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Molecular changes in *Pisum sativum* L. roots during arbuscular mycorrhiza buffering of cadmium stress

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Abstract Molecular responses to cadmium (Cd) stress were studied in mycorrhizal and non-mycorrhizal *Pisum sativum* L. cv. Frisson inoculated with *Glomus intraradices*. Biomass decreases caused by the heavy metal were significantly less in mycorrhizal than in non-mycorrhizal plants. Real-time reverse transcriptase–polymerase chain reaction showed that genes implicated in pathways of Cd detoxification varied in response to mycorrhiza development or Cd application. Expression of a metallothionein-encoding gene increased strongly in roots of Cd-treated non-mycorrhizal plants. Genes encoding γ-glutamylcysteine synthetase and glutathione (GSH) synthetase, responsible for the synthesis of the phytochelatin (PC) precursor GSH, were activated by Cd in mycorrhizal and non-mycorrhizal plants. Cd stress decreased accumulation of GSH/homoglutathione (hGSH) and increased thiol groups in pea roots, whether mycorrhizal or not, suggesting synthesis of PCs and/or homophytochelatins. An hGSH synthetase gene, involved in hGSH synthesis, did not respond to Cd alone but was activated by mycorrhizal development in the presence of Cd. Transcript levels of a glutathione reductase gene were only increased in non-mycorrhizal roots treated with Cd. Studies of three stress-related genes showed that a heat-shock protein gene was activated in mycorrhizal roots or by Cd and chitinase gene transcripts increased under Cd stress to a greater extent in mycorrhizal roots, whilst a chalcone isomerase gene was only up-regulated by Cd. Results indicate that although heavy metal chelation pathways contribute to Cd stress responses in pea, they may not make a major contribution to Cd tolerance strategies operating in the arbuscular mycorrhizal symbiosis.

Keywords Arbuscular mycorrhiza · Cadmium · Detoxification pathways · *Glomus intraradices* · *Pisum sativum* · Thiols

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Introduction

Large areas of land in western Europe are contaminated with heavy metals, including cadmium (Cd), zinc, lead and copper, and their persistence in soil can be of the order of thousands of years (McGrath et al. 2001). Although limit values of Cd have been established for the European Community at 2 mg kg^{-1} (http://www.ademe.fr/partenaires/boues/), Cd content of some polluted soils largely exceed this concentration and can range from 2 to 150 mg kg^{-1} (Wagner 1993; Dubois et al. 2002; Schützendübel and Polle 2002). Plant roots and associated rhizosphere micro-organisms, including saprophytes and mutualistic symbionts beneficial to plant growth and health, are directly exposed to heavy metal pollution in soils. The presence of pollutants can delay or severely reduce root colonization by arbuscular mycorrhizal (AM) fungi, and since these micro-organisms are common root symbionts

in polluted soils (del Val et al. 1999; Turnau et al. 2001), this could have profound effects on ecosystem function and sustainability (Cairney and Meharg 1999). The AM symbiosis has been reported to protect plants against abiotic stress induced by heavy metals, including Cd (Leyval et al. 1997, 2002; Rivera-Becerril et al. 2002). However, the way in which AM can modify metal pollution effects can vary greatly depending on plant growth conditions, the fungal partner and metal concentrations (Heggo et al. 1990; Leyval et al. 1997).

Most information about AM–heavy metal interactions has concerned symbiosis development and metal accumulation, and apart from a recent report of some protein modifications (Repetto et al. 2003), very little research has addressed the question of the molecular basis and physiology of the buffering effect of AM against such stress or the underlying mechanisms. The different strategies that have been proposed to explain avoidance or tolerance of heavy metal toxicity in AM fungi or the mycorrhizal symbiosis are based on nutritional or exclusion mechanisms: (a) increased nutrient supply to the plant by the symbiosis (Kaldorf et al. 1999; Meharg and Cairney 2000), (b) precipitation of metal by soil mycelium (Leyval et al. 1997), (c) metal absorption by extra-radical hyphae and spores (Joner and Leyval 1997; González-Chávez et al. 2002) and (d) heavy metal chelation in fungal cells or AM through the production of chelating molecules such as metallothioneins (MTs) and phytochelatins (PCs) (Leyval et al. 1997; Lanfranco et al. 2002). Previously, it was shown that increased Cd tolerance in 5- to 6-week-old AM pea plants colonized by *Glomus intraradices* or *Glomus mosseae* is characterised by enhanced photosynthetic activity and plant growth in comparison to non-mycorrhizal plants (Rivera-Becerril et al. 2002; Repetto et al. 2003). However, neither reduced Cd uptake nor improved P nutrition in the AM plants could explain this protective effect. The general paucity of information concerning Cd tolerance strategies operating in the AM symbiosis has prompted the present investigation into possible molecular mechanisms that may be operating against Cd in mycorrhizal root systems of pea. Recent studies have indicated modifications in protein metabolism that may be linked to such mechanisms (Repetto et al. 2003).

MT-encoding genes have been isolated from several plant species, including pea (Evans et al. 1990; Laplaze et al. 2002), and recent findings confirm that MT genes also occur in AM fungi (Lanfranco et al. 2002). PCs are synthesized from glutathione (GSH) in a reaction catalysed by a PC synthase (Cobbet and Goldsbrough 2000). Synthesis of GSH involves two steps (Matamoros et al. 1999): (1) a γ-glutamylcysteine (γEC) is formed from glutamate and cysteine by the enzyme γ-glutamylcysteinyl synthetase that is encoded by the gene *gsh1* and (2) a glycine group is added to the C-terminal site of the γEC by the enzyme GSH synthetase, encoded by the gene *gsh2*. Glutathione reductase (GR), an enzyme encoded by the gene *gr*, reduces GSH disulphide to form two GSH (Stevens et al. 1997). Some legumes, including pea, possess another thiol tripeptide, homoglutathione (hGSH), which partially or fully replaces GSH and is the precursor of homophytochelatins (hPCs) (Klapheck 1988; Frendo et al. 1999; Matamoros et al. 1999). hGSH is synthesized from γEC and β-alanine by a specific hGSH synthetase, encoded by the gene *hgsh2* (Moran et al. 2000). Up to now, no AM fungus is known to synthesize PC but high concentrations of GSH, its precursor, have been reported in the ectomycorrhizal fungi *Suillus bovinus* and *Paxillus involutus* (Schützendübel and Polle 2002).

The aim of the present work was therefore to gain insight into the molecular basis of Cd tolerance in AM by investigating plant genes encoding three stress-related proteins (chitinase, chalcone isomerase, heat shock protein 70), MT or enzymes of GSH and hGSH metabolism. Real-time reverse transcriptase–polymerase chain reaction (RT-PCR), based on specific primers designed from homologous pea sequences, has been used to compare plant gene expression in mycorrhizal and non-mycorrhizal roots of *Pisum sativum* L. cv. Frisson grown in the presence and absence of Cd. Accumulation of GSH/hGSH and thiol groups was also quantified in roots since the presence of plant thiol groups above GSH/hGSH level is recognized as an indicator of PC/hPC accumulation. The pea genotype cv. Frisson was chosen because of its high capacity for Cd uptake and the buffering effect by AM under Cd pollution previously observed in this plant (Rivera-Becerril et al. 2002).

Materials and methods

Growth conditions and sampling of plants

Seeds of *P. sativum* L. cv. Frisson (URLEG, INRA, Dijon, France) were surface-disinfected and germinated as previously described (Rivera-Becerril et al. 2002). Five-day-old seedlings were individually transplanted into 400 g of a 1:1 (v/v) soil/sand mix as growth substrate. Soil [clay loam, pH 8.1 (in H_2O), 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 γ-irradiated (10 kGy) and heat sterilized 4 h at 180°C. Sand (Special Aquarium, Quartz, Nr. 3, Zolux, France) was washed and autoclaved three times for 1 h at 121°C.

Experimental treatments (four replicates each) consisted of (a) inoculation with *G. intraradices* Schenck and Smith (BEG 141), (b) added Cd, (c) *G. intraradices* + Cd and (d) control plants (no inoculation with *G. intraradices*, no added Cd). Ground $CdCl_2 \cdot 2.5H_2O$ was added to the soil/sand substrate to obtain 100 mg Cd kg^{-1}, and the mix was humidified for 12 h before planting. The amount of bioavailable Cd at planting, measured using a $CaCl_2$ extraction method (analysis performed by INRA-Arras, France) (Pueyo et al. 2004), gave a final concentration of 2.4+/−0.5 mg bioavailable Cd kg^{-1} soil, which is in the range found in some agricultural soils (Wagner 1993). In the

case of plants inoculated with *G. intraradices*, all the soil in the substrate mix was replaced by inoculum (10-week-old mycorrhizal onion roots, spores and hyphae in the same soil). Non-inoculated plants received filtered (Whatman No. 2) washings of the inoculum to reconstitute the associated soil microflora without *G. intraradices*. Pots were placed in a completely randomized design in a growth chamber (20–24°C night–day, 16 h photoperiod, 330 µmol m^{-2} s^{-1}, 70% relative humidity night–day). Plants received 20 ml of a modified Long Ashton solution (no phosphate, twofold nitrate to compensate for the lack of *Rhizobium*) (Dumas-Gaudot et al. 1994) three times a week and Milli-Q water on other days.

Plants were harvested after a 3-week growth period for gene expression analyses, since preliminary experiments showed frequent RNA degradation in 4- to 6-week-old samples from Cd treatments. Root systems were thoroughly washed in running tap water, then ice-cold deionized water. Shoots and roots were weighed fresh. Mycorrhizal colonization and Cd concentration were quantified in samples of the same root systems analysed for transcript accumulation. Root colonization by *G. intraradices* was determined after staining with trypan blue according to Trouvelot et al. (1986) (http://www.dijon.inra.fr/bbceipm/mychintec/newfiles/new.html), and the extent of cortex colonization in the whole root system (M%) was estimated. Absence of nodules was confirmed visually. Assessment of Cd concentration was carried out by electrothermal atomic absorption spectrometry (Lebourg et al. 1996) by INRA-Arras in root samples dried at 80°C for 72 h.

RNA extraction and DNase treatment

Total RNA was extracted from roots and stored in liquid nitrogen (Franken and Gnädinger 1994). Samples were treated with DNase by incubating 50 µg RNA 30 min at 37°C with 40 U RNase inhibitor (RNasine, Ribonuclease Inhibitor, Promega), 3 U RNase-free DNase (RQ1 DNase, Promega), 6 µl 10× buffer provided by the supplier of the enzyme and diethyl pyrocarbonate (DEPC) water to a final volume of 60 µl. The DNase was removed with 1 vol phenol/chloroform/isoamyl alcohol (25:24:1), and RNA was precipitated overnight at −20°C in 0.1 vol sodium acetate (3 M) and 2.5 vol ethanol (96%). After centrifuging down at 4°C, the RNA pellet was washed with ethanol (70°), centrifuged again, dried and re-suspended in 10 µl DEPC water. Concentration of the DNA-free RNA was estimated by spectrometry and quality checked in denaturing agarose gels.

cDNA synthesis and cloning

DNA-free RNA (1 µg) was denatured 10 min at 65°C, placed on ice for 5 min and reverse transcribed to first-strand cDNA in a 25-µl volume reaction for 15 min at 25°C, followed by 1 h at 37°C, and 2 min at 95°C, in the presence of 200 U reverse transcriptase (Reverse Transcriptase M-MLV RNase H^-, Promega), 80 U RNase inhibitor (RNasine, Ribonuclease Inhibitor, Promega), 2.5 µM deoxyribonucleotide triphosphates (dNTPs), 0.75 µg oligo $(dT)_{15}$ primer (Promega), with the buffer recommended by the enzyme supplier. cDNA was stored at −20°C until use.

One microliter cDNA was used as template for PCR in a final volume of 20 µl containing 100 µM dNTPs, 1× PCR buffer (Gibco-BRL, USA), 2.5 mM $MgCl_2$, 0.25 U *Taq* DNA polymerase (Gibco-BRL) and 0.5-µM specific primers, deduced from available sequences for pea genes encoding β-tubulin (*β-tub*, X54845), heat shock protein 70 (*hsp70*, L03299), γEC synthetase (*gsh1*, AF128455), GSH synthetase (*gsh2*, AF231137), hGSH synthetase

Table 1 Primers used in real-time RT-PCR experiments and annealing temperature (°C)

Gene	Primers (5′-3′)	Expected size (bp)	°C
β-tub	for CAACGAAGCTAGCTGTGGTCG		
	rev AGTAAGCATCATCCTATCAGG	348	62
cht	for GATGGACGGTGTGCTGGTC		
	rev CACGCGTATGGACCGTCTG	190	64
chi	for ATGAGTTCCCAGCGGTGGTTAC		
	rev CCTTCCCATTTAGTGGCAAGAG	168	62
hsp70	for GGTTACTGTGCCTGCTTAC		
	rev CCTCGAGCACTGAGACATC	202	62
$PsMT_A$	for GTCTAGTGGATTGAGCTACTC		
	rev GGGTCACAAGTGCAGTTATC	152	62
gsh1	for GGTTTCCAGCCAAAGTGGGAG		
	rev GCTTCAGAACTAAAGTCCAG	160	62
gsh2	for TCCTGGTGTTGGATTGGTTC		
	rev TCCTGGAGAGAGATTCCTGAAG	160	62
hgsh2	for GATCAGCATTTTGTTTCCGC		
	rev GTTCCATCCGATAGAATTTCTC	109	62
gr	for CGGTTGTTATCCAATGCTGG		
	rev TATTAGGACGCTGAGCCCTG	159	62

(*hgsh2*, AF258319), GR (*gr*, X98274) and MT ($PsMT_A$, Z23097) (Table 1). Primers used for the last gene have the same affinity for a very recently described MT isoform (AB176564). PCR was performed in a T3 Thermocycler (Biometra) with the following parameters: initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 45 s, annealing at respective temperature (Table 1) for 45 s, elongation at 72°C for 45 s and a final elongation at 72°C for 5 min.

Amplified cDNA fragments were separated in 1.2% agarose gels, single bands were extracted by Geneclean (Bio 101, Vista, USA), ligated to pGEM-T vector (Promega) and used to transform competent *Escherichia coli* JM109 cells (Promega). Recombinant clones were screened by PCR (Güssov and Clackson 1989), subcultured and plasmid DNA was isolated (Sambrook et al. 1989). Sequencing was performed using T7 and SP6 primers (Genome Express Company, Grenoble, France or MWG, Ebersberg, Germany). Sequence identity was confirmed by similarity searches in the European Molecular Biology Laboratories database using the BLASTN 2.2.4 program (Altschul et al. 1997) (http://www.ncbi.nlm.nih.gov). cDNA fragments previously cloned into PT7 Blue plasmid (Ruiz-Lozano et al. 1999) were used for chitinase (*cht*) and chalcone isomerase (*chi*) gene expression analyses.

Real-time RT-PCR

To establish a standard curve for each target gene, recombinant plasmids extracted from transformed *E. coli* cells were serially diluted from 10^7 to 10^1 copies. 2.5-µl aliquot from each dilution was used as template in 25-µl final volume PCR reactions using 1.2 µl 10 µM of corresponding primer (Table 1), enzyme and buffer provided in the Smart Kit for Sybr Green I (Eurogentec), as recommended by the supplier. PCR was carried out in a Smart Cycler (Cepheid, Sunnyvale, CA, USA): the first cycle had an extended denaturing step at 95°C for 12 min, followed by 40 cycles at 95°C for 15 s, 62 or 64°C (Table 1) for 15 s and 72°C for 15 s, and finally by 1 cycle with extended polymerization at 60°C for 95 s. Melting curves were realized by increasing the temperature from 60 to 90°C at 0.2°C s^{-1} steps to verify the purity of the real-time RT-PCR products (Devers et al. 2004). Calibration curves representing the log of the concentration of the target gene in function of cycle threshold were obtained for each gene.

Real-time RT-PCR reactions were carried out on cDNA from three replicate root systems per treatment in a 25-µl final volume containing as template 1 µl fourfold diluted cDNA under the same conditions as described above. Cycle threshold was transformed to absolute quantification using the equations from the respective calibration curves. Absolute values for transcript numbers of each gene were normalized against those for the *β-tub* gene, a constitutively expressed gene used as standard control for study of MT-encoding gene expression in *Arabidopsis thaliana* growing in the presence of copper (Murphy and Taiz 1995) and which showed comparative expression in pea roots from the different treatments (Fig. 1).

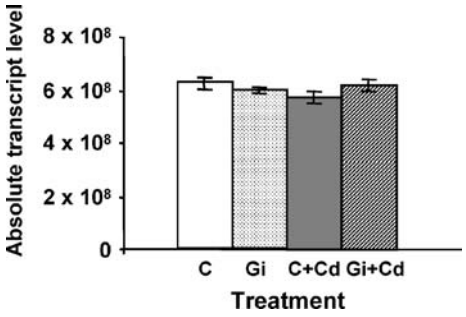

Fig. 1 Absolute transcript accumulation of the *β-tub* gene in non-mycorrhizal (*C*) and mycorrhizal (*Gi*) roots of 3-week-old *P. sativum* cv. Frisson grown in the absence (*C*, *Gi*) or presence (*C + Cd*, *Gi + Cd*) of Cd. Data are the means of three replicates. *Bars* indicate ±SE of mean values

Thiol group and GSH/hGSH measurements

Accumulation of thiol groups and GSH/hGSH was quantified in three replicate root systems per treatment as described by Noctor and Foyer (1998) and Griffith (1980), respectively. Methods applied for GSH/hGSH quantification do not discriminate between both molecules. Briefly, plant roots (100 mg fresh weight) were homogenized in 0.1 M HCl containing 1 mM ethylenediaminetetraacetic acid (EDTA) and the homogenate centrifuged (12,000 rpm, 5 min). For thiol groups, the supernatant (200 µl) was mixed with 700-µl assay buffer containing 120 mM Na phosphate, pH 7.8, 6 mM EDTA, and absorbance (412 nm) was measured following the addition of 100 µl of 6 mM 5′-dithiobis-2-nitrobenzoic acid (DTNB) to the sample. Thiol group concentration was calculated against a standard curve for GSH. Concentration of GSH/hGSH was determined in an assay based on oxidation, then reduction of GSH. GSH was oxidized in a mixture of 120-mM phosphate buffer, pH 7.8, 6 mM EDTA, 0.3 mM nicotinamide adenine dinucleotide phosphate (reduced form), 3 mM DTNB and sample extract (Fadzilla et al. 1997). Reduction of oxidized GSH was initiated by addition of 10 µl GR (5 U ml^{-1}), and change in absorbance was recorded at 412 nm. GSH/hGSH concentrations were deduced from a standard curve for reduction of different concentrations of oxidized GSH.

Statistical analyses

The Student–Newman–Keuls test ($P<0.05$) was used to evaluate differences between means of treatments using the SAS statistical software package (SAS Institute Inc. 1986). Cd concentration and root colonization data were square root and arcsin transformed, respectively, prior to analysis.

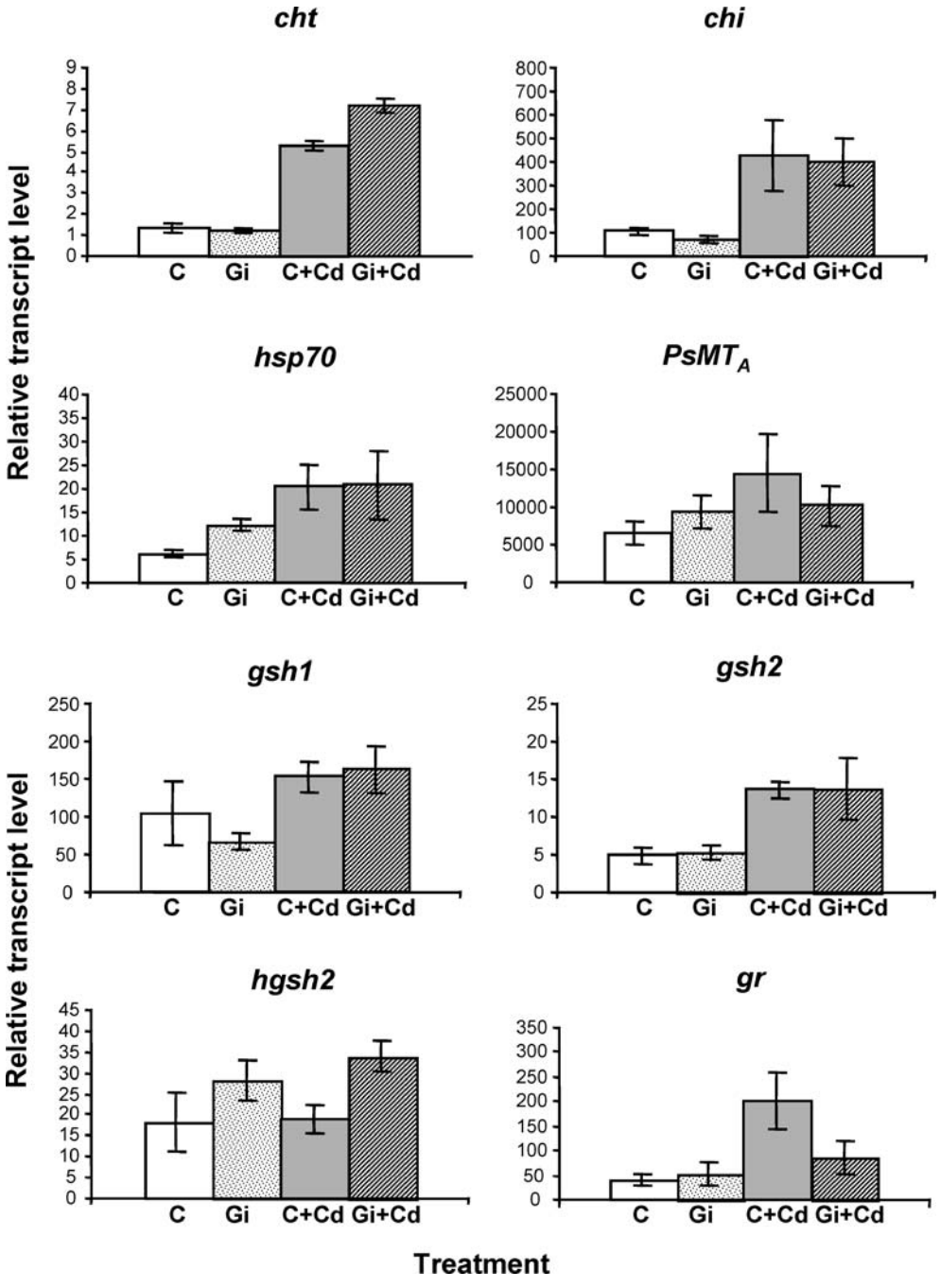

Fig. 2 Expression profiles of genes in non-mycorrhizal (*C*) and mycorrhizal (*Gi*) roots of 3-week-old *P. sativum* cv. Frisson grown in the absence (*C*, *Gi*) or presence (*C+Cd*, *Gi+Cd*) of Cd: *cht* (chitinase), *chi* (chalcone isomerase), *hsp70* (heat-shock protein), *PsMT$_A$* (metallothionein), *gsh1* (γ-glutamylcysteine synthetase), *gsh2* (glutathione synthetase), *hgsh2* (homoglutathione synthetase) and *gr* (glutathione reductase). Data are the means of three replicates. *Bars* indicate ±SE of mean values

Results

Mycorrhiza development and plant growth

Nodules and mycorrhizal colonization were absent from root systems of non-inoculated pea plants. Roots of *G. intraradices*-inoculated cv. Frisson were colonized by the mycorrhizal fungus but not nodulated. All stages of AM fungal development (appressoria, intercellular hyphae, arbuscules, vesicles) were present at 3 weeks after inoculation. The intensity of root cortex colonization (M%) in plants growing in the absence of Cd was higher (45%) than in the presence of the metal (28%). Shoot and root biomass was higher ($P<0.05$) in control (2.94 and 5.76 g) and mycorrhizal plants (2.51 and 5.11 g) growing in the absence of Cd than in non-mycorrhizal (1.69 and 3.34 g) and mycorrhizal (1.91 and 3.78 g) Cd-stressed plants. However, decreases in plant biomass caused by Cd were considerably less ($P<0.01$) in mycorrhizal (−24%) than in non-mycorrhizal (−43%) plants.

Cd accumulation in pea roots

Very low levels of Cd (<5.0 μg g^{-1} dry weight) accumulated in roots of plants growing in the absence of

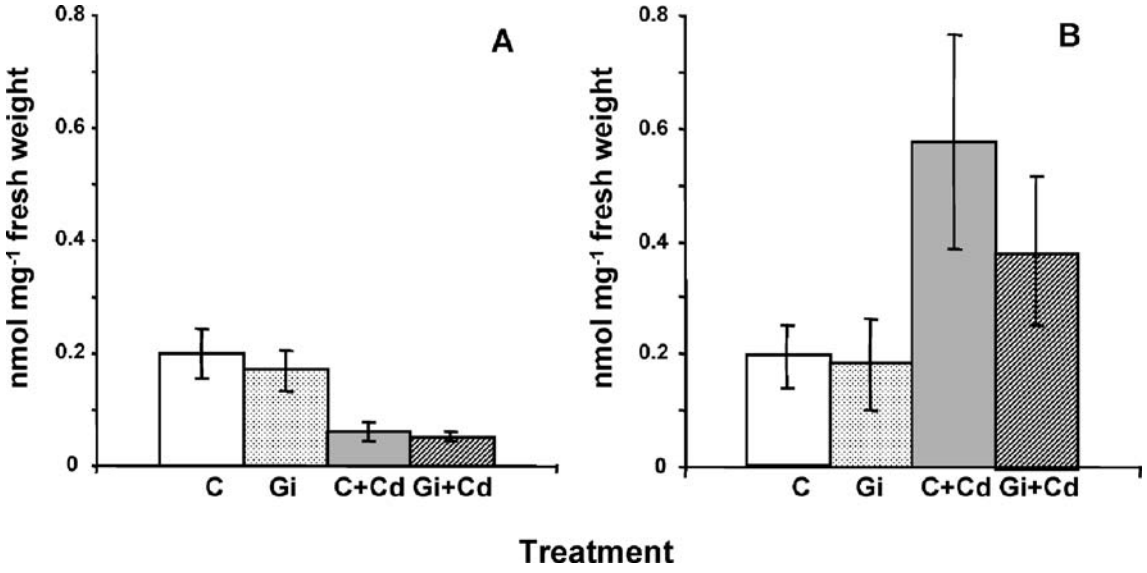

Fig. 3 Accumulation of GSH/hGSH (**A**) and thiol groups (**B**) in non-mycorrhizal (**C**) and mycorrhizal (*Gi*) roots of 3-week-old *P. sativum* cv. Frisson grown in the absence (*C*, *Gi*) or presence (*C+Cd*, *Gi+Cd*) of Cd. Data are the means of three replicates. *Bars* indicate ±SE of mean values

added Cd, and tissue concentrations of the metal tended to decrease in mycorrhizal roots (Table 2). Addition of Cd to the growth substrate greatly increased ($P<0.05$) uptake of the heavy metal by the pea roots (>770 μg g^{-1} dry weight), and the presence of mycorrhiza significantly ($P<0.05$) increased root concentrations.

Gene expression in pea roots in response to mycorrhiza and/or Cd

Expression of the different genes analysed in roots of mycorrhizal and non-mycorrhizal cv. Frisson, growing in the presence and absence of Cd, varied in magnitude. The $PsMT_A$ gene had the highest number of transcripts (10^{10} mRNA molecules μg^{-1} total RNA), whilst between 10^8 and 10^9, copies were detected for *gsh1*, *chi* and *β-tub* genes. Transcripts of the *gr* gene reached a level of 10^8 copies, and those of the *hgsh2* and *hsp70* genes were between 10^7 and 10^8copies. Genes showing the lowest levels of transcripts were *gsh2* (10^7 copies) and *cht* (<10^7 copies).

Gene expression profiles in pea roots of plants treated or not with Cd are presented in Fig. 2. After a 3-week exposure to Cd, transcripts of *cht* accumulated to similar extents in roots of control and mycorrhizal pea plants but levels increased ($P<0.05$) under Cd stress (+305%) and were greatest in mycorrhizal roots exposed to Cd (+447%). Expression of the *chi* gene was activated by Cd (+300%) but not further by AM in comparison with metal-untreated roots, whilst that of *hsp70* increased ($P<0.05$) in mycorrhizal roots (+97%) growing in absence of Cd as compared with controls and reached the greatest levels in roots of Cd-treated plants (+230%) whether these were mycorrhizal or not. Transcripts of $PsMT_A$ increased ($P<0.05$) in response to Cd in non-mycorrhizal roots (+121%) but mycorrhizal colonization had little effect on the expression of this gene either in the absence or presence of Cd. Transcript levels of *gsh1* increased ($P<0.05$) in roots of Cd-treated mycorrhizal (+128%) plants and to a lesser extent in non-mycorrhizal roots. Transcription of *gsh2* in roots increased significantly ($P<0.05$) in plants exposed to Cd (183% more than untreated plants), but AM did not modify expression of this gene. In contrast, the *hgsh2* gene did not appear to be affected by Cd alone, but expression was enhanced ($P<0.05$) by *G. intraradices* colonization of roots, particularly in the presence of Cd (+86%). Finally, transcripts of *gr* presented almost the same levels in mycorrhizal and non-mycorrhizal roots of plants growing in the absence of Cd, and the gene was significantly ($P<0.05$) activated by Cd (+414%) only in non-mycorrhizal roots.

Table 2 Cadmium concentration (μg g^{-1} dry weight) in non-mycorrhizal (C) and mycorrhizal (Gi) roots of 3-week-old *P. sativum* cv. Frisson grown in the absence (C, Gi) or presence (C + Cd, Gi + Cd) of 100 mg Cd kg^{-1} soil/sand mix

Treatment	C	Gi	C + Cd	Gi + Cd
	4.67 c	1.70 c	770.42 b	1013.41 a

Data are the means of three replicates. Mean values followed by the same letter are not significantly different ($P<0.05$)

Effect of mycorrhiza and/or Cd on GSH/hGSH and thiol group concentrations in pea roots

Accumulation of GSH/hGSH and thiol groups in roots of *P. sativum* cv. Frisson was more affected by Cd stress than mycorrhiza development (Fig. 3). Levels of GSH/hGSH were comparable in roots of control (0.20 nmol mg^{-1}) and mycorrhizal (0.17 nmol mg^{-1}) plants, whilst they decreased significantly ($P<0.05$) in Cd-treated non-mycor-

rhizal (−69%) and mycorrhizal (−75%) plants (Fig. 3a). Thiol groups accumulated to similar levels as GSH/hGSH in control (0.20 nmol mg^{-1}) and mycorrhizal (0.18 nmol mg^{-1}) roots, and they considerably increased in concentration in roots of non-mycorrhizal (+193%) and mycorrhizal (+95%) plants grown in the presence of Cd (Fig. 3b).

Discussion

It was previously shown that root colonization by *G. mosseae* or *G. intraradices* can attenuate Cd-induced stress in 5- to 6-week-old pea genotypes, including *P. sativum* cv. Frisson (Rivera-Becerril et al. 2002; Repetto et al. 2003). In the present study, this buffering effect of mycorrhiza was already obvious in 3-week-old plants of the cv. Frisson genotype exposed to Cd where mycorrhizal plant growth was considerably less affected (−24%) by the pollutant than that of non-mycorrhizal plants (−43%). Since Rivera-Becerril et al. (2002) had shown that this protective effect was not due to reduced Cd accumulation or improved P nutrition in mycorrhizal pea, it was decided to explore strategies linked to molecular modifications in the AM symbiosis by expression profiling of plant genes that may be involved.

The present observations of Cd accumulation in roots of 3-week-old pea cv. Frisson growing in soil spiked with the pollutant are similar to those reported previously in older plants (Rivera-Becerril et al. 2002). They confirm the high capacity for Cd sequestration by root tissues of this pea genotype as well as the increased metal accumulation in mycorrhizal roots. The changes in pea gene expression associated with the higher Cd concentrations in roots suggest that metabolic changes occur in these tissues in response to the heavy metal stress.

The expression levels of the *β-tub* gene did not exhibit any significant differences between treatments, including mycorrhiza, and this is consistent with recent studies where this gene is not predicted to be up-regulated by AM (Journet et al. 2002; Küster et al. 2004). The genes encoding chalcone isomerase and chitinase were clearly activated by Cd in roots of *P. sativum* cv. Frisson, and the *cht* gene was further activated by AM development in the presence of the heavy metal. Phytoalexins and pathogenesis-related (PR) proteins are among general stress response molecules in plants, and their production can be stimulated by abiotic stresses, including metals (Cruz-Ortega and Ownby 1993). The role of stress-related genes in heavy metal tolerance of plants is not clear, but it has been suggested that any stress factor that disrupts the integrity of the plasma membrane (including heavy metals) could lead to their activation (Cruz-Ortega and Ownby 1993). Different heat-shock proteins (HSP) have also been reported to increase in leaves and roots as a response to metal stress, and their synthesis has been proposed as a mechanism that may contribute to heavy metal tolerance in plants (Neumann et al. 1994; Wollgiehn and Neumann 1999). Cd can alter membranes via distortion of proteins (Fodor et al. 1995) and HSP, which form a group of chaperones implicated in protein folding (Marshall and Keegstra 1992), bind to denatured proteins and help re-establish their native configuration and reintegration into the membrane complex (Neumann et al. 1994). Transcripts of the *hsp70* gene increased in mycorrhizal roots of pea growing in the absence of Cd, and this may reflect rearrangement of the newly synthesized symbiotic plant membrane during arbuscule development within root cortical cells (Gianinazzi-Pearson 1996). Although expression of *hsp70* reached comparable levels in mycorrhizal and non-mycorrhizal roots of Cd-treated plants, an eventual implication of HSP in maintaining membrane integrity in arbuscule-containing cells could contribute to greater AM tolerance of metal stress, and it would consequently be interesting to locate *hsp70* expression and protein synthesis in AM tissues.

The MT-encoding gene ($PsMT_A$) was activated in non-mycorrhizal pea roots exposed to Cd, but AM development did not significantly affect gene expression. Of the different categories of genes analysed in *P. sativum* cv. Frisson, $PsMT_A$ showed the highest levels of expression which points to a potential role in the tolerance of this pea genotype to Cd stress conditions (Rivera-Becerril et al. 2002; Repetto et al. 2003). These observations concord with a previous report of $PsMT_A$ expression in pea roots (Evans et al. 1990) and after Cd exposure in barley roots (Finkemeier et al. 2003). Transcripts of $PsMT_A$ increased only slightly in mycorrhizal roots of *P. sativum* cv. Frisson growing in the presence or absence of Cd but stronger activation of this MT gene has been observed in *G. intraradices*-colonized roots of another pea genotype, cv. Finale (Rivera-Becerril 2003). No other information is available concerning the activation of MT-encoding genes of plant origin in AM associations. However, *in silico* gene expression analyses do predict activation of different MT-encoding genes in *Medicago truncatula–G. intraradices* mycorrhiza (Journet et al. 2002). Transcripts of an MT-encoding gene (*cgMT1*) are present in symbiotic *Frankia*-infected cells of *Casuarina glauca*, where the corresponding protein could be involved in metal transport and/or homeostasis (Laplaze et al. 2002). In this context, it is interesting to note that elevated concentrations of Zn and Ni have been identified in the inner cortical cell region of maize roots containing arbuscules of a *Glomus* isolate (Kaldorf et al. 1999). More research is needed to elucidate the role of different MT isoforms in AM against Cd pollution.

The genes *gsh1* and *gsh2*, which encode two enzymes involved in GSH synthesis (γEC synthetase and GSH synthetase), were clearly up-regulated by Cd and not by AM. Activation of one or other of the genes by Cd has been observed in *Brassica juncea* roots and shoots (Zhu et al. 1999a,b) and *A. thaliana* shoot and roots (Xiang and Oliver 1998). Cd-induced activity of γEC synthetase has been reported in shoots and roots of maize seedlings (Rüegsegger and Brunold 1992) and transformed poplar overexpressing a bacterial *gsh1* gene (Arisi et al. 2000), and GSH synthetase is active in Cd-stressed *P. sativum* roots (Rüegsegger et al. 1990). Furthermore, Zhu et al. (1999a,b) have demonstrated in Indian mustard overex-

pressing bacterial *gsh1* and *gsh2* genes that PC concentrations increase, which confers a higher tolerance to Cd accumulation in the plant tissues. The GR encoding gene, *gr*, which is involved in the turnover of GSH from the oxidized to its reduced form, was likewise activated in roots of Cd-treated pea plants, in agreement with that reported in *A. thaliana* growing in the presence of the heavy metal (Xiang and Oliver 1998). Induction of this gene is proposed to provide defence against oxidative damage imposed by Cd in pea (Dixit et al. 2001) as well as a higher tolerance to Cd stress in chloroplasts and decreased Cd accumulation in shoots of *B. juncea* (Pilon-Smits et al. 2000). Although increased GR activity has been reported in mycorrhizal roots of soybean in response to drought stress (Porcel et al. 2003), up-regulation of the *gr* gene was not pronounced in mycorrhizal pea roots exposed to Cd. PCs are synthesized from GSH (Grill et al. 1989) and Cd is the strongest inducer of their synthesis (Grill et al. 1987; Cobbett and Goldsbrough 2000). The presence in plant tissues of thiol groups at levels above those of GSH/hGSH is recognized as an indicator of PC/hPC synthesis and accumulation (Rüegsegger et al. 1990; Brune et al. 1995). The Cd-induced expression of *gsh1*, *gsh2* and *gr*, together with decreased GSH/hGSH levels and increased thiol group concentrations, observed in *P. sativum* cv. Frisson roots suggest that Cd chelation by PC and/or hPC plays a role in the tolerance of this pea genotype to Cd.

Activation of *hgsh2* only occurred in mycorrhizal roots of *P. sativum* cv. Frisson, and this was greatest in plants grown in the presence of Cd. This gene, which encodes a hGSH synthetase responsible for the synthesis of hGSH, has already been described in legumes, including pea (Klapheck 1988; Frendo et al. 1999). The GSH/hGSH ratio can differ considerably between legume species as well as between leaves, roots and seeds of a given species (Klapheck 1988). For example, *hgsh2* transcripts are more frequent than those of *gsh2* in roots and nodules of *M. truncatula* (Frendo et al. 1999). This concords with present results on pea roots where the relative transcript level of *hgsh2* transcripts was twofold to threefold higher than that of *gsh2*. The higher expression of *hgsh2* in mycorrhizal tissues may reflect a role of the hGSH pathway rather than GSH synthesis in the symbiosis and its tolerance to Cd.

In conclusion, the AM symbiosis has been shown to buffer Cd stress in different plants (Heggo et al. 1990; Leyval et al. 1997; Rivera-Becerril et al. 2002), and recent proteome analyses have demonstrated that AM modulates the expression of several proteins associated with Cd responses in pea (Repetto et al. 2003). First observations are reported here on molecular modifications in pathways involved in stress responses and Cd detoxification in mycorrhizal, as compared to non-mycorrhizal pea roots. The activation of genes encoding HSP70, an MT and enzymes involved in GSH metabolism in pea roots exposed to Cd, together with the accumulation of thiol groups above GSH/hGSH levels, strongly suggests that roots of *P. sativum* cv. Frisson possess an efficient physiological defence against Cd based on chelating mechanisms. However, only the expression of *hsp70*, *cht* and *hgsh2* genes was significantly enhanced in the pea roots by AM, which indicates that Cd chelation pathways may not make a major contribution to metal tolerance strategies operating in the AM symbiosis, and argues for an alternative action of the symbiosis at the molecular level. Nevertheless, HSP, MT, PC and/or hPC may be operating in AM in a very localized way so that symbiotic functions are protected under Cd stress, especially in arbuscule-containing cells. Further research on GSH, hGSH, PC and hPC characterisation by high-performance liquid chromatography is needed to clarify their specific role in mycorrhizal pea growing in the presence of Cd. Other molecular mechanisms active in this protective effect could involve, for example, transporters of Cd–PC/hPC complexes across the tonoplast (Ortiz et al. 1992) or serine acetyltransferase genes which are involved in the production of cysteine (Howarth et al. 2003), an essential amino acid for the synthesis of MT and PC. Furthermore, AM mycelium has a metal adsorption capacity, which can be higher in heavy metal-tolerant fungal isolates (Joner et al. 2000; González-Chávez et al. 2002) so that sequestration of metals by AM fungal structures in the plant root may also provide an efficient strategy for Cd stress buffering by the symbiosis (Leyval et al. 2002).

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