HEAVY METAL TOLERANCE GENES

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ABSTRACT

Toxic metals released into the environment present an unusual challenge to the ecosystem. Practically indestructible, even low level deposition will accumulate with time. Plant uptake of these elements offers a potential solution for bioremediation, with prospects of engineering nonfood plants to harvest metals from contaminated sources. As plant uptake of heavy metals leads to dietary intake, elucidating the plant mechanisms that govern metal storage might also help partition toxic metals to nonconsumable plant parts. Both of these goals require fundamental knowledge of the molecular mechanisms in the uptake and storage of metals. Since the fission yeast *Schizosaccharomyces pombe* uses the same metal chelators as do higher plants, it can serve as a model organism for elucidating mechanisms for cellular tolerance. Target genes identified in *S. pombe* provide opportunities for engineered expression in a heterologous host or the search for homologous genes in plants.

INTRODUCTION

Plants offer the potential to retrieve metals from contaminated sources. This phytoextraction process, however, requires plants that produce high biomass, grow rapidly and possess high-capacity uptake for the inorganic substrate. Existing hyperaccumulators are generally small slow growing plants that need to be bred for enhanced growth and biomass. Alternatively, hyperaccumulation traits could be engineered into fast growing, high biomass plants. The latter approach requires fundamental knowledge of the molecular mechanisms in metal uptake and storage; and appropriate model systems could expedite basic knowledge in this area.

The fission yeast *Schizosaccharomyces pombe* shares with plants the same cellular response to metal stress. When exposed to certain heavy metals, they induced the production of small metal-binding peptides known as phytochelatins (PCs). PCs are synthesized from glutathione (GSH) by a metal-activated enzyme known as PC synthase [Fig. 1]. The peptides have the general structure of $(\gamma\text{-Glu-Cys})_n$Gly, commonly with 2 to 5 (γ-Glu-Cys) units. Two PC-metal complexes can be isolated from cells exposed to Cd: a low molecular weight (LMW) PC-Cd complex and a more stable high molecular weight (HMW) PC-CdS crystallite complex that contains acid labile sulfide. The addition of sulfide to the LMW complex reduces the approximate Cys:Cd ratio from 4:1 to a more economical 2:1 in the HMW complex.

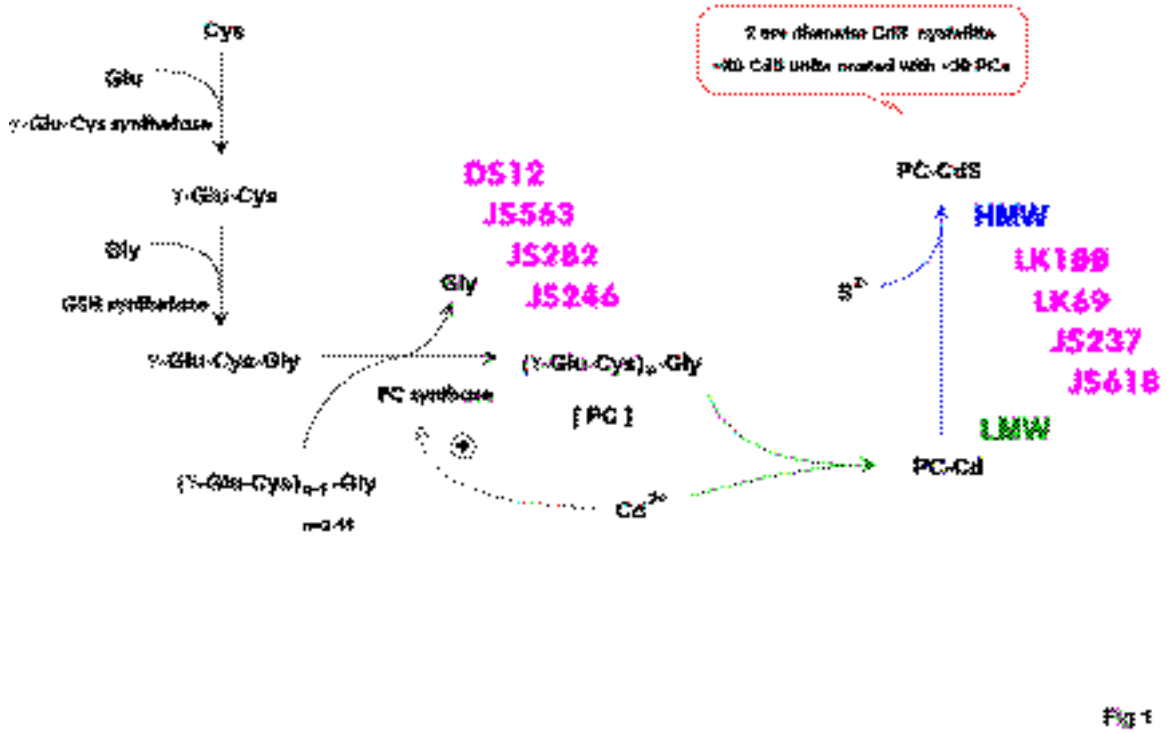

Fig 1

Using *S. pombe* as a cellular model system, we have taken two complementary strategies to identify metal tolerance genes:

STRATEGY I. Complementation of Cd-hypersensitive mutants. The first approach is based on the isolation of DNA that restores tolerance to Cd-hypersensitive mutants. Mutants hypersensitive to Cd were isolated from mutagenesized cultures and further screened for deficient accumulation of one or both of the PC-Cd complexes. Fig. 1 lists 8 Cd-hypersensitive mutants that accumulates deficient amounts of the HMW complex (LK100, LK69, JS237, JS618) or both LMW and HMW complexes (DS12, JS563, JS282, JS246). Current findings are summarized below, including for background purposes, a brief review of published results.

1. PC-Cd transporter. From the analysis of mutant LK100, we discovered a Cd tolerance gene encoding a vacuolar membrane protein. This gene product, which we named HMT1, is a member of the family of ATP

binding cassette (ABC) type transporters.[1] HMT1 transports the cytoplasmic PC-Cd complex into the vacuole[2]. Overproduction of HMT1 enhances Cd accumulation and Cd tolerance. In addition to and independent of the PC-Cd transport activity by HMT1, we also found vacuolar transport activity for Cd and for glutathione-conjugates. The Cd transport activity provides additional Cd ions for the PC-CdS complex [Fig. 2].

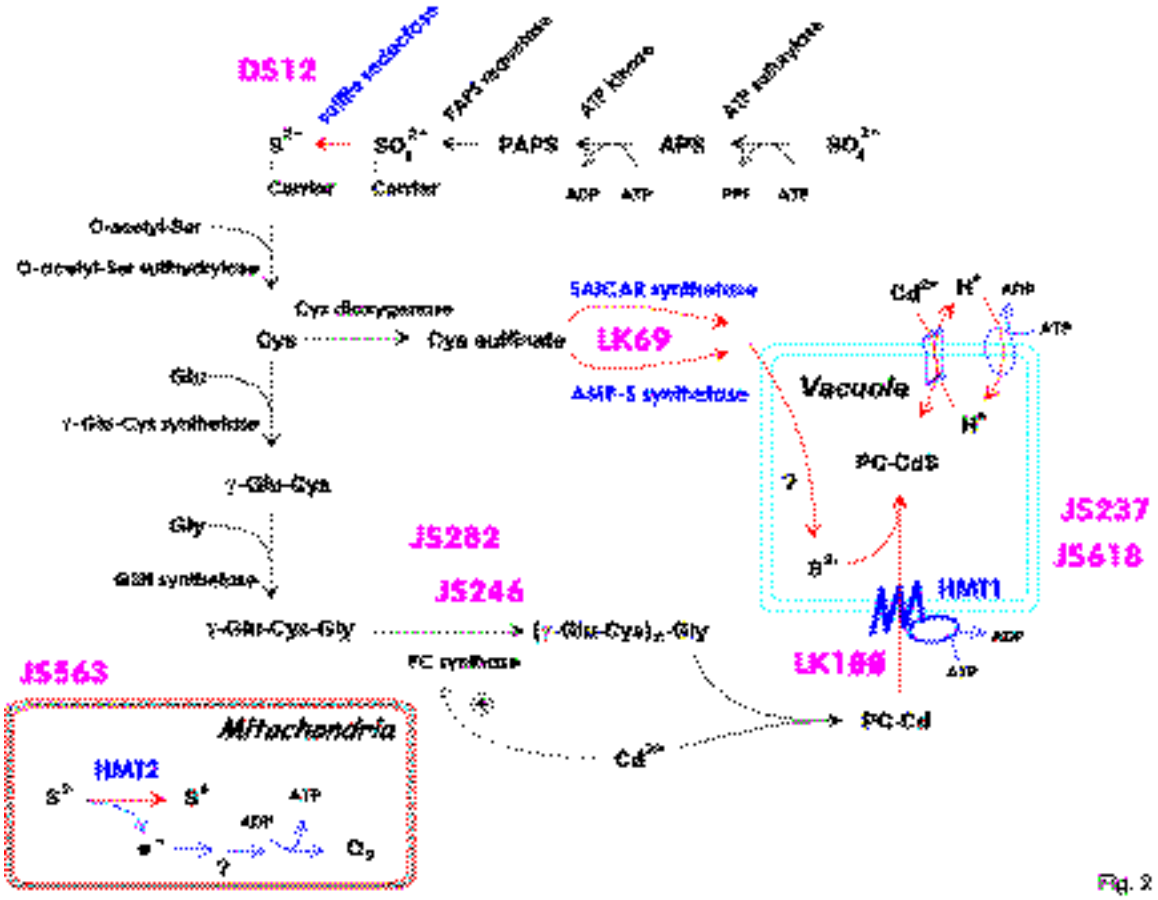

Fig. 2

2. Purine biosynthesis enzymes in sulfide complex formation. Through analysis of mutant LK69, we found that a likely source of the S^{2-} in the HMW PC-CdS complex is cysteine sulfinate, a sulfur analog of aspartate

[1] Ortiz, D.F., Kreppel, L., Speiser, D.M., Scheel, G., McDonald, G., Ow, D.W. 1992. Heavy metal tolerance in the fission yeast requires an ATP binding cassette-type vacuolar membrane transporter. *EMBO J.* 11: 3491-3499.

[2] Ortiz, D.F., Ruscitti, T., McCue, K., Ow, D.W. 1995. Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. *J Biol Chem* 270: 4721-4728.

derived from cysteine oxidation [Fig. 2]. Genetic data suggest that cysteine sulfinate is incorporated into purine intermediates by two enzymes of the purine biosynthetic pathway, AMP-S synthetase and SAICAR synthetase[3] [Fig. 3]. Biochemical studies support this hypothesis. *In vitro*, these enzymes can utilize cysteine sulfinate in place of aspartate[4]. The cysteine sulfinate-derived purine intermediates are probably carriers of another sulfur compound, which then leads to formation of sulfide for the PC-CdS complex.

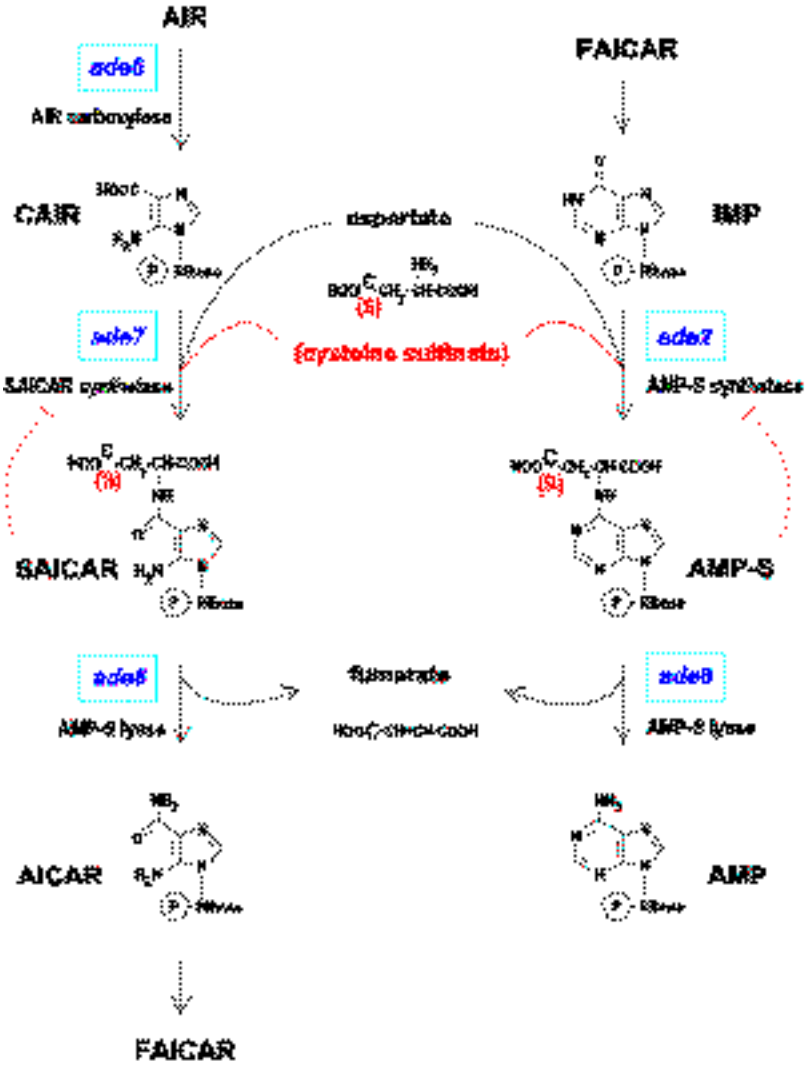

Fig. 3

[3]Speiser, D.M., Ortiz, D.F., Kreppel, L., Scheel, G., McDonald, G., Ow, D.W. 1992. Purine biosynthetic genes are required for cadmium tolerance in *Schizosaccharomyces pombe*. *Mol Cellu Biol* 12: 5301-5310.

[4] Juang, R.-H., McCue, K.F., Ow, D.W. 1993. Two purine biosynthetic enzymes that are required for cadmium tolerance in *Schizosaccharomyces pombe* utilize cysteine sulfinate *in vitro*. *Arch Biochem Biophy* 304: 392-401.

3. Cd-induced sulfide production independent of assimilatory sulfate reduction. The analysis of DS12 revealed a leaky mutation in the sulfite reductase gene [Fig. 1]. This leaky mutation provides adequate cysteine for normal growth, but insufficient cysteine for Cd-induced production of PCs. The cloning of this gene led to the use of the cloned DNA to create a disruption in the sulfite-reductase gene. As expected, when sulfite-reductase function, a key step of the assimilatory sulfate reduction pathway, was abolished, the cell required cysteine supplementation. Under these conditions, it was found that Cd-induced sulfide production was nonetheless operational [Fig. 4]. This indicates that Cd-induced sulfide production is independent of the assimilatory sulfate reduction pathway, and is consistent with the hypothesis that S^{2-} for the HMW complex is channeled through a novel pathway. In contrast to Cd, however, Pb-induced sulfide production was found to require sulfite reductase and the assimilatory sulfate reduction pathway[5] [Fig. 4].

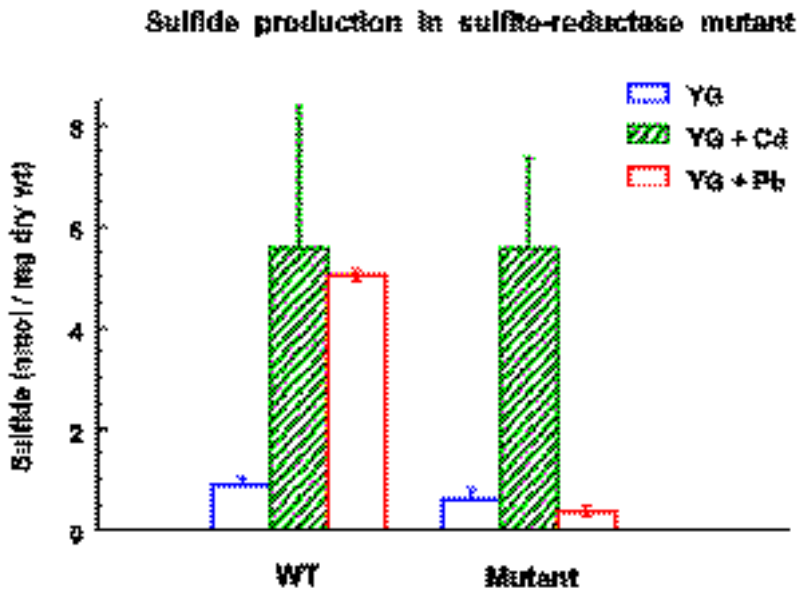

Fig. 4

[5] Ow, D.W. Heavy metal tolerance genes: prospective tools for bioremediation. 1996. *Resources, Conservation and Recycle* 18: 135-149.

4. Sulfide-mediated cisplatin resistance in S. pombe. Cisplatin is one of the most widely used cancer drugs. As with many chemotherapeutic agents, acquired resistance can arise with prolonged use. In a collaborative study, we found that while glutathione and PC levels showed no correlation to cisplatin resistance, the sulfide level is an important determinant[6]. This draws attention to the possibility of sulfide-cisplatin interactions in human cancer cells.

CURRENT FOCUS

5. A mitochondrial gene required for PC production. Mutant JS563 was isolated initially as hypersensitive to Cd with deficient accumulation of PC peptides [Fig. 1]. Further analysis revealed that the mutant is also hypersensitive to Hg^{2+}, heat shock, hydrogen peroxide, and the thiol-oxidizing agent diamide. In the presence of Cd, it forms yellow colonies, suggesting the formation of CdS particles. Indeed the mutant was found to hyperproduce sulfide. The genomic and cDNA clones deduced a 52 kDa protein with an isoelectric point of 9.5 to 10. The sequence of the mutant allele revealed a G to A transition that converted aa 396 from Glu to Lys. The protein, which we have named HMT2, has sequence similarity to flavocytochrome c from *Chromatium vinosum* and sulfide quinone reductase from *Rhodobacter capsulatus*, both of which oxidize sulfide. Indeed, in vitro studies show that HMT2 can bind FAD and catalyze the reaction (Q_2 is a water soluble ubiquinone analog).

$$S^{2-} + \text{coenzyme } Q_{2(oxid)} \rightarrow S^0_{(oxid)} + \text{coenzyme } Q_{2(red)} \quad [K_m = 2 \text{ mM for } S^{2-}]$$

Localization studies showed that HMT2 resides in the mitochondria, loosely associated with mitochondria membranes. It is not yet clear how a mitochondria sulfide:quinone oxidoreductase is involved in PC accumulation. One hypothesis is that during Cd stress, high sulfide levels

[6]Perego, P., VandeWeghe, J., Ow, D.W., Howell, S.B. 1997. The role of determinants of cadmium sensitivity in the tolerance of *Schizosaccharomyces pombe* to cisplatin. *Molecular Pharmacology* 51: 12-18.

accumulate, some of which end up in the mitochondria. HMT2's role could be to oxidize mitochondria sulfide to protect it from poisoning the electron transport chain. It is also possible that the S^{2-} electrons are routed to the electron transport chain [Fig. 2].

6. WASP and an adjacent P-type ATPase Transporter. Mutant JS237 was isolated initially as a Cd hypersensitive mutant that lacks the HMW PC-CdS complex [Fig. 1]. Further analysis revealed that the mutant is also defective in mating, and in tolerance to Ca^{2+}, heat shock, and nutrient stress. Cultures of JS237 in liquid minimal medium reach stationary phase at about half the density of wild type strain. A 7 kb clone was isolated that complements the mating defect but only partially complements the stress defects. This 7 kb genomic fragment contains the sequence for three genes oriented in a head-to-tail fashion. Reading from 5'-to-3', these genes encode a P-type ATPase of the APL/cation transporter family (tentatively named HMT3), an actin-regulatingprotein belongingto the Wiskott-Aldrich Syndrome Protein (WASP) family and a putative transcription/translocation factor of the BTF3/βNAC family [Fig. 5]. WASP, with 575 aa (59.7kD, pI 9.14) is the only gene that is contained intact within this 7 kb clone. The BTF3/βNAC gene is not intact, with only the leader sequence and the first few amino acids represented. The transporter gene lacks about 1 kb from its 5'-end that includes coding sequence. Interestingly, the WASP mRNA has two variants that differ depending on alternate splicing of the fourth intron.

When the mutant allele was sequenced, it was found to harbor a mutation in the splice donor sequence of the first intron of the WASP gene (indicated by * in Fig. 5). RTPCR experiments show that this mutation blocks excision of the intron. Since the unspliced message allows translation of only the first 75 amino acids of WASP, the mutation abolishes WASP function. Consistent with this, JS237 has a severely disrupted actin cytoskeleton, a defect that is exacerbated by stress. GFP-tagging of full-length fission yeast WASP confirms that this protein strictly

colocalizes with cortical actin in living cells. In addition, a two-hybrid experiment using WASP as bait resulted in the isolation of two clones encoding actin-associatedproteins, rvs167p and verprolin, and a third clone encoding a possible signal-transducing adaptor protein, STAM. Transformation of JS237 with a wild-type sequence containing the intact WASP gene restores a wild-type cortical actin phenotype and mating competence, but does not fully restore cadmium-tolerance.

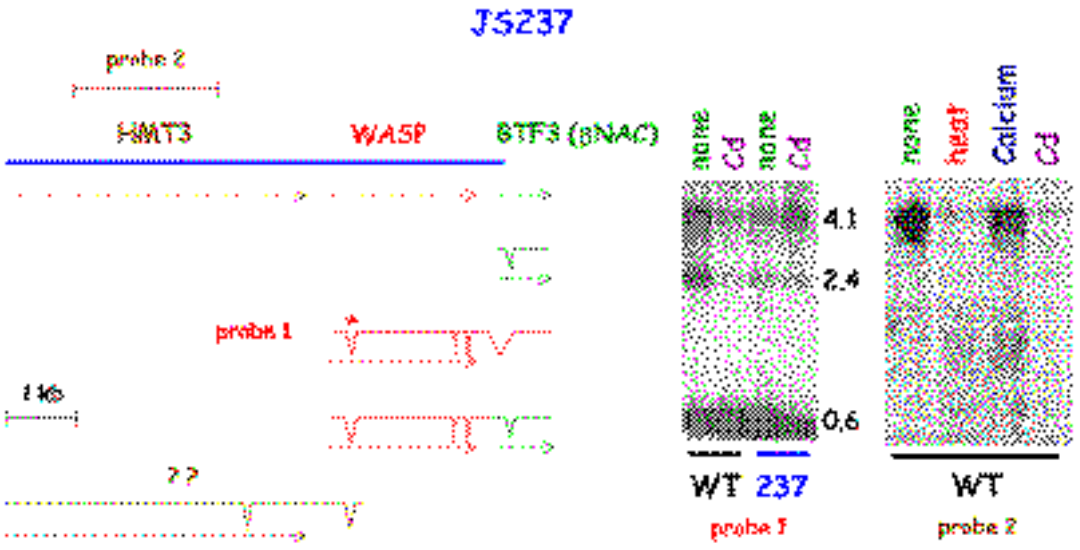

Fig. 6

Our current hypothesis is that the single mutation in JS237 has impaired the expression of both WASP and the P-type transporter. This is consistent with the Northern blot data. As shown in Fig. 5, the WASP cDNA (probe 1) detects three transcripts: the expected 2.4 kb WASP and the 0.6 kb BTF3 transcripts, and unexpectedly, a 4.1 kb message. Only this 4.1 kb message is detected by DNA corresponding to the P-type transporter gene (probe 2). This indicates that the 2.4 kb and the 4.1 kb transcripts overlap. It appears that the single splicing defect has affected the accumulation of the 4.1 kb transcript in addition to the translation of WASP. The Northern data show that the 4.1 kb transcript is misregulated

in the mutant, with higher, as opposed to lower, accumulation during Cd stress. In this model, the mating and growth defects are ascribed to the loss of WASP function, while stress sensitivities, including those to Ca^{2+} and Cd^{2+}, are ascribed to the altered regulation of the transporter transcript. For the time being, we have tentatively named the P-type transporter HMT3. Fig. 6 lists representative P-type transporters with sequence similarity to HMT3. As many of them are involved in ion transport, it is tempting to speculate on a similar role for HMT3. Further experiments are underway to investigate these leads.

HMT3 : Similarity to P-type Transport ATPases

Protein	Organism	BLAST Score	Function
APL ATPase (Bnt2)	S. cerevisiae	5.6 e-208	Spermidine, ethanolamine sensitivity
APL ATPase 27 (Nat1)	S. cerevisiae	6.7 e-206	nourseothricin resistance, mechanism ?
Ca^{2+} ATPase (SMCA4)	Human	7.3 e-58	Plasma membrane, Ca^{2+} pump
Ca^{2+} ATPase (ETA3)	S. pombe	1.5 e-55	Ca^{2+} efflux pump
Cation ATPase (PAT1)	Oryzias latipes	1.2 e-53	Ca^{2+} vacuolar membrane pump
Mg^{2+} ATPase	E. coli	5.0 e-52	Mg^{2+} efflux pump
K+ ATPase (Pma1)	S. cerevisiae	1.2 e-50	plasma membrane K^+ efflux pump
K+ ATPase (gastric)	Human	1.4 e-04	Acid secretion to stomach
Cu^{2+} ATPase (Ccc1)	S. cerevisiae	2.9 e-09	Vacuolar Cu^{2+} ATPase
Na+ ATPase (Ena1)	S. cerevisiae	2.5 e-04	Ca^{2+} pump, protein elimination

Fig. 6

7. A mannose-1-phosphate guanyltransferase-like protein. Mutant JS618 shows deficient accumulation of the HMW complex [Fig. 1]. The DNA fragment that restored tolerance to this mutant encodes a protein with sequence similarity to mannose-1-phosphate guanyltransferase, an enzyme that converts mannose-1-phosphate to GDP-mannose. GDP-mannose is a substrate for glycosyltransferases in protein glycosylation and polysaccharide biosynthesis. Our current thinking is that the mutation abolishes the glycosylation of a protein needed for formation of the HMW

complex. However, we could not link this mutation to an effect on HMT1. Its glycosylation targets remain to be determined.

8. DNA fragment that confer hypersensitivity to Cd and Se. Mutant JS282 hypoproduces phytochelatins [Fig. 1]. A 7 kb genomic DNA fragment complements the Cd hypersensitive phenotype of the mutant. However, the same genomic clone causes hypersensitivity to cadmium or selenium in wild type strains. This effect is not seen with zinc, arsenite, lead, cobalt, chromium or manganese. Presumably this is due to overexpression of a gene when present on a high copy plasmid. Two cDNAs have been isolated and sequenced. A 0.75 kb cDNA is probably not full length. It shows 70% sequence similarity with the N-terminal region of a *S. cerevisiae* protein (Adh4) which is suspected to localize to a membrane, but otherwise has no known function. Expression of this cDNA does not confer hypersensitivity to Cd or Se in wild type strains. The second cDNA is 1.8 kb, and lacks sequence similarity to proteins in the data base. This 1.8 kb cDNA could confer Cd and Se hypersensitivity to wild type cells.

9. DNA fragment that confers Cd- hypertolerance to wild type strains. Mutant JS246 also hypoproduces phytochelatins [Fig. 1]: A genomic clone that restores Cd tolerance has been isolated. Interestingly, wild type strains harboring this genomic clone on a multicopy plasmid become hypertolerant to cadmium. Three cDNAs corresponding to this genomic clone have been sequenced. cDNA#1 is about 1.3 kb and shows sequence similarity with the superoxide-inducible protein from *Bacillus subtilis*, the singlet oxygen resistance protein from the plant pathogen *Cerospora nicotiannae*, and the ethylene-inducible gene from the rubber tree *Hevea brasiliensis*. Surprisingly, when introduced into wild type cells, the cDNA expressed from a plasmid promoter confers cadmium hypersensitivity instead of hypertolerance. cDNA#2 is about 1 kb and shows >60% homology to a *S. cerevisiae* gene of unknown function. cDNA#3 is only 0.4 kb and does not show similarity to genes in the current data base. It is interesting to

note that both mutants JS282 and JS246 have led us to a cDNA that could confer Cd-hypersensitivity to wild type cells. One possibility is that these stress-related proteins must operate at a proper concentration. When their accumulation is either too low, such as due to a mutation, or too high, such as due to plasmid-mediated overproduction, normal tolerance to Cd is adversely affected.

STRATEGY II. Selection for cDNAs that confer Cd hypertolerance. The second approach for identifying genes involved in metal tolerance is based on selecting cDNAs for an ability to impart a Cd-hypertolerant phenotype to the resident host. A cDNA expression library was prepared from *S. pombe* and from *Brassica juncea* (Indian Mustard), a metal accumulating plant. The cDNAs were inserted into a fission yeast vector such that the cDNA would be transcribed from a plasmid promoter.

S. pombe clones. From the *S. pombe* cDNA library, some 40 clones were initially isolated from the wild type strain after selection on high Cd medium. Preliminary sequencing and retransformation showed that 23 unique clones can consistently confer Cd hypertolerance. Based on sequence similarity to the partial 5' sequence of the clones, identity has been assigned to many of them. In addition, many of these show cross resistance to the oxidative stress agents tetra butyl H_2O_2 and diamide. Of particular note is a cDNA that encodes a metallothionein (MT) protein. It is not clear what the native role of this metallothionein might be, as metals induce PC-metal and not MT-metal complex formation. Nonetheless, this MT can function in Cd and Cu resistance when overproduced through artificial means. Other genes encode enzymes in glutathione biosynthesis and proteins that are involved in calcium binding or oxidative stress.

Current efforts are underway to decipher the metal related functions of these proteins. One example is the study of PAP1 (pombe Ap1), which in *S. cerevisiae* is described as a transcription factor for up-regulation of the genes encoding γ-Glu-Cys synthetase and the GSH-conjugate (GSX) pump. The *pap1*$^+$ cDNA indeed complements a *S. pombe pap1*$^-$ mutant, which is hypersensitive to Cd. As in *S. cerevisiae*, the *S. pombe* PAP1 homologue is likely responsible for high-level synthesis of GSH and the GSX pump. We entertained whether this might extend to regulation of HMT1. The *pap1*$^-$ deficiency can be overcome by *hmt1*$^+$ overexpression, but the *hmt1*$^-$ deficiency cannot be compensated by *pap1*$^+$ overexpression. Although this is consistent with *hmt1*$^+$ acting downstream of *pap1*$^+$,

whether this is indeed an effect of epistasis or merely of suppression of independent pathways remains to be determined.

Brassica juncea metallothioneins confer Cd hypertolerance to fission yeast. A cDNA library made from the mRNA of *Brassica juncea* was also introduced into fission yeast. Several Cd-hypertolerantclones were isolated. Three cDNAs were found which encoded distinct members of the class II metallothionein (MT) family. A smaller cDNA (369 bp) encodes a class II type 1 MT (45 amino acids) and two larger cDNAs (492 and 535 bp) encode a class II type 2 MTs (80 amino acids each).

CONCLUDING REMARKS

Completion of the Arabidopsis genome sequence is expected within the next five years, and the rice genome sequence will be another decade behind. The availability of the genome information affords opportunities to engineer expression of target genes and alter the intrinsic characteristics of a plant. The goal to engineer plants for bioremediation will benefit from genome data only if functions can be ascribed to the open reading frames. It is expected that the function of a large percentage of the genes will not be known. As tolerance mechanisms are shared between plants and *S. pombe* at the cellular level, a fission yeast model system serves to expedite the discovery of gene identity and function. Metal tolerance genes found in *S. pombe* provide insight and opportunities to clone and engineer the homologous genes of higher plants.