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Abstract

Following recent indirect evidence suggesting a role for ATP-binding cassette (ABC) transporters in root exudation of phytochemicals, we identified 25 ABC transporter genes highly expressed in the root cells most likely to be involved in secretion processes. Of these 25 genes, we also selected six full-length ABC transporters and a half-size transporter for in-depth molecular and biochemical analyses. We compared the exuded root phytochemical profiles of these seven ABC transporter mutants to those of the wild type. There were three nonpolar phytochemicals missing in various ABC transporter mutants compared to the wild type when the samples were analyzed by high-performance liquid chromatography-mass spectrometry. These data suggest that more than one ABC transporter can be involved in the secretion of a given phytochemical and that a transporter can be involved in the secretion of more than one secondary metabolite. The primary and secondary metabolites present in the root exudates of the mutants were also analyzed by gas chromatography-mass spectrometry, which allowed for the identification of groups of compounds differentially found in some of the mutants compared to the wild type. For instance, the mutant Atpdr6 secreted a lower level of organic acids and Atmrp2 secreted a higher level of amino acids as compared to the wild type. We conclude that the release of phytochemicals by roots is partially controlled by ABC transporters.
Altered Profile of Secondary Metabolites in the Root Exudates of Arabidopsis ATP-Binding Cassette Transporter Mutants

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Following recent indirect evidence suggesting a role for ATP-binding cassette (ABC) transporters in root exudation of phytochemicals, we identified 25 ABC transporter genes highly expressed in the root cells most likely to be involved in secretion processes. Of these 25 genes, we also selected six full-length ABC transporters and a half-size transporter for in-depth molecular and biochemical analyses. We compared the exuded root phytochemical profiles of these seven ABC transporter mutants to those of the wild type. There were three nonpolar phytochemicals missing in various ABC transporter mutants compared to the wild type when the samples were analyzed by high-performance liquid chromatography-mass spectrometry. These data suggest that more than one ABC transporter can be involved in the secretion of a given phytochemical and that a transporter can be involved in the secretion of more than one secondary metabolite. The primary and secondary metabolites present in the root exudates of the mutants were also analyzed by gas chromatography-mass spectrometry, which allowed for the identification of groups of compounds differentially found in some of the mutants compared to the wild type. For instance, the mutant Atatré secreted a lower level of organic acids and Atatrp2 secreted a higher level of amino acids as compared to the wild type. We conclude that the release of phytochemicals by roots is partially controlled by ABC transporters.

The primary functions of roots are the physical anchoring of plants in the soil and the absorption of nutrients and water. Another important function that has attracted much recent attention is the secretion of active phytochemicals by the roots. Root secretions, or root exudates, help roots penetrate the soil (lijima et al., 2003) and orchestrate rhizosphere interactions, including symbiotic, pathogenic, and allelopathic interactions. Hence, exudates play a central role in plant growth in natural habitats (Cobeche et al., 2002; Walker et al., 2003; Bais et al., 2004). Root exudates are composed of both low and high M, components, including an array of primary and secondary metabolites, proteins, and peptides (Bais et al., 2006; Weisskopf et al., 2006). The compounds secreted vary in quantity and chemical structure depending on the plant species, the developmental stage, the interacting organism(s), and a wide variety of environmental factors (Bais et al., 2006). Knowledge of specific genes and mechanisms involved in root secretions remains scant. A plasma membrane H+-ATPase and anion channels were proposed in wheat, lupine, and soybean to account for the root secretion of malate, citrate, and oxalic acid, respectively (Neumann et al., 1999; Yan et al., 2002; Sasaki et al., 2004; Shen et al., 2005). Recently, Loyola-Vargas et al. (2007) and Sugiyama et al. (2007) used a pharmacological approach to provide evidence that root secretion of certain secondary metabolites is an ATP-dependent process and suggested that ATP-binding cassette (ABC) proteins, among other transporters, could be implicated in this process.

The ABC transporters encompass a large protein family found in all phyla and utilize the energy of ATP hydrolysis to translocate solutes across cellular membranes (Higgins, 1992). In bacteria, ABC transporters function...
in both import and export. In eukaryotes, until recently it was thought that ABC transporters function only in export (Saurin et al., 1999). However, recent results provide evidence that some plant ABC transporters also function as importers (Shitan et al., 2003; Santelia et al., 2005; Terasaka et al., 2005). ABC transporters play a central role in many biomedical phenomena, including genetic diseases such as cystic fibrosis, multidrug resistance in cancer patients, and human pathogenic microbes (Borst and Elferink, 2002). These transporters mediate diverse cellular transport processes, such as the excretion of potentially toxic compounds, lipid translocation, heavy metal tolerance, modulation of the activity of ion channels, and nutrient transport and salt stress (Balzi and Goffeau, 1994; Szczypka et al., 1994; Higgins, 1995; Maathuis et al., 2003). Completion of the Arabidopsis (Arabidopsis thaliana) sequence revealed 129 ABC transporter genes that are subdivided into 13 subfamilies (Sanchez-Fernandez et al., 2001; Martinoa et al., 2002; Garcia et al., 2004). Interestingly, the number of Arabidopsis full-size ABC transporters exceeds those reported in yeast (Saccharomyces cerevisiae; Decottignies and Goffeau, 1997) and in humans (Dean et al., 2001), reflecting that the sessile life of plants requires many adaptive strategies and suggests that the higher number of potential substrates (secondary metabolites) produced by plants compared to other organisms may need a higher number of transporters (Dixon, 2001).

In plants, the best-characterized ABC transporters are the full-size subfamilies of transporters, including the multi-drug resistance-related protein (MRP) transporter subfamily, the pleiotropic drug resistance protein (PDR) subfamily, and the multi-drug resistance P-glycoproteins (PGP) subfamily (Martinoba et al., 2002; Crouzet et al., 2006; Geiser and Murphy, 2006; Klein et al., 2006). The MRP transporters are known to function in vacuolar sequestration of glutathionylated compounds, malonylated chlorophyll catabolites and glucuronides (Theodoulou, 2000; Martinoia et al., 2002; Rea, 2007), and in guard cell ion flux (Klein et al., 2003). PDR subfamily members are characterized in fungal systems as efflux transporters of cytotoxic compounds (Balzi and Goffeau, 1994) and in plants they are involved in exporting antifungal diterpene defense compounds to the leaf surface (Jasinski et al., 2001; Stukkens et al., 2005) and also in disease resistance (Kobae et al., 2006; Stein et al., 2006). Members of the third subfamily, the PGP transporters, are characterized to date as being implicated in the transport of the phytohormone auxin (Noh et al., 2001; Geiser et al., 2005; Terasaka et al., 2005; Geiser and Murphy, 2006). The ATH subfamily of half-molecule transporters (16 members in Arabidopsis) is not represented in yeast and little is known about their function in plants (Martinioa et al., 2002).

Generally, the root exudation of phytochemicals is assumed to occur near the root tip and root elongation zone (Hawes et al., 1998; Bais et al., 2002). Therefore, we hypothesized that ABC transporter genes specifically expressed in the epidermis of these regions may likely be involved in the root secretion processes. Here, we have distilled a list of 25 ABC transporters specifically expressed or expressed at higher levels in the cells of the root cap (stage 1), root elongation zone (stage 2), and root hair zone (stage 3) from the data set of Birnbaum et al. (2003), an exhaustive report on the microarray expression analyses of different types of root cells (i.e. lateral root cap cells, endodermis, atrichoblasts, etc.) in Arabidopsis. We correlated this information to other studies that show organ-specific expression of ABC transporters in plants (van den Brulé and Smart, 2002; Santelia et al., 2005). Here, we present a comprehensive report on seven of these ABC transporter genes spanning four subfamilies (MRP, PDR, ATH, and PGP) that are expressed in the root cells and that are involved in the secretion of phytochemicals, employing a combined genetic and biochemical approach.

**RESULTS**

**In Silico Expression Analyses of 25 ABC Transporter Genes Present in the Roots**

After examining the microarray gene expression data set of individual cells of Arabidopsis roots (Birnbaum et al., 2003), we identified 25 ABC transporter genes exclusively or highly expressed in the cells of the endodermis, endodermis-cortex region, atrichoblast, and lateral root cap cells at three different root stages starting from the root tip. Nine subfamilies of ABC transporter genes were found to be expressed in these root cells (Table 1), including PDR, MRP, PGP, and ATH. Among these subfamilies, we did not consider the NAP and GCN subfamilies because they lack a transmembrane domain and either do not function as transporters or the corresponding transmembrane regions are not known (Sanchez-Fernandez et al., 2001). We then analyzed the tissue- and organ-specific and growth-stage-dependent expression pattern of these 25 genes using the Gene Atlas and Gene Chronologer tools of the Genevestigator Web page (Supplemental Tables S1 and S2). The genes of interest belonging to the PDR, MRP, and PGP subfamilies (AtPDR2, AtPDR6, AtPDR8, AtPDR9, AtPDR11, AtMRP8, AtPGP1, and AtPGP4) are highly expressed in the lateral root cap cells and atrichoblasts, except AtMRP2 and AtMRP5, which show high expression in the cells of the endodermis (Table 1). While all these genes are highly or predominantly expressed in roots, they are also expressed in other plant organs (Supplemental Table S1; van den Brulé and Smart, 2002; Santelia et al., 2005). Furthermore, growth-stage-specific expression patterns indicate high transcript abundance in several of the 25 genes (Supplemental Table S2) at days 14 to 17 of plant age, except AtPDR2, AtPDR8, AtPDR9, AtMRP5, AtMRP8, and AtPGP1 in the PDR, MRP, and PGP subfamilies. The expression analysis of these 25 ABC transporter genes throughout Arabidopsis growth reveals three characteristic patterns of expression. Most genes show maximum expression at 14 to 17 after germination (subfamilies PDR, MRP,
Table 1. Array values of 25 ABC transporter genes expressed in the cell types of Arabidopsis root

| Gene ID | Pump Type | Stage 1 | Stage 2 | Stage 3 | Endodermis | Endo-Cortex | Atrichoblast | LRC |
|---------|-----------|---------|---------|---------|------------|-------------|--------------|-----|
| At5g39040 | TAP2 | 452.1 | 333.7 | 228.3 | 223.8 | 73 | 234.3 | 231.8 |
| At4g15230 | PDR2 | 716.9 | 672.2 | 606.6 | 160.4 | 143.9 | 656.5 | 563.8 |
| At2g36730 | PDR6 | 1,656.9 | 2,124.4 | 3790 | 2,647.2 | 1,290.4 | 2,305.1 | 2,568.3 |
| At1g15210 | PDR7 | 1,220.4 | 2,349.3 | 3,853.2 | 3,443.8 | 1,762.6 | 1,655.2 | 1,862.1 |
| At1g9870 | PDR8 | 481.9 | 323.6 | 1,446.8 | 3,920.8 | 2,384.3 | 1,548.4 | 6,630.3 |
| At1g15520 | PDR9 | 62.8 | 82.9 | 70.1 | 1,526.7 | 320 | 664.9 | 3,895.4 |
| At3g33480 | PDR11 | 2,238.7 | 2,524.9 | 2,685.1 | 810 | 542.2 | 2,611.3 | 5,093.3 |
| At2g34660 | MRP2 | 648.9 | 991.1 | 1,452 | 1,117.1 | 514 | 461.9 | 669.5 |
| At1g04120 | MRP5 | 368.5 | 744.5 | 710 | 696.6 | 472.6 | 395.9 | 395.5 |
| At3g32500 | WBC1 | 585.2 | 320.6 | 154 | 1,049.4 | 351.6 | 2,202.3 | 4,087.8 |
| At2g39930 | WBC3 | 755.5 | 435.1 | 211.5 | 209.9 | 399.5 | 298.3 | 282.4 |
| At4g67700 | NAP1 | 635.8 | 568.4 | 750.1 | 493.2 | 323.2 | 326.6 | 253.7 |
| At6g7940 | NAP3 | 185.6 | 296.8 | 237.8 | 123.8 | 211.3 | 330.9 | 334.7 |
| At7g1330 | NAP5 | 230.1 | 190.6 | 164.2 | 154.8 | 192.5 | 239.6 | 281 |
| At3g10670 | NAP7 | 277.8 | 247.7 | 190.5 | 292.4 | 237.8 | 244 | 236.3 |
| At5g02270 | NAP9 | 857.5 | 2,912.4 | 3,597.8 | 1,208.8 | 697.7 | 527.1 |
| At3g47730 | ATH1 | 603.1 | 383.5 | 1,075 | 606.1 | 349.6 | 420.5 | 1,507.7 |
| At3g47780 | ATH4 | 451.5 | 331.1 | 586.3 | 247.7 | 150.6 | 315 | 578 |
| At4g01660 | ATH10 | 1,009.6 | 1,321.8 | 981.2 | 593 | 360.3 | 496 | 358.9 |
| At5g38270 | ATM1 | 605.2 | 484.5 | 420.4 | 421.1 | 253.7 | 294.7 | 237.2 |
| At5g60790 | GCN1 | 1,547.3 | 1,086.8 | 699 | 991.2 | 2,409.6 | 2,022.7 | 2,080.2 |
| At4g4550 | GCN3 | 1,707.6 | 1,236.5 | 581.9 | 572.7 | 567.4 | 723.3 | 504.6 |
| At3g6910 | Pgp1 | 728.2 | 1,280.6 | 1,299.2 | 1,024.3 | 831.2 | 1,121.3 | 274.3 |
| At5g47000 | Pgp1 | 447.6 | 1,005 | 2,106.9 | 1,140.1 | 580.3 | 700.6 | 787.7 |

WBC, NAP, and PGP. Some genes, such as AtTAP2, AtMRP5, AtNAP5, AtATH1, AtATM3, and AtGCN1, show high transcript abundance in the last stage of development (45–50 d). Genes AtMRP8 and AtPGP1 are highly expressed in the preceding stage (36–44 d). Among these 25 ABC transporter genes, we selected seven genes comprising full-length (AtPGP1, AtPGP4, AtPDR2, AtPDR6, AtPDR7, and AtMRP2) and half-size (AtATH6) transporters for detailed genetic and biochemical studies.

Phenotypic Characterization of ABC Transporter T-DNA Knockout Mutants

As detailed in the experimental procedures, the ABC transporter T-DNA knockout (KO) mutants corresponding to the six full-length ABC transporter genes and one half-size transporter were first screened to select homozygous individuals; we then identified the T-DNA insertion region in the gene. Subsequently, RT-PCR assays were conducted to check for complete KO of the gene, and the mutants were screened for visible phenotypes. The T-DNA deletion mutants for the four genes coding for the ABC transporters AtPGP1, AtATH6, AtMRP2, and AtPDR7 were found on the ninth, 12th, 11th, and fifth exons, respectively, in their gene, and the T-DNA insertions for the mutants Atpdr2 and Atpdr6 were found on the 21st intron and the third intron, respectively. All seven ABC transporter mutants show complete KO of the gene, except Atpgp4-2, which has an insertion 133 bp downstream of the stop codon and therefore is able to create a full-length transcript. This transcript appears as a faint band, indicating that RNA stability is affected, probably resulting in a leaky mutant (Fig. 1). Root phenotype analysis showed that the Atmrp2 mutant has significant primary root length reduction (data not shown) and that Atpgp1 has a significant increase in the amount of lateral root formation compared with the wild type. Besides this root phenotype, there is no observable phenotype difference in terms of shoot morphology or bolting or flowering time between the mutants and the wild type under the conditions tested.

HPLC-Mass Spectrometry Analysis of Root Secretion and Root Tissue Metabolite Profiles for the Selected PDR, PGP, ATH, and MRP Arabidopsis Mutants

To explore the role of the seven ABC transporters in the root exudation of phytochemicals, the root exudation profiles of the mutants were analyzed by HPLC-mass spectrometry (MS) and compared with the wild-type profiles at two different time points: 3 and 7 d after continuous secretion of phytochemicals, corresponding to 21- and 25-d-old plants, respectively (see “Materials and Methods”). Based on the extraction method, we found three nonpolar phytochemicals missing in the root exudates of the various mutants compared to those of the wild type after 3 d of continuous root exudation (Table II; Supplemental Fig. S1). Compound 1,
Table II. Retention times and M₆ of the compounds missing in the ABC transporter mutants compared with wild type after 3 d secretion (21-d-old plants) of root exudate profiles analyzed by HPLC-MS

| Compound | Peak Retention Time (min) | Col-0 | pgp4-1 | pgp4-2 | pdr2 | pdr6 | pdr7 | MRP2 | AT16 |
|----------|--------------------------|-------|--------|--------|------|------|------|------|------|
| Compound 1: 33.92 min (208) | + | + | + | - | - | + | - | - | + |
| Compound 2: 38.9 min (387) | + | + | + | + | + | + | - | - | + |
| Compound 3: 39.9 min (647) | + | - | - | + | + | + | - | - | - |

Figure 1. RT-PCR assays of the ABC transporter mutants used in this study. cDNA was prepared from 1.5 μg of root tissue total RNA with superscript reverse transcriptase (Invitrogen) and RT-PCR was performed using gene-specific primers. Below is the actin control for the cDNA of mutants and the wild type. The corresponding Salk line mutants for these ABC transporters are listed in Supplemental Table S3. The primers used for this RT-PCR are listed in Supplemental Table S5. WT, Wild type; M, mutant lines. [See online article for color version of this figure.]

with a retention time of 33.92 min and M₆ of 208, was absent in the exudates of the mutants AtPdr6, AtPdr7, and AtAtp7. Compounds 2 and 3, with retention times of 38.9 and 39.9 min and M₆ of 387 and 647, respectively, were absent in the exudates of the mutants AtMrp2 and AtPgp1. Similarly, compound 3 was also absent in the exudates of the mutants AtPgp4 and AtPdr2. We examined the root exudates in the mutants and wild-type plants for the same three compounds after 7 d of continuous root exudation (Table III; Supplemental Fig. S2). The results were similar to the profiles after 3 d of exudation, except that compounds 1 and 3 were absent in the exudates of the mutants AtPdr7 and AtPdr2 after 3 d of exudation but were present after 7 d of exudation. It is worth noting that these three compounds are present in the wild-type root exudates at both time points.

Additional studies were pursued to validate our data that ABC transporters were involved in root secretion of compounds. First, we performed complementation analyses for one ABC transporter mutant, AtPgp4, by using an overexpresser line in the homozygote AtPgp4-1 mutant background (AtPgp4OX-pgp4-1) to test if the compound missing in the root exudates of the mutants reappears in the overexpresser line. Our results show that compound 3 with M₆ 647, missing in the exudates of the AtPgp4-1 mutant, did reappear in the overexpresser line AtPgp4OX-pgp4-1 after 3 d of secretion of root exudates (Fig. 2), indicating that this compound could be a substrate for this transporter. It is noted that compound 3 accumulates to higher levels in the wild type compared to the AtPgp4OX-pgp4-1. It is possible that the transporter is only expressed in the plasma membrane in the wild type, but that in the overexpresser line the transporter is expressed throughout the plant, with the compounds normally secreted from the root being secreted at other locations, resulting in lower levels of this compound in the root exudates. Second, to prove that root-expressed ABC transporters are involved in the root exudation process, we examined the 3-d root exudate profile of the AtPdr4 mutant, because even though it is highly expressed in the florescence of the wild type, it is not expressed in the roots (van den Brüle and Smart, 2002). This mutant showed no difference in its exudate profile compared to the wild type (Supplemental Fig. S3).

The root tissue phytochemical profiles were further analyzed in all seven mutants at the same time points as those used for the root exudates, and those profiles were compared with those of the wild-type roots. None of the three affected root-secreted compounds were observed in the mutants or wild type, but there were significant differences in the phytochemicals present in the root tissues of the wild type and mutants. For example, compound 4, with a retention time of 19.3 min and M₆ of 398, was present only in the root tissue of mutants AtPgp4, AtPdr6, and AtAtp7 and was not present in that of any other mutants or in wild-type 21-d-old plants (Table IV; Supplemental Fig. S4), but this compound was absent in 25-d-old plants of all mutants and the wild type (Table V; Supplemental Fig. S5). Similarly, compounds 5, 6, and 7, with retention times of 32.0, 45.11, and 46.6 min and M₆ of 186, 314, and 316, respectively, were present in high concentrations in the root tissue of mutants AtPdr2, AtPdr7, and AtMrp2.
Table III. Retention times and \( M_1 \) of the compounds missing in the ABC transporter mutants compared with wild type after 7 d secretion (25-d-old plants) of root exudate profiles analyzed by HPLC-MS

| Compound | Peak Retention Time (Min) | Col-0 | pgp4-1 | pgp4-2 | pdr2 | pdr6 | pdr7 | mpg2 | ath6 | pgp1 |
|----------|---------------------------|-------|--------|--------|------|------|------|------|------|------|
| Compound 1: | 33.92 min (208) | +     | +      | +      | -    | +    | -    | +    | +    | +    |
| Compound 2: | 38.9 min (387) | +     | +      | +      | +    | +    | -    | -    | -    | -    |
| Compound 3: | 39.9 min (647) | +     | -      | +      | +    | +    | -    | -    | -    | -    |

compared with that of wild-type 21-d-old plants. However, the same compounds were present in the root tissue in concentrations similar to those in the wild type in 25-d-old plants except in \( \text{Atmrp2} \), which shows higher concentrations of compounds 6 and 7. In 21-d-old plants of \( \text{Atpg4} \), \( \text{Atpdr6} \), and \( \text{Atathl6} \) mutants, compounds 6 and 7 were similar in concentration in both the exudates of the mutants and the wild type; however, the same compounds were significantly higher in the mutants compared to the wild type in 25-d-old plants. Similarly, in exudates of 21-d-old plants of \( \text{Atpg4} \), \( \text{Atpdr6} \), and \( \text{Atathl6} \) mutants, compound 5 showed a significantly lower concentration compared with its presence in the wild-type exudates: however, the same compound 5 was significantly higher in the exudates of the mutants compared to those of the wild type in 25-d-old plants. In addition, the phytochemical profiles from the root exudates and root tissue of wild-type plants (21 d old) were compared, and these profiles showed at least 80% difference in their profiles (Supplemental Fig. S6).

Partial Characterization of the Missing Compound 1 with \( M_1 \) 208

To identify the missing compounds in the exudates of the mutants, we collected the HPLC eluant corresponding to the peak areas showing the \( M_1 \) corresponding to the compounds 1, 2, and 3 by using HPLC under the conditions described in “Materials and Methods.” Sufficient material was collected for compound 1 to allow further chemical characterization. Accurate \( M_1 \) measurement and tandem MS were performed using hybrid quadrupole time-of-flight tandem MS (QTOF-MS/MS). UV spectral characterization was performed using HPLC coupled with a photodiode array detector (DAD). The HPLC-DAD-detected spectrum showed absorption maximums at 240 nm and 300 nm; the relatively more intense absorption was observed at 300 nm (Supplemental Fig. S7). This type of spectrum can be attributed to a system of four conjugated trans double bonds (Scott, 1964). In addition, a high-resolution MS/MS spectrum in the positive-ion mode showed a molecular ion at 209.1159 (9%: calculated 209.1178 for \( \text{C}_{17}\text{H}_{17}\text{O}_2 \)) and fragment ions at 163.1105 (30%: calculated 163.1123 for \( \text{C}_{17}\text{H}_{16}\text{O} \)), 149.0968 (90%: calculated 149.0967 for \( \text{C}_{17}\text{H}_{15}\text{O} \)), and 131.0861 (100%: calculated 131.0861 for \( \text{C}_{12}\text{H}_{9} \); Supplemental Fig. S7). Based on the fragmentation pattern and UV spectrum data, the following structure is proposed for compound 1: 3-hydroxy-4(Z), 6(Z), 8(Z), 10(Z)-tetraenoic acid (Table II); however, additional proof through NMR analysis is needed to confirm the proposed structure.

Gas Chromatography-MS Analysis of Root Exudate Profiles for the Selected PDR, PGP, ATH, and MRP Arabidopsis Mutants

The primary and secondary metabolites present in the root exudation profiles of the mutants were analyzed by gas chromatography (GC)-MS and compared with the root exudate profiles of the wild type after 3 d of continuous root secretion of phytochemicals corresponding to 21-d-old plants (see “Materials and Methods”). A GC-MS analysis was carried out with the root exudates of the wild type and five mutants (\( \text{Atpdr2} \), \( \text{Atpdr6} \), \( \text{Atmrp2} \), \( \text{Atathl6} \), and \( \text{Atpg4} \)). Principal component analyses (PCAs) showed that the root exudate profiles of mutants \( \text{Atpdr6} \) and \( \text{Atmrp2} \) were clustered separately from other mutants and the wild type (Fig. 3). The identified compounds contributing most to factor 1 in PCA are urea, \( \text{Ty} \), and Asp. Similarly, the identified compounds contributing most to factor 2 are ethanolamine, glycolic acid, and 3-hydroxypropionic acid. On the whole, the exudate profiles of the mutant \( \text{Atpdr6} \) showed significantly lower levels of organic acids (3-hydroxypropionic acid, succinic acid, fumaric acid, etc.) in comparison to the wild type. Figure 2 shows the \( M_1 \) trace of compound 3 (647) in the root exudates of the wild type (Col-0), ABC transporter mutant \( \text{Atpg4-1} \) (\( \text{pgp4-1} \)), and AtPGP4 overexpressor line in the homozygote \( \text{Atpg4-1} \) mutant background (\( \text{Atpg4OX-ppg4-1} \)) after 3 d of continuous root secretion (details in “Materials and Methods”). Arrows indicate the absence of peak, Asterisks indicate the presence of peak. The number indicates the peak number. The results represent experiments repeated two times with three replicates each. [See online article for color version of this figure.]

![Figure 2](image_url)
acid, malic acid, trihydroxybutyric acid, ribonic acid, etc.), while the Atmrp2 mutant showed a significantly high level of amino acids (L-Ser, L-Pro, L-Ile, L-Thr, L-Cys, L-Phe, etc.) compared to the wild-type root exudate profiles (Supplemental Fig. 8). Besides these, there were significant differences in the “unknown” compounds in the exudates of the mutants compared to those of the wild type.

**DISCUSSION**

Generally, in living systems, molecules are transported against a concentration gradient requiring the use of energy. For several transport systems, this energy is directly provided by ATP hydrolysis. Well-known examples of ATP-powered systems are P-type ATPases and ABC transporters. It has been demonstrated that ABC transporters are involved in the membrane transport of endogenous secondary metabolites in plants (Yazaki, 2005). However, until now, only indirect evidence has been presented in support of the involvement of ABC transporters in the release of phytochemicals into the soil (Loyola-Vargas et al., 2007; Sugiyama et al., 2007). No direct genetic evidence that ABC transporters do indeed play a role in root exudation of secondary metabolites has been found. Here, we present direct evidence that ABC-type transporters are involved in the release of secondary compounds from the roots.

A recent report (Birnbaum et al., 2003) provides evidence that ABC transporters are expressed in root cells, including those in the lateral root cap, the epidermis, and the endodermis in the root cap, and the root elongation and root hair zones. We hypothesized that these ABC transporters may play a role in the root exudation process. Among the seven ABC transporters selected for this study, AtPGP1 and AtPGP4 have been shown to be localized to the plasma membrane of mature root epidermal cells, and both are involved in auxin transport (Santelia et al., 2005; Terasaka et al., 2005; Geisler and Murphy, 2006). AtMRP2 has been demonstrated to reside in the vacuolar membrane and to transport glutathione S-conjugates and chlorophyll catabolites (Lu et al., 1998). There are no reports available on the subcellular localization of the other transporters examined in this study (AtPDR2, AtPDR6, AtPDR7, and AtATH6), and only a few reports describe the function of plant PDRs. In these reports, it was proposed that the compounds transported by PDRs are required for plant protection against pathogen attack (Jasinski et al., 2001; Stukkens et al., 2005; Kobae et al., 2006; Stein et al., 2006). Therefore, this subfamily of ABC transporters appears to be particularly important in the root exudation of phytochemicals, and this exudation might influence the microbial composition of the rhizosphere.

HPLC-MS analyses of the seven ABC transporter mutants revealed that the mutant exudates were deficient in either one or two compounds compared with the wild-type profiles (Table II; Supplemental Fig. S1). For example, compound 1, tentatively identified as 3-hydroxy-4(Z),6(Z),8(Z),10(Z)-tetraenoic acid, is lacking in the root exudates of mutants AtPDR6, AtPDR7, and AtATH6, indicating that one compound may act as a substrate for more than one ABC transporter. In contrast, compounds 2 and 3 were absent in the exudates of mutants Atmrp2 and Atpgp1, suggesting that one ABC transporter can transport structurally and functionally unrelated compounds. Previous studies indicate that AtMRP2 and AtMRP3 can simultaneously transport endogenous substrates such as chlorophyll catabolites and glutathione compounds and that neither transport interferes with the other (Lu et al., 1998; Tommasini et al., 1998). ABC transporters can also transport a

### Table IV. Retention times and M of the compounds of the root tissue profiles of 21-day-old wild type and ABC transporter mutants analyzed by HPLC-MS

| Peak Retention Time (M) | Col-0 | pgg4-1 | pgg4-2 | pdr2 | pdr6 | pdr7 | mpg2 | atth6 | pgg1 |
|------------------------|-------|--------|--------|------|------|------|------|-------|------|
| Compound 4: 19.3 min (398) | - | - | - | + | - | - | - | - | - |
| Compound 5: 32.0 min (186) | 12.9 ± 1.0 | **37.3 ± 1.8** | **32.4 ± 1.5** | 13.6 ± 2.5 | **78.9 ± 0.5** | **16.7 ± 0.0** | **31.6 ± 0.5** | **10.9 ± 0.1** |
| Compound 6: 45.11 min (314) | 4.7 ± 1.5 | **9.0 ± 2.2** | **8.3 ± 1.6** | 4.7 ± 2.3 | **9.9 ± 1.0** | **3.8 ± 1.5** | **10.2 ± 1.2** | **8.1 ± 2.2** |
| Compound 7: 46.6 min (316) | 5.0 ± 2.0 | **7.5 ± 0.5** | **8.4 ± 0.3** | 3.8 ± 1.3 | **8.0 ± 1.5** | **2.7 ± 1.0** | **9.3 ± 2.1** | **8.5 ± 1.1** |

Values represented are peak areas of the corresponding compounds in the wild type and mutants. The values in bold are statistically significant when compared to the wild type at P ≤ 0.05, n = 6. +, Absence of compound.
number of chemically different compounds; an example might be PDR5 from yeast, which is active in transport of numerous unrelated compounds (Kolaczkowski et al., 1996). The majority of tetraenoic fatty acids are structural monomers of the lipids present in the mitochondrial membrane, and their levels fluctuate according to the various environmental stimuli, especially low or high temperatures (Makarenko et al., 2003). Additionally, it has been reported that tetraenoic fatty acids are present in root hairs (Qu et al., 2005). We analyzed the root exudation profiles of the seven ABC transporter mutants and compared those profiles with the wild type at two time points. It was interesting to find that compounds 1 and 3 were absent in the 21-d-old plant root exudates but present in the 25-d-old plant root exudates of the mutants Attdr7 and Attdr2, respectively (Table III; Supplemental Fig. S2). Several reasons could account for this observation, such as developmentally dependent redundancy of the ABC transporters in transporting these two compounds outside the cell.

Although only a few plant ABC transporters have been localized so far, it is known that AtPgp1 and AtPgp4 are targeted to the plasma membrane, while AtMRP2 is localized in the vacuolar membrane (Lu et al., 1998; Terasaka et al., 2005; Geisler and Murphy, 2006). Thus, we expected that the phytochemicals not detected in the mutant root exudates would accumulate inside the cell organelles. The phytochemical profile of the root exudates and root tissues of the wild-type plants were not similar and were found to have only 20% overlap based on the $M_r$ of the compounds detected by HPLC-MS (Supplemental Fig. S6). This 80% difference between root tissue and root exudates can be explained by the fact that only a minority of the compounds synthesized in the plant are excreted. The observation that several excreted compounds are not found within the root might be due to the fact that these compounds are excreted very efficiently and that cellular concentrations are kept low. We found significant differences in the root tissue profiles of 21-d-old and 25-d-old mutants with the root tissue profiles of the corresponding wild type (Tables IV and V). Compound 4 was present in the exudates of the mutants Atpgp4, Attdr6, and Atath6 and absent in those of other mutants, including the wild type, suggesting that this compound may be a precursor compound and is accumulating in the vacuoles or other organelles due to the absence of the corresponding ABC transporter. AtMRP2 is localized in the vacuolar membrane and, if impaired, may affect the vacuolar transport of compounds, changing the phytochemical profile of the cytosol and the root exudation profile of the plant.

To identify other compounds (both primary and secondary metabolites) in the exudates, we analyzed the root exudates of the wild-type and ABC transporter mutants by GC-MS. Two mutants, Attdr6 and Attpr2, show a low level of dicarboxylic acid secretion and a high level of amino acid secretion in the exudates compared to the wild type, respectively (Supplemental Fig. S8). Because we found such widespread changes in root secretion of primary and secondary metabolites in the GC-MS analysis of the ABC transporter mutants, we are tempted to suggest that all these changes are not necessarily directly due to the specific ABC transporter but rather that the lack of expression of the transporter could change the expression pattern of other transporting systems leading to changes in the secretion of several other unrelated compounds. This hypothesis is supported by the fact that in the GC-MS data, we did not detect missing compounds compared to the wild-type exudates, as observed in LC-MS analysis, but rather differences in the ratios of the same type of compounds.

It is possible that the missing compounds observed in the ABC transporter mutant root exudates are due to precursor compounds that are not translocated from shoots to roots or between organelles in the cell. ABC transporters located mainly in roots but also expressed in other tissues may be involved not only in the excretion of compounds but also in long-distance transport. In particular, the ABC transporters AtPDR1, AtPDR6, AtPDR7, and AtATH6 do not have subcellular localization data, meaning that they may indeed not be targeted to the plasma membrane and thus that intracellular transport may affect the metabolism and hence the synthesis of the compounds found in the root exudates. On the other hand, the ABC transporters AtPgp4 and AtPgp1, located at the plasma membrane mainly in the root epidermal cells, are quite likely involved in the excretion of compounds at the root level.

Taken together, these data show that ABC transporters are involved in the root exudation process. Based on the results reported here, three nonmutually
exclusive possibilities exist for how that involvement operates: one ABC transporter may transport one specific compound; one compound may act as a substrate for more than one ABC transporter; or one ABC transporter may transport a range of unrelated compounds. Thus, it could be inferred that the additional 18 ABC transporters found in Arabidopsis roots, other types of transporters (i.e., MATE), or other nontransporter mechanisms may account for the secretion of the large number of phytochemicals present in the root exudates. Current efforts were focused on identifying transport systems involved in root secretion and future efforts will focus on complete characterization of these transport systems and compounds. Elucidation of these additional secretion mechanisms will provide tools to overproduce the secretion of phytochemicals for agricultural and biotechnological benefits.

MATERIALS AND METHODS

In Silico Analyses of 25 ABC Transporter Genes

A list of 25 ABC transporter genes expressed in the root cells of Arabidopsis (Arabidopsis thaliana) were derived from the microarray gene expression dataset (kindly provided by Dr. Philip N. Beney, Duke University) of individual root cells such as endodermis, endodermis-cortex region, atrichoblast, and lateral root cap cells at three different stages: stage 1, approximately 0.15 mm from the root tip (root cap); stage 2, approximately 0.30 mm from the root tip (root elongation zone); and stage 3, approximately 0.45-2.2 mm from the root tip (root hair zone; Bimbbaum et al., 2003; Table I). The tissue- and organ-specific expression and growth stage-dependent expression pattern of these 25 genes were analyzed using the Gene Atlas and Gene Chronologer tools of the “Genevestigator” Web interface (https://www.genevestigator.eth.ch/; Zimmermann et al., 2004). The dataset was restricted to the Columbia-0 (Col-0) ecotype representing the sequenced genome and all chips (AIGeneExpress, NASC arrays, GEO, ArrayExpress, Gruissem Laboratory, and the Functional Genomics Centre Zurich) were used for analysis.

Plant Material and Growth Conditions

Arabidopsis seeds were surface sterilized with bleach for 2 min followed by four rinses in sterile distilled water and plated on Murashige and Skoog (Murashige and Skoog, 1962) salts supplemented with 3% Suc and 0.7% bactoagar in petri dishes. Plates were incubated in a growth chamber (Percival Scientific) at 25°C, with a photoperiod of 16 h light/8 h dark for germination. To collect root exudates, 7-d-old seedlings were transferred to 6-well culture plates (Fischer), each containing 5 mL of liquid Murashige and Skoog (Murashige and Skoog) basal salts supplemented with 1% Suc, incubated on an orbital shaker at 90 rpm, and illuminated under cool-white fluorescent light (45 μmol m−2 s−1) with a photoperiod of 16 h light/8 h dark at 25°C. According to the methods of Loyola-Vargas et al. (2007), when plants were 18 d old, they were washed with sterile water to remove the surface-adhering exudates and transferred to new 6-well plates containing 5 mL of MS liquid media and incubated on an orbital shaker at 90 rpm and illuminated under cool-white fluorescent light (45 μmol m−2 s−1) with a photoperiod of 16 h light/8 h dark at 25°C. The exudates were collected 3 and 7 d after transfer (plants were 21- and 25-d-old, respectively). For each replicate analysis, we collected 60 mL exudates from 12 Arabidopsis plants. For vertical plate assays, mutant and wild-type seeds were plated on Murashige and Skoog agar plates supplemented with 0.5%; and 1% Suc and incubated in a growth chamber with a photoperiod of 8 h light/16 h dark and readings were taken after 7 d. The list of ABC transporter T-DNA KO mutants used in this study is provided in Supplemental Table S2.

Screening of T-DNA Mutant Homozygous Lines

Plant lines containing T-DNA insertions for the seven ABC transporter genes used in this study were obtained from the Arabidopsis Biological Resources Center and from the mutant collection of Dr. Enrico Martinoia. The T-DNA insertion and homozygote nature were verified by PCR analysis with a gene-specific primer and left border primer and further confirmed by sequencing. The complete KO of the gene product was confirmed by RT-PCR. Briefly, total RNA was isolated from root tissue using a Qiagen Plant RNAeasy mini kit, and subsequently cDNA was prepared using Superscript Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. PCR was performed by using gene-specific primers. The primers used in this study for homozygote screening and gene expression are listed in Supplemental Tables S2 and S5.

Phytochemical Extraction

Twenty-one-day-old and 25-d-old Arabidopsis seedlings grown in 6-well plates (one plant per well) were frozen in liquid N2. These samples were used for extraction of rhizo-secreted phytochemicals. The collected liquid medium was centrifuged at 8,000 rpm for 20 min followed by filtration using Nylon syringe filters of 0.45-μm pore size (Nalgene) to remove root sheathing and root-border-like cells. After filtration, the liquid was concentrated by freeze-drying (Labconco) to remove water, and the concentrate was dissolved in 5 mL of double-distilled water. The pH was adjusted to 3.0 and the concentrate was partitioned two times with 5 mL of ethyl acetate. The ethyl acetate fractions were pooled and dried under N2 gas. The dried concentrate was then dissolved in 100 μL of methanol and analyzed by HPLC-MS. Root tissue phytochemical extractions were carried out by grinding 50 mg of freeze-dried root tissue in 5 mL of 80% methanol and centrifuging for 20 min at 8,000 rpm to collect the supernatant before drying under N2 gas. The dried concentrate was again dissolved in 1 mL methanol and analyzed by HPLC-MS. The experiment was repeated twice with three replicates.

HPLC and MS Analyses

The extracted phytochemicals from liquid media were chromatographed by gradient elution on a 150-mm × 4.6-mm reverse-phase, C18 column (Supelco). The chromatographic system (Dionex) consisted of two P680 pumps connected to an AS1-100 automated sample injector and was detected at 280 nm with a UV-visible detector. M, determination of the peaks was performed using a quadrupole mass spectrometer detector (MSQ-MS; Thermo Electron). A gradient was used for all separations with a flow rate of 0.7 mL min−1. The gradient was as follows: 0 to 10 min, 90.0% water and 10% methanol, 10 to 60 min, 10.0% to 90% (v/v) methanol and 90% to 10% (v/v) water; and 60 to 70 min, 90.0% (v/v) methanol and 10% water.

GC and MS Analyses

Root exudates for GC-MS analyses were collected in a similar manner as for HPLC-MS analyses, except that the 18-d-old plants were washed in sterile water and transferred to new 6-well culture plates containing 5 mL of sterile water instead of MS liquid media with 1% Suc. After 3 d of continuous secretion, the root exudates were collected, freeze-dried, and processed following the standard methoximation and trimethylsilylation derivative procedure (Brockling et al., 2005). One microliter of each sample was injected onto an Agilent 6890 GC coupled to a 5973 MS at a split ratio of 1:1. The initial oven temperature of 80°C was held for 2 min ramped at 5°C/min to a final temperature of 315°C and then held for 12 min. Separation was achieved using a 60 m DB-5MS (J & W Scientific; 0.25 mm i.d. and 0.25-μm film thickness) at a flow rate of 1.0 mL/min. Peak detection and deconvolution were performed at AMDIS (Halket et al., 1999) for several samples of each treatment, and peak lists were compiled in MET-IDEA (Brockling et al., 2006). MET-IDEA was then used to extract quantitative peak area values for polar and nonpolar data. Redundant peaks were removed from the data set, peak area values were scaled to mean zero, and so 1.0 and the resulting data matrix were analyzed with ANOVA, PCA in JMP (SAS Institute). Only P values of <0.01 were considered significant for ANOVA analyses.

Supplemental Data

The following materials are available in the online version of this article. Please visit the Plant Physiol. Vol. 146, 2008 website.

Supplemental Figure S1. Mass chromatograms of compounds 1 (2038), 2 (387), and 3 (647) in the root exudates of wild-type and ABC transporter mutants for 3 d of continuous root secretion of compounds (details in “Materials and Methods”).
Supplemental Figure S2. Mass chromatograms of compounds 1 (208), 2 (387), and 3 (647) in the root exudates of wild-type and ABC transporter mutants for 7 d continuous root secretion of compounds (details in "Materials and Methods").

Supplemental Figure S3. Root exudates profile of 21-d-old wild-type (Col-0) and ABC transporter mutant AtPrd1 (PDR4) plants analyzed by HPLC-MS at wavelength 280 nm.

Supplemental Figure S4. Metabolic profile of Arabidopsis root tissues of 21-d-old wild-type and ABC transporter mutant plants analyzed by HPLC-MS at wavelength 280 nm.

Supplemental Figure S5. Profile of Arabidopsis root tissues of 25-d-old wild-type and ABC transporter mutant plants analyzed by HPLC-MS at wavelength 280 nm.

Supplemental Figure S6. Chromatogram shows the profiles of root exudates and root tissues from wild-type plants.

Supplemental Figure S7. A, Absorption spectrum of compound 1 showing M, 208 detected by UV coupled with DAD, and B, accurate mass and fragmentation pattern of compound 1 detected using a hybrid QTOF/MS/MS.

Supplemental Figure S8. Graphs illustrating the representative compounds that show different levels in the ABC transporter mutants compared to wild type analyzed by GC-MS.

Supplemental Table S1. Tissue- and organ-specific expression of 25 ABC transporter genes expressed in Arabidopsis roots.

Supplemental Table S2. Growth-stage-dependent expression pattern of 25 ABC transporter genes expressed in Arabidopsis roots.

Supplemental Table S3. List of ABC transporters and their T-DNA KO mutants used in this study.

Supplemental Table S4. List of primers used in this study for screening homozygous lines of ABC transporters T-DNA KO mutants.

Supplemental Table S5. List of primers used in this study for RT-PCR assays.

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ATP-Binding Cassette Transporters Mediate Root Exudation

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