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Expression of a truncated tobacco *NtCBP4* channel in transgenic plants and disruption of the homologous *Arabidopsis CNGC1* gene confer Pb^{2+} tolerance

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Summary

Recently we reported on a plasma membrane tobacco protein (designated NtCBP4) that binds calmodulin. When overexpressed in transgenic plants, NtCBP4 confers Pb^{2+} hypersensitivity associated with enhanced accumulation of this toxic metal. To further investigate possible modulation of Pb^{2+} tolerance in plants, we prepared transgenic plants that express a truncated version of this protein (designated NtCBP4ΔC) from which its C-terminal, with the calmodulin-binding domain and part of the putative cyclic nucleotide-binding domain, was removed. In contrast to the phenotype of transgenic plants expressing the full-length gene, transgenic plants expressing the truncated gene showed improved tolerance to Pb^{2+}, in addition to attenuated accumulation of this metal. Furthermore, disruption by T-DNA insertion mutagenesis of the *Arabidopsis CNGC1* gene, which encodes a homologous protein, also conferred Pb^{2+} tolerance. We suggest that NtCBP4 and AtCNGC1 are components of a transport pathway responsible for Pb^{2+} entry into plant cells.

Keywords: calcium, cyclic nucleotides, heavy metals, ion channel, knockout mutant, T-DNA.

Introduction

The characterization of plant genes that code for proteins involved in the transport of heavy metals should be useful in developing appropriate biotechnologies to clean the environment (e.g. phytoremediation; Raskin, 1996; Terry and Banuelos, 1999), as well as for engineering plant tolerance to toxic metals. Because certain metals such as lead (Pb^{2+}) are non-essential and toxic to plants, plants are unlikely to have transporters specific for these metals. Instead, the permeability of the plasma membrane to these metals is attributed to the inherently limited selectivity of certain ion transporters.

A family of channel proteins from barley (HvCBT1, Schuurink *et al.*, 1998); *Arabidopsis* (AtCNGC1-AtCNGC6, Köhler *et al.*, 1999; Leng *et al.*, 1999); and tobacco (NtCBP4, Arazi *et al.*, 1999) was recently reported. In *Arabidopsis*, at least 19 *CNGC* (cyclic nucleotide-gated ion channel)-related genes are distributed among the five chromosomes (N. Bouché, unpublished results), with some of them clustered (for example, genes *At2g46430*, *At2g46440* and *At2g46450* on chromosome 2). Structurally, all these proteins contain six putative transmembrane domains, a presumed pore region located between the fifth and sixth transmembrane domains, and a putative cyclic nucleotide monophosphate-binding domain coinciding with a calmodulin-binding site located at the C-terminus (Arazi *et al.*, 2000; Köhler and Neuhaus, 2000). These proteins are similar in overall structure to mammalian cyclic nucleotide-gated non-selective cation channels, implicated in Ca^{2+} signal transduction.

Various approaches have been used to investigate the function of this plant protein family. Köhler *et al.* (1999)

reported that two members of the *Arabidopsis* gene family (*AtCNGC1* and *AtCNGC2*) could partially complement a K^+-uptake-deficient yeast mutant. Leng *et al.* (1999), using heterologous expression systems, suggested that AtCNGC2 is a cyclic nucleotide-stimulated cation channel permeable to Ca^{2+} and K^+. Interestingly, Pb^{2+} is a highly toxic metal that can bind to Ca^{2+}-binding sites in regulatory proteins such as calmodulin (Ouyang and Vogel, 1998). The similarity in protein-binding sites for Pb^{2+} and Ca^{2+} is consistent with reports that Pb^{2+} entry into animal cells (Simons and Pocock, 1987; Tomsig and Suszkiw, 1991) as well as into plant cells (Huang and Cunningham, 1996) occurs, at least in part, through Ca^{2+}-permeable channels.

We have taken a reverse genetic approach to studying the function of NtCBPs and AtCNGCs. We reported on the plasma membrane localization of NtCBP4, and on the phenotype of transgenic tobacco overexpressing this protein. We found that overexpression of NtCBP4 conferred tolerance to Ni^{2+} and hypersensitivity to Pb^{2+}, associated with reduced Ni^{2+} uptake and enhanced Pb^{2+} uptake, respectively (Arazi *et al.*, 1999). Because NtCBP4 is, to date, the only identified plant protein capable of modulating tolerance and accumulation of Pb^{2+}, we investigated other possibilities of manipulating Pb^{2+} tolerance in tobacco by engineering NtCBP4.

In addition, we tested whether *NtCBP4* homologues in *Arabidopsis* encode proteins that are potentially involved in Pb^{2+} transport. Comparison of *NtCBP4* with the *CNGC* gene family of *Arabidopsis* on a phylogenetic tree reveals that *NtCBP4* is most related to the *Arabidopsis CNGC1* gene. At the protein level, *NtCBP4* is 82% similar and 75% identical to *CNGC1*. Therefore *AtCNGC1* is a probable orthologue of *NtCBP4* in *Arabidopsis*. Subsequently, we isolated a *CNGC1* knockout mutant and investigated its response to Pb^{2+}. Here we demonstrate that expression of a truncated *NtCBP4* in the transgenic tobacco, and disruption of *CNGC1* in *Arabidopsis* resulted in improved tolerance to Pb^{2+}.

Results

Transgenic tobacco plants expressing a truncated NtCBP4 *exhibit improved tolerance to* Pb^{2+}

The C-terminal half of *NtCBP4* contains a structurally conserved putative cyclic nucleotide-binding domain and a high-affinity calmodulin-binding site (Arazi *et al.*, 2000). In contrast to the mammalian cyclic nucleotide-gated channels, where the calmodulin-binding domain is near the N-terminus of the protein (Varnum and Zagotta, 1997), in *NtCBP4* the calmodulin-binding site coincides with the predicted αC-helix structure of the cyclic nucleotide-binding domain (Arazi *et al.*, 2000). This helix is essential for cyclic nucleotide binding in related proteins from other organisms (Shabb and Corbin, 1992). We reasoned that removal of this important helix should disrupt the binding of both cyclic nucleotides and calmodulin, and could possibly render the channel inactive. Moreover, if plant *NtCBP4*-associated channels function as tetramers, as do mammalian cyclic nucleotide-gated channels (Liu *et al.*, 1996), expression of an inactive subunit might have inhibitory effects on endogenous native *NtCBP4*-associated channel activities. As a first step to test this possibility, we prepared a truncated version of *NtCBP4* from which a major part of the C-terminal half, including the αC-helix, was removed (designated *NtCBP4ΔC*; Figure 1).

Transgenic plants expressing *NtCBP4ΔC* were prepared as described in Experimental procedures, selected on kanamycin, and transferred to the greenhouse for seed production. Seeds were plated on kanamycin-containing medium to select for progeny carrying the transgene. To investigate the possible effects of *NtCBP4ΔC* expression on

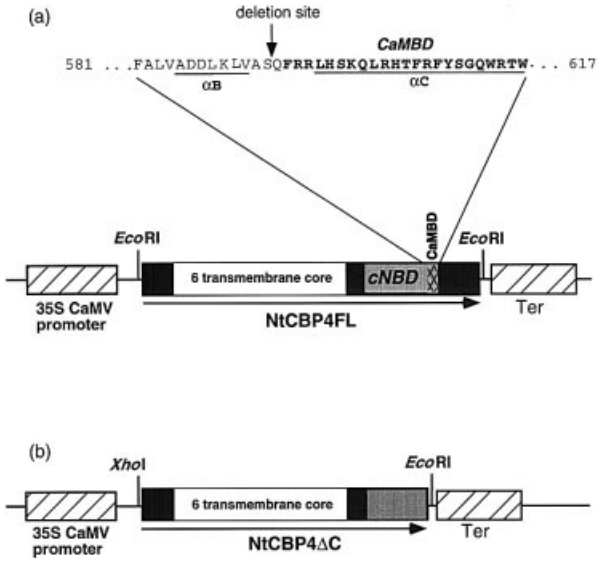

Figure 1. Schematic presentation of DNA constructs for expression of the full-length and C-terminal truncated *NtCBP4* in transgenic tobacco.
(a) Description of the full-length *NtCBP4* cDNA in a binary Ti plant transformation vector, as described by Arazi *et al.* (1999). Regions with the 35S CaMV promoter and the transcription termination sequence are shown in hatched boxes. The NtCBP4 six-transmembrane core, putative cyclic-nucleotide monophosphate-binding domain (cNBD, in grey; Arazi *et al.*, 1999; Arazi *et al.*, 2000) and the overlapping calmodulin-binding domain (CaMBD, cross-hatched; Arazi *et al.*, 2000) are indicated. The amino-acid sequence of part of the cNBD is shown on top. The CaMBD (bold letters) and the αB and αC predicted helices (underlined) of the cNBD are according to Arazi *et al.* (2000). The downward arrow shows the site of the deletion used to create the C-terminal truncated protein designated NtCBP4ΔC (amino acids Met_1–Ser_{593}). Numbers denote the terminal amino-acid residues shown in the sequence, based on Arazi *et al.* (1999) and Arazi *et al.* (2000).
(b) The NtCBP4ΔC cDNA in a binary Ti plant transformation vector. The NtCBP4ΔC cDNA, as described in (a), was prepared by cloning a corresponding PCR-amplified DNA fragment into the *Xho*I and *Eco*RI sites of the same vector used for the full-length NtCBP4.

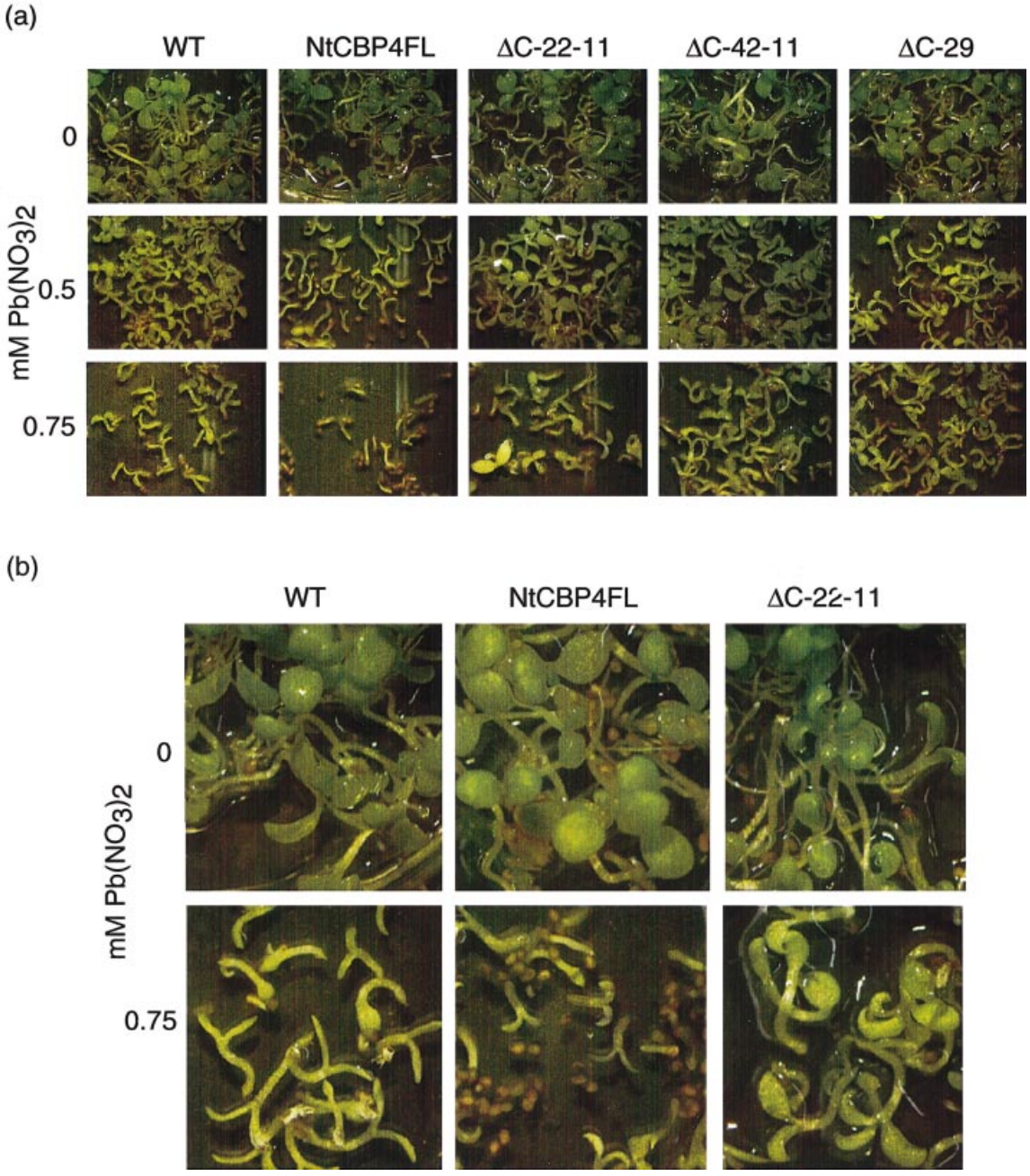

Figure 2. Transgenic tobacco seedlings expressing NtCBP4ΔC are tolerant to Pb^{2+}.
(a) Seedlings of wild-type and transgenic tobacco expressing either the full-length *NtCBP4* (*NtCBP4FL*; Arazi *et al.*, 1999) or the NtCBP4ΔC mRNA (transgenic lines ΔC-22-11, ΔC-42-11 and ΔC-29) were germinated and grown in the presence of the indicated concentrations of $Pb(NO_3)_2$ in modified Blaydes solution (pH 4.5) for 12 days and then photographed.
(b) Photo enlargements of seedlings of wild-type and representative transgenic lines in the presence of 0 and 0.75 mM $Pb(NO_3)_2$.

plant response to Pb^{2+}, *NtCBP4ΔC* transgenic lines were germinated in the presence of $Pb(NO_3)_2$ and their growth was compared with that of wild-type plants and of the transgenic line 49-79, which overexpresses the full-length *NtCBP4* (designated *NtCBP4FL*) and is associated with Pb^{2+} hypersensitivity (Arazi *et al.*, 1999). Eleven independent kanamycin-resistant lines (designated 42-11, 22-11, 29, 33-11, 16-6, 45-16, 52-36, 55-16, 9-2, 36-16, 55-61) that express the *NtCBP4ΔC* transgene were found to be more tolerant to Pb^{2+} than wild-type plants. Figure 2 shows the phenotype of three transgenic lines expressing the *NtCBP4ΔC* gene, and the wild-type and NtCBP4FL control lines, grown with or without Pb^{2+}. The NtCBP4ΔC plants were more tolerant to the toxic metal than the wild-type seedlings, whereas

the NtCBP4FL plants were more sensitive, as previously reported (Arazi *et al.*, 1999). No differences were found between wild-type and tobacco transgenic lines in their response to other metals including Na^+, Zn^{2+}, Mn^{2+}, Cd^{2+} and La^{3+}.

The expression of *NtCBP4FL* and *NtCBP4ΔC* in transgenic plants is driven by the same promoter. However, we wished to confirm that their expression levels were similar, and to exclude the possibility that the expression of the *NtCBP4ΔC* mRNA abolished the expression of the endogenous native gene (for example by a gene silencing mechanism). We also wished to assess the correlation between *NtCBP4ΔC* expression and the phenotype. Northern hybridizations with total RNA samples were used for analysing steady-state mRNA levels (Figure 3a). We tested five independent kanamycin-resistant transgenic lines showing a Pb^{2+}-tolerant phenotype, two kanamycin-resistant transgenic lines without an apparent phenotype, the transgenic line expressing the full-length NtCBP4 (Arazi *et al.*, 1999), and the wild type. In the five transgenic lines that showed a Pb^{2+} tolerance phenotype (ΔC-42-11, ΔC-22-11 and ΔC-29, ΔC-45-16 and ΔC-55-61), the truncated-size mRNA was detected. It was easily distinguishable by its length (727 bases shorter) from the full-length mRNA in line NtCBP4FL. Moreover, the expression levels of the full-length mRNA in NtCBP4, and the truncated mRNA in the NtCBP4ΔC lines were similar, with some variability in mRNA levels among the NtCBP4ΔC lines. Importantly, in the two kanamycin-resistant lines not showing a phenotype (lines ΔC-18-7, ΔC-12-9), expression of the truncated transgene was not detected. In addition, six independent transgenic tobacco lines expressing a non-relevant protein (glutamate decarboxylase) under the control of the 35S CaMV promoter were indistinguishable from wild-type in response to Pb^{2+} (data not shown). These results indicate that the Pb^{2+} tolerance phenotype is associated with expression of the truncated NtCBP4 transgene. It should be noted that the endogenous native NtCBP4 mRNA in wild-type plants was not detected in the total RNA sample, due to its very low abundance (T. Arazi and H. Fromm, unpublished results).

To ascertain that the expression of the endogenous native gene in NtCBP4ΔC transgenic plants was not silenced, we performed RT–PCR amplification with a set of primers that amplify a 404 bp region of the full-length NtCBP4 mRNA, but not of the truncated mRNA. The RT–PCR results (Figure 3b) show that a 404 bp DNA band was amplified from mRNA of the NtCBP4ΔC plants, indicating

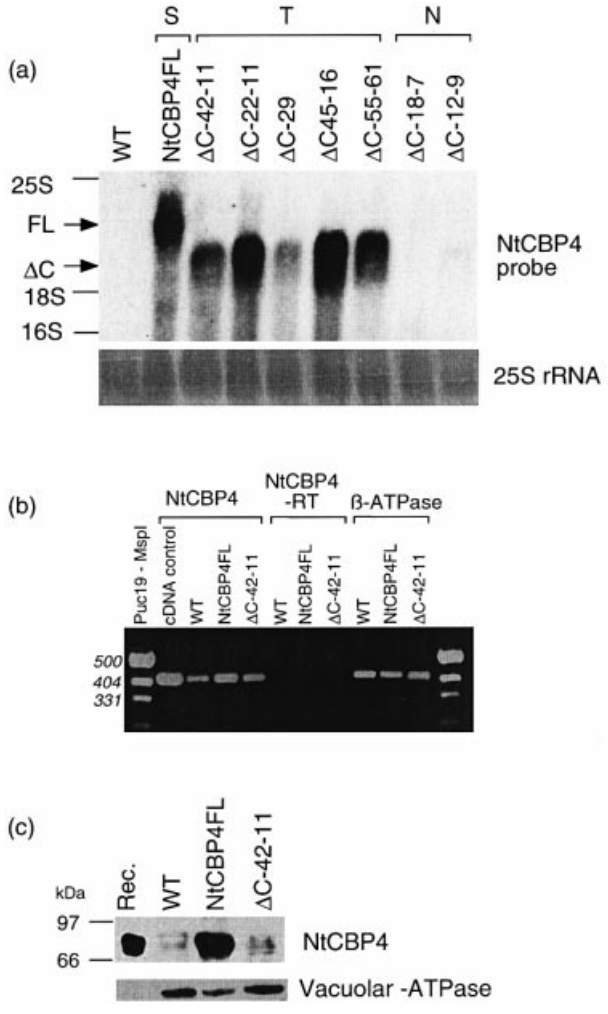

Figure 3. Analysis of gene and protein expression in transgenic tobacco plants.

(a) Total RNA samples (20 μg) from the indicated transgenic plants and wild type were separated by gel electrophoresis, blotted and hybridized with an *NtCBP4*-specific probe (upper panel). Arrows point to the full-length NtCBP4 (FL) and the C-terminal truncated NtCBP4 (ΔC) mRNAs in the upper panel. The letters on top of the upper panel refer, for the transgenic lines indicated, to the hypersensitive (S), tolerant (T) and normal (N) phenotypes regarding response to Pb^{2+} relative to the wild type. The gel positions of the 25S, 18S and 16S ribosomal RNA bands are indicated. The 25S ribosomal RNA band served as an internal standard (lower panel).

(b) Expression analysis of the full-length NtCBP4 mRNA in wild-type, NtCBP4FL and NtCBP4ΔC (line ΔC-42-11) plants by RT–PCR. A 404 bp DNA fragment corresponding to a region of the full-length NtCBP4 mRNA was amplified with the primers designated NtCBP4. Amplification of the corresponding region from the NtCBP4 cDNA clone served as a positive control (cDNA control). The amounts of polyA+ RNA were normalized using the expression of the tobacco gene encoding the β-subunit of mitochondrial H^+-ATPase as a standard. Amplified DNA samples and DNA size markers (indicated in bp on the left) were fractionated by agarose gel electrophoresis, stained with ethidium bromide and photographed.

(c) Immunodetection of the full-length NtCBP4 in microsomes from wild-type, NtCBP4FL and NtCBP4ΔC (line ΔC-42-11) plants. Microsomes (20 μg protein) and a sample of the full-length recombinant NtCBP4 expressed in insect cells (Rec; Arazi *et al.*, 2000) were separated by SDS–PAGE and immunoblotted on a nitrocellulose membrane with polyclonal anti-NtCBP4 antibodies (Arazi *et al.*, 1999; Arazi *et al.*, 2000). The positions of molecular weight markers are indicated. Immunodetection of vacuolar membrane H^+-ATPase (Ward *et al.*, 1992) served as an internal standard.

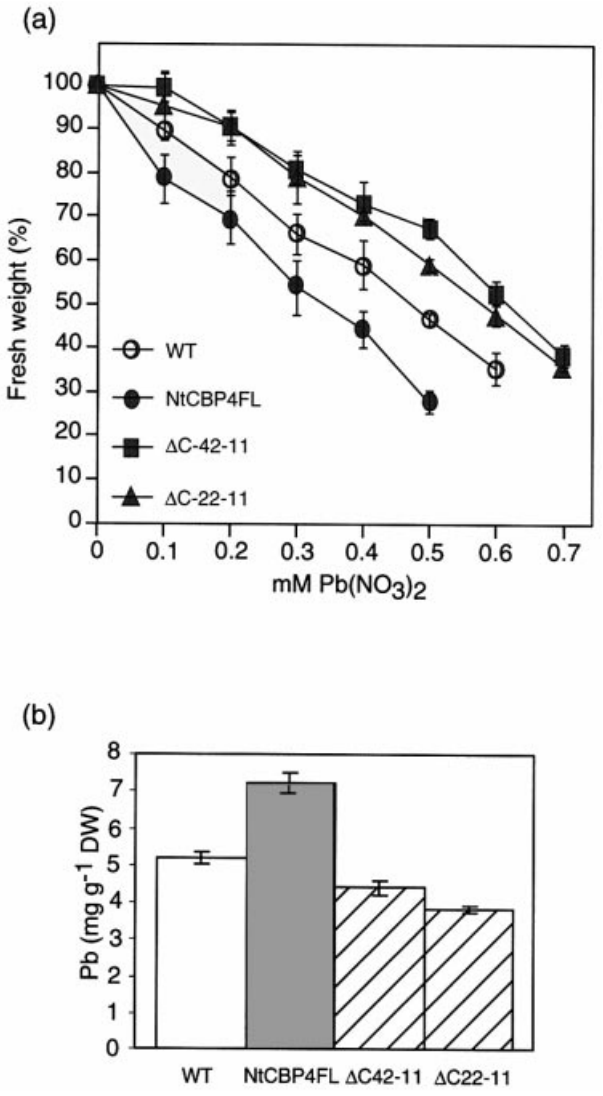

Figure 4. Pb^{2+} tolerance and accumulation in transgenic tobacco seedlings.
(a) Relative fresh weight of wild-type and transgenic tobacco seedlings expressing the full-length *NtCBP4* mRNA (*NtCBP4FL*; Arazi *et al.*, 1999), and two transgenic lines expressing the truncated NtCBP4 mRNA (ΔC-22-11 and ΔC-42-11) in the presence of different concentrations of $Pb(NO_3)_2$. Data are given as the mean FW of 150 seedlings for each concentration ±SD of three independent experiments. Fresh weight of seedlings of each line grown without $Pb(NO_3)_2$ was set as 100%.
(b) Lead accumulation in 12-day-old seedlings grown in the presence of 0.2 mM $Pb(NO_3)_2$. Seedlings were dried at 80°C for 3 days and lead content was determined by ICP-AES as described (Arazi *et al.*, 1999). Data are the mean ±SD of three independent experiments.

that the endogenous native *NtCBP4* mRNA was indeed present in these plants. This amplified band had the same mobility as the control band amplified from the NtCBP4 cDNA clone. Where reverse transcriptase was omitted (–RT), no amplification was detected, indicating that the observed amplifications described above originated from mRNA templates rather than from residual genomic DNA or from contaminating plasmid DNA in the samples. Amplification with primers corresponding to the nuclear gene encoding the β-subunit of mitochondrial H^+-ATPase, as a control, indicated that similar amounts of poly A^+ mRNA were used in all reactions. Furthermore, immunoblot analysis using anti-NtCBP4 antibodies revealed that the endogenous native protein was expressed and present in membranes of NtCBP4ΔC lines, and the level of expression similar to that in the wild type. An example of protein analysis of line ΔC-42-11, the wild type and the NtCBP4FL line is shown in Figure 3(c). It should be noted that as the anti-NtCBP4 antibodies were raised against the C-terminal half of NtCBP4 (Arazi *et al.*, 1999; Arazi *et al.*, 2000), the truncated NtCBP4 protein could not be detected with these antibodies. Nevertheless, this analysis revealed that expression of *NtCBP4ΔC* does not result in silencing of the endogenous *NtCBP4* gene. Rather, the apparent phenotype conferred by *NtCBP4ΔC* expression occurs in the presence of the full-length native endogenous *NtCBP4*.

Quantitative assessment of Pb^{2+} tolerance in NtCBP4ΔC transgenic seedlings, and Pb^{2+} accumulation

The extent of growth inhibition of seedlings by Pb^{2+} was evaluated by determining the fresh weight of seedlings grown in different concentrations of $Pb(NO_3)_2$ (Figure 4a). Whole-seedling fresh weight analysis revealed 50% inhibition at 0.34, 0.47, 0.58 and 0.62 mM Pb^{2+} for NtCBP4FL, wild type, NtCBP4ΔC-22-11 and NtCBP4ΔC-42-11, respectively (Figure 4a). Moreover, statistical analysis revealed that the two NtCBP4ΔC lines were significantly more tolerant ($P < 0.05$) than the wild-type seedlings at Pb^{2+} concentrations of ⩾0.1 mM. Pb^{2+} hypersensitivity of the NtCBP4FL lines was also significantly different from the response of wild-type plants ($P < 0.05$), as previously described (Arazi *et al.*, 1999).

One of the possible explanations for the Pb^{2+}-tolerant phenotype of the NtCBP4ΔC plants may be the attenuated uptake of the metal, as we have already demonstrated that Pb^{2+} hypersensitivity in NtCBP4FL plants was associated with enhanced accumulation of Pb^{2+} (Arazi *et al.*, 1999). To test this possibility, we determined Pb^{2+} accumulation by ICP-AES in 12-day-old seedlings grown in the presence of 0.2 mM $Pb(NO_3)_2$. NtCBP4ΔC plants (lines ΔC-42-11 and ΔC-22-11) showed a marked reduction in Pb^{2+} accumulation compared with wild-type seedlings ($P < 0.05$), whereas NtCBP4FL plants accumulated substantially more Pb ($P < 0.05$) than the wild type (Figure 4b), as previously described (Arazi *et al.*, 1999).

The improved Pb^{2+} tolerance associated with attenuated Pb^{2+} accumulation in tobacco plants expressing a truncated NtCBP4 protein raised the possibility that the truncated protein inhibits the function of endogenous NtCBP4-associated channels. When the NtCBP4 amino-

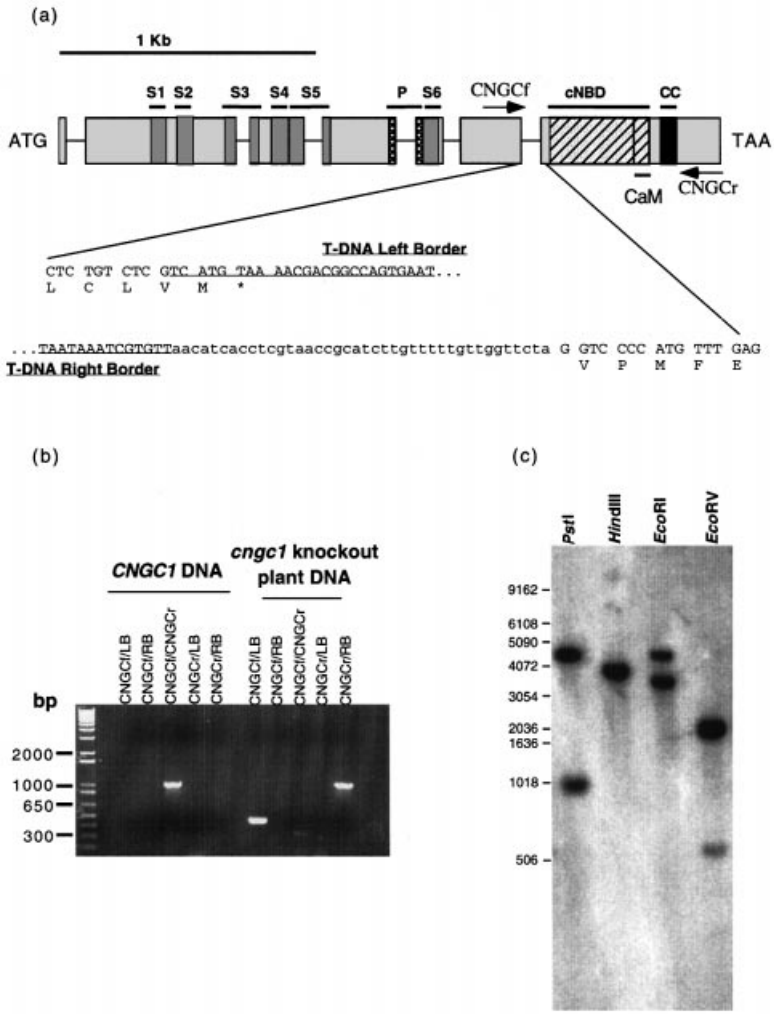

Figure 5. Genomic analysis of the T-DNA insertion in the *Arabidopsis CNGC1* knockout line.
(a) *CNGC1* gene map and T-DNA border sequences (T-DNA sequences are underlined). Exons are represented by boxes. S1 to S6, transmembrane hydrophobic domains; P, predicted pore region; cNBD, cyclic nucleotide-binding site; CC, coiled coil helix; CaM, calmodulin-binding domain; CNGCf, *CNGC1* forward oligonucleotide; CNGCr, *CNGC1* reverse oligonucleotide.
(b) PCR analysis of *cngc1* homozygous plants. Homozygous mutant and wild type (*Arabidopsis thaliana* ecotype Wassilewskija) DNA were subject to PCR amplification using *CNGC1* gene-specific oligonucleotides (CNGCf and CNGCr) and T-DNA left- and right-border oligonucleotides (LB and RB).
(c) Southern blot analysis of homozygous plants. DNA (2 μg per lane) was digested by different restriction enzymes (the T-DNA contains one site for *PstI*, *EcoRI* and *HindIII* and three sites for *EcoRV*), and blotted on nylon membrane. Hybridization was performed with a full-length radiolabelled T-DNA probe.

acid sequence is compared to the amino-acid sequences of the proteins encoded by the *Arabidopsis CNGC* genes, CNGC1 emerges as most similar to NtCBP4. We reasoned that if *AtCNGC1* is the orthologue of *NtCBP4*, then disruption of *AtCNGC1* might confer Pb^{2+} tolerance in *Arabidopsis*. To assess this possibility we chose to isolate and analyse a T-DNA insertion mutant of the *Arabidopsis CNGC1*.

Isolation of a CNGC1 *knockout mutant and its response to* Pb^{2+}

The full *Arabidopsis CNGC1* coding sequence has recently been released and shown to be localized on chromosome 5 (BAC clone number MFH8, accession AB025622). The Versailles T-DNA library was screened by PCR for a knockout mutant using *CNGC1*-specific oligonucleotides, and T-DNA oligonucleotides localized in the left and right borders. One knockout line was isolated and shown to be disrupted in *CNGC1* by sequencing the T-DNA flanking regions (Figure 5a). The T-DNA is inserted in front of the putative cyclic nucleotide-binding domain. The corresponding homozygous line was isolated and characterized by PCR (Figure 5b). Segregation analysis of the T-DNA insertion line revealed one insertion locus. DNA was extracted from homozygous plants, digested by several enzymes, and blotted on nylon membrane. Hybridization with a T-DNA probe revealed the presence of only one T-DNA insertion (Figure 5c). Moreover, DNA from homozygous plants was subjected to a PCR walking method (Devic *et al.*, 1997) to clone any possible T-DNA border present in this line. Importantly, all cloned borders correspond to the *CNGC1* insertion. No new T-DNA flanking regions were cloned from the knockout line, providing evidence for a single insertion site in the *CNGC1* gene.

Subsequently, we analysed the response of seedlings of the homozygous *cncg1* mutant and wild type to different

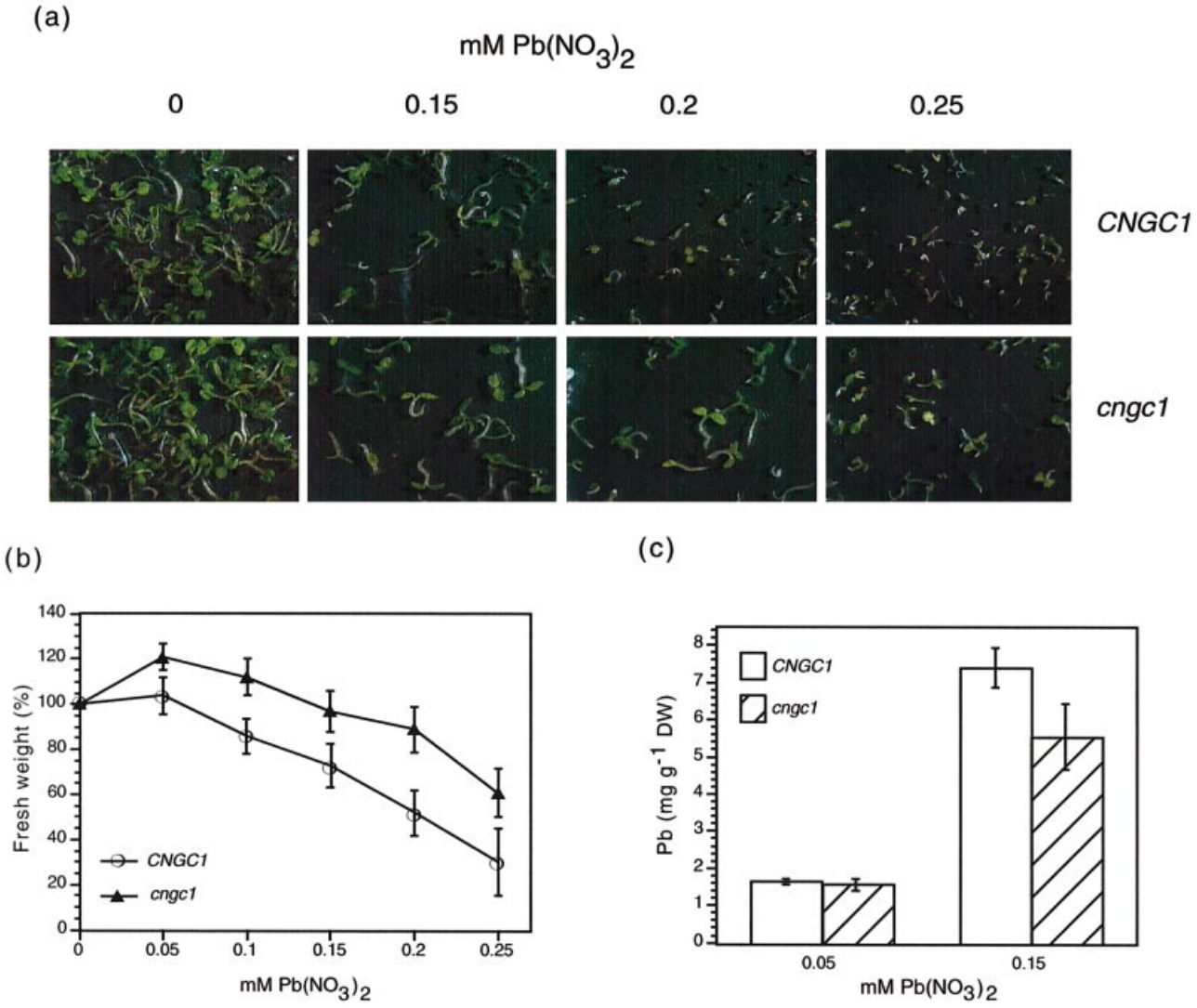

Figure 6. Phenotypic analysis of the *Arabidopsis cngc1* mutant in response to Pb^{2+}.
(a) Photographs of seedlings of wild-type and the *cngc1* mutant germinated and grown in the presence of the indicated concentrations of $Pb(NO_3)_2$ for 2 weeks.
(b) Relative fresh weight of wild-type and *cngc1* mutant in the presence of different concentrations of $Pb(NO_3)_2$. Data are given as the mean FW of 150 seedlings for each concentration ±SD of three independent experiments. Fresh weight of seedlings of each line grown without $Pb(NO_3)_2$ was set as 100%.
(c) Lead accumulation in 10-day-old seedlings grown in the presence of 0.05 and 0.15 mM $Pb(NO_3)_2$. Lead content was determined by ICP-AES as described in Experimental procedures. Data are the mean ±SD of three independent experiments.

concentrations of Pb^{2+}. Increasing $Pb(NO_3)_2$ concentrations adversely affected seedling development of both wild type and *cngc1*. However, in the presence of high concentrations of Pb^{2+} (0.2 and 0.25 mM), the wild-type seedlings were chlorotic and their development was severely retarded compared with that of *cngc1* seedlings (Figure 6a). Similar results were obtained with two batches of wild-type seeds and three batches of mutant seeds from plants grown at different locations.

Quantitative analysis of Pb^{2+} tolerance of the *cngc1* mutant is shown in Figure 6(b). This analysis revealed a statistically significant higher tolerance of the mutant line compared to the wild type. Importantly, analysis of the response of the *Arabidopsis* wild-type and mutant lines to $NiCl_2$ revealed no apparent difference, suggesting that the *cngc1* knockout specifically affects the response to Pb^{2+}. However, this does not exclude possible specific effects on other ions, which have not been tested in this study. In addition we tested seedlings germinated and grown on agar plates. Seedlings were grown in the absence of Pb^{2+} for 7 days and then transferred to agar plates containing Pb^{2+}. This growth regime also revealed a phenotype of

Pb^{2+} tolerance of the *cngc1* mutant in comparison to the wild type. Seedlings of the mutant showed less growth inhibition in the shoot, and retained more chlorophyll than the wild type at high Pb^{2+} concentrations (1 and 5 mM). At these concentrations, the roots of both wild-type and *cngc1* mutant ceased growing (data not shown). After 5 days at 5 mM Pb^{2+}, seedlings of the *cncg1* mutant retained twice as much chlorophyll as wild-type seedlings (42.8 $\pm$ 5.0 and 20.6 $\pm$ 1.6%, respectively), relative to the non-stressed control seedlings.

Furthermore, we tested the Pb content in *Arabidopsis* seedlings exposed to Pb^{2+} for 2 weeks. At low concentrations of Pb^{2+} (0.05 mM) there was no significant difference between the *cngc1* mutant and the wild type. At higher concentrations differences became apparent and significant (Figure 6c). At 0.15 mM Pb^{2+}, the mean content of Pb in the wild type was 30% higher than in the mutant ($P < 0.05$). Thus, with respect to both Pb^{2+} tolerance and accumulation, the phenotype of the *Arabidopsis cngc1* mutants is similar to that of transgenic tobacco plants expressing the truncated NtCBP4 protein.

Discussion

An emerging family of plant plasma membrane channel proteins resembling mammalian cyclic nucleotide-gated non-selective cation channels has been recently described in several plant species (Arazi *et al.*, 1999; Köhler *et al.*, 1999; Leng *et al.*, 1999; Schuurink *et al.*, 1998). We chose to use transgenic plants to study the function of the tobacco NtCBP4 protein, and T-DNA insertion mutagenesis to investigate the *Arabidopsis* homologue *CNGC1*. Our previous studies revealed that overexpression of NtCBP4 in tobacco plants conferred Pb^{2+} hypersensitivity and enhanced accumulation of Pb^{2+}, suggesting the possible involvement of NtCBP4 in Pb^{2+} uptake across the plasma membrane (Arazi *et al.*, 1999). Here we show that by expressing a modified version of NtCBP4 lacking presumed regulatory domains, the phenotype with respect to Pb^{2+} tolerance and accumulation is the opposite of that exhibited by plants overexpressing the full-length protein.

The mammalian cyclic nucleotide-gated channels function as tetrameric complexes (Liu *et al.*, 1996). Therefore the inhibitory effect of NtCBP4ΔC on Pb^{2+} accumulation, and the concomitant improved tolerance to Pb^{2+}, may have resulted from the formation of non-functional NtCBP4/NtCBP4ΔC heteromeric complexes, as the endogenous native NtCBP4 gene was expressed in the background of the NtCBP4ΔC transgene (Figure 3c). However, as indicated, due to the fact that the anti-NtCBP4 antibodies were raised against the C-terminal half of NtCBP4 (Arazi *et al.*, 1999; Arazi *et al.*, 2000), the truncated NtCBP4 protein could not be detected with these antibodies. Further studies are required to assess the possible occurrence of full-length/truncated NtCBP4 heteromeric complexes in the NtCBP4ΔC transgenic plants. Nevertheless, we predicted that if the truncated protein causes the inactivation of endogenous NtCBP4-associated channels, then disruption of the *Arabidopsis CNGC1* gene might cause a similar phenotype. Indeed, we isolated an *Arabidopsis* T-DNA insertion mutant of the *CNGC1* gene. This mutant had characteristics similar to the tobacco plants expressing the truncated NtCBP4 with respect to the response to Pb^{2+}. The phenotype of the *cngc1* mutant was apparent when grown on different nutrient solutions, and irrespective of growth on liquid or solid substrate. Moreover, the phenotype was apparent when seeds were germinated in the presence of Pb^{2+}, or when seedlings were exposed to Pb^{2+} only 1 week after germination. These results suggest the involvement of CNGC1 in Pb^{2+} transport across the plant plasma membrane, consistent with findings regarding NtCBP4 in tobacco.

It is important to note that the analysis described here was confined to the early stages of seedling development in tobacco and *Arabidopsis*. The response of older plants to Pb^{2+} and other metals requires further detailed analysis. In view of the fact that in *Arabidopsis* there are at least 19 *CNGC*-related genes (N. Bouché, unpublished results), the possible formation and complexity of CNGC heteromeric channels cannot be ignored. The composition of such heteromeric complexes may vary between cell types, during development, and in response to external physical and chemical stimuli. Heteromeric complexes might account for variations in regulation as well as in ion selectivity. In addition, CNGC proteins might differentially interact with distinct calmodulin isoforms (Köhler and Neuhaus, 2000), which could further affect their regulatory properties. Elucidating these issues requires a massive effort and a multidisciplinary research approach.

Because Pb^{2+} is a non-essential toxic metal to plants, the presumed NtCBP4- and CNGC1-associated ion transport systems are likely to have other, as yet unknown, physiological roles. On the basis of their primary structure, plant CNGCs are predicted to function as components of non-selective cation channels. The involvement of CNGC1 and CNGC2 in transport of potassium has already been reported (Köhler *et al.*, 1999; Leng *et al.*, 1999, respectively). Of particular interest to us is the possible involvement of NtCBP4, CNGC1 and other related plant proteins in Ca^{2+} signal transduction, either because of their expected regulation by Ca^{2+}-calmodulin, and/or by being permeable to Ca^{2+}, as in the case of mammalian cyclic nucleotide-gated non-selective cation channels. A role in Ca^{2+} transport would be consistent with earlier reports identifying Ca^{2+}-permeable channels as a pathway for Pb^{2+} entry into animal cells (Simons and Pocock, 1987; Tomsig and Suszkiw, 1991) and plants (Huang and Cunningham,

1996), and with the recent demonstration of cyclic nucleotide-dependent Ca^{2+} entry into human embryonic kidney cells transfected with the *Arabidopsis AtCNGC2* gene (Leng *et al.*, 1999). We tested metal content in whole leaf extracts of wild-type and NtCBP4ΔC and NtCBP4FL transgenic tobacco grown in half-strength Hoagland's medium for 4 weeks (without heavy metal stress). There were no significant differences between the wild type and the transgenic lines in their content of a range of metals (Ca, K, Mg, Mn, Zn and Fe). This may indicate that these CNGC channels have subtle effects on metal transport, which could be involved in signalling mechanisms.

Previously we reported that transgenic tobacco overexpressing the full-length NtCBP4 was tolerant to Ni^{2+} and hypersensitive to Pb^{2+}, associated with reduced accumulation of Ni^{2+} and enhanced accumulation of Pb^{2+}, respectively (Arazi *et al.*, 1999). We hypothesized that the hypersensitivity to Pb^{2+} is the result of enhanced NtCBP4 channel activity. The opposite phenotype, resulting from the expression of a truncated NtCBP4 in tobacco and from disruption of *CNGC1* in *Arabidopsis*, is consistent with this hypothesis. On the other hand, it would be difficult to associate the Ni^{2+}-tolerance phenotype with channel activity. First, the *Arabidopsis cngc1* mutant is not different from the wild type in response to $NiCl_2$ (R. Sunkar and H. Fromm, unpublished results). Secondly, we found that transgenic tobacco expressing the truncated NtCBP4 protein retains a certain degree of Ni^{2+} tolerance (R. Sunkar and H. Fromm, unpublished results). Therefore it is likely that the Ni^{2+} tolerance phenotype observed in transgenic tobacco (Arazi *et al.*, 1999) is due to a property of the NtCBP4 protein, which is also present in the truncated protein, but is not directly associated with NtCBP4 channel activity.

In summary, we refined the analysis of the plasma membrane NtCBP4 tobacco protein in relation to Pb^{2+} tolerance. In addition, we found similar characteristics associated with the *Arabidopsis CNGC1* gene. The transgenic tobacco plants and the *Arabidopsis* mutant described here provide unique research tools for addressing questions related to their physiological roles, as well as for elucidating mechanisms of heavy metal uptake and tolerance in plants.

Experimental procedures

Preparation of transgenic plants

Preparation of the DNA construct and of the transgenic tobacco plants for expression of the full-length NtCBP4 protein was recently described by Arazi *et al.* (1999). The DNA construct for the expression of the C-terminal truncated NtCBP4 (NtCBP4ΔC) was prepared by cloning a PCR-amplified DNA fragment using the gene-specific oligonucleotides 5′-CCGCTGAGCTATGAATCACCG-CCAAGACGAG-3′ (sense) and 5′-GAAGGAATTCTTAAGAGGCTA-CAAGCTTTAAATCAT-3′ (antisense), spanning the coding region Met_1–Ser_{593} (Arazi *et al.*, 1999), and containing *Xho*I and *Eco*RI restriction sites on the 5′ and 3′ termini, respectively. The *Xho*I–*Eco*RI-digested DNA fragment was ligated into the corresponding sites of the binary vector described by Arazi *et al.* (1999). Transfer of the transgene to progeny was verified by germination on kanamycin, then by Northern hybridizations to confirm the expression of the *NtCNBP4ΔC* mRNA.

Analysis of transgene expression by Northern hybridizations, RT–PCR and immunoblotting

Total RNA was isolated from 3-week-old seedlings as described (Logemann *et al.*, 1987). RNA fractionation, blotting and hybridization conditions were as described (Chen *et al.*, 1994). A *Hin*dIII fragment from the cDNA of NtCBP4 (nucleotides 1113–2024, GenBank accession number AF079872) was labelled with [^{32}P]dCTP by random priming, and used as a probe. The same membrane was hybridized with a [^{32}P]dCTP-labelled actin probe prepared by amplifying an actin gene fragment using the tomato actin gene (GenBank accession number U60480) specific primers 5′-TTGTGTTGGACTCTGGTGATGG-3′ (sense) and 5′-AGCCAA-GATAGAGCCTCCAATC-3′ (antisense). Total RNA was passed through an oligo-dT column to enrich for polyA mRNA, and reverse transcriptase was used to prepare the corresponding cDNA templates. PCR amplification of a 404 bp NtCBP4 full-length-specific sequence was performed with the primers 5′-GACGACTTCACAGTAAGCAGC-3′ and 5′-CACGACTAAAAATGC-ACTCAATC-3′ (sense and antisense, respectively), which amplify a region spanning nucleotides 2044–2447 of the NtCBP4 cDNA (GenBank accession number AF079872). PCR amplification of the cDNA encoding the β-subunit of *Nicotiana plumbaginifolia* mitochondrial H^+-ATPase (GenBank accession number X02868) served as a control, with the primers 5′-CTTACAGGTTTG-ACCGTGGCTGAGC-3′ (sense), and 5′-TAGTGATCCTCTCCCA-AAATGTGAGG-3′ (antisense). Protein extraction, membrane fractionation and immunoblotting were performed as described (Arazi *et al.*, 1999; Arazi *et al.*, 2000).

Plant culture, metal toxicity and metal accumulation assays

Seeds of tobacco and *Arabidopsis* were sterilized by washing in 70% ethanol, then with chlorine, and subsequently rinsed with distilled water and, unless otherwise indicated, placed in Petri dishes containing modified Blaydes solution (Parrot and Bouton, 1990) pH 4.5, with or without $Pb(NO_3)_2$. The seedlings were grown in a controlled growth room for 10 days (*Arabidopsis*) or 12–13 days (tobacco), with a day/night cycle of 16/8 h at 25°C. Seedlings were collected and their fresh weight measured, after which they were dried at 80°C for 3 days. Lead content was determined by ICP-AES as described (Arazi *et al.*, 1999). Statistical analysis of the data was performed using ANOVA and the sum of squares simultaneous test procedure. For plants grown on agar plates, seeds were sterilized as described above and germinated on plates containing 25% (v/v) Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), 1% (w/v) sucrose and 0.9% (w/v) agar (Sigma A-1296). Plants were grown for 7 days on vertically positioned plates with 16/8 h light/dark cycle with temperature of 25/20°C, respectively, and light intensity of 40 μmol m^{-2} sec^{-1}. Subsequently seedlings were transferred to plates containing 25% (v/v) MS without $CaCl_2$, with phosphate reduced to 0.01 mM and with either 0, 1 or 5 mM $Pb(NO_3)_2$. Shoots

were harvested after 5 days. Chlorophyll was extracted with methanol and spectrophotometrically quantified. Total chlorophyll content (μg chlorophyll ml^{-1} plant extract solution) was calculated as $22.5 \times A_{650} + 4.0 \times A_{665}$.

PCR screening strategy and knockout isolation

We used the T-DNA inserted collection developed by G. Pelletier and co-workers (INRA, Versailles, France) made by infiltrating *Arabidopsis thaliana* ecotype Wassilewskija (Bechtold *et al.*, 1993) with *Agrobacterium tumefaciens* strain C58 carrying the plasmid pGKB5 (Bouchez *et al.*, 1993). This library is composed of 43 584 individual lines, representing about 65 000 independent insertions (±1.5 insertion loci per line). The DNA corresponding to T_2 or T_3 lines was extracted in pools of 48 lines (908 DNA pools). To isolate the *cngc1* knockout, DNA pools were screened by PCR using gene-specific oligonucleotides (CNGCf: 5′-GAAGAGACG-CAGAACAATGG-3′; CNGCr: 5′-CTGGCTCTGCTGGTTTCTGAAG-TAGCATAG-3′) and oligonucleotides anchored in the left and right borders of the T-DNA (RB oligonucleotide: 5′-CTGATACCAGACGTTGCCCGCATAA-3′; LB oligonucleotide: 5′-CTACAAATTGCCTTTTCTTATCGAC-3′). PCR reactions were run on an agarose gel, blotted onto nylon membranes which were hybridized with a T-DNA probe (RB plus LB) and a gene-specific probe (*Arabidopsis* EST 154M3T7 from Ohio Stock Center, corresponding to a partial *CNGC1* cDNA clone). Positive PCR fragments hybridizing with both probes were isolated from agarose gel and sequenced. The 48 individual lines from the positive pool were then subjected to a new PCR and the corresponding knockout line was isolated.

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