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RESEARCH PAPER

Comparative cDNA-AFLP analysis of Cd-tolerant and -sensitive genotypes derived from crosses between the Cd hyperaccumulator *Arabidopsis halleri* and *Arabidopsis lyrata* ssp. *petraea**

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Abstract

Cadmium (Cd) tolerance seems to be a constitutive species-level trait in *Arabidopsis halleri.* In order to identify genes potentially implicated in Cd tolerance, a backcross (BC1) segregating population was produced from crosses between *A. halleri* ssp. *halleri* and its closest non-tolerant relative *A. lyrata* ssp. *petraea*. The most sensitive and tolerant genotypes of the BC1 were analysed on a transcriptome-wide scale by cDNA-amplified fragment length polymorphism (AFLP). A hundred and thirty-four genes expressed more in the root of tolerant genotypes than in sensitive genotypes were identified. Most of the identified genes showed no regulation in their expression when exposed to Cd in a hydroponic culture medium and belonged to diverse functional classes, including reactive oxygen species (ROS) detoxification, cellular repair, metal sequestration, water transport, signal transduction, transcription regulation, and protein degradation, which are discussed.

Key words: *Arabidopsis halleri*, cadmium, cDNA-AFLP, heavy metal tolerance, hyperaccumulator.

Introduction

Cadmium (Cd) is a heavy metal with highly toxic effects on organisms, and Cd pollution is recognized as an environmental problem worldwide. Although not essential for plant growth, Cd^{2+} is readily taken up by roots and can be translocated into aerial organs, where it affects photosynthesis and 'consequently' root and shoot growth. No Cd-specific transporter has been identified, and Cd seems to be transported via several classes of Ca^{2+}, Fe^{2+}, or Zn^{2+} transporters, affecting their uptake and distribution in plants and inducing deficiency (Clemens *et al*., 1998, 2002). Targets of Cd toxicity include zinc metalloenzymes and membrane phospholipids, causing inhibition of enzyme activities, protein denaturation, disruption of cell transport processes, alteration of RNA synthesis (Clemens, 2001; Suzuki *et al*., 2001; Hall, 2002; Schützendübel and Polle, 2002; Deckert, 2005; Kovalchuk *et al*., 2005), and DNA mismatch repair system inhibition (Jin *et al*., 2003; Banerjee and Flores-Rozas, 2005). Cd also induces an oxidative stress via indirect actions such as the disruption of the electron transport chain, the induction of lipid peroxidation, the depletion of glutathione or the displacement of Fe atoms from proteins resulting in increased production of reactive oxygen species (ROS)

* The nucleotide sequences reported in this paper have been submitted to GenBank under accession numbers from DV752011 to DV752315, DV935717, and DV935718, and dbEST Id numbers, from 33963327 to 33963631, 34147988, and 34147989, respectively.

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Abbreviations: AFLP, amplified fragment length polymorphism; BC, backcross; CTR-TDF, Cd tolerance-related transcript-derived fragment; EST, expressed sequence tag; GST, glutathione *S*-transferase; ROS, reactive oxygen species; TDF, transcript-derived fragment; TRX, thioredoxin; VHA, V-type ATPase.

through the Haber–Weiss reaction (Smeets *et al*., 2005). Furthermore, Cd is considered as a mutagen.

Plants have a variety of potential mechanisms at the cellular level that might be involved in the tolerance to heavy metal stress. These are involved primarily in avoiding the build-up of toxic concentrations at sensitive sites within the cell. Within the cytosol, tolerance mechanisms can include the chelation of metals by organic acids, amino acids, or peptides, their sequestration in the vacuole, their efflux to the apoplasm, and the repair of damaged proteins. In the case of Cd, the main mechanism of cellular tolerance in plants described up to now is its chelation by high affinity low molecular weight ligands, such as glutathione, phytochelatins, and metallothioneins (Clemens, 2001; Cobbet and Goldsbrough, 2002; Hall, 2002, Clemens and Simm, 2003), to limit uncontrolled binding to physiologically important functional groups. However, it has been demonstrated that the physiological mechanism of Cd tolerance is not based on an enhanced synthesis of phytochelatins (Ebbs *et al*., 2002; Schat *et al*., 2002) and that in tolerant plants only a small proportion of Cd seems to be co-ordinated by sulphur donor atoms (Küpper *et al*., 2004; Ueno *et al*., 2005). Rather, the majority of Cd was co-ordinated with malate in leaves of *Thlaspi caerulescens*, which was by far the most concentrated vacuolar ligand (Ueno *et al*., 2005). In *Schizosaccharomyces pombe* or *Saccharomyces cerevisiae*, PC-Cd or GS-Cd sequestration in the vacuole is essential for tolerance. Although vacuolar Cd-binding complexes have been measured (Rauser, 2000), the transport pathways of free or complexed Cd through the tonoplast have not been discovered in plants yet. AtCAX2 might function as a Cd^{2+}/H^{+} antiporter at the tonoplast (Hirschi *et al.,* 2000), and ABC members are probably involved in the transport of Cd chelates, as they are in yeast (Bovet *et al*., 2003). Interestingly 17 of the 127 putative ABC *Arabidopsis thaliana* genes were induced after Cd treatments (Bovet *et al*., 2005).

Most plant species are not tolerant to Cd and most of the metallophytes on Cd-contaminated soils are Cd excluders, inhibiting its entry and favouring its retention in roots. However, a rare class of plants, called hyperaccumulators, can accumulate >100 μg of Cd g^{-1} of their shoot dry weight, without showing any sign of phytotoxicity (Brooks, 1998). *Arabidopsis halleri* (L.) O'Kane and Al-Shehbaz has been shown to hyperaccumulate Zn and tolerate Cd, and for some populations to hyperaccumulate Cd. Zn and Cd tolerance traits seem to be constitutive, as revealed by a study on a large number of European populations of *A. halleri* growing on both metal-contaminated and non-contaminated sites (Bert *et al*., 2002). *Arabidopsis halleri* is an emerging model species for the molecular elucidation of plant metal hyperaccumulation (Küpper *et al*., 2000; Dräger *et al*., 2004; Weber *et al*., 2004). *Thlaspi caerulescens* and *A. halleri* are the only two Cd hyperaccumulator species. The active accumulation in the above-ground parts of hyperaccumulator plants provides a promising approach both for cleaning anthropogenically contaminated soils (phytoremediation) and for commercial extraction (phytomining) of metals from naturally metal-rich (serpentine) soils (McGrath *et al*., 1993, 2002). To evaluate the potential of hyperaccumulator-mediated remediation, the genetics and physiology of tolerance and hyperaccumulation have first to be investigated.

The Zn- and Cd-tolerant and hyperaccumulator species *A. halleri* ssp. *halleri* (population of Auby, France) was crossed with the Cd- and Zn-sensitive species *A. lyrata* ssp. *petraea*. One F_1 plant was backcrossed with the sensitive parent species, giving a backcross progeny. The grandparents, the F_1, and some of the backcross genotypes were previously characterized for their Cd and Zn tolerance and hyperaccumulation properties, which appeared to be independent characters (Bert *et al*., 2003). This unique plant material was analysed with an improved cDNA-amplified fragment length polymorphism (AFLP) protocol (Breyne *et al*., 2003) for identifying candidate genes responsible for Cd tolerance, by comparing the transcript profile of the tolerant and sensitive genotypes and selecting the co-segregating transcript-derived fragments (TDFs) expressed only/mostly/differently in the tolerant genotypes. Analysis has been conducted on roots. Root is the first organ to perceive Cd stress and to adapt to its presence in the soil. Moreover, Cd stress signals are first communicated by the root to the shoot. Although *A. halleri* is considered a Cd-hyperaccumulator species, Cd accumulates mainly in the roots (Bert *et al*., 2003). Roots of this population thus have exceptional mechanisms of cellular tolerance allowing them to grow on toxic concentrations (up to 300 μM) of Cd. Therefore, as a first step to unravel the mechanisms involved in Cd tolerance, the transcriptome of roots was studied.

Materials and methods

Plant material, growth conditions

A single cross was performed between one individual from the Zn-tolerant species *A. halleri* ssp. *halleri* (*Ah*: pollen donor) and one from the non-tolerant species *A. lyrata* ssp. *petraea* (*Alp 1*: pollen recipient). The *Ah* individual (2*n*=16) originated from a site highly contaminated with Zn, Cd, and Pb (Auby, France) (Van Rossum *et al*., 2004). The *Alp 1* individual (2*n*=16) originated from an uncontaminated site in the Czech Republic (Unhost, Central Bohemia) (Macnair *et al*., 1999). Both species are self-incompatible and usually outcrossing. One randomly selected F_1 individual was used as male parent to fertilize a second *A. lyrata* ssp. *petraea* individual (*Alp 2*), generating the interspecific backcross progeny (BC1) of >350 individuals. Sixty-six of these genotypes were evaluated for their characters of Cd tolerance and hyperaccumulation (Bert *et al*., 2003).

Between seven and 10 cuttings were generated per parental genotype (*Ah*, *Alp 1* and *2*, and F_1), and per genotype of the BC1 already characterized for tolerance to both Cd and Zn. The 11 most sensitive (BCS) and the 18 most tolerant (BCT) genotypes of the BC1 were selected. Cuttings from mother plants were grown on sand for

6 weeks in a greenhouse. Hydroponic plant culture was performed in a modified MS solution consisting of K_2SO_4 (0.88 mM), KH_2PO_4 (0.25 mM), NaCl (10 μM), $Ca(NO_3)_2$ (2 mM), $MgSO_4$ (1 mM), FeEDDHA (20 μM), H_3BO_3 (10 μM), $ZnSO_4$ (1 μM), $MnSO_4$ (0.6 μM), $CuSO_4$ (0.1 μM), and $(NH_4)_6Mo_7O_{24}$ (0.01 μM) adjusted to pH 5.8, in a climate-controlled growth chamber (temperature cycle of 20/17 °C and a light (100 μmol m^{-2} s^{-1}) cycle of 16 h light/8 h dark). The hydroponic solutions used were continuously aerated and changed every week. After 4 weeks in nutrient solution, 10 μM $CdSO_4$ was added to half of the cuttings belonging to the same genotype. Roots were collected after 72 h of treatment and immediately frozen in liquid nitrogen until use.

RNA extraction and cDNA synthesis

Total RNA was prepared by LiCl precipitation (Sambrook and Russel, 2001). Four pools of RNA were made, by mixing 15–20 μg of total RNA extracted from the sensitive genotypes not treated or treated with 10 μM $CdSO_4$ for 72 h, and tolerant genotypes not treated or treated with 10 μM $CdSO_4$ for 72 h. A 6 μg aliquot of total RNA was used to synthesize first-strand cDNA by reverse transcription with a biotinylated oligo$(dT)_{25}$ primer and Superscript II (N.V. Invitrogen SA, Merelbeke, Belgium). Second-strand synthesis was performed by strand displacement with *Escherichia coli* ligase (New England Biolabs, Westburg, Leusden, The Netherlands), *E. coli* polymerase I (New England Biolabs), and RNase H (Invitrogen). The resulting double-stranded cDNA was purified using a Qiaquick PCR purification kit (Qiagen, Westburg, Leusden, The Netherlands), quantified, and analysed by electrophoresis.

cDNA-AFLP analysis

A 500 ng aliquot of double-stranded cDNA was used for AFLP analysis as described (Vos *et al*., 1995; Bachem *et al*., 1996; Breyne *et al*., 2003) with the following modifications. The restriction enzymes used were *Bst*YI and *Mse*I (New England Biolabs), and the digestion was performed in two separate steps. After digestion with *Bst*YI, the 3′ end fragments were collected on Dyna beads (Dynal, Invitrogen). After digestion with *Mse*I, the restriction fragments released from the beads were collected and used as templates in the subsequent AFLP steps. The following adaptors were used: *Bst*YI-F, 5′-CTCGTAGACTGCGTAGT-3′; *Bst*YI-R, 5′-GATCACTACGCAGTCTAC-3′; *Mse*I-F, 5′-GACGATGAGTCCTGAG-3′; and *Mse*I-R, 5′-TACTCAGGACTCAT-3′; the primers for *Bst*YI and *Mse*I were 5′-GACTGCGTAGTGATC(T/C)N-3′ and 5′-GATGAGTCCTGAGTAANN-3′, respectively, where N represents the selective nucleotides. For pre-amplifications, an *Mse*I primer without selective nucleotides was combined with a *Bst*YI primer containing either a T or a C at the 3′ end. The use of either *BstYI*+T or *BstYI*+C primer in combination with the *Mse*I primer without a selective nucleotide during the non-specific polymerase chain reaction (PCR) amplification reduced by 2-fold the complexity of the mixture of TDFs. The sensitivity of detection was enhanced by reducing the complexity of the mixture of amplified fragments, by the use of primers containing one or two additional selective nucleotides for the second amplification.

A 5 μl aliquot of the amplification mixtures obtained following the non-selective amplification was used for final selective amplifications. Selective [^{33}P]ATP-labelled amplification products were separated on a 5% polyacrylamide gel run at 100 W for 3 h. Gels were dried onto 3MM Whatmann paper, and positionally marked before being exposed for 2 d to Fuji Super RX Medical X-Ray film (Fujifilm Medical Systems, Benelux N.V., Belgium) to obtain the autoradiographs, or 1 d to PhosphorImager screens and scanned in a Storm 860 PhosphorImager (Molecular Dynamics). Amplifications using all the 128 possible *Bst*YI +T/C+N+*Mse*I +NN primer combinations were performed on each genotype and condition.

Isolation and sequencing of amplified cDNA products

The bands of interest were cut from the gels. DNA fragments were extracted from denaturing gels according to Frost and Guggenheim (1999). The gel fragments were rehydrated in 100 μl of 2× PCR buffer (100 mM KCl, 20 mM Tris–HCl, pH 9.0 at 25 °C, 3 mM $MgCl_2$ and 0.2% Triton® X-100) for 10 min at room temperature. The remaining buffer was removed and replaced with a fresh 100 μl aliquot of buffer, samples were incubated at 94 °C for 90 min, and the gel slices were completely crushed by pipetting. A 20 μl aliquot was used for re-amplification using primers with selective nucleotides under the same PCR conditions as in the cDNA-AFLP analysis. Eventually, subsequent PCR amplification was performed to produce sufficient DNA for cloning. Purified DNA fragments were then cloned in pTZ57R/T vector (InsT/Aclone™ PCR Product Cloning Kit; Fermentas GmbH, Germany). Two or three individual clones, corresponding to the major insert and of the expected size, were isolated and sequenced using one plasmid-specific primer (Lac1 pUC18/19: 5′-AGTCACGACGTTGTAAAACGACGGCCAGT-3′). The nucleotide sequences obtained were compared with NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) and TAIR (http://www.arabidopsis.org/Blast/) non-redundant databases using Blastn, Blastx, and tBlastx sequence alignment programs (Altschul *et al*., 1997).

A total of 307 *A. halleri* expressed sequence tag (EST) sequences were submitted to the dbEST National Center for Biotechnology Information, Bethesda, MD, USA. They were assigned GenBank accession numbers, from DV752011 to DV752315, DV935717, and DV935718, and dbEST Id numbers, from 33963327 to 33963631, 34147988, and 34147989, respectively.

Reverse transcriptase–PCR analysis

Reverse primers (Table 3) were designed in the 3′ part of the selected TDF sequences, and the forward primers (Table 3) in regions homologous to the *A. thaliana* corresponding coding sequences. PCR primers were tested on genomic DNA extracted from *Ah*, *Alp 1*, and *2* using the Wizard Genomic DNA Purification Kit (Promega Benelux, Leiden, The Netherlands) to check the specificity and the efficiency. Only PCR primers giving similar results on both *Ah* and *Alp* DNA were used further for reverse transcription (RT)–PCRs. Reverse transcription was performed for 23 candidate genes, using the first-strand cDNA synthesis kit (Fermentas GmbH, Germany) following the manufacturer's instructions (2 μg of total RNA). cDNA fragments were amplified by PCR using the GoTaq DNA polymerase Master Mix (Promega) with the corresponding gene-specific primers, with an annealing temperature of 62 °C or 58 °C. PCR samples were taken at successive cycles; the number of cycles at which an optimum signal was observed is indicated in Table 3. The quantification was performed using the ImageQuant software (Amersham Biosciences, GE Healthcare Europe GmbH, Benelux, Belgium) before saturation. To ensure that equal amounts of cDNA were used in each PCR procedure, two cDNA fragments from housekeeping genes (that showed no expression changes in the cDNA-AFLP analysis), encoding elongation factor 1a and the ADP ribosylation factor (primers given in Table 3), were amplified simultaneously and quantified. Three independent experiments were conducted. Analysis of nine candidate genes (homologous to At3g06130, At2g40140, At1g61640, At5g64350, At2g17480, At4g11010, At5g63470, At5g47030, and At5g56030) did not produce results similar to the cDNA-AFLP.

Results

Selection of the Cd tolerance-related TDF

The objective of this study was the detection of differences in the root transcript profile associated with the Cd tolerance

character. Analysis was performed on plants belonging to *A. halleri* ssp. *halleri*, *A. lyrata* ssp. *petraea*, the F_1, the 11 most sensitive (BCS) and 18 most tolerant (BCT) to Cd^{2+} BC1 genotypes, in control conditions, or following an exposure to $CdSO_4$ (10 μM for 72 h). This treatment was compatible with adaptation of both the sensitive and the tolerant plants (Bert *et al*., 2003).

The improved cDNA-AFLP protocol of Breyne *et al.* (2003) was used that permits transcript profiling for genome-wide screening of differentially expressed genes. The 128 possible *Bst*YI+T/C+N+*Mse*I+NN primer combinations were performed on each genotype or pool of BC genotypes and conditions. A total of 125–175 AFLP bands have been detected per combination on autoradiography, ranging from 70 to 600 bp (Fig. 1).

In order to select TDFs associated with Cd tolerance, a comparison was made of the profiles of BCS and BCT. The TDFs that were differentially abundant between the two types of pools, with or without 10 μM $CdSO_4$ treatment, were selected only when the same tendency was also observed between the original parental species. As a result of cDNA-AFLP analysis, a total of 194 TDFs were identified as differentially expressed and segregating along with the Cd tolerance between the Cd-sensitive and -tolerant genotypes, which were named Cd tolerance-related TDFs (CTR-TDFs). Of these, 97% were more abundant in tolerant than in sensitive genotypes, while the remaining 3% were only detected in sensitive genotypes.

Among the overexpressed CTR-TDFs (188), all were already more abundant in the absence of $CdSO_4$, and most of them (149) were not affected by the $CdSO_4$ treatment. In the presence of $CdSO_4$, 20 CTR-TDFs were further induced, seven only in tolerant and 13 in tolerant genotypes, sensitive genotypes, or pools of genotypes. Nineteen were repressed, five only in tolerant and 14 in both tolerant and sensitive genotypes. The 188 overexpressed CTR-TDFs, which all correspond to *A. halleri* genes, were further analysed. Bands were cut out in duplicate from lanes of tolerant genotypes, and PCR amplified (Fig. 1). The recovered fragments, ranging in length between 90 and 500 bp (250 bp on average), were cloned before sequencing in order to prevent problems associated with direct sequencing of PCR products (Durrant *et al*., 2000; Ditt *et al*., 2001) and the correct sequences were obtained for 156 TDFs. The remaining 32 CTR-TDFs could not be amplified from the polyacrylamide gels.

Based on pairwise comparisons of the 156 sequences of recovered fragments, 134 (86%) showed similarity to reported *Arabidopsis thaliana* genes, but 22 of them did not show homology to any nucleotide or amino acid sequence

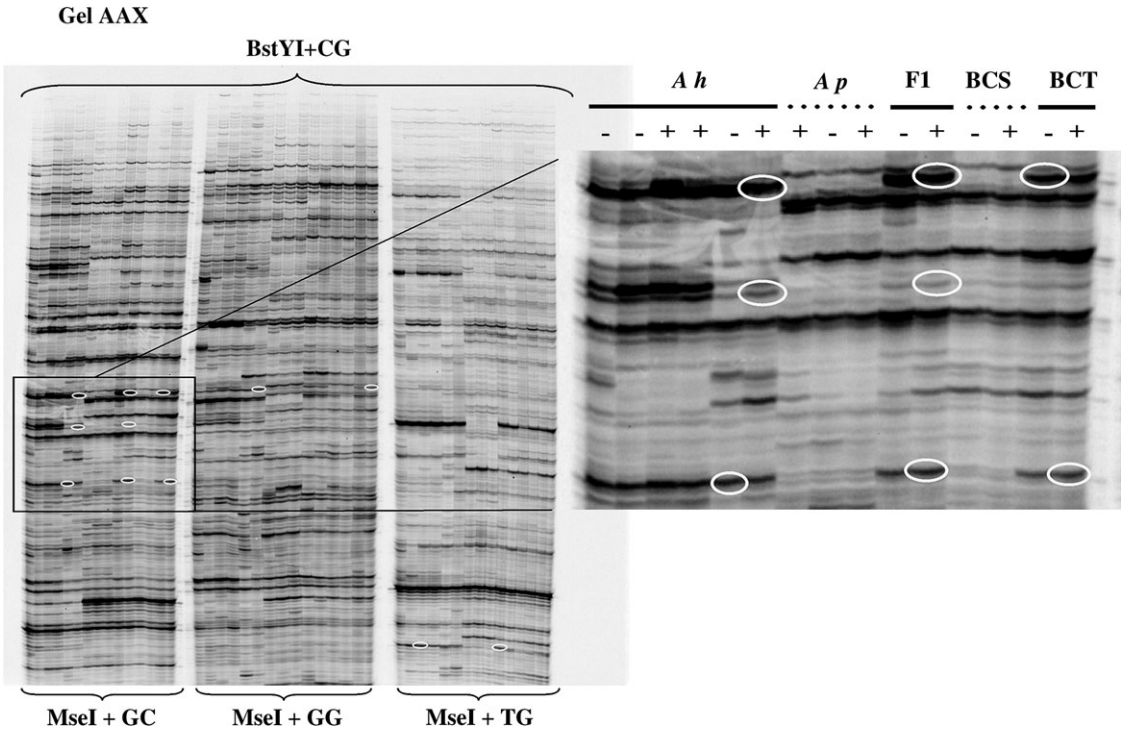

Fig. 1. Left: autoradiography of a representative polyacrylamide gel. Amplicons resulting from three specific primer combinations were separated electrophoretically. There are 15 lanes for one specific primer combination, corresponding to *A. petraea* (Ap), *A. halleri* (Ah), F_1, backcross-sensitive (BCS), and backcross-tolerant (BCT) genotypes, exposed (+) or not (–) to 10 μM $CdSO_4$. Plants were cultivated for 4 weeks in hydroponic conditions and half of the plants 10 μM $CdSO_4$. Roots were harvested after 72 h of were treated with treatment. Right: magnification of a region of the autoradiograph where differentially expressed TDFs between tolerant and sensitive genotypes are clearly visible. Encircled TDFs were cut out of the gel, re-amplified by PCR using the same primer combination, subcloned in the pTZ57R vector, sequenced, and the sequence analysed *in silico*.

in databases. The 134 sequences of TDFs showing homology with *A. thaliana* open reading frames (ORFs), that were isolated following the transcript profiling analysis, are listed in Table 1.

While most CTR fragments were identified only in one primer combination, 5% were present in several. Redundancy seemed to be due to the presence of highly homologous sequences, resulting from mispriming during PCR amplification, or PCR products representing either different alleles from the same gene or different instances of multicopy genes. Bachem *et al*. (1996) reported the occurrence of TDFs with the same mobility in fingerprints obtained with primers having similar sequence extensions and showed this to be due to mismatched primed PCR of highly abundant transcripts. Among these CTR-TDFs are those homologous to *A. thaliana* genes coding for AAA-type ATPases At3g15120 and At2g27600, for transcription factors At2g40140 and At1g78280, for histone H1 At2g18050, enolase At2g36530, mitochondrial inner membrane translocase At2g37410, and heat shock protein (HSP) At5g56030.

The 134 sequences of CTR-TDFs corresponding to *A. thaliana* genes were classified according to functional class based upon MIPS classification (http://mips.gsf.de/proj/thal/db/tables/tables_func_frame.html): transcription, transport, cellular metabolism, signal transduction, cell rescue/stress-related/defence, and unknown function. The largest category of identified genes was in cellular metabolism (53%). CTR-TDFs showed 65–100% nucleotide identity to *A. thaliana* coding sequences, with an average of 91%.

Identification of candidate genes and confirmation by semi-quantitative RT–PCR

In order to validate the results presented above, candidates with possible roles in adaptation to toxic metal concentration were selected for further characterization. All the selected genes were more highly expressed in pools of tolerant genotypes than in the sensitive ones (Table 1). Expression was reassessed by semi-quantitative RT–PCR, with gene-specific primers, on RNA coming from the same genotypes obtained from experiments independent of those analysed by cDNA-AFLP (Fig. 2).

Among the 19 *A. halleri* (*Ah*) genes belonging to the transcription and chromatin remodelling category, putatively involved in the tolerance to Cd, two were chosen for the confirmation by semi-quantitative RT–PCR: histone H1-3 and bZIP23 transcription factor homologues. Transcript levels of the *H1-3* gene (homologous to At2g18050) were ~2.5-fold higher in *Ah* than in *A. petraea* (*Ap*) and 3-fold higher in BCT compared with BCS. The $CdSO_4$ treatment repressed the expression of this gene both in *Ah* (3-fold) and in *Ap* (2-fold). The *bZIP23* gene (homologous to At2g16770) was expressed ~2.5-fold more in *Ah* than in *Ap* and 2-fold more in BCT versus BCS. Gene expression was induced by Cd in *Ap* (2-fold), in *Ah* (1.5-fold), and in F_1 (4-fold).

Several genes identified by this approach have a putative function in transport. Expression profiles of five of them were tested and confirmed as being constitutively up-regulated in the tolerant genotypes. The *Ah* gene homologous to *AtMTP1* (At2g47830) was selected; it belongs to the CDF family and is directly implicated in metal transport. The observed expression was maximal in *Ah* where it was 5-fold higher than in *Ap*. A 2-fold difference was confirmed between the sensitive and tolerant pools of BC1 plants. Expression was stimulated by Cd in BCT plants, but did not reach the level of expression observed in *Ah*.

Furthermore, an up-regulation (5–6-fold) of the proton-ATPase 16 kDa proteolipid *AVA-P1* or *VHA-c* gene (homologous to At2g16510) in the tolerant genotypes compared with the sensitive genotypes was confirmed, with no particular regulation by Cd exposure.

A gene belonging to the H^+/Ca^{2+} exchangers was also selected; this is homologous to *AtCAX8* (At5g17850). The *CAX8* homologue was clearly overexpressed (10-fold) in *Ah* compared with *Ap* and in BCT compared with BCS (5-fold).

As water transport was reported to be a target of Cd, a gene homologous to *PIP2A* (At3g53420; plasma intrinsic protein 2) was selected; this gene encodes a member of the plasma membrane aquaporins. A 2-fold overexpression in Cd-tolerant plants was confirmed between BCT and BCS, and between *Ah* and *Ap* after Cd treatment.

As the last member in the transport category, one mitochondrial protein import component, *TIM17* (homologous to At2g37410), was found to be expressed 2–3-fold more in *Ah* and in BCT, especially after Cd treatment.

Out of 134 CTR-TDFs, 71 fell into the class of cellular metabolism. Five genes have been chosen: two in the lipid modification class (homologous to At2g34690 and At5g65110), two of the AAA-ATPase family (homologous to At2g27600 and to At3g15120), and one in protein turnover (homologous to At5g67250). Several genes putatively implicated in the regulation of lipid composition were found to be overexpressed. Two genes implicated in this control were selected: accelerated-cell-death11 (*ACD 11*, homologous to At2g34690) and acyl-coenzymeA oxidase (*ACX2*, homologous to At5g65110). A 2-fold difference in expression of *ACD11* was observed between *Ah* and *Ap* and between BCT and BCS, and Cd treatment further enhanced expression in *Ah*. A similar difference was observed between *Ah*+Cd and *Ah*+Cd. *ACX2* expression was >10-fold higher in *Ah* and in BCT compared with *Ap* and BCS, but was not regulated by Cd treatment.

Three members of the AAA-type ATPase family (homologous to At2g27600, At3g15120, and At3g09840) were found and two of them were selected for confirmation. The transcript abundance of both genes, homologous to

Table 1. *Properties of cloned TDFs expressed only in tolerant genotypes or with a higher expression in the tolerant genotypes, identified by cDNA-AFLP*

In the last column, modification of TDF abundance after Cd treatment is indicated as plant material/observation. The following abbreviations refer to plant material: S, all sensitive genotypes (*A. lyrata* ssp. *petraea* and pool of BC-sensitive genotypes); BCS, pool of BC-sensitive genotypes; T, all tolerant genotypes (*A. halleri*, F_1, and pool of tolerant BC genotypes); BCT, pool of tolerant BC genotypes; Ap, *Arabidopsis lyrata* ssp. *Petraea*; Ah, *Arabidopsis halleri*. The following symbols refer to expression: +, higher expression; –, lower expression; =, no change in expression; 0, expression not detected.

Clone	Insert size (bp)	Best homology	Assigned functions	Protein ID	BLASTN score	% Nucleotide identity with *At*	Cd treatment
Transcription and chromatin remodelling							
356a	132	At2g16770	AtbZIP23—bzip transcription factor	Q9SLE1	4e-020	90%	S/0; T/+
348-2a	199	At4g02640	bZIP transcription factor family protein	O22763	5e-53	89%	Ap/0; S&T/–
246-1b,e	235 235	At1g79020	Transcription factor-related	Q8GYQ9	1e-078 2e-077	94% 93%	Ap/0; S&T/–
410b,c,d 425a,b,d	348 348	At1g78280	Transcription factor jumonji; similar to apoptotic cell clearance receptor PtdSerR	Q9M9E8	4e-055	90%	Ap/0; Ah/–
403a	253	At1g04850	C2H2 zinc finger; ubiquitin-associated (UBA)/TS-N domain-containing protein	Q9MAT3	3e-090	97%	S/0; T/=
242a	368	At1g11020	Zinc finger (C3HC4-type RING finger) family protein	Q8GYT4	e-115	96%	S/0; T/=
104b	252	At2g42350	Zinc finger (C3HC4-type RING finger) family protein	Q9SLC4	2e-016	96%	S/0; T/=
363e	163	At2g36320	Zinc finger (AN1-like) family protein	Q9SJM6	3e-015	95%	Ap/0; T/=
225d	387	At2g40140	CCCH-type zinc finger protein (zinc finger C-x8-C-x5-C-x3-H type)	Q9XEE6	e-102	89%	Ap/0; Ah/–; BCT/–; BCS/–
230c	390				e-102	89%	
240e	387				e-101	88%	
241b	385				e-104	89%	
245b	389				e-103	89%	
265b	398				1e-096	88%	
259-1a	334	At5g63470	CCAAT-box-binding transcription factor Hap5a (DNA-dependent)	Q9FMV5	6e-052	93%	S/0; T/=
364a	165	At3g01470	ATHB-1—homeobox-leucine zipper protein 5 (HAT5)	Q02283	1e-050	92%	S/0; T/=
365d	159	At5g64340	Basic helix–loop–helix (bHLH) domain-containing protein	Q9FMF4	8e-058	95%	S/0; T/=
411b	690	At5g22650	HD2B—histone deacetylases 2 B (plant-specific class)	Q9FNJ6	e-155	86%	Ap/0; T/=
20a, 107a	284	At2g18050	Histone H1-3 (HIS1-3)	P94109	2e-092	96%	S/0; T/+
42a	278	At5g59950	Transcriptional coactivator-like protein; RNA and export factor-binding protein	Q8L773	6e-078	93%	S/–; T/=
89d	436	At1g79650	DNA repair protein RAD23	Q8LA46	2e-020	96%	S/0; T/=
327b	206	At5g43990	SET-domain transcriptional regulator family (chromatin modification)	Q941L4	2e-077	95%	Ap/0; Ah/–
81b	182	At2g45000	Expressed protein (regulation of transcription, DNA-dependent; DNA binding)	Q9SHD8	2e-042	97%	Ap/0; Ah/–
87d	163	At5g04560	DME1—transcriptional activator DEMETER	Q8LK56	4e-071	97%	S/0; T/=
Transport							
201a	92	At2g16510	AVA-P1—vacuolar H^+-pumping ATPase 16 kDa proteolipid	P59227	3e-011	93%	S/0; T/=
1c	204	At2g47830	MTPc1—cation efflux family protein/metal tolerance protein	O82250	1e-059	95%	Ap/0; T/=
8b	200	At3g53420	PIP2A—plasma membrane intrinsic protein (aquaporin)	P43286	1e-023	91%	Ap/0; T&BCS/+
237c	377	At5g17850	CAX8—cation exchanger, putative; Ca^{2+}:cation antiporter (CaCA) family member	Q9FKP2	9e-077	95%	Ap/0; T/=
256b	354	At3g06130	HMA (heavy-metal-associated) domain-containing protein	Q9M8K5	6e-039	95%	S/0; T/+
325b 326a 329c 345a 386a 393c 394a	380	At2g37410	ATTIM17-2—mitochondrial inner membrane translocase TIM17	Q9SP35	3e-086	89%	S/–; T/=
213	251	At2g34690	ACD11—accelerated cell death 11	O64587	5e-093	98%	Ap/0; T/=
94b	65	At1g61640	ABC1 family protein	Q9SY86	8e-022	95%	Ap/0; T/=
216b	131	At2g48020	Sugar transporter-like protein	Q9ZU87	8e-007	97%	Ap/0; T/=
43a	310	At2g17480	MLO8—membrane protein	O22757	9e-068	91%	S/0; T/=

Table 1. (*Continued*)

Clone	Insert size (bp)	Best homology	Assigned functions	Protein ID	BLASTN score	% Nucleotide identity with *At*	Cd treatment
Cellular metabolism							
256b	354	At5g65110	ACX2—acyl-CoA oxidase 2	Q9FJQ4	3e-077	93%	S/–; T/+
381b	262	At3g06690	ACX3—acyl-CoA oxidase 3	Q9C839	3e-092	95%	S/–; T/–
353a	440	At2g38040	CAC3—acetyl co-enzyme A carboxylase	Q9LD43	e-115	95%	Ap/0; T/=
76c	122	At5g61440	Thioredoxin-like 3	Q9XFI1	2e-026	93%	S/–; T/–
409b	234	At1g45145	ATTRX5—thioredoxin H-type 5 (TRX-H-5, Cytosolic)	Q39241	2e-044	89%	S/–; T/=
98a	299	At5g64120	Peroxidase, putative	Q43387	e-122	97%	Ap/0; T&BCS/–
265a	237	At1g55020	LOX1—lipoxygenase 1	Q06327	1e-080	92%	S/–; T/–
23a	170	At1g48030	Dihydrolipoamide dehydrogenase 1, mitochondrial (MTLPD1)	Q9M5K3	2e-024	95%	S/0; T/=
361b	160	At1g65820	Microsomal glutathione *S*-transferase	Q94BY2	2e-032	89%	Ap/0; T&BCS/–
27c	226	At3g02470	SAMdc—*S*-adenosylmethionine decarboxylase	Q96286	2e-055	100%	Ap/–; T&BCS/+
339b	282	At2g36530	LOS2—enolase (involved in light-dependent cold tolerance)	P25696	2e-089	97%	S/–; T/=
40a	300	At4g21850	Putative methionine sulphoxide reductase	Q84JT6	e-110	95%	S/0; T/=
74a	196	At2g43020	Putative amine oxidase 1	Q9SKX5	7e-009	89%	Ap/0; T&BCS/=
235d	196	At3g43670	Copper amine oxidase, putative	Q9M2B9	9e-005	65%	S/0; Ah/+
249	277	At4g37370	Cytochrome P450, putative	Q9SZT7	5e-058	87%	Ap/0; T&BCS/=
367b	224	At5g56090	Cytochrome oxidase assembly family protein	Q9FKT8	5e-063	92%	S/0; T/=
266d	242	At1g13440	Glyceraldehyde-3-phosphate dehydrogenase (involved in glycolysis and gluconeogenesis)	Q9FX54	2e-037	97%	S/–; T/=
205b	129	At4g17260	L-Lactate dehydrogenase (involved in glycolysis and K^+ transport)	O23569	3e-046	93%	S>T; S,T/+
334d	266	At1g30720	FAD-binding domain-containing protein	Q9SA87	3e-053	87%	Ap/0; T&BCS/=
317b	248	At2g43400	Electron transfer flavoprotein-ubiquinone oxidoreductase family protein	O22854	1e-060	95%	S<T; S,T/=
255e	150	At5g18590	Kelch repeat-containing protein (identical to RanGAP1-interacting protein)	Q8L869	3e-050	93%	S/0; T/=
360a	248	AT1G14330	Kelch repeat-containing protein	Q9M9S9	4e-068	91%	Ap/0; T&BCS/–
15a 37a	445	At2g27600	AAA-type ATPase family protein—DNA replication origin binding activity	Q9ZNT0	e-138	99%	Ap/0; T&BCS/=
266a 305a,e 311a 316 2b 321a 325a 372c,d 380a 384a	348	At3g15120	AAA-type ATPase family protein (chaperone-like ATPase; nucleic acid binding)	Q9LIM2	7e-059	94%	Ap/0; T&BCS/=
251a	250	At3g09840	CDC48A—cell division cycle protein (CDC48), member of AAA-type ATPase family	P54609	2e-084	92%	S/0; T/=
55, 56, 57	300	At5g47030	ATP synthase delta' chain, mitochondrial precursor	Q96252	7e-04	80%	S/0; T/=
59	280	At5g67250	SKIP2—SKP1-interacting partner 2	Q9FE83	6e-078	95%	S/0; T/=
233b	373	At5g39470	F-box family protein; similar to SKP1-interacting partner 2 (SKIP2)	NP_ 198763	6e-017	92%	Ap/0; T&BCS/=
52a	266	At4g33160	F-box protein family (FBX13); (ubiquitin-protein ligase activity; protein catabolism)	Q9SMZ3	3e-039	88%	Ap/0; T&BCS/=
337c	225	At5g20620	UBQ4—polyubiquitin	P59263	2e-053	94%	Ap/0; T/=
403a1	253	At1g04850	Ubiquitin-associated (UBA)/TS-N domain-containing protein	Q9MAT3	3e-090	97%	S<T; S,T/=
336b	242	At1g72370	40S ribosomal protein SA (RPSaA)	Q93V81	8e-055	89%	Ap/0; T/=
249b	164	At3g04840	40S ribosomal protein S3A (RPS3aA)	Q9CAV0	1e-031	96%	Ap/0; T/=
28c	230	At4g39200	40S ribosomal protein S25-2 (RPS25E)	Q9T029	4e-029	88%	Ap/0; T&BCS/=
204	457	At2g47110	40S ribosomal protein S27A (RPS27aB)/ubiquitin extension protein 6 (UBQ6)	P59232	2e-018	82%	S<T; S,T/=
61	147	At2g37270	40S ribosomal protein S5-1	Q9ZUT9	4e-031	100%	Ap/0; T/+

Table 1. (*Continued*)

Clone	Insert size (bp)	Best homology	Assigned functions	Protein ID	BLASTN score	% Nucleotide identity with *At*	Cd treatment
49a	250	At2g01030, At3g41950	28S rRNA gene		8e-060	99%	S/0; T/–
422b	312	At2g24090	Putative chloroplast ribosomal protein L35	Q8VZ55	7e-056	93%	S/0; T/=
358b	280	At1g11650	RNA-binding protein 45 (RBP45)	Q9SAB3	6e-092	93%	Ap/0; T&BCS/=
19a	414	At4g25630	FIB2—fibrillarin 2 (component of SnRNP, functioning in rRNA processing)	Q9SZZ1	e-119	91%	Ap/0; T&BCS/=
89d	436	At5g26710	Glutamate-tRNA ligase/glutamyl-tRNA synthetase, putative	O82462	3e-022	94%	S/0; T/=
94a	462	At1g33520	KOW domain-containing ribosomal protein	Q9C801	5e-049	98%	S/0; T/=
238a	408	At1g64520	26S proteasome regulatory subunit RPN12	Q9SGW3	e-148	95%	Ap/0; T&BCS/=
78b	136	At3g51260	20S proteosomal α subunit D1 (interacts with SnRK, SKP1/ASK1)	P30186	5e-048	93%	Ap/0; T&BCS/=
205b	130	At1g73330	ATDR4—protease inhibitor, putative (involved in response to water deprivation)	Q39091	4e-045	90%	S>T; S,T/+
2a	240	At5g59880	ADF3—actin-depolymerizing factor 3	Q9ZSK4	9e-052	91%	S<T; S,T/=
66a	300						
34a	234	At4g38550	Putative phospholipase	Q9C5F6	3e-011	92%	Ap/0; S,T/=
36a	373	At4g13050	Oleoyl-(acyl-carrier-protein) hydrolase (fatty acid biosynthesis)	Q9SV64	e-106	91%	Ap/0; S,T/=
337b	368	At3g12120	FAD2—ω-6 fatty acid desaturase, endoplasmic reticulum	P46313	8e-044	97%	Ap/0; T/=
60	259	At5g23670	LCB2—encodes the LCB2 subunit of serine palmitoyltransferase	Q9LSZ9	1e-057	94%	Ap/0; T/=
62a	155	At4g11010	NDPK3—nucleoside diphosphate kinase 3, mitochondrial	O49203	2e-039	98%	T>S; S,T/=
228c	348	At5g07080	Transferase family protein similar to 10-deacetylbaccatin III-10-O-acetyl transferase	Q9LYQ7	e-117	93%	Ap/0; T/=
234b	420	At3g05950	Germin-like protein subfamily 1 member 7	Q9SFF9	e-149	94%	Ap/0; T/=
258c	183	At5g39110	Germin-like protein	Q9FID0	6e-055	94%	Ap/0; T&BCS/+
312a	378	At5g62200	Embryo-specific protein-related; similar to embryo-specific protein 3 (ATS3)	Q6NPM5	6e-080	91%	S/0; T/=
304a	274	At1g48420	Desulphydrase family similar to D-cysteine desulphydrase	Q8W4C7	5e-081	94%	S/0; T/=
229d	359	At3g48530	CBS domain-containing protein (a family of magnesium transporters contain CBS domains)	Q9S7W6	3e-072	91%	S/0; T/=
260e	321	At2g46520	Cellular apoptosis susceptibility protein, putative/importin-α re-exporter	Q9ZPY7	3e-078	94%	S/0; T/=
315d	248	At3g61190	BAP1—BON1-associated protein 1 (contains a Ca^{2+}-dependent membrane-targeting module found in many cellular proteins involved in signal transduction or membrane trafficking)	Q941L2	4e-032	87%	Ap/0; T/=
254c	191	At4g35860	ATGB2—GTP-binding protein	Q38922	5e-059	95%	S/0; T/=
261a	359	At3g43300	Guanine nucleotide exchange family protein (ARF family; ADP ribosylation factors)	Q9LXK4	e-115	95%	S/0; T/=
47a	267	At2g24765	ADP-ribosylation factor 3 (ARF3; involved in protein trafficking; may modulate vesicle budding and uncoating within the Golgi apparatus)	P40940	5e-055	90%	Ap/0; T/=
310a	146, 179	At5g11710	Putative clathrin binding protein; involved in endocytosis and cytoskeletal machinery	Q8VY07	4e-036	97%	Ap/0; T/=
108e	191	At3g07680	Emp24/gp25L/p24 family protein similar to Cop-coated vesicle membrane protein p24 precursor	Q9S7M9	1e-083	95%	Ap/0; T/=
108	220	At1g24460	Myosin heavy chain-related (required for protein transport from the ER to the Golgi complex)	Q9FYL7	5e-092	93%	Ap/0; T/=
206b	420	At4g31340	Myosin heavy chain-related (involved in lipid transport; located in endomembrane system)	Q8GUN1	e-141	89%	S/0; Ah/-
421e	206	At5g09760	Pectinesterase family protein (putative pectin methylesterase; cell wall)	Q9LXD9	4e-067	91%	S<T; S,T/=
237	220	At5g53330	Expressed protein (proline-rich cell wall protein-like)	Q8LG11	2e-068	86%	Ap/0; T/=
323c	242	At4g14900	Hydroxyproline-rich glycoprotein family protein; extensin-like protein	Q940H8	3e-063	90%	Ap/0; T/=
362a	174	At1g70620	Cyclin-related; extensin-like protein	Q9CAB4	5e-017	93%	Ap/0; T/=
101a	512	At3g11700	β-Ig-H3 domain-containing protein/fasciclin domain-containing protein	Q93W32	e-140	93%	Ap/0; T/=
371a	381	At5g62700	Tubulin β-2/β-3 chain (TUB3)	P29512	e-141	92%	Ap/0; T/=

Table 1. (*Continued*)

Clone	Insert size (bp)	Best homology	Assigned functions	Protein ID	BLASTN score	% Nucleotide identity with *At*	Cd treatment
Signal transduction							
30d	231	At3g24770	CLE41, putative CLAVATA/ESR-related 41 protein kinase	Q84W98	7e-065	96%	S/0; T/+
251	190	At2g28910	CXIP4—CAX-interacting protein 4; activates the H^+/Ca^{2+} antiporter CAX1	Q9ZV23	3e-031	97%	S<T; S,T/=
400a	205	At5g63610	Putative cyclin-dependent kinase E1; similar to cyclin-dependent kinase cdc2MsE	Q84TI6	4e-033	85%	S/0; T/=
29a	173	At5g49280	Glycosylphosphatidylinositol (GPI)-anchored protein, extensin related	Q9FJ12	6e-012	93%	Ap/0; T/+
313c	350	At1g26270	Phosphatidylinositol 3- and 4-kinase family	Q9C671	4e-010	81%	S/0; Ah/–
350c	221	At5g06140	Phox (PX) domain-containing protein; involved in intracellular signalling pathways	Q9FG38	2e-018	96%	Ap/0; T/=
331a	201	At5g14720	Ste-20-related kinase SPAK; (STE20—kinase regulates Na–K–Cl co-transport)	Q9LER4	1e-64	93%	Ap/0; Ah/–
347c	252	At1g10580	Transducin family protein/WD-40 repeat family protein similar to splicing factor hPRP17	Q9XIJ3	1e-099	96%	Ap<T; S,T/=
64	134	At5g64350	FKBP12—FK506-binding protein/immunophilin	O04263	3e-010	97%	S<T; T/+
24c	249	At3g25230	Peptidyl-prolyl *cis–trans* isomerase/FK506-binding protein (ROF1)	Q9LSF3	3e-024	90%	S/0; T/=
407a	312	At5g57050	ABI2—protein phosphatase 2C ABI2/PP2C ABI2/abscisic acid-insensitive 2 (ABI2)	O04719	3e-060	93%	S<T; T/=
361e1	120	At5g08590	ASK2—serine/threonine-protein kinase	P43292	3e-034	95%	Ap/0; T&BCS/–
95d	250	At2g24360	Serine/threonine/tyrosine kinase, putative	Q9ZQ31	2e-074	95%	S/+; T/–
318d	430	At3g24660	TMKL1—transmembrane kinase-like 1, 7 leucine-rich repeat transmembrane protein kinase	P33543	6e-026	94%	S/0; T/=
340d	121	At1g73080	Leucine-rich repeat transmembrane protein kinase (highly similar to receptor-like protein kinase)	Q9SSL9	1e-042	96%	S<T; S,T/=
17e	172	At5g61560	Receptor-like protein kinase	Q9FKG5	6e-012	100%	S/0; T/=
403d2	185	At5g41260	Protein kinase family protein	Q9FHD7	4e-022	92%	S<T; S,T/=
31a	363	At2g15760	Calmodulin-binding protein	Q9ZQD8	e-114	93%	S<T; T/=
89a	250	At2g41410	Calmodulin-like protein (calcium ion binding; contains EF-hand domain)	P30188	e-120	98%	S/0; T/=
404a	211	At2g45380	Expressed protein; contains EF-hand like domain	O22134	2e-051	88%	S<T; S,T/=
Cell rescue/stress-related/defence							
384 c	258	At4g11650	ATOSM34 – osmotin-like protein	P50700	7e-021	94%	S<T; T/+
226 236a 243d 255 263c	400 382 355	At5g56030	HSP81-2—heat shock protein 81-2	P55737	e-155, e-149, e-155	96%, 96%, 97%	S/0; T/=
102d	161	At3g03270	Universal stress protein (USP) family protein/early nodulin ENOD18 family protein	Q8LFK2	2e-027	98%	Ap<T&BCS; S,T/–
87e	169	At5g54430	Universal stress protein (USP) family protein	Q8VYN9	1e-025	89%	S/0; T/=
250	250	At1g69840	Band 7 family protein (integral membrane protein thought to regulate cation conductance)	Q9CAR7	e-103	95%	Ap/0; T&BCS/=
418e	385	At3g55840	Expressed protein (putative nematode resistance protein)	Q9LY61	e-136	92%	Ap/0; T&BCS/=
Proteins with unknown function							
21a	220	At5g06370	Unknown NC domain-containing protein	Q93V51	3e-034	93%	S<T; T/+
399a	183	At3g59320	Anthocyanin-related membrane protein 2; integral membrane protein	Q948Q8	6e-006	86%	S<T; S,T/=
99a	437	At4g15470	Expressed protein, low similarity to *N*-methyl-D-aspartate receptor-associated protein	Q94A20	e-172	93%	Ap/0; S,T/=
45a	294	At5g55120	Expressed protein	Q9FLP9	7e-050	92%	S<T; T/–
222	246	At5g35525	Expressed protein	NP 680337	3e-037	93%	S=T; S,T/+
232e	220	At1g04590	Expressed protein	Q9C5I2	2e-037	84%	S<T; S,T/=
248c	186	At3g62050	Expressed protein	Q8LC17	2e-048	91%	Ap/0; S,T/=
253b	190	At2g46890	Expressed protein	O81042	3e-010	95%	Ap/0; S,T/=
332c	401	At5g22090	Expressed protein	Q9C579	e-145	91%	Ap/0; S,T/=

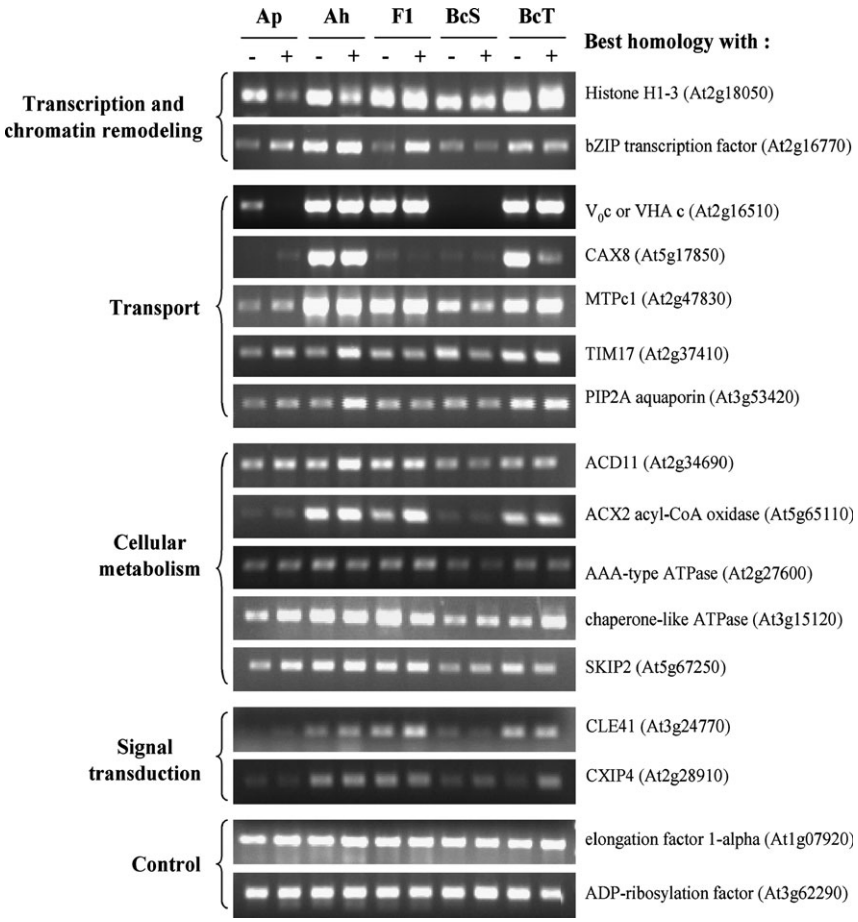

Fig. 2. RT–PCR analysis of the steady-state transcript levels of candidate genes in *A. petraea* (Ap), *A. halleri* (Ah), F_1, backcross-sensitive (BCS), and backcross-tolerant (BCT) genotypes. Plants were cultivated for 4 weeks in hydroponic conditions. Roots were harvested 72 h after being exposed (+) or not (–) to 10 μM $CdSO_4$. Total RNA was extracted and used as a template for cDNA synthesis. PCR products were amplified using the specific primers given in Table 3. The presented photographs are representative of three independent extractions.

At2g27600 and to At3g15120, was 2-fold higher in *Ah* and in F_1 compared with *Ap*. Expression was 1.5-fold higher in BCT compared with BCS as well as in BCT+Cd compared with BCS+Cd. The expression of the putative SKP-interacting partner 2 gene (homologous to *SKIP2*, At5g67250), which is a part of the ubiquitin-protein ligase, was reassessed. A 2-fold induction in *Ah* versus *Ap* and in BCT compared with BCS was confirmed.

In the signal transduction category, two candidates, the *CLE41* and the *CXIP4* homologous genes, were re-analysed. Expression levels of *CLE41* (homologous to At3g24770) showed a 5-fold overexpression in *Ah* and BCT compared with *Ap* and BCS, and a >10-fold over-expression for F_1 versus *Ap*. The presence of Cd induced a 2-fold increase in *CLE41* expression in *Ap*, *Ah*, and F_1, but no significant change in BCS and BCT. The transcript abundance of the homologue of the *A. thaliana* gene encoding the CAX-interacting protein 4 (*CXIP4*; At2g28910), which is implicated in the activation of CAX1, was 3-fold more abundant in *Ah* and in F_1 than in *Ap*. Overexpression in BCT compared with BCS was only visible upon Cd treatment.

The expression of five genes confirmed by semi-quantitiative RT–PCR in four sensitive and four tolerant individual BC1 genotypes was analysed further (Fig. 3). EC_{100} values associated with those genotypes and previously determined by Bert *et al.* (2003) are given in Table 2. The expression of none of the candidate genes strictly co-segregated, but was, on average, more abundant in the BCT than in the BCS.

Discussion

The two Cd-hyperaccumulator species, *T. caerulescens* and *A. halleri* have been previously characterized to a certain extent; both of them belong to the Brassicaceae family and are also Zn hyperaccumulators. *Arabidopsis halleri* is considered as a model species for metal tolerance studies, mostly because of its close relationship to *A. thaliana.* *Arabidopsis halleri* species seems to be constitutively

tolerant to Cd since all accessions of *A. halleri* tested to date showed Cd tolerance (Bert *et al*., 2002). Cd hyperaccumulation is, however, not constitutive in the species but is present in the Auby ecotype, which was used in this study.

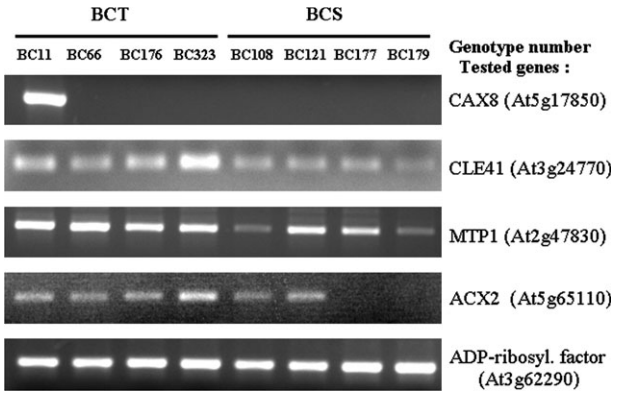

Fig. 3. Expression of six genes confirmed by RT–PCR in four tolerant (BCT; BC11, BC66, BC176, and BC323) and four sensitive (BCS; BC108, BC121, BC177, and BC179) backcross genotypes. Plants were cultivated during 4 weeks in hydroponic conditions. Roots were harvested after 72 h of 10 μM $CdSO_4$ exposure. Total RNA was extracted and used as a template for cDNA synthesis. PCR products were amplified using the specific primers given in Table 3. The presented photographs are representative of three independent extractions.

Table 2. *EC100 values of four tolerant (BC11, BC66, BC176, and BC323) and four sensitive (BC108, BC121, BC177, and BC179) backcross genotypes*

Backcross genotypes	EC100 (in μM Cd)
BC11	150
BC66	133
BC176	133
BC323	150
BC108	25
BC121	25
BC177	25
BC179	25

While hyperaccumulators are usually good translocators of heavy metals from the root to the shoot, *A. halleri* population Auby retains most of the absorbed Cd in the roots. Zn, however, is translocated much more than Cd, with reported shoot:root concentration ratios always higher than 1 (Küpper *et al*., 2000; Bert *et al*., 2003). However, Zn and Cd tolerance characters seem to co-segregate partially in *A. halleri*, underlying the fact that common mechanisms are responsible for both Cd and Zn tolerance (Bert *et al*., 2003). Those mechanisms are thought to rely on detoxification and compartmentation.

Previous transcriptomic studies of *A. halleri* (of the non-Cd accumulator population of Langelsheim) have been performed, using the *A. thaliana* microarray chips containing probes for ~8300 genes, to identify genes highly expressed in the non-treated roots (Weber *et al*., 2004) or Cd^{2+}-exposed roots (Weber *et al*., 2006), and shoots of *A. halleri* grown under low or high Zn supply (Becher *et al*., 2004). For the Cd/Zn hyperaccumulator *T. caerulescens*, a new microarray chip was developed, containing 1900 cDNAs isolated from roots. This array was hybridized with reverse-transcribed RNA from two accessions of Zn-treated *T. caerulescens* plants originating from two different soil types (Plessl *et al*., 2005). This comparative transcript profiling resulted in the identification of genes that are constitutively highly expressed in the hyperaccumulators and genes affected by heavy metals. The current microarray technique, despite its strengths, still has limitations. The principal limitation for many organisms is that only a fraction of the genes for which either the DNA sequence or a cDNA clone is available can be investigated. Another additional disadvantage for microarrays is the difficulty in distinguishing among different transcripts of genes belonging to the same family, when using spotted arrays. Finally, there are two additional technical limitations that restrict the broad use of the current microarray technology, namely the requirement for large amounts of RNA and the sensitivity of hybridization; 40%

Table 3. *PCR primers, annealing temperature, and cycle numbers used in this study to obtain quantitative signals from each gene tested*

Genes	Forward primer 5′–3′	Reverse primer 5′–3′	T (°C)	No. of cycles
At1g07920	CAACATTGTGGTCATTGGCCACGTCGA	CTCCTTCTCAATCTCCTTACCAGAAC	62	24
At3g15120	AAAGTTCTGGAGCTCTTAGTTGGA	TTGTTCAACTGGTTCAGGCATGTT	62	35
At2g16510	CTTCAATGGGAGTGATGAGACCTGAGT	CCAACAAAATAACATCCTACACTC	62	39
At2g16770	TTTGCTTGTTGATATAAGAGGAAG	CACATGAATGATGATACTTCAAAC	58	36
At5g17850	CATTTGCTTCTTCTGTGCTGCT	ACCAAGCCTCATACGGTTACT	62	47
At2g18050	AACCGAAAACCACCACTCATCCTCCAT	GCTGTAGAAAAAGTATTCCTACGG	62	47
At3g24770	CCAGCTTCATCAACAATGGATCTAC	CTCACCGGTAAAAAACCAGATGT	62	30
At2g27600	GGAAACGAGAGTGAAGCTTCAGACGTA	TCAACCTTCTTCTCCAAACTCCTGTGTG	62	27
At2g28910	GATGTTGATTCTGAGATGGAGAGGA	GACCGCTTAGAAGAACCACGTCCA	62	30
At2g34690	ATGGCGGATTCGGAAGCAGATAAGCCA	ATGGTGCCGATGAATTGACATAGCTT	62	27
At2g37410	GGGAACACCAGAGACATCTCG	GGAGGAGCATCAAAACTCTCC	62	27
At2g47830	GGAGTGGTTTGATGATGGCAAATGCT	AAGCTGAATGCTGACACGAGCCACA	62	33
At3g53420	GTGGAAGCCGTTCCCGGAGAAGG	TTAGACGTTGGCAGCACTTCTGAAAGATCA	62	24
At3g62290	GGTCTCGATGCAGCTGGTAAGACTAC	TGTTAGAGAGCCAGTCAAGTCCCTCA	62	24
At5g65110	GCAATTGGTTGCTGATGTCCA	GCTTCGATGAACTTGGCAAGA	62	30
At5g67250	TCGAGGAATTCATGAAGCAGC	ACCACTAGTGTCCTCCTTTGT	62	35

(Breyne and Zabeau, 2001), 53% (Weber *et al*., 2004), or 60% (Becher *et al*., 2004) of the transcripts can be detected, whereas in the case of hybridization of *A.halleri* cDNA to *A. thaliana* chips, 25% (Weber *et al*., 2004) to 35% (Becher *et al*., 2004) of the genes were detected.

In this study, a genome-wide comparative transcriptomic analysis was carried out by cDNA-AFLP between closely related genotypes mainly differentiated by the Cd tolerance character. The principal advantage of cDNA-AFLP compared with microarrays is that it allows genome-wide expression analysis in any species without prior sequence knowledge. In addition, it has the inherent advantage of identifying and assessing new genes. One drawback of this technique is that generating a global overview of gene expression patterns involves a time-consuming series of PCRs and data cannot readily be compared and merged. However, this technique offers the best alternative for performing transcript profiling in non-model plant species, and for in-depth analyses of gene expression, overcoming the problems caused by plant gene redundancy during microarrays studies (Reijans *et al*., 2003; Vuylsteke *et al*., 2006).

In this study, genotypes coming from interspecific crosses were compared. *Arabidopsis halleri* and *A. petraea* are thought to have diverged ~2.9 million years ago (X Vekemans, personal communication), which is half the time for the divergence between *A. halleri* and *A. thaliana* (Koch *et al*., 2001). The polymorphism between *A. halleri* and *A. petraea* probably introduces a bias in the comparison of transcript profiles. However, the comparison of pools of BC plants is thought to have reduced the impact of this problem and favoured the identification of TDFs associated with Cd tolerance. In strong support of this, the proportion of TDFs that were validated (61%; 14 out of 23) was similar to other cDNA-AFLP analyses that compared the same genotype in different growth conditions (Yang *et al*., 2003; Mao *et al*., 2004; Fusco *et al*., 2005). Lack of confirmation may be due to polymorphism problems, but also to difference in growth conditions and physiological status of the plants.

From an estimated 80–85% of the transcriptome, corresponding to ±19 000 transcript tags per genotype and treatment condition scanned by cDNA-AFLP, 188 CTR-TDFs overexpressed in the tolerant genotypes were discovered. Sequences were obtained for 156 CTR-TDFs. Of these, 134 presented significant homology with *A. thaliana* genes with known or putative functions, whereas no homology was found to any nucleotide or amino acid sequences in the database for the remaining 22 CTR-TDFs. Similar findings were reported in cDNA-AFLP studies, even in recent articles on *A. thaliana* (De Paepe *et al.*, 2004). These 22 sequences probably belong to the 3′ untranslated region (UTR) of genes, which is frequently more divergent between the genes of *A. thaliana* and *A. halleri*, or to regions close to the centromeric regions, for which even in *A. thaliana* the whole genomic sequence is as yet unknown.

Several categories of genes expressed more in the tolerant genotypes and possibly involved in Cd tolerance are discussed below.

Cellular detoxification and repair

Cd is not a redox active metal ion; however, it can cause oxidative stress (Smeets *et al*., 2005). Detoxification can occur via proteins that either scavenge the ROS produced by Cd, or repair the cellular components damaged directly by Cd or derived ROS. In this category, sequences of thioredoxin-like 3 (homologous to At5g61440), thioredoxin H type 5 (homologous to At1g45145), glutathione *S*-transferase (GST, homologous to At1g65820), *S*-adenosylmethionine decarboxylase (SAMdc; homologous to At3g02470), a putative methionine sulphoxide reductase (homologous to At4g21850), an HSP (homologous to AtHSP81-2), and AAA-ATPases were identified.

Thioredoxins (TRXs) play direct roles in the antioxidative system by regenerating peroxiredoxins oxidized by peroxides (Foyer and Noctor, 2005). In *S. cerevisiae*, Cd inactivates TRX (Vido *et al*., 2001), and TRX expression is up-regulated during Cd treatment in *S. pombe* (Bae and Chen, 2004). GSTs are considered to play an important role in cellular protection against oxidative stress by synthesizing glutathione-*S*-conjugates, and several members have been shown to be up-regulated during Cd treatment (Marrs and Walbot, 1997; Bae and Chen, 2004; Fusco *et al*., 2005). Dihydrolipoamide dehydrogenase 1 (At1g48030) may also play a role in cellular detoxification. It belongs to a family of enzymes with an oxidoreductase activity, which, besides established roles in the regulation of enzymes of carbon metabolism, may modulate the activity of key antioxidative enzymes (Lutziger and Oliver, 2001).

Another gene associated with antioxidative defences is SAMdc, involved in polyamines synthesis. Higher levels of SAMdC were previously reported in plants during stress conditions (saline, drought, or external abcisic acid application). These plants also had a higher content of polyamines. Their antioxidant properties have been studied in various plant species under Cd-induced oxidative stress (Groppa *et al*., 2001).

HSPs are involved in repair mechanisms of proteins damaged directly by Cd or by ROS indirectly produced by Cd stress (Bae and Chen, 2004). The homologue of HSP81-2 (At5g46030) was constitutively overexpressed in *A. halleri* and in BCT. Methionine sulphoxide reductase repairs oxidative damage to methionine residues arising from ROS and reactive nitrogen intermediates (Taylor *et al*., 2003). Several predicted genes for AAA-ATPases, which are implicated in the folding of proteins, have also been identified in the present screen (homologous to At2g27600, At3g15120, and At3g09840) and may also play a role in protein repair.

Membranes are a main target of ROS, and many genes implicated in the synthesis of fatty acids or in the

metabolism of lipids are regulated during stress, such as acyl-CoA oxidase in response to dehydration (Grossi *et al*., 1995), or to UV (Logemann and Hahlbrock, 2002), or β-hydroxyacyl-ACP dehydratase in response to Cd (Fusco *et al*., 2005). In this study, several genes possibly involved in fatty acid biosynthesis or modifications have been identified, such as an acyl-CoA oxidase homologue (At5g65110), a putative phospholipase (At4g38550), *FAD2* and *LCB2* homologues, an oleoyl-acyl-carrier hydrolase, and a homologue to *ACD11*, which encodes a sphingosine transfer protein (Brodersen *et al*., 2002).

Metal sequestration

There is evidence in the Cd/Zn hyperaccumulator *T. caerulescens* that a considerable proportion of the accumulated Cd is sequestered within the cell vacuole in the form of malate complexes (Ueno *et al.*, 2005). In *A. halleri*, the forms of accumulated Cd have not been determined as yet, but malate has been identified as a main Zn ligand (Sarret *et al*., 2002). Active transport is required to sequester Cd from the cytoplasm into the vacuolar compartment. This might occur via primary active transport, i.e. through an ATP-energized transporter, or by secondary active transport through coupling to the gradient of protons.

Several genes possibly involved in Cd compartmentation that are constitutively overexpressed in tolerant genotypes were identified, such as *Ah*MTP1 (initially named ZAT1 in *A. thaliana*; Van Der Zaal *et al*., 1999), which encodes a vacuolar transporter of the CDF family. The gene has been identified, and is associated with Zn, Mn, Ni, and Co tolerance in a number of plant species (Van der Zaal *et al*., 1999; Assunção *et al*., 2001; Mäser *et al*., 2001; Persans *et al*., 2001; Delhaize *et al*., 2003; Desbrosses-Fonrouge *et al*., 2005), including in *A. halleri* (Dräger *et al*., 2004), where it has been associated with Zn compartmentalization, but not with Cd transport. Because most of the Cd-tolerant BC1 genotypes were also Zn tolerant, MTP1 might be associated with Zn tolerance.

A novel and interesting gene candidate is the 16 kDa V-ATPase component c gene (homologous to At2g16510), for which constitutively higher transcript levels were found in the tolerant genotypes. The H^+-translocating V-type ATPase (VHA) plays a central role in the growth and development of plant cells and has been localized not only in the tonoplast but also in other endomembranes, such as the endoplasmic reticulum (ER) or the Golgi apparatus. The proton electrochemical gradient formed by the VHA provides the primary driving force for the transport of numerous ions and metabolites against their electrochemical gradients, and thus may participate in intracellular membrane trafficking, sorting, and secretion. The 16 kDa proteolipid subunit (component c) is the principal integral membrane protein of the VHA complex that forms the proton channel responsible for translocating protons across lipid bilayers (Sze *et al*., 2002). No other component of this complex has been identified in the present analysis, meaning that they either are not limiting or that they escaped in the present analysis.

Another candidate gene which may be involved in Cd compartmentation is the homologue to At*CAX8* (At5g17850). The transcript of this gene is constitutively overexpressed in *A. halleri* and induced in *A. petraea* following Cd treatment. CAX8 belongs to the H^+/cation exchangers family but, to our knowledge, has not been characterized up to now. CAX2, another member of the family, was however characterized as a high affinity and high capacity H^+/Cd^{2+} antiporter of the tonoplast (Hirschi *et al*., 2000), involved in metal sequestration in the vacuoles (Hirschi *et al*., 2000). In addition, *CAX2* is constitutively overexpressed in the shoots of *A. halleri* compared with *A. thaliana* (Becher *et al.*, 2004). A homologue to *AtCXIP4* (At2g28910) was also identified, which was identified in *A. thaliana* as encoding a CAX1-interacting protein 4 (AtCXIP4). The *CXIP4* homologue was overexpressed *in A. halleri* and further induced by the addition of Cd in BCT.

The fact that members from the CDF and CAX transporter families are constitutively overexpressed in *A. halleri* roots and in tolerant genotypes derived from *A. halleri* supports their important role in the sequestration of metal.

Hydric balance

Cd has been previously reported to inhibit root water transport, and one of the targets of Cd toxicity is the PIP subfamily of aquaporins. In the present study on root transcriptome, an *A. halleri* gene homologous to the aquaporin *AtPIP2A* (At3g53420) was identified. Its expression was induced in sensitive and tolerant plants treated with Cd. RT–PCR confirmed a higher expression in the tolerant genotypes, and an induction by Cd in both *A. petraea* and *A. halleri*. In *A. thaliana*, *PIP2A* is expressed specifically in the vascular bundles, and the protein localizes to the plasma membrane (Kammerloher *et al*., 1994). Interestingly a PIP2 homologue was also induced by Cd treatment in *Brassica juncea* (Fusco *et al*., 2005), supporting that Cd affects water transport.

Signal transduction

Crucial regulatory roles in many aspects of plant development are played by members of the receptor-like kinase (RLK) gene family, but the ligands to which they bind are largely unknown. Two putative RLK genes (homologues to At1g73080 and At5g61560) have been identified in this study, as well as a CLE family member. However, none of the identified sequences have previously been associated with a heavy metal stress response.

A growing body of evidence suggests that the *CLE* genes constitute an ancient, functionally conserved gene family that plays important roles in plant growth and development (Strabala *et al*., 2006). CLE ligands interact with members of the leucine-rich repeat (LRR)-RLK receptor family.

Recently, *CLE41* was partially characterized in *A. thaliana* (Strabala *et al*., 2006). The *CLE41* (At3g24770) homologous gene was overexpressed in tolerant genotypes (*A. halleri*, F_1, BCT).

Transcriptional regulation

In the present study, the histone *Ah*H1-3 gene homologous to At2g18050, which was constitutively overexpressed in the tolerant genotypes in control growth conditions but repressed by Cd in both tolerant and non-tolerant species, was identified. H1-3 was previously associated with the stress response (Scippa *et al*., 2004). Polyamines and histones can influence the DNA structure by causing local DNA bending, which is an effective mechanism for promoting transcriptionally induced stresses (Peng and Jackson, 2000).

Several genes belonging to the bZIP, zinc finger, and basic helix–loop–helix (bHLH) transcription factor families were also identified in the present study. Among them, the gene *Ah*bZIP23 (homologous to At2g16770) is constitutively overexpressed in the tolerant genotypes compared with the sensitive ones, and induced by Cd, in both *A. halleri* and *A. petraea*. There are 75 distinct members of the bZIP family in *A. thaliana*, most of which are not functionally characterized. In fungi, bZIP transcription factors CAD1 and ZIP1 mediate resistance to Cd in *S. cerevisiae* and *S. pombe*, respectively (Wu *et al*., 1993; Harrison *et al*., 2005). The 19 predicted transcription factor genes, which were constitutively overexpressed in *A. halleri* when compared with *A. petraea*, suggest an important up-regulation of transcription. However, post-transcriptional regulations may also present modifications between *A. halleri* and *A. thaliana*.

Protein degradation

Proteolysis often plays a crucial role in the feedback control underlying transcription-dependent stress responses. The ubiquitin−proteasome pathway is one of the major pathways for targeted proteolysis in the eukaryotic cell (Hochstrasser, 1996; Hershko and Ciechanover, 1998). Several genes involved in the ubiquitin–proteasome pathway proteolysis were identified as constitutively more highly expressed in the Cd-tolerant genotypes. Some of them belong to the proteasomal complexes 26S and 20S, others encode F-box family proteins, ubiquitin ligases, polyubiquitin, and other ubiquitin-associated proteins, and three members of the AAA-type ATPase family, similar to those present in the 26S proteolitic complex (Table 1). Such an important number of genes involved in protein degradation processes led to the hypothesis that Cd^{2+} tolerance probably depends on efficient recycling of abnormal proteins.

Some of the reported *A. halleri* genes were previously identified by microarray analysis with *A. thaliana* GeneChips, such as the *AhMTP1* (At2g47830), the homologue to At2g48020, encoding a major facilitator superfamily putative sugar transporter (Becher *et al*., 2004), *Ah*Mlo8 (homologous to At2g17480), or the *Ah* gene encoding a 40S ribosomal protein homologous to At2g16360 (Weber *et al*., 2004, 2006). Others match *A. thaliana* homologues of the same family as those reported in the transcriptomic comparisons published by Becher *et al.* (2004) and Weber *et al.* (2004, 2006), such as the GST At1g65820, HSP At5g56030, zinc finger (AN1-like) family protein At2g36320, C2H2 zinc finger At1g04850, cytochrome P450 family protein At4g37370, zinc finger (C3HC4-type RING finger) family proteins At1g11020 and At2g42350, calcium-binding EF hand family proteins At2g41410 and At2g45380, peroxidase At5g64120, lipoxygenase 1 (At1g55020), or CAX8 (At5g17850). Many other genes were also revealed by this study. There are reasons for those differences; an important reason is that the population of *A. halleri* and the growth conditions are different. For example, the Langelsheim population used for the microarray analysis does not hyperaccumulate Cd, whereas the population from Auby does. Furthermore, the comparisons presented in this study are different and genes have been further investigated only if their corresponding TDF was more abundant in the pool of the tolerant BC1 genotypes than in the sensitive ones. For example, *NAS2* was previously published as being overexpressed in the roots of *A. halleri* compared with *A. thaliana* (Weber *et al*., 2004), but a significant difference in expression between the pools of contrasting BC genotypes was not observed (data not shown).

In addition, genes directly implicated in Cd chelation were not identified in the screen. This is consistent with the observation that there was also no up-regulation of the genes encoding the histidine biosynthetic pathway at the transcript level in *Alyssum lesbiacum* upon exposure to Ni, despite the fact there was a large increase in histidine biosynthesis (Ingle *et al*., 2005). Finally, because the cDNA-AFLP technology is based on a double digest, it can generate short fragments, which are lost in the analysis. This was, for example, the case for *AhHMA4*, which is however expressed more in tolerant genotypes than in sensitive ones (C Courbot, G Willems, P Motte, S Arvidsson, P Saumitou-Laprade and N Verbruggen, unpublished data).

Conclusion

The presented analysis has allowed the identification of new *A. halleri* genes, which are thought to be involved in the adaptation of the roots to tolerate high Cd concentrations. The transcriptome analysis revealed a large variation in genome expression, in controlled growth conditions, resulting from several million years of evolution between *A. halleri* and *A. lyrata*, and profound adaptation of the metabolism to high metal contents present in the environment of the *A. halleri* species. Many of the genes which were identified

as overexpressed in the roots of Cd-tolerant genotypes (*A. halleri*, F_1, and BCT) compared with the sensitive genotypes (*A. lyrata* ssp. *petraea* and BCS) are involved in cellular detoxification, repair, and sequestration, but the largest category belongs to the cellular metabolism pathways.

Despite its limitations, the cDNA-AFLP method has been proven to be a convenient and effective technique for the transcriptomic comparison of different genotypes, without prior knowledge of the genome sequence. Genes implicated in the Cd tolerance of the Zn/Cd hyperaccumulator plant *A. halleri* were revealed and confirmed by the use of an exceptional plant material, and a collection of *A. halleri* ESTs has been generated (dbEST Id numbers, from 33963327 to 33963631, 34147988, and 34147989).

This analysis has identified many putative target genes of Cd toxicity, which may be important to characterize further at the protein level, and it has confirmed the importance of detoxification and metal sequestration. However, the expression of none of the selected genes strictly cosegregates with the tolerance character, but was on average higher in tolerant genotypes of the backcross. Questions remain as to how this large array of genes is overexpressed in the tolerant genotypes. What is the sensing mechanism of the metal concentration in plants, which seems to be deregulated/different in hyperaccumulators?

In the future, it will be crucial to answer these questions and to pinpoint the major genes in order to understand tolerance to a toxic environment and to engineer efficient plants for phytoremediation of Cd-contaminated soils. Quantitative trait locus analysis will be a major tool to address that task.

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