A survey of all 11 ABC transporters in fission yeast: two novel ABC transporters are required for red pigment accumulation in a *Schizosaccharomyces pombe* adenine biosynthetic mutant

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ATP-binding cassette (ABC) proteins transport a wide variety of substrates, including sugars, amino acids, metal ions, lipids, peptides and proteins, across membranes, and most ABC proteins contain transmembrane domains (ABC transporters). Sequencing of the *Schizosaccharomyces pombe* genome has allowed identification of all genes encoding ABC transporters in fission yeast. To date, six such genes have been characterized, and an additional five genes encoding ABC transporters were identified from the genome sequence. In an attempt to characterize all of the ABC transporters in fission yeast, all 11 genes were disrupted. While all the genes were found to be dispensable for cell viability, some disruptants lacked apparent phenotypes. GFP-tagged ABC transporters were localized to membranes as follows: plasma membrane (2), vacuolar membrane (4), mitochondrial membrane (2), endoplasmic reticulum membrane (2), and endosome and Golgi membranes (1). Two Cluster II. 1 proteins, Abc2p (SPAC3F10.11c) and Abc4p (SPAC30.04c), were found to be localized to vacuolar membranes, and to be responsible for accumulation of a characteristic red pigment in the vacuole of an adenine biosynthetic mutant. The doubly disrupted mutant *abc2Δ abc4Δ* exhibited drug sensitivity, and a decreased accumulation of monochlorobimane, suggesting that both of the proteins encoded by these genes are involved in detoxification of xenobiotics, and vacuolar sequestration of glutathione S-conjugates.

INTRODUCTION

The ATP-binding cassette (ABC) protein superfamily is the largest protein family in all organisms for which genomic sequences are known (Henikoff *et al.*, 1997). Most, but not all family members, are membrane proteins (ABC transporters) active in the transport of a broad range of compounds, including xenobiotics, organic anions and cations, and conjugates of glutathione and glucuronic acid (Bauer *et al.*, 1999). All ABC transporters share a similar molecular architecture that includes at least one nucleotide-binding domain (NBD), and several predicted integral membrane domains (transmembrane domains, TMDs). While the TMDs form the transmembrane channel, and are thought

to contain the substrate-binding sites, the NBDs are the molecular motors that transform the chemical energy of ATP into protein conformational changes (Jones & George, 2004). While the NBDs and TMDs are normally arranged in duplicated forward (TMD-NBD)₂ or reverse (NBD-TMD)₂ configurations, half-size transporters have various topologies. The complete core structures for half-size transporters can be homo- or heterodimers.

Thirty-one ABC transporter genes are known in humans, and 55 in *Caenorhabditis elegans* (Dean *et al.*, 2001a, b; Sheps *et al.*, 2004). Animal ABC proteins are classified into eight subfamilies: ABCA–ABCH (Dean *et al.*, 2001a, b). ABCA has no homologues in yeast, and ABCE and ABCF have no TMDs. ABCH proteins are not found in humans, but are found in *Drosophila* and budding yeast. The ABCH protein YDR061w in yeast possesses no TMDs (Dean *et al.*, 2001b). *Arabidopsis* has over 100 ABC transporter genes (Sánchez-Fernández *et al.*, 2001). Fewer ABC transporters are found in unicellular eukaryotes. *Saccharomyces cerevisiae* has 29 ABC proteins, and 23 of these have TMDs (Bauer *et al.*, 1999;

Abbreviations: ABC, ATP-binding cassette; BfA, brefeldin A; CHX, cycloheximide; ER, endoplasmic reticulum; GS-X, glutathione S-conjugate; GS-X pump, glutathione S-conjugate transporting ATPase; MCIB, monochlorobimane; NBD, nucleotide-binding domain; 4-NQO, 4-nitroquinoline oxide; TMD, transmembrane domain; V-ATPase, V-type ATPase.

Decottignies & Goffeau, 1997). *Candida albicans* has 21 ABC transporters (Gaur *et al.*, 2005).

In *Sac. cerevisiae*, ABC proteins are classified into six families corresponding to clusters of amino acid sequence similarity (Decottignies & Goffeau, 1997). Cluster I is equivalent to the mammalian ABCG family. Most Cluster I proteins have (NBD-TMD)₂ configurations, and are divided into three subfamilies. Cluster I. 1 has eight members, including Pdr5p and Snq2p, which confer multidrug resistance (Decottignies & Goffeau, 1997; Bauer *et al.*, 1999), while Cluster I. 2 has one member, YOL075C. Members of Cluster I. 1 and 2 are full-size molecules. Cluster I. 3 has one member, Adp1p, with an unknown function, but with a unique TMD-NBD-TMD topology.

Cluster II members comprise three subfamilies consisting of full-size molecules with a forward orientation, (TMD-NBD)₂. Cluster II. 1 is equivalent to the mammalian ABCC family, while the ABCB family includes Cluster II. 2 and 3. Cluster II. 1 contains Ycf1p, Bpt1p and Ybt1p/Bat1p, which have been shown to transport glutathione conjugates, bile acid and the characteristic red pigment (ade-pigment) of mutants affected in the adenine biosynthetic pathway (Chaudhuri *et al.*, 1996; Li *et al.*, 1996; Sharma *et al.*, 2002, 2003; Klein *et al.*, 2002; Ortiz *et al.*, 1997). Another Cluster II. 1 member, Yor1p, is known to provide resistance to oligomycin and other compounds (Katzmann *et al.*, 1995; Cui *et al.*, 1996; Decottignies *et al.*, 1998). Cluster II. 2 has one member, Ste6p, which exports the α -factor pheromone (Kuchler *et al.*, 1989). Cluster II. 3 proteins are half-size ABC transporters, and three members have been found in *Sac. cerevisiae*: Atm1p, Mdl1p and Mdl2p. Atm1p functions in the export of Fe-S clusters from mitochondria (Leighton & Schatz, 1995; Kispal *et al.*, 1997). Mdl1p localizes to mitochondria, mediates peptide transport from mitochondria, and has a known homologue, Mdl2p (Young *et al.*, 2001; Dean *et al.*, 1994).

Cluster III proteins are also half-size ABC transporters equivalent to mammalian ABCD family members. Two members of this family, Pxa1 and Pxa2, play roles in the transport of long-chain fatty acids into peroxisomes (Shani *et al.*, 1995; Shani & Valle, 1996). Cluster IV and VI members have no TMDs. Cluster V consists of an RNase L inhibitor homologue Rli1p, which was predicted to have TMDs (Decottignies & Goffeau, 1997), but has since been found to localize to the cytoplasm and nucleus, and to function in translation initiation and ribosome biogenesis (Dong *et al.*, 2004).

Prior to the present study, six ABC transporters of *Schizosaccharomyces pombe* had been previously identified. Bfr1p confers brefeldin A (BfA) resistance (Nagao *et al.*, 1995; Turi & Rose, 1995). Mam1p is known to function as a pheromone M-factor transporter (Christensen *et al.*, 1997a), and Pmd1p is a multidrug efflux transporter that recognizes leptomycin B (Nishi *et al.*, 1992). The sequence and expression pattern of *abc1*⁺ has been analysed, but its

precise role remains to be defined (Christensen *et al.*, 1997b). A half-size molecule, Atm1p, has a role in mitochondrial function (Chen & Cowan, 2003; Iwaki *et al.*, 2005). Hmt1p is the best-characterized ABC transporter in fission yeast to date, and is required for cadmium tolerance, and sequestration of phytochelatin-Cd²⁺ complexes in vacuoles (Ortiz *et al.*, 1995, 1992). Although phytochelatin belongs to the family of glutathione-related peptides having the structure (γ -Glu-Cys)*n*-Gly (where *n* is $\sim$ 2–11) (Cobbett, 2000), it has been shown that Hmt1p does not catalyse glutathione (GS) transport (Ortiz *et al.*, 1995).

Identification of ABC transporter genes in fission yeast has been facilitated by the recent completion of the *Sch. pombe* genome sequencing project (Wood *et al.*, 2002). A BLAST search of protein databases indicated that *Sch. pombe* contains 11 ABC transporters, which were assigned to subfamilies according to the *Sac. cerevisiae* classification system (Fig. 1a; Decottignies & Goffeau, 1997). Gene disruption was used to characterize the ABC transporters. Localization of the ABC transporters was performed by GFP tagging. The results of the present study demonstrate that two ABC transporters are major vacuolar glutathione S-conjugate (GS-X) pumps in fission yeast.

METHODS

Database searches and classification of ABC transporters.

The fission yeast ABC transporters were identified by screening the *Sch. pombe* genome database for new homologues of the known yeast ABC transporters using the BLAST program of sequence alignment. BLAST scores lower than e^{-10} were considered significant. The fission yeast ABC transporters were classified based on the most similar *Sac. cerevisiae* protein (Decottignies & Goffeau, 1997).

Strains, media and materials. *Escherichia coli* strain XL-1 Blue (Stratagene) was used for all cloning procedures. The *Sch. pombe* gene disruption mutants were constructed using wild-type strain YF016 (*h⁻ leu1-32 ura4-C190T ade7::ura4*), which is a derivative of ARC039 (*h⁻ leu1-32 ura4-C190T*). Standard rich medium (YES), synthetic minimal medium (MM) and YE for *Sch. pombe* cells were used as described (Moreno *et al.*, 1991). FM4-64, MitoTracker Red CMXRos and monochlorobimane (MClB) were from Molecular Probes. All other chemicals were from Sigma or Wako Pure Chemicals.

Cloning and disruption of genes encoding ABC transporters.

Genes encoding ABC transporter proteins were cloned into pGEM-T EASY or pGEM-T vectors (Promega) following PCR amplification using the primers listed in Table 1. The resulting plasmids were digested with the restriction enzymes listed in Table 1, followed by insertion of a *ura4*⁺ gene cassette to generate gene disruption constructs (Fig. 1b). The fission yeast *ade7* strain YF016 was transformed by electroporation (Suga & Hatakeyama, 2001), with the PCR products amplified from these constructs. Gene disruptions were confirmed by colony-PCR, using appropriate primers.

Resistance assays. Cells cultured overnight in 5 ml YES were diluted with water to an OD₆₀₀ of 0.5, corresponding to about 10⁷ cells ml⁻¹, and used as an inoculum. The cell suspension was serially diluted 1:10 and 1:100. Aliquots (5 μ l) were spotted onto YES plates containing potential inhibitors at the indicated concentrations

Fig. 1. Relatedness of *Sch. pombe* ABC transporters and construction of disruption alleles. (a) Phylogenetic tree of *Sch. pombe* ABC transporters. Protein sequences were aligned using CLUSTALW 1.7, and subjected to phylogenetic analysis by the distance with neighbour-joining method (www.ddbj.nig.ac.jp/search/clustalw-j.html). (b) Construction of gene disruptions. Open bar, *ura4⁺* cassette; arrow, ORF; asterisks, primers listed in Table 1; B, *Bam*HI, Bg, *Bgl*II, C, *Cl*al; Ei, *Eco*RI; EV, *Eco*RV; H, *Hind*III; K, *Kpn*I; S, *Sma*I; X, *Xho*I.

(Fig. 2). SDS, at a concentration of 0.006% (w/v), was added to medium containing BFA to enhance the toxicity of this inhibitor (Nagao *et al.*, 1995). Growth was scored after 3 days.

C-terminal GFP tagging. The *Sch. pombe* ABC transporter ORFs were amplified from YF016 genomic DNA by PCR, using the oligonucleotides listed in Table 1. The corresponding PCR products were digested with the appropriate restriction enzymes, and cloned into the corresponding sites of pTN197 derived from pREP41 (Nakamura *et al.*, 2001), which is a thiamine-repressible expression vector.

Organelle staining and fluorescence microscopy. Nuclei were stained by using the DNA-specific dye Hoechst 33342 (Dojindo Laboratories). Cells were collected by centrifugation, and suspended in $1 \mu\text{g ml}^{-1}$ Hoechst 33342 solution. After a 10 min incubation at room temperature, cells were washed twice with distilled water, and visualized by fluorescence microscopy.

Mitochondria were stained using the mitochondrion-specific dye MitoTracker Red CMXRos. Cells were suspended in 10 mM HEPES, pH 7.4, containing 3% glucose, and MitoTracker Red CMXRos was added to yield a final concentration of 100 nM. After a 15 min incubation at room temperature, cells were visualized by fluorescence microscopy.

To visualize the fission yeast vacuole, cells were labelled with the lipophilic dye FM4-64, as described by Vida & Emr (1995). Briefly, 1 ml of exponentially growing cells in YES was harvested by centrifugation, and suspended in 0.5 ml YES medium containing $16 \mu\text{M}$ FM4-64, followed by incubation at 30°C for 30 min, with shaking for pulse labelling. The labelled cells were then washed once with fresh medium, resuspended in 1 ml YES medium without dye, and incubated at 30°C for 90 min. The cells were then pelleted by centrifugation, resuspended in distilled water to induce vacuolar fusion under hypotonic stress, and examined by fluorescence microscopy.

Table 1. ABC transporter genes analysed, and oligonucleotides used

Family	Systematic name	Gene	<i>Sac. cerevisiae</i> [†]	Oligonucleotide	Sequence (5'–3')	Primer [‡]	Site [§]
I	SPCC18B5.01c	<i>bfr1</i>	SNQ2	bfr1FW	CTCTTCAGTGTACAGTGAGCTCGGTTGC	D	<i>EcoRI</i>
				bfr1RV	GCAGGATCATTGATATATCTCTCCCATGAC	D	<i>HindIII</i>
				bfr1N	GTTTCTCGAGATGAATCAAAATTCGGATAC	T	<i>XhoI</i>
				bfr1C	GTTTGCGGCCGCCACCAGTTCGGTAATC	T	<i>NotI</i>
	SPAPB24D3.09c	<i>pdr1</i> [*]	SNQ2	pdr1FW	ACCAGGTGACAAACTACGATCTACGCAAC	D	<i>BamHI</i>
				pdr1RV	GAGACTGGAACCCATCAATGCTGCAGACGG	D	<i>HindIII</i>
				pdr1N	GTTCTCGAGATGAGTGAACAAGAAAAAGGG	T	<i>XhoI</i>
				pdr1C	GTGCGGCCGCCTGGAACAAAACGAGCAGCC	T	<i>NotI</i>
II. 1	SPAC3F10.11c	<i>abc2</i> [*]	<i>YCF1</i>	mrp1FW	TTCAAGAACTAGCTAGGGAGAACGCACGG	D	<i>HindIII</i>
				mrp1RV	GCGCAATTCTCTGGTATTCCTTAACTGCAG	D	<i>EcoRI</i>
				mrp1N	GTTTTAGATCTAAATGGTTCTTGAACAGG	T	<i>BglII</i>
				mrp1C	GTTTGCGGCCGCCCAGCAGCTTTTTCGTTG	T	<i>NotI</i>
	SPBC359.05	<i>abc3</i> [*]	<i>YCF1</i>	mrp2FW	TCAAGTGAGTTCAACTAATTGTTGTGAGGC	D	<i>Clal</i>
				mrp2RV	GCAGGAAATACTATATCCGCAGTGAGAGC	D	<i>EcoRV</i>
				mrp2N	GTTCTCGAGAATGATCACCGCAAACAAAG	T	<i>XhoI</i>
				mrp2C	GTTGCGGCCGCCAATGAGACCGCTTTCITTT	T	<i>NotI</i>
	SPAC30.04c	<i>abc4</i> [*]	<i>YBT1</i>	mrp3FW	GGCTTTTGTAAGATTCTCCACCAGCGAAC	D	<i>BglII</i>
				mrp3RV	CGACCAAAGGTCCTAAAACAATAGGGACGG	D	<i>HindIII</i>
				mrp3N	GTTTCTCGAGATGGAACAACTTTCACCATCG	T	<i>XhoI</i>
				mrp3C	CGAACCGCGGCCGCCAGGAGCAGAAAAAG	T	<i>NotI</i>
	SPAC9E9.12c	<i>abc1</i>	<i>YBT1</i>	abc1FW	TCATGCCAATCACACCCAACACCCC	D	<i>BamHI</i>
				abc1RV	TGACTTCCCAGAACCTGTTGAACCGACCAC	D	<i>EcoRI</i>
				abc1N	GTTTGTGACGTGATGAAAATGTTTCAGTC	T	<i>SalI</i>
				abc1C	GTTTGCGGCCGCTAATATGACTTTCCTTGC	T	<i>NotI</i>
II. 2	SPBC25B2.02c	<i>mam1</i>	<i>STE6</i>	mam1FW	GAGTGCTATTGCTGGAAGAGAGCTCGATC	D	<i>XhoI</i>
				mam1RV	TGGTGAATAATGACTCTCTGGATAACTGCG	D	<i>HindIII</i>
				mam1N	GTTTGTGCACTTTAATGCACATTCATTCAG	T	<i>SalI</i>
				mam1C	GTTTGCGGCCGCCTAATTTCCAAAAATGTG	T	<i>NotI</i>
	SPCC663.03	<i>pmd1</i>	<i>STE6</i>	pmd1FW	GGATGAGAAGGAGAACGATTGAGATGCTGC	D	<i>HindIII</i>
				pmd1RV	GATCGTGGATTGTCCGCTACCACTGGCACC	D	<i>KpnI</i>
				pmd1N	GTTGGATCCCATAATCATGTCTTTGCATAG	T	<i>BamHI</i>
				pmd1C	GTTGCGGCCGCCTTTGTTCAATCCCTGTTC	T	<i>NotI</i>
II. 3	SPAC15A10.01	<i>atm1</i>	<i>ATM1</i>		See Iwaki <i>et al.</i> (2005)		
	SPCC737.09c	<i>hmt1</i>	<i>ATM1</i>		See Iwaki <i>et al.</i> (2003), Iwaki & Takegawa (2004)		
	SPBC9B6.09c	<i>mdl1</i> [*]	<i>MDL1</i>	mdl1FW	CCTTACAGAGCCGATTTCCTCTCCAAACCG	D	<i>BamHI</i>
				mdl1RV	GAGTCTCATCGATACGTAGAGCATCATCCC	D	<i>BglII</i>
				mdl1N	GTCTCGAGATGGATCCAATACGTTTTGGAC	T	<i>XhoI</i>
				mdl1C	GTGCGGCCGCGTTCAACTTTTCCAAGCTGC	T	<i>NotI</i>

*Genes named in this study.

[†]The most similar *Sac. cerevisiae* homologues.[‡]Primers for gene disruption and GFP-tagging are indicated as D and T, respectively.[§]Cloned PCR products were digested with the indicated restriction enzymes in order to construct gene disruptions through insertion of *ura4⁺* as a selectable marker. To tag GFP, amplified PCR products were digested with the indicated restriction enzymes, and cloned into pTN197.

Uptake of MClB was visualized after harvesting exponential-phase cells, resuspending them in 1 ml YES medium containing 100 µM MClB, and incubating at 30 °C for 5 h. The cells were then pelleted by centrifugation, and washed twice with YES medium. The cells were viewed by fluorescence microscopy, and photographed using exposure times of 10 ms.

Stained cells were observed under a fluorescence microscope (model BX-60; Olympus) using a U-MWIG filter set (Olympus) for FM 4-64 and MitoTracker Red CMXRos, a U-MGFPHQ filter set (Olympus) for GFP, and a U-MWU filter set (Olympus) for Hoechst 33342 and MClB. Images were captured with a Sensys Cooled CCD camera using MetaMorph (Roper Scientific).

Fig. 2. Phenotypic characterization of fission yeast strains harbouring disrupted ABC transporter alleles. An overnight culture in YES was diluted to an OD₆₀₀ of 0.5, and then serially diluted 1:10. Aliquots (5 μl) were plated on YES containing: 0.1 μg amphotericin B ml^{-1} , 0.1 μg cerulenin ml^{-1} , 1 or 5 μg BfA ml^{-1} , 0.2 mM CdCl₂, 30 μg CHX ml^{-1} , 0.2 μg clotrimazole ml^{-1} , 0.1 μg 4-NQO ml^{-1} or 2 mM diamide. Plates were photographed after 3 days at 30 °C. YE was used to monitor pigment accumulation.

RESULTS

Fission yeast ABC transporters

We analysed the complete genome sequence of *Sch. pombe* in order to identify ABC transporters, and found that *Sch. pombe* contains 11 transporters only. These proteins were classified into four subfamilies: Cluster I (2 ORFs), Cluster II. 1 (4 ORFs), Cluster II. 2 (2 ORFs), and Cluster II. 3 (3 ORFs) (Table 1, Fig. 1a). *Sch. pombe* has no homologues of Cluster I. 3 or Cluster III members. In addition to the six ABC transporters identified in fission yeast prior to this study, five new ABC transporters were revealed by this analysis. These uncharacterized genes are named in Table 1.

Phenotypic characterization of all ABC transporters

All 11 *Sch. pombe* ORFs were cloned, and disruption mutants were constructed (Fig. 1b). All were found to be viable. The *atm1* mutants grew slowly, and exhibited sensitivity to diamide (Fig. 2), H₂O₂ (0.01 %), DTT (5 mM), CuCl₂ (1 mM), NaCl (0.3 M), LiCl (5 mM), G418 (10 µg ml⁻¹), hygromycin B (15 µg ml⁻¹), nalidixic acid (180 µg ml⁻¹), caffeine (6 mM) and high temperature (37 °C) (data not shown). Growth of the other deletants was not inhibited by these compounds, or by high temperature. The growth of *bfr1Δ* cells was severely inhibited by BfA, cerulenin and clotrimazole, and slightly inhibited by cycloheximide (CHX; Fig. 2). The growth of *pmd1Δ* cells was also slightly inhibited by CHX, but less so by BfA than the wild type (Fig. 2). The *hmt1Δ* strain was found to be more sensitive to cadmium than any other deletants, and its growth was inhibited by clotrimazole (Fig. 2). Most of these mutants exhibited partial sensitivity to cerulenin, while *atm1Δ*, *pmd1Δ* and *abc2Δ* were found to be tolerant. Other ions, including Ca²⁺ (0.1 M), Mn²⁺ (1 mM), Zn²⁺ (2 mM), Mg²⁺ (10 mM), Hg²⁺ (10 µM) and Fe³⁺ (2 mM), chelators including EGTA (10 mM) and *o*-phenanthroline (30 µg ml⁻¹), an alkaloid (caffeine, 2 mM), antibiotics, including amphotericin B (0.1 µg ml⁻¹), nystatin (2 µg ml⁻¹), staurosporine (1 µg ml⁻¹) and tunicamycin (0.1 µg ml⁻¹), a weak acid (acetic acid, 80 mM), and 4-nitroquinoline oxide (4-NQO) (Fig. 2), had no observed effect on the growth of these mutants (data not shown).

Mutations in *ade7* of *Sch. pombe* (equivalent to *ADE1* in *S. cerevisiae*) led to accumulation of characteristic red pigments (ade pigments) in vacuoles. The precursor of the ade pigments is toxic, and is conjugated by glutathione prior to transport to the vacuole mediated by a GS-X-transporting ATPase (GS-X pump) (Chaudhuri *et al.*, 1996; Smirnov *et al.*, 1967). These intermediates accumulate only when the mutants are grown in adenine-limiting medium. When cells are grown in adenine-limiting YE, *atm1* mutants have been observed to form pale-pink colonies because of a mild petite phenotype (Iwaki *et al.*, 2005). Other deletants were indistinguishable from the wild type, suggesting that

multiple proteins may contribute to *Sch. pombe* GS-X pump activity.

Localization of ABC transporters in fission yeast

To confirm localization of fission yeast ABC transporters, GFP was fused to their C termini, and the tagged constructs were expressed under control of the attenuated *nmt1* promoter (Fig. 3).

Two ABC transporters involved in drug resistance, Pmd1p and Bfr1p, were found in the cell periphery, consistent with a plasma membrane location. Localization of Mam1p was inconclusive, yielding small intracellular dots of fluorescence. This pattern of fluorescence was similar to that observed for the *Sac. cerevisiae* a-factor transporter Ste6p, which localizes mainly to endosomes, and possibly to some Golgi (Kelm *et al.*, 2004; Huyer *et al.*, 2004). Atm1-GFP and Mdl1-GFP co-localized with the mitochondrion, as visualized by MitoTracker Red CMXRos. Hmt1-GFP has previously been shown to localize to the vacuolar membrane (Iwaki *et al.*, 2003). The patterns of fluorescence of three Cluster II. 1 proteins, Abc2-GFP, Abc3-GFP and Abc4-GFP, were similar to that of Hmt1-GFP, and co-localized with FM4-64, a vacuolar membrane-specific dye. Abc1-GFP and Pdr1-GFP were found in the cell perimeter and around the nucleus, as visualized by Hoechst 33342, in a typical endoplasmic reticulum (ER) pattern.

Search for ABC transporters required for red pigment accumulation

A defect in the *ADE1* gene of *Sac. cerevisiae* leads to accumulation of the purine intermediate phosphoribosyl-aminimidazole carboxylate (Fisher, 1969). Glutathione conjugates of the ade pigments are transported to the vacuole mediated by the Cluster II. 1 proteins Ycf1p, Bpt1p and Ybt1p/Bat1p (Chaudhuri *et al.*, 1996; Sharma *et al.*, 2002, 2003). Studies in *Sac. cerevisiae* have shown that colony colour of *ade1* and *ade2* mutants is a good indicator of GS-X pump activity.

Mutations in *gcs1*⁺, encoding the first enzyme in glutathione biosynthesis, γ-glutamylcysteine synthetase, exhibit a defect in pigment formation, indicating that the ade pigment is probably a glutathione conjugate in fission yeast (Chaudhuri *et al.*, 1996). However, the ade-pigment pump of *Sch. pombe* remains to be characterized. To date, the only finding is that it is distinct from the vacuolar pump Hmt1p (Chaudhuri *et al.*, 1996).

Phenotypes of single-gene deletants, localization of GFP-tagged proteins, and similarity to budding yeast Ycf1p, strongly suggest that ade-pigment transport is mediated by two or more Cluster II. 1 proteins. Therefore, we constructed a quadruple deletion mutant, *abc1Δ abc2Δ abc3Δ abc4Δ*. This mutant formed paler colonies than the wild type (Fig. 4a). While the *abc2Δ* mutant formed a somewhat pale colony, its tolerance for xenobiotics was indistinguishable

Fig. 3. Intracellular localization of ABC transporters. GFP-tagged proteins were expressed in wild-type cells. Cells were cultured in MM-Leu medium without thiamine for 18 h, and then stained with Hoechst 33342 to label the nucleus, MitoTracker Red CMXRos to label mitochondria, or FM4-64 to label the vacuolar membrane.

Fig. 4. Phenotypic characterization of fission yeast strains harbouring disrupted Cluster II. 1 alleles. (a) Cells were spotted on YE, and incubated at 30 °C for 3 days. (b) Serial dilutions of cells were plated on YES containing 0.5 mM CdCl_2 , 25 μg CHX ml^{-1} , or 0.2 μg 4-NQO ml^{-1} , and then incubated at 30 °C for 3 days. (c) Serial dilutions of cells were plated on YES containing 0.1 mM CdCl_2 , and then incubated at 30 °C for 4 days.

from that of the wild type. When two Cluster II. 1 proteins, *abc2* and *abc4*, were deleted, the double disruption mutant formed pale colonies similar to the quadruple mutant. This double deletion caused sensitivity to CHX and 4-NQO (Fig. 4b). Unlike *Sac. cerevisiae ycf1Δ* cells, Cd^{2+} had no effect on growth of the double disruptant. Although all Cluster II. 1 proteins share a high degree of similarity to one another, disruption of *abc3* or *abc1* had no additional effect on colony colour, or tolerance for xenobiotics. From these results, we conclude that two Cluster II. 1 proteins, Abc2p

and Abc4p, are responsible for ade-pigment transport into vacuoles.

To determine if Abc2p and Abc4p are responsible for Cd^{2+} tolerance by mediating transport of glutathione- Cd^{2+} , as seen in *Sac. cerevisiae*, a triple disruption mutant, *abc2Δ abc4Δ hmt1Δ*, was constructed. While this mutant was found to be more sensitive to 0.1 mM CdCl_2 than *hmt1Δ*, the double mutant *abc2Δ abc4Δ* was able to grow as described above (Fig. 4c). This observation suggests that

transport of glutathione–Cd²⁺ plays a significant role in Cd²⁺ tolerance, but a minor role relative to phytochelatins–Cd²⁺ transport by Hmt1p.

Vacuolar accumulation of MCIB-GS *in vivo*

MCIB, a membrane-permeable nonfluorescent compound, is specifically conjugated with glutathione by cytosolic glutathione S-transferases to generate the intensely fluorescent membrane-impermeable S-conjugate MCIB–glutathione (Shrieve *et al.*, 1988; Oude Elferink *et al.*, 1993; Ishikawa *et al.*, 1994). GS-X pumps exhibit activity toward a broad range of S-conjugates, including MCIB–glutathione (Ishikawa *et al.*, 1994; Li *et al.*, 1996). Thus, MCIB is commonly used as a sensitive probe for monitoring the intracellular transport and localization of its S-conjugate. In *Sac. cerevisiae*, vacuolar transport of MCIB–glutathione is mediated by Ycf1p (Li *et al.*, 1996).

To determine if Cluster II. 1 proteins play a role in vacuolar accumulation of MCIB–glutathione in intact cells, wild-type and mutant cells were observed by fluorescence microscopy after incubation in medium containing MCIB (Fig. 5). Wild-type cells exhibited an intense punctate fluorescence, corresponding to vacuoles, as determined by Nomarski optics. A similar pattern was observed in the *abc4Δ* mutant, although fluorescence was slightly lower in the *abc2Δ* mutant. Accumulation of MCIB decreased remarkably when both *abc2* and *abc4* were deleted. Deletion of the two Cluster II. 1 genes *abc3* and *abc1* had little effect on accumulation of MCIB under these conditions. These results indicate that activity of the GS-X pump was affected significantly by loss of Abc2p and Abc4p.

GS-X transporter(s) other than ABC transporters

In *Sac. cerevisiae*, mutants lacking V-type ATPase (V-ATPase) activity form pale colonies, even in an *ade2* background (Supek *et al.*, 1994). Thus, it is believed that a factor depending on the H⁺ gradient across the vacuolar membrane is partly responsible for GS-X transport into vacuoles, although the ABC transporters are thought to play the major role (Sharma *et al.*, 2003; Penninckx, 2002). To test the contribution of V-ATPase to pigmentation, we constructed a *vma3Δ* disruptant in an *ade7* background. As shown in Fig. 6(a), the *vma3Δ* mutant formed white colonies. This was partly a consequence of a petite (respiration-defective) phenotype (data not shown), although less pigmentation was observed in the *vma3Δ* mutant than in the mild petite *atm1Δ* mutant.

To observe GS-X transporter activity by microscopy, distribution of MCIB was also determined (Fig. 6b). While intense fluorescence was observed in the vacuoles of *vma3Δ*, MCIB–glutathione was also detected in the cytosol. This cytosolic fluorescence was not observed in other strains examined (compare with Fig. 5). These observations suggest that an unknown transporter may be involved in GS-X transport to vacuoles, and that its activity may depend on

Fig. 5. Vacuolar accumulation of MCIB in mutant cells. Cells were incubated for 5 h at 30 °C in YES containing 100 μM MCIB, and then incubated in water for 1 h to induce fusion of vacuoles. Cells were observed by fluorescence microscopy and photographed.

Fig. 6. Mutation of V-ATPase affects GS-X transport into vacuoles. (a) Effect of *vma3* deletion on ade pigmentation. Cells were spotted on YE, and incubated at 30 °C for 4 days. (b) Vacuolar accumulation of MCIB in *vma3Δ* cells. Cells were incubated for 5 h at 30 °C in YES containing 100 μM MCIB. Cells were observed by fluorescence microscopy and photographed.

the H⁺-gradient generated by V-ATPases. Sorting to the vacuoles may also be affected because of the severe defect reported in vacuolar protein sorting (Iwaki *et al.*, 2004).

DISCUSSION

ABC transporters constitute a superfamily of proteins responsible for transporting a variety of substrates across cell membranes. Impaired function of ABC transporters in yeast leads to various phenotypes, including sensitivity to heavy metals, antibiotics, weak acids and oxidative stress (Bauer *et al.*, 1999; Decottignies & Goffeau, 1997).

In *Sch. pombe*, a phenotypic survey of all ABC transporter gene disruptants revealed that only the *atm1Δ* cells showed a decrease in vacuolar accumulation of the ade pigment. Further characterization demonstrated that *atm1Δ* exhibited a mild petite phenotype, and was defective in vacuolar function, thereby affecting colony colour (Iwaki *et al.*, 2005). This observation strongly suggested that multiple transporters are involved in vacuolar accumulation of ade pigment and GS-X, raising the question of which is the actual pump

that transports ade pigment directly into vacuoles. We speculated that it might be one of the Cluster II. 1 proteins, similar to Ycf1p, Bpt1p and Ybt1p/Bat1p of *Sac. cerevisiae*. While four Cluster II. 1 proteins were found in fission yeast, notable differences in colour were not observed in these single-gene disruptants. Because Cluster II. 1 proteins have redundant functions, significant reduction in pigmentation may not be detectable in the single deletants. To determine if Cluster II. 1 proteins transport ade pigment into vacuoles, all Cluster II. 1 genes were disrupted. Quadruple disruption of Cluster II. 1 proteins revealed that transport of GS-X is mediated by Cluster II. 1 proteins. Moreover, a major role for Abc2p and Abc4p in fission yeast was discovered, because there was almost no difference in pigmentation and uptake of MCIB between the *abc2Δ abc4Δ* mutant and the quadruple mutant. These observations indicate that the contributions of Abc1p and Abc3p are minor, and that the ade pigment is not a preferred substrate of these Cluster II. 1 proteins.

While loss of the ABC transporters caused a reduction in colony colour, pigmentation was not completely lost in these mutants. Pigmentation may derive from the contribution of a transporter dependent on the vacuolar H⁺-ATPase-generated pH gradient. In *Sac. cerevisiae*, the contributions of this transporter are thought to be minor compared with the contributions of the Cluster II. 1 proteins (Sharma *et al.*, 2003; Penninckx, 2002). However, these contributions may not be negligible in *Sch. pombe*, because loss of V-ATPase activity gave rise to white colonies lacking pigmentation. As previously reported, deletion of *vma3* encoding V-ATPase subunit c causes a severe defect in vacuolar protein sorting of carboxypeptidase Y, although vacuolar ABC transporter Hmt1p was correctly sorted to vacuoles (Iwaki *et al.*, 2004). These results suggest that loss of V-ATPase activity results in loss of colony colour due to a combination of effects. In V-ATPase mutants, unknown H⁺-gradient-dependent mechanisms will be impaired. Elevated vacuolar pH may also affect ABC transporter activity, thereby reducing vacuolar accumulation of GS-X. Potential sorting defects of these transporters must also be considered.

The Cd²⁺ sensitivity of the *abc2Δ abc4Δ hmt1Δ* mutant indicates that GS-X transport also plays a role in heavy-metal tolerance. It has been shown that GS-X transport by Ycf1p is required for detoxification of heavy metals in budding yeast (Li *et al.*, 1996). However, GS-metal transport in fission yeast is not yet well understood because *Sch. pombe* has been observed to synthesize phytochelatin to chelate heavy metals with subsequent transport of the phytochelatin-metal complex into vacuoles by Hmt1p (Cobbett, 2000; Ortiz *et al.*, 1992, 1995). *hmt1Δ* was found to be Cd²⁺ sensitive, and to form faint pink colonies on rich medium containing 20 μM Cd²⁺ (data not shown). This suggests that GS-X transport by Abc2p and Abc4p is activated in *hmt1Δ* cells. A double disruption mutant, *abc2Δ abc4Δ*, and a quadruple deletion mutant, *abc1Δ abc2Δ abc3Δ*

abc4Δ, were able to grow in the presence of 0.1 mM Cd²⁺, strongly suggesting that the contribution of glutathione-metal transport to heavy-metal tolerance is much less significant than phytochelatin-metal transport by Hmt1p.

The number of ABC transporters found in fission yeast is about half that found in *Sac. cerevisiae*, which has 23 ABC transporters, although this number contains the duplicated ORFs *YKR103w/YKR104w*. The Cluster III proteins, including Pxa1 and Pxa2, necessary for transport of long-chain fatty acids into peroxisomes in budding yeast (Shani *et al.*, 1995; Shani & Valle, 1996), and the Cluster I. 3 proteins, were not found in the fission yeast genome. Recently, it was reported that budding yeast takes up exogenous sterol, and that two Cluster I proteins, Aus1p and Pdr11p, facilitate sterol cycling between the plasma membrane and ER (Li & Printz, 2004). In contrast, exogenous sterol cannot be incorporated into fission yeast cells (Hughes *et al.*, 2005), presumably due to lack of the corresponding Cluster I proteins. The budding yeast contains eight Cluster I. 1 proteins, and one Cluster I. 2 protein, involved in multidrug resistance (Bauer *et al.*, 1999; Decottignies & Goffeau, 1997), while only two Cluster I proteins were found in the *Sch. pombe* genome. However, fission yeast has one additional Cluster II. 2 protein, Pmd1p, which is involved in leptomycin B resistance (Christensen *et al.*, 1997a). Pmd1p may functionally substitute for Cluster I proteins, some of which have apparently been lost during evolution. These findings indicate that most fission yeast ABC transporters may have multiple specificities or functions, except for the phytochelatin transporter Hmt1p (Ortiz *et al.*, 1995, 1992).

In this report, all ABC transporters were characterized and localized by GFP tagging and fluorescence microscopy. ER-localized ABC transporters within the Cluster I. 1, I. 2 and II. 1 families have not been reported in budding yeast to date, but two fission yeast proteins exhibited an ER pattern of fluorescence. ER localization of Adp1p, a Cluster I. 3 protein, has been inferred from direct assay (Kumar *et al.*, 2002), and human Cluster III (ABCD) proteins are known to localize in the ER (Bresnahan *et al.*, 1997). In fission yeast, overexpression on a multicopy plasmid might cause aberrant mislocalization of Abc1p and Pdr1p to the ER. Abc1-GFP and Pdr1-GFP function could not be confirmed by complementation of the disruption mutants because these mutants exhibited no apparent phenotypes relative to the tested inhibitors. Further analysis is needed to confirm correct localization of the tagged proteins, and expression levels of the ABC transporter genes.

The present study also detected an additional and intriguing phenotype of a fission yeast strain lacking Pmd1p. While Pmd1p was originally isolated as a homologue of human P-glycoprotein, which catalyses efflux of leptomycin B (Nishi *et al.*, 1992), we found that the *pmd1Δ* mutant had an increased tolerance for BfA, indicating that Pmd1p is directly or indirectly involved in uptake of BfA. *Lactococcus lactis* LmrA, which is a homologue of human P-glycoprotein, can take up and efflux ethidium bromide (Balakrishnan

et al., 2004). Ethidium uptake by LmrA has been found to be mediated by proton-ethidium symport, without a direct requirement for ATP (Venter *et al.*, 2003). Similar mechanisms may be conserved in Pmd1p. The present results provide an important starting point for future detailed analysis of the functions of fission yeast ABC transporter proteins.

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