



Influence of ATP-binding cassette transporters in root exudation of phytoalexins, signals, and in disease resistance

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The roots of plants secrete compounds as a way to exchange information with organisms living in the soil. Here, we report the involvement of seven root-expressed ATP-binding cassette (ABC) transporters corresponding to both full and half-size molecules (*Atabcg36*, *Atabcg37*, *Atabcc5*, *Atabcf1*, *Atabcf3*, *Atnap5*, and *Atath10*) in root exudation processes using *Arabidopsis thaliana*. Root exuded phytochemicals were analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS) and gas chromatography-mass spectrometry (GC-MS), and it was determined that some of the root exudates from the corresponding ABC transporter mutants were significantly different compared to the wild type. For example, *Atabcg37* and *Atabcc5* secreted higher levels of the phytoalexin camalexin, and *Atabcg36* secreted higher levels of organic acids, specifically salicylic acid (SA). Furthermore, we analyzed the root tissue metabolites of these seven ABC transporter mutants and found that the levels of SA, quercetin, and kaempferol glucosides were higher in *Atabcg36*, which was correlated with higher expression levels of defense genes in the root tissues compared with the wild type. We did not observe significant changes in the root exudates of the half-size transporters except for *Atabcf1* that showed lower levels of few organic acids. In summary, full-size transporters are involved in root secretion of phytochemicals.

Keywords: ABC transporters, phytoalexin, root exudates, disease resistance, salicylic acid, defense proteins

INTRODUCTION

ATP-binding cassette (ABC) transporters are one of the largest protein families in bacteria, fungi, plants, and animals, including both membrane bound and soluble proteins (Henikoff et al., 1997; Yazaki, 2005; Rea, 2007; Verrier et al., 2008; Yazaki et al., 2009). In prokaryotes, most members of this family are involved in high-affinity uptake of small molecules like maltose or histidine (Higgins, 1992). In humans, ABC transporters play a key role in multidrug resistance of cancer cells, by detoxifying chemotherapeutic drugs; and in genetic diseases such as cystic fibrosis (Gottesman and Pastan, 1993; Borst and Elferink, 2002). In plants, ABC transporters are responsible for the transportation of a multitude of compounds (Kang et al., 2010). They are involved in the excretion (extracellular over the plasma membrane or intracellular into the vacuoles of plants) of potentially toxic compounds, lipid translocation, transport of steroids and their derivatives, conferring heavy metal tolerance, and transporting phytohormones (Yazaki, 2005; Rea, 2007; Yazaki et al., 2009; Kang et al., 2010). Recent reports have shown that the *Arabidopsis* ABC transporters AtPDR8, AtPDR12, and NpPDR1 are involved in plant defense responses against *Pseudomonas syringae* pv. *Lycopersici* (DC3000), *Peronospora parasitica*, *Phytophthora infestans*, *Golovinomyces orontii*, and *Plectosphaerella cucumerina* (Lipka et al., 2005; Kobae et al., 2006; Stein et al., 2006). It has also been demonstrated that AtPDR8 and AtPDR9 are involved in regulation of auxin homeostasis and plant development by directional transport of the auxin precursor

indole-3-butyric acid (IBA; Strader and Bartel, 2009; Ruzicka et al., 2010). In addition, it was demonstrated that AtPDR8 was required for the extracellular accumulation of the glucan polymer callose, in response to treatment with a microbe-associated molecular pattern (MAMP) derived from bacterial flagellin (Clay et al., 2009). Recent literature has also reported that some ABC transporters are involved in the secretion of compounds by roots (Badri et al., 2008, 2009).

Roots secrete phytochemicals into the soil that mediate complex interactions with soil-borne organisms (Badri et al., 2009). Recently, it has been reported that root-specific ABC transporters of *Arabidopsis* are involved in exudation of phytochemicals (Badri et al., 2008), and that mutants of a specific transporter (*Atabcg30*) when grown in natural soils of *Arabidopsis* promote a complete overhaul of the microbial community found in association with wild-type *Arabidopsis* roots (Badri et al., 2009). Specifically, the *Atabcg30* mutant promoted the growth of beneficial bacteria such as plant growth promoting rhizobacteria (PGPRs), nitrogen fixers, and bacteria involved in heavy metal remediation. Metabolomic profiling revealed an increase in phenolic compounds and a decline in sugars in the root exudates of the *Atabcg30* mutant compared to the wild type and other ABC transporter mutants. Therefore, it was hypothesized that the modified ratios of sugars to phenolics in the root exudates of the mutant promote the growth of specific soil microbes at the expense of others.

The *Arabidopsis* genome encodes 129 ABC transporter proteins that are sub-divided into 13 subfamilies (Sanchez-Fernandez et al., 2001; Martinoia et al., 2002; Garcia et al., 2004; Rea, 2007); exceeding the numbers reported in yeast (Decottignies and Goffeau, 1997) and in humans (Dean et al., 2001). Interestingly, 25 of these transporters are highly expressed in roots, and the root exudate profiles of seven of those mutants have been determined (Badri et al., 2008). Based on previous studies highlighting the dramatic changes that one mutation in an ABC transporter can have on the overall diversity of compounds secreted by roots, we report here the effect of seven additional root-expressed ABC transporter mutants, corresponding to both full-size and half-size molecules (*Atabcg36*, *Atabcg37*, *Atabcc5*, *Atabcf1*, *Atabcf3*, *Atnap5*, and *Atath10*), in terms of root exudates secretion.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Arabidopsis seeds were surface-sterilized with laundry bleach for 1 min followed by four rinses in sterile distilled water and plated on Murashige and Skoog (MS; Murashige and Skoog, 1962) salts supplemented with 3% sucrose and 0.7% bactoagar in Petri plates. Petri plates were incubated in a growth chamber (Percival Scientific) at 25°C, with a photoperiod of 16 h light/8 h dark for germination. For vertical plate assays, mutant and wild-type seeds were plated on MS agar plates supplemented with 0.5 and 1% sucrose and incubated in a growth chamber with a photoperiod of 8 h light/16 h dark and readings were taken after 14 days. The list of ABC transporter T-DNA KO mutants used in this study is provided in **Table A1** in Appendix.

SCREENING OF T-DNA MUTANT HOMOZYGOUS LINES

T-DNA mutants for the seven ABC transporter genes used in this study were obtained from the *Arabidopsis* Biological Resources Center (ABRC) and from the mutant collection of Dr. Enrico Martinoia. The T-DNA insertion and homozygote nature of the mutants were verified by PCR analysis with a gene-specific primer and left border primer, and further confirmed by sequencing. The complete knockout of the gene product was confirmed by RT-PCR. Briefly, total RNA was isolated from root tissue using a Trizol reagent and subsequently cDNA was prepared using Superscript Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. PCR was performed using gene-specific primers. The primers used for homozygote screening and gene expression are listed on **Tables A2** and **A3** in Appendix.

ROOT EXUDATE COLLECTION AND PHYTOCHEMICAL EXTRACTION

To collect root exudates, 7-day-old seedlings were transferred to six-well culture plates (Fischer Co.) each containing 5 ml of liquid MS (MS basal salts supplemented with 1% sucrose), incubated on an orbital shaker at 90 rpm and illuminated under cool white fluorescent light ($45 \mu\text{mol m}^{-2} \text{s}^{-1}$) with a photoperiod of 16 h light/8 h dark at $25^\circ\text{C} \pm 2$. According to the methods of Badri et al. (2008, 2009), when plants were 18 days old they were washed with sterile water to remove the surface adhering exudates and transferred to new six-well plates containing fresh 5 ml MS liquid media and incubated on an orbital shaker at 90 rpm and illuminated under cool white fluorescent light ($45 \mu\text{mol m}^{-2} \text{s}^{-1}$) with a

photoperiod of 16 h light/8 h dark at $25^\circ\text{C} \pm 2$. The exudates were collected 3 days after transfer (plants were 21 days old). For each replicate analysis, we collected 60 ml exudates from 12 individually grown *Arabidopsis* plants. The collected root exudates were filtered using nylon filters of pore size $0.45 \mu\text{m}$ (Millipore) to remove root sheathing and root-border-like cells. After filtration, the exudates were freeze-dried (Labconco), dissolved in 5 ml double distilled water and the pH was adjusted to 3.0 with 1 N HCl and partitioned two times with equal volume of ethyl acetate (Fisher Scientific). The ethyl acetate fractions were pooled and dried under nitrogen gas. The dried concentrate was again dissolved in 100 μl absolute methanol (Fisher Scientific) and analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS). Root tissue metabolites were extracted by grinding 50 mg of freeze-dried root tissue in 5 ml of 80% methanol and centrifuging for 20 min at 8000 rpm to collect the supernatant before drying under N_2 gas. The dried concentrate was again dissolved in 1 ml methanol and analyzed by HPLC-MS. The experiment was repeated twice and each experiment had three replicates.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY ANALYSES

The phytochemicals extracted from root exudates were chromatographed by gradient elution on a 150 mm \times 4.6 mm reverse phase, C18 column (Dionex). The chromatographic system (Dionex Co., Sunnyvale, CA, USA) consisted of two P680 pumps connected to an AS1-100 automated sample injector and detected at 280 nm with a UV-visible detector. Mass determination of the peaks was analyzed by an MSQ-MS detector system (Thermo Electron Co., Waltham, MA, USA). A gradient was applied for all separations with a flow rate of 0.7 ml min^{-1} . The gradient was as follows: 0–3 min, 90% (v/v) water and 10% (v/v) methanol; 3–43 min, 10–90% (v/v) methanol, 90–10% (v/v) water; 43–51 min, 90% (v/v) methanol and 10% (v/v) water.

GAS CHROMATOGRAPHY AND MASS SPECTROMETRY ANALYSES

Root exudates for gas chromatography-mass spectrometry (GC-MS) analyses were collected similarly as for the HPLC-MS analyses except that the 18-day-old plants were washed in sterile water and transferred into new six-well plates containing 5 ml sterile water instead of MS liquid media with 1% sucrose. This procedure was adopted to prevent the interference of exogenously supplemented salts and sucrose present in the MS liquid media for the subsequent GC-MS analyses. After 3 days' continuous secretion, the root exudates were collected, freeze-dried, and processed following the standard methoximation and trimethylsilylation derivative procedure (Broeckling 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 was 80°C and was held for 2 min, ramped at 5°C min^{-1} 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 ID and 0.25 μm film thickness) at a flow rate of 1.0 ml min^{-1} .

DATA TRANSFORMATION AND STATISTICAL ANALYSIS

Data from HPLC-MS analysis of root exudates and root tissue metabolite profiles were analyzed by calculating the retention

times in minutes and peak area for each sample. Data detected at wavelengths of 254, 280, and 310 nm were analyzed but only data from 280 nm are shown here because the results were similar in all three wavelengths. The most prominent 25 peaks that were consistently present in all replicates of a given mutant were considered for further multivariate statistical analysis. For GC-MS analysis, peak detection and deconvolution was performed with automated mass spectral deconvolution and identification system (AMDIS; Halket et al., 1999) for several samples of each treatment and peak lists were compiled in a metabolomics ion-based data extraction algorithm (MET-IDEA; Broeckling et al., 2006). MET-IDEA was then used to extract quantitative peak area values for polar and non-polar data. Redundant peaks were removed from the data set, peak area values were scaled to mean zero and standard deviation 1.0 and the resulting data matrix analyzed for further multivariate analyses. Compound identifications were performed by matches with the mass spectral library developed by Dr. Lloyd Sumner laboratory, Samuel Roberts Noble Foundation. Relative Sorenson distances were calculated between GC-MS or LC-MS profiles and visualized by Bray–Curtis ordination (endpoint selection: Bray–Curtis original, Axis Projection and Residual Distances: Euclidean). Statistical differences between mutants were determined by using the non-parametric multi-response permutation procedure (MRPP) assuming a completely randomized design with one factor (i.e., mutant). All analyses were conducted in PC-ORD (McCune and Grace, 2002).

IDENTIFICATION OF CANDIDATE PEAKS SHOWING QUANTITATIVE AND QUALITATIVE DIFFERENCES IN ABC TRANSPORTER MUTANT ROOT EXUDATES AND ROOT TISSUE METABOLITES BY HPLC-MS

In an effort to identify the missing compounds in the ABC transporter mutants compared to the wild type, we identified compound 4 in the root exudates with retention time 40.0 and positive ESIMS201 as camalexin by running standard camalexin that was obtained as a gift from Dr. Eugene Nester, University of Washington. Similarly, we identified four additional compounds from the root tissue metabolites profiles: peak retention time 24.3 and positive ESIMS: m/z 741 is kaempferol-3-*O*-rutinoside-7-*O*- α -L-rhamnopyranoside (C₃₃H₄₀O₁₉); peak retention time 27.1 and positive ESIMS: m/z 611 is quercetin 3-*O*-glucoside-7-*O*-rhamnoside (C₂₇H₃₀O₁₆); peak retention time 29.0 and positive ESIMS: m/z 595 is quercetin 3,7-di-*O*- α -L-rhamnopyranoside (C₂₇H₃₁O₁₅); and peak retention time 31.8 and positive ESIMS: m/z 579 is kaempferol 3,7- α -L-dirhamnoside (C₂₇H₂₉O₁₄) by running authentic standard compounds isolated from *Arabidopsis* root tissues and characterized by proton nuclear magnetic resonance (NMR), liquid chromatography-mass spectrometry (LCMS; low resolution), and finally by LCMS/MS (high resolution; Badri et al., 2008). Total salicylic acid (SA) extractions were performed on root tissues of 18-day-old plants as described by Bowling et al. (1994) and measured by GC-MS.

RNA ISOLATION AND SEMI-QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Total RNA was isolated from frozen root tissues collected from different mutants and wild type by using the RNeasy midi kit

(Qiagen Inc.) and quantified by Nanodrop ND-1000 Spectrophotometer. RNA integrity was checked on a formamide denaturing agarose gel. Two micrograms of purified total RNA from root tissues were reverse-transcribed using Superscript III RT and poly (T) primer at 42°C for 1 h (Invitrogen) according to the manufacturer's instructions. The reaction product was diluted to a concentration of 50 ng μ l⁻¹ and 1 μ l used for each polymerase chain reaction (PCR) reaction. The reaction mix (20 μ l) contained 0.4 μ mol of each gene-specific primer, 200 μ mol of dNTPs, 1 \times reaction buffer, and one unit of Taq DNA polymerase (Takara). PCR included 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min in an Applied Biosystems thermal cycler (GeneAmp PCR system 2700). Actin primers were used as a control to determine the uniformity of the concentration of cDNA. The gene-specific primers used for RT-PCR assays are listed in **Table A4** in Appendix.

RESULTS

PHENOTYPIC CHARACTERIZATION OF ABC TRANSPORTER MUTANTS

Organ-specific expression of the seven ABC transporters was determined by RT-PCR in root, leaf, and flower tissues of the wild-type Col-0. Among the seven ABC transporters, only *AtABCF1* was expressed equally in roots, leaves, and flowers. In contrast, the other six ABC transporters (*AtABCG36*, *AtABCG37*, *AtABCC5*, *AtABCF3*, *AtNAP5*, and *AtATH10*) showed higher levels of expression in the roots compared to leaves and flowers (**Figure 1A**). Further, we determined the T-DNA insertions for the ABC transporter mutant's *Atabcf1*, *Atabcf3*, *Atnap5*, and *Atath10* were found on the first, fifth, third, and second exons, respectively, in their genes (data not shown). RT-PCR assays on root tissues from all seven mutant homozygous lines verified the complete knockout of the corresponding gene, except *Atabcf3*, which had a faint band because the primers designed to amplify the fragment are upstream of the T-DNA insertion sequence (**Figure 1B**). Root phenotype analysis showed that the *Atath10* mutant has a significant increase in the number of lateral roots compared with other mutants and the wild type (**Figure 1C**), when grown under MS media supplemented with 0.5% sucrose as a carbon source. In contrast, there were no significant differences observed in primary root length compared with the other mutants and the wild type (**Figure 1D**). Besides this difference, there were no observable differences in terms of shoot morphology, bolting, and flowering time between the mutants and the wild type under the conditions tested. We did not perform the root phenotype assays for mutant's *Atabcc5*, *Atabcg36*, and *Atabcg37* because these were already described in the literature. Mutants *Atabcg36* and *Atabcg37* did not show any phenotypic differences when compared with wild type (Ito and Gray, 2006; Strader and Bartel, 2009), but *Atabcc5* exhibits decreased root growth and increased lateral root formation (Gaedeke et al., 2001).

ANALYSIS OF SECONDARY METABOLITES IN THE ROOT EXUDATES OF ABC TRANSPORTER MUTANTS BY HPLC-MS

We examined the role of these ABC transporters in the root secretion of phytochemicals by examining the root exudation profiles of the mutants by HPLC-MS and comparing those to the wild-type profiles (see Materials and Methods). Comparison of the

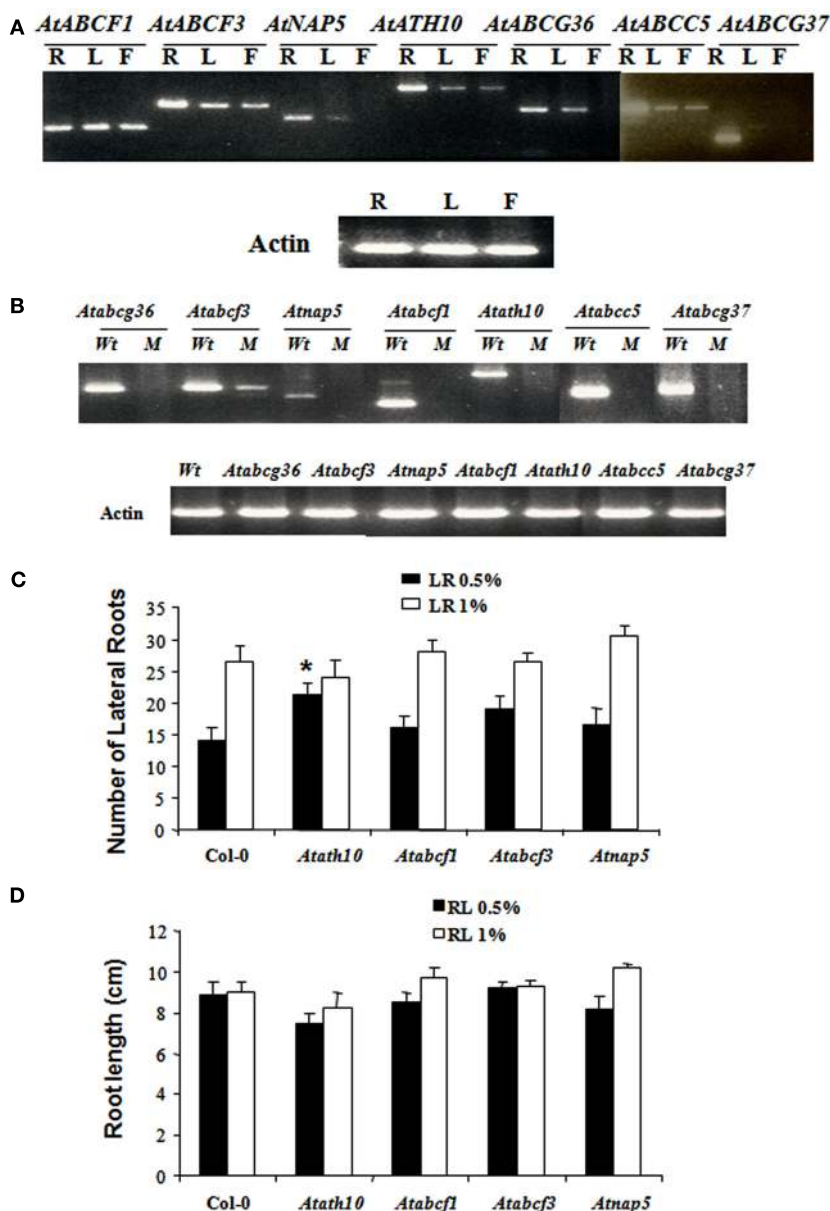


FIGURE 1 | Phenotypic characterization of ABC transporter T-DNA KO mutants used in this study. (A) Organ-specific expression of ABC transporters in wild type roots, leaves, and flowers. **(B)** RT-PCR assays of the ABC transporter mutants used in this study. **(C)** Characterization of root phenotype by growing wild type and mutants in MS agar medium supplemented with 0.5 and 1% sucrose. Number of lateral roots was measured when the seedlings were 14 days old. **(D)** Characterization of root

phenotype by growing wild type and mutants in MS agar medium supplemented with 0.5 and 1% sucrose. Root length was measured when the seedlings were 14 days old. cDNA was prepared from respective tissues. Total RNA and RT-PCR was performed using gene-specific primers. The lower panel is the actin control for the cDNA of wild type root, leaf, and flower. R, root; L, leaf; F, flower. The results represent experiments repeated two times with three replicates each.

root exudates' profiles by MRPP analysis showed that the mutants *Atabcg37*, *Atabcc5*, *Atabcf3*, *Atnap5*, and *Atath10* were significantly different from the wild type (Figure 2; Tables A5 and A6 in Appendix). In addition, a careful peak-by-peak analysis allowed determining four non-polar phytochemicals missing in the root exudates of the various mutants compared to that of the wild type. (Table 1; Figure A1 in Appendix). Compound 1, with a retention time of 13.8 min and a molecular mass of 277, was absent only in

the exudates of *Atabcg37*. Similarly, compound 2 with retention time of 35.58 and molecular mass of 176 was only absent in the exudates of *Atabcc5*. In contrast, compound 3 with retention time of 39.3 and molecular mass of 224 was present only in *Atabcg37* and absent in the other mutants and wild type. Compound 4 with a retention time 40.0 and a molecular mass of 200 was identified as camalexin and was present only in the exudates of *Atabcc5* and *Atabcg37*, but absent in the other mutants and wild type.

ANALYSIS OF PRIMARY AND SECONDARY METABOLITES IN THE ROOT EXUDATES OF ABC TRANSPORTER MUTANTS BY GC-MS

We also analyzed the primary and secondary metabolites present in the root exudates profiles of the mutants by GC-MS and compared the results with wild type's root exudate profiles. Comparison of the GC-MS profiles of the mutants and wild type by MRPP revealed that all of the mutants were significantly different from the wild type (Figure 3; Tables A5 and A6 in Appendix). Further quantitative analysis of the identified compounds showed that these mutants exuded varying levels of organic acids and sugars compared to the wild type. Mutant *Atabcc5* showed significantly lower levels of organic acids (oxalic acid, succinic acid, fumaric acid, malic acid, trihydroxybutyric acid, *p*-hydroxy benzoic acid, etc.). In contrast, *Atabcg36* exudates contained significantly higher levels of organic acids (oxalic acid, benzoic acid, succinic acid, fumaric acid, nicotinic acid, SA, vanillic acid, *p*-hydroxy benzoic acid, etc.) compared to the wild type and the other mutants (Figure A2 in Appendix). We did not observe any significant differences in the levels of sugars between the mutants and wild type except that *Atabcc5* had significantly decreased levels of ribose and D-galactose in the root exudates (Figure A3 in Appendix). In addition, there

were significant differences in the “unknown” compounds between mutants and wild type.

ROOT TISSUE METABOLITES PROFILES OF ABC TRANSPORTER MUTANTS BY HPLC-MS ANALYSIS

The root tissue metabolite profiles were also analyzed in all seven mutants at the same time point (21 days old) as those used for the root exudates collection. The LC-MS profiles utilized only the retention times and peak areas of the 17 most prominent peaks present in all of the samples. Based on the MRPP analysis, we found that the profiles of all the mutants, except *Atabcc5*, were significantly different from those of wild type and the other mutants (Figure 4; Tables A5 and A6 in Appendix). Among those 17 peaks, four peaks were identified based on the retention time (R_t), positive-ion molecular mass (M^+), and UV absorbance by running the authentic standard compounds as follows: kaempferol-3-*O*-rutinoside-7-*O*- α -L-rhamnopyranoside ($R_t = 24.3$, $M^+ = 741$), quercetin 3-*O*-glucoside-7-*O*-rhamnoside ($R_t = 27.1$, $M^+ = 611$), quercetin 3,7-di-*O*- α -L-rhamnopyranoside ($R_t = 29.0$, $M^+ = 595$), and kaempferol 3,7- α -L-dirhamnoside ($RT = 31.8$, $M^+ = 579$). We further analyzed the profiles with peak-by-peak analysis and found both quantitative and qualitative differences in the mutants compared with wild type. For example, the compounds with retention time 25.58, 49.31, and 51.24 were absent in *Atabcg37* but those compounds were present in wild type and other mutants (Table 2). Similarly, we observed that several other compounds differed quantitatively in the mutants compared with wild type and some are worth noting here (Table 2). For example, the concentrations of kaempferol-3-*O*-rutinoside-7-*O*- α -L-rhamnopyranoside ($R_t = 24.4$ and $M^+ = 741$), quercetin 3-*O*-glucoside-7-*O*-rhamnoside ($R_t = 27.1$ and $M^+ = 611$), and quercetin 3,7-di-*O*- α -L-rhamnopyranoside ($R_t = 29.0$ and $M^+ = 595$) were significantly higher in *Atabcg36* compared with the wild type. Similarly, kaempferol-3-*O*-rutinoside-7-*O*- α -L-rhamnopyranoside concentration was also higher in *Atath10* compared with the wild type. Interestingly, we did not observe camalexin in the root tissue metabolites profiles of wild type and the other transporter mutants used in this study but we observed camalexin only in the root exudates profiles of the mutants *Atabcg37* and *Atabcc5*.

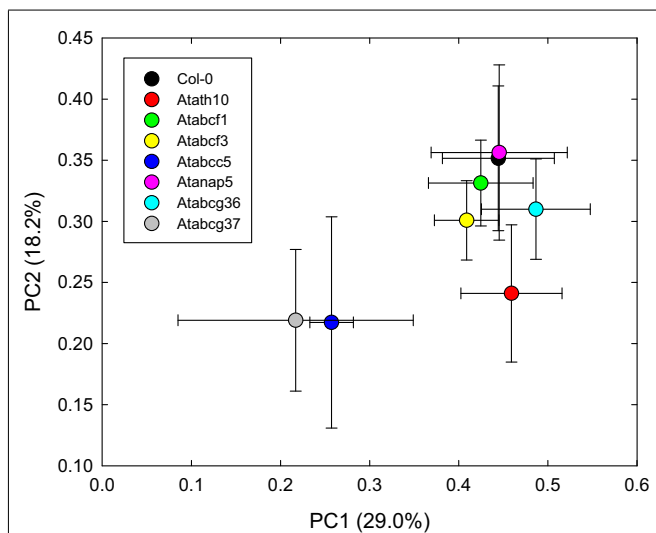


FIGURE 2 | Bray-Curtis ordination analyses with Sorenson distance of the root exudates of wild type and ABC transporter mutants constructed from HPLC-MS data obtained from root exudates profiles.

FURTHER CHARACTERIZATION OF *Atabcg36* IN DEFENSE RESPONSE

Based on our primary and secondary metabolites' analyses, *Atabcg36* mutants (both *Atabcg36-1* and *Atabcg36-2*) secreted

Table 1 | Retention times and molecular masses of the compounds missing in the ABC transporter mutants compared with wild type after 3 days' secretion (21-day-old plants) of root exudate profiles analyzed by HPLC-MS.

Retention time (min), molecular mass	Col-0	<i>Atath10</i>	<i>Atabcg36</i>	<i>At abcf3</i>	<i>Atabcf1</i>	<i>Atnap5</i>	<i>Atabcc5</i>	<i>Atabcg37</i>
13.8, 277	+	+	+	+	+	+	+	-
35.58, 176	+	+	+	+	+	+	-	+
39.3, 224	-	-	-	-	-	-	-	+
40.0, 200	-	-	-	-	-	-	+	+

+, indicate presence of compound; -, indicate the absence of compound.

higher concentrations of organic acids such as SA, vanillic acid, oxalic acid, nicotinic acid, and other unknown compounds in the root exudates. Previous published information has involved *Atabcg36* in non-host resistance and heavy metal tolerance (Kobae et al., 2006; Stein et al., 2006; Kim et al., 2007). Taking this information together, we hypothesized that higher levels of SA secretion by roots indirectly indicates the increase in the concentration of SA in tissues that contributes to the disease resistance response exhibited by *Atabcg36*. Therefore, we measured the relative SA

concentrations in the root tissues of wild type and *Atabcg36* mutants (*Atabcg36-1* and *Atabcg36-2*) and found that there was a significant increase in SA concentration in the mutant plants of *Atabcg36* compared to wild type (Figure 5). The increase in concentration of SA in *Atabcg36* is probably due to a pleiotropic effect in the gene mutation. The observed variability in the SA concentrations between the mutant lines is unexplainable and warrants further studies in order to dissect the specific pleiotropic effects.

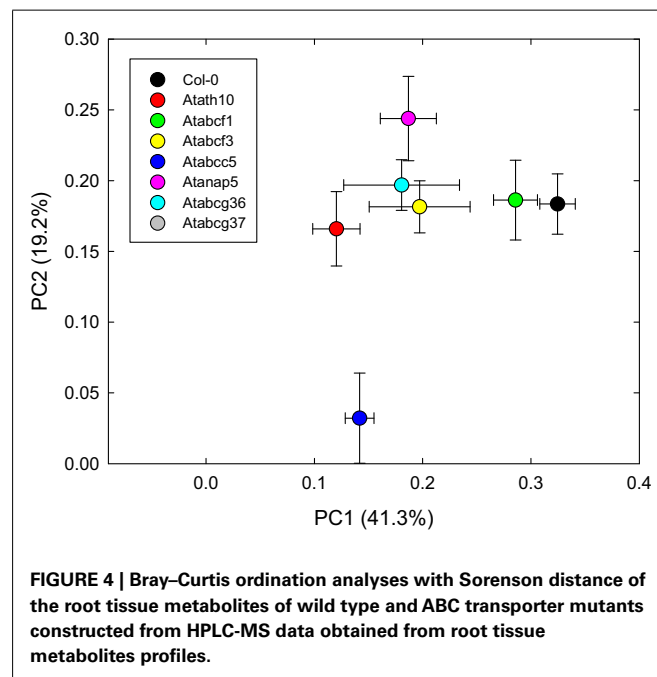
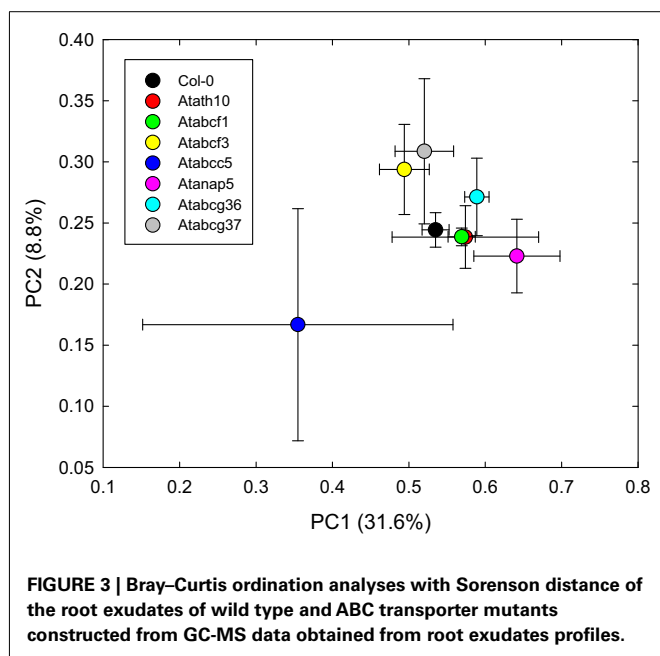
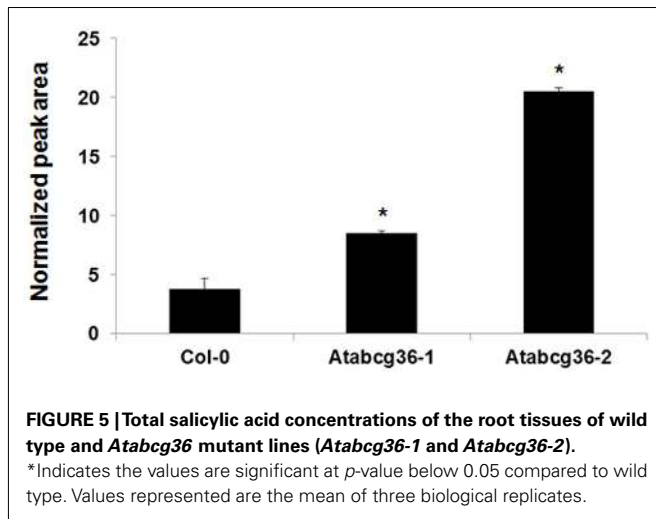


Table 2 | Retention times of the compounds of the root tissue profiles of 21-day-old wild type and ABC transporter mutants analyzed by HPLC-MS.

RT	Col-0	<i>Atabcf1</i>	<i>Atabcf3</i>	<i>Atath10</i>	<i>Atabcc5</i>	<i>Atanap5</i>	<i>Atabcg36</i>	<i>Atabcg37</i>
10.42	132.3 (8.9)	145.5 (4.9)	105.1 (6.7)	59.5 (5.2)	42.8 (2.9)	94.3 (7.6)	135.7 (15.2)	44.6 (2.7)
14.91	4.9 (0.2)	10.3 (0.5)	10.3 (0.1)	12.4 (0.7)	5.7 (0.1)	6.4 (0.5)	11.6 (0.3)	7.5 (0.7)
19.0	11.0 (1.3)	18.4 (0.7)	25.3 (0.7)	30.8 (2.6)	33.5 (1.3)	20.5 (1.9)	28.8 (0.3)	18.5 (1.5)
20.36	16.9 (1.1)	23.5 (0.8)	35.8 (0.8)	36.6 (2.7)	31.7 (0.8)	28.5 (3.0)	40.5 (0.9)	20.1 (1.2)
23.0	6.5 (1.7)	9.7 (0.5)	3.6 (0.5)	8.4 (2.0)	3.1 (0.8)	10.5 (0.7)	17.5 (1.3)	2.5 (0.3)
24.33	20.7 (1.7)	28.1 (0.7)	25.9 (1.4)	34.3 (0.7)	23.3 (0.6)	24.2 (1.8)	32.7 (0.8)	33.4 (2.6)
25.58	26.1 (2.9)	27.8 (0.7)	16.4 (1.3)	21.4 (1.5)	21.6 (1.3)	16.8 (1.2)	22.6 (0.8)	0 (0)
27.11	18.1 (1.5)	23.1 (0.5)	28.5 (0.7)	35.4 (2.6)	11.0 (1.0)	30.2 (2.3)	48.1 (1.5)	29.5 (1.7)
29.0	29.1 (2.6)	39.7 (1.0)	39.8 (1.3)	49.1 (3.1)	28.1 (1.1)	41.1 (2.3)	57.9 (1.2)	37.0 (2.6)
30.59	17.3 (1.5)	22.1 (0.4)	14.7 (0.7)	19.3 (1.2)	10.8 (0.3)	15.3 (0.9)	18.1 (0.6)	12.4 (0.8)
31.38	18.8 (4.6)	37.7 (1.5)	10.0 (0.4)	37.7 (0.7)	36.1 (1.1)	12.2 (0.7)	17.9 (1.9)	10.9 (0.7)
34.86	17.3 (2.0)	27.5 (1.9)	17.2 (1.7)	33.1 (0.6)	16.7 (0.6)	16.5 (1.4)	26.7 (1.2)	5.3 (0.5)
36.6	2.4 (0.0)	3.9 (0.5)	2.9 (0.2)	4.55 (0.7)	1.3 (0.0)	1.5 (0.0)	1.7 (0.0)	2.8 (0.3)
37.32	48.0 (4.6)	74.1 (3.7)	59.3 (2.9)	70.1 (0.5)	33.5 (1.5)	65.1 (4.8)	59.2 (1.5)	32.6 (2.0)
40.49	7.6 (1.3)	11.2 (1.8)	6.5 (1.2)	15.6 (0.9)	4.8 (0.7)	14.2 (1.1)	7.9 (0.8)	9.7 (1.6)
49.31	6.7 (0.8)	10.4 (1.3)	7.8 (0.7)	12.5 (0.9)	4.7 (0.4)	9.4 (1.2)	10.4 (0.8)	0 (0)
51.24	10.2 (1.4)	15.9 (2.3)	8.1 (0.7)	14.2 (1.8)	6.5 (0.6)	8.4 (0.9)	13.6 (1.1)	0 (0)

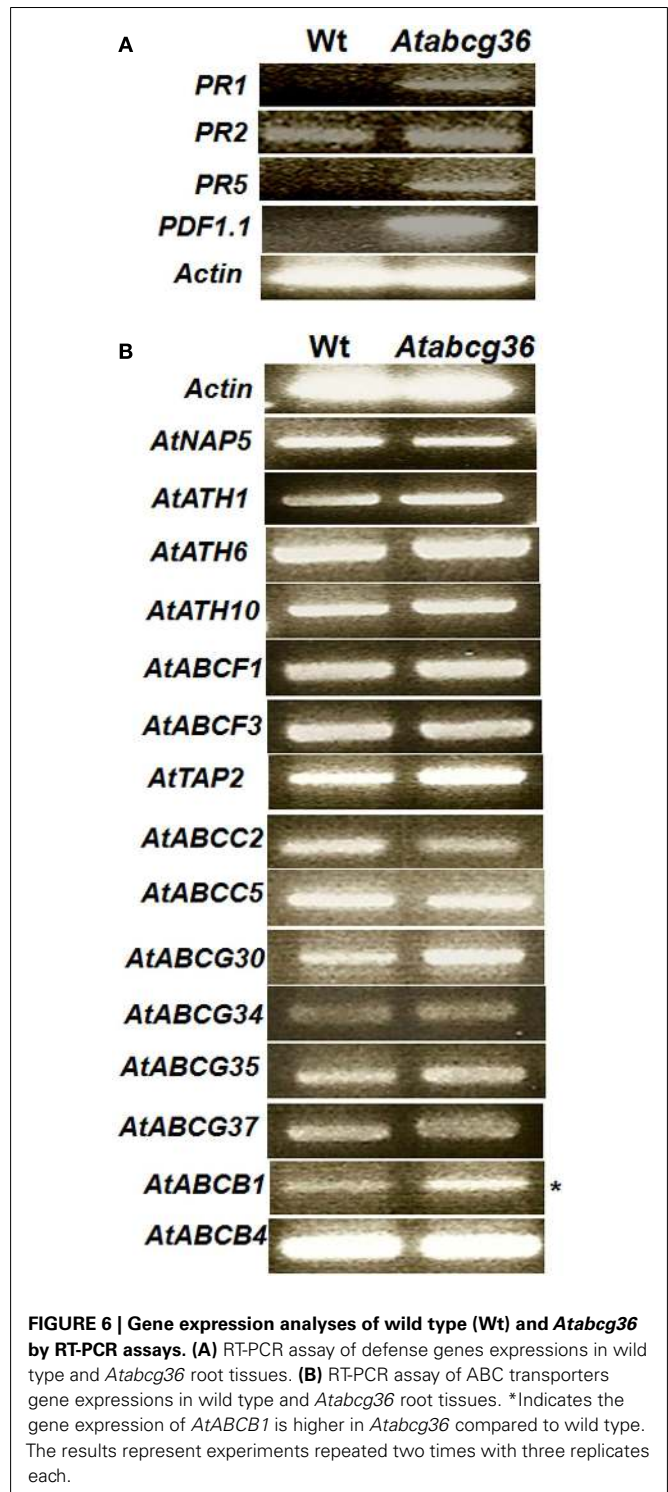
Values represented are peak areas of the corresponding compounds in the wild type and mutants. The values in bold are statistically significant when compared to the wild type at $p \leq 0.05$, $n = 8$. Values represented in parentheses are SE values.



We further investigated the basal resistance level in *Atabcg36* by determining the expression of defense genes in the root tissues compared with that of the wild type under aseptically grown conditions. We found that *Atabcg36* showed higher levels of expression of defense genes' (*PR1*, *PR2*, *PR5*, and *PDF1.1*) in the root tissues compared with the wild type (**Figure 6A**). We did not observe the expression of *PR1*, *PR5*, and *PDF1.1* in wild type roots. It was reported that mutations in certain transporters could modify the otherwise normal expression of other transporters in *Arabidopsis* (Badri et al., 2009). Thus, we analyzed the expression of other ABC transporters that could be highly expressed in roots (Badri et al., 2008) of *Atabcg36* to take over the function of ABCG36. We checked the expression of 15 ABC transporters which includes both full-size and half-molecule transporters. Among those, only AtABCB1 (full-size transporter) expression was up-regulated in *Atabcg36* compared to the wild type (**Figure 6B**). However, further evaluation is required to corroborate these gene expression patterns by real-time quantitative PCR analysis.

DISCUSSION

The ABC proteins encompass a large protein family ubiquitous in all organisms, which includes both membrane bound and soluble proteins (Higgins, 1992; Yazaki et al., 2009). These proteins are classified as primary transporters which are directly energized by ATP hydrolysis to translocate solutes across cellular membranes (Higgins, 1992), a process that is independent of membrane potential and proton gradients across the membrane. ABC proteins are involved in various biochemical and physiological functions in plants, few are involved in defense mechanisms against biotic stresses (Yazaki et al., 2009) and others are involved in basic functions necessary for plant life such as hormone transport or phytol accumulation (Geisler and Murphy, 2006; Nagy et al., 2009). A recent report demonstrated the influence of some full-size ABC transporters in root exudation processes (Badri et al., 2008) by employing a mutant approach. In an expansion of that study, here we presented a detail characterization of a distinct set ABC transporters highly expressed in root cells (Badri et al., 2008) and their role in root exudation.



ABC TRANSPORTERS ARE INVOLVED IN ROOT SECRETION OF PHYTOCHEMICALS

Based on our metabolomic analyses (HPLC-MS and GC-MS) the root exudates profiles of the mutants *Atabcg36*, *Atabcg37*, *Atabc5*, *Atabc3*, and *Atath10* were significantly different from those of the wild type (**Figures 2 and 3**). Qualitative analyses of HPLC-MS

root exudate profiles revealed that one compound ($R_t = 13.8$ and $M^+ = 277$) was completely absent in *Atabcg37* compared with the wild type and the other mutants used in this study. Similarly, another compound ($R_t = 35.58$ and $M^+ = 176$) was absent in *Atabcc5* compared with the other mutants and the wild type (Table 1). These results reinforce the previous notion describing that one transporter could transport a particular type of compound (Badri et al., 2008). Also, the differences observed in the secretion of phytochemicals by different ABC transporters could be attributed to their localization in the cell. For example, AtABCG36 and AtABCG37 are localized in the plasma membrane and AtABCC5 is localized in the vacuolar membranes (Dunkley et al., 2006; Knoller and Murphy, 2011). Previously, ABCG36 was reported to be involved in resistance to heavy metal (cadmium) and auxinic herbicides (Ito and Gray, 2006; Kim et al., 2007). It has also been reported that ABCG36 and ABCG37 are involved in regulation of auxin homeostasis and plant development by directional transport of the auxin precursor IBA (Strader and Bartel, 2009; Ruzicka et al., 2010). On the other hand, ABCC5 is involved in root development and stomata movement by transporting inositol hexakis phosphate (Gaedeke et al., 2001; Nagy et al., 2009). Based on the earlier published results and the results of the present study, it is assumed that one ABC protein might be involved in different kinds of functions depending upon their expression in particular plant organs. In contrast, we did not observe any qualitative differences in the root exudates profiles of secondary metabolites in the half-size transporters mutants (AtATH10, AtABCF1, AtABCF3, and AtNAP5) compared with the wild type. Therefore, these half-size transporters might not be involved in exporting secondary metabolites from the root cells. This is probably true, because these half-size transporters are thought to be soluble since they lack any detectable transmembrane domain and probably function in processes other than transport, as is the case for their yeast and human orthologs, which participate in ribosome recycling and translational control (Vazquez de Aldana et al., 1995; Tyzack et al., 2000; Braz et al., 2004; Dong et al., 2004; Pisarev et al., 2010). However, the phenotype differences observed in the root exudates profiles of these half-size transporter mutants need further confirmation by examining other allelic mutants.

Interestingly, we found one compound ($R_t = 40.0$ and $M^+ = 201$), identified as camalexin, which is present significantly only in the root exudates of *Atabcc5* and *Atabcg37* (Table 1; Figure A4 in Appendix). However, we observed the camalexin mass trace in other mutant lines and wild type but they were relatively very low and close to the threshold levels (background noise; Figure A4 in Appendix). Camalexin is a phytoalexin produced by the plant in response to pathogen infection and MAMPs but absent in plants without stress (Consonni et al., 2010; Millet et al., 2010). The secretion of camalexin in *Atabcc5* and *Atabcg37* is probably due to the pleiotropic effect of these mutations resulting in an elevated expression of genes involved in indolic metabolite biosynthesis, as reported in the case of *Arabidopsis* MLO2, 6, and 12 mutants (Consonni et al., 2010). We also analyzed the root tissue metabolite profiles of all the mutants by HPLC-MS analyses and did not find camalexin in the root tissues of any of the mutants or the wild type, which indicates that this compound might be toxic to the cells and extruded outside the cells by other

means of transportation (Rogers et al., 1996; Glawischnig, 2007). The genetics and biochemistry of the biosynthesis and secretion of camalexin in *Atabcc5* and *Atabcg37* root exudates warrant further experimentation.

Besides the absence of camalexin in the root tissue metabolite profiles of all mutants and wild type, we observed significant quantitative differences in other compounds between the wild type and the mutants used in this study (Table 2). Among those compounds, the concentrations of kaempferol-3-*O*-rutinoside-7-*O*- α -L-rhamnopyranoside, quercetin 3-*O*-glucoside-7-*O*-rhamnoside, and quercetin 3,7-di-*O*- α -L-rhamnopyranoside were significantly higher in the root tissues of *Atabcg36*. Circumstantial evidence indicates that flavonoids function in plant defense against bacterial and viral pathogens; however specific molecule interactions have yet to be identified (Shirley, 1996; Iriti and Faoro, 2009). Specifically, glycosides of the flavonols, kaempferol, and quercetin have been shown to induce the *vir* genes of *Agrobacterium tumefaciens* (Zerback et al., 1989). In addition, *Atabcg36* root exudates showed higher concentrations of organic acids like SA, vanillic acid, *p*-hydroxybenzoic acid, nicotinic acid, etc. in our GC-MS analyses (Figure A2 in Appendix). We found higher concentrations of SA in the root tissues compared to wild type, which partly explains *Atabcg36* role in non-host resistance (Kobae et al., 2006; Stein et al., 2006).

BASAL DEFENSE LEVEL IS HIGHER IN *Atabcg36*

Previous reports have shown that the *Arabidopsis* ABC transporters AtABCG36, AtABCG40, and NpPDR1 are involved in plant defense responses (Lipka et al., 2005; Kobae et al., 2006; Stein et al., 2006). For example, Stein et al. (2006) demonstrated that SA genes are hyperinduced in *Atpen3/Atabcg36* after pathogen inoculation. In addition, it was demonstrated that AtABCG36 was required for the extracellular accumulation of the glucan polymer callose, mediated by the glucan synthase like enzyme PMR4/GSL5, in response to treatment with a MAMP derived from bacterial flagellin (Clay et al., 2009). However, microbes can trigger callose formation in *Arabidopsis* to restrict further pathogen growth via the plant hormone SA-dependent pathway (DebRoy et al., 2004). In the present study, we found higher concentrations of SA in the root exudates, and SA, kaempferol, and quercetin glucosides in the root tissues of *Atabcg36* which suggested that the basal defense levels of *Atabcg36* are higher than the wild type. It is worth noting here that the biosynthesis of these three compounds (SA, kaempferol, and quercetin) is derived from phenylalanine involved in the phenylpropanoid pathway, though SA can also be synthesized from the isochlorismate pathway (Iriti and Faoro, 2009). Our results showed that the basal expression level of defense genes *PR1*, *PR2*, *PR5*, and *PDF1.1* are higher in *Atabcg36* compared to the wild type (Figure 6A). Previously, it was reported that the expression of PR proteins is higher in the leaves of *Atabcg36* grown under non-sterile conditions without challenging with pathogen inoculation, but might be induced by beneficial microbes present in the soil (Kobae et al., 2006). In addition, *AtABCG36* expression was also up-regulated upon pathogen inoculation (Kobae et al., 2006). In the present study, we showed that the basal defense proteins (PR proteins) expression is higher in the roots of *Atabcg36* grown under sterile conditions without challenging the plant with any

pathogen or beneficial microbe. This higher expression of PR proteins is likely induced by the over-production of SA in the roots of *Atabcg36* plants. It is worth noting that callose formation in *Arabidopsis* in response to MAMPs is initiated by SA-dependent and SA-independent pathways (DebRoy et al., 2004; Bednarek et al., 2009). Further mechanistic studies are needed to dissect the specific pathways modified by the AtABCG36 mutation. In addition, *Atabcg36* will serve as a good model system to study the regulatory networks associated with elevated basal defense response in the host compared with those induced upon pathogen attack.

Overall this study draws the following conclusions: (1) Full-size ABC transporters that are highly expressed in the root cells play a role in the root secretion of secondary metabolites. (2) Basal

defense gene expression levels are higher in the *Atabcg36* mutant due to overproduction of SA. (3) Mutations of ABC transporters like *ABCC5* and *ABCG37* induce the secretion of the phytoalexin camalexin. (4) This study opens a new dimension for the role of ABC transporters in defense response and plant-microbe interaction in addition to their function in the transportation of substances across cellular membranes. This study also provides clues for developing strategies for disease resistance.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Lloyd W. Sumner (Noble Foundation, Ardmore) for providing his laboratory facility service to run GC-MS. This work was supported by a grant from the National Science Foundation to Jorge M. Vivanco (MCB-0950857).

REFERENCES

- Badri, D. V., Loyola-Vargas, V. M., Broeckling, C. D., De-La-Pena, C., Jasinski, M., Santelia, D., Martinoia, E., Sumner, L. W., Banta, L. M., Stermitz, F., and Vivanco, J. M. (2008). Altered profile of secondary metabolites in the root exudates of *Arabidopsis* ATP-binding cassette transporter mutants. *Plant Physiol.* 146, 762–771.
- Badri, D. V., Quintana, N., El Kassib, E. G., Kim, H. K., Choi, Y. H., Sugiyama, A., Verpoorte, R., Martinoia, E., Manter, D. K., and Vivanco, J. M. (2009). An ABC transporter mutation alters root exudation of phytochemicals that provoke an overhaul of natural soil microbiota. *Plant Physiol.* 151, 2006–2017.
- Bednarek, P., Pislewska-Bednarek, M., Svatos, A., Schneider, B., Doub-sky, J., Mansurova, M., Humphry, M., Consonni, C., Panstruga, R., Sanchez-Vallet, A., Molina, A., and Schulze-Lefert, P. (2009). A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science* 323, 101–106.
- Borst, P., and Elferink, R. O. (2002). Mammalian ABC transporters in health and disease. *Annu. Rev. Biochem.* 71, 537–592.
- Bowling, S. A., Guo, A., Cao, H., Gordon, A. S., Klessig, D. F., and Dong, X. (1994). A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired resistance. *Plant Cell* 6, 1845–1857.
- Braz, A. S., Finnegan, J., Waterhouse, P., and Margis, R. (2004). A plant orthologue of RNase L inhibitor (RLI) is induced in plants showing RNA interference. *J. Mol. Evol.* 59, 20–30.
- Broeckling, C. D., Huhman, D. V., Farag, M. A., Smith, J. T., May, G. D., Mendes, P., Dixon, R. A., and Sumner, L. W. (2005). Metabolic profiling of *Medicago truncatula* cell cultures reveals the effects of biotic and abiotic elicitors on metabolism. *J. Exp. Bot.* 56, 323–336.
- Broeckling, C. D., Reddy, I. R., Duran, A. L., Zhao, X., and Sumner, L. W. (2006). MET-IDEA: data extraction tool for mass spectrometry-based metabolomics. *Anal. Chem.* 78, 4334–4341.
- Clay, N. K., Adio, A. M., Denoux, C., Jander, G., and Ausubel, F. M. (2009). Glucosinolate metabolites required for an *Arabidopsis* innate immune response. *Science* 323, 95–101.
- Consonni, C., Bednarek, P., Humphry, M., Francocci, F., Ferrari, S., Harzen, A., Van Themaat, E. V. L., and Panstruga, R. (2010). Tryptophan-derived metabolites are required for antifungal defense in the *Arabidopsis* mlo2 mutant. *Plant Physiol.* 152, 1544–1561.
- Dean, M., Hamon, Y., and Chimini, G. (2001). The human ATP-binding cassette (ABC) transporter superfamily. *J. Lipid Res.* 42, 1007–1017.
- DebRoy, S., Thilmony, R., Kwack, Y. B., Nomura, K., and He, S. Y. (2004). A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9927–9932.
- Decottignies, A., and Goffeau, A. (1997). Complete inventory of the yeast ABC proteins. *Nat. Genet.* 15, 137–145.
- Dong, J., Lai, R., Nielsen, K., Fekete, C. A., Qiu, H., and Hinnebusch, A. G. (2004). The essential ATP-binding cassette protein RLI1 functions in translation by promoting preinitiation complex assembly. *J. Biol. Chem.* 279, 42157–42168.
- Dunkley, T. P., Hester, S., Shadforth, I. P., Runions, J., Weimar, T., Hanton, S. L., Griffin, J. L., Bessant, C., Brandizzi, F., Hawes, C., Watson, R. B., Dupree, P., and Lilley, K. S. (2006). Mapping the *Arabidopsis* organelle proteome. *Proc. Natl. Acad. Sci. U.S.A.* 103, 6518–6523.
- Gaedeke, N., Klein, M., Kolukisaoglu, U., Forestier, C., Muller, A., Ansoerge, M., Becker, D., Mamnun, Y., Kuchler, K., Schulz, B., Mueller-Roeber, B., and Martinoia, E. (2001). The *Arabidopsis thaliana* ABC transporter AtMRP5 controls root development and stomata movement. *EMBO J.* 20, 1875–1887.
- Garcia, O., Bouige, P., Forestier, C., and Dassa, E. (2004). Inventory and comparative analysis of rice and *Arabidopsis* ATP-binding cassette (ABC) systems. *J. Mol. Biol.* 343, 249–265.
- Geisler, M., and Murphy, A. S. (2006). The ABC of auxin transport: the role of p-glycoproteins in plant development. *FEBS Lett.* 580, 1094–1102.
- Glawischnig, E. (2007). Camalexin. *Phytochemistry* 68, 401–406.
- Gottesman, M. M., and Pastan, I. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* 62, 385–427.
- Halket, J. M., Przyborowska, A., Stein, S. E., Mallard, W. G., Down, S., and Chalmers, R. A. (1999). Deconvolution gas chromatography/mass spectrometry of urinary organic acids – potential for pattern recognition and automated identification of metabolic disorders. *Rapid Commun. Mass Spectrom.* 13, 279–284.
- Henikoff, S., Greene, E. A., Pietrokovski, S., Bork, P., Attwood, T. K., and Hood, L. (1997). Gene families: the taxonomy of protein paralogs and chimeras. *Science* 278, 609–614.
- Higgins, C. F. (1992). ABC transporters: from microorganisms to man. *Annu. Rev. Cell Biol.* 8, 67–113.
- Iriti, M., and Faoro, F. (2009). Chemical diversity and defence metabolism: how plants cope with pathogens and ozone pollution. *Int. J. Mol. Sci.* 10, 3371–3399.
- Ito, H., and Gray, W. M. (2006). A gain-of-function mutation in the *Arabidopsis* pleiotropic drug resistance transporter PDR9 confers resistance to auxinic herbicides. *Plant Physiol.* 142, 63–74.
- Kang, J., Hwang, J. U., Lee, M., Kim, Y. Y., Assmann, S. M., Martinoia, E., and Lee, Y. (2010). PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2355–2360.
- Kim, D. Y., Bovet, L., Maeshima, M., Martinoia, E., and Lee, Y. (2007). The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. *Plant J.* 50, 207–218.
- Knoller, A. S., and Murphy, A. S. (2011). “ABC transporters and their function at the plasma membrane,” in *The Plant Plasma Membrane*, eds A. S. Murphy, W. Peer, and B. Schulz (Berlin: Springer-Verlag), 353–377.
- Kobae, Y., Sekino, T., Yoshioka, H., Nakagawa, T., Martinoia, E., and Maeshima, M. (2006). Loss of AtPDR8, a plasma membrane ABC transporter of *Arabidopsis thaliana*, causes hypersensitive cell death upon pathogen infection. *Plant Cell Physiol.* 47, 309–318.
- Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiermer, M., Stein, M., Landtag, J., Brandt, W., Rosahl, S., Scheel, D., Llorente, F., Molina, A., Parker, J., Somerville, S., and Schulze-Lefert, P. (2005). Pre- and postinvasion defenses both contribute to non-host resistance in *Arabidopsis*. *Science* 310, 1180–1183.
- Martinoia, E., Klein, M., Geisler, M., Bovet, L., Forestier, C., Kolukisaoglu, U., Muller-Roeber, B., and Schulz, B. (2002). Multifunctionality of plant ABC transporters – more than just detoxifiers. *Planta* 214, 345–355.

- McCune, B., and Grace, J. B. (2002). *Analysis of Ecological Communities*. Gleneden Beach, OR: MjM Software.
- Millet, Y. A., Danna, C. H., Clay, N. K., Songnuan, W., Simon, M. D., Werck-Reichhart, D., and Ausubel, F. M. (2010). Innate immune responses activated in *Arabidopsis* roots by microbe-associated molecular patterns. *Plant Cell* 22, 973–990.
- Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15, 473.
- Nagy, R., Grob, H., Weder, B., Green, P., Klein, M., Frelet-Barrand, A., Schjoerring, J. K., Brearley, C., and Martinoia, E. (2009). The *Arabidopsis* ATP-binding cassette protein AtMRP5/AtABCC5 is a high affinity inositol hexakisphosphate transporter involved in guard cell signaling and phytate storage. *J. Biol. Chem.* 284, 33614–33622.
- Pisarev, A. V., Skabkin, M. A., Pisareva, V. P., Skabkina, O. V., Rakontondrafara, A. M., Hentze, M. W., Hellen, C. U., and Pestova, T. V. (2010). The role of ABCE1 in eukaryotic posttermination ribosomal recycling. *Mol. Cell* 37, 196–210.
- Rea, P. A. (2007). Plant ATP-binding cassette transporters. *Annu. Rev. Plant Biol.* 58, 347–375.
- Rogers, E. E., Glazebrook, J., and Ausubel, F. N. (1996). Mode of action of the *Arabidopsis thaliana* phytoalexin camalexin and its role in *Arabidopsis*-pathogen interactions. *Mol. Plant Microbe Interact.* 9, 748–757.
- Ruzicka, K., Strader, L. C., Bailly, A., Yang, H., Blakeslee, J., Langowski, L., Nejedla, E., Fujita, H., Itoh, H., Syono, K., Hejatko, J., Gray, W. M., Martinoia, E., Geisler, M., Bartel, B., Murphy, A. S., and Friml, J. (2010). *Arabidopsis* PIS1 encodes the ABCG37 transporter of auxinic compounds including the auxin precursor indole-3-butyric acid. *Proc. Natl. Acad. Sci. U.S.A.* 107, 10749–10753.
- Sanchez-Fernandez, R., Davies, T. G., Coleman, J. O., and Rea, P. A. (2001). The *Arabidopsis thaliana* ABC protein superfamily, a complete inventory. *J. Biol. Chem.* 276, 30231–30244.
- Shirley, B. W. (1996). Flavonoid biosynthesis: “new” functions for an “old” pathway. *Trends Plant Sci.* 1, 377–382.
- Stein, M., Dittgen, J., Sanchez-Rodriguez, C., Hou, B. H., Molina, A., Schulze-Lefert, P., Lipka, V., and Somerville, S. (2006). *Arabidopsis* PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. *Plant Cell* 18, 731–746.
- Strader, L. C., and Bartel, B. (2009). The *Arabidopsis* pleiotropic drug resistance8/ABCG36 ATP binding cassette transporter modulates sensitivity to the auxin precursor indole-3-butyric acid. *Plant Cell* 21, 1992–2007.
- Tyzack, J. K., Wang, X., Belsham, G. J., and Proud, C. G. (2000). ABC50 interacts with eukaryotic initiation factor 2 and associates with the ribosome in an ATP-dependent manner. *J. Biol. Chem.* 275, 34131–34139.
- Vazquez de Aldana, C. R., Marton, M. J., and Hinnebusch, A. G. (1995). GCN20, a novel ATP binding cassette protein, and GCN1 reside in a complex that mediates activation of the eIF-2 alpha kinase GCN2 in amino acid-starved cells. *EMBO J.* 14, 3184–3199.
- Verrier, P. J., Bird, D., Burla, B., Dassa, E., Forestier, C., Geisler, M., Klein, M., Kolukisaoglu, U., Lee, Y., Martinoia, E., Murphy, A., Rea, P. A., Samuels, L., Schulz, B., Spalding, E. J., Yazaki, K., and Theodoulou, F. L. (2008). Plant ABC proteins – a unified nomenclature and updated inventory. *Trends Plant Sci.* 13, 151–159.
- Yazaki, K. (2005). Transporters of secondary metabolites. *Curr. Opin. Plant Biol.* 8, 301–307.
- Yazaki, K., Shitan, N., Sugiyama, A., and Takanashi, K. (2009). Cell and molecular biology of ATP-binding cassette proteins in plants. *Int. Rev. Cell Mol. Biol.* 276, 263–299.
- Zerback, R., Dressler, K., and Hess, D. (1989). Flavonoid compounds from pollen and stigma of *petunia hybrida* – inducers of the vir region of the *Agrobacterium tumefaciens* Ti plasmid. *Plant Sci.* 62, 83–91.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 March 2012; accepted: 16 June 2012; published online: 05 July 2012.
 Citation: Badri DV, Chaparro JM, Manter DK, Martinoia E and Vivanco JM (2012) Influence of ATP-binding cassette transporters in root exudation of phytoalexins, signals, and in disease resistance. *Front. Plant Sci.* 3:149. doi: 10.3389/fpls.2012.00149
 This article was submitted to *Frontiers in Plant Metabolism and Chemodiversity*, a specialty of *Frontiers in Plant Science*.
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APPENDIX

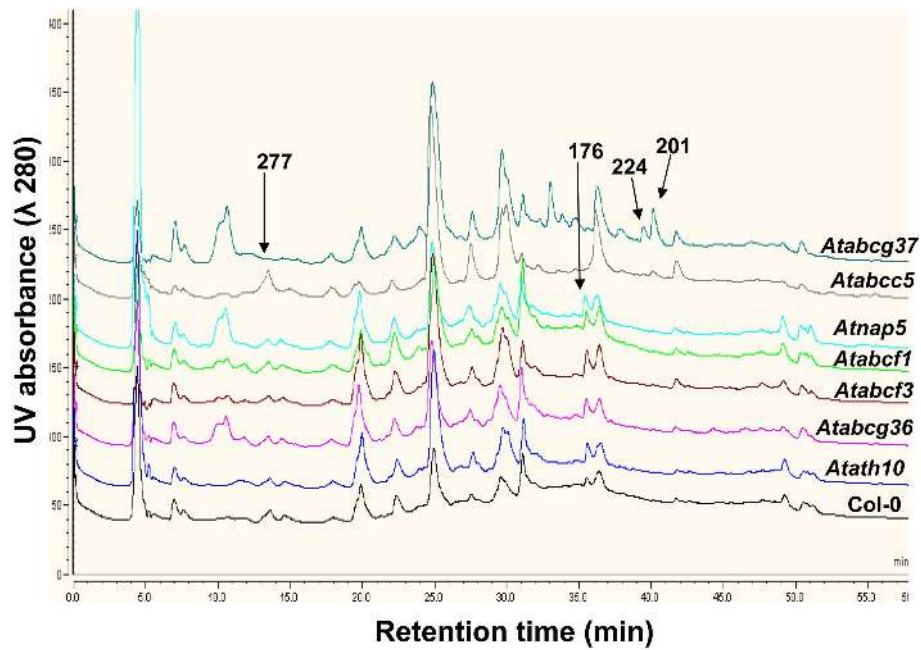


FIGURE A1 | Root exudates profile of 21-day-old wild type (Col-0) and ABC transporter mutant plants analyzed by HPLC-MS at wavelength 280 nm. Arrows indicate the peaks present or absent in respective

mutants. The numbers indicate the positive ESIMS of the peaks. The chromatogram represent experiments repeated two times with three replicates each.

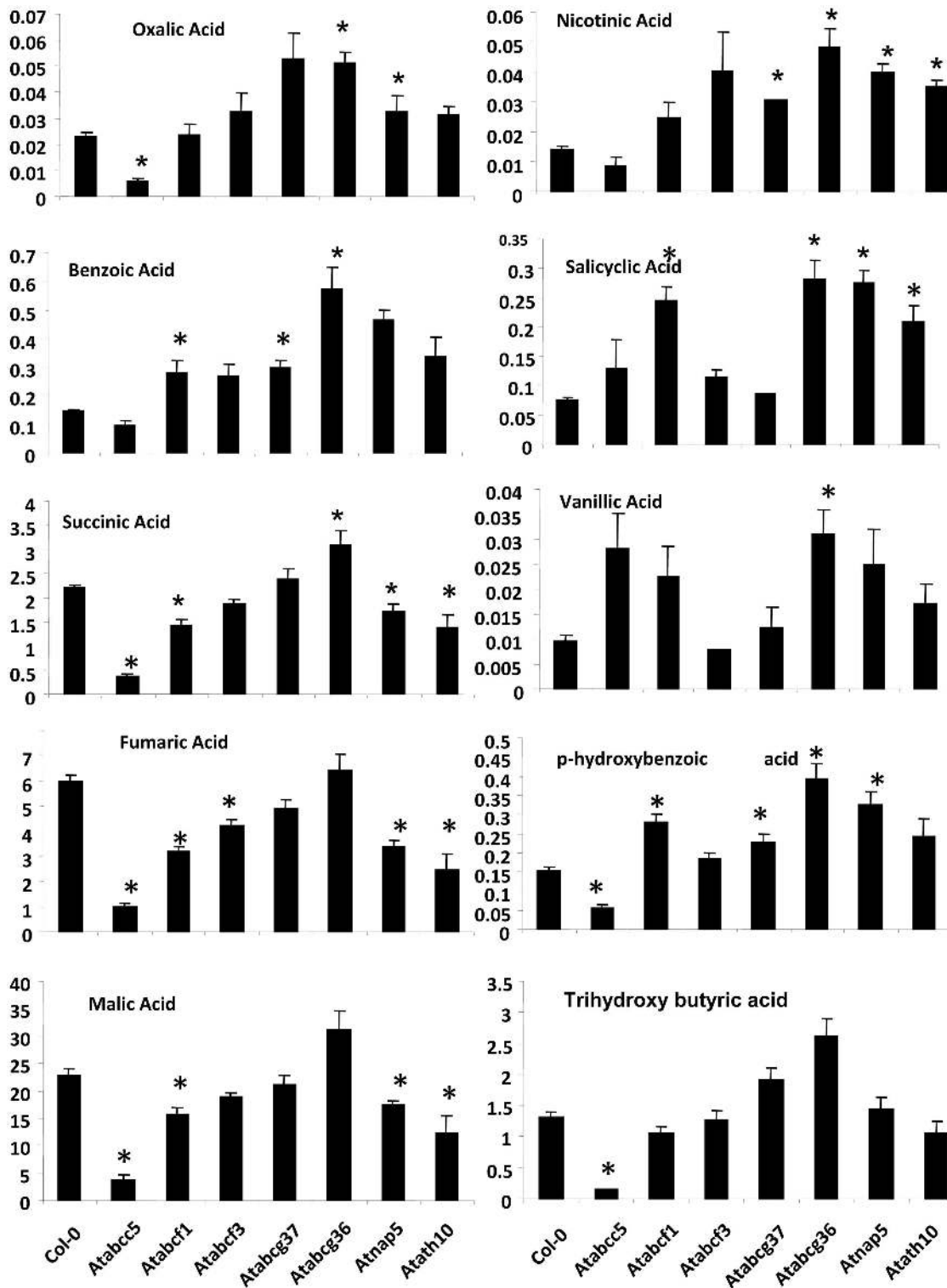


FIGURE A2 | Graphs illustrating the representative phenolic compounds that show different levels in the ABC transporter mutants compared to wild type analyzed by GC-MS. *Indicates the values are statistically significant ($p < 0.05$) compared with wild-type (Col-0) line ($n = 8$).

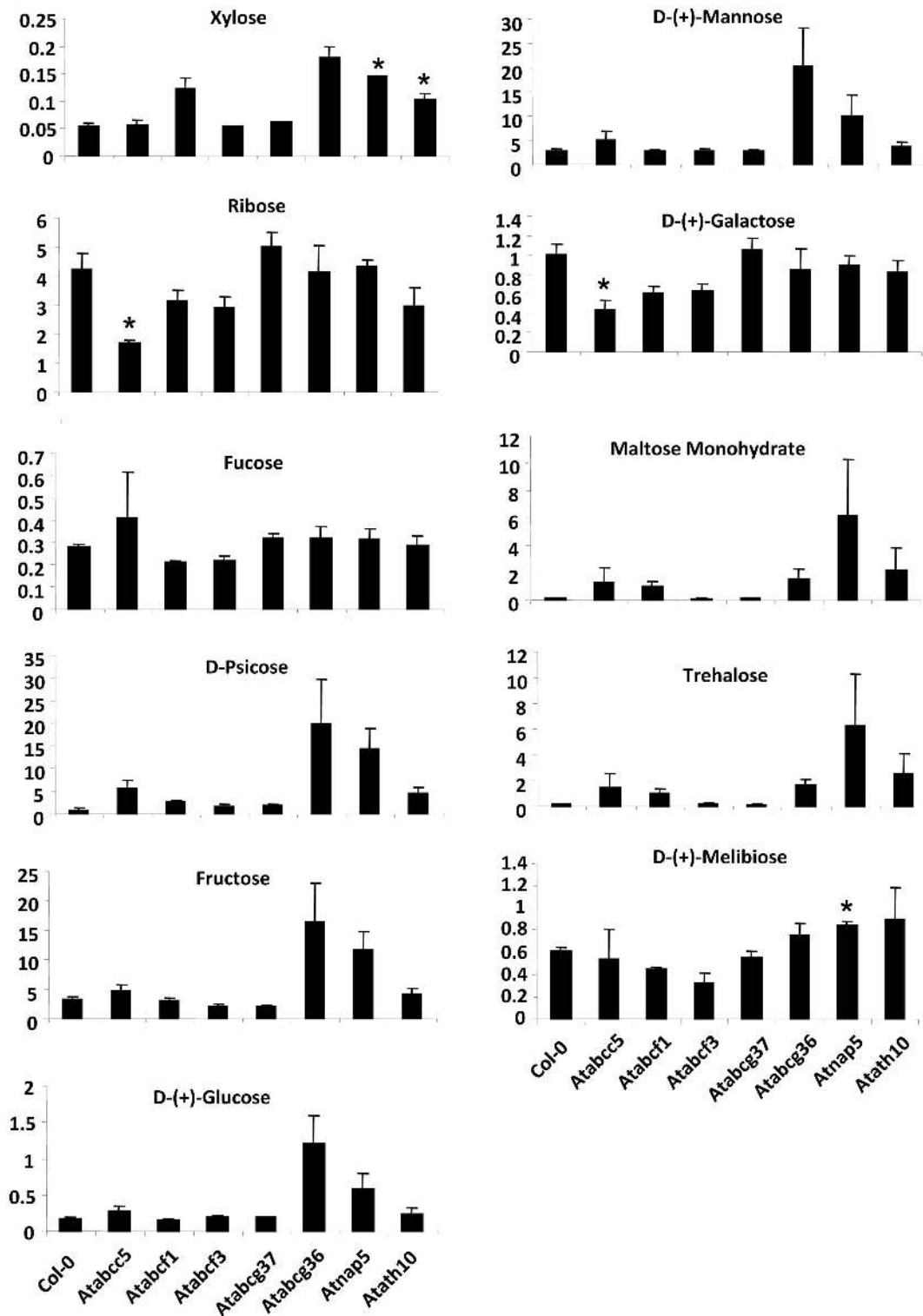


FIGURE A3 | Graphs illustrating the representative sugars that show different levels in the ABC transporter mutants compared to wild type analyzed by GC-MS. *Indicates the values are statistically significant ($p < 0.05$) compared with wild-type (Col-0) line ($n = 8$).

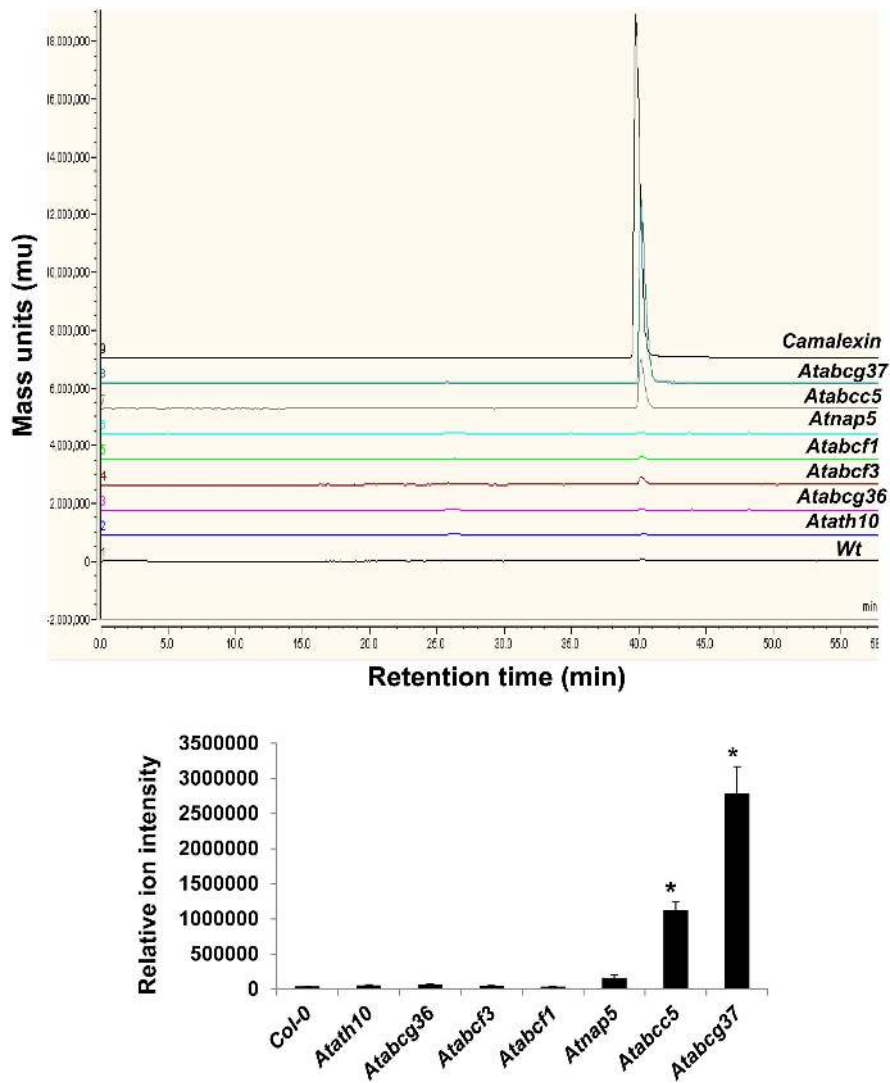


FIGURE A4 | Mass trace of compound camalexin (positive ESIMS 201) in the root exudates of wild type (Wt) and ABC transporter mutants used in this study overlayed with authentic camalexin.

Table A1 | List of ABC transporters and their T-DNA KO mutants used in this study.

AGI code	New systemic name	Mutant name	Reference
At1g59870	<i>AtABCG36</i>	Salk_000578 (<i>Atabcg36-1</i>)	Kobae et al. (2006)
		Salk_142256 (<i>Atabcg36-2</i>)	Kobae et al. (2006)
At3g53480	<i>AtABCG37</i>	<i>pdr9-1</i>	Kim et al. (2007)
At1g04120	<i>AtABCC5</i>	<i>Mrp5-1</i>	Knoller and Murphy (2011)
At5g60790	<i>AtABCF1</i>	Salk_129048	In this study
At1g64550	<i>AtABCF3</i>	Salk_055035	In this study
At1g71330	<i>AtNAP5</i>	Salk_047823	In this study
At4g01660	<i>AtATH10</i>	Salk_105250	In this study

Table A2 | List of primers used in this study for screening homozygous lines of ABC transporters T-DNA KO mutant lines.

AGI code new systemic name	Mutant name	Primer pairs ^a
At5g60790 <i>AtABCF1</i>	Salk_129048	F: 5'-TGCTCGTCAAGCACAGAGTAA-3' R: 5'-CTGAGTCACCTTTCCAACGAA-3'
At1g64550 <i>AtABCF3</i>	Salk_055035	F: 5'-CCGGTTTTGAAGTGAAGAATTTG-3' R: 5'-CAAAGAACATGCAACGATTTTC-3'
At1g71330 <i>AtNAP5</i>	Salk_047823	F: 5'-CCAAGTCAAGATTTTGGC-3' R: 5'-CTTGACTTTCTGTTTCCCGTC-3'
At4g01660 <i>AtATH10</i>	Salk_105250	F: 5'-GCTCGTTTGCAATACATCAGAG-3' R: 5'-TGTTGAATCAGGTACGGTAACG-3'

^aF indicates left gene-specific primer and R indicates right gene-specific primer.

Table A3 | List of primers used in this study for screening the expression of ABC transporter genes in T-DNA KO lines.

AGI Code	New systemic name	Primer pairs ^a
At1g59870	<i>AtABCG36</i>	F: 5'-AGAGCAGCGGCTATTGTGATGA-3' R: 5'-TGCCGTAGACGATGAGTGAGAT-3'
At3g53480	<i>AtABCG37</i>	F: 5'-TGAGGAGAGGTATAACAGGAGGTC-3' R: 5'-GAGAGATTCAAAGAACGAGAGAGG-3'
At1g04120	<i>AtABCC5</i>	F: 5'-CCTGCTCTGGGTTTCTCTGCT-3' R: 5'-ATGGTGGGCAGACACTAAGGCC-3'
At5g60790	<i>AtABCF1</i>	F: 5'-CTGCGATTGGTAGGTTTGGT-3' R: 5'-CTCTCAAGTGTGTGGCTTTA-3'
At1g64550	<i>AtABCF3</i>	F: 5'-AAAGCATGGATTGGTGAAGC-3' R: 5'-CAATTCGCATTCTCCATCCT-3'
At1g71330	<i>AtNAP5</i>	F: 5'-TACATATTGCACGCGCTCTCTACC-3' R: 5'-TTCACAGGAGCTTGCACATCCTCA-3'
At4g01660	<i>AtATH10</i>	F: 5'-ACGGTAACGGAAGCATCATCTC-3' R: 5'-GTTGTTGGTTCGAAAGCATAG-3'

^aF indicates forward and R indicates reverse.

Table A4 | List of primers used in this study for screening the expression of genes for RT-PCR assays.

AGI Code	Gene	Primer pairs ^a
At2g14610	<i>AtPR1</i>	F: 5'-GTAGGTGCTCTTGTCTTCCC-3' R: 5'-CACATAATCCCACGAGGATC-3'
At3g57260	<i>AtPR2</i>	F: 5'-CTACAGAGATGGTGTCA-3' R: 5'-AGCTGAAGTAAGGGTAG-3'
At1g75040	<i>AtPR5</i>	F: 5'-CACATTCTCTCCTCGTGTTC-3' R: 5'-TAGTTAGCTCCGGTACAAGTG-3'
At5g44420	<i>AtPDF1.2</i>	F: 5'-CACCCCTATCTTCGCTGCTC-3' R: 5'-TGTTTGGCTCCTCAAGGTT-3'
At1g71330	<i>AtNAP5</i>	F: 5'-TACATATTGCACGCTCTCTACC-3' R: 5'-TTCACAGGAGCTTGACATCCTCA-3'
At3g47730	<i>AtATH1</i>	F: 5'-TCAAGTGGGTTTCCCTATGC-3' R: 5'-CCCGGTATCCCGATAAATCT-3'
At3g47780	<i>AtATH6</i>	F: 5'-GGGAAACTTGAGAGGGGAAG-3' R: 5'-CTTCAGCTCTTTGGTTGC-3'
At4g01660	<i>AtATH10</i>	F: 5'-ACGGTAAACGAAGCATCATCTC-3' R: 5'-GTTGTTGGTTTCGGAAAGCATAG-3'
At5g39040	<i>AtTAP2</i>	F: 5'-ATGGAAGCGAGAATGGTTTG-3' R: 5'-CTACTAGCGCTGCAGCTTT-3'
At5g60790	<i>AtABCF1</i>	F: 5'-CTGCGATTGGTAGGTTTGGT-3' R: 5'-CTCTCAAGTGTGTGGCTTA-3'
At1g64550	<i>AtABCF3</i>	F: 5'-AAAGCATGGATTGGTGAAGC-3' R: 5'-CAATTCGCATTCTCCATCCT-3'
At2g34660	<i>AtABCC2</i>	F: 5'-CCGCAGAAATCCTCTTGGTCTTGATG-3' R: 5'-CCTTGTAAGTGGTGTGAGTCATCTTTGG-3'
At1g04120	<i>AtABCC5</i>	F: 5'-CCTGCTCTTGGGTTTCTCTGCT-3' R: 5'-ATGGTGGCAGACACTAAGGCC-3'
At2g36380	<i>AtABCG34</i>	F: 5'-AGATGTTGACGTCACGAATCTTGCT-3' R: 5'-GTTGCCCTGCGTAAAAGAATTG-3'
At1g15210	<i>AtABCG35</i>	F: 5'-GGACATACACGCTTCCCACT-3' R: 5'-AAGCACACTTGTCCCAACC-3'
At3g53480	<i>AtABCG37</i>	F: 5'-TGAGGAGAGGTATATCAGGAGGTC-3' R: 5'-GAGAGATTCAAAGAACGAGAGAGG-3'
At2g36910	<i>AtABC1</i>	F: 5'-AGACCTGGAAGCGGCACATGC-3' R: 5'-TAGAGTCGCGGCTTGATGAT-3'
At2g47000	<i>AtABC4</i>	F: 5'-TTCATCAGTGGTCTGCAACAG-3' R: 5'-TGAAGCTGAACTAACGAAGCA-3'

^aF indicates forward and R indicates reverse.

Table A5 | Multi-response permutation procedure (MRPP) analysis of the root exudates and root tissue profiles of the wild type and mutants analyzed by GC-MS and HPLC-MS methods.

Statistic	GC-MS exudates	HPLC-MS exudates	HPLC-MS tissues
Test statistic (<i>T</i>)	-12.044	-12.562	-11.834
Observed delta	0.194	0.192	0.095
Expected delta	0.256	0.248	0.192
Variance of delta	<0.001	<0.001	<0.001
Skewness of delta	-0.440	-0.481	-0.513
<i>p</i> -Value	<0.001	<0.001	<0.001

Table A6 | Pairwise comparisons of the root exudates and root tissue profiles of the wild type and mutants analyzed by GC-MS and HPLC-MS methods.

Comparison		p-Values		
Mutant 1	Mutant 2	GC-MS exudates	HPLC-MS exudates	HPLC-MS tissues
<i>Col-0</i>	<i>Atabcc5</i>	0.001	0.001	0.084
<i>Col-0</i>	<i>Atabcf1</i>	0.001	0.052	0.006
<i>Col-0</i>	<i>Atabcf3</i>	0.002	0.002	0.006
<i>Col-0</i>	<i>Atabcg37</i>	0.003	0.013	0.006
<i>Col-0</i>	<i>Atabcg36</i>	0.002	0.066	0.007
<i>Col-0</i>	<i>Atnap5</i>	0.002	<0.001	0.010
<i>Col-0</i>	<i>Atath10</i>	0.002	<0.001	0.009
<i>Atabcc5</i>	<i>Atabcf1</i>	0.001	0.058	0.008
<i>Atabcc5</i>	<i>Atabcf3</i>	0.001	0.040	0.006
<i>Atabcc5</i>	<i>Atabcg37</i>	0.002	<0.001	0.006
<i>Atabcc5</i>	<i>Atabcg36</i>	0.001	<0.001	0.010
<i>Atabcc5</i>	<i>Atnap5</i>	0.002	<0.001	0.007
<i>Atabcc5</i>	<i>Atath10</i>	0.004	<0.001	0.009
<i>Atabcf1</i>	<i>Atabcf3</i>	0.003	0.011	0.020
<i>Atabcf1</i>	<i>Atabcg37</i>	0.002	0.025	0.007
<i>Atabcf1</i>	<i>Atabcg36</i>	0.001	0.095	0.141
<i>Atabcf1</i>	<i>Atnap5</i>	0.008	<0.001	0.068
<i>Atabcf1</i>	<i>Atath10</i>	0.082	<0.001	0.012
<i>Atabcf3</i>	<i>Atabcg37</i>	0.145	0.009	0.006
<i>Atabcf3</i>	<i>Atabcg36</i>	0.006	0.001	0.007
<i>Atabcf3</i>	<i>Atnap5</i>	0.003	<0.001	0.007
<i>Atabcf3</i>	<i>Atath10</i>	0.021	<0.001	0.010
<i>Atabcg37</i>	<i>Atabcg36</i>	0.010	0.132	0.006
<i>Atabcg37</i>	<i>Atnap5</i>	0.003	<0.001	0.006
<i>Atabcg37</i>	<i>Atath10</i>	0.020	<0.001	0.010
<i>Atabcg36</i>	<i>Atnap5</i>	0.030	<0.001	0.088
<i>Atabcg36</i>	<i>Atath10</i>	0.162	<0.001	0.009
<i>Atnap5</i>	<i>Atath10</i>	0.322	0.466	0.009