Evidence for copper homeostasis function of metallothionein (MT3) in the hyperaccumulator *Thlaspi caerulescens*☆

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Abstract Metallothioneins chelate metals and consequently may be a control point of metal homeostasis. Homologous to type 3 metallothioneins, *TcMT3 cDNA* was identified in the Cd/Zn hyperaccumulator, *Thlaspi caerulescens*. TcMT3 amino acid sequence showed modifications in the Cys positions when compared with its *Arabidopsis* orthologue. A structural model established that the MT3 carboxyterminal domain is similar to the β domain of animal metallothioneins and predicts a smaller cavity to chelate metals for *A. thaliana* than for *T. caerulescens*. Functional testing in yeast and Northern blot analysis added further evidence for adaptative variations of MT3 for the maintenance of Cu homeostasis in a metal hyperaccumulator.

Keywords: Heavy metal stress; Cadmium; Metallothionein; Metal homeostasis; Metal hyperaccumulation

1. Introduction

Plants, like other organisms have evolved a range of mechanisms to maintain the concentration of essential metals within a physiological range and control the toxicity of non-essential heavy metal ions [1–4]. Chelation and subsequent transport of the metal–ligand complexes represent one of the principal mechanisms of metal homeostasis and detoxification [1–4].

Metallothioneins (MTs) have been found throughout all the kingdoms and constitute a superfamily of Cys rich polypeptides with a low molecular mass (4–8 kDa) [1,4]. MTs are able to chelate metal ions as Cd(II), Zn(II) and Cu (I) [5,2,6]. The only three-dimensional structures of MTs that have been published are those of animals and yeast. A number of animal MT structures with Zn(II) and/or Cd(II) ions are available and reveal that MTs are composed of two domains (α and β), each presenting a metal–thiolate cluster [5,2,6]. In animals, MTs are known to be involved in metal homeostasis and some can protect cells against Cd toxicity [7,2,6]. Similarly, information obtained by gene expression analysis and functional expression in microorganisms suggests that plant MTs may be involved in metal homeostasis [8–12] and Cu tolerance [13,14]. However, in contrast to animals, direct information about the role of MT proteins from plants is scarce because of difficulties in purifying them. Only one group succeeded in the identification of peptide fragments derived from *Arabidopsis* MTs and immunoblot analysis demonstrated that the levels of MT1 and MT2 proteins corresponded to the observed RNA levels [4,13]. Difficulties in identifying and purifying plant MTs may arise from instability of these proteins in the presence of oxygen. There is however, a critical need for more protein data.

A rare class of plants named hyperaccumulators are able to tolerate elevated concentrations of heavy metals, accumulating extremely high concentration of heavy metals such as Zn, Cd and Ni in their shoot (more than 100-fold the concentration found in other plants) [15]. In particular, a population of *Thlaspi caerulescens* from the Ganges region in South of France presents a level of Cd hyperaccumulation (concentration reach 7 g kg^{-1} DW) and tolerance probably unprecedented in living organisms [16–18]. The physiological and molecular mechanisms responsible for the metal hyperaccumulation phenotype are still poorly understood. A possible role for MTs in metal tolerance in *T. caerulescens* or any other hyperaccumulator has not been explored so far. This work presents the functional properties of the metallothioneins (MT3) from a hyperaccumulator compared to a non-accumulator related orthologue.

2. Materials and methods

2.1. Yeast strains and expression vector

Cup2Δ (*MATa his3 leu2 met15 ura3 cup2::URA3*), *zrc1cot1*Δ (*MATa his3 leu2 met15 ura3 zrc1::kanMX cot::1natMX*) and the parental strain BY4741 (*MATa his3 leu2 met15 ura3*) were grown at 30 °C on minimal medium (Yeast Nitrogen Base, Sigma) supplemented with appropriate amino acids. pYX212 (Ingenius, Madison, WI) were used to express cDNAs in yeast.

2.2. Functional screening of the cDNA library of T. caerulescens

To identify *T. caerulescens* cDNAs conferring Cd tolerance to yeast, transformants were selected for their ability to grow on minimal medium containing 15 μM $CdSO_4$. About 430 000 *T. caerulescens* cDNA clones were screened for growth on high Cd medium [19]. Plasmids were isolated from Cd tolerant yeast and restransformed into BY4741 to confirm the functional tolerance.

☆ The sequence data reported in this paper have been submitted to the GenBank via Bankit under Accession Nos. AY531114 (TcMT3) and AY540104 (TcPCS).

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2.3. Cloning of the MT3 cDNA from Arabidopsis *in the pYX212 yeast*

The cDNA sequence of *A. thaliana* MT3 (*AtMT3*) was amplified by RTPCR using primers (AACTCAATCATGTCAAGCA and CGCATTAGTTGGGGCAGCAA). The resulting fragment was cloned in the *EcoR1* and *Xho1* sites of pYX212.

2.4. Structural analysis

A search of the PDB sequences using Blast [20] was performed to find structural homologues of the TcMT3 deduced amino acid sequence. The fold recognition server 3D-PSSM [21] was also used, as an alternative approach, to search for compatible folds with TcMT3. The 3D-PSSM server automatically generates the models by mapping the query sequence onto the folded candidate based on the deduced alignment. The 3D-PSSM method relies on the structural classification of proteins (SCOP). SCOP defines structural domains from known protein structures and classifies them according to similarities at the structural, sequence and functional levels. The 3D-PSSM fold library is built by taking a SCOP domain representative from each Superfamily (distant homologues) found in the classification to generate position-specific scoring matrices (PSSM). These include aligned sequences from a non-redundant sequence database (NRPROT), structurally aligned SCOP domains from the same Superfamily, and secondary structures and solvent accessibility derived from the SCOP domain structures. The query sequence is then compared to the 3D-PSSM library allowing the recognition of distant structural and functional relationships.

2.5. Measurement of the yeast metal tolerance

The yeasts were transformed by the lithium acetate procedure [22]. The overnight cultures of yeast strains containing the pYX212 vector and the different pYX212 recombinants were diluted 10-fold in a fresh liquid minimal medium and incubated at 30 °C until the density OD_{600} 0.8. For the metal-tolerance test on agar plates, 5 μl of successive culture dilutions was dropped on the minimal medium-plate supplemented with the appropriate concentration of heavy metals. The concentration to test Cd tolerance remains unchanged. For the metal-tolerance test on liquid medium, yeasts were adjusted to OD_{600} 0.1 in fresh liquid medium supplemented with the appropriate concentration of heavy metals.

2.6. Measurement of the expression of TcMT3 and AtMT3 recombinant proteins in yeast

To detect the MT proteins in yeast, the MT3 cDNAs of *T. caerulescens* and *A. thaliana* were cloned in frame with the V5 epitope (TcMT3:V5; AtMT3:V5) and the recombinant plasmids (pYES-TcMT3:V5, pYESAtMT3:V5) were transformed in C*up2*Δ. The C*up2*Δ yeast transformants were grown in liquid medium supplemented with heavy metals as described before. After 16 h, the OD_{600} of the yeast was determined and their Cd and Cu tolerance was verified. The cells were centrifuged and resuspended in 50 mM sodium phosphate (pH 7.4) to reach an OD_{600} equal to 250. The cells were lysed by sonication in the presence of antiprotease (protease inhibitor mix, Amersham Biosciences) and 5 mM tributylphosphine (TBP). The reduced disulfide groups were then alkalised with 15 mM iodoacetamide using the protocol described in ready prep reduction alkylation kit (Bio-Rad). Proteins were quantified using Bradford procedure and 30 μg of proteins was then electrophoresed using standard procedure on SDS–PAGE (Tris–glycine 15%). Proteins were electroblotted onto PVDF membranes (Amersham Biosciences) and the fusion proteins were detected using Anti-V5-HRP (horseradish peroxidase) antibodies (Invitrogen, Life Technologies) and the Enhanced Chemiluminescence reagent kit (Amersham Biosciences). Membranes were stained with Comassie blue to verify the equal amount of protein transferred on the membrane.

2.7. Plant materials and growth conditions

Seeds of *Thlaspi caerulescens* J. & C. Presl (Brassicaceae) were obtained from populations in Prayon (Belgium), St Félix-de-Pallières (Ganges region – France) and Puente Basadre (Spain). Metal concentrations of soils sampled from the natural habitat are shown by Roosens et al. (2003) [18]. These three populations were previously characterised [18] and greatly varied in Cd tolerance and hyperaccumulation. *T. caerulescens* and *A. thaliana* were grown as described by Roosens et al. (2003) [18]. After 5 weeks and 18 days in hydroponic culture, respectively, for *T. caerulescens* and for *A. thaliana*, the nutrient solution was supplemented with an appropriate concentration of $CdSO_4$ or $CuSO_4$.

2.8. Measurement of the MT3 expression in plant

For Northern blot analysis, total RNA was extracted and transferred to membranes using standard procedures [23]. Membranes were hybridised overnight at 57 °C or 64 °C with ^{32}P radiolabelled *TcMT3* and *AtMT3* coding sequences or 18S rRNA probes and washed at room temperature 2× SSC for 20 min and two times more in 0.2× SSC/0.1% (w/v) SDS at 65 °C. Similar pattern of expression was observed in both temperatures of hybridisation (data not shown). The blot was scanned with a phosphoimager. To verify that differential expression of *MT3* observed in Northern blot analysis was not due to differences in *MT3* sequence within populations, a RT-PCR analysis was performed. First strand cDNA was synthesised from 1 μg of total RNA with the Superscript II reverse transcriptase (Invitrogen Life Technologies) using poly-T primer. Primers designed in the *TcMT3* sequence were: ATGTCAAGCAACTGCGGAAGCTG and GTTGGGGCAGCAAGTGCAGTTGAC. To verify the similar annealing of the primers, PCR was also performed in parallel on genomic DNA extracted from each population. PCR programme was 94 °C for 5 min, followed by 29 cycles at 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min 15 s and finished by 72 °C for 5 min.

2.9. Measurements of Cd hyperaccumulation and Cd tolerance in T. caerulescens

Plants cultivated as described above were washed, oven-dried and weighed and Cd was measured by atomic absorption spectroscopy as described by Roosens et al. (2003) [18].

2.10. Statistical analysis

One-way ANOVA followed by a Tukey test allowing multiple comparisons between treatments was used to analyse the data regarding MT3 RNA quantification. The differences are mentioned as significant for P smaller or equal to 0.05.

3. Results

3.1. Sequence analysis of T. caerulescens *MT3*

Screening of *T. caerulescens* cDNAs that confer functional Cd tolerance to *S. cerevisiae* resulted in the isolation of 139 cDNAs including 60 cDNAs similar to the phytochelatin synthase 1 (*TcPCS1*) (bankit599609 AY540104), well known to be involved in the chelation of Cd. The second most represented sequence type was isolated 57 times and is homologous to plant Class II MTs sequences. Among these cDNAs, sequences homologous to the MTs of type 3 are the most represented, as they were isolated 54-fold. The deduced amino acid sequence of TcMT3 (bankit597144 AY531114) was aligned to plant MT sequences defined in the SwissProt database [24] and TrEMBL database (http://us.expasy.org). The deduced amino acid sequence of TcMT3 presents the best identity of 80% with AtMT3 (O22433). The deduced MT3 protein of *T. caerulescens* and a comparison with those of *A. thaliana* is given in Fig. 1A. The TcMT3 shows the same number of Cys residues as the AtMT3 sequence but the positions of some of these residues are modified. The shift in positions results from two amino acids substitutions in the *A. thaliana* protein (Ala_{11} for Cys and Cys_{64} for Gly).

3.2. Structural analysis

As no data about the three dimensional structure of plant MTs are available, a computer-based study has been undertaken in order to propose folding candidates of the plant MT based only on their primary structure. No evidence of structural homologues to the TcMT3 and the AtMT3 deduced amino acid sequences was found when a search was performed

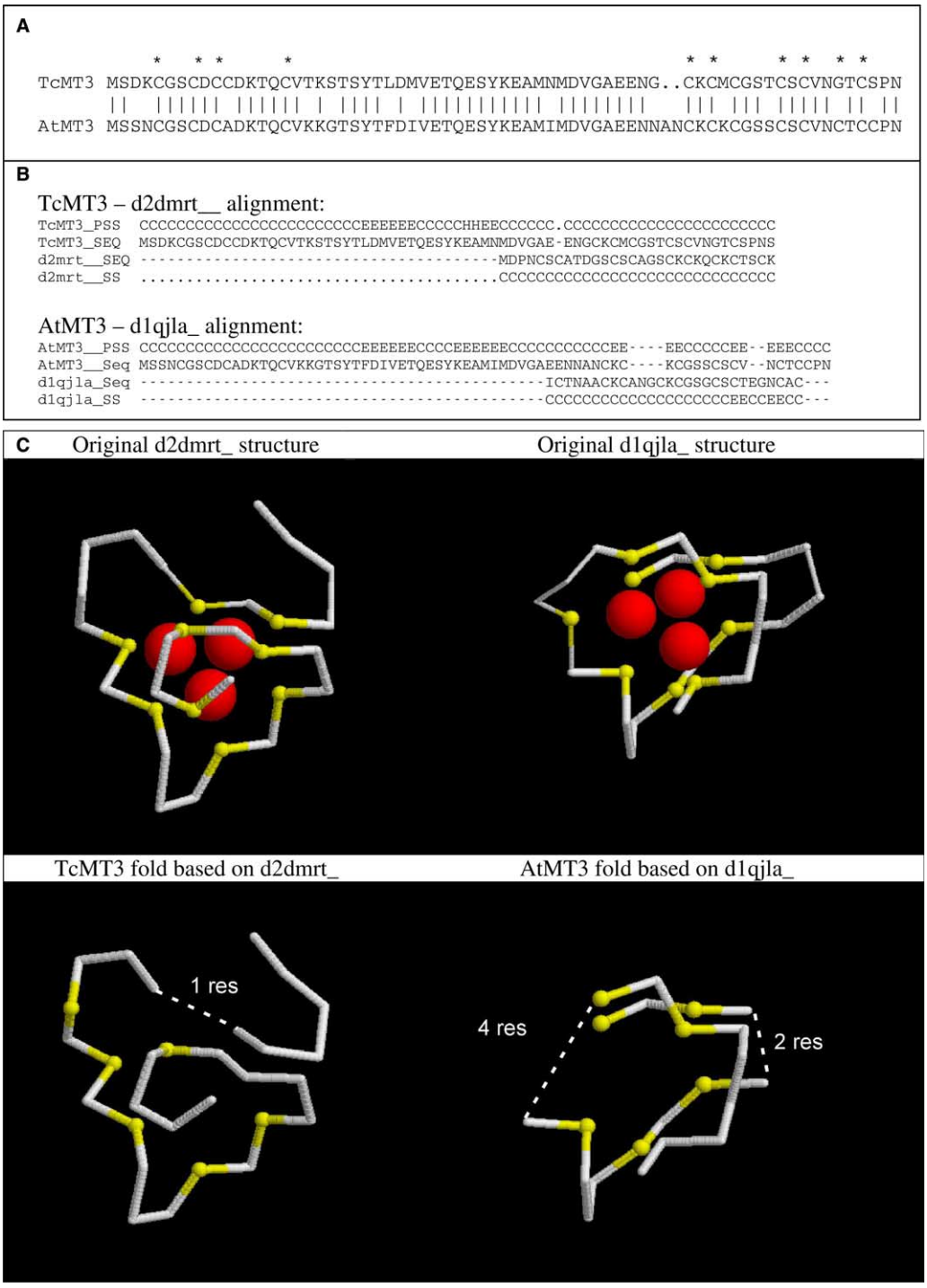

Fig. 1. Characterization of the *T. caerulescens* MT3 sequence. (A) Comparison of the deduced amino acid sequence of the *T. caerulescens* MT3 (TcMT3) with the corresponding isoform in *A. thaliana* (AtMT3). Asterisks indicate conserved cysteines in Type 3 MT of plants. (B) Sequence-structure alignment between TCMT3 and d2mrt_ SCOP domain and between the ATMT3 and d1qjla_ SCOP domain. Above the alignment is shown the TcMT3 or the AtMT3 predicted secondary structures and below the alignment is shown the derived one from d2mrt_/d1qjla_ structure. C, E and H are, respectively, assigned for coil, β-strand and α-helix. (C) Representation of the original d2mrt_ structure and TcMT3 structural model on the left and the original d1qjla_ structure and the AtMT3 structural model on the right. The dotted lines on the models represent deletions annotated with the number of residues deleted from the original templates d2mrt_ and d1qjla_. These regions are shown with thinner lines on the original structures. Small spheres on the backbone represent the Cys residues. Large spheres in the original structures represent the Cd atoms found in the cavity.

using Blast. The deduced amino acid sequences were then submitted to the fold recognition server 3D-PSSM. Significant scoring hits for the C-terminal but not for the N-terminal part of both MT3 were obtained using this method. The highest significant scoring hit for TcMT3 (*E*-value of 0.00278) was obtained with the SCOP domain d2mrt_ of the rat liver [25],

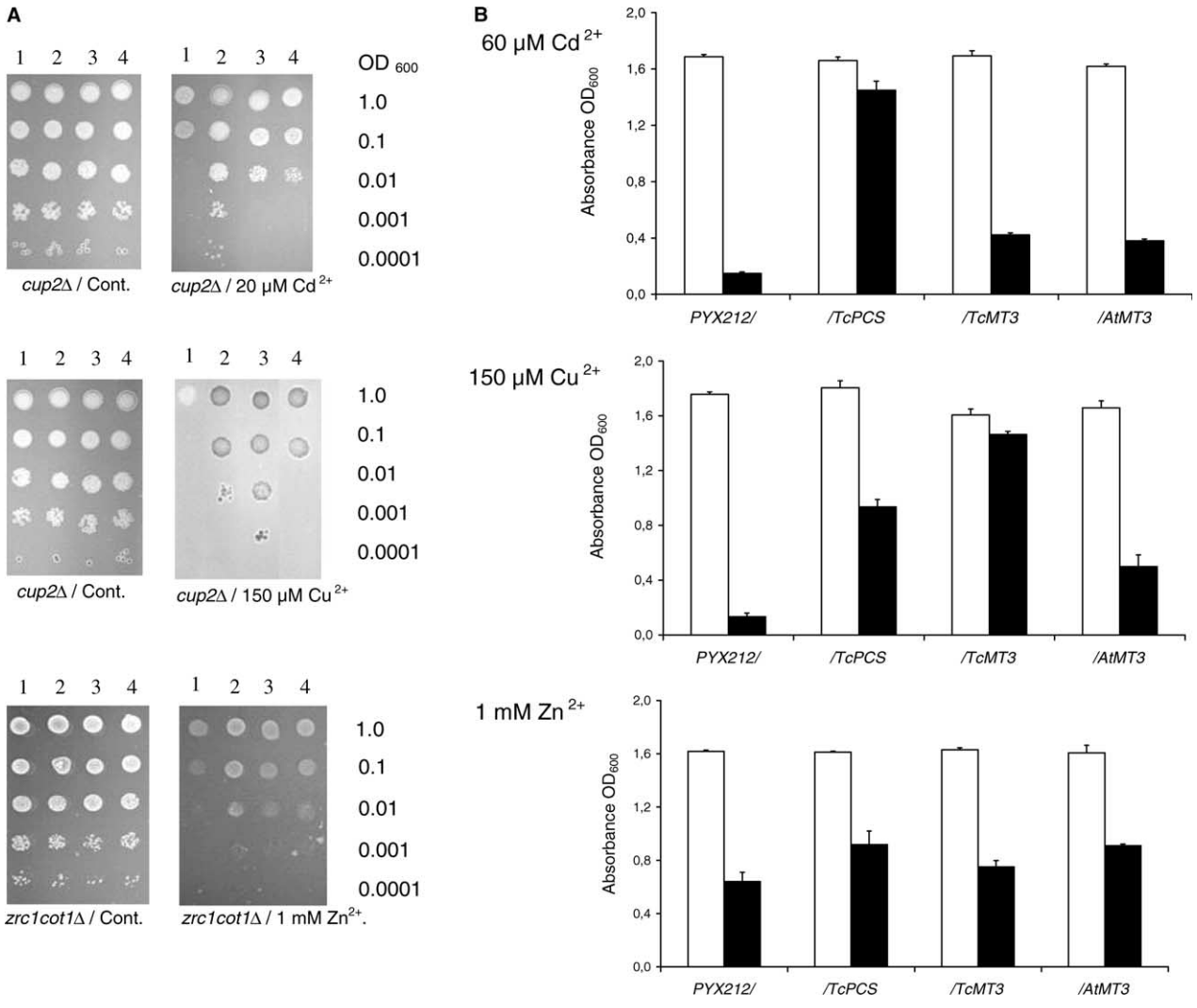

Fig. 2. Comparison of the metal tolerance of yeast expressing *MT3* cDNAs of *T. caerulescens* and *A. thaliana*. Three-day-growth in solid medium (A) not supplemented (Cont) or supplemented by heavy metals (Cd^{2+}, Cu^{2+}, and Zn^{2+}) of yeast transformed by pYX212 (1), pYX212-TcPCS (2), pYX212-TcMT3 (3), and pYX212-AtMT3 (4). Fourteen-hours-growth in liquid medium (B) not supplemented (white bars) or supplemented by heavy metals (Cu, Cd or Zn) (black bars) of yeast transformed by pYX212, pYX212-TcPCS, pYX212-TcMT3, and pYX212-AtMT3. Each tolerance test was repeated three times with four independent transformants for each recombinant plasmid. Vertical bars represent standard error.

while AtMT3 presented the best hit (E-value of 0.0943) with the SCOP domain d1qjla_ from the purple sea urchin [26]. These two domains belong to the same SCOP Metallothionein family and therefore are structurally equivalent. They both correspond to the β-domain containing a three-metal cluster (Cd_3Cys_9) of the two-domain Cd_7MT proteins elucidated for animals. No significant hit was obtained for other SCOP domains within or outside the MT family. Fig. 1B shows the alignment between the two plant MT3 sequences and the matching SCOP domains, including the predicted secondary structure for TcMT3 (TcMT3_PSS) and AtMT3 (AtMT3_PSS). Fig. 1C shows the original d2mrt_ and d1qjla_ SCOP domain structures on the first row and the deduced TcMT3 and AtMT3 models on the second row. The models have been provided by the 3D-PSSM web server which generates them by taking the original structures on which the TcMT3 and AtMT3 sequences have been mapped, based on the alignment shown in Fig. 1B. Both original d2mrt_ and d1qjla_ β-domain structures have nine Cys residues involved in the chelation of 3 Cd(II) ions. The same mammalian protein if demetalled and titrated with Cu(I) can be loaded with six-Cu(I) ions in this domain [6]. The TcMT3 and the AtMT3 domain models show, respectively, only 6 and 7 Cys residues that can be involved in metal binding, suggesting a reduced capacity to chelate metal atoms by comparison with the original SCOP domains (d2mrt_ and d1qjla_) found in animals. Moreover, the alignment of AtMT3 on d1qjla showed a total of six residues that were deleted, while the TcMT3 alignment on d2mrt presented only one deletion. The localisation of these deletions on the AtMT3 3D model suggests a smaller central cavity able to chelate less metal atoms compared to TcMT3.

3.3. *Comparison of the metal tolerance of yeast expressing MT3 cDNAs of* T. caerulescens *and* A. thaliana

To test whether differences in amino acid sequences between the MT3 of *T. caerulescens* and its *A. thaliana* orthologue

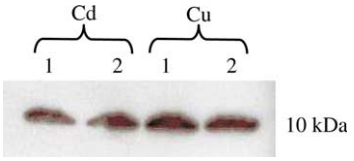

Fig. 3. Analysis of plant MT3 production in yeast. Western blot analysis was performed with total protein extract of yeast expressing AtMT3:V5 (1) and TcMT3:V5 (2) grown 14 h in liquid minimal medium supplemented by heavy metals (60 μM $CdSO_4$ (Cd) or 150 μM $CuSO_4$ (Cu)). The experiment was repeated three times with similar results. Immunoblot was hybridised with anti-V5-HRP antibody.

result in changes in metal binding, the MT3 from both plants was overexpressed in yeast. *TcPCS1* was also expressed in yeast, as phytochelatins are a well-known heavy metal ligand and have the greatest affinity for Cd [4]. The use of the *cup2*Δ mutant and of the double mutant *zrc1cot1*Δ allowed us to use lower concentrations than BY4741 to test the Cu and Zn tolerance. Indeed, the *cup2*Δ mutant has a deletion in *CUP2* which encodes a transcriptional regulator that upregulates *CUP1* (which encodes a metallothionein). The double mutant *zrc1cot1*Δ has deletions in *ZRC1* and in *COT1*, which both encode vacuolar transporters able to transport Zn. In solid and in liquid medium supplemented with Cd, the *cup2*Δ mutant overexpressing *TcPCS1* showed the highest tolerance ($P < 0.01$) (Fig. 2). The expressions of *TcMT3* and *AtMT3* or *TcMT3:V5* and *AtMT3:V5* (data not shown) were both able to increase to a similar level the Cd tolerance of *cup2*Δ but neither to as much as *TcPCS1* (Fig. 2). Similar results for Cd tolerance were obtained when *TcMT3* and *AtMT3* were overexpressed in the BY4741 wild-type strains (data not shown). Although overexpression of *TcPCS1* enhanced the Cu-tolerance of the *cup2*Δ mutant, this increase is lower than the one conferred by the expression of the *TcMT3* ($P < 0.01$) (Fig. 2). Moreover, *TcMT3* conferred strikingly higher Cu tolerance on the yeast cells than *AtMT3* ($P < 0.001$) (Fig. 2).

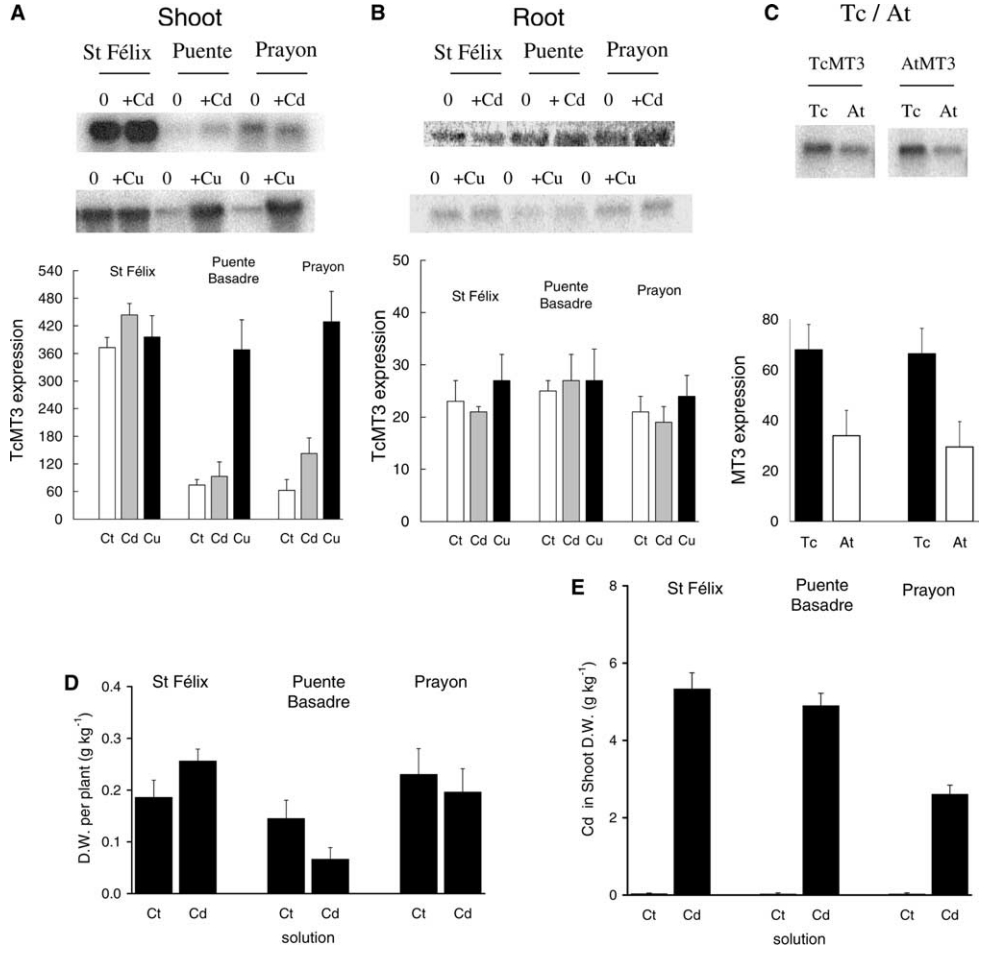

Fig. 4. Expression analysis of *MT3* in 3 populations of *T. caerulescens* and in *A. thaliana*. Total RNA was isolated from shoot (A) and root (B) of three contrasting populations of *T. caerulescens*: St Félix-de-Pallière (France), Puente Basadre (Spain) and Prayon (Belgium). Plants were grown in control solution (0) or in solution supplemented for 72 h by 100 μM $CdSO_4$ (+Cd) or 50 μM $CuSO_4$ (+Cu) (A, B). Comparison of the *MT3* expression in the shoot of *T. caerulescens* (Prayon) (Tc) and *A. thaliana* (At) (C). Both hybridisations confirm a 2.5-fold difference in *MT3* expression level between *T. caerulescens* and *A. thaliana*. Northern blots were hybridised overnight at 57 °C with ^{32}P radiolabelled *TcMT3* (A, B) or with ^{32}P radiolabelled *TcMT3* and *AtMT3* (C) coding sequences. *MT3* expression level was normalised to the one of 18S rRNA and expressed in arbitrary units (graph in A, B, C). Cd tolerance as measurement of the dry biomass (D) and Cd concentration (E) in the shoot after 10 days of 30 μM Cd treatment, both confirming [18]. Results are the average of at least three independent experiments. Vertical bars represent standard error.

Similar results were obtained when *TcMT3:V5* and *AtMT3:V5* were expressed in the *cup2*Δ mutant (data not shown). No difference in Zn tolerance between BY4741 or *cup2*Δ carrying the vector pYX212 alone (control) and the one expressing the *MT3* genes could be detected (data not shown). The use of the Zn sensitive *zrc1cot1*Δ mutant allowed us to greatly reduce the selective concentration of Zn. In these conditions, a slight increase of Zn tolerance was noticed upon expression of the *TcMT3*, the *AtMT3* and the *TcPCS1* in solid medium but this increase was not significant in liquid medium (Fig. 2).

3.4. Measurement of the expression of TcMT3:V5 and AtMT3:V5 fusion proteins in yeast

Production of plant MT in yeast was monitored using the V5 tag. The *TcMT3:V5* and *AtMT3:V5* fusions were expressed in *cup2*Δ yeast. The amount of TcMT3 and AtMT3 proteins expressed was then assessed in immunoblots probed with antibodies raised against the V5 epitope. A protein band of 10 kD was observed, which corresponds to the expected size of the MT3:V5 fusion protein (Fig. 3). In all the tested conditions (minimal medium supplemented by Cd and minimal medium supplemented by Cu), production of TcMT3:V5 and AtMT3:V5 fusion proteins was similar.

3.5. TcMT3 expression in contrasting populations of T. caerulescens in relation to their Cd tolerance and hyperaccumulation

TcMT3 expression was investigated by Northern blot hybridisation in three populations of *T. caerulescens* with contrasting levels of Cd tolerance and hyperaccumulation (Fig. 4C and D) [18]. Our data indicated that the *MT3* gene was preferentially expressed in the shoots compared to the roots in all the three populations ($P < 0.01$) (Fig. 4). Results showed that the expression of *TcMT3* was 3–7-fold higher ($P < 0.001$) in the shoot of the St Félix-de-Pallières population compared to the ones of Prayon (which accumulates less Cd) or Puente Basadre (which tolerates less Cd) (Fig. 4A, D, and E). This result was confirmed by RT-PCR (data not shown). In contrast, *TcMT3* expression was similar in the root of the 3 populations (Fig. 4B). Exposures of 6, 24 (data not shown) and 72 h (Fig. 4A and B) to 100 μM Cd did not affect significantly ($P > 0.05$) the level of *MT3* expression in any population. On the contrary, 50 μM Cu treatment increased the *MT3* expression in the shoot of Prayon and Puente Basadre populations, while its expression in St Félix-de-Pallières remains high but was unaffected (Fig. 4A and B). The expression of *MT3* was compared in *T. caerulescens* and *A. thaliana* (Fig. 4C). *TcMT3* transcript was about 2.5-fold more abundant in the shoot of the population Prayon than the *AtMT3* transcript was in the shoot of *A. thaliana*.

4. Discussion

A *T. caerulescens* cDNA displaying high homology to type 3 MT was identified by a functional screening to isolate cDNAs that increased the Cd tolerance of yeast. The fact that this cDNA was isolated with high frequency probably reflected a high expression in the Ganges population used to make the cDNA library and may be related to the role of MT3 in Cd tolerance. Thus, it was of primary interest to analyse further MT3 gene and its potential role in the Cd tolerance and hyperaccumulation of *T. caerulescens*.

The deduced amino acid sequence of TcMT3 shows an identity of 80% with AtMT3. The divergence between the two deduced sequences included variations in the Cys positions, which are proposed to be the crucial residues for metal chelation [2,4,6]. These modifications occurring in the C-terminal cysteine-rich domain of TcMT3 resulted in the loss of some of the conserved motifs that are seen among the plant MT type 3 (six Cys residues present as Cys–Xaa–Cys) [4]. Interestingly, among the 13 deduced plants MTs homologous to type 3 isolated from the database (Accession No. for *A. thaliana*: O22433; *Oryza sativa*: O04185, O22376; for *Gossypium hirsutum*: Q9XET5; *Brassica juncea*: Q852U1, Q852U2, Q852U3; *Actinadia chinensis*: P43389; *Carica papaya*: Q96386; *Malus domestica*: O24059; *Musa acuminata*: Q40256; *Prunus avium*: O48951; *Picea glauca*: Q40854) none of them show these modifications.

To get more information about the potential effect of these modifications and about the structure of the MT3 protein, we attempted to construct a model. Three-dimensional structures of MTs with Zn(II) and/or Cd(II) ions have been elucidated by crystallographic and NMR methods in mammalian and crustacean [2,6], but no information is available about plant MT as their purification has been reported to be particularly difficult [3,4]. The lack of known plant MT structures and the important evolutionary distance between plant and animal MT sequences make it difficult to understand the role of the Cys residues in the chelation mechanism at the molecular level for the AtMT3 and TcMT3 proteins. By using the 3D-PSSM fold recognition method, it has been possible to assign the AtMT3 and TcMT3 sequences to a structural template with good confidence. However, due to the weak similarity in sequence, the proposed models must be seen only as a rough estimation of the possible fold they adopt.

The structural model established for both AtMT3 and TcMT3 suggests that the structure of their carboxy-terminal domains is similar to the β domain of mammalian and crustacean MTs, which are structurally related. The presence of similar domain architecture of the metal–thiolate clusters in evolutionary-distant MTs emphasises the unique role of the Cys residues in defining the structural organisation of MTs and the evolutionary importance of this organisation. In addition, the MT3 model predicts a smaller cavity to chelate metals for *A. thaliana* than for *T. caerulescens* suggesting a lower capacity for trapping metal ions. As no structural model exists for Cu(I)MTs from animals, this model suffers from a lack of information regarding the coordination properties of an original matching fold for Cu(I). Moreover, it was demonstrated that MT isoforms containing 18 conserved Cys residues but differing in other amino acids are dedicated specifically to Cd(II) or Cu(I) chelation [27] and at the present time no model even in animals can take into account these differences. Our results show that there are several differences observed in the predicted amino acid sequences of the MT3 proteins of the closely related species *T. caerulescens* and *A. thaliana*, but it is difficult to assess whether these changes could result in modification of the functional properties of these proteins.

To address this question, *TcMT3* and *AtMT3* cDNAs were expressed in the *cup2*Δ yeast mutant with downregulated endogenous MT. Firstly, the increase of metal tolerance of the

yeast transformants is consistent with MTs of type 3 that function in vivo, as proteins that can bind Cd, Cu and Zn. These results are comparable with those previously published for MT type 1 and 2 of *A. thaliana* [8,11,13]. Secondly, the differences in the level of metal tolerance observed among the different yeast transformants suggest a difference in the metal-binding properties of the overexpressed proteins. The yeast expressing *TcPCS* were the most tolerant to Cd but not to Cu, which supports the idea that the observed metal tolerance probably reflects the capacity of the peptides for chelating metal. TcMT3 increased by far more the tolerance of yeast to Cu than its corresponding isoform from *A. thaliana*, although its protective effect on Cd or Zn was similar. Since the better growth on Cu of yeast expressing *TcMT3* than *AtMT3* was not due to differences in MT3 protein levels, we propose that the TcMT3 is able to chelate more Cu than AtMT3. Furthermore, our data support the hypothesis that the differences observed between the primary sequences of these two MTs result in modification of the metal-binding ability of these proteins and that the capacity for Cu binding of the TcMT3 is increased.

To provide some insight on the function of MT3 in *T. caerulescens*, *TcMT3* expression was analysed. Most of the type 3 genes characterised in other plants are expressed primarily in fruits as they ripen [28–30]. Type 3 MTs are also expressed at high levels in the leaves of plants that do not produce fleshy fruit such as *Arabidopsis* [4,12]. The pattern of expression of *TcMT3* in the shoot and in the root is comparable to the one observed in *A. thaliana* [12]. However, all the studied populations of the hyperaccumulator, *T. caerulescens*, present a constitutive higher *MT3* expression than *A. thaliana*. This provided valuable information into the potential function of *MT3*. Recently, comparative microarray analysis of the *A. thaliana* and *Arabidopsis halleri*, which is a Cd and Zn hyperaccumulator, suggested that adaptation to high intracellular Cd/Zn concentration is related to a large change in gene expression [31,32]. In *T. caerulescens*, MT3 overexpression and putative change in structure are likely to be a part of the adaptation to Cd/Zn hyperaccumulation. Moreover, the St Félix-de-Pallières population from Ganges showed a much higher expression of *TcMT3* in the shoot than the other *T. caerulescens* populations analysed. This population is the only one characterised to date that possesses both high levels of Cd hyperaccumulation and tolerance [18]. High levels of *MT3* expression in the shoot may be required for the detoxification of Zn and Cd. Another hypothesis is that MT3 may be involved in Cu homeostasis and/or the delivery of this essential metal. Some observations support the role of MT in Cu homeostasis. In many plants including *A. thaliana*, MT RNAs (including MT3) are strongly induced by Cu and slightly by Cd and Zn [8,10,12,33]. In *T. caerulescens*, the induction of *MT3* expression by Cu was similarly confirmed, while no change by Cd was observed. In addition, the level of type 2 MT gene expression is correlated with the Cu tolerance across *A. thaliana* ecotypes [13] and in *Silene vulgaris* with a Cu tolerant background [14].

In conclusion, the primary sequence of the type 3 MT of *T. caerulescens* displays modifications that are proposed to increase its Cu binding properties when compared to the non-hyperaccumulator, *A. thaliana* orthologue. *TcMT3* seems not to be responsible for Cd tolerance by itself, rather it may be an adaptation of this plant to ensure adequate Cu homeostasis in the context of competition with high Cd and Zn content in the shoot. This putative role is further suggested by the constitutively higher expression of this gene in all the tested populations of *T. caerulescens* when compared to *A. thaliana* and the induction of the expression of the *MT3* transcript by Cu. The particular high expression level of the *T. caerulescens* population from the Ganges region remains to be analysed in further details. Analysis of crosses between St Félix-de-Pallières (Ganges) and Prayon populations will allow to assess whether Cd accumulation/tolerance and MT3 overexpression cosegregates. This work emphasises that complexity of the different plant MTs is necessary to deal with the variety of metals present in their environment.

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