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Accumulation of metal-binding peptides in fission yeast requires *hmt2*$^+$

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Summary

The fission yeast *Schizosaccharomyces pombe* detoxifies cadmium by synthesizing phytochelatins, peptides of the structure (γ-GluCys)$_n$Gly, which bind cadmium and mediate its sequestration into the vacuole. The fission yeast protein HMT2, a mitochondrial enzyme that can oxidize sulphide, appears to be essential for tolerance to multiple forms of stress, including exposure to cadmium. We found that the *hmt2*$^-$ mutant is unable to accumulate normal levels of phytochelatins in response to cadmium, although the cells possess a phytochelatin synthase that is active *in vitro*. Radioactive pulse–chase experiments demonstrated that the defect lies in two steps: the synthesis of phytochelations and the upregulation of glutathione production. Phytochelatins, once formed, are stable. *hmt2*$^-$ cells accumulate high levels of sulphide and, when exposed to cadmium, display bright fluorescent bodies consistent with cadmium sulphide. We propose that the precipitation of free cadmium blocks phytochelatin synthesis *in vivo*, by preventing upregulation of glutathione production and formation of the cadmium–glutathione thiolate required as a substrate by phytochelatin synthase. Thus, although sulphide is required for phytochelatin-mediated metal tolerance, aberrantly high sulphide levels can inhibit this pathway. Precise regulation of sulphur metabolism, mediated in part by HMT2, is essential for metal tolerance in fission yeast.

Introduction

Heavy metals such as Cd^{2+}, Cu^{2+} and Pb^{2+} are toxic, and different organisms have evolved a variety of mechanisms to detoxify metals. In eukaryotes, the route of detoxification involves synthesis of chelating molecules that bind to the metal ions and mediate their transport out of the cells or their sequestration into subcellular compartments. The fission yeast *Schizosaccharomyces pombe* has become a well-developed model system for understanding metal detoxification pathways common to fungi and plants (Fig. 1).

S. pombe responds to the presence of cadmium and some other heavy metals by producing phytochelatins, peptides of the structure (γ-EC)$_n$G, $n = 2-11$, which chelate and sequester free metal ions (Murasugi *et al*., 1981; Kondo *et al*., 1984; Grill *et al*., 1986; Cobbett, 2000). During cadmium exposure, phytochelatin (PC) peptides are synthesized from glutathione (GSH) by the enzyme phytochelatin synthase, a dipeptidyl transpeptidase (Clemens *et al*., 1999; Ha *et al*., 1999; Vatamaniuk *et al*., 1999). This enzyme is present constitutively in the cell, but requires heavy metal ions for activity. Cadmium exerts its effect by forming a metal–glutathione thiolate that acts as the acceptor molecule in the transpeptidation reaction (Vatamaniuk *et al*., 2000).

Once synthesized, phytochelatins bind cadmium ions to form a low-molecular-weight complex (LMW PC–Cd), which is transferred to the vacuole by the ABC-type transporter HMT1 (Ortiz *et al*., 1992; 1995). In the vacuole, stoichiometric addition of sulphide and additional free cadmium ions leads to the formation of a high-molecular-weight phytochelatin–cadmium–sulphide complex (HMW PC–CdS), in which the metal is bound with higher affinity (Murasugi *et al*., 1983; Reese and Winge, 1988). The additional cadmium appears to enter the vacuole via a Cd^{2+}/H^+ antiport (Ortiz *et al*., 1995), whereas the sulphide ion appears to be derived from cysteine sulphinate incorporation into purine precursors by SAICAR synthetase and AMP-S synthetase, two enzymes of the purine biosynthesis pathway (Speiser *et al*., 1992; Juang *et al*., 1993).

HMT2, a mitochondrial sulphide dehydrogenase (Vande Weghe and Ow, 1999), also appears to play an important role in metal tolerance. The *hmt2*$^-$ mutant is extremely cadmium-sensitive; exposure to concentrations as low as 5 μM reduces its growth rate relative to wild type. Mutant cells accumulate an abnormally high amount of sulphide, which further increases during cadmium stress. This association between high sulphide levels and metal sensitivity was surprising. It contrasts with previous studies in *S. pombe* (Perego *et al*., 1997) and *Candida glabrata* (Mehra *et al*., 1994). In those studies, mutants with high levels of sulphide form correspondingly high amounts of the high-molecular-weight sulphide-rich

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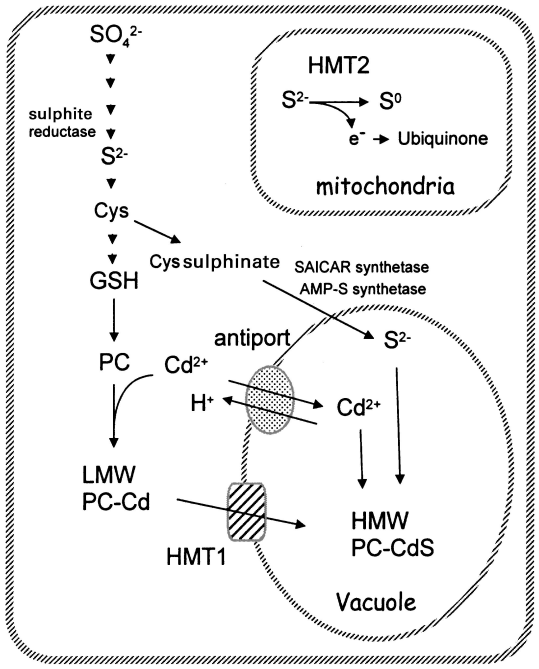

Fig. 1. Model of phytochelatin-mediated cadmium detoxification in fission yeast. Cd^{2+} induces production of PC peptides that in turn chelate Cd^{2+} to form a low-molecular-weight (LMW) PC–Cd complex with a Cys:Cd^{2+} ratio of 2:1–4:1. The LMW complex is sequestered into the vacuole by the ABC-type transporter HMT1. Within the vacuole, a more stable high-molecular-weight (HMW) complex is formed incorporating S^{2-} and additional Cd^{2+}, reducing the Cys:Cd^{2+} ratio to 1:1. The additional Cd^{2+} enters the vacuole through a cation antiport. The source of S^{2-} for the HMW complex appears to be derived from Cys via Cys sulphinate incorporation into purine precursors by two purine biosynthesis enzymes, SAICAR synthetase and AMP-S synthetase. Whether this S^{2-} source is formed outside or within the vacuole is not known. The proposed role for mitochondrial HMT2 is to convert excess S^{2-} to S^0. This source of S^{2-} is not known, but could be derived directly from SO_4^{2-} reduction and/or from the cysteine sulphinate pathway. Without functional HMT2, excess S^{2-} may inhibit mitochondrial respiration and permit toxic CdS formation. The lack of free Cd^{2+}, in turn, prevents a higher production of GSH and formation of the Cd-GSH thiolate substrate for PC synthesis.

phytochelatin–cadmium complex, and are hypertolerant to cadmium.

To account for the cadmium-hypersensitive phenotype in an *hmt2*$^-$ mutant, we had previously proposed that HMT2 is needed for the detoxification of sulphide that would otherwise inhibit mitochondrial respiration (Vande Weghe and Ow, 1999). In this article, we report that phytochelatin accumulation is also severely deficient in an *hmt2*$^-$ mutant. This links HMT2 to a role wider than the one previously proposed. Regulation of sulphide levels by HMT2 is required in order for the cell to synthesize phytochelatins in response to metal stress.

Results

Phytochelatin deficiency in the hmt2$^-$ *mutant*

The growth of the *hmt2*$^-$ mutant is not more sensitive than wild type to $NiCl_2$ (1–800 μM), $Bi(NO_3)_2$ (1 μM–2 mM) or $Pb(NO_3)_2$ (1 μM–4 mM) across the range of concentrations tested. However, it is more sensitive to $CdSO_4$ (> 5 μM), $HgCl_2$ (> 50 μM), $CuSO_4$ (> 2 mM), and $ZnSO_4$ (> 4 mM). Sensitivity to multiple heavy metals could indicate a defect in the phytochelatin-mediated metal detoxification pathway. Cadmium-induced wild-type cells, such as JS23, produce two characteristic cadmium-containing peaks on gel filtration chromatography, representing high- and low-molecular-weight phytochelatin–cadmium complexes (Fig. 2A). A strain bearing a point mutation in the *hmt2* coding sequence, JS563, produces neither phytochelatin–cadmium complex. A second strain, JV5, in which an engineered disruption of the *hmt2* promoter causes reduced gene expression, forms only low levels of the high-molecular-weight phytochelatin–cadmium complex. Correspondingly, overexpression of *hmt2*$^+$ on a multicopy expression plasmid (pJV26) restores the ability of an *hmt2*$^-$ strain to accumulate the high- and low-molecular-weight phytochelatin–cadmium complexes (Fig. 2B).

To determine whether failure to produce phytochelatin–cadmium complexes resulted from deficient synthesis of the phytochelatin peptide or from a problem in Cd complex assembly, we assayed for the presence of apo-phytochelatins. Extracts of cadmium-induced cells were fractionated by reversed-phase high performance liquid chromatography (HPLC). Thiol-containing molecules

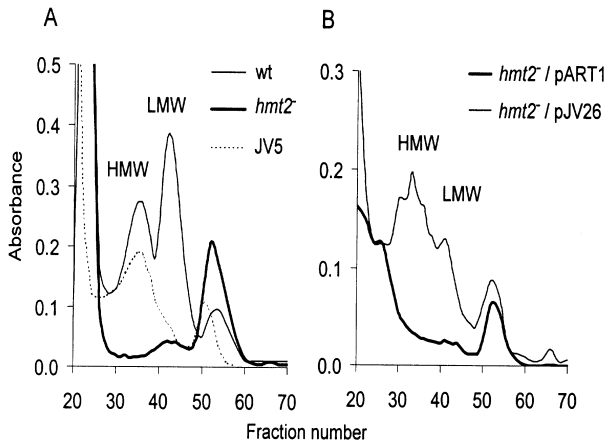

Fig. 2. Gel filtration analysis of cadmium-binding complexes.
A. JS23 (wild type), JS563 (*hmt2*$^-$ mutant) and JV5 (*hmt2* disruption mutant) were grown in rich (YG) media with 200 μM cadmium for 30 h. Extracts containing 2 mg of protein were fractionated on a G-50 column.
B. JS563 bearing the empty vector pART1 or the complementing *hmt2*$^+$ plasmid pJV26 were grown in minimal (SG + uracil) media and induced with 200 μM cadmium for 24 h. Extracts containing 0.6 mg of protein were fractionated on a G-50 column. Cadmium content was measured by atomic absorbance spectroscopy. Peaks corresponding to high- and low-molecular weight phytochelatin–cadmium complexes are marked HMW and LMW respectively. Free Cd appears after fraction 50.

were detected after post-column derivatization with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), which reacts specifically with -SH groups to create a yellow compound. Wild-type cells exposed to cadmium accumulate large amounts of the apo-phytochelatins $(\gamma\text{-EC})_2$G and $(\gamma\text{-EC})_3$G (referred to as PC_2 and PC_3 respectively) (Fig. 3A); mutant cells accumulate only minor amounts of these peptides (Fig. 3B). As *hmt2*$^-$ cells lack a mitochondrial sulphide dehydrogenase activity (Vande Weghe and Ow, 1999), a possibility may exist that the redox balance in mutant cells was altered such that phytochelatins accumulated in an oxidized form, which would be non-functional as well as undetected by the DTNB assay. To see if this could be the case, $NaBH_4$ was added to reduce the extracts chemically prior to HPLC. As a control, $NaBH_4$ was tested on a sample of cell extract spiked with 10 nM oxidized glutathione. The addition of $NaBH_4$ caused a quantitative conversion of the oxidized glutathione to the reduced form (data not shown). When $NaBH_4$ was added to extracts from *hmt2*$^-$ cells, however, the amount of phytochelatins detected by DTNB failed to increase (Fig. 4).

We also searched for oxidized phytochelatins using ^{35}S labelling. Extracts from radiolabelled *hmt2*$^-$ cells were examined simultaneously by DTNB detection (Fig. 5A) and flow scintillation counting (Fig. 5B). While oxidized phytochelatins would escape DTNB detection, they should still be detectable via the ^{35}S label. Therefore, if the *hmt2*$^-$ mutant possesses a significant pool of oxidized phytochelatins, it should display ^{35}S-labelled peaks absent from the DTNB-detection profile. As a result of formation of interchain and intrachain disulphide bonds (Meuwly *et al*., 1995), retention times of oxidized phytochelatins could also be slightly altered from those of the reduced forms, so any ^{35}S peaks at unusual retention times in the mutant would also bear consideration. However, in extracts from *hmt2*$^-$ cells, each radiolabelled HPLC peak corresponded to a peak visible by DTNB. The ^{35}S-labelled peaks at the retention times of reduced PCs were much smaller than those from the Cd^{2+}-exposed wild-type strain (not shown), and there were no additional peaks at novel retention times that could correspond to a hidden pool of oxidized PCs. We conclude that there is not a significant amount of oxidized phytochelatins in *hmt2*$^-$ cells.

Position of the defect in the phytochelatin biosynthetic pathway

S. pombe and *A. thaliana* mutants lacking normal levels of glutathione, the biosynthetic precursor of phytochelatins, fail to produce phytochelatins and are hypersensitive to heavy metals (Mutoh and Hayashi, 1988; Glaeser *et al*., 1991; Howden *et al*., 1995a). However, *hmt2*$^-$ cells grown in the absence of cadmium have normal levels of reduced

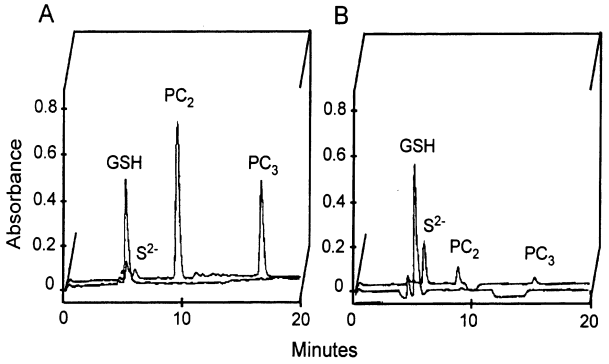

Fig. 3. High performance liquid chromatography (HPLC) analysis of acid-soluble thiols. Extracts from wild-type JS23 (A) and *hmt2*$^-$ JS563 (B) cells grown in rich (YG) media in the absence of cadmium (foreground) or with 200 μM Cd for 24 h (background) were separated by reverse-phase HPLC and detected by reaction with DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)). GSH: glutathione; S^{2-}: sulphide; PC_2, PC_3: $(\gamma\text{-EC})_2$G and $(\gamma\text{-EC})_3$G phytochelatins respectively.

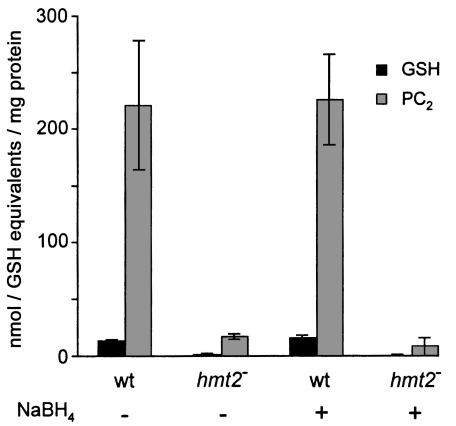

Fig. 4. High performance liquid chromatography (HPLC) analysis of thiols after chemical reduction. Extracts from wild-type JS23 and *hmt2*$^-$ JS563 cadmium-induced cells were analysed by HPLC with DTNB (5,5′-dithiobis-(2-nitrobenzoic acid) detection before (left) and after (right) $NaBH_4$ reduction.

glutathione, as measured by HPLC (Figs 3A and B), and of total glutathione, as measured by the glutathione reductase recycling assay (wild type: 19.0 nmol GSH/mg protein, ± 1.5 SE; mutant: 18.6 nmol GSH/mg protein, ± 2.6 SE; $n = 8$). Therefore, the *hmt2*$^-$ mutant phenotype is not due to a basic deficiency in glutathione production.

It remained possible that mutant cells can synthesize phytochelatins at normal rates but quickly run out of substrate, or degrade the phytochelatins soon after synthesis. To evaluate these possibilities, we examined the time-course of cadmium induction. Cells were fed $^{35}SO_4^{2-}$ in the absence of cadmium to label the glutathione pool. Excess unincorporated label was then removed and cells were placed in media containing unlabelled SO_4^{2-} along with 200 μM cadmium. Prior to cadmium induction, mutant and wild-type cells have similar amounts of glutathione (Fig. 6A and B), which is consistent with

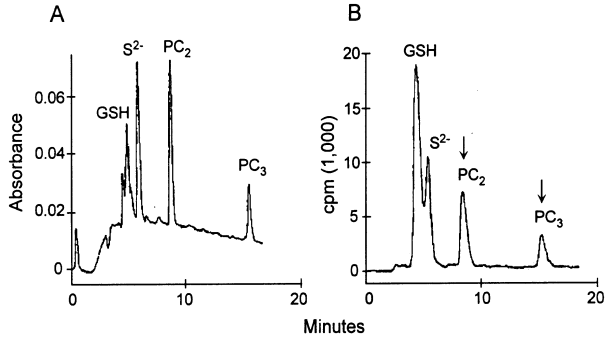

Fig. 5. Detection of radiolabelled thiols. An extract from ^{35}S-labelled *hmt2*$^-$ JS563 cells induced with 200 μM cadmium for 8 h was separated using high performance liquid chromatography (HPLC). Peaks were detected simultaneously by reaction with DTNB (5,5′-dithiobis-(2-nitrobenzoic acid) (A) and flow scintillation counting (B). Arrows indicate the retention times of PC_2 and PC_3 from wild-type cells.

previous assays. However, exposure to cadmium induces synthesis of additional glutathione in wild-type cells but not in *hmt2*$^-$ cells.

The two strains also differ in rates of glutathione turnover. The ^{35}S-labelled glutathione peak diminishes in wild-type cells after 2 h of cadmium exposure, while ^{35}S-phytochelatins rise steadily (Fig. 6B). In the mutant, glutathione levels decrease only slightly, while phytochelatins increase much more slowly than in the wild type. The slow rate of phytochelatin accumulation in the mutant could be consistent with either slower rates of phytochelatin synthesis, or normal rates of synthesis coupled with higher rates of degradation. If the latter explanation were true, we would expect a pulse of ^{35}S label to enter the phytochelatin pool at equal rates in the mutant and wild-type cells, but to 'chase' out of the pool faster in the mutant than in wild type. In fact, the converse is true: ^{35}S-labelled phytochelatins appear more rapidly in the wild-type cells than in the mutant, and levels in both strains remain elevated, with no evidence of differential degradation rates.

Phytochelatin synthase activity was assayed in extracts from wild-type and mutant cells. Both samples were capable of producing phytochelatins *in vitro* when supplied with excess glutathione and 500 μM cadmium. The reaction failed to occur in the absence of added heavy metal (not shown). The activity appears to be rather low compared with synthesis rates *in vivo*, but is not significantly different between the two strains (wild type: 6.25 nmol phytochelatin (as GSH equivalents)/mg protein $h^{-1} \pm 2.77$ SE; *hmt2*$^-$ mutant: 5.91 nmol phytochelatin/mg protein $h^{-1} \pm 1.39$ SE; $n = 3$). Extracts prepared from cadmium-induced wild-type and mutant cells both displayed *in vitro* phytochelatin synthase activity, although at lower levels (16% and 17% of activity from non-induced cells respectively). As *hmt2*$^-$ cells possess a phytochelatin synthase that appears fully active *in vitro*, the HMT2 protein is probably not directly involved in PC biosynthesis. This leaves us to postulate that the HMT2 protein may be indirectly required for the reaction to occur *in vivo*, either by activating phytochelatin synthase or by removing an inhibitor of the reaction.

Possible mechanism of phytochelatin synthase inhibition

We hypothesized that the high level of sulphide accumulated in *hmt2*$^-$ cells might be responsible for inhibition of phytochelatin synthesis. To test this possibility, phytochelatin synthase activity of wild-type extracts was tested in the presence of increasing levels of sodium sulphide and either 500 μM or 2 mM cadmium. When added sulphide was at least of equal molar concentration to added cadmium, phytochelatin synthase activity was inhibited by over 90% (Fig. 7). To determine the relevance of these *in vitro* data to actual conditions in cells, we measured cadmium and sulphide concentration in metal-exposed cells (Table 1). Wild-type cells accumulated cadmium and sulphide at a ratio of approximately 2:1. In contrast, mutant cells accumulated cadmium and sulphide at a ratio of 1:1 or lower. Based on *in vitro* data, in wild-type cells the moderate sulphide levels might be expected to inhibit phytochelatin synthase by about 50%. In mutant cells, however, the high sulphide levels should almost completely inhibit phytochelatin synthase activity. High sulphide levels therefore may be sufficient to account for the low accumulation of phytochelatins in *hmt2*$^-$ cells.

The 1:1 stoichiometry of cadmium and sulphide required for inhibition suggested that cadmium and sulphide might be directly interacting. Formation of an insoluble CdS complex could prevent formation of glutathione–cadmium thiolate, the physiological substrate of phytochelatin synthase, and thus prevent synthesis of phytochelatins. Metal–sulphide complexes appear to form in *hmt2*$^-$ cells,

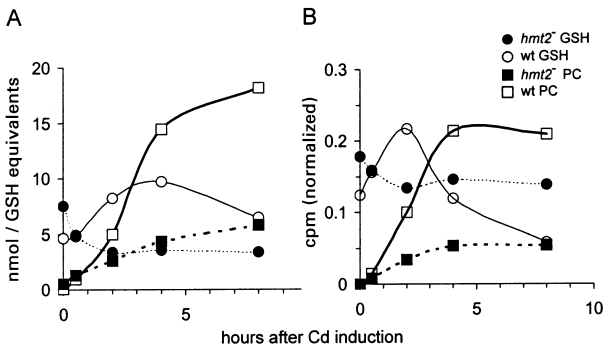

Fig. 6. Time-course of phytochelatin synthesis. Radiolabelled wild-type JS23 and *hmt2*$^-$ JS563 cells were exposed to 200 μM cadmium. At the designated time-points, aliquots were analysed for thiol content using high performance liquid chromatography (HPLC) with DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) detection (A) and flow scintillation counting (B). Values in (B) were normalized to the total acid-soluble radioactivity of the sample at time = 0.

as the colonies turn bright yellow in the presence of cadmium, brown in the presence of copper and grey in the presence of lead or bismuth (not shown). Fluorescence microscopy, using UV illumination, showed that when *hmt2*$^-$ cells are exposed to cadmium for a few hours, a faint, diffuse orange material on the cell surface is seen. This material is not seen with wild-type cells. After longer exposure of *hmt2*$^-$ cells to cadmium, the diffuse staining pattern gives way to discrete, apparently intracellular, bodies that luminesce white, blue, yellow or orange (Fig. 8). Wild-type cells contain a small number of similar-looking, but much fainter, fluorescent bodies. DAPI (4,6-diamidino-2-phenylindole) staining of these samples gives better visualization of the cells, but the cadmium-specific fluorescence patterns could also be seen in the absence of DAPI.

The bodies that are visible in cadmium-exposed *hmt2*$^-$ cells probably consist of or contain cadmium sulphide because of the following: (1) they occur only in the presence of cadmium, (2) they disappear upon treatment with 0.05N HCl that would liberate sulphide, and (3) they are reactive with a silver stain for sulphide. Silver staining also quenches the previously observed fluorescence (data not shown). These objects appear to pellet with the insoluble fraction of a cell-free extract after centrifugation at 15 000 *g*. Fluorescent particles were partially purified from cadmium-exposed *hmt2*$^-$ cells by differential centrifugation in the presence of 1% Triton X-100. This fluorescent material contains cadmium and sulphide in an approximately 1:1.45 ratio. All indications therefore suggest that, in *hmt2*$^-$ cells, cadmium and sulphide interact to form insoluble cadmium–sulphide complexes.

Discussion

A defect in sulphide dehydrogenase activity causes hypersensitivity to Cd^{2+}, Hg^{2+}, Cu^{2+} and Zn^{2+} in *hmt2*$^-$ cells. The spectrum of metal sensitivity suggested the failure of a phytochelatin-mediated tolerance pathway, as Cd^{2+}, Hg^{2+}, Cu^{2+} and Zn^{2+} all induce phytochelatin synthesis (Grill *et al*., 1987). Phytochelatins have been shown to bind these metals (Maitani *et al*., 1996; Mehra *et al*., 1996a, b) and phytochelatin-deficient *Arabidopsis thaliana* mutants are hypersensitive to these metals (Howden and Cobbett, 1992; Howden *et al*., 1995b). Recent studies of *S. pombe* phytochelatin synthase mutants have confirmed the hypersensitivity to cadmium, disagreed on copper, and failed to show hypersensitivity to zinc or mercury (Clemens *et al*., 1999; Ha *et al*., 1999). Differences in concentrations and specific growth conditions tested could potentially account for the reported discrepancies.

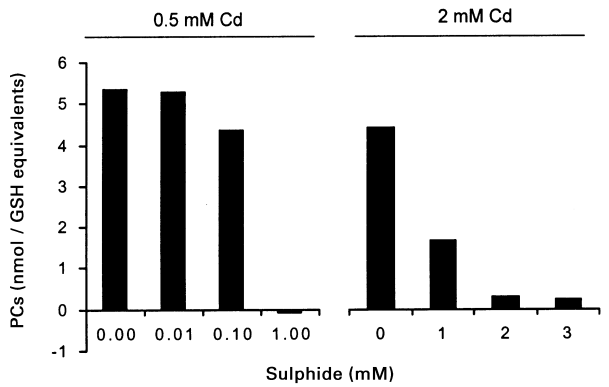

Fig. 7. Phytochelatin synthase activity in the presence of sulphide. Extracts from wild-type cells grown in minimal (SG) media without cadmium were assayed for phytochelatin synthase activity in the presence of various concentrations of Na_2S and 0.5 mM (left) or 2 mM (right) $CdCl_2$. Each column represents net PC synthesis in 4 h by an extract corresponding to 50 ml (left) or 10 ml (right) of cell culture.

Table 1. Cadmium and sulphide content of cadmium-induced cells.

Strain	Trial	mM Cd	mM S^{2-}	Cd:S ratio
JS23	I	3.1	1.3	2.38
JS563	I	1.8	2.0	0.9
JS23	II	2.7	1.6	1.69
JS563	II	3.3	5.7	0.58

Wild-type and *hmt2*$^-$ mutant cells were exposed to cadmium for 5 h, and intracellular cadmium and sulphide were assayed independently. Concentrations were calculated based on the wet-cell pellet volume of 75 ml.

HMT2 deficiency is associated with deficient accumulation of phytochelatins. Our initial hypothesis was that this oxidoreductase, in addition to acting on sulphide, might be capable of reducing glutathione or phytochelatins. Therefore, the absence of this function might result in an accumulation of an oxidized form that would fail to bind metals. However, we found no evidence for perturbation of the redox state of thiol pools in *hmt2*$^-$ cells, and have been unable to demonstrate direct reduction or oxidation of thiols by the HMT2 protein. Instead, pulse–chase labelling experiments suggest an indirect effect that operates at two different levels. First, the *hmt2*$^-$ mutant fails to upregulate production of glutathione when exposed to cadmium. Second, the mutant fails to initiate phytochelatin synthesis, as judged by both a lack of newly made phytochelatin peptides and a lack of glutathione turnover. It is known that phytochelatin synthesis requires metals such as Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}, Zn^{2+} or Pb^{2+} in *S. pombe* (Ha *et al*., 1999). In the *hmt2*$^-$ mutant, high levels of intracellular sulphide appear to precipitate out large amounts of cadmium as cadmium sulphide. Therefore, the free cadmium concentration may be too low to induce additional glutathione synthesis, or to form the cadmium–glutathione thiolate required by phytochelatin synthase.

If the above model is correct and most of the cadmium in mutant cells is present as an insoluble CdS precipitate, then why are the cells sensitive to cadmium? Previously, we had proposed that HMT2 prevents excess sulphide

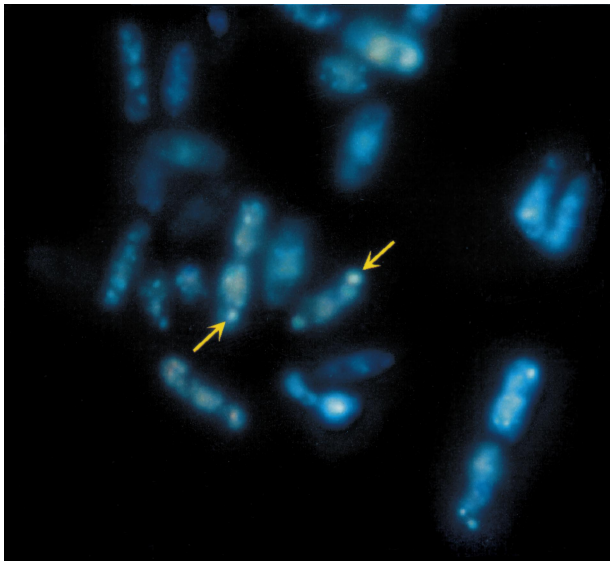

Fig. 8. Fluorescence micrographs of cadmium-exposed cells. The *hmt2*$^-$ mutant JS563 was grown in rich (YG) media with 1 mM cadmium for 24 h. Whole, unfixed cells were counterstained with DAPI (4,6-diamidino-2-phenylindole; 1 μg ml^{-1}), mounted in glycerol containing phenylene diamine (1 mg ml^{-1}) and viewed by fluorescence microscopy with UV excitation. Deep blue staining is by DAPI. Cadmium exposure gave rise to diffuse pale orange fluorescence and to discrete luminescent bodies (arrowheads). This effect is not seen with wild-type cells (not shown).

from inactivating mitochrondrial respiration, as sulphide is a potent inhibitor of cytochrome *c* oxidase. Now we propose an additional explanation, that unregulated intracellular deposition of CdS itself could interfere with cell metabolism. Although wild-type fission yeast cells also form intracellular cadmium sulphide, it is less luminescent. Previous studies of *Candida glabrata* have correlated the luminescence intensity of cadmium sulphide with particle size (Mehra *et al*., 1994). Mutant cadmium sulphide particles may not only be larger but, because of the failure to produce phytochelatins, the complex would also remain unshielded. In wild-type cells, cadmium–sulphide complexes are surrounded by a shell of phytochelatin peptides, and this entire high-molecular-weight complex is enclosed within the vacuole (Vogeli-Lange and Wagner, 1990; Ortiz *et al*., 1992; Mehra *et al*., 1994). Vacuolar sequestration would minimize potential toxic effects of the precipitate on the rest of the cell.

Previous studies have shown that the ability to reduce sulphur is absolutely required for *S. pombe* to achieve cadmium tolerance. A sulphite reductase (Fig. 1) mutant that cannot assimilate the sulphur required to produce cysteine, glutathione and phytochelatins, is cadmium-hypersensitive. The demands for reduced sulphur during cadmium detoxification are such that even supplying exogenous cysteine in the medium fails to restore cadmium tolerance to the mutant (D. Speiser, unpublished observations). Similarly, strains with certain mutations in the purine biosynthesis pathway are unable to increase sulphide production during cadmium stress. These strains fail to accumulate the high-molecular-weight cadmium–sulphide–phytochelatin complex and are also cadmium-hypersensitive (Speiser *et al*., 1992). In the same vein, some *S. pombe* and *C. glabrata* mutants that hyperaccumulate sulphide have been shown to be cadmium-hypertolerant, accumulating large amounts of high-molecular-weight cadmium–sulphide–phytochelatin complex (Mehra *et al*., 1994; D. W. Ow *et al*., unpublished observations).

The present study demonstrates that, although sulphide production is necessary for cadmium tolerance in *S. pombe*, it is not sufficient. In fact, the ability to appropriately modulate sulphide levels, controlled by *hmt2*$^+$, is required in order for normal phytochelatin-mediated metal detoxification to proceed. It remains to be resolved why high sulphide levels apparently preclude phytochelatin synthesis in the *hmt2*$^-$ mutant but not in the cadmium-hypertolerant mutants described above. Little is known about the details of cadmium-induced sulphide production. One might speculate that the cell finely controls the timing, level or location of sulphide production, coupling it tightly to cadmium exposure. If sulphide accumulation were increased only within the vacuole, or only after phytochelatin synthesis was initiated, the sulphide could be used productively to form the high-molecular-weight complex. Early, excessive or inappropriately located sulphide accumulation in the *hmt2*$^-$ strain could lead to abnormal CdS deposits in the cell. This model would indicate that spatial and temporal regulation of sulphur metabolism plays a key role in metal tolerance. Better genetic and biochemical characterization of the Cd-hypertolerant, sulphide-hyperaccumulating mutants, and genetic crosses with other strains defective in sulphur metabolism, including the *hmt2*$^-$ mutant, may help to address this question.

Experimental procedures

Genotypes, plasmids and culture conditions

JS23 (wild type) is *ura4.294, leu1.32, h*$^+$; JS563 is *ura4.294, leu1.32, h*$^+$, *hmt2*$^-$; JV5 is *ura4.294, leu1.32, h*$^-$, *hmt2::URA3* (Vande Weghe and Ow, 1999). pJV26 contains the minimal *hmt2*$^+$ genomic clone in the pART1 (McLeod *et al*., 1987) fission yeast expression vector. Cells were grown at 30°C in YG (2% glucose, 0.5% yeast extract) or in SG (2% glucose, 0.67% yeast nitrogen base without amino acids) supplemented with 100 μg ml^{-1} leucine and/or 20 μg ml^{-1} uracil as appropriate.

Analysis of phytochelatins

Protein content of samples was measured by the Bio-Rad

Protein Assay (Bio-Rad Laboratories). Phytochelatin–Cd complexes were separated by gel filtration chromatography as described (Ortiz *et al*., 1992), and the cadmium content of resulting fractions was measured by atomic absorption spectroscopy (Model 3110 atomic absorption spectrometer, Perkin Elmer). For high performance liquid chromatography (HPLC) analysis of phytochelatins, cell pellets resuspended in 100 μl of distilled water were vortexed with glass beads, then centrifuged at 15 000 *g*, 4°C for 2 min. An equal volume of ice-cold 10% 5-sulphosalicylic acid was added. After a 5 min incubation on ice, centrifugation was repeated and supernatants filtered through a 0.2 μm syringe filter. Non-radioactive samples contained 200 μg of protein prior to deproteinization; radioactive samples corresponded to 4 ml of the radiolabelled cultures. Samples (50 μl) were injected onto a Betasil Basic-18 HPLC column (Keystone Scientific) equilibrated in 5% acetonitrile/95% (0.05% trifluoroacetic acid in water), and eluted by a linear gradient to 12.5% acetonitrile/87.5% (0.05% trifluoroacetic acid in water) over 20 min, at a rate of 1 ml min^{-1}. DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) (0.7 mg ml^{-1}) in 0.3 M KPO_4 pH 7.8/7.5 mM EDTA was mixed with post-column effluent at a rate of 1 ml min^{-1} (for non-radioactive samples) or 0.1 ml min^{-1} (for radioactive samples), and absorption of the derivatized effluent was monitored at 405 nm. For radioactive samples, effluent was then mixed with an equal amount of Ultima-Flo M scintillation cocktail (Packard Instrument), and radioactive peaks were monitored by flow scintillation counting. Purified GSH, $(\gamma\text{-EC})_2G$, $(\gamma\text{-EC})_3G$, and sulphide were used as standards for the identification of peaks; purified GSH was used for quantification of peaks.

For reduction of thiols with $NaBH_4$, cell-free extract corresponding to 2 mg of protein was adjusted to 1 M HCl and centrifuged to remove precipitated protein. The supernatant was adjusted to neutral pH with NaOH and brought to 10 mM Tris-Cl pH 7.0. $NaBH_4$ was added to 0.3 M and extracts were incubated at 37°C for 30 min. 5-sulphosalicylic acid was then added to 5% and extracts were analysed using HPLC.

For *in vivo* ^{35}S labelling, 200 ml YG cultures at $A_{550} = 0.25$ were centrifuged at 4000 *g* for 5 min. Pellets were washed once in 1x phosphate-buffered saline (PBS) and incubated for 20 min in 4 ml of low-sulphur medium (MM medium (Alfa *et al*., 1993) minus sodium sulphate). After adding 80 μCi of $Na_2^{35}SO_4$ for 15 min, cells were harvested and washed once with 1x PBS, and a time = 0 sample was collected. The remaining cells were diluted into 200 ml of YG containing 200 μM cadmium and, at various time-points, aliquots were spun down and frozen in liquid nitrogen for subsequent HPLC analysis.

Enzyme assays

For the glutathione reductase recycling assay, cells grown to saturation in SG were harvested at 4000 r.p.m., 4°C and washed once with 1x PBS, then resuspended in 1x PBS to a total volume of approximately 0.8 ml. Cells were frozen in liquid nitrogen and ground with 1.2 g of alumina in a mortar and pestle, then resuspended in 500 μl of 10 mM $NaPO_4$, pH 7.0/1 mM EDTA. A 10 μl aliquot was reserved for protein quantitation, and the remainder was deproteinized with 2 ml of 3% 5-sulphosalicylic acid. After a 5 min incubation on ice and a 5 min centrifugation at 15 000 *g*, aliquots of the supernatant were adjusted to pH 7.0 with NaOH and assayed for glutathione as described (Akerboom and Sies, 1981).

Phytochelatin synthase assays were performed essentially as described in Howden *et al*. (1995b). A typical assay contained 4 mg of protein extract from non-induced cells, 0.2 M Tris pH 8, 10 mM beta-mercaptoethanol, 3.3 mM glutathione and 500 μM $CdCl_2$. The reaction mixture was incubated at 30°C, and aliquots were removed at various time-points for analysis of phytochelatin production by HPLC.

Measurement of sulphide and cadmium

YG-grown cells exposed to 200 μM cadmium for 5 h were harvested, washed once in 1x PBS, resuspended in 10 mM Tris-Cl pH 8.0, and broken by vortexing with glass beads. Extract amounts were normalized by total cell number, cell pellet size and soluble protein content; all three methods yielded the same conclusion. The extract was divided in half, and the cadmium concentration of one half was measured by atomic absorption spectroscopy; the sulphide concentration of the other half was determined using the methylene blue assay as described in Speiser *et al*. (1992), after deproteinization with 5% 5-sulphosalicylic acid and centrifugation.

The silver stain for sulphide is a modification of the procedure described in Pearse (1972). Cells were incubated briefly in 3% $AgNO_3$, washed once with water, mounted in a mixture of 0.0185% formaldehyde/0.005% citric acid, and examined under UV and visible light.

Fluorescent particles were prepared from JS563 cells grown to saturation in YG and induced with 800 μM $CdCl_2$ for 48 h. Cells were washed once in distilled water and lysed by vortexing with glass beads, followed by 2 min of sonication. The cell lysate was subjected to three rounds of differential centrifugation, in which the fraction that pelleted between 1000 *g* and 15 000 *g* was collected. For the last round of centrifugation, Triton X-100 was added to a final concentration of 1%. The final 15 000 *g* pellet was resuspended in distilled water and analysed for cadmium and sulphide content.

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