Adaptative Evolution of Metallothionein 3 in the Cd/Zn Hyperaccumulator *Thlaspi caerulescens*

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A functional screening in yeast allowed to identify various cDNAs from the Cd/Zn hyperaccumulator *Thlaspi caerulescens. TcMT3* displayed high identity with its closest homologue in *Arabidopsis thaliana* but variation in its Cys residues. Functional analysis in yeast supported a higher binding capacity for Cu, but not for Cd or Zn, of *TcMT3* compared to *AtMT3*. Expression analysis in plants indicated that metallothionein 3 (*MT3*) like all the other *T. caerulescens* genes from the screen studied is overexpressed in all studied populations of *T. caerulescens* compared to *A. thaliana*. *TcMT3* was induced by Cu, but not by Cd. Moreover significant variation in expression within *T. caerulescens* populations that have contrasting tolerance and accumulation capacities indicated a possible local adaptation of MT3.

Key words: Metallothionein, Metal Homeostasis, Metal Hyperaccumulation

Introduction

Some plants named hyperaccumulators are able to tolerate elevated concentrations of heavy metals, together with high accumulation of heavy metals such as Zn, Cd and Ni in their shoot (more than 100-fold the concentration found in other plants) (Baker, 1981). The physiological and molecular mechanisms responsible for the metal hyperaccumulation phenotype are still poorly understood. Metallothioneins (MTs) are a superfamily of Cys rich polypeptides with a low molecular mass (4 to 8 kDa) able to chelate metal ions as Cd(II), Zn(II) and Cu(I) (Cobbett and Goldsbrough, 2002; Romero-Isart and Vasak, 2002). In animals, MTs are known to be involved in metal homeostasis and some can protect cells against Cd toxicity (Klaassen *et al*., 1999; Romero-Isart and Vasak, 2002). Similarly, information obtained by gene expression analysis and functional expression in microorganisms suggests that plant MTs may be involved in metal homeostasis (Zhou and Goldsbrough, 1994, 1995; Robinson *et al*., 1996; Guo *et al*., 2003) and Cu tolerance (Murphy *et al*., 1997; van Hoof *et al*., 2001). However, in contrast to animals, direct information about the role of MT proteins from plants is scarce because of difficulties in purifying them. A role for MT type 2 in Cu tolerance has already been published in the Cu tolerant non accumulator *Silene vulgaris* (van Hoof *et al*., 2001). A possible role for MTs in metal tolerance in *T. caerulescens* or any other hyperaccumulator has not been investigated so far. This work presents evidences for a possible role of MT in the adaptation to Cd/Zn hyperaccumulation.

Functional screening of a cDNA library of *Thlaspi caerulescens* in yeast and identification of *TcMT3*

Functional screening of *T. caerulescens* cDNAs that confer Cd tolerance (15 μM $CdSO_4$) to *S. cerevisiae* (BY4741) resulted in the isolation of 139 cDNAs (Table I). The most isolated cDNAs contained Cys rich motifs in their deduced amino acid sequence and were predicted to encode cytosolic peptides and to chelate metals. The database search assigned putative function to these cDNAs. The first group encodes proteins known to be involved in metal chelation, the phytochelatin synthase 1 (PCS1) and the metallothioneins (MTs). Among these cDNAs, sequences homologous to PCS1 and MT3 were the most represented, as they were respectively isolated 60- and 54-fold. A first

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Table I. Summary of the cDNAs identified by functional Cd tolerance screen in yeast.
No of cDNA: number of times that each cDNA has been identified during the screening. Identity (%): highest identity of the deduced aa (amino acid) sequence corresponding to the cDNA isolated in *T. caerulescens* with the deduced aa of a *A. thaliana* cDNA. Accession: number of the corresponding *A. thaliana* clone in the NCBI database; the number between brackets corresponds to the size of the cDNA coding sequence. SAM: salicylic acid carboxyl methyl transferase.

No of cDNA	Putative function	Identity (%) with *A. thaliana*	Accession *A. thaliana*
I. Metal detoxification			
60	Phytochelatin synthase (AY540104)	78% on 485 aa	At5 g44070 (485)
54	Metallothionein type 3 (AY531114)	80% on 69 aa	At3 g15353 (69)
2	Metallothionein type 2	91% on 81 aa	At3 g09390 (81)
1	Metallothionein type 1	69% on 45 aa	At1 g07600 (45)
7	Metallothionein related protein	/	/
II. Metal transport			
4	Cd/Zn transp. P-type ATPase (AJ567384)	79% on 259 aa	At2 g19110 (1172)
III. Signalling pathway			
2	Heat shock transcription factor	91% on 187 aa	At4 g18880 (401)
1	General transcription factor IID	93% on 134 aa	At4 g31720 (134)
IV. Others			
1	SAM (1)	71% on 197 aa	At5 g66430 (354)
1	Chl A-B binding protein	98% on 169 aa	At1 g29920 (267)
1	40S ribosomal protein	98% on 90 aa	At5 g35530 (248)
1	Photosystem I subunit	96% on 101 aa	At4 g12800 (219)
V. Unknown			
2	Unknown protein	92% on 268 aa	At3 g15840 (268)
1	Unknown protein	71% on 232 aa	At5 g15790 (232)
1	Unknown protein	94% on 56 aa	At3 g12140 (327)

characterization of the MT3 cDNA (AY531114) has been recently published (Roosens *et al.*, 2004). The second group contains truncated cDNAs encoding a peptide homologous to the Cys and His rich carboxy terminal domain of a heavy metal ATPase (HMA4) (Bernard *et al.*, 2004). The peptide was devoid of transporter function but retained potential Cd binding motifs, and could protect the cell from toxic effects of free Cd ions. cDNAs of groups three and four encoded proteins without clear relationship to heavy metal tolerance but for most of them contain motifs susceptible to chelate metals.

From the amino acid analysis to the prediction of a structural model

T. caerulescens is closely related to *A. thaliana* displaying about 87% of its coding sequence with it (Peer *et al.*, 2004). In strong agreement, most of the deduced amino acid sequences identified in *T. caerulescens* share between 70 and 98% identity with *A. thaliana* (Table I). Nevertheless, divergence in sequences may be related to the adaptation of metallicolous populations of *T. caerulescens* to its environment. These potential adaptative variations are described in Bernard *et al.* (2004) and Roosens *et al*. (2004). For example, a MT3 structural model predicts a smaller cavity to chelate metals for *A. thaliana* than for *T. caerulescens* suggesting a lower capacity for trapping metal ions (Roosens *et al*., 2004).

Functional analysis of plant MT3 in yeast

To investigate whether differences in amino acid sequences between the MT3 of *T. caerulescens* and its *A. thaliana* orthologue result in changes in metal binding, the MT3 cDNA from both plants was overexpressed in yeast (Table II). The *pYX212* vector (Ingenius, Madison, WI) was used to express cDNA in yeast and yeasts were transformed by the lithium acetate procedure (Gietz and Schiestl, 1995). First tests were performed in the BY4741 wild-type strain. The critical concentration used to test tolerance of yeast on Cd, Cu and Zn ions was 60 μM, 600 μM and 15 mM, respectively. A similar protective effect against Cd was

Table II. Comparison of the metal tolerance of yeast (BY4741, wild type; *cup2Δ*, copper hypersensitive mutant; *zrc1cot1Δ*, zinc hypersensitive mutant) expressing cDNAs of *T. caerulescens* and *A. thaliana* (14 h). The growth in liquid minimal medium (M.M.) not supplemented or supplemented by heavy metals ($CuSO_4$, $CdSO_4$ or $ZnSO_4$) of yeast transformed by *pYX212*, *pYX212-TcPCS*, *pYX212-TcMT3*, *pYX212-AtMT3* is shown. Values are density OD_{600} measured by a spectrophotometer ($1.0 = 5 \times 10^4$ cells/cm^3) after 16 h of growth. Values are means ± SE ($n = 4$). Superscripts are the type of BY4741 mutant used for the experiment ([a]*cup2Δ*; [b]*zrc1cot1Δ*).

BY4741				BY4741 mutant			
pYX212/	*/TcPCS*	*/TcMT3*	*/AtMT3*	*pYX212/*	*/TcPCS*	*/TcMT3*	*/AtMT3*
M.M.				M.M.[a]			
1,6 ± 0,0	1,7 ± 0,0	1,6 ± 0,0	1,6 ± 0,0	1,7 ± 0,0	1,7 ± 0,0	1,7 ± 0,0	1,6 ± 0,0
M.M. + 60 μM Cd^{++}				M.M. + 60 μM Cd^{++}[a]			
0,2 ± 0,0	1,4 ± 0,0	0,6 ± 0,0	0,6 ± 0,0	0,2 ± 0,0	1,5 ± 0,1	0,4 ± 0,0	0,4 ± 0,0
M.M. + 600 μM Cu^{++}				M.M. + 150 μM Cd^{++}[a]			
0,4 ± 0,1	0,5 ± 0,1	0,5 ± 0,0	0,5 ± 0,1	0,1± 0,0	0,9 ± 0,1	1,5 ± 0,0	0,5 ± 0,1
M.M. + 15 μM Zn^{++}				M.M. + 1 mM Zn^{++}[b]			
0,8 ± 0,2	0,8 ± 0,2	0,8 ± 0,2	0,8 ± 0,2	0,6 ± 0,1	0,9 ± 0,1	0,8 ± 0,1	0,9 ± 0,0
M.M + 1 mM diamide				M.M. + 1 mM diamide[a]			
0,3 ± 0,1	0,4 ± 0,1	0,3 ± 0,1	0,4 ± 0,1	0,4 ± 0,1	0,4 ± 0,1	0,4 ± 0,1	0,3 ± 0,1

provided to BY4741 by the expression of *TcMT3* and *AtMT3*. For both Cu and Zn ions, no improved growth of yeast overexpressing MT3 proteins was observed, suggesting that MT3 has no affinity for both elements or that the metal tolerance of BY4741 could mask a phenotype. The use of the copper sensitive *cup2Δ* mutant lacking the *CUP2* transcriptional regulator of the *CUP1* metallothionein allowed to reduce the critical concentration for Cu ions to 150 μM. In these conditions, an increase of tolerance to Cu of yeast overexpressing *MT3* could be observed. To decrease the critical concentration for Zn, the mutant *zrc1Δ* lacking the Zn *ZRC1* vacuolar transporter was used. This Zn sensitive mutant allowed to reduce the critical concentration for Zn ions to 7 mM. In these conditions however no difference in Zn tolerance could be observed whether *zrc1Δ* overexpressed plant *MT3* or not. The use of the double mutant *zrc1cot1Δ* lacking 2 vacuolar transporters able to transport zinc allowed to reduce the critical concentration to 1 mM of Zn ions. On this concentration, *zrc1cot1Δ* had an improved growth upon overexpression of *AtMT3* or *TcMT3*. Moreover, *TcMT3* provided an increased Cu tolerance compared to *AtMT3*. This difference was not related to changes in MT3 protein levels as revealed by a V5-tag fusion. As plant MTs may have a role as antioxidants (Hall *et al.*, 2002), tolerance to oxidative stress of MT3 was tested by adding 1 mM diamide in growth medium as described in Babiychuk *et al.* (1995). No improvement of growth was observed on diamide either by using the BY4741 or the *cup2Δ* mutant. An hypersensitive mutant to oxidative stress may be used in future analysis to better study the possible role of MTs in protecting cells against oxidative stress.

The expression in yeast indicated that MTs functions *in vivo* as proteins that can bind Cd, Cu and Zn. The use of an appropriate mutant was crucial to reveal the metal binding properties of the proteins. The differences in the level of metal tolerance observed among the different yeast transformants suggest metal-binding properties for TcMT3 and AtMT3. TcMT3 increased by far more the tolerance of yeast to Cu than its corresponding isoform from *A. thaliana* although its protective effects on Cd or Zn were 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 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 TcMT3 is increased. Similarly, variations in the amino acid sequence of HMA4 were observed in particular in the cytosolic *C*-terminal domain (containing several heavy metal binding motifs) between *A. thaliana* and *T. caerulescens*. This divergence was associated with a stronger Cd binding capacity of the TcHMA4 C-domain than the one of *A. thaliana,* when studied by heterologous expression in yeast (Bernard *et al*., 2004).

Analysis of gene expression in *T. caerulescens* and *A. thaliana*

In order to investigate the potential role in Cd tolerance of the genes identified in yeast (group 1 metal detoxification, group 2 metal transport), the expression of these genes was analysed in *T. caerulescens* (Table III). In first, comparison with *A. thaliana* showed that all the studied populations of the hyperaccumulator *T. caerulescens* present a constitutive higher expression of all the *MTs* (2- to 4-fold in the shoot) and of *HMA4* (20-fold in the root) than *A. thaliana*. Moreover, the St Félix de Pallières population from Ganges showed a much higher expression of *TcMT3* but not of the other genes in the shoot (3- to 7-fold higher) than other *T. caerulescens* populations with contrasting levels of Cd tolerance and hyperaccumulation (Roosens *et al*., 2004). This population is the only one characterized to date, that possesses both high levels of Cd hyperaccumulation and tolerance (Roosens *et al*., 2003). Exposures of 6 h, 24 h and 72 h to 100 μM Cd ions did not affect significantly the level of *MT* or *TcHMA4* expression in any population. On the contrary, 50 μM Cu treatment increased the MT of type 1 and 3 expression in the shoot of *Thlaspi* populations.

Table III. Expression analysis of *MT1*, *MT2*, *MT3* and *HMA4* in *T. caerulescens* (Prayon) and *A. thaliana*. The expression of the genes was compared between *T. caerulescens* and *A. thaliana* (*T.c./A.t.*). In *T. caerulescens*, the expression of the genes was compared between the shoot and the root (shoot/root), in plants treated with 30 μM $CdSO_4$ for 24 h relatively to the control (Ct/Cd) and in plants treated with 30 μM $CuSO_4$ for 24 h relatively to the control (Ct/Cu). The symbols “+”, “++” and “+++” represent an induction of the gene expression of respectively a 2- to 10-fold, 10- to 20-fold and more than 20-fold. The symbol “–” represents a 2- to 10-fold decrease. The symbol “/” represents no modification of the gene expression. N.M., not measured. All the gene expressions were normalized to the one of the 18S rRNA.

Gene	*T.c./A.t.*	shoot/root	Ct/Cd	Ct/Cu
MT1	+	+++	/	+
MT2	+	/	/	/
MT3	+	+	/	+
HMA4	++	–	/	N.M.

High levels of *MTs* expression in the shoot may be required for the detoxification of Zn and Cd. Another hypothesis is that MTs may be involved in Cu homeostasis and/or the delivery of this essential metal. The induction of *MT* of type 1 and 3 expression by Cu while no change by Cd was observed supports this hypothesis. The study of *MT* expression in plants gives first indication on a potential role of *MT* in the hyperaccumulation. It will be essential to be able to determine corresponding protein levels.

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