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RESEARCH PAPER

Genotypic variation of the response to cadmium toxicity in *Pisum sativum* L.

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Abstract

This work evaluates the (cor-)relations between selected biochemical responses to toxic Cd and the degree of Cd sensitivity in a set of pea genotypes. Ten genotypes were analysed that differ in their growth response to Cd when expressed as root or shoot tolerance indices (*TI*s). Concentrations of non-protein thiols (NPTs) and malondialdehyde (MDA), activity of chitinase, peroxidase (POX), and catalase significantly increased in all pea genotypes treated with Cd. Cd-sensitivity of genotypes was correlated with relative increases in MDA concentration as well as activities of chitinase and POX, suggesting similar Cd stress effects. Activities of ascorbate peroxidase (APX) decreased, but concentrations of glutathione (GSH) increased in the less Cd-sensitive genotypes. Differences in root and leaf contents of Cd revealed no correlation with *TI*, metabolic parameters, and enzyme activities in Cd-treated plants, respectively, except that shoot Cd concentration positively correlated with shoot chitinase activity. Toxic Cd levels inhibited uptake of nutrient elements such as P, K, S, Ca, Zn, Mn, and B by plants in an organ- and genotype-specific manner. Cd-sensitivity was significantly correlated with decreased root Zn concentrations. The results show both similarities, as well as distinct features, in Cd toxicity expression in genotypes of one species, suggesting that independent and multi-factorial reactions modulate Cd sensitivity on the low-tolerance level of plants. The study illustrates the biochemical basis of earlier detected genotypic variation in Cd response.

Key words: Ascorbate peroxidase, cadmium, catalase, glutathione, heavy metals, peroxidase, *Pisum sativum* L.

Introduction

Cadmium (Cd), being a highly toxic metal pollutant of soils, inhibits root and shoot growth and yield production, affects nutrient uptake and homeostasis, and is frequently accumulated by agriculturally important crops including pea with a significant potential to impair animal and human health (Sanita di Toppi and Gabrielli, 1999). In pea, a number of toxic effects of Cd on metabolism has been reported, such as decreased uptake of nutrient elements (Sandalio *et al.*, 2001), inhibition of various enzyme activities (Obata *et al.*, 1996; Chugh *et al.*, 1992), and induction of oxidative stress (Lozano-Rodriguez *et al.*, 1997; Romero-Puertas *et al.*, 1999; Sandalio *et al.*, 2001) including alterations in enzymes of the antioxidant defence system (Dalurzo *et al.*, 1997; Sandalio *et al.*, 2001; Romero-Puertas *et al.*, 2002; Skorzynska-Polit *et al.*, 2003/4).

The sensitivity of plants to heavy metals (HMs) depends on an interrelated network of physiological and molecular mechanisms such as (i) uptake and accumulation of metals through binding to extracellular exudates and cell wall constituents; (ii) efflux of HMs from cytoplasm to extraplasmatic compartments including vacuoles; (iii) complexation

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Abbreviations: APX, ascorbate peroxidase; GSH, glutathione; HM, heavy metals; MDA, malondialdehyde; NPT, non-protein thiols; POX, peroxidase; ROS, reactive oxygen species; *TI*, tolerance index.

of HM ions inside the cell by various substances, for example, organic acids, amino acids, ferritins, phytochelatins, and metallothioneins; (iv) general biochemical stress defence responses such as the induction of antioxidative enzymes and the accumulation of free proline; and (v) activation or modification of plant metabolism to allow adequate functioning of metabolic pathways and rapid repair of damaged cell structures (Verkleij and Schat, 1990; Brune *et al.*, 1995; Prasad, 1999; Sanita di Toppi and Gabrielli, 1999; Hall, 2002; Sanita di Toppi *et al.*, 2002; Cho *et al.*, 2003).

Plant species significantly differ in tolerance to and uptake of Cd and other HMs. Legume crops are less tolerant to Cd toxicity than cereals and grasses and frequently encounter strong inhibition of biomass production in the less than micromolar range of Cd (Inouhe *et al.*, 1994). Considerable variability among 99 pea genotypes, including those studied in the present paper, in tolerance to Cd and uptake of different heavy metals has been found (Belimov *et al.*, 2003). Intraspecific genetic variation in tolerance and uptake of Cd, Zn, Cu, and Mn also exists in other legume species (Polson and Adams, 1970; White *et al.*, 1979; Horst, 1983; Bell *et al.*, 1997). However, little is known about the biochemical mechanisms underlying the intraspecific variation in sensitivity versus tolerance to Cd and other heavy metals in plants.

The aim of the present work was to study the relationships between physiological and biochemical reactions to Cd-stress in pea genotypes differing in Cd-sensitivity, in order to contribute to an understanding of genotypic variation of environmental adaptation.

Materials and methods

Plant material

Ten wild-growing or primitive varieties of pea (*Pisum sativum* L.) were obtained from the Pea World Collection of the Vavilov Institute for Plant Industry (VIPI), St Petersburg. Their catalogue collection numbers (188, 1658, 1693, 3273, 3445, 3429, 4788, 6875, 8456, 7128) are given in the text, tables, and figures throughout this paper. Information about these pea genotypes can be found in the VIPI catalogue (http://www.dainet.de/genres/vir) and Belimov *et al.* (2003).

Hydroponic culture

Seeds were surface-sterilized and scarified by treatment with 98% H_2SO_4 for 15 min, rinsed carefully with tap water and germinated in Petri dishes covered with filter paper for 3 d at 25 °C in the dark. The germinated seeds were transferred to plastic pots (4 seeds per pot; 4 pots per pea genotype and per treatment) containing 500 ml of nutrient solution (μM): KNO_3, 1500; $Ca(NO_3)_2$, 700; $MgSO_4$, 500; $(NH_4)_2HPO_4$, 250; H_3BO_3, 11; Fe-tartrate, 12; $MnSO_4$, 1.3; $ZnSO_4$, 0.2; Na_2MoO_4, 0.3; $CuSO_4$, 0.8; pH 5.5. Cadmium was added as $CdCl_2$ at a concentration of 5 μM. The plants were cultivated for 10 d in a growth chamber at a day/night cycle of 12/12 h, at 23/18 °C, respectively, a relative humidity between 50% and 60% and a photon flux density of 170 μmol quanta m^{-2} s^{-1}. Nutrient solution was constantly aerated and replaced twice during each experiment. The plants were harvested, and root and shoot fresh weight (FW) of each plant measured. Root and leaf samples were immediately frozen in liquid nitrogen and stored at −80 °C for biochemical analyses, or were dried at 80 °C for elemental analysis. Cd-tolerance of the genotypes was calculated as the tolerance index (*TI*) which gives the percentage of shoot and/or root fresh biomass (g per plant) of Cd-treated (*FWt*) over untreated control (*FWc*) plants according to the following equation:

$$\frac{FWt}{FWc} \times 100 - 100 = TI[\%]$$

Sand culture

Four pea genotypes having a maximum *TI* (genotypes 1658 and 3429) and a minimum *TI* (genotypes 188 and 4788) in hydroponics were tested for their response to Cd toxicity in sand culture. Seeds were surface-sterilized and scarified as described above and planted in pots containing 3 kg quartz sand fertilized with 300 ml of nutrient solution (μM): KH_2PO_4, 7250; $MgSO_4$, 2100; $CaCl_2$, 2200; NH_4NO_3, 750; $Na_2FeEDTA$, 10; H_3BO_3, 5; NaCl, 3.5; $MnSO_4$, 0.7; $ZnSO_4$, 0.6; KBr, 2.5; KJ, 1.8; $CuSO_4$, 1.2; $Al_2(SO_4)_3$, 0.6; $CoCl_2$, 0.8; $NiSO_4$, 0.6; Li_2SO_4, 0.1; pH 5.5. The seeds were inoculated with a commercial strain of symbiotic nodule bacterium *Rhizobium leguminosarum* bv. *viciae* 1026 (the ARRIAM Collection, St Petersburg) in an amount of 10^8 cells per seed. Cadmium at 5 mg kg^{-1} sand was added as $CdCl_2$ solution 18 d after planting. Six pots with eight plants each (two plants of each pea genotype per pot) were prepared for Cd-treatment and untreated control. The plants were cultivated for 37 d in a polyethylene greenhouse during summer under natural illumination (June–July, St Petersburg). Pots were watered daily to 60% water-holding capacity of the quartz sand. The plants were sampled, harvested, and dried as described above. Dry weight (DW) of plants was used for calculation of *TI* as described above.

Biochemical analyses

For the determination of total non-protein thiols (NPTs) and glutathione (GSH) the frozen plant material (100 mg FW) was homogenized in 100 mM HCl/1 mM EDTA solution and centrifuged at 12 000 *g* for 5 min at 4 °C. Concentrations of NPTs were determined as described by Noctor and Foyer (1988) using 5′-dithio*bis*-2-nitrobenzoic acid (DTNB) reagent. Contents of glutathione were determined with an enzyme cycling assay based on sequential oxidation of GSH by DTNB and reduction by NADPH in the presence of glutathione reductase (Griffith, 1980). Chitinase activity was measured using the substrate carboxy-methyl chitin remazol brilliant violet (CM-chitin-RBV, Blue Substrates, Gőttingen, Germany) according to the method described by Wirth and Wolf (1990).

A more detailed characterization of stress-related biochemical responses was performed with six pea genotypes: the more tolerant genotypes (3429 and 1658), the more sensitive genotypes (4788 and 188), and the genotypes with intermediate *TI*-values (7128 and 8456). For the determination of catalase, peroxidase (POX), and ascorbate peroxidase (APX) the plant material (100 mg FW) was homogenized in 100 mM HEPES/KOH buffer (pH 7.4) and centrifuged at 10 000 *g* for 10 min at 4 °C. Catalase activity was determined polarographically by measuring the rate of H_2O_2 conversion to O_2 at room temperature (Dat *et al.*, 1998). Guaiacol-dependent POX activity was determined according to Adam *et al.* (1995). APX activity was measured in the presence of 0.25 mM ascorbic acid and 0.5 mM H_2O_2 by monitoring the decrease in absorption at 290 nm (Janda *et al.*, 1999). For determination of chitinase activity the plant material (200 mg FW) was homogenized in 100 mM K-acetate buffer (pH 5.5) and centrifuged at 12 000 *g* for 10 min at 4 °C. For measurement of the level of lipid peroxidation 250 mg FW of plant tissue was homogenized in

0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 10 000 *g* for 5 min at 4 °C. The level of lipid peroxidation was quantified by determining malondialdehyde (MDA), a breakdown product of lipid peroxidation, using a modified method of Heath and Packer described by Metwally *et al.* (2003).

Elemental analysis

Element composition of the genotypes used for detailed biochemical characterization was determined. Dried shoots and roots were ground and digested in 10% HNO_3 (v/v) at 165 °C under pressure. The extracts were analysed with an inductively coupled plasma atomic emission spectrometer (Jobin Yvon JY 70, Instruments SA, Longjumeau, France). The element contents of the samples were quantified by comparison with standard solutions at appropriate dilutions (Merck, Darmstadt, Germany): 10 μg ml^{-1} P and K, 2 μg ml^{-1} Mg, S, and Ca, and 0.1 μg ml^{-1} Cd, Fe, Mn, Zn, Na, B, and Al.

Statistical analysis

The data were processed by analysis of variance (MANOVA) and Pearson correlation using the software STATISTICA version 5.5 (StatSoft, Inc., 1995, USA), and standard error (SE) and standard deviation (SD) were calculated. LSD stands for Fisher's least significant difference.

Results

Growth response to Cd toxicity

In the presence of Cd in the nutrient solution all pea genotypes exhibited reduced root and shoot growth; roots were more sensitive to Cd toxicity than shoots (Fig. 1). In Fig. 1 and all subsequent figures the genotypes from left to

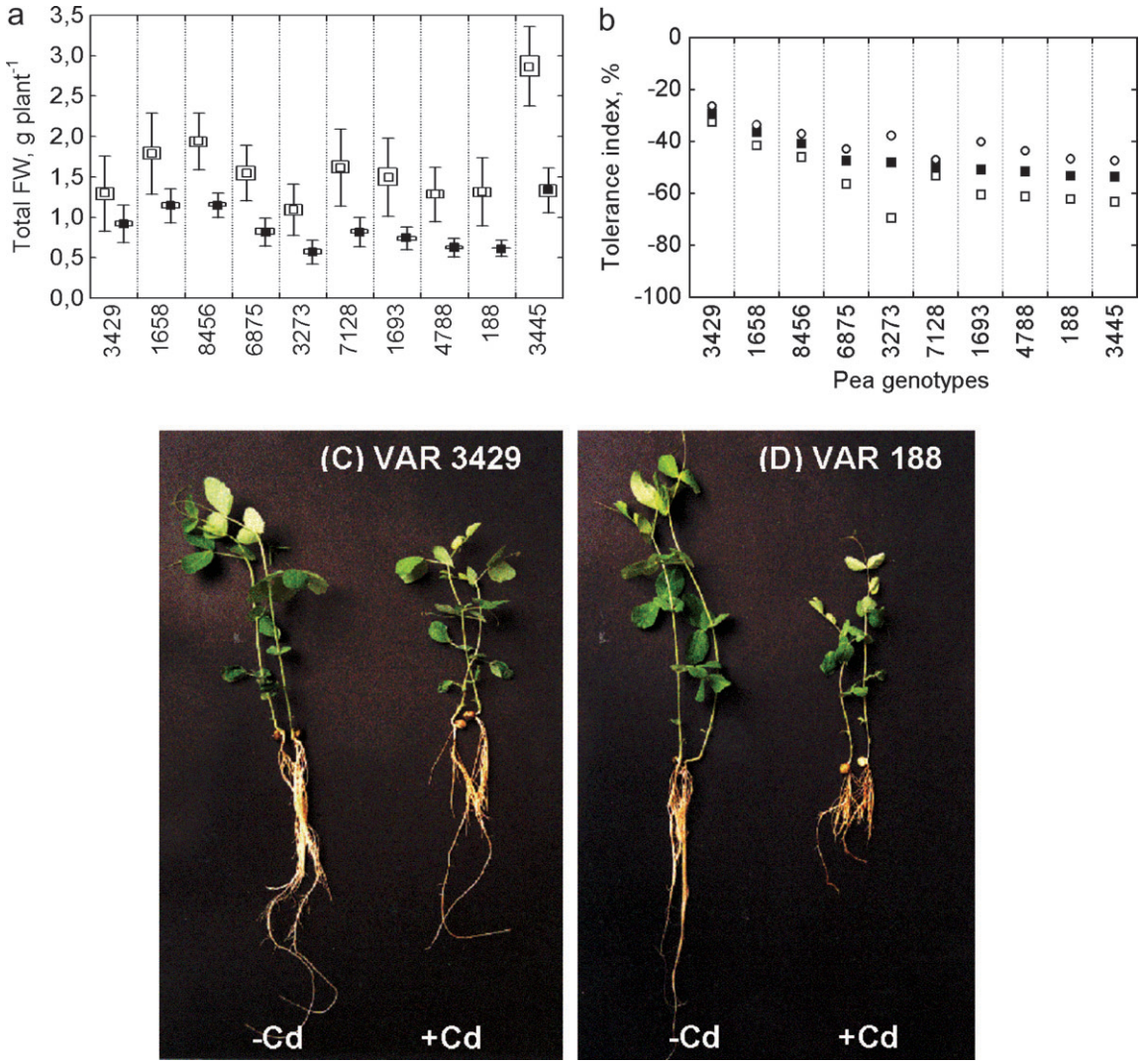

Fig. 1. Plant fresh weight (A) and tolerance index (B) of pea genotypes grown in hydroponics culture. Data in (A) refer to untreated control (open squares) and treatment with 5 μM $CdCl_2$ (filled squares) ±SE (box), ±SD (bars) from 24 determinations of three experiments. In (B), the tolerance index, i.e. growth in the presence of Cd related to control, is shown for fresh weight of whole plant (filled squares), root (open squares) and shoot (open circles) as calculated from the data shown in (A). A set of control (left) and Cd-treated (right) plants of the less sensitive genotypes 3429 (C) and 188 (D) is shown at the time of harvest, 13 d after sowing.

right are listed in the order of decreasing *TI* as calculated from total plant FW. Root *TI* positively correlated with shoot *TI* (r=+0.74; P=0.014; n=10). Only two genotypes revealed an irregular behaviour in their growth response to Cd toxicity: the genotype 3273 had a low root *TI* value but a relatively high shoot IT value, whereas the genotype 7128 showed the opposite response of root and shoot growth. Genotypes 3429, 1658, and 8456 were found to be the most tolerant genotypes with *TI*s in the range of −26% to −45%. In a converse manner, 4788, 188, and 3445 were the most Cd-sensitive genotypes with *TI*-values between −48% and −69%. Figure 1C exemplarily shows the plant habitus of genotypes 3429 (left) and 188 (right) at the end of the 13 d growth period when grown in the absence (left plants of each set) and presence of Cd (right plants). The increased sensitivity to Cd of 188 compared with 3429 is clearly discernible by the level of root length and leaf size. From each tolerance group, two genotypes were selected for further characterization of biochemical stress parameters (the more tolerant genotypes: 3429 and 1658; the more sensitive genotypes: 4788 and 188; and the genotypes with intermediate *TI*-values: 7128 and 8456). The Cd-sensitive genotype 3445 was not included in further experiments because this genotype significantly differed from the other genotypes studied by its high biomass production that probably could affect its response to Cd through dilution of Cd during fast growth of the plants.

Significant differences between the selected two Cd-tolerant and two Cd-sensitive pea genotypes in growth response to toxic Cd concentration were observed when the plants were grown in quartz sand culture (Fig. 2A, B). The root biomass of genotypes 3429 and 1658 was slightly affected by Cd, whereas it was significantly reduced in genotypes 4788 and 188. Shoot biomass of Cd-treated plants of all genotypes was significantly reduced and shoot growth inhibition caused by Cd was more pronounced in genotypes 4788 and 188. The results on growth response of pea genotypes to Cd in sand culture (Fig. 2A, B) correlated with the data obtained in hydroponic culture (Fig. 1), suggesting that genotypic differences in Cd tolerance are significant and reproducible under different experimental conditions.

NPTs and GSH contents

Ten genotypes grown in hydroponic culture were tested for root accumulation of NPT and GSH. Treatment with Cd increased NPT contents by factors of 2–3 compared with untreated control plants (Fig. 3A, B). Genotypes 3273 and 188 were characterized by the maximum root content of NPT from all Cd-treated plants. A correlation between

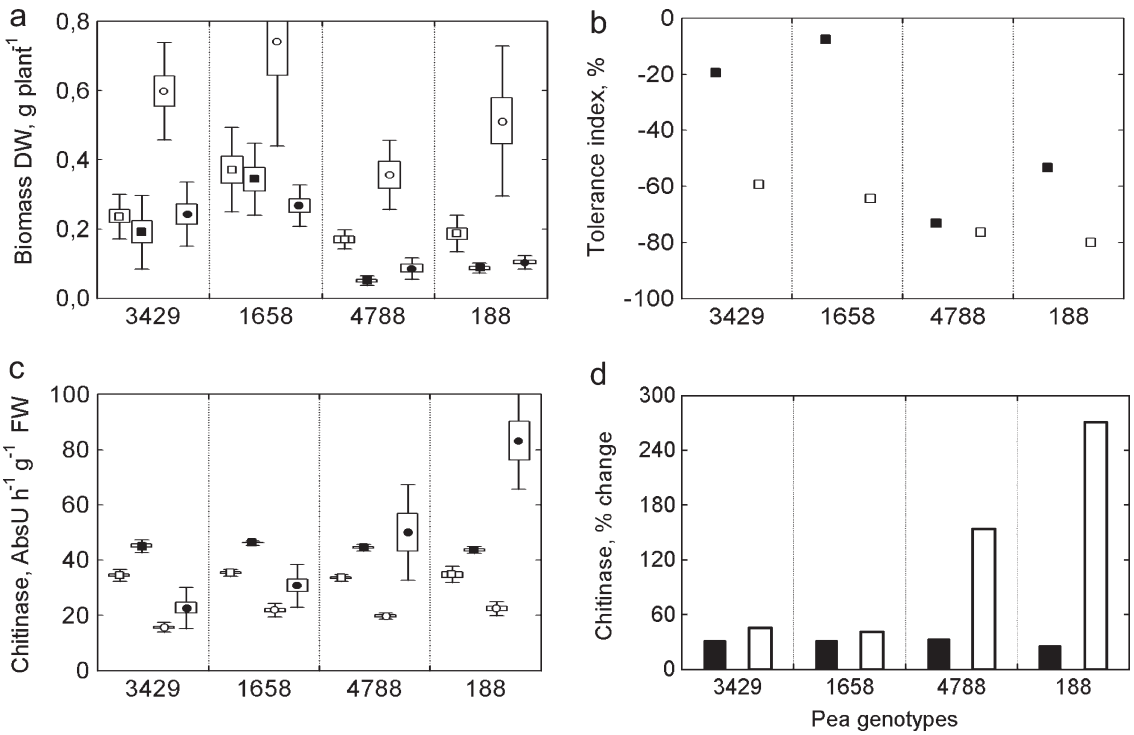

Fig. 2. Plant dry weight (A), tolerance indices (B), and chitinase activities (C, D) of pea genotypes grown in quartz sand culture with and without Cd. Data in (A) and (C) represent data from roots of untreated (open squares) and Cd-treated plants (filled squares), shoot (A) and leaves (C) of untreated (open circles) and Cd-treated plants (filled circles), respectively, ±SE (box) and ± SD (bars) from 12 determinations of one experiment. Symbols in (B) correspond to *TI* of roots (filled squares) and shoots (open squares). The percentage changes between treatment and control are given in (D) for roots (filled squares) and leaves (open squares).

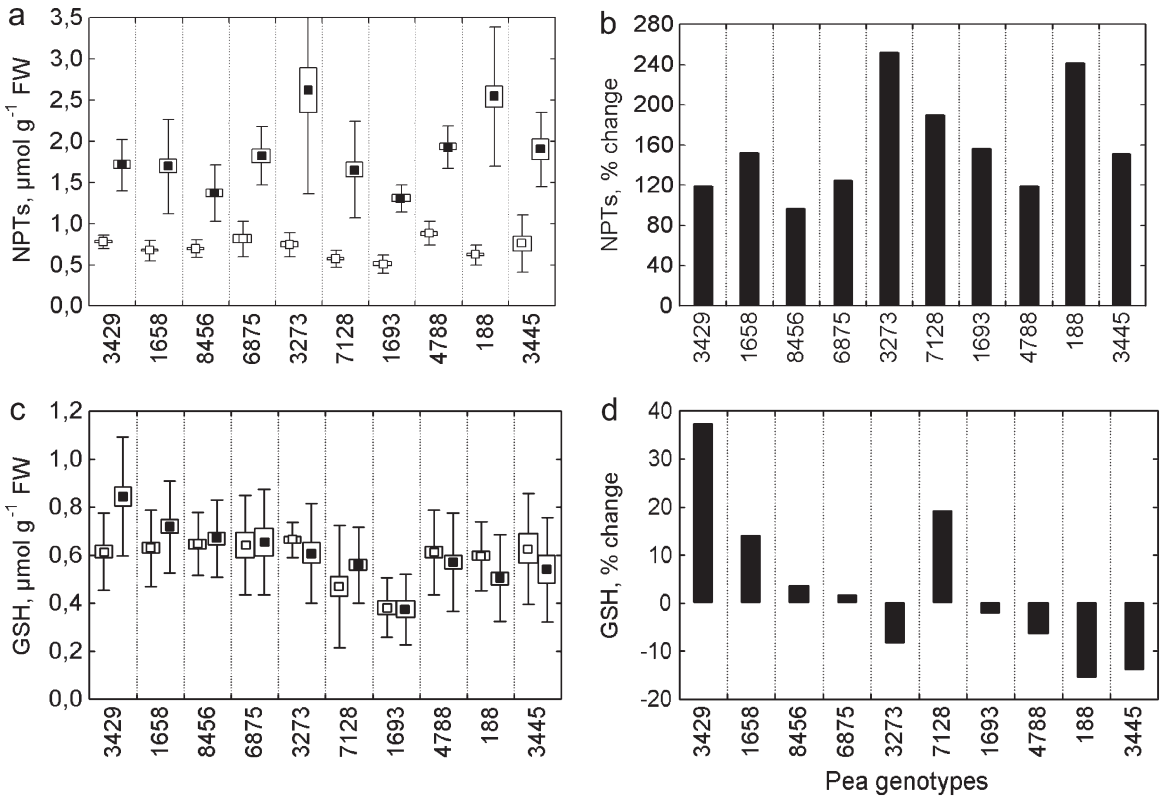

Fig. 3. Contents of non-protein thiols (NPTs) (A, B) and glutatione (GSH) (C, D) in roots of pea genotypes grown in hydroponics culture with or without Cd. Data in (A) and (C) correspond to control (open squares) and Cd-treatment (filled squares), ±SE (box) and ±SD (bars) from 12 determinations of two experiments. The percentage changes between treatment and control are given in (B) and (D).

growth response and NPT contents of pea genotypes in the presence of Cd was not observed (r varied between −0.18 and −0.57; P >0.05; n=10). Concentrations of GSH slightly increased in roots of Cd-treated genotypes 3429, 7128, and 1658, decreased in roots of genotypes 188 and 3445, and were unchanged in the other genotypes (Fig. 3C, D). Glutathione contents as well as the percentage change of GSH contents in Cd-treated roots were positively correlated with root, shoot, and total plant *TI*s (r varied between +0.69 and +0.88; P varied between 0.03 and 0.001; n=10), suggesting significant differences in GSH accumulation between Cd-tolerant and Cd-sensitive genotypes. Root contents for NPTs other than GSH in the presence of Cd were calculated as the difference of NPTs and GSH contents (data not shown); the values tentatively quantify phytochelatins (PC_{tent}). Contents of [PC_{tent}]=[NPT]–[GSH] were negatively correlated with root *TI* (r= −0.76; P =0.01; n=10).

In sand culture, NPT concentrations in roots and leaves of Cd-treated plants significantly increased by 320–420%, and 10–30%, respectively, compared with the corresponding control tissues (data not shown). A significant correlation was not observed between root or leaf concentrations of NPTs and the effect of Cd on growth of pea genotypes. Concentration of GSH in plants grown in sand culture was not determined.

Enzyme activities

Upon Cd exposure, chitinase activities in both roots and leaves increased in all pea genotypes (Fig. 4). The percentage changes induced by Cd administration differed between the genotypes as well as between roots and leaves. The increase in chitinase activity caused by Cd in roots (Fig. 4C) was negatively correlated with *TI* of roots (r= −0.93; P <0.001; n=10), shoots (r= −0.64; P=0.04; n=10) and total plant biomass (r= −0.83; P=0.003; n=10), suggesting that the less sensitive genotypes responded to Cd exposure with a lower degree of relative enzyme induction. Positive correlation was observed between [PC_{tent}] and increase in chitinase activity caused by Cd in roots (r= +0.77; P =0.009; n=10). Chitinase activity was also determined in roots and leaves of four pea genotypes grown in sand culture (Fig. 2C, D) and responded to Cd similarly as in hydroponics. In leaves of the less sensitive genotypes 3429 and 1658 induction of chitinase caused by Cd was as low as 45% and 41%, respectively, whereas in leaves of the more sensitive genotypes 4788 and 188 chitinase activity dramatically increased by 154% and 271%, respectively (Fig. 2D).

Six genotypes differing in growth response to toxic Cd were tested for activity of antioxidative enzymes such as peroxidase (POX), ascorbate peroxidase (APX), and catalase in roots (Fig. 5). The less sensitive pea genotypes 3429

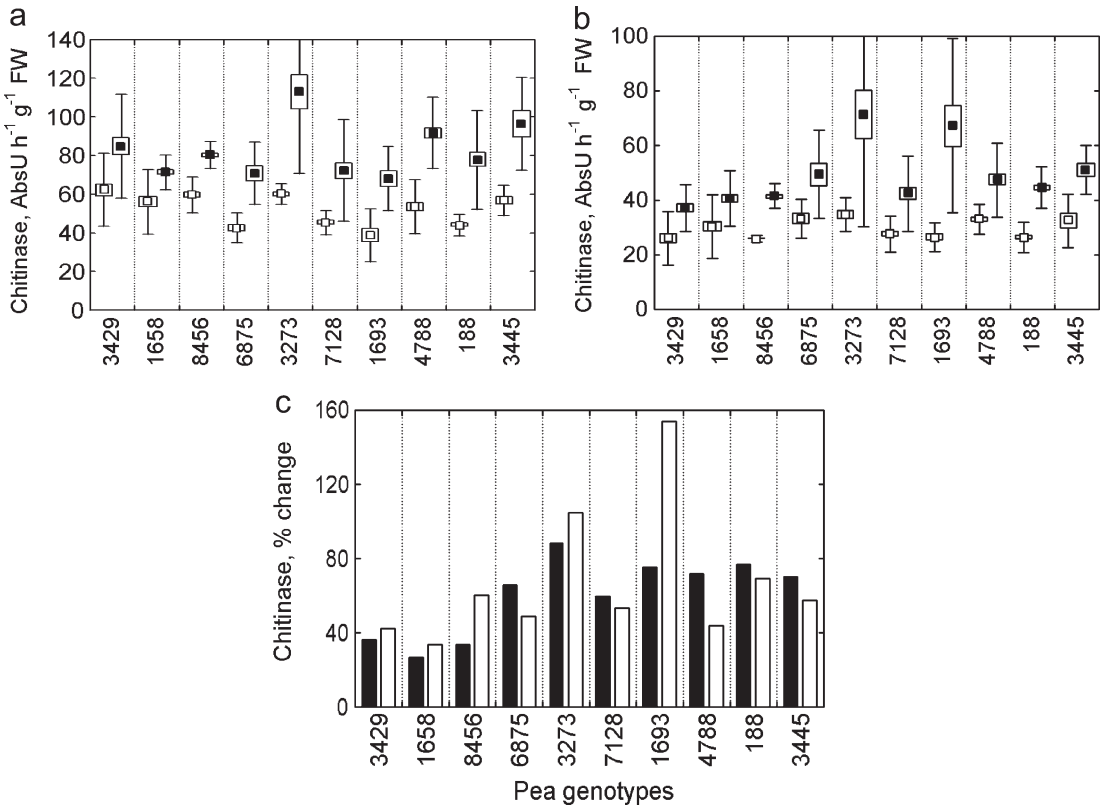

Fig. 4. Chitinase activities in roots (A, C) and leaves (B, C) of pea genotypes grown in hydroponics culture with or without Cd. Data in (A) and (B) correspond to control (open squares) and Cd-treatment (filled squares), ±SE (box) and ±SD (bars) from 12 determinations of two experiments. The percentage changes between treatment and control are given in (C) for roots (filled squares) and leaves (open squares).

and 1658 responded to Cd toxicity with a small increase in POX activity by about 15%, whereas the more sensitive genotypes 4788 and 188 revealed significant increases in POX activity by about 65%. The observed increase in POX activity (Fig. 5B) negatively correlated with the root *TI* (r= −0.89; P=0.017; n=6) and the total *TI* (r= −0.82; P=0.045; n=6). Activity of APX in the Cd-treated plant roots was reduced in genotypes 3429 and 1658, increased in genotype 188, and was not affected in the other genotypes (Fig. 5C, D). The effect of Cd on APX activity was highly correlated with root, shoot, and total plant *TI*, respectively (r_{root}= −0.94; P=0.006; n=6; r_{shoot}= −0.89; P=0.017; n=6; r_{plant}= −0.93; P=0.008; n=6) and PC_{tent} content in roots (r= +0.85; P=0.03; n=6). Catalase activity increased in roots of all genotypes after treatment with Cd (Fig. 5E, F). The increase in catalase activity caused by Cd varied from 23% to 100% depending on pea genotype, but did not correlate with Cd sensitivity of pea genotypes.

Lipid peroxidation

Treatment with Cd stimulated accumulation of lipid peroxides in roots of all tested pea genotypes (Fig. 6). The Cd-induced increase of MDA level was lower in the less sensitive genotypes 3429 and 1658 compared with the more sensitive genotypes 4788 and 188. However, a maximum value of lipid peroxides was observed in genotype 8456, which showed relatively high tolerance to Cd as deduced from growth parameters. Therefore, correlation between the increase in root MDA levels and root *TI* (r= −0.97; P=0.007; n=5) and total *TI* (r= −0.94; P=0.02; n=5) was significant only when the genotype 8456, having particular high MDA concentration, was excluded from the analysis.

Element composition

Cd levels in untreated control plants varied from 2.9 μg g^{-1} to 5.1 μg g^{-1} DW in roots and from 0.1 μg g^{-1} to 0.4 μg g^{-1} DW in shoots, but differences between genotypes were not significant (data not shown). Upon Cd treatment, root and shoot Cd concentrations dramatically increased in all genotypes and Cd concentrations in roots were approximately 10-fold higher than in shoots (Fig. 7A). High variation between the genotypes was observed in Cd concentrations in roots and shoots. To characterize the extent of Cd transport from root to shoot, a ratio between shoot and root Cd concentrations was calculated for each genotype showing that relatively low proportions of the Cd taken up by roots were transferred to the shoots in the less sensitive genotypes 3429 and 1658 compared with other genotypes (Fig. 7B). However, Cd contents of treated plants as well as shoot:root ratio of Cd contents were unrelated to *TI*s, NPTs, and GSH contents, and enzyme activities with some exceptions: shoot Cd contents correlated with (i) the

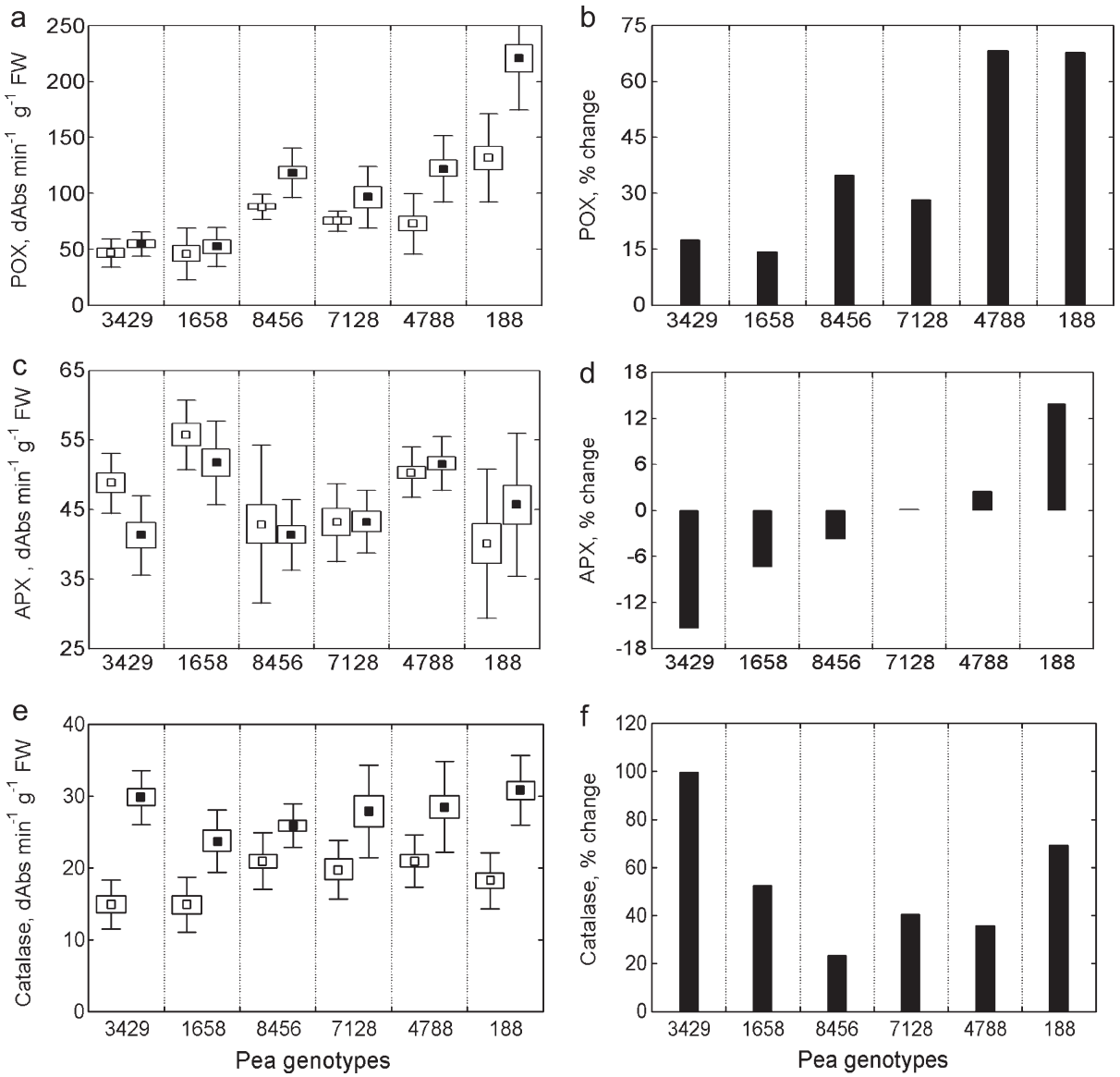

Fig. 5. Root activities of antioxidant enzymes in pea genotypes grown in hydroponics culture with or without Cd. (A, B) Peroxidase (POX), (C, D) ascorbate peroxidase (APX), (E, F) catalase. Data in (A), (C), and (E) correspond to control (open squares) and Cd-treatment (filled squares), ±SE (box) and ±SD (bars) from 8–12 determinations of two experiments. The percentage changes between treatment and control are given in (B), (D), and (F).

increase in shoot chitinase activity (r= +0.84; P=0.04; n=6) and (ii) the root APX activity of untreated plants (r= −0.91; P=0.01; n=6). (iii) The shoot:root ratios of Cd contents were negatively correlated with root GSH concentration of untreated plants (r= −0.82; P=0.04; n=6). In addition, the particularly low root Cd content combined with high Cd translocation to the shoot of genotype 7128 may account for the peculiar feature of having high root *TI* and low shoot *TI*, respectively.

Treatment with Cd also affected nutrient element composition of both roots and shoots. The Cd-induced modulation of element composition varied between pea genotype and plant organs (Table 1). Common symptoms of Cd toxicity were a reduction in root and shoot K and Mn contents, reduction of root levels of P and S, decreased B concentration in shoots, and increased root Mg and shoot S contents. A significant decrease in Zn concentration of Cd-treated plants was also observed, particularly in genotypes 3429 and 1658, which are considered as being less sensitive to Cd toxicity. Interestingly, shoot concentrations of P, S, Mg, Ca, and Zn in Cd-treated plants correlated with shoot Cd concentration (r varied from +0.85 to +0.99; P varied from 0.03 to 0.0001; n=6), but did not correlate with root Cd concentration. The effect of Cd on root and shoot concentrations of Fe, Na, and Al was insignificant for all genotypes (data not shown).

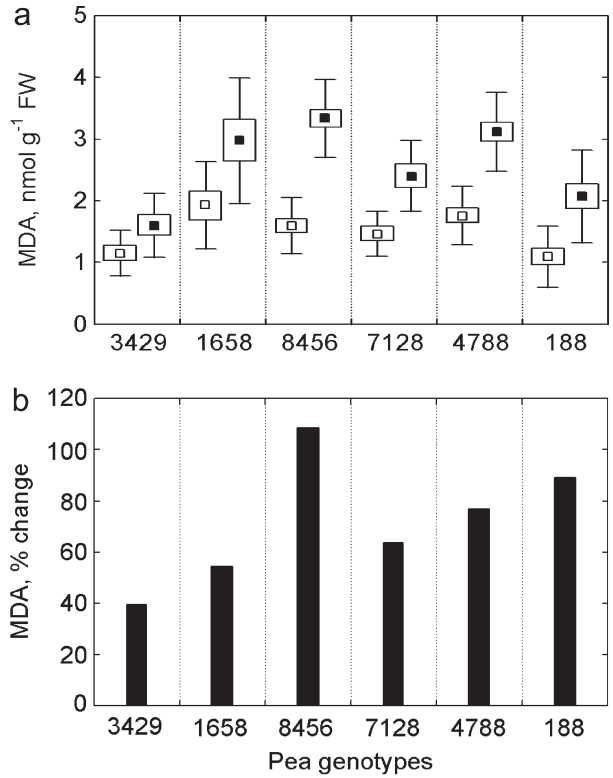

Fig. 6. Contents of malondialdehyde (MDA) in roots of pea genotypes grown in hydroponics culture with or without Cd. Data in (A) correspond to control (open squares) and Cd-treatment (filled squares), ±SE (box) and ±SD (bars) from 8–12 determinations of two experiments. The percentage changes between treatment and control are given in (B).

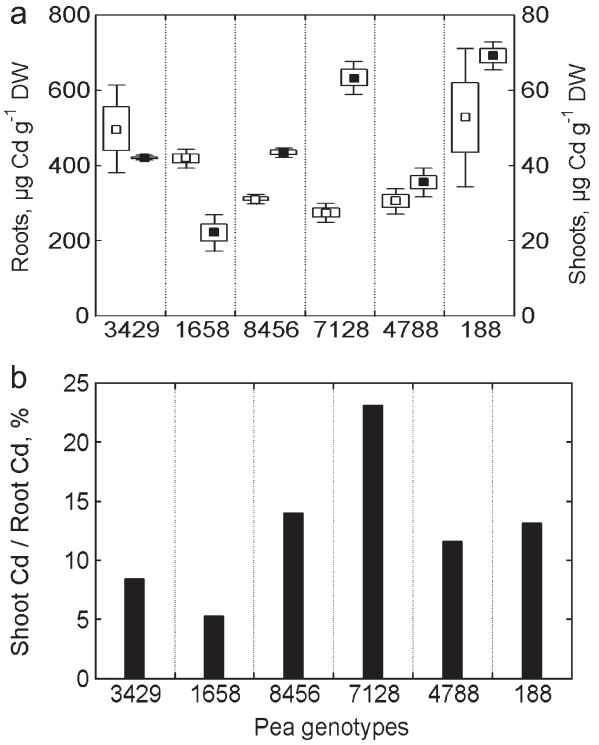

Fig. 7. Cd contents (A) and root:shoot ratio of Cd (B) in pea genotypes grown in hydroponics culture in the presence of 5 µM $CdCl_2$. Data in (A) correspond to Cd contents in roots (open squares) and shoots (filled squares), ±SE (box) and ±SD (bars) from four determinations of two experiments.

Discussion

Growth response to Cd toxicity

For the present study ten pea genotypes with varying levels of Cd sensitivity were selected on the basis of previous findings concerning the genetic variation between 99 pea genotypes. The first study characterized Cd tolerance of the pea genotypes during growth in sand culture and included the accumulation of heavy metals from contaminated soil (Belimov *et al.*, 2003). The results presented here on pea growth in hydroponics and sand culture in short-term experiments correlate with previous observations and further elaborate that the selected genotypes significantly differed in growth response to toxic Cd. Cd inhibited biomass production of roots to a greater extent than of shoots, whereas an opposite effect on biomass production was observed in sand culture. Apparently, Cd toxicity varies with growth conditions and experimental design, and depending on Cd availability, duration of Cd treatment, and plant age. Although there was a positive correlation between root and shoot *TI*s of the genotypes studied, two genotypes, 3273 and 7128, showed a peculiar feature in growth response. The genotype 3273 exhibited a much higher shoot than root *TI*. In a converse manner, 7128 revealed a low shoot *TI*; but its root *TI* was relatively high compared with other sensitive genotypes. This points to the facts that (i) different mechanisms of Cd tolerance may be prevailing in these genotypes and (ii) in toxicological studies, exclusive determination of root *TI* or shoot *TI* may be insufficient to evaluate the Cd tolerance of plants. The results obtained are in agreement with the findings of other authors describing the existence of intraspecific genetic variation of different legume species in the tolerance to heavy metals such as Zn, Cu, and Mn (Polson and Adams, 1970; White *et al.*, 1979; Horst, 1983). Table 2 summarizes the correlations between *TI*s and biochemical characteristics observed in the study and discussed below.

Chitinase

Chitinases belong to the pathogenesis-related proteins and hydrolyse chitin, a major component of fungal cell walls and insect exoskeletons. They accumulate in plants in response to pathogens (Graham and Sticklen, 1993) and different abiotic stressors including cadmium (Metwally *et al.*, 2003). An increase in chitinase activity in plants subjected to abiotic stresses may be a result of the induction of cross-tolerance via cross-talking signalling pathways. In the present study, treatment with Cd increased chitinase activity in roots and shoots of all pea genotypes and the highest induction was observed in roots (hydroponic

Table 1. *Concentration of nutrient elements in pea genotypes grown in the absence (upper row) and presence (lower row) of 5 μM cadmium*

Data are given in mg g^{-1} DW for P, K, S, Mg, and Ca, and in μg g^{-1} DW for Zn, Mn, and B. Differences between means of Cd-treated and untreated plants are significant at $P < 0.05$ (a), $P < 0.01$ (b), and $P < 0.001$ (c) for a given genotype or all genotypes, respectively (Fisher's LSD test, three-way MANOVA; four determinations from two experiments).

Genotype	Concentration of elements							
	P	K	S	Mg	Ca	Zn	Mn	B
Roots								
3429	13.5	66	10.1	1.8	2.9	788	19	12
	10.9 c	60 a	10.2	2.3 b	3.2	555 c	14 a	13
1658	12.8	63	16.0	2.2	2.5	656	38	12
	7.4 c	36 c	6.9 c	1.6 b	2.7	357 c	10 c	11
8456	10.9	66	12.8	1.2	2.9	799	13	14
	8.8 c	54 c	9.4 c	1.7 a	2.8	644 b	8 a	12
7128	11.9	72	17.0	2.0	3.1	777	17	13
	9.6 c	55 c	12.5 c	2.9 c	3.0	842	12 b	14
4788	10.0	62	8.4	1.4	2.6	579	11	15
	8.4 c	69 b	11.6 c	2.0 b	3.0	562	10	18 a
188	14.4	76	16.9	2.5	3.5	830	16	15
	8.8 c	63 c	13.8 c	2.7	3.4	787	12 a	15
All genotypes	12.2	68	13.5	1.9	2.9	738	19	14
	9.0 c	56 c	10.7 c	2.2 c	3.0	625 c	11 c	14
Shoots								
3429	7.3	40	3.7	2.4	3.6	196	19	12
	7.4	31 b	4.5	2.6	3.5	158	13 b	8 b
1658	7.7	37	4.5	2.6	3.1	137	23	13
	6.0 c	20 c	4.8	2.7	3.0	70 a	16 c	7 c
8456	6.3	32	4.8	2.7	4.0	232	16	15
	6.1	26 a	5.3	2.9	3.3 b	137	12 a	13
7128	7.5	36	6.0	3.5	5.1	284	18	20
	8.0	24 c	6.2	3.5	4.7	254	12 b	16 b
4788	5.7	30	3.8	2.7	3.7	191	16	13
	6.3	26	4.7	2.8	3.1 a	151	13	11
188	6.9	46	6.2	3.5	5.0	342	20	19
	7.7	31 c	8.0 a	3.7	5.3	312	13 c	9 c
All genotypes	6.9	37	4.8	2.9	4.1	231	19	15
	6.9	26 c	5.6 a	3.0	3.8 a	181 a	13 c	11 c

Table 2. *Summary of significant coefficients of correlation found between tolerance indices (TI) and biochemical characteristics of pea genotypes in their response to toxic Cd*

Correlations are significant at $P < 0.05$ (a), $P < 0.01$ (b), and $P < 0.001$ (c), respectively; *n* means the number of observations, and nc means that no correlation was found.

Characteristics	Root *TI* (%)	Shoot *TI* (%)	Total plant *TI* (%)
GSH content in Cd-treated roots, μM g^{-1} FW (*n*=10)	+0.77 b	+0.73 a	+0.87 c
GSH, % change (*n*=10)	+0.88 c	+0.69 a	+0.83 b
PC_{tent} content, $[PC_{tent}]=[NPT]-[GSH]$ (*n*=10)	−0.76 b	nc	nc
Chitinase in roots, % change (*n*=10)	−0.93 c	−0.64 a	−0.83 b
POX, % change (*n*=6)	−0.89 b	nc	−0.82 a
APX, % change (*n*=6)	−0.94 b	−0.89 a	−0.93 b

culture) and shoots (sand culture) of the more Cd-sensitive genotypes. The negative correlation between chitinase activity and Cd tolerance of pea genotypes probably reflects the increased relative level of stress caused by Cd in the more sensitive genotypes. This assumption is supported by recent data of Metwally *et al.* (2003) demonstrating that chitinase activity was not stimulated after the alleviation of Cd toxicity in barley seedlings by treatment with salicylic acid.

Lipid peroxidation

Lipid molecules in general and unsaturated lipids in particular are sensitive to oxidation by reactive oxygen species (ROS) generated under stress conditions. Consequently, the presence of elevated levels of lipid peroxides is generally accepted as an indicator of severe oxidative stress. The induction of oxidative stress in plants by heavy metal stress has been extensively documented (Dietz *et al.*, 1999), and the accumulation of ROS and increased lipid peroxidation in pea caused by Cd toxicity were also reported (Lozano-Rodriguez *et al.*, 1997; Romero-Puertas *et al.*, 1999; Sandalio *et al.*, 2001). Among the pea genotypes, the less sensitive line 3429 showed a lower level of lipid peroxides in roots compared with the highly sensitive genotypes 4788 and 188, suggesting that oxidative stress caused by Cd varied between pea genotypes. At the same time, the maximum MDA content was observed in roots of genotype 8456 that was characterized by relatively high *TI*s, indicating that this genotype has a particularly high ability to counteract or to cope with the oxidative stress. The latter explanation is more likely, since the increased MDA content was not associated with specifically high glutathione concentrations or the induction of antioxidative enzymes such as catalase, POX, and APX in this genotype.

Antioxidative enzymes

Oxidative stress caused by Cd in plants leads to increased expression and activities of antioxidative enzymes such as POX, APX, and catalase (Chaoui *et al.*, 1997; Metwally *et al.*, 2003; Skorzynska-Polit *et al.*, 2003/4). Skorzynska-Polit *et al.* (2003/4) also showed that antioxidant activities change depending on enzyme and external Cd concentration. However, at very high heavy metal toxicity the activities of POX and APX were inhibited (Sandalio *et al.*, 2001; Luna *et al.*, 1994). In particular, catalase activity often decreased following exposure to elevated Cd concentrations (Dalurzo *et al.*, 1997; Sandalio *et al.*, 2001; Fornazier *et al.*, 2002). There is also evidence that a decrease in catalase activity may occur as a result of oxidative stress in different plant species (Shim *et al.*, 2003).

In roots, the increase in POX activity correlated with the Cd-sensitivity of the pea genotypes, supporting previous suggestions to use POX activity as a biomarker for metal toxicity in plants (Radotic *et al.*, 2000). Opposite responses were observed for root APX to Cd in the less and more Cd-sensitive pea genotypes. Such a response may be related to the higher oxidative stress occurring in the more sensitive genotypes. On the other hand, the observed genotypic differences in APX activity may be associated with

differences in the effect of Cd on GSH concentrations in the treated plants. APX is a key enzyme in the so-called ascorbate/glutathione cycle, which is a major hydrogen peroxide-detoxifying system in plant chloroplast and cytosol (Asada, 1992). APX-mediated detoxification of H_2O_2 is coupled with ascorbate oxidation. Oxidized ascorbate is then regenerated via the oxidation of glutathione. Therefore, it is interesting that glutathione contents were decreased in the more sensitive genotypes whereas APX activity increased. These observations support the hypothesis that the sensitive genotypes experience more oxidative stress and rely on a more active ascorbate/glutathione cycle. Activity of catalase significantly increased in all pea genotypes after treatment with Cd, and no correlation was observed between catalase activity and growth parameters, Cd uptake, and biochemical changes caused by Cd in plants, respectively. Catalase activity is a function of ROS-triggered gene activation and Cd-induced enzyme inhibition (Dalurzo *et al.*, 1997; Sandalio *et al.*, 2001; Fornazier *et al.*, 2002; Shim *et al.*, 2003). Romero-Puertas *et al.* (2002) showed that in leaves of Cd-treated pea plants catalase was oxidized and subjected to increased proteolytic degradation caused by Cd toxicity. The complex interaction between both counteracting processes may account for the absence of a correlation between *TI* and catalase activity. This is the first report describing such intraspecific genotypic variation in the response of antioxidant enzymes to heavy metal toxicity in a large(r) set of diverse genotypes. Such analysis was restricted to two parameters (POX and superoxide dismutase) in only two barley genotypes (Guo *et al.*, 2004), excluding the possibility of identifying significant correlations and to evaluate intraspecific diversity.

NPT and glutathione

Cadmium binding to sulphydryl groups of phytochelatins (PCs) is a fundamental component mechanism of Cd detoxification (Prasad, 1999; Sanita di Toppi *et al.*, 2002). PCs are synthesized from glutathione, which also serves numerous other physiological functions in plant metabolism such as storage and transport of sulphur, the regulation of gene expression and synthesis of proteins, the decrease of oxidative stress via inactivation of AOS, and binding of toxic metals (Bae and Mehra, 1997; May *et al.*, 1998; Dietz, 2003). Therefore, glutathione plays a significant role in various biochemical processes related to the adaptation of plants to stressful conditions via counteracting the toxic effects of heavy metals.

Although the role of PCs in heavy metal detoxification is well documented (Hall, 2002), some studies have shown that increased tolerance is not explained by the accumulation of PC-heavy metal complexes. For example, the synthesis of PCs in *Silene vulgaris* as induced by toxic metal concentrations was increased to a greater extent in Cd-sensitive than in Cd-tolerant genotypes (de Knecht *et al.*, 1994), as well as in Zn-sensitive compared with Zn-tolerant genotypes (Harmens *et al.*, 1993). Similar results were observed in roots of maize seedlings exposed to Cd (Tukendorf and Rauser, 1990). Here, pea genotypes responded to Cd toxicity with a significant increase in sulphydryl groups (NPTs) that was not correlated with their tolerance to Cd. Moreover, in accordance with the observations of Harmens *et al.* (1993), the accumulation of PCs were negatively correlated with root tolerance to Cd and positively correlated with an increase in chitinase and APX activities. Therefore, it can be excluded that the more Cd-tolerant genotypes had increased PCs synthesis, and that PCs were responsible for the distinct degree of Cd tolerance.

Formation of Cd-GSH and Cd-PC complexes reduces free Cd-concentration in the cytoplasm and contributes to suppress the activation of stress-related responses in plant metabolism. Both reactions contribute to a depletion of cellular GSH. Decreased GSH contents in pea plants treated with Cd have been reported repeatedly (Ruegsegger *et al.*, 1990; Klapheck *et al.*, 1995). Pea is a rather Cd-sensitive plant species and shows very low GSH levels in the presence and absence of toxic Cd when compared with other plant species (Obata and Umebayashi, 1993). In the present study the more Cd-sensitive pea genotypes had decreased root GSH concentrations, whereas the less sensitive genotypes had increased root GSH concentrations in response to Cd treatment. This result is in agreement with observations made by De Knecht *et al.* (1994) that GSH concentrations are higher in Cd-tolerant genotypes than in Cd-sensitive genotypes of *Silene vulgaris*. These results suggest that GSH availability was not a critical factor limiting PC formation in the less sensitive pea genotypes. However, the GSH depletion could weaken the cellular antioxidative response and defence strength against stress in the more sensitive genotypes, and thereby contribute to the genotypic differences in Cd sensitivity.

Element composition

Significant differences between pea genotypes in Cd accumulation in roots and shoots were observed in Cd-treated plants grown in hydroponic culture. Although a correlation of Cd concentration and *TI*s of genotypes was insignificant, the less sensitive genotypes were characterized by lower translocation of Cd from roots to shoots. These results are in agreement with previous findings showing a complex and genotype-dependent relationship between Cd accumulation and Cd tolerance in pea. From that two tentative hypotheses can be derived: (i) the ability of some pea genotypes to prevent excessive uptake of Cd in shoots may partially account for their tolerance to this toxic metal, (ii) however, some more tolerant genotypes accumulate Cd at high concentrations (Belimov *et al.*, 2003). Particular, a high root-to-shoot translocation and high shoot Cd contents in genotype 7128 (described here) and in genotype 3273 (Belimov *et al.*, 2003), could result in strong inhibition of

shoot and root growth, respectively. An increased Cd tolerance of the genotype 1658 could partially be the result of low accumulation and translocation of Cd.

It is known that Cd and other heavy metals affect the element composition of plants, mostly by inhibiting the normal uptake and utilization of macro- and micronutrients (Brune and Dietz, 1995; Krupa *et al.*, 2002). In the present study, significant decreases in the concentrations of P, K, S, Ca, Zn, Mn, and B in plants treated with Cd were observed. At the same time concentration of Mg in roots and S in shoots increased. For the most part, the changes in element concentrations in the pea genotypes studied correlated with previous findings describing Cd-induced disturbances in the element composition of pea (Sandalio *et al.*, 2001) and barley (Metwally *et al.*, 2003) as outlined in the Results section. These distinct observations, as well as the positive correlation between shoot Cd and some nutrients, suggest that the differences in Cd tolerance are not or not exclusively explained by genotypic compensation of Cd-induced disturbances in element composition.

Conclusion

Pea lines express significant variation in growth response to toxic Cd. Physiological and biochemical responses to Cd varied between the different pea genotypes, suggesting that diverse mechanisms contribute to the specific Cd tolerance level of different genotypes. Apparently, independent and multi-factorial reactions modulate Cd sensitivity on the low-tolerance level of plants such as pea. Correlations established between growth parameters and metabolic responses such as GSH and MDA contents, chitinase, POX, and APX activities indicate as expected that the Cd-induced stress is more pronounced in the more Cd-sensitive genotypes. In addition to the suitability of these biochemical determinants as molecular and biochemical markers that indicate the stress level induced by toxic metals, the results suggests that oxidative stress is a significant factor in Cd toxicity expression as has been demonstrated by other authors in pea plants (Sandalio *et al.*, 2001; Romero-Puertas *et al.*, 2002). These conclusions are supported by the finding that presoaking of pea seeds with salicylic acid similar to previous experiments in barley (Metwally *et al.*, 2003) partly relieved Cd inhibition of growth in the sensitive lines 188 and 4788, but not in the more tolerant line 3429, although salicylic acid tissue levels were similar (A Metwally, unpublished results). The differential effect points to distinct regulatory networks in the pea genotypes that contribute to establish the particular level of Cd sensitivity.

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