Functional Characterization of AtATM1, AtATM2, and AtATM3, a Subfamily of Arabidopsis Half-molecule ATP-binding Cassette Transporters Implicated in Iron Homeostasis*

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The functional capabilities of one of the smallest subfamilies of ATP-binding cassette transporters from Arabidopsis thaliana, the AtATMs, are described. Designated AtATM1, AtATM2, and AtATM3, these half-molecule ABC proteins are homologous to the yeast mitochondrial membrane protein ATM1 (ScATM1), which is clearly implicated in the export of mitochondrially synthesized iron/sulfur clusters. Yeast ATM1-deficient (atm1) mutants grow very slowly (have a petite phenotype), are respiration-deficient, accumulate toxic levels of iron in their mitochondria, and show enhanced compensatory high affinity iron uptake. Of the three Arabidopsis ATM(s), AtATM3 bears the closest functional resemblance to ScATM1. Heterologously expressed AtATM3 is not only able to complement the yeast atm1 petite phenotype but is also able to suppress the constitutively high capacity for high affinity iron uptake associated with loss of the chromosomal copy of ScATM1, abrogate intramitochondrial iron hyperaccumulation, and restore mitochondrial respiratory function and cytochrome c levels. By comparison, AtATM1 only weakly suppresses the atm1 phenotype, and AtATM2 exerts little or no suppressive action but instead is toxic when expressed in this system. The differences between AtATM3 and AtATM1 are maintained after exchanging their target peptides, and these proteins as well as AtATM2 colocalize with the mitochondrial fluor MitoTracker Red when expressed in yeast as GFP fusions. Although its toxicity when heterologously expressed in yeast, except when fused with GFP, precludes the functional analysis of native AtATM2, a common function, mitochondrial export of Fe/S clusters or their precursors for the assembly of cytosolic Fe/S proteins, is inferred for AtATM3 and AtATM1.

The ATP-binding cassette (ABC)5 protein superfamily is one of the largest protein families known, and many but not all are membrane proteins (“ABC transporters”) competent in the transport of a broad range of materials across membranes. ABC proteins are designated as such, because each possesses one or two ATP-binding cassettes or nucleotide binding folds (NBFs) sharing 30–40% identity between family members and one or more transmembrane domains (TMDs) (1). Each NBF encompasses ~200 amino acid residues and contains three idiotypic sequence motifs. These are a Walker A box and Walker B box separated by ~120 amino acid residues and an ABC signature (alias C) motif situated between the two Walker boxes (1). The NBFs catalyze ATP hydrolysis, whereas the TMDs, each of which contains multiple transmembrane spans, mediate solute transport across the phospholipid bilayer (or from one leaflet of the bilayer to the other).

A feature of ABC transporters, evident from a survey of the superfamily, is their modular construction. The four core domains or modules, two NBFs and two TMDs, may be expressed as separate polypeptides or as multidomain proteins (1). In some transporters (e.g. many of those from bacterial sources), the four domains reside on different polypeptides (“quarter molecules”). In others the domains are fused in various combinations as half-molecules (NBF-TMD) or full molecules (NBF1-TMD1-NBF2-TMD2 or the reverse).

Plants are a particularly rich source of ABC proteins. For instance, the genomes of Arabidopsis thaliana and rice (Oryza sativa) each contain in excess of 120 ORFs for ABC proteins (2–4). With only a few exceptions, these proteins, which fall into 12 or more subfamilies, pose a challenge, because many of the subfamilies contain upward of 13 members. One of the few exceptions is the AtATM (A. thaliana “ABC transporter of the mitochondrial” homolog) subfamily of forward orientation half-molecule ABC transporters. It contains only three ORFs: two that are immediately adjacent to each other on chromosome IV (AtATM1 and AtATM2) and one other on chromosome V (AtATM3) (2, 5). To date, only one ORF in rice (3024.m00134) and two in poplar (Populus trichocarpa)
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(Eugene 3.00131305 and Genewise1-v1.C_LG_XIX2542), all three of which most closely resemble AtATM3, have been assigned to this subfamily in the genomes from other plant sources (3).7

The prototypical ATM, Saccharomyces cerevisiae ATM (ScATM1), localizes to the inner mitochondrial membrane (6) and is inferred to participate in the elaboration of cytosolic iron/sulfur proteins by catalyzing the transport of iron/sulfur centers from the mitochondrial matrix, where they are synthesized, into the cytosol (7). Deletion of the yeast ATMI gene yields petite mutants that grow slowly, lack cytochromes, are deficient in respiration, exhibit constitutive activation of high affinity iron uptake across the plasma membrane, and establish anomalously high mitochondrial iron levels (6–8). Likewise, cellular depletion of ScATM1 by promoter shut-off markedly attenuates cytosolic iron/sulfur enzyme activity while leaving mitochondrial iron/sulfur enzyme activity unaffected (7, 9).

In those that have been investigated in sufficient detail, albeit indirectly, a basic equivalence of function among the ATMs from different eukaryotes has been demonstrated. In humans, two genes are able to functionally complement yeast atm1 mutants. One is HsABC7, mutation of which is responsible for X-linked sideroblastic anemia and ataxia (XLSA/A), an iron storage disease associated with mitochondrial hyperaccumulation of this metal (10). The other is HsMTABC3, which has been mapped to the same segment of chromosome 2 as the locus for lethal neonatal metabolic syndrome, a mitochondrial disorder that is also associated with mitochondrial iron hyperaccumulation (11). In plants, specifically Arabidopsis, AtATM3 (alias STA1, the starikl gene product), whose deficiency causes pronounced dwarfism and chlorosis (12), is also implicated in the transport of iron/sulfur clusters as well as tolerance toward heavy metals (14). AtATM3 has a mitochondrial localization in planta, complements yeast atm1 mutants, and when ectopically overexpressed enhances cadmium and lead tolerance (13, 14). However, except for one experiment indicating that AtATM1 (alias STA2) only partially suppresses the sta1 phenotype of AtATM3-deficient plants (13), very little is known of the plant ATMs as a family or of the cellular biochemical basis of the effects they exert.

In this paper, we describe the cloning and functional definition of all three members of the Arabidopsis ATM subfamily. In so doing, it is determined that they all target to the mitochondrial region when ectopically expressed in Arabidopsis or heterologously expressed in yeast but have different catalytic competencies and expression patterns. AtATM3, which is ubiquitously expressed in planta, satisfies all of the requirements of a canonical ATM inclusive of participation in iron transport and the restoration of respiratory function at the level of the electron transport chain in yeast atm1 mutants regardless of the ATM-type targeting peptide with which it is associated. The same applies to AtATM1 but at a lower level in all respects. AtATM2, by contrast, which shows only low levels of expression in planta under standard growth conditions, is toxic except when fused with GFP and exerts little or no suppression of the yeast atm1 phenotype regardless of how the phenotype is monitored.

MATERIALS AND METHODS

Yeast Strains and Yeast Transformation—S. cerevisiae atm1 mutants were selected from strain CM3262 (MATa, leu2-3,112, ura3-52, gen4-101, his3-609, ino1-13) by screening for small colony size (a petite phenotype) and constitutive high-affinity cellular iron uptake as described by Dancis et al. (15). After establishing a 2+/−2− segregation ratio for the mutants in backcrosses, a tetrad from one of these crosses, designated x392b-4c (MATa, leu2-3,112, ura3-52, gen4-101, his3-609, atm1-21), which was shown to be allelic to an atm1 deletion strain provided by Dr. Jonathan Leighton (6) was employed for the experiments described here. This strain and its isogenic wild-type, CM3262, were maintained in YPD liquid medium (2% (w/v) glucose, 2% (w/v) bactopeptone, and 1% (w/v) yeast extract). All yeast transformations were performed as described previously (16), and the transformants were selected on complete synthetic drop-out medium lacking uracil (CSM − Ura) (2% (w/v) glucose or galactose, 0.67% (w/v) yeast nitrogen base without amino acids (Difco), 0.08% (w/v) CSM − Ura (Bio101, Inc.), and 2% (w/v) agar).

Cloning of AtATM1, AtATM2, and AtATM3—The cDNAs corresponding to AtATM1, AtATM2, and AtATM3 were cloned by PCR amplification. Total RNA was isolated from 15-day-old Arabidopsis seedlings that had been grown in standard liquid MS medium, in the case of AtATM1 and AtATM3, or in liquid MS medium containing 50 μM CdCl2, in the case of AtATM2, using TriZol Reagent (Invitrogen) according to the manufacturer’s recommendations. cDNA was synthesized from the RNA extracts using the SuperScript Premplification System (Invitrogen). All of the PCR amplifications were performed using Pfu Turbo DNA polymerase with proofreading activity (Stratagene). The gene-specific primers were designed to add a NotI restriction site at the 5′-end of the gene and a BamHI restriction site at the 3′-end. The primer combinations were AtATM1-F and AtATM1-R for AtATM1, AtATM2-F and AtATM2-R for AtATM2, and AtATM3-F and AtATM3-R for AtATM3 (Table 1). The thermal profile used for PCR was as follows: 1 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 4 min at 72 °C. For heterologous expression in yeast, the amplified AtATM1 and AtATM3 cDNAs were subcloned into the multiple cloning site between the constitutive PGK (3-phosphoglycerate kinase) gene promoter and CYC1 (cytochrome c1 gene) termination sequences of the Escherichia coli yeast shuttle vector pYES3 (16). Because of its toxicity in both E. coli and yeast when subcloned into pYES3, the amplified cDNA for AtATM2 was first cloned into NotI and BamHI double-digested pBluescript and then into pYES2 (Stratagene), the equivalent of pYES3 but containing the galactose-inducible yeast GAL1 gene promoter instead of the PGK gene promoter. The fidelity of the pYES3-AtATM1, pYES2-AtATM2, and pYES3-AtATM3 constructs was established by sequencing.

Ectopic Expression and Visualization of AtATM::GFP Fusions in Arabidopsis and Yeast—Using the pYES3-AtATM or pYES2-AtATM constructs described above as templates, the AtATM1, AtATM2, and AtATM3 cDNAs, excluding their stop

7 R. Sánchez-Fernández and P. A. Rea, unpublished results.
8 A. Dancis, unpublished results.
codons, were PCR-amplified with Pfu Turbo DNA polymerase (Stratagene). The primer combinations were as follows: AtATM1-GFP(F) and AtATM1-GFP(R) for AtATM1, AtATM2-GFP(F) and AtATM2-GFP(R) for AtATM2, and AtATM3-GFP(F) and AtATM3-GFP(R) for AtATM3 (Table 1). After double digestion with NcoI and BglII, the AtATM1 and AtATM3 amplification products were cloned into double-digested binary vector pCAMBIA1302 (accession number AF234298) to generate an in-frame C-terminal GFP translational fusion. The corresponding amplification product of AtATM2 was digested with NcoI and cloned into the NcoI site of pCAMBIA1302. The fidelity of the constructs, designated pCAMBIA-AtATM1-GFP, pCAMBIA-AtATM2-GFP, and pCAMBIA-AtATM3-GFP, was confirmed by sequencing.

For subcellular localization of the ATM::GFP fusions, the constructs were transformed into Agrobacterium tumefaciens strain C58C/pGV3850, and Agrobacterium-mediated transformation of Arabidopsis (ecotype Columbia) by the floral dip method was performed as described by Clough and Bent (17). To select transformants harboring the appropriate ATM::GFP construct, T1 seeds were germinated on MS medium containing 30 μg/ml hygromycin. Homozygous T3 and T4 plants were employed for all of the analyses reported here.

The subcellular distributions of the AtATM1, AtATM2, and AtATM3 GFP fusions, specifically their association with mitochondria, was assessed by fluorescence microscopy of the transformants after infiltration with MitoTracker Red. MitoTracker Red is a cell-permanent probe that is concentrated by active mitochondria and retained through interaction of its thiol-reactive chloromethyl moiety with intramitochondrial proteins and peptides (Molecular Probes). Ten-day-old seedlings were submerged in MS medium containing 500 nM MitoTracker Red for 20 min, rinsed three times in MS medium minus dye for 15 min before fluorescence microscopy and further processed using a Leica DM IRB fluorescence microscope (Wetzlar, Germany) equipped with a rhodamine filter and fluorescein filter for the visualization of the MitoTracker Red and GFP fluorophores, respectively. The images collected were exported as TIFF files and further processed using Adobe Photoshop (version 7.0; Adobe Systems, San Jose, CA).

For expression of the ATM::GFP fusions in yeast, the AtATM-GFP insertions of the pCAMBIA-AtATM1-GFP, pCAMBIA-AtATM2-GFP, and pCAMBIA-AtATM3-GFP constructs were amplified by PCR using the forward primers AtATM1-F, AtATM2-F, and AtATM3-F, respectively, and the reverse primer GFP-Bam(R) (Table 1). After double digestion with NotI and BamHI, the amplification products were sub-cloned into pYES3 and, after confirmation of the fidelity of the constructs by sequencing, transformed into yeast strain H9262 for selection on CSM – Ura solid medium. For fluorescence microscopy, the transformants were inoculated into CSM – Ura liquid medium, grown at 30 °C overnight at 245 rpm, pelleted by centrifugation at 2000 rpm for 5 min, and rinsed once with water. The cells were stained for 45 min with 100 nM Mitotracker Red dissolved in 10 mM HEPES buffer, pH 7.5, containing 5% (w/v) glucose and rinsed in the same medium minus dye for 15 min before fluorescence microscopy as described above.

RT-PCR of AtATM1, AtATM2, and AtATM3—For RT-PCR analyses of the tissue specificity of expression of AtATM1, AtATM2, and AtATM3, poly(A) RNA was isolated from fully expanded rosette leaves, flowers, roots, stems, siliques, and cauline leaves of 21-day-old Arabidopsis plants using a MicroPoly(A)Pure Kit (Ambion). Twenty-ng aliquots of the poly(A) samples were reverse-transcribed and subjected to PCR as described by Hansen et al. (18) using the gene-specific primers AtATM1-F and AtATM1-R for AtATM1, AtATM2-F and AtATM2-R for AtATM2, and AtATM3-F and AtATM3-R for AtATM3 (Table 1). To verify that equivalent amounts of RNA had been amplified, the same RNA samples were also subjected to RT-PCR using primers Actin1-F and Actin1-R (Table 1) for Arabidopsis Actin-8, a constitutively expressed gene. The thermal profile used for PCR was as follows: 2 min at 94 °C; 28 cycles for the AtATMs or 23 cycles for Actin-8 of 30 s at 94 °C, 30 s at 57 °C, and 1 min at 72 °C. All of the RT-PCRs were done in triplicate, and the PCR products were analyzed by agarose gel electrophoresis. The gels were documented using a Kodak DC120 EDAK gel imager (Eastman Kodak Co.).

Exchange of Putative Targeting Peptides of AtATM1 and AtATM3—The coding sequences corresponding to the putative N-terminal targeting peptides (TPs) of AtATM1 (270 bp) and AtATM3 (375 bp) were identified using SignalP (available on the World Wide Web) and PCR-amplified from pYES3-AtATM1 and pYES3-AtATM3 using Pfu Turbo DNA polymerase and the primer combinations AtATM1-F and AtATM1-Sma(R) and AtATM3-F and AtATM3-Sma(R), respectively (Table 1). After double digestion with NsiI and SmaI, the PCR fragments were cloned into pYES3 to yield the constructs pYES3-TP1 and pYES3-TP3. To generate pYES3 derivatives encoding TP1-AtATM1, TP3-AtATM1, TP1-AtATM3, or TP3-AtATM3, the AtATM1 and AtATM3 genes lacking their endogenous putative targeting peptides were amplified by PCR using the primer combination AtATM1-Sma(F) and AtATM1-R and the combination AtATM3-Sma(F) and AtATM3-R (Table 1), respectively. The constructs designated pYES3-TP1-AtATM1, pYES3-TP1-AtATM3, pYES3-TP3-AtATM1, and pYES3-TP3-AtATM3 were generated by double digesting the PCR products with SmaI and BamHI and cloning them into double-digested pYES3-TP1 or pYES3-TP3 as appropriate. The fidelity of the constructs was confirmed by sequencing.

Measurement of High Affinity Iron Uptake by Intact Yeast Cells—For the measurements of high affinity iron uptake by intact yeast cells, 96-well microtiter plates containing 100 μl of YPD medium/well were inoculated with triplicate samples of pYES3-AtATM1, or pYES3-AtATM3-transformed yeast atm1 strain x392b-4c or wild-type strain CM3262 and grown at 30 °C for 12 h. The cultures were then diluted 1:10 into 50 μl of YPD in a fresh microtiter plate and incubated for a further 3 h under the same conditions before initiating uptake by the addition of 50 μl of a solution containing 4 mg/ml sodium ascorbate, 5% (w/v) glucose, 50 mM sodium citrate, pH 6.5, and 1.0 μM radioactive iron (55FeCl3; 37–50 mCi/mg total iron). After uptake for 2 h, cell density was estimated by measuring the OD720 nm of each well in an EL800 microplate reader.
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TABLE 1
Sequences of PCR primers used in these investigations

| Primer name                        | Sequence                                                                 |
|-----------------------------------|--------------------------------------------------------------------------|
| AtATM1-F                          | TTA AGC GCC GGC ATG ATG AGG GTA TCT C                                    |
| AtATM1-R                          | TTA AGC GCC GGC ATG ATG AGG GTA TCT C                                    |
| AtATM1-Sma(F)                     | ATG CCC GGG GTA TGG AGG AAA GAC AAC CCA GAA                             |
| AtATM1-GFP(F)                     | GCT AAG ATC TTC CAA GAT AGG AGT TTG TGG TGG TGG TGG TGG TGG TGG TGG TGG TGG |
| AtATM2-F                          | TTC ACC ATG GTA GAT GAT GAG TTT CTC AA                                  |
| AtATM2-R                          | TTC ACC ATG GTA GAT GAT GAG TTT CTC AA                                  |
| AtATM3-GFP(F)                     | GCT AAG ATC TTC CAA GAT AGG AGT TTG TGG TGG TGG TGG TGG TGG TGG TGG TGG TGG |
| AtATM3-GFP(R)                     | GCT AAG ATC TTC CAA GAT AGG AGT TTG TGG TGG TGG TGG TGG TGG TGG TGG TGG TGG |
| AtATM3-Sma(R)                     | ATA CCC GGG GTA TGG AGG AAA GAC AAC CCA GAA                             |
| Actin1-F                          | TGG AAC TGG AATGGT TAA GGC TGG                                         |
| Actin1-R                          | TGG AAC TGG AATGGT TAA GGC TGG                                         |

(BIO-TEK Instruments, Winooski, VT), and the suspensions were vacuum-filtered using a PHD harvester (Brandel, Gaithersburg, MD). The vacuum filters were washed four times with water, and the radioactivity retained was determined by liquid scintillation counting. Iron uptake was enumerated as accumulation in pmol/OD720 nm/h after calibration against 55Fe standards of known radiospecific activity.

Measurement of Yeast Mitochondrial Iron Accumulation—For the measurements of mitochondrial iron uptake by yeast atm1 mutant strain x392b-4c after transformation with pYES3, pYES3-AtATM1, pYES2-AtATM2, pYES3-AtATM3, pYES3-AtATM1-GFP, pYES3-AtATM2-GFP or pYES3-AtATM3-GFP, and wild-type strain CM3262 after transformation with pYES3, the transformants were grown in CSM — Ura liquid medium containing 1 μM copper sulfate and 2 μM ferric ammonium sulfate at 30 °C to an A600 nm of 0.2 before 4-fold dilution of the cultures into the same medium containing the appropriate carbon source (15). Glucose (2% (w/v)) was the carbon source in all cases except for the pYES2-AtATM2/x392n-4c transformants, which were grown in media containing raffinose (1% (w/v)) and galactose (1% (w/v)) instead of glucose. To initiate iron uptake, 0.2 μM FeCl3 (37–50 μCi/mg) was added to the media, and the cultures were incubated at 30 °C for 15 h, after which time the cells were harvested by centrifugation, and their mitochondria were purified as described by Murakami et al. (19). Mitochondrial 55Fe content was estimated by liquid scintillation counting.

Measurement of Respiratory Competence and Mitochondrial Cytochrome Content—Mitochondrial respiratory competence was assessed by the tetrazolium overlay technique for the identification of respiration-deficient yeast (20). Aliquots of liquid growth medium containing ~30 or 1000 cells from cultures of pYES3, pYES3-AtATM1-1, pYES2-AtATM2, or pYES3-AtATM3-transformed atm1 x392b-4c cells or from pYES3-transformed wild-type CM3262 cells were spotted onto the appropriate selective medium, grown for 3 days at 30 °C, and overlaid with 0.1% (w/v) 2,3,5-triphenyltetrazolium chloride. After incubation for 3 h, the overlays were photographed. Mitochondrial cytochrome content was assessed by both difference spectrophotometry and Western analysis. In the former case, reduced minus oxidized difference spectra from ~800-μg aliquots of mitochondria purified from pYES3-, pYES3-AtATM1-, pYES2-AtATM2-, or pYES3-AtATM3-transformed atm1 x392b-4c cells or from pYES3-transformed wild-type CM3262 cells were recorded. The mitochondrial suspensions in 100 mM Tris-HCl buffer, pH 7.5, containing 0.5% (w/v) Triton X-100 were first oxidized with potassium ferricyanide and then reduced with potassium dithionite for difference spectrophotometry. In the case of the Western analyses, the mitochondrial suspensions from the same controls and transformants were subjected to denaturation, SDS-PAGE, electrophoresis, and reaction with polyclonal antibody raised against yeast cytochrome c (a gift from Dr. Debkumar Pain) or monoclonal antibody raised against yeast mitochondrial porin (Molecular Probes). The blots were probed with mitochondrial porin to verify that equivalent amounts of total mitochondrial protein had been loaded and electrotransferred in each case. Immunoreactive bands were visualized by ECL using a SuperSignal System (Pierce).

Chemicals—All of the general reagents were obtained from Fisher, Research Organics, Inc., and Sigma.

RESULTS

Cloning and Sequence Analysis of AtATM1, AtATM2, and AtATM3—There are three ORFs, designated AtATM1, AtATM2, and AtATM3, in the genome of Arabidopsis capable of encoding proteins bearing a close similarity to S. cerevisiae ATM1 (ScATM1) (2). Two of these, AtATM1 and AtATM2, map to within 582 bp of each other on chromosome IV (5). The other, AtATM3, maps to a portion of chromosome V that is otherwise devoid of ABC protein ORFs (5). For the investigations described here, the coding sequences for all three of these
genes were cloned by RT-PCR of total RNA isolated from 15-day-old Arabidopsis seedlings grown in standard liquid MS medium (AtATM1 and AtATM3) or MS medium containing 50 µM CdCl₂ (AtATM2). In the first instance, all three PCR clones were subcloned into pYES3 vector under control of the PGK promoter (16) for constitutive heterologous expression in yeast. Subsequently, however, because constitutively expressed pYES3-borne AtATM2 was found to be toxic in E. coli and yeast, this PCR clone was subcloned into pBluescript for secondary cloning into pYES2 vector (Invitrogen) for expression from the galactose-inducible yeast GAL1 promoter instead of the constitutive PGK promoter.

Phylogenetic analyses of the sequences of the AtATMs against those of representative ATM subfamily members from yeast and mammals firmly establish their membership of a common subfamily but one in which AtATM1 and AtATM2 group as a subcluster distinct from AtATM3 and their homologs in yeast and mammals (Fig. 1). AtATM1 and AtATM2 are 85% sequence-identical and 89% sequence-similar to each other but no more than 73% sequence-identical and 81% sequence-similar to their nearest equivalent, AtATM3, which in turn is no more than 56% identical and 73% sequence-similar to the nearest equivalent of the ATMs from a source other than Arabidopsis, HsABC7 (Table 2). As detailed under “Discussion,” it is therefore conceivable in the light of the immediate proximity of AtATM1 and AtATM2 to each other on chromosome IV (5) that they arose through the direct tandem duplication of an ancestral AtATM gene after their divergence from AtATM3.

Although AtATM1, AtATM2, and AtATM3 share only ~64% overall sequence similarity with ScATM1, their hydrophobicity profiles are nearly identical (data not shown). Moreover, all three of the Arabidopsis ATMs possess putative N-terminal mitochondrial targeting sequence cleavage sites: ARV/FFF in AtATM1, ARV/MFF in AtATM2, and GRL/FST in AtATM3 (2). This further reinforces their equivalence with their half-molecule homologs from other sources, such as HsABC7, MmABC7, and HsMTAB3, which also localize to this organelle. The closest homolog to the ATMs that does not localize to the mitochondrion is Schizosaccharomyces pombe HMT1 (Table 2), a half-molecule ABC transporter implicated in the transport of phytochelatins (glutathione polymers synthesized in response to heavy metal stress) that localizes to the vacuolar membrane (22, 23).

Subcellular Localization of AtATMs in planta—To explore their intracellular distributions and determine if they indeed have a mitochondrial localization as indicated by their possession of putative mitochondrial targeting sequence cleavage sites, the coding sequences of AtATM1, AtATM2, and AtATM3 were fused in frame and upstream of the GFP reporter gene and transformed into Arabidopsis.

Fluorescence microscopy of the roots of the AtATM::GFP transformants yielded results consistent with a predominantly mitochondrial localization for all three ATMs. In all cases, the GFP fluorescence was restricted to round or elliptical structures of between 0.5 and 2 µm diameter distributed throughout the cytoplasm (Fig. 2). As would be expected if the structures visualized were mitochondria, counterstaining of the same cells with the mitochondrion-specific dye MitoTracker Red demonstrated colocalization of this reagent’s red fluorescence with the green fluorescence of GFP (Fig. 2). Where there was a difference between the three ATMs was in terms of the density and definition of the structures that fluoresced. Whereas the GFP fluorescence associated with the localization of AtATM3::GFP was punctate and well defined, the corresponding fluores-

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**TABLE 2**

| Percentage similarities (numbers above 100 in each column) and identities (numbers below 100 in each column) among AtATM1, AtATM2, and AtATM3 and examples of ATM-like half-molecule ABC transporters from other sources |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| AtATM1                          | AtATM2          | AtATM3          | HsABC7          | HsMTAB3         | ScATM1          | SpHMT1          |
| AtATM1                          | 100             | 89             | 81             | 73             | 61             | 65             | 54             |
| AtATM2                          | 85              | 100            | 78             | 70             | 61             | 64             | 53             |
| AtATM3                          | 73              | 70             | 100            | 73             | 64             | 62             | 56             |
| HsABC7                          | 53              | 50             | 56             | 100            | 61             | 68             | 55             |
| HsMTAB3                         | 43              | 43             | 46             | 41             | 100            | 58             | 62             |
| ScATM1                          | 48              | 45             | 46             | 49             | 39             | 100            | 61             |
| SpHMT1                          | 36              | 36             | 37             | 36             | 46             | 38             | 100            |

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**FIGURE 1. Phylogenetic analysis of ATM subfamily sequences from yeast, mammals and Arabidopsis.** The full amino acid sequences were aligned using ClustalX (21) and subjected to phylogenetic analysis by the distance with neighbor-joining method using the phylogenetic analysis program PAUP (version 4.7b10) (available on the World Wide Web). The structure of the tree was confirmed by the bootstrap analysis of 1000 replicates. The bootstrap percentages are shown at each branch point. Branch lengths are proportional to phylogenetic distance. The protein sequences employed for this analysis (accession numbers in parenthesis) were as follows: AtATM1 (At4g28630), AtATM2 (At4g28620), AtATM3 (At5g58270), HsABC7 (AF133659), HsMTAB3 (AB039371), ScATM1 (X82612), SpHMT1 (Q02592), and MmABC7 (XM907304). The first two letters of the acronym denote the organisms, A. thaliana (At), Homo sapiens (Hs), S. cerevisiae (Sc), S. pombe (Sp), or M. musculus (Mm), from which the sequences were derived.
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A. ATM1::GFP

B. ATM2::GFP

C. ATM3::GFP

RT-PCR Analyses of AtATM Expression—Given the high sequence identity among AtATM genes at the DNA level, especially between AtATM1 and AtATM2, whose coding sequences are 85% identical, it was anticipated, and indeed found, that standard Northern analyses of the steady state levels of gene-specific transcripts would not be practicable. For this reason and because AtATM1 and AtATM2 are in tandem on chromosome IV such that the 3′-untranslated region of the former might overlap with the 5′-untranslated region of the latter, the expression analyses were conducted by RT-PCR using gene-specific primers. In this way, it was determined that the expression patterns of the three genes were distinguishable. Of the three classes of transcript, those derived from AtATM3 were expressed at the highest levels in all of the tissues examined, particularly in flowers, siliques, stems, and roots (Fig. 3). Comparison of these results with the microarray data compiled at Genevestigator (available on the World Wide Web) disclosed the same basic expression pattern for this gene. By contrast, the RT-PCRs with primers specific for AtATM2, despite indications of a slight increase in expression in flowers and roots, yielded weak signals regardless of the tissue from which the RNA samples were derived (Fig. 3). An interesting feature of AtATM2 was its susceptibility to increased expression in the rosette leaves of plants after a 7-day period of iron deprivation (data not shown). The expression patterns for AtATM1 were generally intermediate between those of AtATM3 and AtATM2 with relatively high levels in rosette leaves and roots and diminished levels in cauline leaves (Fig. 3).

Heterologous Expression of AtATM1, AtATM2, and AtATM3 in Yeast atm1 Mutants—The functional properties of the Arabidopsis AtMs were probed through their heterologous expression in yeast atm1 mutant strain x392b-4c. Yeast strain x392b-4c has four distinctive traits: it grows very slowly (has a petite phenotype) when grown on minimal medium, is deficient in respiration, mediates constitutive high affinity iron uptake across the plasma membrane, and accumulates high mitochondrial levels of iron. Each of these properties was monitored in the AtATM transformants of strain x392b-4c to assess the capacity of AtATM1, AtATM2, and/or AtATM3 to simulate the action of wild-type yeast ATM1.

Although a basic equivalence, with some quantitative differences, between AtATM1 and AtATM3 was discernible at all four levels, AtATM2 presented a number of problems that complicated interpretation of its effects. Transformation of strain x392b-4c with pYES3-AtATM1 or pYES3-AtATM3 restored colony size of the atm1 mutant strain x392b-4c. Yeast strain x392b-4c has four distinctive traits: it grows very slowly (has a petite phenotype) when grown on minimal medium, is deficient in respiration, mediates constitutive high affinity iron uptake across the plasma membrane, and accumulates high mitochondrial levels of iron. Each of these properties was monitored in the AtATM transformants of strain x392b-4c to assess the capacity of AtATM1, AtATM2, and/or AtATM3 to simulate the action of wild-type yeast ATM1.

In investigating this effect further, heterologously expressed AtATM2 was determined to be toxic for yeast. Not only strain x392b-4c but also the wild-type parental yeast strain CM3262 grew poorly when transformed with pYES2-AtATM2, and this effect was seen even when the GAL1 promoter of the expression
vector should be maximally repressed when glucose is the sole carbon source (Fig. 4).

Exchange of Putative Targeting Sequences of AtATM1 and AtATM3—The differential effects of at least two of the three Arabidopsis ATMs when heterologously expressed in yeast were not attributable to differences in their putative targeting sequences. After estimating the length of their putative TPs (90 and 125 amino acid residues, respectively, for AtATM1 and AtATM3, using the SignalP program (available on the World Wide Web), the coding sequences for both TPs and the endogenous TPs were amplified and ligated together into pYES3 to yield constructs pYES3-TP1-AtATM1, pYES3-TP3-AtATM1, pYES3-TP1-AtATM3, and pYES3-TP3-AtATM3. Colony morphology after growth on minimal medium was then screened after transforming these constructs into yeast strain x392b-4c.

As is evident from the results shown in Fig. 5, the capacity of AtATM3 to suppress the petite phenotype of atm1 yeast strain x392b-4c was similar regardless of whether it retained its endogenous TP, TP3, or had had it replaced by TP1. An equivalent pattern was seen for AtATM1; it conferred partial suppression either with its own TP, TP1, or that from AtATM3, TP3. The implication is that the differential effects of AtATM1 and AtATM3 are attributable to their core structures rather than their TP sequences.

Subcellular Localization of AtATMs after Heterologous Expression in Yeast—If the properties of the Arabidopsis ATMs after heterologous expression are to have a direct bearing on their physiological role, it is crucial that they localize to the same subcellular structures in yeast as they do in plant cells. This was investigated by the expression of AtATM C-terminal GFP fusion proteins from the PGK promoter of pYES3 in atm1 mutant strain x392b-4b and the isogenic wild-type strain CM3262. By this approach, it was determined that the activities of the fusions were similar to those of the corresponding native proteins except that the toxicity of AtATM2 was alleviated. Although heterologous expression of AtATM3::GFP suppressed the petite colony phenotype of strain x392b-4b (Fig. 5), as did AtATM1::GFP, albeit at lower efficacy, AtATM2::GFP did not (data not shown). However, the C-terminal fusion of AtATM2 with GFP abolished the former’s capacity to inhibit growth of the yeast transformants and yielded a polypeptide...
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that, like AtATM1::GFP and AtATM3::GFP, assumed a punctate intracellular localization coincident with that of MitoTracker Red (Fig. 6). As was found for the in planta distribution of the same fusions, the mitochondrial localization of AtATM3::GFP in the yeast transformants was better defined than those of AtATM1::GFP and AtATM2::GFP, the latter of which had the most diffuse distribution (Fig. 6).

Cellular Iron Uptake—By comparison with those of wild-type CM3262 yeast, cells of the atm1 mutant mediated constitutive high affinity iron uptake (Fig. 7), which was suppressed by the AtATMs to extents commensurate with suppression of the atm1 petite phenotype. High affinity iron uptake by the pYES3-AtATM3/X392b-4c transformants was diminished from a level 6–7 times greater to a level only 2 times greater than wild type (Fig. 7). In contrast, high affinity uptake by the pYES3-AtATM1/X392b-4c transformants was diminished by only 13% versus untransformed or empty pYES3 vector-transformed controls (Fig. 7).

Recovery of Yeast Mitochondrial Function—Direct participation of AtATM1, AtATM2, and/or AtATM3 in alleviation of the yeast atm1 phenotype at the mitochondrial level was examined in three ways: by assaying for mitochondrial respiratory function, mitochondrial cytochrome levels, and mitochondrial iron accumulation.

Mitochondrial respiratory function was assessed by the tetrazolium overlay technique (20). In this way, it was determined that colonies derived from wild-type controls or pYES3-AtATM3/X392b-4c transformants were competent in the reduction of tetrazolium and stained a deep red color (Fig. 8). A similar pattern, albeit less clear cut, was determined for the colonies derived from pYES3-AtATM1/X392b-4c transformants (Fig. 8). By contrast, the majority of the colonies derived from pYES2-AtATM2/X392b-4c transformants and those from untransformed and empty pYES3 vector-transformed X392b-4c cells lacked this activity and failed to stain red (Fig. 8). The origin of the few atm1 strain X392b-4c colonies that stained red is not known but may be attributable to rho− conversion and/or nuclear reversion, two events that occur at relatively high frequency in this strain.8

During the course of these investigations of AtATM1 and AtATM3, it was noted that although the mitochondria purified from wild-type yeast and those from pYES3-AtATM1/X392b-4c and pYES3-AtATM3/X392b-4c transformants were pale brown in color, those from untransformed strain X392b-4c were white. Suspecting that this might be attributable to gross differences in cytochrome content, these mitochondrial preparations were subjected to reduced minus oxidized difference spectrophotometry for cytochromes, in general, and Western analysis for cytochrome c, in particular. The results of these analyses are summarized in Figs. 9 and 10, respectively.

Although the difference spectra of mitochondria purified from atm1 mutant strain x392b-4c and the same strain after transformation with empty pYES3 vector were devoid of cytochrome absorbance maxima, the equivalent preparations from wild-type strain CM3262 and from pYES3-AtATM3/X392b-4c or pYES3-AtATM3::GFP/X392b-4c transformants, and to a lesser extent from pYES3-AtATM1/X392b-4c transformants, yielded clear absorbance maxima at 550 nm characteristic of c-type (c and c1) cytochromes (Fig. 9). Accordingly, Western blots of the same preparations after SDS-PAGE, electrophoresis, and immunoreaction with polyclonal antibody raised against yeast cytochrome c demonstrated near wild-type levels of cytochrome c in the mitochondrial preparations from CM3262 cells and pYES3-AtATM3/X392b-4c cells and an attenuated but readily discernible cytochrome c signal in the preparations from pYES3-AtATM1/X392b-4c cells (Fig. 10). Western blots of the same preparations from untransformed or empty pYES3 vector- or pYES2-AtATM2-transformed X392b-4c cells, by contrast, lacked cytochrome c (Fig. 10). These differences were not attributable to a diminution of the intracellular mitochondrial titer or differences in membrane protein yield, because the same blots when probed with monoclonal antibody raised against yeast mitochondrial porin yielded a band of similar intensity in all of the preparations (Fig. 10).

Since excessive mitochondrial iron accumulation has been clearly implicated in the atm1 mutant phenotype (6, 7, 8, 11,
24), the capacity of AtATM1, AtATM2, and/or AtATM3 to suppress mitochondrial accumulation of this metal was investigated. For this purpose, mitochondria were purified from yeast cells that had been incubated in media containing $^{55}$Fe and subjected to liquid scintillation counting to determine their steady state levels of radioactive iron (15). The results of these analyses were highly instructive. They demonstrated that although the steady levels of $^{55}$Fe in the mitochondrial fractions from empty pYES3 vector- or pYES2-AtATM2-transformed x392b-4c cells were high ($48.5 \pm 1.8$ and $45.19 \pm 4.69$ nmol/mg protein, respectively), the corresponding values for the wild-type strain CM3262 or for the pYES3-AtATM3 transformants were low ($2.51 \pm 0.49$ and $3.81 \pm 0.21$ nmol/mg protein, respectively) (Fig. 11). By comparison, the steady state levels of mitochondrial $^{55}$Fe established by the pYES3-AtATM1/c392b-4c transformants were intermediate in magnitude ($21.71 \pm 3.64$ nmol/mg protein) (Fig. 11).

**DISCUSSION**

The investigations described here substantiate the conclusions drawn by Kushnir et al. (13), namely that AtATM3 (STA1) is a mitochondrially localized functional ortholog of yeast ATM1. However, through the application of more refined complementary cellular biochemical approaches, the findings presented here extend the analysis to show that AtATM3 and to a lesser extent AtATM1, but not AtATM2, exert their effects at the level of the central role played by the mitochondrion in cellular iron homeostasis. AtATM1, AtATM2, and AtATM3 clearly belong to the same small subfamily of forward orientation, half-molecule ABC transporters, but AtATM3 closely followed by AtATM1 bears the closest resemblance to the yeast prototypical ATM at a functional level. AtATM2, by contrast, has properties that clearly distinguish it from the other two Arabidopsis ATMs and preclude its detailed analysis by the...
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FIGURE 11. Mitochondrial $^{55}$Fe contents of empty pYES3 vector-transformed wild-type yeast strain CM3262 (wild type:pYES3), or yeast atm1 strain x392b-4c after transformation with the vector (atm1::pYES3)), pYES3-AtATM1 (atm1::[ATATM1]), pYES2-AtATM2 (atm1::[ATATM2]), pYES3-AtATM3 (atm1::[ATATM3]), pYES3-AtATM1::GFP (atm1::[ATATM1::GFP]), pYES3-AtATM2::GFP (atm1::[ATATM2::GFP]), or pYES3-AtATM3::GFP (atm1::[ATATM3::GFP]). Steady state mitochondrial $^{55}$Fe uptake was established and estimated as described under "Materials and Methods."

approaches described. Heterologously expressed AtATM3 and to a lesser extent AtATM1 suppress the severe petite phenotype of yeast atm1 mutants, and in both cases this effect closely parallels the degree to which each alleviates the specific losses of function that accompany mutation. AtATM3 not only fully reverses the impaired growth but also all of the other properties of yeast atm1 mutants. When heterologously expressed in yeast atm1 mutants, AtATM3 restores respiratory competence and mitochondrial cytochrome levels concomitant with the reestablishment of wild-type levels of cellular high affinity iron uptake and steady state mitochondrial iron content. The effects exerted by heterologously expressed AtATM1 are similar but less pronounced. Moreover, regardless of whether AtATM3::GFP or AtATM1::GFP is heterologously expressed in yeast or ectopically expressed in planta, their GFP fluorescence colocalize with that of MitoTracker Red, although the signal from the AtATM3 fusion is more punctate, less diffuse, than that from the AtATM1 fusion. Of the three Arabidopsis ATM transcripts, those derived from AtATM3 are most widely and highly expressed, as would be expected of a gene encoding a core mitochondrial transporter. By comparison, the steady state levels of AtATM1 transcripts are generally lower and more restricted in their tissue distribution. As indicated by the results from the heterologous expression of derivatives of AtATM3 and AtATM1, in which the coding sequence for the putative targeting peptide of each was substituted by the coding sequence for the putative targeting peptide of the other, the patterns of localization and phenotypic repercussions of these two AtATMs, at least in yeast, are the same irrespective of the identity of their putative targeting sequences.

The properties of AtATM2 are quite distinct from those of AtATM3 and AtATM1. Despite its possession of an N-terminal mitochondrial targeting sequence cleavage site and its mitochondrial localization, as judged by the intracellular distribution of AtATM2::GFP fusions in both yeast and Arabidopsis, AtATM2 does not appear to suppress the atm1 phenotype at any level. Instead, it is toxic, not only for the yeast atm1 mutant but also for the CM3262 parental strain.

The mechanistic basis of the toxicity of AtATM2 is not known, but its effects are not restricted to yeast but extend to E. coli. Neither yeast nor E. coli AtATM2 transformants grow well even at the low levels of expression achieved in the former when it is expressed from the yeast GAL1 promoter or in the latter system when expression is from the yeast PGK promoter (25). Presumably, the toxicity of AtATM2 prohibits its participation in yeast ATM1-like processes, or this particular member of the Arabidopsis ATM subfamily participates in processes other than mitochondrial iron/sulfur cluster export. Although this phenomenon and the finding that the C-terminal fusion of AtATM2 with GFP alleviates its toxicity but has no effect on its inability to suppress the yeast atm1 phenotype warrants further investigation, it is complicated by a number of other factors.

Among these are the immediate adjacency of AtATM2 and AtATM1 on chromosome IV, the high sequence identities of these two genes, and the lack of abiotic and biotic factors known to modulate expression of the former. In combination, these factors seriously compromise the application of reverse genetic approaches to this gene.

It is noteworthy that of all of the eukaryotes for which comprehensive genomic sequence information is available, Arabidopsis is currently the only one known to contain three ATM homologs; all of the others contain only one or two homologs. When account is taken of their chromosome distributions and sequence relatedness, it is therefore probable that the three Arabidopsis ATM homologs arose from an ancestral gene, that corresponding to AtATM3, by two gene duplication events. The first was a duplication of AtATM3 to give AtATM1; the second, the more recent, was a tandem duplication of AtATM1 to give AtATM2. It remains to be determined if this phenomenon is peculiar to Arabidopsis, but the rice genome contains only one ATM homolog (3), and BLAST searches and phylogenetic analyses of the recently sequenced poplar genome disclose only two homologs. Thus, if it is assumed that AtATM3 is representative of the ancestral gene, which is supported by the fact that all three of the homologs from other plant sources most closely resemble this Arabidopsis ATM, and AtATM3, alone, appears to be sufficient for mitochondrial iron/sulfur cluster transport, it might explain how the other two AtATM genes to some extent escaped the functional constraints to which AtATM3 is subject and acquired some new functions or relaxed their old ones. AtATM1 and AtATM2 may represent different stages in the molecular drift or decay of AtATM3, which, in turn, could account for the inability of AtATM2 to suppress the yeast atm1 mutant phenotype and the diffuse intracellular targeting of its GFP fusions.

A facet of plant ATMs that has drawn some attention is their differential susceptibility to expression activation by heavy metals. Although AtATM1 appears to be expressed constitutively, AtATM3 has a requirement for exposure to heavy metals, such as cadmium, for maximal expression (14). These findings in combination with others showing that atatm3 mutants are more sensitive to cadmium in the growth medium than wild-
type controls and that ectopic overexpression of AtATM3 confers enhanced tolerance toward cadmium and lead (14) indicate that this AtATM is capable of contributing to heavy metal detoxification. AtATM3 is not unique among the ATMs in this regard in that another member of this subfamily from the unicellular green alga Chlamydomonas reinhardtii, CrCDS1, has been identified that also contributes to cadmium tolerance (26). Designated CrCDS1, this gene, which when insertion-mutagenized, confers a cadmium-hypersensitive phenotype, is subject to induction by cadmium, and encodes a 1062-amino acid residue mitochondrially localized protein bearing 40–50% sequence identity (60–70% similarity) to the yeast and Arabidopsis ATMs (26). The key question here is whether AtATM3 and/or CrCDS1 participate directly in the transport of cadmium or exert their effects indirectly. Do AtATM3 and/or CrCDS1 alleviate toxicity directly by contributing to the efflux of cadmium from the mitochondrial matrix, possibly by catalyzing the ATP-energized efflux of iron/sulfur-like cadmium thiolates, or do they exert their effects indirectly by maximizing the efflux of iron/sulfur clusters from the mitochondrial matrix? For instance, do they abrogate the accumulation of toxic levels of free iron that would otherwise arise in the matrix by the formation of cadmium-thiol adducts consequent on displacement of iron from the iron/sulfur clusters fabricated in this compartment? In this way, AtATM3 and/or CrCDS1 might act to maintain free iron at levels insufficient to promote the generation of highly reactive hydroxyl (OH•) radicals by Fenton’s reaction. Of these two alternatives, which are not necessarily mutually exclusive, the latter is at least consistent with the findings reported here, which demonstrate the impact AtATM3 and, to a lesser extent, AtATM1 have on cellular iron homeostasis, as indicated by the suppression of high affinity cellular iron uptake concomitant with the abolition of intramitochondrial iron hyperaccumulation.

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