Functional Cloning and Characterization of a Plant Efflux Carrier for Multidrug and Heavy Metal Detoxification*

Received for publication, September 12, 2001, and in revised form, December 5, 2001
Published, JBC Papers in Press, December 5, 2001, DOI 10.1074/jbc.M108777200

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We have identified a detoxifying efflux carrier from Arabidopsis using a functional cloning strategy. A bacterial mutant, KAM3, is deficient in multidrug resistance and does not survive on medium containing norfloxacin. After transformation of KAM3 cells with an Arabidopsis cDNA library, transformants were selected for restored growth on the toxic medium. One cDNA clone that complemented KAM3 codes for a novel protein with twelve putative transmembrane domains and contains limited sequence homology to a multidrug and toxin efflux carrier from bacteria. We named this Arabidopsis protein AtDTX1 (for Arabidopsis thaliana Detoxification 1). A large gene family of at least 56 members encoding related proteins was identified from the Arabidopsis genome. Further functional analysis of AtDTX1 protein in KAM3 mutant demonstrated that AtDTX1 serves as an efflux carrier for plant-derived alkaloids, antibiotics, and other toxic compounds. Interestingly, AtDTX1 was also capable of detoxifying Cd^{2+}, a heavy metal. Further experiments suggest that AtDTX1 is localized in the plasma membrane in plant cells thereby mediating the efflux of plant-derived or exogenous toxic compounds from the cytoplasm.

Plants, as well as other organisms, synthesize and accumulate a diverse array of natural products, which can serve many functions, including defense against or attraction to various insects and microbes in its environment. On the other hand, plants are also exposed to exogenous toxins, including agrochemicals (e.g. pesticides) and toxic compounds secreted by other plants or pathogenic microbes. Disposal and detoxification of toxic compounds of both endogenous and exogenous origin are important processes for plant survival and development. Several possible mechanisms of detoxification include modification of toxic compounds by endogenous enzymes (1), sequestration into vacuole (2, 3), and transport outside of the cell (4, 5).

A large number of transport proteins that mediate the efflux of a broad range of compounds have been identified from various bacteria (6). Four families of detoxifying efflux transporters have previously been described that include major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the resistance/nodulation/compartment (RND) family, and the ATP-binding cassette (ABC) superfamily. The MFS, SMR, and RND families are secondary transporters, typically energized by the proton-motive force. In contrast, ABC is utilized as the energy donor for members of the ABC family of multidrug efflux pumps that are often considered primary transporters (4, 6). To date, ABC family of multidrug efflux pumps have been identified in organisms ranging from bacteria, yeast, animals, and plants. The SMR and RND are only found in bacteria (4). Perhaps the most diverse of all is the MFS type of transporters, which have 12–14 transmembrane domains (TMDs) and are involved in the symport, antiport, or uniport of various substrates, such as sugars, Krebs cycle intermediates, phosphate esters, oligosaccharides, and antibiotics (7). More recent studies have identified two new multidrug efflux proteins, NorM from Vibrio parahaemolyticus and a homologue in Escherichia coli, YdhE (8). Both transporters are required for the efflux of a broad range of toxic compounds. NorM requires Na⁺ for their activity, suggesting that these new systems are Na⁺/toxin antiporters (9). The NorM homologues contain 12 predicted transmembrane domains and are therefore grouped into the MFS family. However, these proteins share no sequence similarity with any member of the MFS and do not exhibit any of the signature sequences specific to the 18 MFS families identified by Pao et al. (7). NorM and YdhE are therefore defined as a new family of secondary transporters, which was referred to as the MATE (multidrug and toxic compound extrusion) family (6).

Although extrusion of toxic compounds by efflux transporters is the major route for detoxification in bacteria, most plant cells possess large vacuoles that can serve as a disposal compartment for toxic compounds. Indeed, at least one member of the plant ABC family, AtMRP1, has been shown to transport glutathione conjugates into vacuoles (10), providing a molecular basis for detoxification by vacuole sequestration in plant cells. Further studies identified a large family of ABC-type proteins from Arabidopsis referred to as AtMRPs (multidrug resistance protein) (3). At least 60 genes in the Arabidopsis genome are identified to encode MRP-like proteins (3, 11). It is speculated that these gene products are localized to various cell membranes and may play different roles in plant cells. For example, a plasma membrane protein, AtPGP1, is highly homologous to animal multidrug resistance protein and is involved in light-dependent hypocotyl elongation (12). A plastid ABC protein is

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* This work was supported by National Institutes of Health Grant GM-52826 (to S. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ The abbreviations used are: MFS, major facilitator superfamily; SMR, small multidrug resistance family; RND, resistance/nodulation/compartment division family; ABC, ATP-binding cassette family; TMD, transmembrane domain; MATE, multidrug and toxic compound extrusion family; MRP, multidrug resistance protein; MOPS, 4-morpholinepropanesulfonic acid; MIC, minimal inhibitory concentrations; GFP, green fluorescence protein; CCCP, carbonyl cyanide m-chlorophenylhydrazone; AcrAB, acrifiavine resistance protein AB.
involved in the plastid-nucleus communication mechanism (13). Although all these AHC-related proteins are presumed to play a role in membrane transport, the transport function of AtPGP1 and the plastid ABC protein has yet to be identified. Concerning detoxification by a possible efflux mechanism, none of the plant proteins have been shown to function as an efflux carrier for multidrug transport. Using a functional cloning strategy, this study identified a MATE-related efflux protein from Arabidopsis (AtDTX1). Transport assays have demonstrated that AtDTX1 protein functions as an efflux transporter that detoxifies a number of lipophilic cations and cadmium, a heavy metal. We also provide evidence that AtDTX1 is localized in the plasma membrane in Arabidopsis plants.

EXPERIMENTAL PROCEDURES

Isolation of AtDTX1 cDNA by E. coli KAM3 Mutant Complementation—E. coli mutant strain KAM3 harbors a deletion in the chromosomal AcrAB genes of TG1 strain and was sensitive to many drugs that are known as substrates of the AcrAB system (14). This mutant strain does not grow on medium containing 0.05 μm norfloxacin (8). We expressed an Arabidopsis cDNA library CD4–7 (Arabidopsis Biological Resource Center, Columbia, OH) in the KAM3 mutant and selected transfectants on the norfloxacin-containing medium. The cDNA clones that supported growth of KAM3 mutant on the toxic medium were sequenced and subcloned into pTrc99A vector (Amersham Biosciences, Inc.). We focused on one cDNA, AIDTX1, for further analyses. The cDNA sequence of AIDTX1 encodes the same protein as annotated under the accession number AAD28687 in the Arabidopsis genomic sequence data base (available at www.mips.biochem.mpg.de/proj/thal/).

For confirmation of complementation, AtDTX1 cDNA was constructed into pTrc99A vector and transformed into KAM3 cells by electroporation. Transformants were selected on the LB medium supplemented with 100 μg/ml ampicillin. Individual transformants were grown in liquid medium containing the same concentration of antibiotics and 1 mM isopropyl-β-D-thiogalactopyranoside for induction of AtDTX1 expression. The cultures were adjusted to 1.0 A600, diluted to 10-fold gradient series, and spotted (2 μl) onto LB solid medium supplemented with 0.05 μg/ml norfloxacin or different concentrations of heavy metal or antibiotics. The growth of different strains was scored after incubation at 37 °C for 24 h. The KAM3 mutant transformed with pTrc99A was used as a control.

Assay of Drug Accumulation In Cells—Assay of norfloxacin and ethidium bromide accumulation was performed as described previously (8, 9). Briefly, E. coli KAM3 containing pTrc99A empty vector or pTrc99A-AtDTX1 were grown in the LB broth supplemented with 40 mM potassium lactate to the late, exponential phase of growth under aerobic condition at 37 °C, harvested, and washed with 0.2 M MOPS-Tris pH 7.0 buffer containing 10 mM potassium lactate to 50 mg (wet weight)/ml. The assay mixture contained cells (10 mg (wet weight)/ml) in the same buffer and 10 mM potassium lactate. After incubation at 37 °C for 5 min, norfloxacin (100 μM, final concentration) was added to initiate the assay. Samples (1 ml each) were taken at intervals, centrifuged at 10,000 rpm for 30 s at 4 °C, and washed once with the same buffer. After 15 min of incubation, ethidium cyanide m-chlorophenylhydrazine (CCCP) was added to the assay mixture at 100 μM to disrupt the proton gradient across the membrane. The pellet was suspended in 1 ml of 100 mM glycine-HCl (pH 3.0). The suspension was shaken vigorously for 1 h at room temperature to release their fluorescent contents and then centrifuged at 15,000 rpm for 10 min at room temperature. The fluorescence of supernatants was measured (excitation at 277 nm and emission at 448 nm) with a Hitachi fluorometer. The amount of maximum fluorescence was normalized to 100%.

Susceptibility Tests and Growth Curves—Minimal inhibitory concentrations (MICs) of heavy metals and drugs were determined by culturing cells in the LB broth containing a particular heavy metal or drug at various concentrations (18, 19). Aliquots of the culture were taken at different time points to measure the growth rates that were used to make a growth curve as indicated in figure legends.

RESULTS

Identification of AtDTX1 by Functional Complementation—We designed a functional cloning strategy to identify putative multidrug efflux carriers from Arabidopsis. An E. coli mutant strain, KAM3, lacks AcrAB systems that are multidrug efflux carriers required for multidrug resistance (14). As a result, KAM3 cells do not grow on medium containing 0.05 μg/ml norfloxacin, a substrate of AcrAB efflux systems. We transformed KAM3 by an expression cDNA library of Arabidopsis plants and isolated those cDNA clones that restored the growth of KAM3 mutant on the medium supplemented with norfloxacin. Among the sequenced cDNA clones, one showed limited homology to the MATE family efflux transporters in the cell (17). The amount of maximum fluorescence was normalized to 100%.

Efflux of Ethidium Bromide and Berberine—For determining ethidium bromide efflux, cells from an overnight culture (5 ml, A600 = 2) were pelleted and washed twice with 100 mM MOPS-Tris (pH 7.0), 2 mM MgSO4 buffer. To load cells with ethidium bromide, the cells were incubated in the same buffer supplemented with 10 μg/ml ethidium bromide and 20 μM CCCP by incubation at 37 °C for 30 min as previously described (9, 17). Cells were pelleted, washed twice, and resuspended at the same ice-cold buffer (A600 = 4.0). Suspension was placed into a flowmeter cuvette and diluted 100-fold with solutions indicated in each figure legend. Because only nucleic acid-bound ethidium bromide gives significant fluorescence, efflux of ethidium bromide from the cells was determined as a decrease in fluorescence. Measurement of berberine efflux was performed by a similar procedure with excitation at 355 nm and emission at 517 nm. The concentration of berberine for cell loading was 30 μg/ml (18).

Subcellular Localization of AtDTX1-GFP Fusion Protein—The subcellular localization of AtDTX1 protein was addressed by GFP fusion and confocal microscopy. The coding region of AIDTX1 was fused to GFP coding region in binary vector pMD1 that contains the cytomegalovirus−JS promoter followed by a short polylinker, GFP-coding region, and the nopaline opine synthesizer terminase region (21). The AtDTX1 cDNA without a stop codon was inserted into the polylinker region to form a frame fusion with the GFP-coding region. The construct was used to transform wild type Arabidopsis plants (Columbia ecotype) by floral dip method (22). Transformants were selected on 0.5× Murashige-Skoog medium containing 1% (w/v) sucrose, 0.8% (w/v) agar, and 60 μg/ml kanamycin and were propagated in the soil. T2 seedlings were used to localize GFP fluorescence by a confocal microscope (Zeiss 510 UV-visible). The images were processed by Adobe Photoshop.

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FIG. 2. Sequence analyses of AtDTX1 and related genes in the Arabidopsis genome. A, phylogenetic tree showing the relationship of AtDTX1 and other members in the AtDTX superfamily. The five clusters are indicated and numbered. The tree was built upon a multiple alignments of polypeptide sequences of AtDTX genes using the ClustalX program (DNAstar DNA analysis software). The horizontal scale indicates the probability (%) of substitutions per amino acid position. B, AtDTX members from different clusters (or subfamilies) are compared. Only limited homology is shared by these members. C, sequence alignment of AtDTX1 with NorM and other putative MATE family transporters. The putative TMDs of AtDTX1 protein, as produced by the TMHMM program, are indicated by horizontal lines above the sequence. NorM, NorM protein of V.
parahaemolyticus (AB04063); YdhE, YdhE protein of *E. coli* (P37340); MEP, multidrug efflux protein of *Xylella fastidiosa* (XP2686); ERI, ethionine resistance protein of *Saccharomyces cerevisiae* (NP_382954); DinF, DNA damage-inducible protein of *Pyrococcus abyssi* (A75151).

Sequence alignments in B and C were performed using the DNastar program with default parameters. Identical residues in the majority of sequences are framed. Gaps were represented by dashes and introduced for optimal alignment. D, phylogenetic tree showing the evolutionary relationship of AtDTX1 and related proteins from bacteria and yeast. See A for interpretation.
AtDTX1 represents a large multigene family in Arabidopsis—A search for sequences with homology to the AtDTX1 gene led to the identification of a large family of genes in the Arabidopsis genome. At least 56 distinct genes were identified that encode proteins with significant sequence similarity to AtDTX1. We refer to these genes as AtDTX1 to AtDTX56. Phylogenetic analysis of this family revealed that members can be further divided into at least five clusters or subfamilies (Fig. 2A). The first cluster includes AtDTX1 through AtDTX19, the second cluster includes AtDTX20 through AtDTX41, and the third cluster includes AtDTX42 through AtDTX49. The fourth cluster may include AtDTX50 through AtDTX55. The fifth group contains only AtDTX56. The putative proteins in this family range in size from 414 to 539 residues, and hydropathy analyses revealed that they all possess 12 putative transmembrane domains (TMD) except for the third cluster of genes, which encode proteins with 8–13 TMDs. Sequence alignments among the genes in different clusters indicated that the most conserved sequences are CGQA located between TMD2 and 3, and RVSNXLGA located in TMD8 (Fig. 2B). Concerning the location of AtDTX genes in the Arabidopsis genome, 21 members are located on chromosome 1; 10 members on chromosome 2; 7 members on chromosome 3; 9 members on chromosome 4; and 9 members on chromosome 5. The Arabidopsis genome initiative numbers showed their chromosome location as listed in Fig. 2A. Some of these genes are present as tandem arrangements on the chromosomes. The genes in the same tandem array often show higher homology and are grouped in the same cluster/subfamily, suggesting that they may be derived from gene duplication.

AtDTX1 also showed limited homology to several genes in other organisms, including NorM, YdhE, and DinF from bacteria, and ERC1 from yeast (Fig. 2C). As discussed earlier, NorM and YdhE are MATE family multidrug extrusion transporters. It is not known whether DinF protein functions as an efflux transporter in bacteria (23). Its expression has been shown to be induced by DNA damage (24). ERC1 confers ethionine resistance in yeast, although a direct transport function of ERC1 protein has not been addressed (25). The phylogenetic relationship of AtDTX1 and these MATE-related proteins is presented in Fig. 2D. According to this phylogeny “tree,” AtDTX1 shares the highest similarity with NorM gene and its homologues.

AtDTX1 gene expression appeared to be ubiquitous. As shown in Fig. 3, AtDTX1 RNA was detected in all organs examined. Its mRNA accumulated in flowers and stems at higher levels as compared with those in leaves and roots.

AtDTX1 reduces accumulation of norfloxacin in KAM3 cells—AtDTX1 complemented norfloxacin-sensitive mutant of E. coli and showed sequence similarity to MATE efflux transporters from bacteria, suggesting that AtDTX1 may function as a norfloxacin transport protein. To further determine the functional properties of AtDTX1, we measured norfloxacin accumulation in the KAM3 mutant cells and cells expressing AtDTX1. If AtDTX1 serves as an efflux carrier, it is expected that KAM3 cells expressing AtDTX1 would accumulate less norfloxacin. As shown in Fig. 4A, a high level of norfloxacin accumulation was observed with the mutant cells harboring the empty vector pTrc99A (KAM3–pTrc99A). In contrast, KAM3 transformed by a plasmid expressing AtDTX1 showed much lower levels of norfloxacin accumulation. This result suggests that AtDTX1 is capable of reducing the net accumulation of the drug by inhibiting norfloxacin influx or increasing efflux. Because efflux through secondary transporters often depends on the transmembrane electrochemical gradient of proton or ions (5), we measured norfloxacin accumulation in the presence of CCCP, a
Cells were first loaded with ethidium bromide. Reduction of fluorescence was used as a measure of the efflux (see "Experimental Procedures").

Function of AtDTX1 as a multidrug transporter was examined further by assaying accumulation of ethidium bromide, another putative substrate for MATE transporters. Fig. 4B indicates that AtDTX1-transformed KAM3 cells accumulated much less ethidium bromide as compared with cells transformed by empty vector. After CCCP was added to the assay mixture, the fluorescence of AtDTX1 transformants was rapidly increased to the similar level in KAM3-pTrc99A cells, suggesting that ethidium bromide, like norfloxacin, was likely to be extruded from KAM3 cells by AtDTX1 protein.

AtDTX1 Mediates Efflux of Ethidium Bromide from KAM3 Cells—To further support that AtDTX1 mediates the efflux of the drugs, we performed ethidium bromide efflux assay according to a previously described procedure (10, 26). Cells were first loaded with ethidium bromide under de-energized conditions in the presence of CCCP. After removing external ethidium bromide and CCCP from the cells, potassium lactate, an energy donor, was added to the efflux buffer to initiate drug efflux process. KAM3 transformed by pTrc99A vector did not show significant efflux of ethidium bromide whereas KAM3-AtDTX1 cells displayed rapid efflux in the same buffer (Fig. 5).

Ethidium Bromide Efflux through AtDTX1 Is Regulated by External pH—It was recently reported that bacterial NorM protein functions as a Na⁺/H⁺ antiporter (9). We tested whether AtDTX1 also depends on external Na⁺ for drug transport. Our results showed that external sodium does not affect AtDTX1 function (data not shown). In both bacteria and higher plants, H⁺ is a common currency for energizing secondary transport processes (27). The fact that CCCP inhibited efflux process revealed a requirement for proton-motive force in AtDTX1-mediated transport. Proton-dependent transport is often regulated by external pH values. We assayed ethidium bromide efflux at various extracellular pH values (Fig. 6). From pH 4.0 to 8.0, activity of AtDTX1 increased significantly. When the external pH increased to 9.0, the efflux activity sharply decreased. The pH regulation observed with AtDTX1 is very similar to regulation of EmrE, a multidrug efflux carrier that mediates proton/drug antiport in bacteria (28).

If an inward proton gradient is the only parameter that is altered by extracellular pH values, increasing extracellular pH values would decrease such a gradient thereby inhibiting drug efflux. Our results on AtDTX1 and from EmrE studies are exactly opposite to this prediction. Clearly, a more complicated mechanism is involved in the pH regulation of AtDTX1- or EmrE-mediated efflux. A model interpreting this pH effect has been proposed with EmrE (28). In particular, studies on EmrE suggest that proton and drug substrates share a common binding site in the EmrE protein. Extracellular pH not only changes the proton gradient but also regulates the charge state of the drug, the affinity of the drug and proton to the common binding site of EmrE, and membrane potential. The combination of these effects determines a net result on the regulation of EmrE-mediated drug efflux by extracellular pH (28). More studies are required to determine if AtDTX1 regulation by pH follows a similar model.

The reversal of pH effect at pH 9 has been reported earlier by Hsieh et al. (26) with a different multidrug transporter that depends on proton gradient. At alkaline pH, an electrogenic Na⁺/H⁺-antiporter in bacteria leads to the acidification of the cytoplasm to maintain the pH homeostasis (29). Hence, at pH 9.0, the pH gradient is inverted. The inverted pH gradient will act to increase the accumulation of weak base and strongly inhibit efflux activity (26).

Efflux of Plant Alkaloids through AtDTX1—Berberine and palmatine are common alkaloids produced in plants. Both exhibit antibiotic properties and serve as substrates for the multidrug resistance pump (26). Recent studies also show that plant alkaloids such as berberine are common substrates for MATE family efflux carriers such as NorM and YdhE (8). The fact that AtDTX1 shares sequence similarity with NorM protein suggests that AtDTX1 may transport plant alkaloids as well. If AtDTX1 mediates efflux of plant alkaloids, these compounds may competitively inhibit the efflux of ethidium bromide. We tested this possibility by using berberine and palmatine. As shown in Fig. 7A, several concentrations, including 1, 5, 10, 50, 100, 250 μM, of berberine were added to the efflux assay mixture. When berberine concentration increased to 100 μM, it significantly inhibited the efflux of ethidium bromide through AtDTX1. Palmatine also inhibited efflux, although it was less effective as compared with berberine (Fig. 7B).

To determine whether AtDTX1 utilizes plant alkaloids as substrates, we examined alkaloid efflux directly using a similar procedure for ethidium bromide efflux assay. Berberine is a planar cationic molecule that resembles ethidium bromide and binds to DNA (30). Similar to ethidium bromide, DNA-bound berberine has enhanced fluorescence. This property of berberine was used to examine berberine efflux as was done with ethidium bromide efflux. The fluorescence was measured with...
excitation at 355 nm and emission at 517 nm as described earlier (18). Cells were first loaded with berberine by adding CCCP in loading mixture. Washed cells were resuspended, and efflux was initiated by adding cells into fluorescence assay buffer. As shown in Fig. 7C, KAM3 transformed by AtDTX1 showed significantly more efflux as compared with KAM3 cells transformed by pTrc99A vector under the same conditions (Fig. 7C). These results indicate that AtDTX1 mediates efflux of plant alkaloids.

AtDTX1 Confers Cadmium Resistance to KAM3 Cells—Heavy metals are toxic to plant growth, and relatively little is understood on the mechanism of heavy metal detoxification in plants (31). In bacteria, several studies suggest that some multidrug resistance proteins are involved in heavy metal resistance (32). For example, disruption of mdrL, a multidrug efflux carrier in Listeria monocytogenes, was associated with reduction in heavy metal resistance (19). To test whether AtDTX1 is involved in heavy metal detoxification, we cultured KAM3-pTrc99A and KAM3-AtDTX1 strains on the media supplemented with different heavy metals, including Al^{3+}, Co^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}, and Zn^{2+}, respectively. The two strains grew differently on the medium containing Cd^{2+} (Fig. 8A) but not on media containing other heavy metals (data not shown). The mutant strain, KAM3-pTrc99A, did not grow on the medium containing 10 μM or higher concentrations of Cd^{2+}. In contrast, KAM3 transformed with AtDTX1 tolerated Cd^{2+} up to 100 μM (Fig. 8A). More analysis using liquid cultures further confirmed that the KAM3-AtDTX1 strain is significantly more tolerant to Cd^{2+} as compared with KAM3-pTrc99A strain (Fig. 8B). During a 24-h culture period, KAM3-pTrc99A and KAM3-AtDTX1 cells grew similarly in the absence of Cd^{2+}. However, 10 and 100 μM Cd^{2+} almost completely inhibited the growth of the KAM3-pTrc99A strain. In contrast, 10 μM Cd^{2+} had little effect on the overall growth of KAM3-AtDTX1 cells. At 100 μM, Cd^{2+} inhibited early growth of KAM3-AtDTX1 cells, but these cells recovered dramatically and nearly reach the density of the control at the end of a 24-h culture. When Cd^{2+} concentration reached 1 mM, growth of AtDTX1 cells was inhibited. This result clearly showed that AtDTX1 confers Cd^{2+} tolerance to KAM3 cells.

GFP Fusion Analysis Suggests a Plasma Membrane Localization of AtDTX1 in Arabidopsis Plants—The functional analyses of AtDTX1 in a bacterial model demonstrated that AtDTX1 serves as an efflux carrier that extrude a number of toxic compounds and possibly heavy metals from cells. To perform the similar cellular function in plants, AtDTX1 protein must be localized to the plasma membrane. To determine the subcellular localization of AtDTX1, we fused AtDTX1 cDNA to a gene coding for green fluorescence protein (GFP) in a binary vector containing GFP fused with a nuclear localization signal (NLS). The transgenic Arabidopsis plants were assayed for expression of GFP by a fluorescence microscope. Figure 8C shows that AtDTX1 was localized to the plasma membrane, and no signal was detected in the nucleus or cytoplasm. These results suggest that AtDTX1 is localized to the plasma membrane, which is consistent with the efflux function of AtDTX1 in plants.
the same material as in Arabidopsis plants. We determined the subcellular localization of AtDTX1-GFP fusion protein in the transgenic plants by laser confocal microscopy. In all cell types examined, fluorescence of AtDTX1-GFP was associated with the plasma membrane. Fig. 9 presents confocal images of root tip and elongated root cells from the controls and AtDTX1-GFP-expressing plants. For the background controls, wild-type non-transformed plants and plants transformed with GFP alone were analyzed. Background signals associated with the root tip region are shown in Fig. 9A. Localization of GFP only is shown in Fig. 9 (B and G), indicating a ubiquitous localization pattern in plant cells. As a positive control for plasma membrane localization, plants transformed with a plasma membrane marker fused to GFP were examined (Fig. 9C, and available on the web at deepgreen.stanford.edu). Fig. 9C shows a defined periphery localization pattern of this fusion protein. As in the positive control, the fluorescence detected in cells of root tip region and elongation zone shows that AtDTX1-GFP fusion is also localized to the cell wall or plasma membrane (cell wall or plasma membrane) not the nucleus or other intracellular compartments (Fig. 9, D and H). Because elongated root cells contain a large central vacuole, the plasma membranes and tonoplasts are closely located and may not be distinguishable by our procedure. However, cells in the root tip region do not contain large central vacuoles, and the fluorescence pattern indicates plasma membrane or cell wall association. To determine if AtDTX1 is localized to the cell wall or the plasma membrane, a plasmolysis experiment was performed. When roots from transgenic seedlings were placed in 0.5 M mannitol, fluorescence in both the positive control and the AtDTX1-GFP-transformed plants was internalized with the cytoplasm (Fig. 9, E and F). This localization pattern indicates that AtDTX1 is localized in the plasma membrane but not in the cell wall, consistent with the presence of 12 putative transmembrane domains in the protein sequence.

**DISCUSSION**

Although several families of detoxification efflux carriers have been described in bacteria, little is known on the efflux system in higher plants. Using a functional cloning strategy, our study has identified and characterized the transport properties of a multidrug efflux carrier from Arabidopsis (AtDTX1). To our knowledge, this is the first plant protein that has been shown to function as a detoxifying efflux carrier. Together with the evidence of plasma membrane localization of AtDTX1 protein, our study shows that plant cells possess an efflux mechanism, in addition to vacuole sequestration, for multidrug and heavy metal detoxification.

The sequence of AtDTX1 protein bears a limited homology to MATE family of multidrug resistance efflux carriers from bacteria. In particular, AtDTX1, like the NorM protein in the MATE family, contains 12 putative transmembrane domains suggesting that AtDTX1 and NorM protein share a similar topology in the membrane. Consistent with this structural similarity, AtDTX1 and NorM both function as an efflux carrier that mediates the extrusion of lipophilic cations such as ethidium bromide and berberine alkaloids. Regarding the mechanism underlying efflux through AtDTX1, our results suggest that a proton-motive force is required for AtDTX1-mediated efflux as supported by CCCP inhibition. This is different from the mechanism of NorM-mediated transport that requires a sodium gradient (9). The conclusion on NorM transport is based on the finding that ethidium bromide efflux through NorM was specifically dependent on the presence of an inward Na⁺ gradient. In addition, Na⁺ efflux was elicited by the influx of ethidium bromide. We tested possible regulation of AtDTX1 by Na⁺ but did not observe any effect. In contrast, CCCP inhibition of efflux through AtDTX1 is a typical property of proton-dependent transporters in bacteria (4, 29, 30). Further studies are required to shed light on the mechanism of AtDTX1-mediated efflux. Another important difference between AtDTX1 and NorM is the capability of AtDTX1 to detoxify cadmium. There are studies showing heavy metal detoxification by other types of MDRs in bacteria (31). For instance, mdrl in L. monocytogenes was required for heavy metal resistance (19). The mechanism for cadmium detoxification is unknown. Comparing with ABC type transporters that use glutathione conjugates as substrates, MATE-type transporters such as NorM and AtDTX1 may transport the substrates that are not conjugated. Finding of AtDTX1 transporters as detoxifying efflux carriers expands the repertoire of plant transporters for removing toxic compounds from important compartments such as cytoplasm.

Although NorM-type transporters often exist as a single gene in bacteria, AtDTX1 is a member of a large multigene family with at least 56 related members in the Arabidopsis genome that is widely accepted as the simplest genome of flowering plants. When this report was in preparation, two genetic studies in Arabidopsis identified two genes that belong to the AtDTX superfamily (34, 35). One gene, referred to as TT12, identical to AtDTX41 in Fig. 2A, was identified by phenotypic screening for mutants with altered seed coat color. Mutation in the TT12 gene reduces the color in seed coat. Because the seed color is largely the result of flavonoid accumulation in vacuoles, and the TT12 sequence contains similarity to MATE transporters, TT12 is speculated to play a role in sequestration of flavonoids into the vacuole of endothelium in the seed coat (34). The second gene, ALF5 (corresponding to AtDTX19 in Fig. 2A), was identified in search for genes...
involved in lateral root formation. The gene product of ALF5 also bears homology to MATE family members and confers resistance to certain toxic compounds in yeast (35). It is hypothesized that ALF5 may be involved in detoxifying toxic compounds in the growth medium and, therefore, is required for lateral root formation and growth. Because transport function and cellular localization of TT12 and ALF5 are not studied, it is not known whether these proteins indeed play a role in membrane transport. It is also unclear whether they are involved in vacuole sequestration or extrusion of toxic compounds from cytoplasm. Nevertheless, both studies reveal the importance of MATE-related proteins in plant development.

Although we doubted that our screening has reached saturation, it is intriguing that complementation screening in this study failed to isolate other members in the AtDTX superfamily if most of these genes function in a similar manner as AtDTX1. One plausible explanation is that different members of the family may function differently. This possible functional diversity can be reflected by several factors. First, the sequence diversity among the members in different clusters of the large family implies diversity in structural features. For example, AtDTX1 represents a cluster of 19 members in the family. In the same cluster, the sequence identity is rather high. If AtDTX1 were compared with those members in other clusters, the sequence identity would drop significantly (Fig. 2B). This structural diversity may lead to functional variations. It will be interesting to test whether members in the same or different cluster function equivalently or differently as compared with AtDTX1 using the RAM3 mutant as a model.

The second factor that could determine the functional diversity is the gene expression pattern and subcellular localization of each member in the family. In this study, we attempted to determine the expression pattern and subcellular location of AtDTX1 protein. Although the AtDTX1 gene did not appear to display much specificity in different organs, we speculate that various members of the AtDTX gene family may differ in their expression pattern in various tissues and at different developmental stages of plants. Different expression pattern would reflect different function. Using GFP fusion and confocal microscopy, we identified plasma membrane as a putative location for AtDTX1. This location is consistent with the functional property of AtDTX1 as a detoxifying efflux carrier characterized in a bacterial model. This also suggests that AtDTX1 may function as an efflux carrier to extrude toxic compounds, including exogenous chemicals (such as pesticides) and secondary metabolites synthesized in plant cells. If some family members highly related to AtDTX1 are also localized to the plasma membrane, it is likely that these members would play a similar function as AtDTX1 does. However, if highly related members are localized to the different compartments of the cell, they must function differently. Study on TT12 could suggest that members of the AtDTX family are localized to different membranes in plant cells. It is speculated that TT12 may serve as a tonoplast transporter for flavonoid sequestration into the vacuole, because t12 mutation reduces seed coat color development (34). Analysis of transport properties and cellular localization of TT12 protein will test this hypothesis. Some other members of the AtDTX family contain presequences that predict location in different membrane systems. For example, AtDTX48 may target chloroplast according to the prediction using the program PSORT (available at psort.nibb.ac.jp). Plant cells have a number of compartments that may serve as a storage space for secondary metabolites or that require such compounds in a metabolic process. It is logical to target AtDTX members to the membrane of various cellular compartments to coordinate the cellular trafficking of their substrate compounds.

The third factor that can result in functional diversity of DTX members is the substrate specificity. Although our study here does not exhaust the list of possible substrates for AtDTX1, it is clear that AtDTX1 transports a broad range of substrates, including norfloxcin, ethidium bromide, berberine alkaloids, and possibly heavy metals. It is speculated that different substrates are transported by different members. If TT12 indeed transports flavonoids into the vacuole as speculated (34), it would have different substrate specificity from AtDTX1 that does not appear to transport flavonoids.2 As shown in this study (Fig. 7), AtDTX1 mediates the efflux of alkaloids such as berberine. This finding could be physiologically relevant, because it has been shown that alkaloids are transported from one plant organ to another through the xylem stream (36). We speculate that transport proteins such as AtDTX1 would be required to export the compounds into the xylem for long distance transport. Because plants produce a large number of secondary metabolites, it will be important to determine if AtDTX1 and other AtDTX members are indeed involved in the distribution of alkaloids and other secondary metabolites. The large number of genes in the AtDTX family and their possible substrate diversity are consistent with their role in the transport of secondary metabolites.

REFERENCES

1. Dixon, D. P., Cummins, I., Cole, D. J., and Edwards, R. (1998) Curr. Opin. Plant Biol. 1, 255–266
2. Yelin, R., Rotem, D., and Schuldiner, S. (1999) J. Bacteriol. 181, 949–956
3. Liu, G., Sanchez-Fernandez, R., Li, Z.-S., and Rea, P. A. (2001) J. Biol. Chem. 276, 8646–8656
4. Putman, M., Veen, H. W., and Konings, W. N. (2000) Microbiol. Mol. Biol. Rev. 64, 672–693
5. Zgurskaya, H. I., and Nikaido, H. (2000) Mol. Microbiol. 37, 219–225
6. Brown, M. H., Paulsen, I. T., and Skurray, R. A. (1999) Mol. Microbiol. 31, 394–395
7. Pao, S. S., Paulsen, I. T., and Saier, M. H. (1998) Microbiol. Mol. Biol. Rev. 62, 323–344
8. Morita, Y., Kodama, K., Shiota, S., Mine, T., Kataoka, A., Mizushima, T., and Tsuzuki, T. (1998) Antimicrob. Agents Chemother. 42, 1778–1782
9. Morita, Y., Kataoka, A., Shiota, S., Mizushima, T., and Tsuzuki, T. (2000) J. Bacteriol. 182, 6694–6697
10. Lu, Y.-P., Li, Z.-S., and Rea, P. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8243–8248
11. Davies, T. G. E., and Coleman, J. O. D. (2000) Plant Cell Environ. 23, 431–443
12. Sideris, M., Haasa, P., Hasan, S., Ringli, C., and Rudler, R. (1998) Plant Cell 10, 1623–1636
13. Müller, S. G., Kunkel, T., and Chua, N.-H. (2001) Genes Dev. 15, 90–103
14. Okusu, H., Ma, D., and Nikaido, H. (1996) J. Bacteriol. 178, 306–308
15. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
16. Bolhuis, H., Molenaar, D., Poelarends, G., Van Veen, H. W., Poelman, B., Driessen, A. J. M., and Konings, W. N. (1994) J. Bacteriol. 176, 6957–6964
17. Neyfakh, A. A., Bidnenko, V. E., and Chen, L. B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4781–4785
18. Sierczynski, F. R., Lorenz, P., Tawara, J. N., Zewenzie, L., and Lewis, K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1433–1437
19. Mata, M. T., Baquero, F., and Perez-Diaz, J. C. (2000) FEMS Microbiol. Lett. 187, 185–188
20. Gupta, R., Huang, Y., Kieber, J., and Luan, S. (1998) Plant J. 16, 581–589
21. Sheen, J., Huang, S., Niwa, Y., Kobayashi, H., and Galbraith, D. W. (1995) Plant J. 8, 777–784
22. Clough, S. J., and Bent, A. F. (1998) Plant J. 16, 735–743
23. Thomas, D., and Sardin-Kerjan, Y. (1997) Microbiol. Mol. Biol. Rev. 61, 503–532
24. Thomas, B., and Wackenagel, W. (1987) J. Bacteriol. 169, 1731–1736
25. Shiomi, N., Fukuda, H., Morikawa, H., Fukuda, Y., and Kimura, A. (1988) Appl. Microbiol. Biotech. 29, 302–304
26. Haish, P.-C., Siegel, S. A., Rogers, B., Davis, D., and Lewis, K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6602–6606
27. Miechtle, B., and Boutry, M. (1995) Plant Physiol. (Rockville) 106, 1–6
28. Yurusultanli, H., and Schuldiner, S. (2000) Biochemistry 39, 14711–14719
29. Padan, L., and Schuldiner, S. (1994) Biochim. Biophys. Acta 1187, 206–210
30. Jennings, B. R., and Riddle, P. J. (1983) Biophys. Struct. Mech. 10, 71–79
31. Silver, S. (1996) Gene (Amst.) 179, 9–19
32. Cobbett, C. S. (2000) Plant Physiol. (Rockville) 123, 825–832
33. Cutler, S. R., Ehrhardt, D. W., Griffis, J. S., and Somerville, C. R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3718–3723
34. Debeaujon, I., Peeters, A. J. M., Leon-Kloosterziel, K. M., and Koomene, M. (2001) Plant Cell 13, 853–871
35. Diener, A. C., Gaxiola, R. A., and Fink, G. R. (2001) Plant Cell 13, 1625–1637
36. Facchini, P. (2001) Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 29–66

2 L. Li and S. Luan, unpublished results.