Running Head: Plant transporter mutations confer antibiotic resistance

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Multiple antibiotic resistance in Arabidopsis thaliana is conferred by mutations in a chloroplast-localized transport protein

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ABSTRACT

Widespread antibiotic resistance is a major public health concern, and plants represent an emerging antibiotic exposure route. Recent studies indicate that crop plants fertilized with antibiotic-laden animal manure accumulate antibiotics, however, the molecular mechanisms of antibiotic entry and subcellular partitioning within plant cells remain unknown. Here we report that mutations in the Arabidopsis locus Multiple Antibiotic Resistance (MAR1) confer resistance, while MAR1 overexpression causes hypersensitivity to multiple aminoglycoside antibiotics. Additionally, yeast expressing MAR1 are hypersensitive to the aminoglycoside, G418. MAR1 encodes a protein with 11 putative transmembrane domains with low similarity to ferroportin1 from Danio rerio. A MAR1:YFP fusion protein localizes to the chloroplast, and chloroplasts from plants overexpressing MAR1 accumulate more of the aminoglycoside, gentamicin, while mar1-1 mutant chloroplasts accumulate less than wild type. MAR1 overexpression lines are slightly chlorotic, and chlorosis is rescued by exogenous iron. MAR1 expression is also downregulated by low iron. These data suggest that MAR1 is a plastid transporter that is likely to be involved in cellular iron homeostasis, and allows opportunistic entry of multiple antibiotics into the chloroplast.

INTRODUCTION

The amount of antibiotics used non-therapeutically in agriculture is estimated to be eight times greater than the amount used in all of human medicine (Mellon et al., 2001), and accounts for about 70% of total antibiotic use in the United States (Florini et al., 2005). It is also estimated that approximately 75% of antibiotics are not absorbed in the gut and are excreted largely unchanged (Mackie et al., 2006; Sarmah et al., 2006). Many of these antibiotics retain activity in soil for long periods of time (Chander et al., 2005). Agricultural crops are routinely fertilized with livestock waste, which has led to widespread antibiotic contamination of the environment and contributed to the selection of resistant bacteria, threatening human health. Two obvious reservoirs of residual antibiotics in the environment are farm soil and groundwater, and recent studies have shown that crop plants accumulate antibiotics after growth on antibiotic-contaminated soils (Kumar et al., 2005; Boxall et al., 2006). This poses a public health concern, as continual, low-level exposure to antibiotics through produce consumption may foster the development of resistant bacteria (Hocquet et
al., 2003). Despite concerns, virtually nothing is known about how plants are capable of taking up and distributing antibiotics, both within the plant body and on a subcellular level. Endogenous mechanisms of antibiotic resistance in plants have not been well studied.

Multiple drug resistance in bacteria is often conferred by multidrug efflux transporters encompassing several families, including (but not limited to) the ATP binding cassette (ABC) transporters, the major facilitator superfamily (MFS), and the multidrug and toxic compounds efflux (MATE) family (Paulsen, 2003). There are only a few reports of antibiotic resistance in plants that are not based on expression of prokaryotic resistance genes, and three recent reports involve transporters. Overexpression of AtWBC19, from Arabidopsis thaliana, confers resistance to kanamycin in plants (Mentewab and Stewart, 2005). Our lab has found that overexpression of the Arabidopsis multi-drug resistance 1 (MDR1) confers resistance to multiple herbicides and a single antibiotic, cycloheximide (Thomas et al., 2000; Windsor et al., 2003). The most recent report reveals that mutations in and RNAi-based downregulation of the putative transporter gene RTS3 confer resistance to kanamycin (Aufsatz et al., 2009).

The sensitivity of plants to antibiotics that target prokaryotic translational machinery, such as spectinomycin, tetracycline, lincomycin, and the aminoglycosides, is attributed to the similarity of chloroplast ribosomes to bacterial ribosomes (Ellis, 1970; Kasai et al., 2004). In fact, it has been shown that mutations in chloroplast ribosomal subunits can confer resistance to various aminoglycosides (Kavanagh et al., 1994; Rosellini et al., 2004). This indicates that these antibiotics must not only enter the cell, but must also gain entry to the chloroplast in order to function – a process which requires passage across the plasma membrane as well as the chloroplast double membrane. Movement across membranes can be difficult for hydrophilic antibiotics such as the aminoglycosides (Scholar and Pratt, 2000), and therefore may be facilitated by membrane transport proteins. Interestingly, the RTS3 putative transport protein is predicted to be chloroplast localized, indicating that it may be acting as an entry point for antibiotics into this subcellular compartment. However, no experimental evidence has yet been provided to support this prediction (Aufsatz et al., 2009). We have isolated three independent rts3 mutants that also confer resistance to kanamycin, and we have found that this resistance extends to other aminoglycoside antibiotics as well. Additionally,
we have expanded on the work of Aufsatz et al. to show chloroplast localization and transport functionality of RTS3.

Here, we refer to RTS3 as MAR1 (Multiple Antibiotic Resistance 1). Both a single nucleotide change (mar1-1) and two independent T-DNA insertions (mar1-2 and mar1-3) are able to confer resistance, which is highly specific to aminoglycosides that affect prokaryotic translational machinery. This resistance does not extend to antibiotics of other classes, or to aminoglycosides that affect eukaryotic translational machinery. MAR1 is most likely a chloroplast envelope protein, and appears to be a means by which antibiotics are able to opportunistically access their intracellular targets in a plant system. While the natural function of MAR1 remains unknown, our preliminary experiments indicate that it may play a role in cellular iron homeostasis.

RESULTS

Isolation and Map-based Cloning of Multiple Antibiotic Resistant Mutant mar1-1

mar1-1 was generated via EMS mutagenesis and was found to be resistant to several aminoglycoside antibiotics including kanamycin, streptomycin, gentamicin, amikacin, tobramycin, and apramycin (Fig. 1A and B). Interestingly, mar1-1 was not found to be resistant to the aminoglycosides hygromycin, G418, or paromomycin (Suppl. Fig. 1 and 2). These compounds, while structurally similar to other aminoglycosides, are distinct in that they inhibit both prokaryotic and eukaryotic protein synthesis (Eustice and Wilhelm, 1984). No resistance was found to antibiotics of other classes, including spectinomycin (an aminocyclitol), tetracycline, chloramphenicol, and lincomycin (Fig. 1D). Each of these antibiotics target prokaryotic translational machinery, but are structurally distinct from the aminoglycosides.

A backcross of mar1-1 to wild type revealed that the mutation is nuclear and monogenic. The mutant locus was isolated via map-based cloning. A single nucleotide change (C to T) was found in the 10th exon of the locus At5g26820 (Fig. 2A), which is annotated as having low similarity to Ferroportin1 from Danio rerio. At5g26820 has been described as AtIREG3 based on sequence similarity to AtIREG1 and AtIREG2, two iron-regulated transporters in Arabidopsis (Schaaf et al., 2006). More recently, it has been described as
RTS3, and two mutations in the gene (rts3-1 and rts3-2; Fig. 2A and B) were shown to confer kanamycin resistance at 40 mg/L (Aufsatz et al., 2009).

Native expression of At5g26820 was able to complement the mutant marl-1 (data not shown). Here, we will refer to At5g26820 as MAR1 (Multiple Antibiotic Resistance 1, since the gene name RTS3 (RNA-mediated Transcriptional gene Silencing) does not accurately describe the functionalities we have uncovered for At5g26820. The single nucleotide change in marl-1 leads to a single amino acid change (alanine to valine) at position 441 in the protein (A441V, Fig. 2B). This particular alanine residue lies in the middle of a putative transmembrane domain of the protein, and is highly conserved among MAR1 homologs (Fig. 2C).

As mentioned earlier, MAR1 has only two homologs in Arabidopsis – AtIREG1 (At2g38460) and AtIREG2 (At5g03570). However, there are three MAR1 homologs in rice (Oryza sativa; Os12g3570, Os05g04120, Os06g36450) and two homologs in grape (Vitis vinifera; A5AS54, A5BT51). MAR1 is more closely related to rice homologs that are predicted to be chloroplast localized (Os12g37530 and Os05g04120), and to its grape homolog that is predicted to function in secretory pathways (A5AS54) (Fig. 2D) (Schwacke et al., 2003). This could indicate that the MAR1 protein may localize to an intracellular compartment, as opposed to the plasma membrane of the cell.

The T-DNA Insertion Mutants marl-2 and marl-3 Phenocopy marl-1

We obtained two T-DNA insertion lines for MAR1 (Salk_034189 and Salk_009286) from the Arabidopsis Biological Resource Center. We have designated Salk_034189 as marl-2 and Salk_009286 as marl-3. Both lines show an extreme reduction in MAR1 transcript, as measured by quantitative real-time PCR (Suppl. Fig. 3A), and both were found to be nearly phenotypically identical to marl-1, with respect to antibiotic resistance (Fig. 1 A and C). Note that Salk lines are expected to be kanamycin and paromomycin resistant due to expression of nptII, but this does not lead to cross-resistance to other antibiotics, as illustrated by an unrelated kanamycin resistant, nptII-expressing Salk insertion line (Salk_030942; Fig. 1 A and C).
Overexpression of MARI Confers Multiple Antibiotic Hypersensitivity

Since the T-DNA insertion lines mar1-2 and mar1-3 phenocopy the EMS mutant mar1-1, and all mutations confer multiple antibiotic resistance, we hypothesized that overexpressing MARI would lead to the opposite phenotype – hypersensitivity to multiple antibiotics. We expressed the MAR1 genomic locus from start to stop codon under control of the CaMV35S promoter in wild type plants, and found that it did confer a phenotype of hypersensitivity to both kanamycin and gentamicin, based on severe chlorosis and stunted growth of seedlings (Fig. 3A and B). MARI expression in two independent 35S overexpression lines was found to be at least 48-fold higher than wild type (Suppl. Fig. 3B).

To further confirm that mutations in At5g26820 are responsible for the phenotype of mar1, we expressed 35S::At5g26820(MARI) in the mar1-1 background. Analysis of several independent transgenic lines revealed that this construct led to a reversal of the kanamycin resistance phenotype of mar1-1, i.e., mutant mar1-1 plants overexpressing MARI were found to be hypersensitive to kanamycin (Fig. 3C). Additionally, native expression of MARI in a mar1-2 background reverted the phenotype back to approximately wild-type levels of resistance (data not shown).

MARI Localizes to the Chloroplast Envelope

The ARAMEMNON plant membrane protein database (Schwacke et al., 2003) utilizes data from 17 individual programs to arrive at a consensus prediction for subcellular location. This consensus prediction method (Schwacke et al., 2007) predicts that the MAR1 protein is targeted to the chloroplast. According to the ChloroP program (Emanuelsson et al., 1999), the predicted chloroplast transit peptide of MAR1 includes the first 54 amino acids of the protein (Fig. 2B). A C-terminal YFP fusion to the putative transit peptide of MAR1 was transiently expressed in Arabidopsis protoplasts. Chloroplast transit peptides are known to effectively mediate transport across the chloroplast membrane (Inaba and Schnell, 2008) so the expected localization of YFP fused to a transit peptide would be the stroma. This is what
was observed in our experiment, based on distinct YFP colocalization with red (autofluorescent) chloroplasts (Fig. 4F, G, H).

C-terminal and N-terminal translational fusions between full-length \textit{MAR1} cDNA and YFP were also expressed. In C-terminal fusions, YFP fluorescence was clearly associated with chloroplasts (Fig. 4J, K, L), and in N-terminal fusions, fluorescence was cytoplasmic (Fig. 4O, P).

The \textit{MAR1}-YFP C-terminal translational fusion described above was also used to transform plants. Expression of this fusion protein was able to complement the resistance phenotype of \textit{mar1-2} (data not shown). Leaves of these plants were examined by confocal microscopy (Fig. 5A – L), and compared to untransformed controls (Fig. 5M, N, O). YFP fluorescence in transformed lines colocalized with chloroplast autofluorescence (Fig. 5C, F, I, L), and appeared particularly enhanced at the periphery of these organelles, indicating that \textit{MAR1} may be associated with the chloroplast envelope.

**Expression of \textit{MAR1} in Yeast Confers Hypersensitivity to the Aminoglycoside, G418**

To further test the function of the \textit{MAR1} putative transport protein, we expressed this protein in the yeast strain BY4700 under control of the strong PGK promoter. Both wild-type and \textit{mar1-1} mutant alleles were utilized for these experiments. BY4700 was utilized because it is only slightly sensitive to the aminoglycoside G418 (authors’ observations). Yeast expressing wild-type \textit{MAR1} were found to be hypersensitive to G418 when compared to empty vector controls (Fig. 6A). Interestingly, yeast expressing the mutant allele \textit{mar1-1} were also hypersensitive, but to a lesser extent than the \textit{MAR1} yeast (Fig. 6A). To eliminate the possibility that hypersensitivity was due to a general toxicity effect, the experiment was repeated using varying concentrations of cycloheximide (CHX), which is highly toxic to yeast. No growth differences were seen, at any CHX concentration, among yeast expressing either \textit{MAR1}, \textit{mar1-1}, or empty vector controls (Fig. 6B).

To ensure that \textit{MAR1} protein was being properly expressed, and to determine its localization pattern in yeast, we also expressed a GFP tagged version of \textit{MAR1}. Yeast expressing \textit{MAR1}-GFP were hypersensitive to G418, indicating functionality of the fusion protein (data
not shown). While GFP alone was clearly cytoplasmic (Figure 6D), GFP-tagged MAR1 localized to the yeast mitochondria (Figure 6C), which is typical for chloroplast membrane proteins expressed in yeast (Versaw and Harrison, 2002; Jeong et al., 2008). Since the aminoglycoside G418 acts on both prokaryotic and eukaryotic ribosomes (Vicens and Westhof, 2003), G418 is likely to be inhibiting yeast growth in MAR1-expressing strains by accumulating in mitochondria.

Since MAR1 was found to localize to the yeast mitochondria, we did an additional control using chloramphenicol, which is known to inhibit yeast mitochondrial translation (Ilbrahim et al., 1974). We did not find resistance to chloramphenicol in mar1-1 or mar1-2 mutant plants (Fig. 1D), therefore it is not likely to be a substrate for the MAR1 transporter. In support of this, we did not observe major growth differences between yeast lines expressing MAR1 (or mar1-1) and empty vector controls when grown for 48 hours in the presence of 0.5, 1, or 2 mg/mL chloramphenicol (Suppl. Fig. 4).

**MAR1 Regulates Gentamicin Entry into Chloroplasts.**

Since MAR1 appeared to be a chloroplast-localized transport protein, and its disruption and overexpression caused antibiotic resistance and hypersensitivity, respectively, we decided to test its functionality as a transporter for antibiotics. To accomplish this, we developed both a short-term uptake assay using isolated chloroplasts and a longer-term uptake assay using whole seedlings. For short-term uptake, isolated chloroplasts were exposed to high levels of antibiotic (12.5 mg/mL) for short periods of time (1 and 5 min) (Fig. 7A). For longer-term uptake, whole seedlings were exposed to lower levels of antibiotic (70 mg/L) for two days (Fig. 7D). Excess antibiotic was washed away, and chloroplasts were lysed to release their antibiotic content. Lysates were then spotted onto nitrocellulose in dot-blot fashion (Fig. 7C) along with gentamicin standards (Fig. 7B), and gentamicin was detected via anti-gentamicin antibody. This allowed for a simple yet quantitative method for measuring the gentamicin content of chloroplasts – each dot was analyzed using the integrated density function of ImageJ64 to determine a relative intensity value, which correlated positively with amount of antibiotic in the lysate.
In short-term uptake experiments with isolated chloroplasts, it was found that chloroplasts from mar1-1 mutant plants accumulated less gentamicin than wild type (Ler) controls, while chloroplasts from MAR1 overexpressors accumulated the most gentamicin (Fig 7A and C). This experiment was performed a total of three independent times with the same result. In uptake experiments using whole seedlings, it was found that chloroplasts from mar1-1 and mar1-3 mutant seedlings accumulated less gentamicin than the wild type (Col-0) control (Fig. 7D). Evidence from these experiments demonstrates the role of MAR1 as a chloroplast-associated transporter that is capable of importing aminoglycoside antibiotic.

**MAR1 May Have a Role in Iron Homeostasis.**

It is unlikely that evolutionary pressures would have selected for a means of entry for toxic antibiotics into plant chloroplasts. Therefore, we propose that MAR1 has a more “conventional” role in the plant, and the transport of antibiotics is an opportunistic effect. The expression pattern of MAR1 does not yield many clues as to its potential function. Promoter-reporter fusion experiments using MAR1::GUS transgenic plants demonstrated that MAR1 is expressed throughout the plant body in young seedlings (Suppl. Fig. 5A and B). In addition, MAR1 appears to be fairly evenly expressed in most tissue types based on AtGenExpress data (Suppl. Fig. 5C). Given its sequence similarity to ferroportin, it is possible that MAR1 could be involved in some aspect of iron transport. In an attempt to test this possibility, we expressed MAR1 cDNA in the yeast double mutant fet3fet4, which is defective in low and high affinity iron uptake (Dix et al., 1994). We found that MAR1 expressed in vector pVV214 was unable to complement fet3fet4 on SD media ± 5, 10, or 20 μM FeCl3 (data not shown). This result is probably not surprising, given MAR1’s localization to the yeast mitochondria (Fig. 6D).

A common symptom of iron deficiency in plants is chlorosis, since iron is essential for chlorophyll biosynthesis (Vert et al., 2002). Interestingly, we observed a visible and quantifiable phenotype of chlorosis in 35S::MAR1 seedlings when grown for two weeks on plain MS media (Fig. 8B) or MS media supplemented with 50μM Fe-EDTA (Fig. 8A, first plate). Additionally, when 35S::MAR1 plants were grown in soil, they appeared slightly more chlorotic than wild-type, both in leaves and stems. Chlorosis was especially prominent
along the midvein and older areas of cauline leaves, which also displayed an altered leaf shape, pinched towards the tip, compared to Ler (Fig. 8D). Chlorosis of plate-grown seedlings persisted until media was supplemented with 300μM Fe-EDTA (Fig. 8A and C). Our results here suggest that the overexpression of *MAR1* creates an iron-limiting condition for the plant.

One of the *MAR1* homologs in *Arabidopsis*, *AtIREG2*, was found to be upregulated under iron deficiency (Schaaf et al., 2006). With this in mind, we examined *MAR1* for transcriptional changes under iron limitation and iron excess. Plants were grown in liquid culture for 14 days, and baseline tissue samples were taken before addition of either 600 μM Fe-EDTA (iron excess) or 300 μM ferrozine (iron limitation). We observed a 60% decrease in *MAR1* expression after 4 days of growth under iron deficiency (Fig. 9A), along with the expected upregulation of *Iron Regulated Transporter 1 (IRT1)*, which encodes for a major high-affinity iron transporter (Stacey et al., 2008). We also observed a downregulation of *MAR1* when plants were grown for two weeks on plates containing a lower concentration of ferrozine (100 μM; Fig. 9B). A subsequent increase in *MAR1* transcription was not observed when iron levels were elevated for four days (Fig. 9C), despite the expected downregulation of *IRT1* under these conditions (Fig. 9C). Because the chlorosis of 35S::*MAR1* can be rescued by excess iron, and *MAR1* is downregulated under limiting iron conditions, we postulate that *MAR1* may play a role in iron chelation, storage, or sequestration.

**DISCUSSION**

We have uncovered a mutant, *mar1-1*, which was found to be resistant to multiple aminoglycoside antibiotics (Fig. 1 A and B) based on a single point mutation in a putative transporter gene (Fig. 2 A and B). The resistance of *mar1-1* is highly specific for aminoglycosides that target prokaryotic translational machinery and does not extend even to the structurally similar aminocyclitol, spectinomycin (Fig. 1D). Thus, MAR1 is an example of a transporter capable of recognizing a very specific group of drugs.
The change of alanine to valine in the mar1-1 protein, given its location in the middle of a predicted transmembrane domain and the residue’s high level of conservation among homologs in *Arabidopsis* and other plants (Fig. 2C), is likely to be very important to the function of *MAR1*. Additionally, *MAR1* homologs that do not have alanine at position 441 replace this residue with either serine or glycine (Fig 2C) – two amino acids with small R-groups. It is therefore likely that the addition of two relatively bulky methyl groups at this position in the mar1-1 mutant protein is enough to substantially alter MAR1 function. The nearly identical phenotypes of *mar1-1*, *mar1-2*, and *mar1-3* (Fig. 1A and C) indicate that all alleles are probably hypomorphic mutations, and since all confer multiple resistance, the *MAR1* transporter must be a means of entry for antibiotics. We confirmed this hypothesis by overexpressing *MAR1* in both wild-type and *mar1-1* backgrounds, which conferred hypersensitivity to multiple antibiotics (Fig. 3).

Since G418 and hygromycin do not cause chlorosis in plants, we were not able to do chlorophyll assays to determine resistance/sensitivity. However, we tested a wide range of concentrations and examined seedlings closely for phenotypic differences. We saw no difference in growth between *mar1-1* and wild-type Ler at any concentration (representative images are shown in Suppl. Fig. 1). Additionally, *mar1-1* was not resistant to the aminoglycoside paromomycin and appeared just as sensitized as the wild type Col-0 (Suppl. Fig. 2), while plants expressing NPTII do show significant resistance (Suppl. Fig. 2, bottom row).

Since *mar1* mutants are sensitive to those particular aminoglycoside antibiotics that act in the cell cytoplasm (hygromycin, G418, and paromomycin; Suppl. Fig. 1 and 2) but resistant to those that act only in the chloroplast (kanamycin, tobramycin, gentamicin, streptomycin, amikacin, and apramycin; Fig. 1A), we would predict that these mutations act to keep antibiotics out of the chloroplast. In support of this hypothesis, we have successfully demonstrated that MAR1-YFP fusions localize to the chloroplast in both protoplasts (Fig. 4) and whole plants (Fig. 5). Additionally, we have shown that the MAR1 transit peptide coupled to YFP delivers the fluorophore to the chloroplast stroma (Fig. 4F, G, H), while the addition of YFP at the N-terminus blocks proper localization of the transporter (Fig. 4N, O,
P). Since the transit peptide is the site of specific interactions with TIC (Translocon at the Inner envelope membrane of Chloroplasts) and TOC (Translocon at the Outer envelope membrane of Chloroplasts) complexes of the chloroplast envelope, it is likely that the addition of a bulky YFP fluorophore ahead of this domain may interfere with these interactions, which are necessary for import (Dixit et al., 2006; Inaba and Schnell, 2008). Although we have yet to experimentally confirm whether MAR1 localizes to the inner or outer membrane of the chloroplast, the presence of an N-terminal transit peptide indicates that MAR1 is likely to localize specifically to the inner envelope, since most plastid proteins of the outer envelope do not possess these transit peptides (Hofmann and Theg, 2005; Jarvis, 2008).

In the yeast strain BY4700, it was found that MAR1 localized to the mitochondria (Fig. 6C), and its expression caused a strong increase in sensitivity to G418 (Fig. 6A). The mutant allele \textit{mar1-1} also conferred sensitivity, albeit to a lesser extent. We hypothesize that the A to V mutation in \textit{mar1-1} causes a structural change in the transporter, such that its function is reduced. This reduced ability to function could be due to many factors, including reduced ability of the mutant transporter to bind or release substrate, or reduced ability to bind or release a co-transported ion (such as Na+ or H+) used as an energy source for transport. Future experiments will enable us to distinguish between these and other possibilities.

To test the import function of MAR1 in a plant system, we performed uptake experiments using both isolated chloroplasts (Fig. 7A) and whole seedlings (Fig. 7D). To date, there is no report on uptake studies of aminoglycoside antibiotics in a plant system, and therefore no convenient assay was available. The assay developed here allows for inexpensive, non-radioactive detection of antibiotic, and is based on the ability of aminoglycosides to adsorb onto nitrocellulose membrane without the need for fixation (Mihelic-Rapp and Giebel, 1996). Differences are clearly seen in lysate spots from \textit{mar1-1} mutant chloroplasts, as compared to wild type chloroplasts and chloroplasts from an overexpression line (Fig. 7C). These experiments provide evidence for the function of MAR1 as a transport protein.
A recent paper describes independent mutations of the \textit{MAR1} locus (At5g26820) that are sufficient to achieve kanamycin resistance in \textit{Arabidopsis} (Aufsatz et al., 2009). These findings agree with our data, however, Aufsatz \textit{et al.} report that resistance is kanamycin-specific and does not carry over to gentamicin or hygromycin. We did not see hygromycin resistance in any of our \textit{mar1} mutants, which was expected, because hygromycin has effects against eukaryotic ribosomes and therefore acts in the cytoplasm of the plant cell (Eustice and Wilhelm, 1984). However, we do show that \textit{mar1} mutants are multiply resistant to several aminoglycosides, including gentamicin (Fig. 1A). A possible reason for this discrepancy could be that the Aufsatz \textit{et al.} mutations are distinct from our \textit{MAR1} mutations (Fig. 2A and B), and thus may confer slightly different phenotypes. We also note that Aufsatz \textit{et al.} tested for gentamicin resistance at a concentration of 100 mg/L, while we test at 70 mg/L. Furthermore, Aufsatz \textit{et al.} only mentioned the testing of kanamycin, hygromycin, and gentamicin – resistance to other aminoglycosides is not discussed.

The chlorosis phenotype of the \textit{MAR1} overexpression line gives insight into the natural function of the \textit{MAR1} protein. Since this phenotype is rescued by iron feeding (Fig 8A and C), \textit{MAR1} may play a role in the chelation, storage, and/or sequestration of iron. If so, we might expect a decrease of \textit{MAR1} transcript under iron limiting conditions, which is what was observed (Fig. 9A and B). Under limiting conditions, we also saw the expected increase in the transcript of the major root iron transporter \textit{IRT1}, which is highly upregulated under iron limitation to increase the supply of iron to the plant cell (Eide et al., 1996; Korshunova et al., 1999; Rogers et al., 2000; Connolly et al., 2002). This upregulation leads to an increase in cytoplasmic iron, but due to the poor substrate specificity of \textit{IRT1}, it also results in increasing cytoplasmic levels of other toxic divalent metal cations, such as nickel. One of the \textit{MAR1} homologs in \textit{Arabidopsis}, \textit{AtIREG2}, is proposed to play a role in the vacuolar sequestration of excess nickel accumulated under iron-limiting conditions, due to the action of \textit{IRT1} (Schaaf et al., 2006). Schaaf \textit{et al.} showed that \textit{AtIREG2} was upregulated under iron deficiency, in contrast to \textit{MAR1}, which is downregulated (Figure 9A and B). Thus, we suggest that \textit{MAR1} and \textit{AtIREG2} play distinct roles in the plant cell. Their expression patterns are quite different – \textit{AtIREG2} is mainly expressed in the root (Schaaf et al., 2006), while \textit{MAR1} is highly expressed in all tissues (Suppl. Fig. 4C). \textit{AtIREG2} localizes to the
vacuole (Schaaf et al., 2006), while MAR1 localizes to the chloroplast (Fig. 4). Despite these differences, we hypothesize that MAR1 and AtIREG2 both act to transport metal – AtIREG2 transports nickel into the vacuole, while MAR1 may be transporting iron in the chloroplast.

AtIREG1 was postulated to be involved in vessel loading of iron (Curie and Briat, 2003), and its downregulation in DwMYB2 overexpressors (Chen et al., 2006) may be the cause of the disruption in iron translocation (from root to shoot) observed in these plants. Because citrate appears to be the major chelator for iron in the xylem (Haydon and Cobbett, 2007), it is possible that AtIREG1 exports citrate (or an iron-citrate conjugate) from root cells into the vasculature, playing a role similar to FRD3, which mediates citrate efflux into root vasculature (Durrett et al., 2007). With this in mind, we postulate that MAR1 may also be acting to transport an iron chelator, such as citrate or nicotianamine.

Nicotianamine (NA) plays a key role in iron homeostasis by ensuring iron solubility in the weakly alkaline environment of the plant cytoplasm (Douchkov et al., 2005; Weber et al., 2008). Plants lacking NA (such as the tomato mutant chloronerva) show phenotypes of interveinal chlorosis in young tissues (Cassin et al., 2009). However, NA overaccumulation can paradoxically increase the sensitivity of Arabidopsis to iron deficiency by sequestering iron (Cassin et al., 2009). Since cytosolic iron homeostasis depends on NA (Hell and Stephan, 2003), and NA appears to be present in chloroplasts (Becker et al., 1995; Stephan, 1995), it may be possible that MAR1 transports NA into the chloroplast, where it likely is required to maintain iron solubility in the weakly alkaline environment of the stroma (Wu and Berkowitz, 1992).

The chlorosis phenotype of 35S::MAR1 plants could be due to excess NA accumulating in the chloroplast, where it may sequester iron, creating the phenotype of iron deficiency. The phenotype observed in leaves of mature 35S::MAR1 plants is the opposite of that seen in plants lacking NA (such as chloronerva) – instead of interveinal chlorosis in young tissues, chlorosis arises in the midvein and in older tissues. This unusual chlorosis pattern may be
the result of a re-distribution of the cytoplasmic NA pool to the chloroplast. This has the dual effect of restricting NA from performing its role in phloem transport of iron and other metals (von Wiren et al., 1999), and also sequestering iron itself, thus preventing it from being re-distributed throughout the plant body. Iron, applied in excess (300 μM), is able to rescue the chlorosis phenotype, and the \textit{MAR1} gene is downregulated under iron deficiency to prevent sequestration of needed iron. It may be that there is no increase in \textit{MAR1} expression under iron excess (600 μm) due to the negative effects of NA over-accumulation (Cassin et al., 2009).

It is well known that aminoglycosides mimic polyamines, and can use their inward transport systems for entering both bacteria and eukaryotic cells (Van Bambeke et al., 2000). Since NA is a polyamine (Ling et al., 1999), it may be a good potential candidate for a natural substrate of MAR1. This hypothesis will require further investigation. Since MAR1 is classified as a ferroportin, the possibility also remains that MAR1 is transporting iron, and the chlorosis seen in \textit{MAR1} overexpressors is a result of oxidative damage caused by excess iron accumulation in the chloroplast. If this is the case, one possibility is that chlorosis of overexpressors is relieved in the presence of high levels of exogenous iron (300 μM) because at this level, the plant is likely to activate its many defense mechanisms against iron toxicity, such as downregulation of \textit{IRT1} and \textit{AtNRAMP3} (Vert et al., 2002; Ravet et al., 2009), upregulation of ferritin (Gaymard et al., 1996), increasing NA production (Pich et al., 2001), and activation of responses against oxidative stress (Fourcroy et al., 2004). If \textit{MAR1} does act to transport iron into the chloroplast, it may be regulated like \textit{AtSBL}, a putative transporter hypothesized to import iron into chloroplasts for storage in ferritins (Wintz et al., 2003).

Both the chloroplast and mitochondria require metalloproteins for photosynthesis and respiration, respectively, though the question of how iron and other metals are allocated between the two organelles has not yet been addressed (Merchant et al., 2006). Since most photosynthetic components are down-regulated under iron limitation (Tognetti et al., 2007), one possibility is that under limiting conditions, iron is preferentially allocated to the mitochondria to maintain respiration. If this is the case, and MAR1 is acting to transport
iron, we would expect to see a decrease in its expression under iron limitation, which is what was observed (Fig. 9A and B).

*mar1* represents an interesting example of plant antibiotic resistance that is based on the restriction of antibiotic entry into a subcellular compartment. Knowledge about this process – and other processes of antibiotic entry – could enable the production of crop plants that are incapable of antibiotic accumulation, aid in development of phytoremediation strategies for decontamination of water and soils polluted with antibiotics, and further the development of new plant-based molecular markers. The work described here also contributes to our understanding of how plants interact with the antibiotics they encounter, both in the laboratory (where aminoglycosides such as kanamycin are used heavily to select for transgenics) and in the natural environment.

The data presented here indicate that MAR1 is a transport protein likely to be located on the chloroplast envelope, which appears to be capable of subcellular transport of multiple aminoglycoside antibiotics (Fig. 10). MAR1 is highly specific for aminoglycosides that act on prokaryotic translational machinery, since *mar1* mutants are not resistant to antibiotics of other classes, including those that act specifically in the chloroplast (Ellis, 1970; Kasai et al., 2004). Based on lack of sequence similarity, *MAR1* does not appear to belong to the ABC class of transporters previously implicated in *Arabidopsis* single antibiotic resistance. Instead, MAR1 may be transporting iron or a molecule involved in iron homeostasis. MAR1 is not able to distinguish between this molecule and the aminoglycosides. Further investigation is necessary to uncover the native function(s) of MAR1 in plant growth and development.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions.** The original antibiotic resistant mutant, line E2-123 (*marl-1*), was generated via EMS mutagenesis of line E2-6 (*Ler* background, antibiotic sensitive) (Kilby et al., 1992). The E2-6 line contains a methylation silenced *nptII* gene at an
unknown location. The original mutagenesis and screen was performed to try to identify components of the DNA methylation pathway. However, the T-DNA containing the silenced nptII gene segregates away from the MARI locus. The original E2-123 (mar1-1) line was outcrossed to wild type Ler one time, and one subsequent homozygous mar1-1 F2 progeny, without any T-DNA (verified by Southern blot and PCR analysis), was used as the parent in the experiments presented here.

Plants were grown either in a growth room at 21°C, ambient humidity, under constant fluorescent illumination, or on Petri dishes in a Percival chamber under similar conditions.

**Plant Transformation.** All constructs to be used in plant transformation experiments were transferred to *Agrobacterium tumefaciens* GV3101 via electroporation. *Arabidopsis thaliana* plants were transformed by *Agrobacterium*-mediated transformation using the floral dip method (Clough and Bent, 1998). Primary transformants were selected in soil or on MS plates using the herbicide Basta (1.5 μl/mL; AgrEVO, Germany). The progeny of at least three selfed, primary transformants were used for experiments.

**Map-Based Cloning of MARI.** A total of 608 kanamycin-resistant F2 progeny from a cross of the mar1-1 mutant (F2 minus T-DNA as described above) to Col-0, and the mar1-1 and Col-0 parents were genotyped using microsatellite loci polymorphic between Col-0 and Ler. Resistant seedlings were selected after two weeks of growth on MS media plus kanamycin (25 mg/L). Genotype data were analyzed using MetaPhor® agarose gels (Cambrex, Rockland ME) and by fragment analysis using the Applied Biosystems 3730 Genetic Analyzer and GeneMapper® software. Additional details are available in Supplementary Methods.

**Gene Cloning and Plasmid Construction.** All clonings were done using the Gateway™ system (Invitrogen). All attB-tailed PCR products were initially cloned into pDONR222 using BP Clonase, and sequence verified before subcloning into various plant and yeast expression vectors (using LR Clonase) mentioned below.

35S::MARI

The MARI locus (At5g26820) was amplified by PCR (TripleMaster PCR system;
Eppendorf) from Ler (wt) genomic DNA using attB-tailed gene specific primers (Suppl. Table 1). *MAR1* was then subcloned into the plant overexpression vector pB7WG2 (Karimi et al., 2002) for subsequent *Agrobacterium*-mediated transformation of Col-0, Ler, and mar1-1 plants. At least three independent Basta resistant transformed lines were isolated for analysis for each vector-genotype combination.

35S::*MAR1-YFP* and 35S::*YFP-MAR1*

*MAR1* cDNA in vector pENTR/SD-DTOPO was obtained from the ABRC stock center through TAIR (www.arabidopsis.org, clone name: U16896). *MAR1* cDNA was amplified by PCR from this vector using specific primers (Suppl. Table 1). *MAR1* cDNA lacking a stop codon was subcloned into vector pH7YWG2 (Karimi et al., 2005) in frame to *YFP* for subsequent expression in *Arabidopsis* Col-0 protoplasts and mar1-2 plants. N-terminal fusions (35S::*YFP-MAR1*) were constructed exactly as above, except that MAR1 cDNA was cloned into the vector pB7WGY2 (in frame with YFP) and the stop codon was retained.

35S::*MAR1tp-YFP*

The first 162 nucleotides of MAR1 were amplified by PCR using specific primers (Suppl. Table 1). Vector pH7YWG2 (Karimi et al., 2005) was used for subsequent expression in *Arabidopsis* Col-0 protoplasts.

**Arabidopsis Protoplast Transformation.** Protoplasts were isolated from 20 day-old seedlings and transformed according to methods previously described (Weigel and Glazebrook, 2002). Constructs used for transformation included 35S::*MAR1-YFP*, 35S::*YFP-MAR1*, 35S::*MAR1tp-YFP* (all described above), and 35S::*YFP* (pCL-eYFP-FL; a gift from Dr. Enamul Huq).

Transformed protoplasts were allowed to incubate overnight under continuous light at 22°C prior to confocal microscopy.

**Confocal Microscopy Analysis of 35S::*MAR1-YFP*, 35S::*MAR1tp-YFP*, and 35S::*YFP*.** A Leica SP2 AOBS confocal laser scanning microscope was used for visualizing fluorescence images from *Arabidopsis* protoplasts and leaves. Excitation was at 514 nm and the emission signal was collected between 525 nm and 590 nm for YFP fluorescence, and between 622 nm and 700 nm for chlorophyll autofluorescence. Untransformed protoplasts and leaves were also
examined as controls.

**T-DNA Knockout Lines mar1-2 and mar1-3.** T-DNA insertion alleles were identified from the SIGnAL (Salk Institute Genomic Analysis Laboratory) collection. *mar1-2* carries a T-DNA insertion in the 11th exon of At5g26820 (Salk_034189, position 9436545 on chromosome V). *mar1-3* carries a T-DNA insertion in the 9th exon of At5g26820 (Salk_009286, position 9436095 on chromosome V). Lines were confirmed homozygous by PCR and by segregation analysis on kanamycin.

**Quantification of Antibiotic Resistance in Plants.** Titrations of the antibiotics kanamycin, gentamicin, streptomycin, tobramycin, amikacin, and apramycin were established to determine the concentration at which the greatest difference in resistance could be observed between wild type and mutant *mar1-1* when plated on MS media plus antibiotic. These concentrations were determined to be kanamycin 25 mg/L, gentamicin 70 mg/L, streptomycin 75 mg/L, tobramycin 40 mg/L, amikacin 100 mg/L, and apramycin 200 mg/L. Seeds of mutant lines *mar1-1* and *mar1-2* along with a corresponding wild type line (Col x Ler, F4) and an unrelated kanamycin resistant T-DNA insertion line (Salk_030942, which interrupts *MYB5*) were surface sterilized and plated onto MS media and MS plus antibiotic. After 48 hrs vernalization, plates were moved to a 22°C incubator under constant light conditions for 14 days.

**Measurement of Seedling Chlorophyll Content.** Chlorophyll was extracted and quantified in triplicate according to methods described previously (Porra et al., 1989).

**Yeast Transformation.** *MAR1* and *mar1-1* cDNAs were cloned into vector pVV214 (Van Mulfem et al., 2003) via the Gateway™ method, and the *Saccharomyces cerevisiae* strains BY4700 (MATa ura3Δ0) and fet3fet4 (DEY 1453; MATα trp1 ura3 Δfet3::LEU2 Δfet4::HIS3; (Eide et al., 1996)) were transformed with these constructs (or empty vectors) using standard methods (Elble, 1992). The fet3fet4 strain was always maintained in dropout media containing 0.2 mM FeCl₃ prior to testing for complementation on media supplemented with 0-50 μM FeCl₃ or Fe-citrate.
Yeast Antibiotic Susceptibility Assays.
Eight individual clones from each line (described above) were selected from -URA dropout plates and PCR checked for the presence of the transgene. BY4700 transformed with pVV214 alone served as a control. Of the positive clones, three were selected and grown overnight at 30°C in 5 mL of -URA liquid dropout media. Cultures were then standardized to 0.01 OD (λ.600) before addition of various concentrations of G418 or cycloheximide (Fig. 6). After 48 hours of growth at 30°C, OD_{600} was recorded for each culture. The experiment was carried out in triplicate.

MAR1 Localization in Yeast. MAR1 cDNA (with stop codon removed) was cloned into vector pAG426GPD-ccdB-EGFP (Addgene plasmid 14204) via the Gateway™ method, and the yeast strain BY4700 was transformed as described above. pAG426GPD-ccdB-EGFP alone was used as a control. A mixed population of transformed and untransformed cells was incubated in a 500 nM solution of MitoTracker Red CMXRos (Invitrogen) for 20 minutes at room temperature. A Leica SP2 AOBS confocal laser scanning microscope was used for visualizing fluorescence images. Excitation was at 514 nm, and the emission signal was collected between 525 nm and 540 nm for GFP fluorescence, and between 600 nm and 650 nm for MitoTracker Red.

Chloroplast Isolation and Antibiotic Uptake Assays. Intact chloroplasts were isolated basically according to previous methods (Weigel and Glazebrook, 2002), with several modifications (Aronsson and Jarvis, 2002) to ensure that chloroplasts were import-competent (See Suppl. Methods). Chloroplasts were consistently determined to be >80% intact based on photoreduction of ferricyanide (Sigma Chloroplast Isolation Kit Technical Bulletin, 2002).

Chloroplasts were counted using a hemocytometer, and a standard number was used for each reaction (Fig. 7 legend). The uptake reaction buffer was HMS (see Suppl. Methods) + 10 mM carbonate + 0.2% w/v BSA. Gentamicin was added to a final concentration of 12.5 mg/mL, and uptake reactions were carried out on a rotator in a Percival chamber under constant fluorescent illumination for given time periods (Fig. 7A). Negative controls were incubated in HMS uptake buffer without gentamicin. To stop the uptake reaction, tubes were spun at 1000 x g for 2 minutes in a microcentrifuge, supernatant was decanted, and chloroplasts were washed with 500
μl HMS buffer. This was repeated for a total of three washes. Chloroplasts were then incubated in 150 μl CP lysis buffer (20 mM HEPES pH 7.5, 5 mM KCl, 1.5 mM MgCl₂, 10 mM DTT, 10% v/v glycerol, and 1% w/v PVP) on ice for one hour with occasional vortex. Supernatants were collected after centrifugation (3000 x g for 5 minutes) and stored at -20°C until use in dot blot.

Dot blots for antibiotic detection in chloroplast lysates were performed as follows: 2 μl of each lysate was spotted onto nitrocellulose membrane (pore size 0.2 μM) in triplicate (Fig. 7C), along with 2 μl of each of a set of standard gentamicin solutions (in CP lysis buffer) as positive controls (Fig. 6B). Spotted membranes were allowed to dry for 45 minutes before blocking with 1X PBS pH 7.4 + 0.05% v/v Tween20 + 5% w/v nonfat dry milk. Blocking time was 1 hour on a rotary shaker at RT. After the block, mouse anti-gentamicin antibody (AbCam, Cambridge MA) was applied (in blocking solution) at 1:1000 dilution, and incubation was carried out at 4°C overnight. The membrane was then washed 2X for 15 mins each with PBS, 3X for 15 mins each with PBS + 0.05% v/v Tween 20, and 1X for 15 mins with PBS.

Goat anti-mouse HRP-conjugated secondary antibody (Santa Cruz Biotechnology) was applied (in blocking solution) at a dilution of 1:5000 and allowed to incubate for 1.5 hours on a rotary shaker at RT. The above washes were then repeated. The membrane was allowed to incubate for 1 minute in Western Lighting™ Plus-ECL solution (Perkin Elmer) before exposure to film (Kodak BIOMAX Light) for 10 sec – 1 min. Images of developed film were analyzed using ImageJ64 (NIH). The image was inverted, and background was subtracted using a rolling ball radius between 60-80 pixels, depending on the blot (rolling ball radius should be equivalent to the size of the largest dot on the blot). The integrated density function was then used to measure the intensity of each dot. The average of three replicate dots (±SD) was graphed (Fig. 7A).

Whole Seedling Uptake. Approximately 2000 seeds were sterilized for each line and vernalized for 2 days at 4°C in 100 mL volumes of liquid MS growth media. Flasks were then moved to a shaker in a Percival chamber (22°C, continuous fluorescent light). On day 11, the media was changed to fresh liquid MS. On day 13, gentamicin was added to a final concentration of 70 mg/L. On day 15, media was decanted and seedlings were washed with 300 mL of ddH2O.
Chloroplasts were isolated from seedlings exactly as described above, and $3 \times 10^8$ chloroplasts from each line were lysed. The lysis protocol was the same as above and dot blots were also performed as above, except that lysates were diluted 1:30 before spotting.

**Gene Expression Analysis.** Seeds from *Ler* (wild-type) were grown in Erlenmeyer flasks containing 200 mL liquid MS media supplemented with 1% sucrose at 21°C under continuous white light on a shaker set to constant RPM in a Percival growth chamber. After 14 days of growth, several whole seedlings (roots and shoots) were removed, and RNA was extracted using the QIAgen RNeasy Plant mini kit with on-column DNAse treatment. Media was then supplemented with either 600 μM Fe-EDTA (iron excess) or 300 μM ferrozine (iron restriction), and remaining seedlings were allowed to incubate for a further 4 days. On day 4, RNA was extracted from remaining whole seedlings as above.

RNA (4 μg) from each sample was used in 40 μl reverse transcription reactions containing 250 nM *actin*, *IRT1*, and *MARI* gene-specific reverse primers. For each target (*actin*, *IRT1* and *MARI*), five PCR reactions containing 400 nM primers and 2 μl first strand cDNA as a template were performed using SYBR® green master mix (ABI) and a spectrofluorometric thermal cycler (ABI 7900HT). The comparative cycle threshold method was used to analyze the results (User Bulletin 2, ABI PRISM Sequence Detection System).

For gel-based RT-PCR, plants were grown for two weeks on plates containing 0 or 100 μM ferrozine. On day 14, whole seedling tissue (root and shoot) was harvested and RNA extracted as above. 2 μg of RNA was used as a template for each cDNA reaction (containing both *MARI* and *APRT* primers), and equal amounts of cDNA reactions were loaded on a gel. Products were visualized with UV and ethidium bromide.

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FIGURE LEGENDS

Figure 1. Resistance phenotypes of *mar1-1, mar1-2, and mar1-3*. (A) Chlorophyll content of seedlings grown on aminoglycoside antibiotics. Wild type (wt) seedlings and an unrelated homozygous T-DNA line, Salk_030942 (30942), were used as controls. Antibiotic concentrations were: kanamycin (Kan) 25 mg/L, tobramycin (Tob) 40 mg/L, gentamicin (Gent) 70 mg/L, streptomycin (Strep) 75 mg/L, amikacin (Ami) 100 mg/L, apramycin (Apr) 200 mg/L. GM was plain growth media (no antibiotic). (B) Phenotypes of seedlings grown on MS media + kanamycin (25 mg/L) for 7 days. (C) Phenotypes of the Salk T-DNA knockout mutants *mar1-2* (two individual homozygotes are indicated as (a) and (b)) and *mar1-3*, along with control line (30942) and Col-0, grown on MS media + tobramycin (40 mg/L) for 14 days. (D) Chlorophyll content of seedlings grown as in (A) on media containing four non-aminoglycoside antibiotics. Antibiotic concentrations were: spectinomycin (Spec) 8 mg/L, chloramphenicol (Cm) 10 mg/L and 30 mg/L, lincomycin (Linc) 25 mg/L, tetracycline (Tet) 10 mg/L. GM was plain growth media (no antibiotic).

Figure 2. Analysis of the MAR1 gene and protein. (A) The MAR1 gene in *Arabidopsis*. Exons are depicted as solid black boxes. The mutation sites for *mar1-1, rts3-1*, as well as insertion sites for SALK lines *mar1-2, mar1-3*, and GABI-KAT line *rts3-2* (Aufsatz et al., 2009) are shown. (B) The MAR1 protein. Transmembrane domains in MAR1 are shown along with consensus score values (Schwacke et al., 2003). Domains with consensus scores above 0.42 are counted in the total number of transmembrane domains. A putative chloroplast transit peptide is predicted (with 11.7 consensus score value) (Schwacke et al., 2003; Schwacke et al., 2007). The chloroplast transit peptide cleavage site (as predicted by ChloroP) is indicated with a green arrowhead. The amino acid changes in mutant *mar1-1* and *rts3-1* are also shown. (C) Alignment of MAR1 (At5g26820) with its homologs in *Arabidopsis* (IREG2 – At5g03570, IREG1 – At2g38460), *Oryza sativa* (Os12g37530, Os05g04120, Os06g36450), and *Vitis vinifera* (A5AS54, A5BT51). Degree of conservation of various amino acids is indicated below the alignment by periods (highly conserved), colons (very highly conserved), and grey asterisks.
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Figure 3. **MAR1 overexpression results in hypersensitivity to antibiotics.** (A) Seeds were plated on kanamycin (10 mg/L). After 14 days, two representative seedlings of each line were photographed. Phenotypes of three independent overexpression lines are shown (a, b and c). All lines are in the Ler background. (B) Chlorophyll content (μg of chlorophyll per mg fresh weight) of MAR1 overexpression lines grown for two weeks on media containing gentamicin (70 mg/L). OE-A, OE-B, and OE-C are three independent MAR1 overexpression lines. Measurements represent the average chlorophyll content (±SD) of three separate batches of seedlings. (C) Overexpression of MAR1 in mar1-1 background reverses the kanamycin resistance phenotype of mar1-1. Mutant mar1-1 plants were transformed with 35S::MAR1 and seeds were plated on kanamycin (25 mg/L). After 14 days of growth, two representative seedlings were photographed.

Figure 4. **MAR1-YFP localizes to chloroplasts in protoplasts.** Confocal microscopic images depict the localization of YFP alone under control of the CaMV35S promoter (35S::YFP; first column), MAR1 chloroplast transit peptide fused to YFP (35S::MAR1tp-YFP; second column), full-length MAR1 cDNA with YFP at the C-terminus (35S::MAR1-YFP; third column), full-length MAR1 cDNA with YFP at the N-terminus (35S::YFP-MAR1; fourth column), and an untransformed protoplast (fifth column). A bright field image (A, E, I, M, Q), Chlorophyll autofluorescence (B, F, J, N, R), YFP fluorescence (C, G, K, O, S), and a merge of the two channels (D, H, L, P) are included for each protoplast. We note that (T) is a merge of all three channels (transmitted, chlorophyll, and YFP).

Figure 5. **MAR1-YFP localizes to chloroplasts in leaves of transformed plants.** Plants were transformed with the C-terminal fusion construct 35S::MAR1-YFP as described in Methods.
Confocal single-slice images of the leaves of two individually transformed plants (A – F and G – L) were compared to an untransformed leaf (M, N, O). (D, E, F) and (J, K, L) are close-up images of (A, B, C) and (G, H, I), respectively. Chlorophyll autofluorescence (A, D, G, J, M), YFP fluorescence (B, E, H, K, N), and a merge of the two channels (C, F, I, L, O) are shown for each leaf section. Scale bar = 8 μM.

**Figure 6. Expression of **MAR1** in yeast confers hypersensitivity to G418.** (A and B) MAR1 and mar1-1 were expressed in yeast under control of the PGK promoter (vector pVV214). Cultures were standardized to OD 0.01 at 600 nm before addition of antibiotic ((A) G418 at 0, 200, 300, and 400 mg/L; (B) Cycloheximide at 0, 0.2, 0.3, and 0.5 mg/L). Cultures were analyzed spectrophotometrically after 48 hours of growth, and ODs were plotted. For both graphs, each bar represents the average absorbance of three independent cultures (±SD). (C and D) Yeast strain BY4700 was transformed using MAR1 cDNA (lacking a stop codon) fused in-frame with EGFP (C) or using EGFP alone (D). MitoTracker Red was used to visualize mitochondria, and a transmitted image is included to illustrate integrity of the cells.

**Figure 7. MAR1 regulates gentamicin entry into chloroplasts.** (A) Plants were grown for 15 days before chloroplast isolation, and 8.5 x 10⁷ chloroplasts were incubated in 12.5 mg/mL gentamicin for each uptake reaction (1 minute and 5 minutes). (B) Gentamicin standards (dissolved in chloroplast lysis buffer) were spotted as positive controls for every dot blot. Numbers above each dot indicate gentamicin concentration in mg/mL. (C) Representative data from a 1 minute uptake experiment. Left panel – triplicate lysate spots from chloroplasts incubated with 12.5 mg/mL gentamicin for 1 minute (+Gent). Right panel – triplicate lysate spots from chloroplasts incubated in uptake buffer alone for 1 minute (-Gent). In each panel, the left-hand column shows lysate from wild-type Ler chloroplasts (wt), the middle column shows lysate from mar1-1, and the right-hand column shows lysate from 35S::MAR1 overexpressor chloroplasts (35S). (D) Whole seedling uptake results. Seedlings were exposed to 70 mg/L gentamicin for 2 days, washed, and chloroplasts isolated. 3 x 10⁸ chloroplasts from each line were lysed. For (A) and (D), each bar represents the average relative intensity of three triplicate spots (±SD).
Figure 8. Chlorosis of 35S::MAR1 is rescued by 300 $\mu$M Fe-EDTA. (A) Plants were grown for two weeks on varying concentrations of Fe-EDTA (as indicated) before photographing. For each plate, the upper left section contains 35S::MAR1 seedlings, the upper right contains mar1-1 seedlings, and the lower section contains Ler wild-type seedlings. (B) Chlorophyll content of three MAR1 overexpression lines (OE-A, OE-B, and OE-C), Ler and mar1-1 grown on MS plates supplemented with 1% sucrose for two weeks (as described in Methods). (C) Chlorophyll content of 35S::MAR1, mar1-1, and Ler seedlings after two weeks growth on MS supplemented with varying concentrations of Fe-EDTA (as indicated). Chlorophyll was extracted and quantified as in (B). (D) Chlorosis phenotype of 35S::MAR1 leaves from plants grown in soil for 32 days.

Figure 9. MAR1 is downregulated under iron deficiency. (A) Plants were grown for two weeks in liquid MS supplemented with 1% sucrose, and seedling tissue samples were taken before and after four days of incubation in 300 $\mu$M ferrozine. Expression levels of IRT1 and MAR1 are expressed as fold-changes relative to their expression prior to ferrozine treatment. (B) Plants were grown for two weeks on media containing 100 $\mu$M ferrozine prior to RNA extraction and RT-PCR. Equal amounts of each reaction were loaded on an agarose gel, and APRT was included as an internal control. (C) Plants were grown exactly as in (A), except that 600 $\mu$M of Fe-EDTA was added on day 14 (instead of ferrozine). Expression levels of IRT1 and MAR1 are expressed as fold-changes relative to their expression prior to Fe-EDTA treatment.

Figure 10. Model for function of MAR1. Aminoglycoside antibiotics enter the chloroplast through the MAR1 transporter in order to gain access to their ribosomal targets (aminoglycosides bind the 30S ribosomal subunit where they induce misreading and/or premature termination (Recht et al., 1999)). The mutant mar1-1 (indicated as mar1) is less functional, thus minimizing entry of antibiotics and conferring resistance. OM, chloroplast outer membrane; IM; inner membrane; 30S, small ribosomal subunit; 50S, large ribosomal subunit; Ab, aminoglycoside antibiotic.
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