT by 25% or 46%, mostly due to the increased biomass.

In general, toxic metal contents of SGECd^t roots were significantly greater (Wilcoxon matched pairs test, n = 15, $P = 0.013$) than in WT roots. However, there was no such difference when comparing shoot metal contents of SGECd^t and WT plants (n = 15, $P = 0.24$).

Treatments with toxic metals altered nutrient element contents of root (Fig. 1S) and shoot (Fig. 2S) in both pea genotypes. For the most part, metal toxicity decreased nutrient element contents although opposite effects were also observed. Particularly, Hg treatment increased root Cr, Cu, K, Ni, P and S contents (Fig. 1S) and shoot Co, Cr, Cu, Fe, K, Mg, Ni, P and S contents in both genotypes, and Zn content in SGECd^t shoots (Fig. 2S). Treatment with Ag increased root Ca and K contents in both genotypes and shoot Cu and Ni contents of WT. Plants grown in acidic (pH = 4.5) nutrient solution had increased root B and Cu content (Fig. 1S) and shoot Co, Cu, K, Mg, Ni, P, S and Zn content (Fig. 1S). Eliminating phosphate from the nutrient solution increased root Ca, Co, Cr, Cu, Na, Ni and Zn content (Fig. 1S) and shoot Ca, Co, Cr, K, Mg, Mn, Ni, S and Zn content (Fig. 1S) of both pea genotypes.

Quantitative elemental composition of both pea genotypes was similar when the plants were grown in complete nutrient solution at pH = 5.5 without adding toxic metals (Figs. 1S and 2S), with exception for the 13% higher root Mg content of SGECd^t compared to WT plants (Fig. 1S). Adding toxic metals to the nutrient solution induced genotypic differences in the content of nutrient elements in plants (see Figs. 1S and 2S for details), with significant differences summarized in Table 2. Compared to WT plants, the

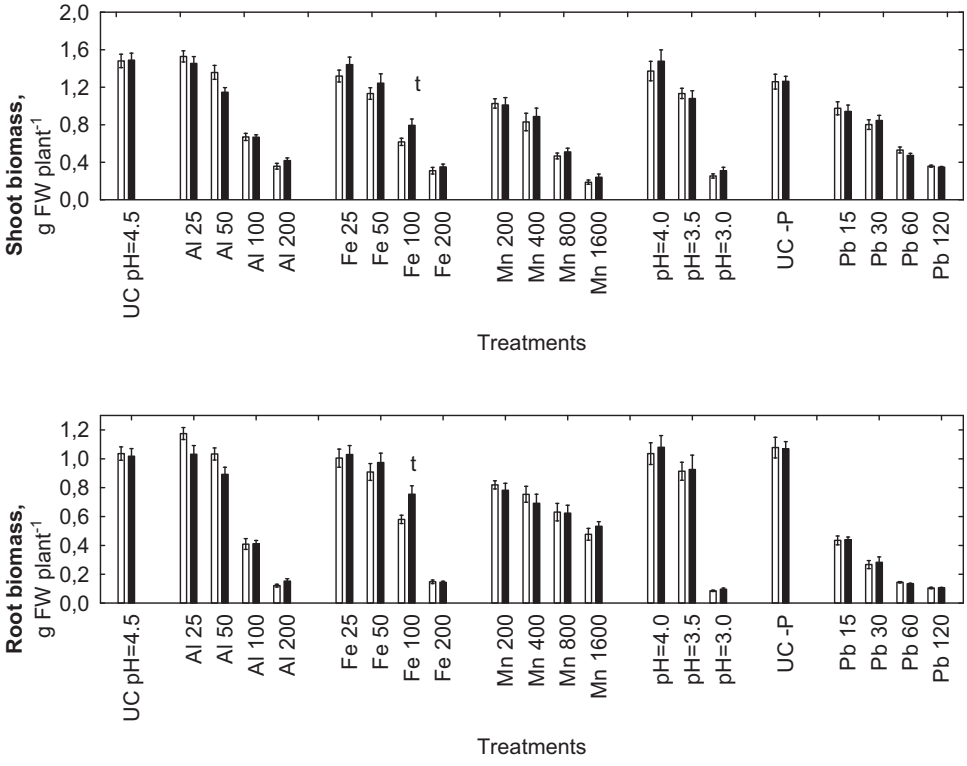

Fig. 3. Growth response of pea plants to toxic concentrations of Al, Fe, Mn, Pb and low pH.
Pea genotypes: wild type SGE (□), mutant SGECdt (■).
The plants treated with 100 μM Al, 100 μM Fe or 400 μM Mn were grown in the solution having pH = 4.5.
The plants treated with 30 μM Pb were grown in the minus P solution.
The axis of abscissa listed elements and their concentration in μM.
UC pH = 4.5 and UC – P stand for untreated controls in nutrient solutions having pH = 4.5 and minus P, respectively.
Asterisks show significant difference between pea genotypes at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) as determined by Fisher's LSD test (two way ANOVA; n ≥ 10).
t shows significant difference between pea genotypes at $P < 0.05$ as determined by Student's test (n = 15) for a given treatment.

Table 1
Content of toxic metals in root and shoot of the treated plants.

Treatment	Concentration of the corresponding metal in plants, μg g^{-1} DW			
	Root		Shoot	
	SGE	SGECd^t	SGE	SGECd^t
10 μM Ag	309 ± 15	358 ± 34	1.9 ± 0.9	3.7 ± 1.0
100 μM Al	14109 ± 1160	14429 ± 401	78 ± 10	83 ± 7
4 μM Cd	361 ± 24	821 ± 79 *	59 ± 4	169 ± 10 *
50 μM Co	1271 ± 221	1314 ± 241	206 ± 47	224 ± 39
40 μM Cr	1069 ± 47	1125 ± 47	6.4 ± 0.7	6.3 ± 0.5
8 μM Cu	932 ± 24	944 ± 29	39 ± 5	29 ± 1
100 μM Fe	7832 ± 278	8260 ± 299	141 ± 10	149 ± 16
2 μM Hg	7158 ± 345	7552 ± 378	71 ± 8	45 ± 1 *
240 μM La	3179 ± 283	3492 ± 649	20 ± 2	22 ± 1
400 μM Mn	4546 ± 787	7514 ± 700 *	1759 ± 22	1840 ± 96
50000 μM NaCl	17768 ± 343	17896 ± 485	7247 ± 59	7328 ± 269
20 μM Ni	554 ± 13	511 ± 12	121 ± 10	110 ± 6
30 μM Pb	14476 ± 656	13910 ± 574	138 ± 5	137 ± 27
200 μM Zn	3385 ± 86	3645 ± 179	958 ± 57	997 ± 83
400 μM Zn	5535 ± 116	5987 ± 143 *	1283 ± 52	1262 ± 33

The data are means ± SE.
Asterisks show significant difference between pea genotypes for root or shoot respectively (Student's *t*-test; $P \leq 0.05$; n = 4).

Table 2
Nutrient elements having genotypic difference in plant tissue contents after treatment with toxic metals.

Treatment	Nutrient elements			
	Increased concentration in SGECd^t		Decreased concentration in SGECd^t	
	Root	Shoot	Root	Shoot
10 μM Ag	=	=	Mg (11), Ni (25)	=
100 μM Al	B (13)	=	=	=
4 μM Cd	Ca (21), Fe (222), Mg (37), Mn (101), S (10)	B (101), Ca (18), Mg (28), Mn (33), Na (112), Zn (25)	=	=
50 μM Co	=	=	=	=
40 μM Cr	=	=	=	=
8 μM Cu	=	=	=	=
100 μM Fe	=	=	=	=
2 μM Hg	Co (57), Cr (64), Cu (15)	=	Ca (38), K (31), Mg (22), S (18)	Ca (65), K (17), Mg (22), S (19), Zn (44)
240 μM La	S (33)	=	=	Cu (54), Na (76), Ni (90), Zn (24)
400 μM Mn	Co (46)	=	=	=
50000 μM NaCl	=	=	=	=
20 μM Ni	=	=	P (15)	Cu (14), K (14), P (16)
30 μM Pb	=	=	=	=
200 μM Zn	Cr (48)	Co (28)	=	=
400 μM Zn	=	=	=	=

Element symbols show significant difference between pea genotypes in the content of this element (Student's *t*-test; $P < 0.05$; n = 4).
Percent change between WT and SGECd^t shown in brackets.
Equal signs show similar content of all elements for both pea genotypes. See supplemental Figs. 1 and 2 for details.

SGECd^t mutant had increased root Ca, Fe, Mg, Mn and S content and shoot B, Ca, Mg, Mn, Na and Zn content following Cd-treatment. Similarly, the mutant had increased root Co, Cr and Cu content, but lower root and shoot Ca, K, Mg and S content following Hg-treatment. Treatment with La increased root S content but decreased shoot Cu, Na, Ni and Zn content of SGECd^t. Treatment with Ni decreased root P content and shoot Cu, K and P content of SGECd^t. Differences in individual elements were also observed in plants treated with Ag, Al, Mn or Zn (see Table 2 and Figs. 1 and 2S for details).

4. Discussion

After growing the SGE pea line and its SGECd^t mutant in nutrient solutions containing a range of toxic concentrations of 14 different metals, only three metals (Cd, Hg and Co) caused genotypic differences in root and/or shoot biomass. Previous hydroponic experiments of similar design revealed the increased tolerance of SGECd^t mutant to 1, 3 or 4 μM Cd (Tsyganov et al., 2007; Belimov et al., 2015) and to 0.5, 1 or 2 μM Hg (Belimov et al., 2015). These genotypic differences in biomass were retained at 8 μM Cd (Fig. 1) and 4 μM Hg (Fig. 2) when plant growth was almost completely inhibited. Genotypic differences between SGECd^t and WT growth in response to Co, Fe and Zn are described here for the first time. Interestingly, the genotypic difference in Co tolerance was more pronounced in the shoots whereas Cd tolerance was expressed especially in the roots at all Cd concentrations tested. This feature distinguished the tolerance of the mutant to Co and Cd. In addition, the SGECd^t mutant tended to grow better after treatment with 100 μM Fe and 400 μM Zn., but only at these particular concentrations. Therefore it is unlikely that the mutant possess higher tolerance to these metals. While the

SGECd^t mutant shows similar growth responses to salinity, Al^{3+}, Mn^{2+} and H^+, it shows specific tolerance to a number of toxic heavy metals (Cd, Co and Hg).

Generally, root and shoot toxic metal contents were similar between genotypes (Table 1), but there were some exceptions. Firstly, Cd-treatment increased root and shoot Cd content of SGECd^t plants, as seen previously (Tsyganov et al., 2007; Belimov et al., 2015). Secondly, Hg-treatment decreased shoot Hg content of SGECd^t plants, as seen previously (Belimov et al., 2015). Thirdly, the mutant had increased root (but not shoot) Mn and Zn content after treatment with these metals. Interestingly, although SGECd^t had increased tolerance to Co, the content of this element in Co-treated plants was similar to WT. These results suggest that the ability to prevent uptake and translocation of toxic metals from root to shoot is not a mechanism of metal tolerance in SGECd^t mutant.

Moreover, SGECd^t roots were better able to (non-specifically) take up various toxic elements (as estimated by Wilcoxon matched pairs test,), including Cd, Co and Hg, the metals to which the mutant exhibits different or opposing growth responses. This agrees with previous observations of reciprocally grafted plants, with the roots having a crucial role in increased Cd-tolerance and Cd-accumulation of SGECd^t (Malkov et al., 2007). The mutant also had increased root xylem exudation in the absence of Cd and following Cd exposure, potentially enhancing water uptake and transport from root to shoot (Belimov et al., 2015). These features might facilitate greater diffusion of metal ions into the root with water flow.

Plants accumulate or tolerate metals such as Cd, Co and Hg according different mechanisms in plants (Clemens, 2006; Patra and Sharma, 2000; Nagajyoti et al., 2010) but there are few comparisons of physiological or biochemical effects and/or uptake. In the metal hyperaccumulating species *Allusum murale,* the main mechanism of Co storage was exocellular sequestration (Tappero et al., 2007), but little is known about sequestration of this metal in other plant species. However, it was repeatedly shown that vacuolar sequestration is important for storage of Cd in different plant species (Clemens, 2006; Morel et al., 2009). Radioisotope techniques demonstrated that ^{57}Co was retained in wheat (*Triticum aestivum*) roots without being loaded into the xylem, whereas ^{109}Cd was transported from root to leaf (Page and Feller 2005). In our experiments the root/shoot concentration ratio of Cd and Co for WT plants was similar (16%), but differed for SGECd^t plants (21% and 17% respectively – Table 1). Biosynthesis of phytochelatins in roots was induced after exposure of *Zea mays* seedlings to Cd, but not to Hg (Rellan-Alvarez et al., 2006). Toxic Cd or Hg concentrations elicited different dynamics of oxidative stress in *Medicago sativa* seedlings (Ortega-Villasante et al., 2005). A Cd-tolerant cell line of tomato was isolated and showed slightly higher tolerance to Cu but not to Hg, Zn, Ag and Pb (Huang et al., 1987). Since SGECd^t shows opposite phenotypic responses depending on the toxic metal or even independent of metal toxicity (e.g. increased root xylem exudation of metal untreated plants as shown by Belimov et al., 2015), we propose that the mutation is not directly related to tolerance/uptake of toxic metals, but affects some regulatory genes or transcription factors. In this respect new phenotypic differences between SGECd^t and WT worth to be discovered.

Although tolerance and accumulation of Cd (Zha et al., 2004) or Zn (Macnair et al., 1999) may be genetically independent characters, they seem inter-related. The proteins responsible for uptake and transport of metal ions are also often involved in plant growth responses to metal toxicity (Patra and Sharma, 2000; Hall, 2002; Clemens, 2006; Nagajyoti et al., 2010). In *A. thaliana*, overexpressing the heavy metal associated transporter AtHMA3 increased tolerance to Cd, Co, Pb and Zn as well as Cd accumulation via vacuolar sequestration of these metals (Morel et al., 2009), whereas another transporter AtHMA4 increased Cd, Co and Zn tolerance while enhancing metal loading into the xylem (Verret et al., 2004). Transgenic *A. thaliana* plants overexpressing the yeast ABC binding cassette transporter YCF-1 had increased tolerance and accumulation of Cd and Pb (Song et al., 2003). A chemically induced *A. thaliana* mutant MCR-32 showed higher Cd tolerance and Cd content compared to WT plants, but was characterized by slow growth rate and altered leaf development (Watanabe et al., 2010). Introducing a mercury uptake pump merC from *Acidithiobacillus ferrooxidans* into *A. thaliana* caused hypersensitivity to Hg^{2+}, probably due to increased Hg uptake, but did not change Cd tolerance (Sasaki et al., 2006). Overexpressing the Zn-transporter ZAT in *A. thaliana* increased root Zn content and Zn tolerance to toxic Zn concentration (Van der Zaal et al., 1999). Overexpressing the ABC transporter AtPDR8 (efflux pump at the plasma membrane) improved Cd and Pb tolerance and decreased root and shoot Cd content of *A. thaliana* (Kim et al., 2007). However, these results with transgenic plants do not correlate with phenotype of SGECd^t mutant, suggesting that other genes (proteins) are involved in response of this genotype to toxic metals.

Among the metals tested, the SGECd^t mutant differed from WT in tolerance and/or uptake of Cd, Co, Fe, Hg, Mn and Zn. Many cation channels in plants are not selective and differently regulated in different organs and tissues, but information about their selectivity may be incomplete (Demidchik et al., 2002). For example, little is known about transport of Co, Fe, Hg and Mn by various families of Cd and Zn transporters (Verkleij et al., 2009). Several Mn transporters are also involved in Cd, Co, Fe and Zn transport, but their selectivity to Hg was not studied (Socha and Guerinot, 2014). Proteins within the cation diffusion facilitator (CDF) family, also called metal tolerance proteins (MTPs), transport heavy metals such as Cd, Co, Fe, Mn and Zn, but no information is available about transport of Hg by these cation channels (Montanini et al., 2007; Gustin et al., 2011). In the bacterium *Wautersia metallidurans,* an MTP protein WmFieF is involved in detoxifying Cd, Co, Fe, Ni and Zn (Munkelt et al., 2004). Heterologous expression of OsMPT1 in yeasts *Saccharomyces cerevisiae* conferred resistance to Cd, Co and Zn (Menguer et al., 2013). Some proteins of CDF family may be involved in the heavy metal relations of SGECd^t, since exactly the listed metals (Cd, Co, Fe, and Zn) showed differences between WT and the mutant (Table 2). It should be mentioned, that Ni was the metal that decreased nutrient elements in root and shoot of Ni-treated SGECd^t plants compared to WT. It seems worthwhile to explore the role of MTP transporters in plant tolerance to toxic Hg concentrations.

Interestingly, the Group 12 elements (Cd, Hg and Zn) which show common trends in their physical and chemical behavior (Housecroft and Sharpe, 2008), show genotypic differences between WT and SGECd^t plants. Indeed, plant responses to Cd and Zn toxicity may be similar, because of common transport channels, mechanisms of tolerance and uptake, and localization in plant tissues (Ma et al., 2005; Clemens, 2006; Nagajyoti et al., 2010; Lin and Aarts, 2012). However, Hg has generally been ignored in comparative studies of the effects of these metals on plants. Here, the Cd-tolerant and Hg-sensitive SGECd^t mutant had increased tolerance to Co and a concentration dependent tendency for increased tolerance to Fe and Zn. This is the first report of direct and inverse relationships between plant tolerance to these heavy metals, probably mediated by the same gene, allowing the prospect of understanding mechanisms of metal co-tolerance and correlations between metal toxicity and metal transport, particularly using genetically modified plants.

A comprehensive elemental analysis allowed the effects of many heavy metals, Al^{3+}, Fe^{3+}, Mn^{2+} and H^+ on the uptake of macro- and micronutrients by the two pea genotypes to be compared (Figs. 1S and 2S). Cadmium decreased root Ca, Co, K, Mn, Ni, P and Zn contents in both pea genotypes, while root Cu and Fe contents

and shoot Ca, Mg, Mn, Na, Ni and Zn contents decreased in WT plants only. These results agree with other reports showing that Cd inhibited nutrient uptake of pea (Sandalio et al., 2001; Metwally et al., 2005; Rodriguez-Serrano et al., 2009) and other plant species (Larsson et al., 2002; Zornoza et al., 2002; Yang et al., 2004). It was confirmed that Cd-treated SGECd^t mutant had higher root Ca and Mg content and shoot B, Mg, Mn and Zn content (Tsyganov et al., 2007). Moreover, Cd-treated SGECd^t mutant also showed increased root Fe and Mn content and shoot Ca and Na content, compared to WT. These results indicate the important role of nutrient homeostasis in the tolerance of the SGECd^t mutant to Cd toxicity.

Contrary to the plant response to Cd, there were no genotypic differences in the contents of nutrient elements in Co-treated pea plants, suggesting that nutrient homeostasis is not involved in Co tolerance of SGECd^t. Interestingly, genotypic difference in growth response were more pronounced in Cd-treated (than Co-treated) plants and Co was not taken up by the mutant as compared with Cd. However, little is known about the effects of Co on plant nutrient composition and the role of nutrient homeostasis in plant Co tolerance. Therefore the SGECd^t mutant is a promising genetic model for the study of plant tolerance to Co.

Among the metals studied, a distinct feature of Hg treatment was that the contents of many nutrient elements in plants increased, demonstrating specific mechanisms of action on plants. The Hg-sensitive mutant SGECd^t had decreased Ca, K, Mg, S and Zn contents in root or/and shoot as compared with WT, but the absolute values were similar to, or even higher than for the untreated plants. This suggests there was no nutrient deficiency in SGECd^t caused by Hg treatment and the nutrients did not mediate Hg tolerance, contrary to Cd-treated plants. The only important exception may be Ca, because Ca content decreased in Hg-treated plants and SGECd^t had lower root (Fig. 1S) and shoot (Fig. 2S) Ca content compared with WT plants. Ca plays key roles in structural and signalling processes and plant responses to various stimuli, including stress factors (Lecourieux et al., 2006; Dodd et al., 2010; Hey et al., 2010). In addition, Ca is closely associated with water transport in plants (Gilliham et al., 2011) and this can have a direct relation to our results. Indeed, comparison the response of SGECd^t to Cd and Hg revealed a positive correlation between growth parameters (this report; Belimov et al., 2015), Ca content in root or shoot (this report) and root sap water flow (Belimov et al., 2015). Therefore, we propose that maintaining Ca homeostasis can contribute to opposite growth responses of SGECd^t to both Cd and Hg.

Information about the effects of heavy metals such as Co, Cr, Cu, Ni, Pb and Zn on the uptake of nutrient elements is limited. Cu phytotoxicity was associated with limited root-to-shoot Fe and Mn transport (Marschner, 1995) while Pb treatment did not alter macro- and microelement nutrient content in lettuce (Michalska and Asp, 2001). Treating the metal hyperaccumulating plant *Thlaspi caerulescens* with Cu and Pb decreased shoot Zn content while Zn treatment decreased root and shoot Cu, Fe and Mn content (Walker and Bernal, 2004). Our results showed that investigating the elemental composition of plants treated with toxic metals gives mechanistic insights into metal tolerance and differentiates the responses of plants to various metals.

5. Conclusion

Thus, among 14 toxic metals tested, only Cd, Hg and Co induced significant biomass differences between WT and the SGECd^t mutant. This is the first report showing increased Co tolerance of SGECd^t and a tendency for increased tolerance to Fe and Zn. Genotypic differences in root or shoot contents of toxic metals were found only for the plants treated with Cd, Hg, Mn or Zn. Changes in element composition of plants caused by metal toxicity depended on the toxic metal but usually were similar for both genotypes, except for Cd and Hg treatments. This suggests a high specificity in phenotypic responses of this mutant exposed to toxic concentrations of various heavy metals, as well as of Al^{3+}, Fe^{3+}, Mn^{2+} and H^+. This specificity is supported by observations showing that the SGECd^t mutant: (i) exhibits altered tolerance to 3 of 14 metals and altered content of 4 toxic metals only; (ii) these metals exert specific effects, namely increased tolerance and content (Cd), increased tolerance and unchanged content (Co), unchanged tolerance and increased content (Mn and Zn) and decreased tolerance and content (Hg). Such a complete characterization of the SGECd^t mutant in terms of its metal tolerance and elemental composition will further the use of this genetic model to study mechanisms of plant responses to metal toxicity.

To our knowledge this is a first report of the detailed relationships between plant tolerance to toxic metal concentrations, probably mediated by the same gene. A complex behavior of SGECd^t in relation to different metals, varying from increased (Cd and Co), decreased (Hg), but neutral to many other metals tested, along with the increased water transport from root to shoot (Belimov et al., 2015), suggests that the mutation might affect regulatory genes, thereby determining unknown mechanisms of plant metal tolerance and uptake. The important role of Ca homeostasis for the tolerance of SGECd^t to Cd (Tsyganov et al., 2007; present report) and to Hg (present report) supports this hypothesis, assuming that Ca is responsible for many signalling processes in plants. It is also possible that the mutated gene can be involved in regulating ion transporters, most probably belonging to the CDF family and permeable for water. These hypotheses will be tested in further studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2016.04.009.

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