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ORIGINAL PAPER

Cadmium effects on populations of root nuclei in two pea genotypes inoculated or not with the arbuscular mycorrhizal fungus *Glomus mosseae*

Ombretta Repetto · Nadia Massa · Vivienne Gianinazzi-Pearson · Eliane Dumas-Gaudot · Graziella Berta

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Abstract Plants possess a broad range of strategies to cope with cadmium (Cd) stress, including the arbuscular mycorrhizal (AM) symbiosis. In cell responses towards Cd, the contribution of changes in ploidy levels is still unclear. We used flow cytometry to investigate if nuclear ploidy changes are involved in response mechanisms toward Cd and to analyze the effect of the symbiotic status on populations of nuclei. The impact of Cd was investigated in roots of two pea (*Pisum sativum* L.) genotypes differing in their Cd-sensitivity (Cd-sensitive VIR4788 and Cd-tolerant VIR7128). In pea seedlings grown under hydropony, 25 and 250 μM Cd concentrations lead to an increase in 4 C together with a decrease in 2 C nuclei. The same genotypes, grown in soil/sand substrate, were inoculated or not with the AM fungus *Glomus mosseae* BEG12 and treated or not with Cd at transplanting (Cd_1) or 2 weeks after (Cd_2). The Cd_2 increased the proportion of 6 and 8 C nuclei in the mycorrhizal VIR4788 and in the non-mycorrhizal VIR7128 genotypes. Thus, changes in ploidy levels reflect pea responses towards Cd, which are modulated by the symbiotic interaction. The Cd-induced increase in ploidy may account for changes in DNA transcription and/or translation.

O. Repetto · N. Massa · G. Berta
Department of Environmental and Life Science,
University of Piemonte Orientale 'Amedeo Avogadro',
Via Bellini 25G,
15100 Alessandria, Italy

O. Repetto · V. Gianinazzi-Pearson · E. Dumas-Gaudot (✉)
UMR 1088 INRA/CNRS5484/UB, PME
(Plante-Microbe-Environnement) INRA-CMSE,
Domaine d'Epoisses,
BP 86510,
21065 Dijon Cedex, France
e-mail: dumas@epoisses.inra.fr

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Abbreviations

AM	arbuscular mycorrhiza
Cd	cadmium
DAPI	4′6 diamidino-2-phenylindole
DNA	deoxyribonucleic acid
HM	heavy metal
FW	fresh weight
ANOVA	analysis of variance
SE	standard error

Introduction

Pea (*Pisum sativum* L.) is an important crop plant, establishing beneficial root interactions with microbes including arbuscular mycorrhizal (AM) fungi of the phylum Glomeromycota (Schussler et al. 2001). Colonization by these fungi induces several changes in root architecture and longevity, together with nuclear modifications at the level of shape, position, and chromatin organization (Lingua et al. 2001a,b; Berta et al. 2002). Different beneficial roles, including an enhanced plant tolerance to heavy metals (HMs), have been attributed to the AM symbiosis (Leyval et al. 2002; Rivera-Becerril et al. 2002; Liao et al. 2003). Plant associations with AM fungi are suggested as potential biological solution to ameliorate plant resistance to metal toxicity and restore fertility of soils polluted by HMs such as cadmium (Cd) (Vivas et al. 2005).

Cadmium is a highly toxic pollutant of large environmental concern. Different sources, both natural and anthropogenic, are responsible for its release into the

environment (Alloway 1990; Greger 1999; Sanità di Toppi and Gabbrielli 1999). Even in traces, this metal can cause serious health hazards to most living organisms of both eukaryotic and prokaryotic kingdoms (Duxbury 1985; Giller et al. 1998; Waalkes 2000; Palus et al. 2003). In plants, Cd may interfere with RNA metabolism (Marcano et al. 2002), induces chromosomal aberrations (Zhang and Xiao 1998; Fusconi et al. 2005), influences DNA integrity and triggers programmed cell death, leading to chromatin fragmentation (Fojtová and Kovarik 2000; Behboodi and Samadi 2004). In the last 20 years, several works have shown that specific biochemical pathways are seriously affected by Cd in pea plants (Chugh and Sawhney 1999a,b; Sandalio et al. 2001; Romero-Puertas et al. 2002; Romero-Puertas et al. 2004). Moreover, different molecules associated with pea responses towards Cd have been characterized (Klapheck et al. 1995; Lozano-Rodríguez et al. 1997; Mori and Leita 1998; Repetto et al. 2003). The AM symbiosis has recently been reported to modulate the expression of certain Cd-induced pea root proteins (Repetto et al. 2003).

In this context, a growing field of interest in plant biology is represented by the impact of environmental stresses on genome organization. Plant cell genome is characterized by a particular plasticity, which is related to genome amplification by polyploidization (Bachmann 1993; Soltis and Soltis 1995). This process is mainly due to either endomitosis or endoreduplication, and the corresponding polyploid cells possess a multiple doubling (2^n) of nuclear DNA (Joubès and Chevalier 2000). During endoreduplication, which occurs in more than 90% of angiosperms (D'Amato 1984), nuclei retain the capability of DNA replication without going through mitosis, so that cells do not divide and increase in ploidy level as well as in size and metabolic activity (Galbraith et al. 1991; Kondorosi et al. 2000). Among biotic factors leading to plant genome reorganization, symbiotic interactions have been shown to induce polyploidization (Berta et al. 2000; Kondorosi and Kondorosi 2004). Up to now, nuclear ploidy changes in plants exposed to Cd and their possible involvement in plant response/detoxification mechanisms towards Cd have received little attention. As ploidy can control gene expression and can be related to modifications in root differentiation (D'Amato 1998; Galitski et al. 1999), Cd-induced effects on plant root metabolism may be correlated with changes in DNA ploidy.

Therefore, the aim of the present work was to check by means of flow cytometry whether Cd exposure changes the proportion of nuclear populations with different ploidy in roots of two Cd-treated pea (*Pisum sativum* L.) genotypes VIR4788 and VIR7128, selected for their sensitivity and tolerance to Cd, respectively (Belimov et al. 2003; http://dainet.de/genres.vir). Moreover, we investigated if the symbiotic interaction with the AM fungus *Glomus mosseae* BEG12 modulates the Cd effects on pea nuclear populations. Results are discussed in relation to the genetic variation between the two pea genotypes and the possible AM-modulation of pea nuclear responses towards Cd.

Materials and methods

Plant material and growth conditions

The *Pisum sativum* L. Cd-sensitive VIR4788 (Mongolia) and Cd-tolerant VIR7128 (Daghestan) genotypes were obtained from the Pea World Collection of the Vavilov's Institute for Plant Industry (VIPI; St. Petersburg, Russia). Their responses towards Cd have been characterized at different levels (growth, nutrient uptake, genes, proteins, Cd accumulation) (Rivera-Becerril et al. 2002; Repetto et al. 2003; Metwally et al. 2005). Therefore, they represent potential plant models for analysing the Cd effects on pea plants.

Hydroponic culture

The Cd effects upon populations of root nuclei were first investigated in VIR4788 and VIR7128 pea genotypes grown under hydropony. Seeds were surface-sterilized as described by Repetto et al. (2003) and germinated in Petri dishes on sterile filter paper at 20°C/24°C in the dark. Three-day-old germinated seeds were individually transferred into 1-l plastic pots containing 0.5 l of mineralized water for control seedlings, or 0.5 l of a $CdCl_2$ solution for Cd-treated ones. Particular attention was given in placing seedlings of comparable root length in each pot.

Three increasing Cd concentrations were tested (i.e., 2.5, 25, and 250 μM). They were chosen in view of pilot experiments, which revealed that they affected primary root growth as well as mitotic activity of pea seedlings (Repetto et al. unpublished results). Ten replicates for each treatment were grown for 48 h in a growth chamber at 20°C/24°C day/night temperatures, under a 16-h day photoperiod at 175 μmol m^{-2} s^{-1} light intensity with 60% relative humidity. All solutions were replaced once at 24 h. After 48 h, pea seedlings were harvested, the length of primary roots measured and roots immediately processed to flow cytometry analyses.

Soil/sand culture

With the aim of studying the pea responses to longer Cd exposure and AM colonization, experiments were then carried out with a soil/sand substrate. Seeds were sterilized and germinated as described above. Five-day-old seedlings

were individually planted in 0.4-l pots containing 1:1 (v/v) soil/sand mix growth substrate for control plants. Soil (clay loam, pH 8.1, 16.6 g C kg^{-1}, 1.8 g N kg^{-1}, 26 mg Olsen P kg^{-1}, 0.9 μg Cd kg^{-1}) was steam-sterilized for 1 h at 100°C and for 30 min 3 days later. Sand (Special Aquarium, quartz sand 40/1, Torino, Italy) was washed and heat-sterilized for 2 h at 160°C.

Inoculation with *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe (BEG 12) was performed by incorporating 20% (v/v) of quartz sand-based *G. mosseae* BEG12 inoculum (mycorrhizal roots of *Sorghum*, spores and hyphae in quartz sand, BIORIZE, Dijon, France) into 30% (v/v) of quartz sand and 50% (v/v) of soil. For Cd-treated plants, a $CdCl_2 \cdot 2.5\ H_2O$ solution was added to a final amount of 100 mg Cd kg^{-1} substrate per pot (corresponding to around 5 μM $CdCl_2$ concentration). Cadmium was applied once at two different times, either at the time of transplanting (Cd_1) or 2 weeks later (Cd_2), as described by Repetto et al. (2003). The amount of bioavailable Cd after adding $CdCl_2$ solution was measured using a $CaCl_2$ extraction method (analysis performed by INRA-Arras, France). This gave a final concentration of 2 mg bioavailable Cd kg^{-1}, which is in agreement with previous data (Repetto et al. 2003). In the case of Cd-untreated plants, the soil/sand mix was humidified with sterile milli-Q water a day before planting.

Experimental treatments consisted of control (C), Cd-treated (Cd), *G. mosseae*-inoculated (Gm), *G. mosseae*-inoculated and Cd-treated (GmCd) plants. Six replicates for each treatment were grown under controlled conditions, as previously described. Pots were watered to saturation with a modified Long Ashton nutrient solution (Dumas-Gaudot et al. 1994) three times a week. Plants were harvested 5 weeks after transplanting and root samples were subjected to flow cytometry analyses. A sample of roots per mycorrhizal plant, treated or not with Cd, was randomly taken from the whole root system for estimation of mycorrhizal colonization after trypan blue staining (Trouvelot et al. 1986). The extent of root cortex colonization (M%) together with the frequency of colonized root fragments (F%), and arbuscule abundance in the whole root system (A%) were used as parameters of AM infection to assess the degree of *G. mosseae* intraradical colonization and its modulation upon Cd treatment.

Flow cytometry

Nuclear extraction and sample preparation

Nuclei from Cd-untreated and treated seedlings grown under hydropony (ten seedlings per treatment) were extracted from primary roots deprived of the apices (about 0.07 g of root fresh weight "FW" per seedling), and therefore only root differentiated zones were considered. To avoid any nucleus disturbances and preserve DNA integrity, all operations were carried out on ice. Samples were individually chopped with a razor blade in about 1.5 ml extraction solution (0.1 M citric acid, 0.5% [w/v] Tween 20) and incubated in this solution for 30 min. The resulting suspensions were filtered through a 20-μm nylon mesh, centrifuged (1,800 rpm for 14 min) on a 1.5-M sucrose cushion, and the pellet was resuspended in 1 ml of 0.1 M phosphate-buffered saline (PBS, pH 7.4). Nuclear suspensions were then adjusted with PBS to obtain 10^4 nuclei ml^{-1} per sample, stained with DAPI at a saturating concentration (5.6 μM) and kept in the dark for 5 min before flow cytometry analysis. Nuclei of chicken erythrocytes (DNA content equal to 2.3 pg) were used as internal biological standard (IS) for each sample. In this condition, after detecting the DNA fluorescence intensity (FI) of the IS, the FI value relates to DNA content and for each nuclear population it is thus possible to calculate its DNA content from the corresponding FI value (Kapuscinski 1995).

Nuclei from Cd-treated and untreated plants grown in soil/sand cultures (six plants per treatment) were extracted from a standard amount of root systems (1.5 g of root FW per plant), which was randomly taken from the whole root system deprived of the apices. Nuclear extraction and sample preparation were performed as previously described, with the exception that after filtration nuclear suspensions were fixed [3:1 (v/v) ethyl alcohol/acetic acid solution] and kept at −20°C until flow cytometry analyses and that 10^5 nuclei ml^{-1} were analyzed per sample.

Data acquisition and histogram analysis

The Partec PAS instrument (Partec GmbH, Münster, Germany), equipped with a mercury arc lamp, was used. UV excitation (DAPI excitation λ=359–461 nm) employed KG1, DUG11 filters, and a TK420 dichroic mirror. DAPI fluorescence was detected using a EM455 barrier filter (DAPI emission λ=460–488 nm). For evaluation, flow cytometry data were displayed in the form of frequency histograms, in which the counts of nuclei (*y* axis) are plotted against the relative FI expressed in logarithmic scale (*x* axis), and each peak corresponds to a population of nuclei with the same ploidy level (Givan 2001). For illustration, histograms for populations with different ploidy levels from pea nuclei are shown in Fig. 1. From histograms, three parameters were acquired: (1) the 'count of nuclei', calculated as peak integer; (2) the 'mean FI', that is the average of FI, and (3) the 'coefficient of variation, CV %', calculated as the ratio of the FI of the peak to the FI of the internal biological standard, providing information about data dispersion. For each peak, the 'percentage of

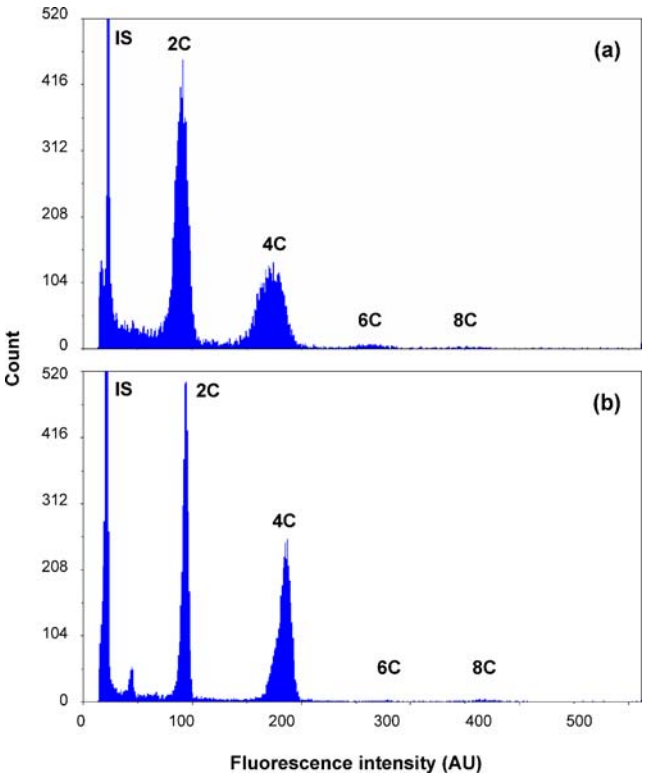

Fig. 1 Characteristic DNA histograms of the distribution of nuclei with different DNA content (2, 4, 6, and 8 C). DAPI-stained nuclei extracted from a standard amount of control roots (1.5 g of root fresh weight) of 5-week-old VIR4788 (**a**) and VIR7128 (**b**) pea genotypes. Count, *y*-axis: number of analysed nuclei; AU, *x*-axis: intensity of fluorescence (arbitrary units). IS internal standard (nuclei of chicken erythrocytes)

population of nuclei' was obtained as [(count of nuclei in the peak) × (total count of nuclei in the sample)$^{-1}$ × 100]. The mean FI and the CV% were estimated with FloMax software (Partec GmbH, Münster, Germany). The mean DNA content of each nuclear population was calculated as ratio between the mean FI of the peak and the FI of the internal standard.

Statistical analyses

All analyses were done with STATVIEW 4.5 software package (Abacus Concepts, Berkeley, CA, USA). Data were analyzed by one-way analysis of variance (ANOVA) followed by a monofactorial analysis with Fisher's protected least significant difference (PLSD) test. This test allows to determine which populations of data are significantly different from the others by performing pair-wise comparisons of the means. In the hydroponic experimental design, the populations of data analyzed were the four treatments (0, 2.5, 25 and 250 µM Cd). In the soil/sand experimental design, the populations of data corresponded to the six treatments (C, Gm, Cd_1, Cd_2, $GmCd_1$, and $GmCd_2$). The output variables analyzed were: (1) 'mycorrhizal colonization', (2) 'DNA content' and (3) 'population of root nuclei (2, 4, 6, or 8 C)'. For each of them, the Fisher PLSD test compared the variance of data from the treatments of the same pea genotype. Indeed, since the two pea genotypes showed significantly different DNA content values in each population of root nuclei (Results, Table 1), global statistical analysis was restricted within one genotype. All data are presented as means±standard error (SE). The level of significance for all analyses was set at 0.05 and differences were considered statistically significant at $P<0.05$.

Results

Mycorrhizal colonization

In the absence of Cd treatment, the intensity of root cortex AM colonization was not significantly different between VIR4788 and VIR7128 plants (M%=40.09±2.90 and 31.38±1.86). Compared to mycorrhizal plants grown without Cd, mycorrhizal development was significantly decreased by the addition of Cd_1 (M%=21.78±1.21 and 12.56±1.10 in VIR4788 and VIR7128, respectively),

Table 1 DNA contents in distinct populations of control root nuclei in pea

		DNA contents of nuclear populations			
Cultures	Genotypes	2 C	4 C	6 C	8 C
Hydropony	VIR4788	9.40±0.30 (6)^a	18.82±0.42 (6)^a	n.d.	37.13±0.87 (6)^a
	VIR7128	8.57±0.13 (6)^b	16.84±0.28 (6)^b	n.d	33.16±0.50 (6)^b
Soil/sand	VIR4788	9.60±0.10 (6)^a	18.61±0.28 (6)^a	28.26±0.37 (6)^a	37.11±0.48 (6)^a
	VIR7128	8.82±0.11 (6)^b	17.01±0.28 (6)^b	26.07±1.23 (2)^b	34.14±1.35 (2)^b

Nuclei of the Cd-sensitive VIR4788 and Cd-tolerant VIR7128 pea genotypes grown in hydropony or soil/sand cultures were extracted from roots and the DNA contents were determined for each nuclear population (2, 4, 6, and 8 C), as described in Materials and methods. DNA content data are expressed in picograms (pg). Values are the mean±SE of six different plants (n=number of counts in parentheses). In each column, different superscripts denote DNA content values significantly different between the VIR4788 and VIR7128 genotypes within the same culture at the $P<0.05$ level. *n.d.* not detected

while it was not significantly affected by Cd_2 in both pea genotypes (M%=39.07±2.18 and 29.21±3.11 in VIR4788 and VIR7128, respectively). This was also observed for the two other mycorrhizal parameters (F% and A%).

Nuclear population and DNA content analysis

Whatever the pea genotype and the plant growth conditions, flow cytometry analysis of the extracted nuclei gave characteristic peaks, the lowest one corresponding to 2 C nuclei and the others to 4 and 8 C nuclei. Only 5-week-old plants grown in soil/sand substrates showed the existence of an additional population of root nuclei with a 6 C ploidy level (Fig. 1). Among the different populations of nuclei, those with the 4 and the 2 C ploidy levels were predominant in 5-day-old seedlings from hydropony and 5-week-old plants from soil/sand cultures, respectively.

Whenever the culture, significant differences were found in the DNA content (pg) of each population of nuclei between control roots of the two pea genotypes (Table 1). The DNA content values of control root nuclei did not vary depending on plant age and growth conditions, the VIR4788 genotype being always characterized by higher DNA content values in comparison to VIR7128 (Table 1). The VIR4788 and VIR7128 genotypes grown in soil/sand substrate differed for the relative DNA abundance in their populations of root nuclei even after Cd and/or Gm treatments. Moreover, the VIR7128 genotype showed a lower amount of 6 and 8 C populations in C, Cd_1, and $GmCd_1$ roots compared to those observed in the VIR4788 genotype (data not shown).

In primary roots of pea seedlings grown in hydroponic cultures, the lowest Cd concentration of 2.5 μM only modified the populations of nuclei in the VIR4788 genotype, in which an increase in 4 C nuclei was observed (Fig. 2, 2.5 μM). On the contrary, the other two concentrations of 25 and 250 μM modified the distribution of nuclei and led to a decrease in 2 C together with an increase in 4 C nuclei. This was also true for the VIR7128 pea genotype (Fig. 2, 25 and 250 μM). On the contrary, the

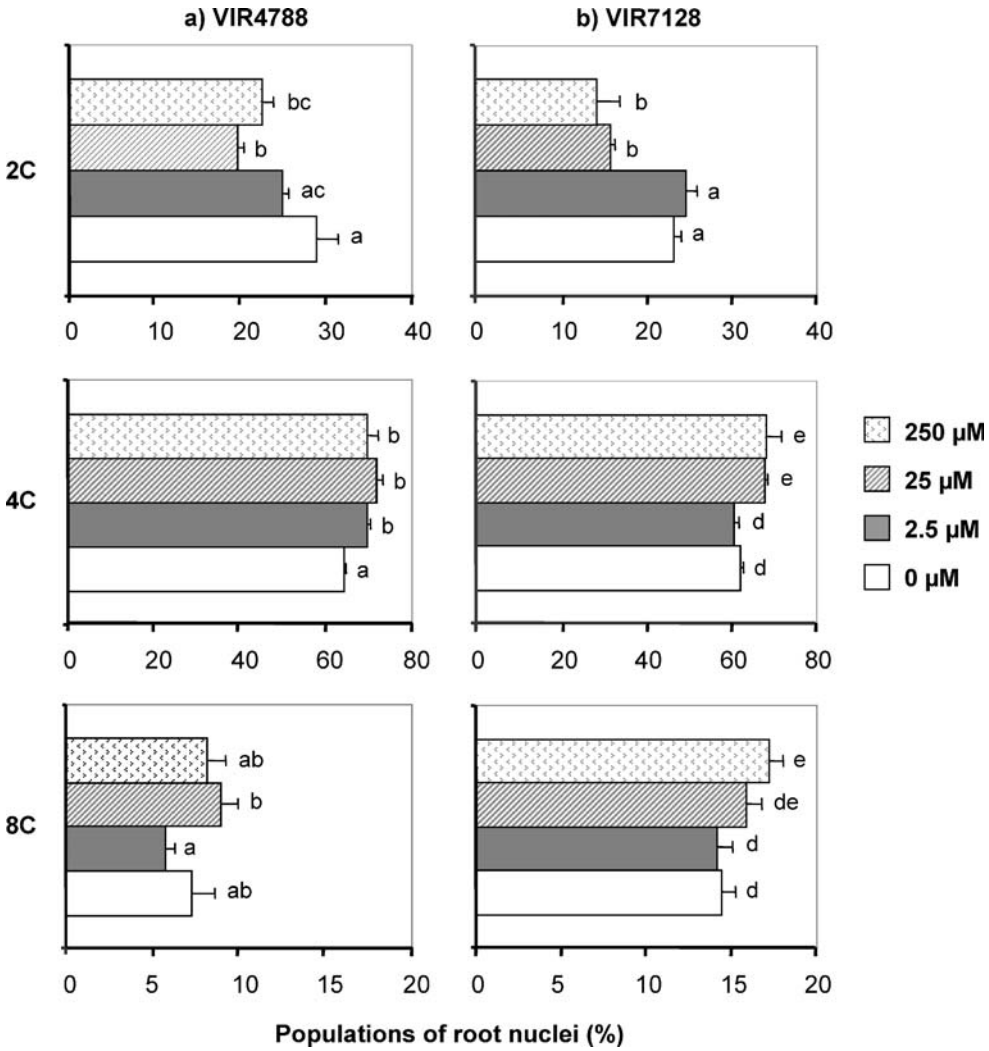

Fig. 2 Effects of cadmium (Cd) on the populations of root nuclei in the Cd-sensitive VIR4788 (**a**) and Cd-tolerant VIR7128 (**b**) pea genotypes from hydroponic culture. The effects of three Cd concentrations (2.5, 25, and 250 μM) were evaluated on the 2, 4, and 8 C populations of nuclei in differentiated zones of primary roots (10^4 nuclei ml^{-1} per sample). Data are the average of ten repetitions. Means of the same genotype followed by different letters are significantly different ($P<0.05$) for 2, 4, 6, or 8 C data. *x*-axis: root population nuclei %. *Bars* indicate SE

VIR7128 only showed Cd-induced modifications in 8 C nuclei after treatment with the highest Cd concentration of 250 μM (Fig. 2, 250 μM).

In 5-week-old plants, the distribution of nuclear populations was differentially affected by inoculation with *G. mosseae* depending on the pea genotype, with a decrease in 2 C and an increase in 4 and 8 C nuclei in VIR4788, and an increase in 6 C nuclei in VIR7128 (Fig. 3, Gm). Moreover, mycorrhization enhanced, although not significantly, 8 C nuclei in VIR7128 (Fig. 3b, Gm). While nuclear populations of both genotypes were not affected by Cd_1 treatment in 5-week-old plants, they were significantly influenced by Cd_2 application. In Cd_2-treated nonmycorrhizal roots, a reduction of 2 C and an increase in 4 C nuclei were found in VIR4788 (Fig. 3a, Cd_2), while a significant increase in 6 and 8 C nuclei was observed in VIR7128 (Fig. 3b, Cd_2). An increase in both 6 and 8 C nuclei was observed in roots of mycorrhizal Cd_2-treated plants of

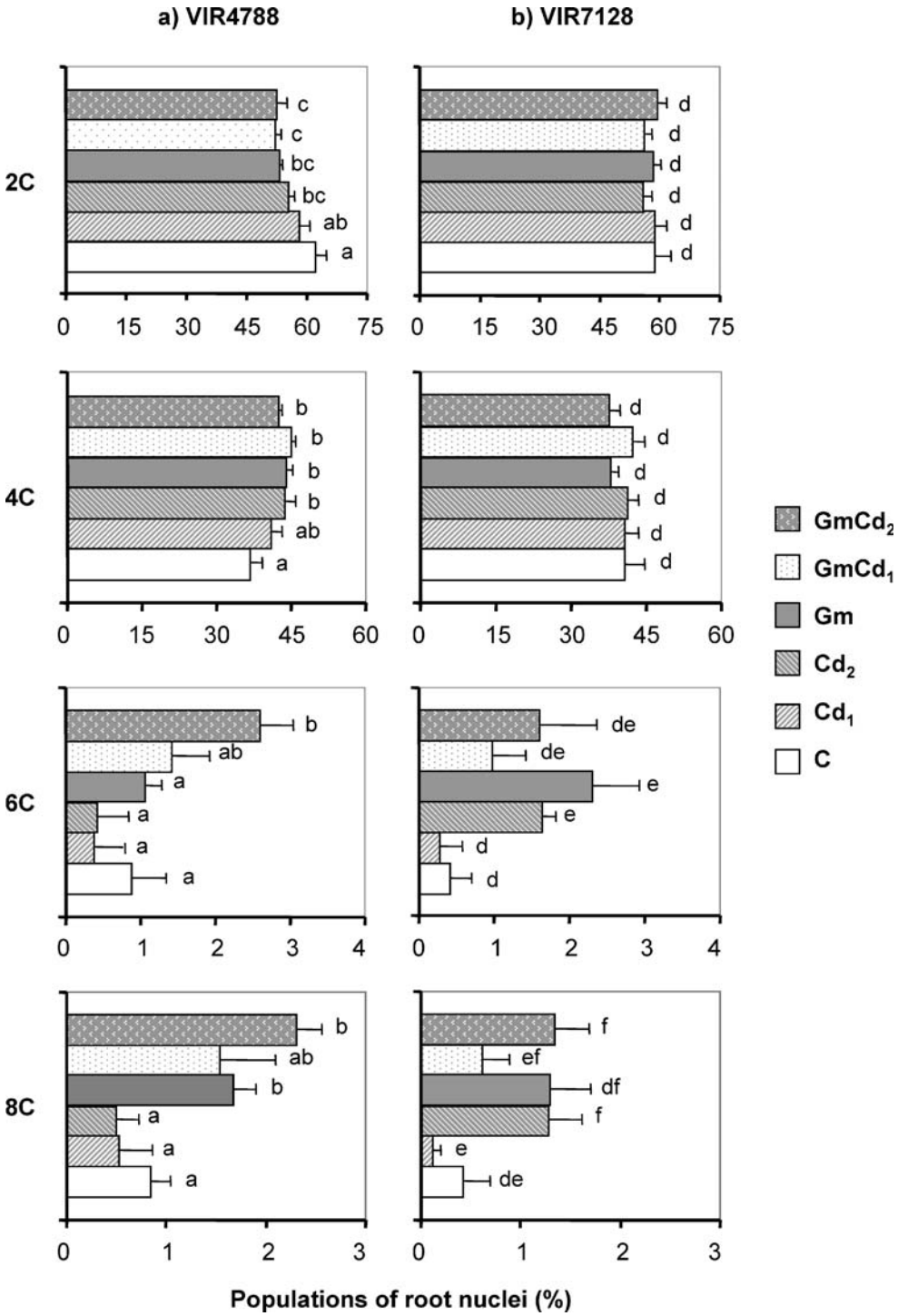

Fig. 3 Effect of cadmium (Cd) and mycorrhization on the percentages of 2, 4, 6, and 8 C nuclei populations of roots (10^5 nuclei ml^{-1} per sample) of the Cd-sensitive VIR4788 (**a**) and Cd-tolerant VIR7128 (**b**) pea genotypes from soil/sand culture. Plants were untreated (C) or treated with 100 mg kg^{-1} cadmium at transplanting (Cd_1) or 2 weeks after transplanting (Cd_2), and inoculated with *Glomus mosseae* in the absence (Gm) or in the presence of Cd ($GmCd_1$ and $GmCd_2$). Data are the average of six repetitions. Means of the same genotype followed by different letters are significantly different ($P<0.05$) for 2, 4, 6, or 8 C data. *x*-axis: root population nuclei %. *Bars* indicate SE.

VIR4788 compared to nontreated mycorrhizal roots (Fig. 3a, Gm, $GmCd_2$), and *G. mosseae* colonization enhanced both 6 and 8 C nuclei in Cd_2-treated plants of VIR4788 compared to nonmycorrhizal ones (Fig. 3a, Cd_2, $GmCd_2$). No interactions were observed between mycorrhiza and Cd_2 treatments in the VIR7128 pea genotype.

Discussion

Whatever the pea genotype, root exposure to Cd_1 inhibited *Glomus mosseae* intraradical colonization. This is in agreement with our previous results (Repetto et al. 2003) and with findings showing that heavy metals can delay, reduce, or even eliminate spore germination (Weissenhorn et al. 1993; del Val et al. 1999), confirming the stronger inhibitory effect of the metal when applied concomitantly to the fungal inoculum. In spite of the negative impact of Cd_1 on *G. mosseae* development in pea plants, the presence of mycorrhiza attenuated the inhibitory effect of Cd_1 on root biomass of the Cd-sensitive VIR4788 pea genotype (data not shown). This result agrees with previous observations (Rivera-Becerril et al. 2002; Repetto et al. 2003), and may argue in favour of a protective effect of AM symbiosis towards Cd in pea plants. Different mechanisms have been proposed to explain mycorrhiza alleviation of Cd stress (Joner and Leyval 2001; Rivera-Becerril et al. 2002; Schutzendubel and Polle 2002), but the exact molecular pathways involved in this protection remain unknown.

Flow cytometry analyses showed that in both cultures, the two pea genotypes possessed four distinct populations of nuclei with different DNA content (2, 4, 6, and 8 C), the 2 C population being predominant. This partly agrees with previous findings from Wojtyla-Kuchta and Gabara (1991), where 2, 4, 8, and 8–16 C populations were observed in differentiated root zones of *P. sativum* cv. De Grace. In contrast, only 2 and 4 C nuclei were detected by Sgorbati et al. (1993) in roots of three other *P. sativum* cultivars. The DNA contents of the four populations of root nuclei differed between the two pea genotypes, the VIR4788 being characterized by higher DNA content values. Interestingly, we report in this paper for the first time the existence of 6 C nuclei. Nuclei of 6 C DNA content resulting from autopolyploidization have been observed within populations of *Dianthus* species (Weiss et al. 2002). The DNA content of nuclei with 6 C ploidy level may be ascribed to extra rounds of DNA replication. As endoreduplicated nuclei possess a $(2\ C)^n$ DNA content (where C is the haploid DNA content) (Joubès and Chevalier 2000), it seems unlikely that 6 C nuclei originate from endoreduplication and, therefore, further research will be necessary to interpret their presence.

Cadmium effects upon these populations of root nuclei were first investigated in 3-day-old pea seedlings submitted to a range of metal concentrations under hydroponic short-term culture, where Cd inhibitory effects on primary root growth were more evident. Cd differentially affected the populations of root nuclei, depending on both its concentration and the pea genotype. The lowest Cd concentration of 2.5 μM only modified the populations of nuclei in the Cd-sensitive VIR4788, in which an increase in 4 C nuclei was observed. On the contrary, the other two concentrations of 25 and 250 μM modified the distribution of nuclei and led to a decrease in 2 C together with an increase in 4 C nuclei whatever the pea genotype, possibly by a mechanism of restitutional mitosis, as observed by Fusconi et al. (2005) in a short experiment on another pea cultivar treated with a number of Cd concentrations.

The Cd-induced modifications in 8 C nuclei were only observed in the Cd-tolerant VIR7128 genotype after treatment with the highest Cd concentration of 250 μM. The decrease in 2 C with the increase in 4C as well as the increase in 8 C nuclei observed in the tolerant genotype may be somehow involved in Cd response/tolerance mechanisms. The sensible genotype responds more weakly to Cd application (4 C nuclei), while the tolerant one more strongly increases the ploidy level of its nuclei (8 C). A large pool of 8 C cells as well as the occurrence of 8-16 C DNA and an increase in nuclear size have been previously reported in 6-day-old roots of *P. sativum* cv. De Grace treated with 10^{-4} M $CdCl_2$ for 24 h under hydropony (Romaniuk and Gabara 1988; Wojtyla-Kuchta and Gabara 1991).

In pea plants grown in soil/sand long-term experiment, the pattern of populations in root nuclei was again differentially influenced by Cd_2 application depending on the pea genotype. A reduction in 2 C together with an increase in 4 C nuclei characterized the Cd-sensitive VIR4788, while an increase in 8 C nuclei occurred in the Cd-tolerant VIR7128. Interestingly, pea nuclear ploidy was not affected by Cd_1 in spite of its inhibitory effects upon root growth. However, the two Cd administrations were applied to root systems characterized by different growth rates and differentiation programs, which are known to affect cell cycle (Larkins et al. 2001). Therefore, this differential response of root nuclei to Cd may reflect a variation in cell cycle sensitivity to the metal, depending on the pea developmental stage at which Cd stress occurred. Cadmium effects on the expression of genes regulating cell division have been recently shown (Yeh et al. 2004; Sobkowiak and Deckert 2003).

However, to our knowledge, there are no reports about Cd stress and a regulation of genes known to be involved in endocycle control (Joubès and Chevalier 2000). The differential response of root nuclei towards Cd application

in the two pea genotypes may result from differences in their root system structure, which has been shown to play a role in plant tissue protection against Cd toxicity (Lux et al. 2004). Interestingly, in contrast with biotic stresses such as pathogen attacks that increase 2 C root nuclei (Lingua et al. 2001a, 2001b), Cd stress never increased the percentage of this nucleus population. This suggests that biotic and abiotic stresses have different impacts on nucleus populations.

Endoreduplication has long been thought to be linked to cell metabolic activity (D'Amato 1998), and more recent findings have shown that polyploidy genomes are not always a sum of their constituent genomes but products of dynamic genetic and epigenetic changes, resulting in alterations of gene expression without a change in DNA sequencing (Madlung et al. 2005). Pea plants respond to Cd by an increased synthesis of specific molecules, including phytochelatins, glutathione, and stress proteins (Klapheck et al. 1995; Lozano-Rodríguez et al. 1997; Mori and Leita 1998; Repetto et al. 2003). Therefore, it may be hypothesized that the observed Cd-induced increase in nuclei with the highest ploidy levels may be related to a larger nuclear activity in root cells, leading to an active synthesis of molecules involved in response/detoxification mechanisms towards Cd. In this context, the transcription of a specific subset of genes has been shown to be activated in pea root responses towards Cd (Rivera-Becerril 2003; Sävenstrand and Strid 2004).

In plants, increased nuclear ploidy levels frequently result in increased cell/organ cell size (Sugimoto-Shirasu and Roberts 2003). An increase in cell elongation was observed after Cd treatment in other plants than pea (Bisoka et al. 2003; Kuthanova et al. 2004), but the relative variation in nuclear DNA contents was not considered. Furthermore, a role of endopolyploid cells in repairing DNA damage has been reported in p53 mutant tumor cells subjected to irradiation (Ivanov et al. 2003), and Cd genotoxic effects are well documented in plants (Behboodi and Samadi 2004; Gichner et al. 2004). Consequently, the increased number of nuclei with endopolyploid nuclei we observed in the present study in Cd-treated roots may be somehow related to a plant response counteracting Cd-induced damage to DNA. Recent observations of mitotic aberrations in Cd-treated pea seedlings grown in hydropony may support this hypothesis (Fusconi et al. 2005).

In plants inoculated with *G. mosseae*, modification in the populations of root nuclei was found to be an AM-symbiosis-responsive parameter. The proportion of nuclei with different DNA content of AM roots differed between the two genotypes. While increases in both 6 and 8 C nuclei may be a general response to mycorrhization in pea, the decrease in 2 C nuclei appears to be characteristic of the Cd-sensitive VIR4788 since no modifications in 2 or 4 C nuclei were observed in mycorrhizal roots of the Cd-tolerant VIR7128. This differential response of the two pea genotypes towards AM symbiosis may partly result from their nuclei having different DNA content and partly from their genetic variability (Belimov et al. 2003; Metwally et al. 2005). As the decrease in 2 C nuclei observed in mycorrhizal VIR4788 pea plants was concomitant with an increase in 4 C nuclei, it may result from a mycorrhiza-mediated up-regulatory effect on the endocycle, leading to both 4 and 8 C nuclei. An increase in 8 C nuclei, resulting from a direct fungus-mediated increase in metabolic activity in arbuscule-containing cortical cells, was also found in *G. mosseae*-colonized roots of *Lycopersicon esculentum* (Berta et al. 2000). However, the nature of 4, 6, and 8 C nuclei and, indirectly, the putative process leading to their DNA augmentation after *G. mosseae* inoculation need to be better defined by *in situ* cytometric analyses of pea roots.

The effects of Cd_2 application in presence of *G. mosseae* were only significant in the Cd-sensitive VIR4788, where an increase in both 6 and 8 C nuclei was observed. As the VIR4788 genotype was more dependent than VIR7128 on AM symbiosis for alleviation of Cd-induced stress, it is tempting to suggest that the increase in 6 and 8 C nuclei in the former may be related to pea response mechanisms towards Cd. The AM symbiosis was previously shown to particularly modulate the expression of Cd-induced proteins in the VIR4788 pea genotype (Repetto et al. 2003). In the present work, one-dimensional electrophoresis analyses showed the same AM-induced regulation of protein profiles in Cd-treated pea roots (data not shown). The AM protective effect in the Cd-sensitive VIR4788 pea genotype could result from a higher capacity of this genotype to respond to the AM symbiosis, compared to the Cd-tolerant VIR7128 one (Repetto et al. 2003; Rivera-Becerril 2003). The buffering effect of the AM symbiosis vis-à-vis Cd pollution, which has been linked to enhanced photosynthetic activity (Rivera-Becerril et al. 2002) and modulation of root protein profiles (Repetto et al. 2003) may also be related to an increase in nuclear populations with highest ploidy levels.

In conclusion, our study shows for the first time that differential responses to Cd occurred at the root DNA ploidy level, depending on: (1) the pea genotype, (2) the AM symbiotic status, and (3) plant growth conditions (hydropony vs soil/sand substrate; short-term vs long-term experiments). The Cd-sensitive VIR4788 and Cd-tolerant VIR7128 pea genotypes were characterized by a plastic genome. They both responded to Cd exposure by increasing the content of root nuclei with highest ploidy levels and these Cd-induced changes were modulated by the symbiotic interaction. A Cd-induced increase in 6 and 8 C nuclei was observed in the mycorrhizal VIR4788 and in the non-mycorrhizal VIR7128 pea genotypes.

Thus, changes in ploidy levels may be part of the basis of Cd tolerance in pea plants, a process that can be modulated by the AM symbiotic interaction. These changes in ploidy may be related to an increased transcription of genes leading to synthesis of proteins involved in response/detoxification mechanisms towards Cd toxicity.

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