An Allelic Mutant Series of ATM3 Reveals Its Key Role in the Biogenesis of Cytosolic Iron-Sulfur Proteins in Arabidopsis

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The ATP-binding cassette transporters of mitochondria (ATMs) are highly conserved proteins, but their function in plants is poorly defined. Arabidopsis (Arabidopsis thaliana) has three ATM genes, namely ATM1, ATM2, and ATM3. Using a collection of insertional mutants, we show that only ATM3 has an important function for plant growth. Additional atm3 alleles were identified among sirtinol-resistant lines, correlating with decreased activities of aldehyde oxidases, cytosolic enzymes that convert sirtinol into an auxin analog, and depend on iron-sulfur (Fe-S) and molybdenum cofactor (Moco) as prosthetic groups. In the sirtinol-resistant atm3-3 allele, the highly conserved arginine-612 is replaced by a lysine residue, the negative effect of which could be mimicked in the yeast Atm1p ortholog. Arabidopsis atm3 mutants displayed defects in root growth, chlorophyll content, and seedling establishment. Analyses of selected metal enzymes showed that the activity of cytosolic aconitase (Fe-S) was strongly decreased across the range of atm3 alleles, whereas mitochondrial and plastid Fe-S enzymes were unaffected. Nitrate reductase activity (Moco, heme) was decreased by 50% in the strong atm3 alleles, but catalase activity (heme) was similar to that of the wild type. Strikingly, in contrast to mutants in the yeast and mammalian orthologs, Arabidopsis atm3 mutants did not display a dramatic iron homeostasis defect and did not accumulate iron in mitochondria. Our data suggest that Arabidopsis ATM3 may transport (1) at least two distinct compounds or (2) a single compound required for both Fe-S and Moco assembly machineries in the cytosol, but not iron.

Plant cells contain more than 50 iron-sulfur (Fe-S) enzymes that carry out important redox and catalytic functions in many aspects of metabolism (Imsande, 1999; Balk and Lobréaux, 2005). The assembly of Fe-S cofactors is mediated by dedicated machinery of ancient evolutionary origin. In plants, mitochondria harbor homologs of the bacterial ISC (for iron sulfur cluster) proteins, while plastids have inherited the sulfur mobilization machinery from their cyanobacterial ancestor (Balk and Lobréaux, 2005; Kessler and Papenbrock, 2005; Pilon et al., 2006). Plant cytosol contains homologs of the cytosolic Fe-S assembly proteins that have recently been identified in yeast (Lill and Mühlenhoff, 2008), including the scaffold protein AtNBP35 (Bych et al., 2008; Kohbushi et al., 2009) and the hydrogenase-like AtNAR1 (Cavazza et al., 2008). It is thought that cytosolic Fe-S cluster assembly is dependent on at least one of the organelles, because the Cys desulfurases that generate sulfur for Fe-S clusters, CpnIIIS and NFS1, are strictly localized in the plastids and mitochondria, respectively (Kushnir et al., 2001; Frazzon et al., 2007; Van Hoewyk et al., 2007).

In yeast, cytosolic and nuclear Fe-S cluster assembly depends on the mitochondrial ISC pathway and on the ATP-binding cassette (ABC) transporter of the mitochondria, Atm1p (Kispal et al., 1999). Atm1p is classified as a “half-transporter” that functions as a homodimer and is localized in the mitochondrial inner membrane with the ATPase domains at the matrix side (Leighton and Schatz, 1995). The orientation indicates that the direction of transport is from the mitochondrial matrix to the intermembrane space and cytosol. In accordance, mutations of yeast ATM1 cause a defect in cytosolic/nuclear Fe-S cluster assembly but not in mitochondrial Fe-S cluster assembly (Kispal et al., 1999). The substrates of Atm1p, however, or of its functional orthologs in other eukaryotes have not been identified thus far.

Yeast atm1 mutations also disrupt iron homeostasis: iron uptake transporters are constitutively expressed independent of the iron concentration, and iron accumulates 10- to 30-fold in the mitochondria (Kispal...
et al., 1997, 1999). Mutations in the human ortholog ABCB7 are the cause of X-linked sideroblastic anemia with ataxia, in which one of the symptoms is mitochondrial iron overload (Rouault and Tong, 2008). Moreover, ATMs are widespread and highly conserved in β-proteobacteria and could be involved in nickel and cobalt resistance (Mikolay and Nies, 2009).

The Arabidopsis (Arabidopsis thaliana) genome contains more than 100 ABC transporters, of which the ATMs form a small subfamily in the B group consisting of three genes: ATM1, ATM2, and ATM3 (Rea, 2007). The genes were first identified in Arabidopsis by Kushnir et al. (2001) and were named STA2, STA3, and STA1, respectively. (A proposal for a unified nomenclature [Verrier et al., 2008] has suggested the gene symbols AtABCB24, AtABCB23, and AtABCB25 for AtATM1, AtATM2, and AtATM3, respectively. However, for consistency with previous publications, we have adhered to the ATM gene symbols in this paper.) Expression of GFP fusions showed that all three ATM proteins localized to mitochondria (Kushnir et al., 2001; Chen et al., 2007). Arabidopsis ATM3 could functionally complement the yeast atm1 phenotype (Kushnir et al., 2001; Chen et al., 2007), whereas Arabidopsis ATM1 complemented poorly and ATM2 expression was toxic in yeast (Chen et al., 2007). Until now, functional analysis of the ATM genes in Arabidopsis has been restricted to one atm3 mutant, called starik (Kushnir et al., 2001), in which the protein lacks the C-terminal ATPase domain. The atm3-1 (sta1) mutant is dwarfed and chlorotic, but the biochemical basis of its pleiotropic phenotypes is unknown. In addition, induced expression of ATM3 in response to cadmium and lead, as well as sensitivity of the atm3-1 (sta1) mutant to cadmium, have implicated the ATM3 transporter in heavy metal tolerance (Kim et al., 2006).

We have analyzed a comprehensive set of mutants in the Arabidopsis ATM genes and found that ATM3 plays a key role in plant metabolism, while mutants in ATM1 and ATM2 did not display an obvious phenotype. Genetic and biochemical evidence from an atm3 allelic series showed that ATM3 is important for the activity of cytosolic Fe-S and molybdenum cofactor (Moco) enzymes, but it does not play a major role in metal homeostasis.

RESULTS
ATM3, But Not ATM1 and ATM2, Has a Critical Function under Normal Growth Conditions

To investigate the functions of ATM1, ATM2, and ATM3 in Arabidopsis, insertion mutants were obtained from the Arabidopsis stock centers (Fig. 1A; for details, see Table I). Homozygous plants were analyzed by reverse transcription (RT)-PCR, confirming the absence of ATM1 transcript in the atm1-1 and atm1-2 lines and the absence of ATM3 transcript in the atm3-2 insertion line (Fig. 1B). The expression of ATM2 in the atm2 line could not be assessed, as no RT-PCR product of ATM2 could be detected in the wild type or any other line. However, the ATM2 primer set gave a positive PCR with genomic DNA. When grown on soil under long-day conditions, the atm1-1, atm1-2, and atm2 insertion mutants grew like the wild type, while the atm3-2 insertion mutant was dwarfed and pale green. The atm1-1 atm3-2 double mutant was very...
similar to atm3-2 plants, indicating that ATM1 plays a
minor role compared with ATM3 (Fig. 1D).

Interestingly, we found new alleles of Arabidopsis
ATM3 in screens for resistance to sirtinol. This com-
 pound mimics auxin in the phenotypic responses it
causes in plants and has been used for genetic screens
that have uncovered mutants in genes involved in
auxin signaling (Zhao et al., 2003) and Moco biosyn-
thesis (Dai et al., 2005). Mapping and cloning of the
atm3-3 mutation identified a point mutation (G-1,835
to A) causing a substitution of a highly conserved Arg
to Lys (R612K). The atm3-4 mutant line was allelic with
atm3-3 and was found to have a 39-nucleotide deletion
in the promoter extending into the 5′ untranslated
region, resulting in approximately 90% lower tran-
script levels as determined by quantitative RT-PCR
(Fig. 1C).

The occurrence of atm3 mutant alleles in genetic
screens, higher expression levels, and the strong pheno-
type of the atm3-2 insertion mutant indicate that
ATM3 plays a crucial role in plant growth and devel-
opment, whereas ATM1 and ATM2 have negligible
functions under normal conditions.

Phenotypic Analysis of atm3 Alleles

Next, we focused on the atm3 mutant alleles to better
understand the function of ATM3 in plants. Together
with the previously described starik mutant (Kushnir
et al., 2001), the sirtinol mutants and insertion mutant
formed an allelic series ranging from mild to severe
(Fig. 2A). In this paper, the alleles will be indicated by
the nature of their mutation to immediately link this to
each phenotype, as follows: R612K (Arg-612 to Lys
substitution), Δprom (promoter deletion), ΔNBD (de-
letion of the nucleotide-binding domain), and T-DNA
(insertion mutant); for further details, see Table 1.

Inspection of rosette growth showed that only the
ΔNBD and T-DNA mutants had a dwarfed stature
(Fig. 2A). The same mutant alleles were also severely
chlorotic, with total chlorophyll levels decreased by
more than 50% (Fig. 2B). In contrast, the R612K and
Δprom alleles had a more modest but significant 10%
decrease in chlorophyll content. Further analysis of the
atm3-1 allele indicated that the lower chlorophyll
levels are attributable to lower chloroplast numbers
with normal chlorophyll content (Supplemental Fig. S1).

Although the R612K mutant grew as well as the
wild type aboveground, the average root length was
shortened by nearly 60% in seedlings 8 d after germi-
nation (Fig. 2C). The average root lengths of the
Δprom, ΔNBD, and T-DNA seedlings were even fur-
ther diminished. The atm3 alleles were fertile except
for the T-DNA (atm3-2) mutant, which did not produce
seeds (Fig. 2D). Anthers were shrunk and failed to
release pollen, but female fertility could be demon-
strated in crosses, for instance with atm1-1 (Fig. 1D).

In addition to the previously described chlorosis
and dwarfism (Kushnir et al., 2001), we observed a
defect in seedling establishment under low-light con-
ditions. In particular, the R612K, ΔNBD, and T-DNA
atm3 alleles showed a decrease in the percentage of
seedlings that developed true foliage after 14 d (Fig.
2E). Seedling establishment was fully rescued to wild-
type levels by 1% (w/v) Suc in the medium, but
addition of Suc did not reverse the chlorosis (data not
shown).

Interestingly, the T-DNA insertion mutant, which is
likely to represent a null mutant, has a stronger
phenotype than the ΔNBD mutant. The latter expresses
a fusion protein of the membrane domain and neom-
ycin phosphotransferase. If the membrane domain is
correctly folded and inserted, this may function as a
pore for passive transport of the substrate.

Taken together, broad phenotypic analysis of the
atm3 allelic series shows that chlorosis and decreased
root growth are primary defects, while dwarfism and
male sterility are probably secondary phenotypes.

ATM3 Is Required for the Activities of Cytosolic
Fe-S Enzymes

To investigate the underlying biochemical causes of
the observed phenotypes, we first analyzed the activi-
ties of aldehyde oxidases (AO). These enzymes have
been implicated to catalyze the conversion of the
sirtinol-derivative 2-hydroxy-1-naphthaldehyde to the
auxin analog 2-hydroxy-1-naphthaldehyde (Dai et al.,
2005), and the R612K and Δprom alleles were isolated
as sirtinol-insensitive lines. In-gel analysis of AO ac-
tivities in leaves showed that all three active isoforms
were decreased by 10-fold or more in the Δprom,
ΔNBD, and T-DNA mutants (Fig. 3A; quantification by
densitometry not shown). The levels of AO activities

Table 1. Summary of mutant alleles used in this study

| Locus          | Allele | This Study | Polymorphism                  | Mutation(s)                      |
|---------------|--------|------------|--------------------------------|----------------------------------|
| AT4G28630 = ATM1, STA2 | atm1-1 | atm1-1     | SALK_090939                    | T-DNA insertion (+34)            |
| atm1-2        |        |            | SALK_121795                    | Tandem T-DNA insertion (+517)    |
| AT4G28620 = ATM2, STA3 | atm2   | atm2       | WiscDsLox293-296invB11         | T-DNA insertion (+498)           |
| atm3-1, sta1  | ΔNBD   |            | star1 (Babiychuk et al., 1997) | T-DNA insertion (+1,458) resulting in NPTII fusion protein |
| atm3-2        |        |            |                                | T-DNA insertion (+426)           |
| atm3-3        | R612K  |            |                                | Point mutation, G-1,835 → A     |
| atm3-4        | Δprom  |            |                                | Deletion of –63 to –25           |

Fe-S Enzymes

ATM3 Is Required for the Activities of Cytosolic Fe-S Enzymes

To investigate the underlying biochemical causes of the observed phenotypes, we first analyzed the activities of aldehyde oxidases (AO). These enzymes have been implicated to catalyze the conversion of the sirtinol-derivative 2-hydroxy-1-naphthaldehyde to the auxin analog 2-hydroxy-1-naphthaldehyde (Dai et al., 2005), and the R612K and Δprom alleles were isolated as sirtinol-insensitive lines. In-gel analysis of AO activities in leaves showed that all three active isoforms were decreased by 10-fold or more in the Δprom, ΔNBD, and T-DNA mutants (Fig. 3A; quantification by densitometry not shown). The levels of AO activities...
in the R612K allele of ATM3 may be decreased slightly, but this is difficult to assess with the semiquantitative in-gel method. In the atm2 and atm1-1 mutants, the upper two AO isoforms were similar in activity to the wild type, while the lower isoform was increased in activity. Xanthine dehydrogenase (XDH) activity, an enzyme that is similar in domain structure to AO, was also decreased, correlating with the severity of the atm3 mutant allele.

AO and XDH are cytosolic enzymes containing two [2Fe-2S] clusters, Moco, and FAD. The first steps of the biosynthetic pathways of both Fe-S clusters and Moco are localized in the mitochondrial matrix (Balk and Lobréaux, 2005; Mendel and Bittner, 2006); therefore, the abundance of either or both cofactors could depend on ATM3. To unravel these possibilities, we first measured the activity of nitrate reductase (NR), a cytosolic enzyme depending on Moco, heme, and FAD. NR activity was decreased to about 50% in the ∆NBD and T-DNA alleles (Fig. 3B), while the activity of catalase, a peroxisomal heme-dependent enzyme, was not significantly lower (Fig. 3C). These data may indicate a defect in Moco biosynthesis resulting in a 2-fold decrease in Moco, but this cannot account for the ≥10-fold lower AO and XDH activities.

Second, we analyzed the activity of cytosolic aconitase, an enzyme with one [4Fe-4S] cluster. For this purpose, a non-denaturing gel system was developed to separate the three highly similar aconitase isoforms from small leaf samples. The activity was visualized by coupling the aconitase activity to NADP-dependent isocitrate dehydrogenase and the reduction of tetrazolium salt, forming a blue-purple formazan precipitate. Analysis of insertional knockout mutants of ACO1, ACO2, and ACO3 showed that the activity with the lowest electrophoretic mobility (the upper band) corresponded to ACO1 protein (Fig. 4A, left panel). ACO1, which lacks a mitochondrial targeting sequence, has previously been shown to provide the majority of aconitase activity in the cytosol (Arnaud

Figure 2. Phenotypic analysis of an allelic series of atm3 mutants. A, Rosette growth of the wild type and atm3 mutant alleles after 4 weeks in 16 h of light/8 h of dark at 20°C. B, Chlorophyll (Chl) levels (µg mm⁻²) are decreased in atm3 alleles. The proportions of chlorophyll a and b levels are indicated by light and dark gray, respectively, and as numerical values below the graphs. n = 6 for the wild type (WT), R612K, and ∆prom; n = 4 for ∆NBD; n = 3 for T-DNA. Error bars represent S.D. C, Root length of wild-type and atm3 seedlings 4 and 8 d after planting. Seedlings were grown on vertical agar medium containing half-strength Murashige and Skoog medium and 1% (w/v) Suc, 18°C ± 2°C, except for the T-DNA line, where n = 5. Error bars represent S.D. D, Fertility in the wild type, atm3-1 (∆NBD), and atm3-2 (T-DNA) alleles. Bars = 5 mm in the siliques (left), 1 mm in the flowers (middle), and 0.3 mm in the anthers (right). Anthers were stained with Alexander's stain to visualize viable pollen. E, The effect of low light (8 h of light/16 h of dark, 70 µmol m⁻² s⁻¹) on the frequency of establishment in wild-type and atm3 seedlings. Seedlings were grown on Murashige and Skoog medium plus or minus 1% (w/v) Suc and scored for the development of true foliage leaves after 14 d (inset); n ≥ 50.
et al., 2007). Its cytosolic localization was confirmed by protein blot analysis of supernatant and pellet fractions prepared by gentle disruption of leaf protoplasts (Fig. 4A, right panel). ACO2 and ACO3 fractionated in the pellet fraction that contains mitochondria, in agreement with proteomics data (see the SUBA database at http://www.plantenergy.uwa.edu.au). Next, we analyzed the in-gel aconitase activities in wild-type and atm leaves. The cytosolic ACO1 activity was strongly decreased in atm3 mutant alleles, whereas the mitochondrial aconitase activities were equal (Fig. 4B, top panel; note that the ACO3 activity is low relative to its protein abundance). The intensity of activity staining of ACO1 was decreased by approximately 20% in R612K, 40% in Δprom, and 60% in ΔNBD (see quantification in Supplemental Fig. S2). The decrease in ACO1 activity was paralleled by the abundance of holoprotein, as shown by protein-blot analysis of the native gel (Fig. 4B, middle panel). In contrast, the transcript levels of ACO1 were equal in all atm3 alleles (Fig. 4C).

To corroborate the in-gel aconitase results, cytosolic and mitochondrial fractions were prepared from root callus. Photometric enzyme analysis showed a decrease in aconitase activity in the cytosolic fractions of the atm3 lines, correlating with the severity of the atm3 mutation, but similar mitochondrial aconitase activity (Fig. 4D). At first, the magnitude of decrease seemed less than that observed in-gel, but we found that a large proportion of the aconitase activity in the cytosolic fraction can be attributed to contamination with mitochondrial matrix proteins. The activity of fumarase, an exclusively mitochondrial enzyme, was approximately 20% in the cytosolic fractions of all atm3 callus lines, which would correspond to 40 milliunits mg\(^{-1}\) aconitase activity from mitochondrial origin (Fig. 4D, indicated by the asterisk). Subtracting this showed that the actual cytosolic aconitase activity was decreased by approximately 30% in the Δprom and by approximately 70% in the T-DNA insertion mutant, corresponding to the in-gel analysis. The activities of cytosolic AO and XDH were also decreased in the callus lines (data not shown). In summary, ATM3 is primarily required for the activity of cytosolic Fe-S enzymes and to some extent for Moco enzymes but not for heme-dependent enzymes.

**ATM3 Is Not Required for Mitochondrial and Plastid Fe-S Enzymes**

To further investigate the impact of ATM3 mutations on organellar Fe-S proteins, key enzymes and processes that are dependent on Fe-S clusters were analyzed in the mitochondria and plastids. First, electron transfer from succinate to cytochrome c, using complex II (three Fe-S clusters) and complex III (one Rieske-type Fe-S cluster, heme) of the mitochondrial respiratory chain, was measured in callus mitochondria. Cytochrome c reduction rates were similar in
atm3 alleles, atm1-1, and wild-type mitochondria (Fig. 4E). Second, leaf extracts were analyzed for the activity of nitrite reductase (NiR), a Fe-S- and heme-dependent enzyme exclusively localized in the plastids. NiR activity was not decreased in the atm3 phenotypic series (Fig. 4F), although variations in its activity occurred, most notably an increase in NiR activity in ΔNBD correlating with lower NR activity (Fig. 3B).

To assess the function of Fe-S proteins in the photosynthetic machinery, fluorescence and P700 absorbance parameters were recorded with a Dual-PAM-100 measuring system in intact leaves (Table II). P700 oxidation is a measure of PSI activity, which is dependent on three [4Fe-4S] clusters. Maximal P700 oxidation (ΔA820/A820) was halved in atm3-1 (ΔNBD) and decreased to one-third in nfu2.1 compared with the wild type. However, when normalized to chlorophyll content, the P700 absorbance change in atm3-1 was similar to that in the wild type, in agreement with normal PSI activity measured in isolated chloroplasts (Supplemental Fig. S3). In the nfu2.1 mutant, in contrast, normalized P700 absorbance was 2-fold lower than in the wild type, reflecting the lower PSI levels reported previously (Touraine et al., 2004; Yabe et al., 2004). Furthermore, a 3-fold increase of the parameter Y(NA) indicated that P700 oxidation in nfu2.1 is acceptor limited (i.e. P700 cannot be oxidized by a saturation pulse due to a lack of available electron acceptors, including ferredoxin [2Fe-2S]). In contrast, no P700 acceptor-side limitation is observed in atm3 mutants.

Taken together, the normal activity of key mitochondrial and plastid Fe-S enzymes indicates that ATM3 is not required for Fe-S cluster assembly in these organelles.

**ATM3 Plays a Minor Role in Metal Homeostasis**

In yeast and human, dysfunction of Atm1p and ABCB7, respectively, leads to constitutive expression of iron-uptake genes and accumulation of iron in the mitochondria (Lill and Mühlenhoff, 2008; Rouault and Tong, 2008). To investigate whether this is the case in leaf protoplasts were disrupted and centrifuged at 12,000g to obtain pellet (P12) and supernatant (S12) fractions enriched in mitochondrial and cytosolic proteins, respectively. After separation on starch/PA gels, the fractions were blotted and labeled with aconitase antibodies (gray scale). B, In-gel aconitase activities (purple) and protein levels (gray scale) in wild-type, atm3, and atm1-1 leaves. For quantification, see Supplemental Figure S2. An equal volume (5 µL) of the native sample was separated on a denaturing gel, blotted, and labeled for actin as a loading control. C, RT-PCR of ACO1 and ACT8 in wild-type, atm3, and atm1-1 leaves. D and E, Activities of Fe-S enzymes in the cytosolic fraction (D) and purified mitochondria (E) of callus generated from roots of wild-type, atm3, and atm1-1 leaves (n = 3). Based on fumarase activity measurements, approximately 40 milliunits of aconitase in the cytosolic fraction is estimated to be of mitochondrial origin (indicated by the asterisk). Error bars represent s.d. F, NiR activity in leaf extracts of the wild type, atm3, and atm1-1 (n = 3). Error bars represent s.d.
atm3 mutants in Arabidopsis, the expression of two iron-regulated genes, encoding the root iron transporter IRT1 and ferritin, was analyzed at the protein level in the atm3-1 (ΔNBD) mutant. Seedlings were grown on minimal medium with 1, 10, 20, and 40 μM iron. As expected, the levels of IRT1 in wild-type roots were strongly increased under iron-limiting conditions (1 μM iron) but nondetectable under iron-replete conditions (10 μM iron and higher; Fig. 5A, top panels). In atm3-1 roots, IRT1 was also strongly induced under iron-limiting conditions, but contrary to the wild type, low levels of the protein were detectable at 10 and 20 μM iron. Ferritin levels in roots correlated inversely with those of IRT1 (Fig. 5A, top panels). In particular, atm3-1 roots had lower ferritin levels than the wild type, atm3 mutants, and nfu2.1 (Fig. 5A, bottom panels) but were overall 1.5 fold (Fig. 6D) and could cause the loss of cytochromes different from yeast cells upon down-regulation of the protein were strongly increased under iron-limiting conditions (1 μM iron) but nondetectable under iron-replete conditions (10 μM iron and higher; Fig. 5A, top panels). In atm3-1 roots, IRT1 was also strongly induced under iron-limiting conditions, but contrary to the wild type, low levels of the protein were detectable at 10 and 20 μM iron. Ferritin levels in roots correlated inversely with those of IRT1 (Fig. 5A, top panels). In particular, atm3-1 roots had lower ferritin levels than the wild type, corresponding to attenuated IRT1 expression at 10 to 20 μM medium iron. At 40 μM medium iron, ferritin levels were approximately 2-fold lower in atm3-1 roots compared with the wild type (densitometric quantification). In rosette leaves, the levels of ferritin increased with the iron concentration in the medium (Fig. 5A, bottom panels) but were overall 1.5 times less in atm3-1 leaves, correlating with lower chlorophyll content and chloroplast numbers (Fig. 2B; Supplemental Fig. S1). Taken together, the expression patterns of IRT1 and ferritin in the atm3-1 mutant are not constitutive but generally respond to external iron levels. Nevertheless, the attenuated IRT1 and ferritin levels suggest that atm3-1 seedlings may experience a mild iron deficiency.

Next, the iron content of mitochondria was measured in purified organelle preparations. Neither mitochondria from atm3-1 callus lines nor from atm3-1 (ΔNBD) leaves contained elevated levels of iron (Fig. 5B). Although Kushnir et al. (2001) found 1.5 to 1.8 times more iron in mitochondria purified from atm3-1 (sta1) cell culture, both results are clearly different from yeast cells upon down-regulation of ScATM1, where iron levels are increased at least 10-fold (Fig. 6D) and could cause the loss of cytochromes and mitochondrial DNA (Kispal et al., 1999). Chloroplast iron levels were also not altered in the atm3-1 mutant (Fig. 5B).

Furthermore, it was investigated whether the phenotype of atm3-1 seedlings could be alleviated by applying iron directly to the leaves, bypassing uptake and xylem transport. While spraying with ferric ammonium significantly improved growth and chlorophyll biosynthesis in the irt1-1 mutant (Varotto et al., 2002), this treatment had no effect on atm3-1 seedlings (Fig. 5C). Either the atm3 mutants cannot use iron properly, resulting in functional iron deficiency, or the chlorosis is not caused by iron deficiency.

Following a report that the atm3-1 mutant roots showed a growth reduction in the presence of cadmium (Kim et al., 2006), the effect of cadmium on root length was tested for all atm3 alleles. Under our conditions, cadmium concentrations of 20 to 40 μM inhibited root growth in all atm3 alleles to a similar extent as in the wild type (Fig. 5D, top). When the data are rendered as a percentage of wild-type root length, root growth is only slightly more inhibited by cadmium in atm3 alleles than in the wild type (Fig. 5D, bottom). The effect of cadmium observed by Kim et al. (2006) in the atm3-1 mutant (C24 background) is also relatively weak (85% growth reduction in the mutant compared with 49% in the wild type) and very minor compared with that observed for mutants in the bona-fide cadmium extrusion pump AtPDR8 (Kim et al., 2007). These data suggest that ATM3 may play an indirect, rather than a direct, role in heavy metal resistance.

In summary, metal homeostasis is only marginally affected in atm3 mutants, supporting the general view that iron regulation differs in plants from fungi/metazoa (Curie and Briat, 2003). Importantly, iron sensing may not depend on a Fe-S cluster protein as in yeast and mammals (Lill and Mühlhoff, 2008).

Arg-612 Has a Conserved Function in B-Group ABC Transporters

To confirm the effect of the R612K substitution in ATM3, especially since AO activities were not dramatically decreased in the Arabidopsis mutant (Fig. 3A), we engineered the equivalent amino acid substitution in yeast Atm1p (R569K). The Arg is strictly conserved in all ATM protein sequences, although it can be a Lys, Ala, or Gly in other ABC transporters (Supplemental Fig. S4). Comparison with the Sav1866 structural model showed that Arg-612 (Arg-569 in yeast Atm1p) is located on the so called x-loop connecting the membrane domain and the ATPase

### Table II. Chlorophyll fluorescence of PSII, redox state of P700 (PSI), and acceptor-side limitation in the wild type, atm3 mutants, and nfu2.1

| Allele       | PSII     | PSI       | Acceptor-Side Limitation Y(NA) |
|--------------|----------|-----------|-------------------------------|
|              | Fv/Fm    | ΔA680/A650 (ΔH × 10^{-1}) | Normalized to Chlorophyll per Leaf Area |                          |
| Wild type (Col-0) | 0.79 ± 0.005 | 4.94 ± 0.65 | 4.94 ± 0.65 | 0.06 ± 0.02 |
| atm3-3 (R612K)   | 0.78 ± 0.006 | 4.44 ± 0.77 | 5.21 ± 0.90 | 0.06 ± 0.02 |
| atm3-4 (prom)    | 0.79 ± 0.007 | 4.67 ± 0.35 | 5.25 ± 0.39 | 0.06 ± 0.02 |
| atm3-1 (ΔNBD)    | 0.74 ± 0.007 | 2.45 ± 0.11 | 4.72 ± 0.21 | 0.06 ± 0.01 |
| nfu2.1          | 0.58 ± 0.035 | 1.38 ± 0.52 | 2.66 ± 0.10 | 0.20 ± 0.04 |
domain (Dawson and Locher, 2006). We expressed mutant versions of ScATM1 from a p426 plasmid in cells in which the endogenous ATM1 gene is under the control of the GAL1-10 promoter, which is activated by Gal but repressed by Glc or lactate (Kispal et al., 1999). Depletion of endogenous Atm1p strongly decreased the growth of cells containing the empty vector, whereas production of the R569K- or R569A-substituted proteins maintained growth (Fig. 6A). Protein blot analysis showed that the amino acid substitutions did not affect the stability of Atm1p (Fig. 6B).

Next, we analyzed the effect of the R569K and R569A substitutions in Atm1p on the activity of two cytosolic Fe-S enzymes, isopropyl malate isomerase (the second step in Leu biosynthesis) and sulfite reductase. Interestingly, Lys substitution of Arg-569 resulted in an approximately 50% decrease of both activities, whereas Ala substitution had little effect (Fig. 6C). The activity of alcohol dehydrogenase (ADH), a zinc-dependent enzyme that was analyzed as a control, was not altered in the mutants compared with wild-type ATM1. Note that ADH activity is strongly increased in the empty-vector control, because Atm1p-depleted cells switch their metabolism to alcohol fermentation (Kispal et al., 1997).

To investigate mitochondrial iron accumulation, cells were grown with Glc or with lactate to deplete endogenous Atm1p, and mitochondria were purified by differential centrifugation. In the presence of Glc, little iron accumulated in mitochondria, as reported previously (Kispal et al., 1999). However, in the presence of lactate, a nonfermentative carbon source that...
requires mitochondrial respiration, approximately 10-fold more iron was associated with mitochondria from Atm1p-depleted cells (p426; Fig. 6D). A 2-fold increase in mitochondrial iron content was observed in the R569K and R569A mutants.

These results show the importance of the x-loop Arg for the function of eukaryotic ATM proteins. Clearly, the positive charge of this residue is not critical, but the Arg performs a specific role in ATM proteins, at the interface of the membrane domain to ATP hydrolysis.

DISCUSSION

Arabidopsis has three closely related ATM genes, whereas other plant species like rice (Oryza sativa) and yeast have only one gene (Chen et al., 2007). Here, we show that only mutations in ATM3 affected plant growth, while T-DNA insertions into ATM1 and ATM2 did not result in an obvious phenotype (Fig. 1C). The T-DNA insertions in atm1-2, atm2, and atm3-2 lines are all in a similar position at the 5’ end of the open reading frame, corresponding to the first transmembrane helix. Therefore, it is unlikely that partial transcription of ATM1 or ATM2, but not ATM3, could lead to a functional protein. We investigated whether the function of ATM1 allowed slow growth of the atm3-2 mutant, but mutation of atm1-1 in the atm3-2 background did not further enhance the phenotype of atm3-2 (Fig. 1D). Likewise, atm2 atm3-1 double mutants resembled the atm3-1 parental line (data not shown). Either the expression levels of ATM1 and

Figure 6. Arg-569 to Lys substitution in yeast Atm1p results in impaired activity of cytosolic Fe-S proteins. A, Ten-fold serial dilutions of Gal-ATM1 cells transformed with p426, p426-ATM1, p426-ATM1_R569K, or p426-ATM1_R569A were grown on minimal medium containing Gal or Glc as a carbon source and incubated at 30°C for 4 d. B, R569K and R569A substitutions do not modify the stability of Atm1p. Immunoblot analysis of Atm1p or Nfs1p (as a loading control) in mitochondria purified from cells as described in A grown on Glc. C, The activities of isopropyl malate isomerase (Leu1p) and sulfite reductase (SIR) are decreased when Arg-569 is replaced by a Lys in Atm1p. Enzyme activities were measured using cell extracts prepared from cells described in A grown in the presence of Glc to deplete endogenous Atm1p. ADH activity was monitored as a control. Graphs represent data from one typical experiment (repeated three times with reproducible results). D, Iron accumulation in mitochondria from cells expressing mutant versions of ATM1. Yeast cells as described in A were grown in minimal Glc or rich lactate medium. Iron content was measured in samples of 150 μg of mitochondrial protein using the colorimetric iron chelator ferene (n = 2). Error bars represent so.
ATM2 are too low to compensate for the absence of ATM3 (Fig. 1B; Chen et al., 2007) or sequence divergence prevents complementation of atm3. Both arguments may be valid. First, accumulated microarray data suggest that the combined mRNA levels of ATM1 and ATM2, which are not listed separately because of high sequence identity, are approximately 5% of ATM3 (Genevestigator). Moreover, the expression of ATM1 or ATM2 is not up-regulated in the atm3-2 mutant (Fig. 1B). Second, Kushnir and colleagues (2001) showed that overexpression of ATM1 (STA2) from the 35S promoter did not rescue the pleiotropic phenotypes of atm3-1 (sta1), although it did improve growth. Taken together, our data and those of others suggest that ATM3 is likely to function as a homodimer rather than a heterodimer with ATM1 or ATM2. It is further of interest that ATM3 is not essential for viability (assuming that atm3-2 is a true null mutant; Fig. 1D). Yeast ATM1 also is not an essential gene, despite the fact that a number of cytosolic/nuclear Fe-S proteins are essential (Lill and Mühlenhoff, 2008). It has been suggested that other mitochondrial ABC transporters have overlapping substrate specificities with AtATM3/ScAtm1p or that some membrane diffusion of the substrate can occur. The mammalian ortholog ABCB7, on the other hand, is essential for early embryonic development in mice (Pondarré et al., 2006).

How can the phenotypes of the atm3 mutant allele be explained? Slow root growth can be the result of many biochemical defects, not in the least in plant hormone biosynthesis. The identification of weak atm3 alleles in sirtinol screens suggests lower activities of AO, as confirmed in the atm3-4 allele, which will affect abscisic acid and possibly auxin biosynthesis (Mendel and Bittner, 2006). Indeed, abscisic acid levels are strongly decreased in atm3-1 mutants and do not respond to drought treatment (J. Teschner and F. Bittner, unpublished data). Poor seedling establishment in atm3 alleles (Fig. 2E) may be the consequence of impairment of the glyoxylate cycle (Eastmond et al., 2000), due to lower activities of cytosolic aconitate (Fig. 4, B and D). However, the percentage of seedling establishment did not correlate fully with the levels of cytosolic aconitase activity in the atm3 alleles, and other factors may affect this biological process.

As more and more Fe-S enzymes are being identified, especially in DNA repair, transcription, and translation (for review, see Lill and Mühlenhoff, 2008), it is no surprise that ATM3, which we show is primarily important for the function of cytosolic (and by extension nuclear) Fe-S enzymes, is important for many aspects of plant metabolism. For example, the previously observed 2- to 10-fold up-regulation of DNA repair enzymes (Kushnir et al., 2001) could be a response to lower activities of Fe-S-dependent analogs (Balk and Lobréaux, 2005). However, so far we have been unable to explain why atm3 mutants are chlorotic. The chloroplasts, although much lower in number, appear normal in atm3-1 (sta1), with extensively developed thylakoid membranes stacked into granas (Kushnir et al., 2001), corresponding to mostly unaltered enzyme activities and photosynthesis parameters (Fig. 4F; Table II; Supplemental Fig. S3). Furthermore, chlorophyll levels and iron content in isolated chloroplasts are similar in atm3-1 and the wild type. Together with only subtle changes in the expression of iron-regulated genes (Fig. 5A) and no effect of foliar iron application (Fig. 5C), it also seems unlikely that the chlorosis is caused by iron deficiency. Therefore, we hypothesize that an as yet unknown cytosolic or nuclear Fe-S protein is important for developing full photosynthetic capacity in the leaves. Alternatively, there is a possibility that the substrate of ATM3 is required for a specific plastid function, other than Fe-S cluster assembly by the stromal assembly machinery. This would implicate plastid localization of ATM3, for which there is indeed some proteomic evidence. In addition to studies confirming mitochondrial localization (Brugiére et al., 2004; Heazlewood et al., 2004), ATM3 has been found in two proteomic studies of the plastids (Froehlich et al., 2003; Zybaïlov et al., 2008) but not in the study of Ferro et al. (2003). GFP and immunological studies are currently being undertaken in our laboratory to revisit the previously published GFP localization data (Kushnir et al., 2001; Chen et al., 2007) and to investigate whether a minor fraction of ATM3 is dual localized.

Our data provide fresh clues about the substrate of ATM3 that will form the basis for future investigations. Although ATM3, and therefore the substrate, is required for cytosolic Fe-S clusters, it is unlikely to be an Fe-S cluster. First, free Fe-S is unstable and would need to be liganded, for instance by a small peptide (Kuhnke et al., 2006). Second, iron does not accumulate in the mitochondria of atm3 mutants (Fig. 6C). Possibly, ATM3 could transport a chemical form of sulfur, generated by the mitochondrial Cys desulfurase NFS1, which then assembles with iron in the cytosol to form Fe-S clusters, mediated by the cytosolic Fe-S assembly machinery. Persulfide from Cys desulfurases, rather than hydrogen sulfide generated by sulfite reductase, appears to be the form of sulfur required for Fe-S cluster assembly (Kessler, 2006). It is interesting in this respect that the activity of NR, a Moco enzyme, is decreased in atm3 mutants. ATM3 may also provide sulfur to the second step of Moco biosynthesis localized in the cytosol (Dai et al., 2005; Mendel and Bittner, 2006). However, other possibilities cannot be ruled out, for example that ATM3 transports multiple substrates, including the pterin precursor of Moco. In support of a sulfide derivative as substrate, it was shown that the ATPase activity of yeast Atm1p is stimulated by thiol compounds in vitro (Kuhnke et al., 2006). Although it has been suggested that ATM3 transports cadmium, our finding that cadmium has little effect on root length in atm3 mutants (Fig. 6D) does not support this idea.

The analysis of ATM3 sheds further light on the compartmentalization of Fe-S cluster assembly in plant cells and the cross talk between these pathways.
(Xu and Möller, 2008). While ATM3 links the mitochondrial ISC machinery with the cytosol, Fe-S cluster assembly in the plastids appears to be an independent process.

Enzyme Assays
In-gel activity assays for aldehyde oxidases and XDH were performed as described previously (Koshiba et al., 1996). For in-gel analysis of aconitate activities, proteins were extracted by grinding 50 to 100 mg of leaf tissue with 1.5 volumes of 50 mM Tris-HCl, pH 8.0, 30 mM KCl, 0.2% (v/v) Triton X-100, 2 mM sodium citrate, and 1 mM diethanolamine. Samples were centrifuged for 15 min at 4°C at maximum speed in a microfuge. The supernatant was mixed with 0.25 volume of loading buffer (20 mM Tris-HCl 8.0, 80% [v/v] glycerol, and 0.1% [w/v] bromophenol blue), and 80 to 100 μg of protein was loaded per lane. The proteins were separated on a minigel with a stacking gel of 4% (w/v) polyacrylamide (PA) and a running gel of 2% (w/v) hydroyzed potato starch (Starch Art), 8% (w/v) PA in 0.13 ionic strength buffer, pH 8.6, and 3.6 mM sodium citrate. Gels were run in 0.025 M Tris-Gly buffer, pH 8.3, and 3.6 mM sodium citrate at 120 V for 3.5 h. The activity of aconitates was visualized by incubating the gels in 0.1 μl Tris-HCl, pH 8.0, 2.5 mM MgCl2, 0.5 mM NADP+ 2.5 mM cis-aconitate, 5 mM L-1,4-thiazol blue tetrazolium bromide, 50 μg mL−1 phenazine methosulfate, and 0.13 units mL−1 porcine isocitrate dehydrogenase for 10 to 30 min at room temperature.

Aconitase activity in cell extracts and mitochondria was assayed by coupling the activity to isocitrate dehydrogenase and the formation of NADPH (Stehling et al., 2007). Fumarase activity was measured following the conversion of 1-malate (50 mM) into fumarate at 240 nm. Complex II (succinate dehydrogenase) and complex III activities were assayed as the reduction of cytochrome c at 550 nm after addition of succinate (12 mM) in intact mitochondria (Stehling et al., 2007). For catalase, the disappearance of hydrogen peroxide (12.5 mM) was monitored at 240 nm. NR activity was measured following nitrite production, detected as in the NiR activity assay (Takahashi et al., 2001). The yeast enzymes isopropyl isomerase and sulfit reductase were assayed as described previously (Kispal et al., 1999; Rutherford et al., 2005).

Cell Fractionation and Protein-Blot Analysis
Cytosolic and mitochondria-enriched fractions were prepared from protoplasts derived from calli and Arabidopsis ecotype Somerville and Ogren (1981) and Sweetlove et al. (2007), respectively, except that differential centrifugation steps were deemed to give sufficient purity and density gradients were omitted. Mitochondria were purified from leaves following Day et al. (1985) and from yeast according to Daum et al. (1982). Chloroplasts were isolated following the method of Aronsson and Jarvis (2002). Protein concentrations were determined with Coomassie Dye Reagent (Bio-Rad). Proteins were separated by PA gel electrophoresis, transferred to nitrocellulose membrane by electroblotting, and labeled with antibodies and chemiluminescence detection. Polyclonal antibodies against purified His-ATACO1 (amino acids 120–898) were raised in rabbits courtesy of Sabine Molnik and Roland Lil. Antibodies against IRT1 were a kind gift from Erin Connolly (Connolly et al., 2002), and antibodies against ferritin were kindly provided by Frédéric Gaymard (Dellagi et al., 2005). The monoclonal antibody against Arabidopsis actin (MA1-744) was from Affinity BioReagents; antisera against Atm1 and Nfs1 were described previously (Kispal et al., 1999).

Dual-PAM Analysis
Chlorophyll fluorescence parameters and the redox change of P700 were assessed with a Dual-PAM-100 measuring system (Walz) in 4-week-old plants. The maximum PSII quantum yield, Fv/Fm, was measured in dark-adapted plants with Fv/Fm = (Fm − F0)/Fm.
P700 oxidation was monitored by absorbance changes at 820 nm (ΔA820) relative to an A820 calibration (ΔA820/ΔA700). The maximal ΔA820 (Pm; P700 fully oxidized) was determined by application of a saturation pulse after 10 s of far-red preillumination. Y(NA), the quantum yield of nonphotochemical energy dissipation due to acceptor-side limitation, was calculated based on a Pm' determination at 220 μmol m−2 s−1 actinic light according to: Y(NA) = (Pm − Pm')/Pm.

Miscellaneous Methods
Chlorophyll extraction in 80% (v/v) acetone (Lichtenthaler, 1987), Alexander’s stain for pollen viability (Johnson-Brousseau and McCormick, 2004), quantification of total iron using the iron chelator ferene (Hennessy et al., 1984) or inductively coupled plasma-mass spectrometry, as indicated;
root length measurements and densitometry were carried out with ImageJ software.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. Plastid chlorophyll levels and chloroplast counts in atm3-1.

Supplemental Figure S2. Quantification of ACO1 in-gel activity.

Supplemental Figure S3. PSI activity in isolated chloroplasts in atm3-1.

Supplemental Figure S4. Sequence alignment of Arabidopsis ATM1, ATM2, and ATM3 and related proteins.

Supplemental Table S1. List of primers.

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