An ABC Transporter Mutation Alters Root Exudation of Phytochemicals That Provoke an Overhaul of Natural Soil Microbiota[1][C][W][OA]

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Root exudates influence the surrounding soil microbial community, and recent evidence demonstrates the involvement of ATP-binding cassette (ABC) transporters in root secretion of phytochemicals. In this study, we examined effects of seven Arabidopsis (*Arabidopsis thaliana*) ABC transporter mutants on the microbial community in native soils. After two generations, only the Arabidopsis *abcg30* (*Atpdr2*) mutant had significantly altered both the fungal and bacterial communities compared with the wild type using automated ribosomal intergenic spacer analysis. Similarly, root exudate profiles differed between the mutants; however, the largest variance from the wild type (Columbia-0) was observed in *abcg30*, which showed increased phenolics and decreased sugars. In support of this biochemical observation, whole-genome expression analyses of *abcg30* roots revealed that some genes involved in biosynthesis and transport of secondary metabolites were up-regulated, while some sugar transporters were down-regulated compared with genome expression in wild-type roots. Microbial taxa associated with Columbia-0 and *abcg30* cultured soils determined by pyrosequencing revealed that exudates from *abcg30* cultivated a microbial community with a relatively greater abundance of potentially beneficial bacteria (i.e. plant-growth-promoting rhizobacteria and nitrogen fixers) and were specifically enriched in bacteria involved in heavy metal remediation. In summary, we report how a single gene mutation from a functional plant mutant influences the surrounding community of soil organisms, showing that genes are not only important for intrinsic plant physiology but also for the interactions with the surrounding community of organisms as well.

The diversity of the microbial (bacterial and fungal) communities in soil is extraordinary; 1 g of soil contains more than 10 billion microorganisms belonging to thousands of different species (Roselló-Mora and Amann, 2001). Soil microbial populations are involved in a framework of interactions known to affect key environmental processes like biogeochemical cycling of nutrients, plant health, and soil quality (Pace, 1997; Barea et al., 2004; Giri et al., 2005). Most of the dynamic soil microbial interactions happen near the plant roots and root soil interface, an area called the rhizosphere (Lynch, 1990; Barea et al., 2002; Bais et al., 2006; Prithiviraj et al., 2007). Rhizosphere microbial communities differ between plant species (Priha et al., 1999; Innes et al., 2004; Batten et al., 2006), between ecotypes/chemotypes within species (Kowalchuk et al., 2006; Micallef et al., 2009), between different developmental stages of a given plant (Mougel et al., 2006; Weisskopf et al., 2006), and from those present in bulk soil (Broz et al., 2007). Different root types can also cultivate specific microbes (Lilijeroth et al., 1991; Yang and Crowley, 2000; Baudoin et al., 2002), a response that has generally been attributed to the microenvironments surrounding a root and the varying ability of specific root types to uptake nutrients from soils and secrete exudates. Recent evidence

suggests that specific plant species support a highly coevolved soil fungal community, and this process is mediated by root-secreted compounds (Broeckling et al., 2008). Rhizosphere interactions are initiated by the release of compounds from different organisms, and it is believed that carbon compounds secreted by roots act as substrates for certain species of microbes in the rhizospshere (Morgan et al., 2005).

Root exudates are released into the rhizosphere by three major pathways: diffusion, ion channel, and vesicle transport (Bertin et al., 2003). Recent evidence has implicated ATP-binding cassette (ABC) transporters in the secretion of phytochemicals present in the root exudates of Arabidopsis (*Arabidopsis thaliana*) and other plants (Loyola-Vargas et al., 2007; Sugiyama et al., 2007; Badri et al., 2008; Badri and Vivanco, 2009). ABC transporters are the largest family of membrane transport proteins found in all organisms from bacteria to humans (Higgins, 1992). These transmembrane proteins use the energy of ATP to pump a wide variety of substrates across the membranes, including peptides, carbohydrates, lipids, heavy metal chelates, inorganic acids, steroids, and xenobiotics (Goossens et al., 2003). ABC transporters are also involved in plant disease resistance at the leaf level (Kobae et al., 2006; Stein et al., 2006).

There is accumulating evidence that root exudates play a role in establishing specific interactions with particular microbes in the rhizosphere (legume's symbiotic interaction with rhizobia, interaction of plants with mycorrhizae, and plant-growth-promoting rhizobacteria [PGPR]; Nagahashi and Douds, 2000; Bais et al., 2006, 2008; Prithiviraj et al., 2007; Rudrappa et al., 2008). However, how root exudation processes that result in large-scale changes to the surrounding soil microbial community compared to individual microbes have not been determined, although some recent reviews have referred to it as a biological frontier (O'Connell et al., 1996; Kuiper et al., 2004; Ryan et al., 2009). In contrast, gene deletions and overexpression of specific genes in plants have been shown to attract or deter specific microbes (Widmer, 2007), herbivores, or their predators (Baldwin et al., 2006; Pandey and Baldwin, 2007; Mitra and Baldwin, 2008), and recently it has been shown that mutations in nonpigment floral chemistry genes affect flower visitation by native pollinators (Kessler et al., 2008). Thus, it is possible that gene expression manipulation leading to an altered spectrum of root exudates can influence the widespread community of soil organisms surrounding a plant. Using all available information described above, we present the most comprehensive study on the effect of a single gene mutation in an ABC transporter involved in root secretion of phytochemicals by Arabidopsis on the natural and coevolved soil microbial composition. We further determine the compounds that are likely to have an effect on moderating the microbial composition and characterized specific and natural microbes that interact with Arabidopsis in the soil by employing pyrosequencing technology.

RESULTS

Microbial Diversity Analyses

We analyzed the soil microbial community structure supported by Arabidopsis wild type (Columbia-0 [Col-0]) and seven ABC transporter mutants (*abca7*, *abcc2*, *abcg30*, *abcg34*, *abcg35*, *abcb1*, and *abcb4*; Verrier et al., 2008) grown in Arabidopsis-accustomed soil for two subsequent generations. At the onset of this experiment (generation 0), significant differences in the soil microbial community structure were observed (Fig. 1; Supplemental Tables S1 and S2), presumably associated with natural spatial heterogeneity in the soil, despite our attempts to homogenize the starting soil. However, after one generation, this heterogeneity disappeared and there were no significant differences in either bacterial or fungal community structure between any of the treatments (Fig. 1; Supplemental Tables S1 and S2). Following the second generation, we found that the ABC transporter mutant *abcg30* significantly affected both fungal and bacterial microbial community structure compared with other ABC transporter mutants and the wild type based on an multiresponse permutation procedure (MRPP) analysis of the entire automated ribosomal intergenic spacer analysis (ARISA) profile (Fig. 1; Supplemental Table S1).

Based on the ARISA analysis, eight fungal operational taxonomic units (OTUs) decreased significantly in the soil when *abcg30* was grown, compared to the wild type and the negative control (no plant). Similarly, 14 bacterial OTUs decreased and four bacterial OTUs increased when *abcg30* was grown (Supplemental Fig. S1). Among the eight fungal OTUs that decreased significantly, four of them also decreased in the negative control (no plant; Supplemental Fig. S1A). This result indicates that the other four OTUs (H72, H117, H123, and H129) decreased specifically due to the absence of *abcg30*. Similarly, among the 14 bacterial OTUs that decreased significantly with *abcg30*, three OTUs (H116, H170, and H241) increased in the negative control compared with the wild type (Supplemental Fig. S1B). These results suggest that the three OTUs increased in the negative control due to environmental factors present in the greenhouse. Two other OTUs (H155 and H255) decreased in both *abcg30* and the negative control compared to the wild type. In addition, four OTUs (H90, H19, H141, and H164) increased significantly in *abcg30* compared with the wild type, but one OTU (H90) also increased in the negative control. These results suggest that the root exudates of the wild type and the ABC transporter mutant *abcg30* might have a different composition of phytochemicals, which impacted the microbial community composition of the native soils.

Chemical Analysis of Root Exudates

The root exudates of Arabidopsis wild type and all ABC transporter mutants were analyzed in this study via NMR spectroscopy coupled with multivariate data

Figure 1. NMS analysis of fungal and bacterial community profiles of Arabidopsis wild type and ABC transporter mutant rhizospheric soil samples conducted using the relative Sorenson distance measure. All generation samples were analyzed simultaneously. A, Fungi; B, bacteria. G0, zero generation (before seeding); G1, first generation; G2, second generation; L*er*, Landsberg *erecta* ecotype; T, negative control (soil without plant).
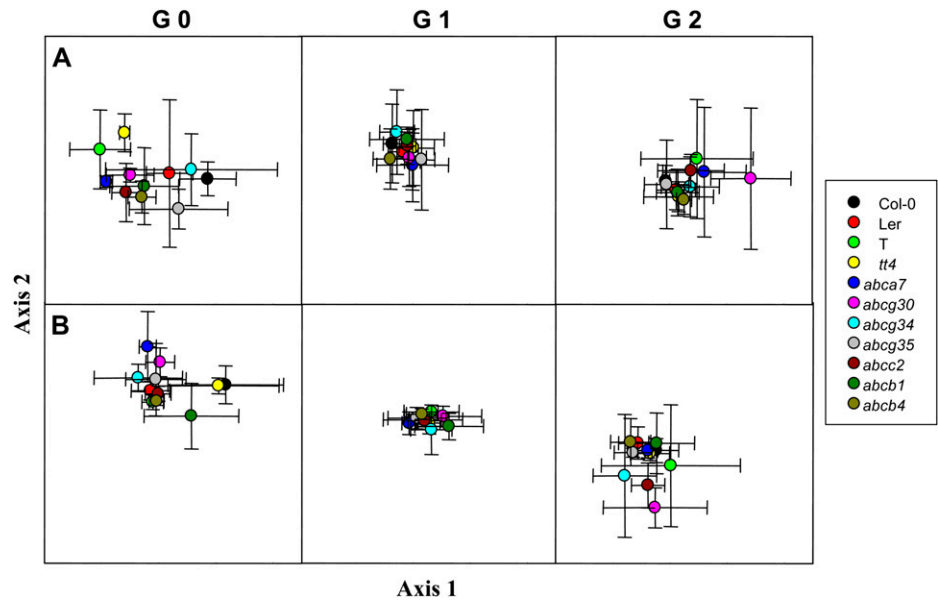
analyses. In total, based on ^{1}H-NMR and other spectral analyses, we identified 33 compounds in the wild-type root exudates, including organic acids, amino acids, sugars, flavonols, phenolics, anthocyanidins, and indole compounds (Supplemental Figs. S2–S10). Principal component analysis (PCA) of the root exudates using the *J*-resolved NMR signals showed that the 10 Arabidopsis lines used in this study fell into seven statistically different groups (Fig. 2A). The root exudates of *abcg30* appeared to be unique (Fig. 2A) and showed the lowest similarity to the wild type (Fig. 2B). Compared to the wild type, the following compounds were present in higher concentrations in the root exudates of *abcg30*: benzoic acid, salicylic acid, syringic acid, tartaric acid, lactic acid, α-linolenic acid, cyanidin, sinapoyl malate, Val, and indole 3-acetic acid. In contrast, lower amounts of some sugars (raffinose, Glc, Fru, and mannitol) were present (Supplemental Table S3).

Analyzing the Pleiotropic Effect of *abcg30* Mutation at the Genome Level

Based on the comparison of the root exudates profiles of all ABC transporter mutants, we found that *abcg30* had increased phenolics and fewer sugars compared to the wild type. We hypothesized that the observed differences (phenolics versus sugars) in *abcg30* root exudates might not be under the direct control of the ABCG30 transporter but are probably the pleiotropic effects of the mutation. To elucidate the pleiotropic effects of abcg30 mutation, we performed whole-genome expression analyses on *abcg30* roots compared with wild-type roots using the Affymetrix GeneChip Arabidopsis ATH1 genome array chip. Out of 22,810 genes on the chip, 355 (1.5%) genes were up-regulated >1.5-fold, and 156 (0.7%) genes were down-

regulated to <0.5-fold in *abcg30* compared to the wild type. We focused our analyses on selected genes involved in transport, secondary metabolism biosynthesis, and transcription factors (Supplemental Table S4). We observed that 10 genes involved in transport, including lipid transporters (At4g22460 and At3g22120) and ABC transporters (At5g44110 and At2g26910), and 16 genes involved in secondary metabolism, including phenylproanoid (At2g23910, At1g65060, and At1g67980) and flavonoid biosnthesis genes (At3g55120, At5g08640, At5g05270, At5g07990, and At5g13930) were up-regulated significantly in *abcg30* compared to the wild type. Similarly, six genes involved in transport, including sugar transporters (At1g08920 and At4g04760) and a mannitol transporter (At4g36670), and nine genes involved in secondary metabolism, such as terpene biosynthesis (At4g20230, At3g31415, and At5g42600), were down-regulated in *abcg30* compared to the wild type. In addition to transporters and secondary metabolism biosynthesis genes, we found that some transcription factors and genes belonging to different functional categories, such as primary metabolism, signal transduction, cell growth, and cell division, defense responses and genes of unknown function were also differentially (both up- and down-regulated) expressed in *abcg30* compared with the wild type (Supplemental Table S4 and Figure S11). The microarray data were also deposited to a permanent public repository, ArrayExpress, under the accession number E-TABM-821.

In Vitro Analysis of the Effect of Arabidopsis Wild-Type and *abcg30* Exudates on Native Soil Microbes

Due to the differences in phytochemical composition in the root exudates, we examined whether wild-

terized the soil microbial communities after the 2nd generation of plant growth by pyrosequencing of rRNA libraries. A total of 160 fungal (Fig. 3A) and 2,489 bacterial (Fig. 3B) sequence reads were obtained
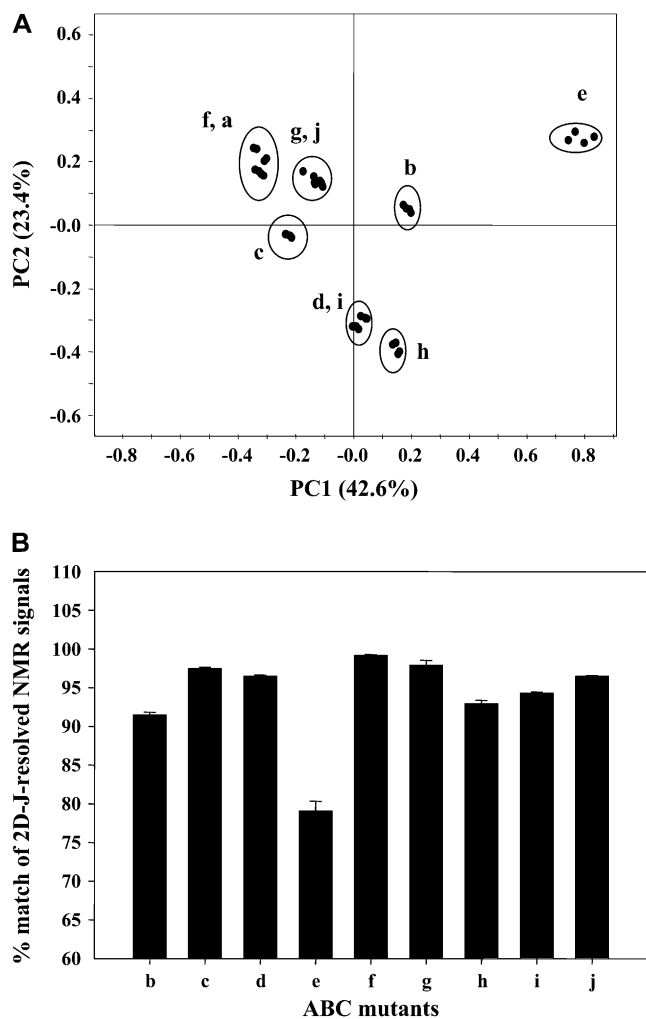

Figure 2. A, PCA of Arabidopsis wild type (Col-0) and ABC transporter mutant root exudates using projected one-dimensional *J*-resolved spectrum. B, Percentage of matched two-dimensional *J*-resolved signals between wild type (Col-0) and Arabidopsis ABC transporter mutant root exudates. a, Col-0; b, *abca7*; c, *dtx12*; d, *abcc2*; e, *abcg30*; f, *abcg34*; g, *abcg35*; h, *abcb1*; i, *abcb4*; j, *abcb27*.

type and *abcg30* root exudates affected the in vitro growth of natural Arabidopsis soil microbes using standard serial dilution techniques. The *abcg30* exudates significantly reduced both the number and growth rate of the fast-growing, culturable soil microbes. For example, after 24 h, 2.8×10^7 colonies were visible on Col-0 exudates amended plates, and no colonies were observed on *abcg30* exudates amended plates, whereas, at the termination of the experiment (48 h), only 7.1×10^6 colonies could be observed on the *abcg30* amended plates, but Col-0 amended plates showed complete bacterial lawns (Supplemental Table S5).

Taxa Identification Using Pyrosequencing

To further characterize the specific taxa associated with *abcg30* and wild-type root exudates, we charac-

C

	Fungi		Bacteria	
	Col-0	*abcg30*	Col-0	*abcg30*
ACE	425	464	10,206	9,857
Chao	405	327	9,176	8,882
H	4.42	4.24	7.51	7.48

D

	Homologous	Heterologous	
	X	**Y**	**P-value**
Fungi	Col-0	*abcg30*	0.072
	abcg30	Col-0	0.000
Bacteria	Col-0	*abcg30*	0.000
	abcg30	Col-0	0.000

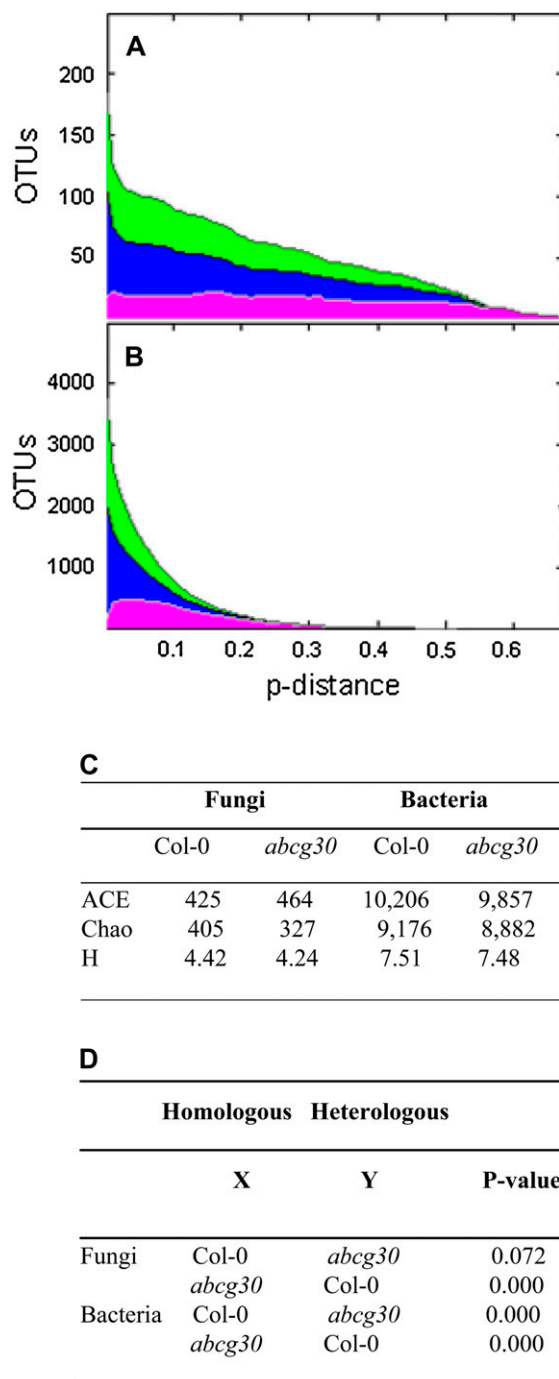**Figure 3.** Fungal (A) and bacterial (B) OTUs present in the 2nd generation of *Atabcg30* and wild-type soils determined by rRNA pyrosequencing. Green, *abcg30*; blue, wild type (Col-0); pink, shared. C, Total estimated species richness (ACE and Chao) and Shannon diversity index (H). D, Significance of microbial libraries community profile comparison using ʃ-libshuff. [See online article for color version of this figure.]

from the wild-type and *abcg30* soils. Although there appeared to be no difference in either fungal or bacterial total OTU richness or diversity (Shannon-diversity index) between the two soils (Fig. 3C), each library was characterized by a high number of unique (i.e. below the detection level of 1 per 2,689 reads or a relative abundance ≤0.037%) taxa resulting in statistically different communities (Fig. 3D). For example, at a genetic distance of 1%, 84 fungal and 1,661 bacterial OTUs were unique to the *abcg30* community, whereas 90 fungal and 1,879 bacterial OTUs were unique to the wild-type community.

From this analysis, the relative abundance of 105 bacterial OTUs differed significantly ($P < 0.05$) be-

tween the wild type and *abcg30* (Fig. 4A, B). Unambiguous identification of the various OTUs to the species/strain level is frequently not possible due to either high homology of the rRNA sequences to multiple species or missing reference sequences in the relevant databases. In this study, however, many of the *abcg30* up-regulated OTUs appear to be closely related to known PGPRs or otherwise beneficial bacteria. These include one (species) *Microbacterium* sp., one Nocardioidaceae sp., one *Pseudonocardia* sp., one Flexibacteraceae sp., three (species) Sphingobacteriales spp., one *Methylibium* sp., two *Duganella* spp., one *Pseudoxanthomonas* sp., one *Flavobacterium* sp., six *Brevundimonas* spp., one *Cystobacter* sp., three Rhodo-

Figure 4. Relative abundance of bacterial OTUs (1% dissimilarity) significantly up- (A) or down-regulated (B) in the *abcg30* mutant soils compared to the wild type. Taxonomic assignments were made with the RDP II naïve Bayesian classifier (80% confidence threshold) using a single representative sequence for each OTU. Col-0, black bars; *abcg30*, gray bars.
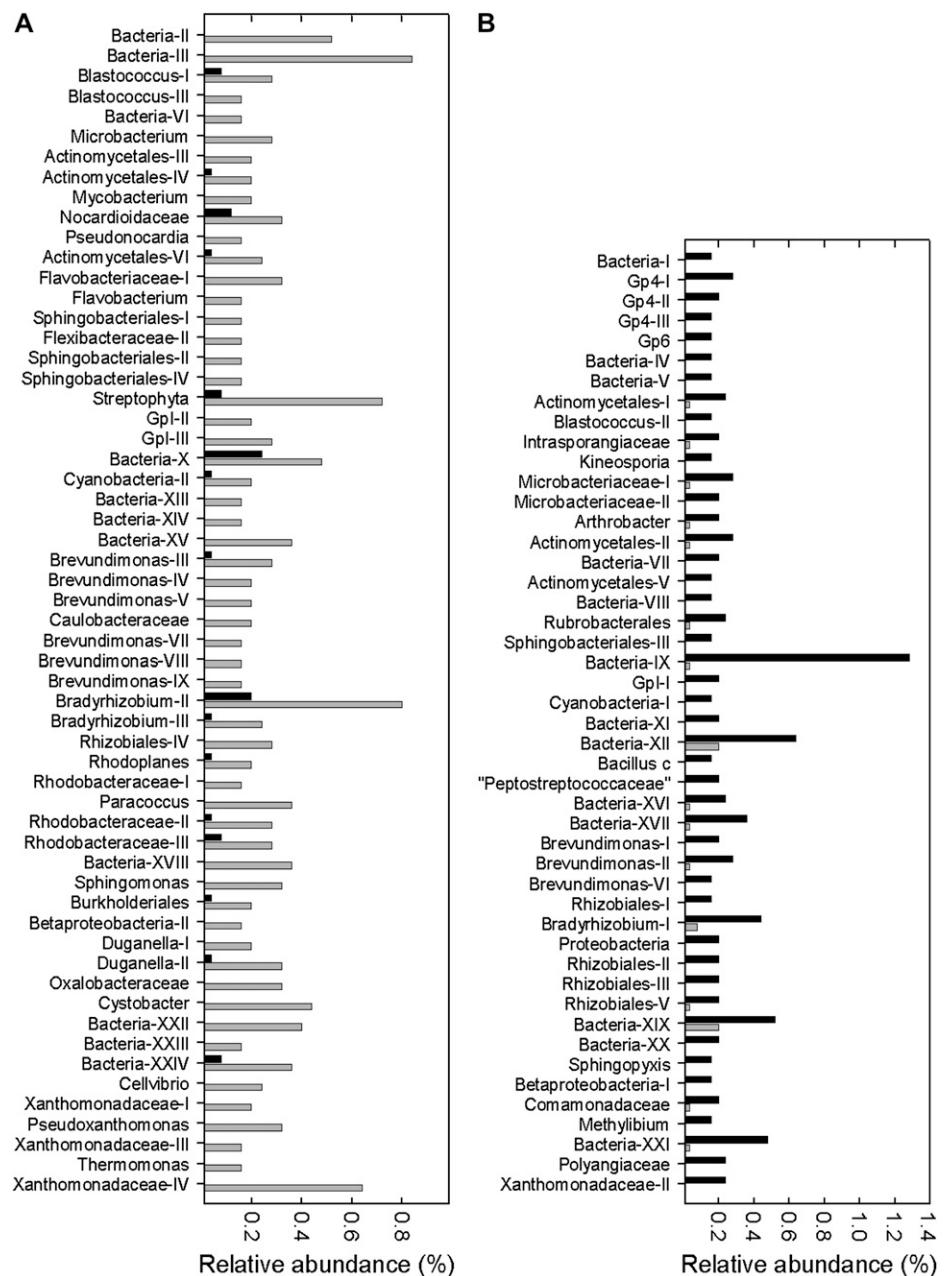
bacteriaceae spp., two *Bradyrhizobium* spp., and one *Paracoccus* sp. The *Microbacterium*, Nocardioidaceae, Flexibacteraceae, Sphingobacteriales, and *Flavobacterium* spp. are of special interest because they play a role in heavy metal (Ni and S) remediation (Idris et al., 2004), whereas the *Pseudonocardia*, *Methylibium*, *Duganella*, and *Pseudoxanthomonas* spp. are involved in detoxifying toxic compounds like ether pollutants, dinitrotoluene, bioplastics, and methylated aromatic compounds (Grech-Mora et al., 1996; Kaplan and Kittis, 2004; Vainberg et al., 2006; Kane et al., 2007; Kim et al., 2008). A variety of bacteria associated with nitrogen fixation were also up-regulated in the *abcg30* soil, including six *Brevundimonas* spp., two *Bradyrhizobium* spp., and one *Paracoccus* sp. In contrast, the wild-type soil preferentially supported a much smaller number of potential PGPBs or otherwise beneficial bacteria. These include three *Brevundimonas* spp., one *Bradyrhizobium* sp., one *Lysobacter* sp., and one *Bacillus* sp., which are different OTUs than the ones cultivated by *abcg30* based on their genetic distance (>1%) and phylogenetic analysis with their nearest known neighbors (Supplemental Fig. S12). Differences were also observed for the fungal community structure in the soils surrounding the wild type and the *abcg30* mutant (Fig. 5). Four fungal OTUs were significantly up-regulated (Ciliophora sp., one Basidiomycota sp., one *Scenedesmus* sp., and one Trebouxiophyceae sp.), and five were significantly down-regulated (two Ascomycota spp., two *Mortierella* spp., and one *Ciliophora* sp.). Additionally, we determined that one *Xylella* sp. was enriched in the soil cultured by *abcg30* compared to wild-type-grown soil, and a similar observation was found for *Mycobacterium* sp.
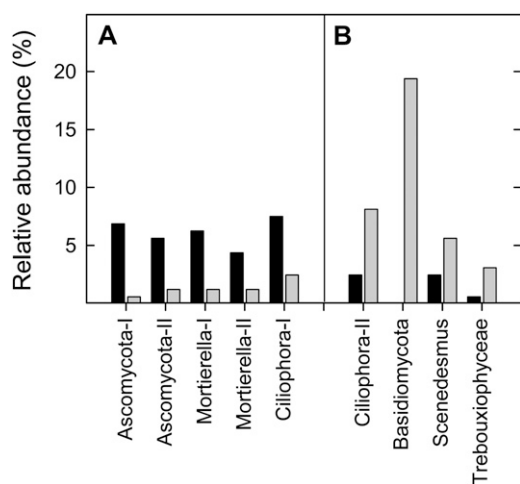

Figure 5. Relative abundance of fungal OTUs (1% dissimilarity) significantly down- (A) or up-regulated (B) in the *abcg30* mutant soils compared to the wild type. Taxonomic assignments were made based on the nearest neighbor in GenBank (homology >80%) using a single representative sequence for each OTU. Col-0, black bars; *abcg30*, gray bars.

DISCUSSION

The impact of plant species and closely related variants (i.e. ecotypes) on rhizobacterial communities has been well documented (Dalmastri, 1999; Mazzola et al., 2004; Micallef et al., 2009). In this study, we showed that a single gene mutation (ABC transporter, *abcg30*) involved in root exudation influences the soil microbial community. We analyzed seven Arabidopsis ABC transporter mutants, whose putative transporters were highly expressed in root cells (Badri et al., 2008), for differences in their root exudates and their ability to influence the microbial community in Arabidopsis-accustomed soil. Previously, it has been shown that Arabidopsis fails to support the fungal community of soils to which it is an unfamiliar transplant but sustains the fungal community in native soils (Broeckling et al., 2008), and this process was partly mediated by root exudates. In this study, we demonstrate that one ABC transporter mutant, *abcg30*, significantly affects the soil microbial community compared with the wild type. The function of *abcg30* (also known as *Atpdr2*) is unknown; however, the organ-specific expression pattern of this gene shows that it is highly expressed in root epidermal cells (Birnbaum et al., 2003; Badri et al., 2008). In plants, the pleiotropic drug resistance gene family has been shown to be involved in extruding the antifungal diterpene from leaves (Jasinski et al., 2001), heavy metal detoxification (Lee et al., 2005), herbicide detoxification (Ito and Gray, 2006), and nonhost resistance (Stein et al., 2006). Although *abcg30* caused the most dramatic changes in overall microbial composition in the soil compared to the wild type, it should be noted that the other ABC transporter mutants tested in this study generated changes in certain microbial OTUs (data not shown).

The changes observed in soil microbial diversity between the generations could be due to a direct plant effect or to soil microbial population dynamics. Based on our results and experimental design, we conclude that the observed changes in soil microbial diversity are due to the effect of the plant because (1) no microbial changes in the soil were observed in the control (no plant) from the first to second generation, and (2) significant changes in soil microbial diversity from the first to second generation were observed in *abcg30* grown soil but not in the wild type. Additional studies are needed to determine the longevity of the *abcg30*-induced microbial community changes or if the other mutants may eventually significantly alter the microbial community.

We further analyzed the root exudates profiles of seven ABC transporter mutants using two-dimensional NMR analyses and found that *abcg30* has increased phenolics and fewer sugars compared to the wild type. This result is not in agreement with the previous report by Badri et al. (2008) where *abcg30* root exudates did not show significant differences with the wild type and the other ABC transporter

mutants based on PCA analysis. This observed discrepancy is because in Badri et al. (2008), root exudates were extracted with water and analyzed by gas chromatography-mass spectrometry to study the differences predominantly in the composition of hydrophilic compounds such as primary metabolites. However, in this study, the root exudates were extracted with ethyl acetate and analyzed by NMR to study the differences primarily of hydrophobic compounds and hence predominantly in the secondary metabolites between the mutants and the wild type. In both studies, the contributing factors (primary versus secondary metabolites) for PCA analyses were different, which explains why *abcg30* root exudates were significantly different from the wild type and other mutants used in this study.

As stated above, a wide variety of compounds exhibited an altered level of secretion in *abcg30*; however, this does not necessarily mean that secretion of all of these compounds is under the direct control of ABCG30. Instead, the pleiotropic effect of the single gene (*abcg30*) mutation may be linked to the coregulation of metabolic processes or other transport systems. Both possibilities were evident in our whole-genome expression analyses, which showed that the genes involved in secondary metabolite biosynthesis and transporting systems (lipid transporters and ABC transporters) are up-regulated in *abcg30* but not in the wild type. Conversely, we observed that sugar and mannitol transporters were down-regulated in *abcg30* compared with the wild type due to the pleiotropic effect of the gene mutation. Because we observed relatively more salicylic acid and sinapic acid in the root exudates of *abcg30* compared with the wild type, the use of other gene mutations like *sid2* (salicylic acid deficient) and *fah1* (sinapate deficient) may provide insight about the role of salicylic or sinapic acids in regulating soil microbial composition. In this study, we used the *tt4* (defective in flavonoid synthesis) mutant but found no significant effect in reshaping soil fungal or bacterial communities compared to the wild type (Supplemental Tables S1 and S2). These data suggest that flavonoids are not common, or widespread, signals regulating the overall soil microbial community, despite their ability to induce nodulation of legumes by Rhizobium (Peters et al., 1986).

In vitro serial dilution plating showed that *abcg30* root exudates significantly reduced total microbial growth (i.e. colony-forming units). From this experiment, it is impossible to determine whether the lack of sugars, increase in phenolics, or both were responsible for the reduced microbial growth; however, like the study of Broeckling et al. (2008), this experiment provides additional proof of a direct link between root exudates and soil microbial growth and survival.

Pyrosequencing analyses revealed that the *abcg30* soil microbial community is enriched with rhizobacteria that may be related to heavy metal remediation, detoxification of toxic chemicals, and nitrogen fixation. Unfortunately, the pyrosequencing analysis as employed here was semiquantitative and could not be used to accurately estimate the absolute abundance of specific microbes or OTUs. However, it is clear that the *abcg30* soil microbial community is shifting and that the relative abundance of several OTUs closely related to known PGPRs or otherwise beneficial bacteria is increasing. Additional studies are needed to determine the absolute abundance of these OTUs (e.g. species-specific quantitative PCR) and to what degree these community shifts may affect such processes as nitrogen fixation and/or heavy metal remediation.

We hypothesize two possible explanations for the ability of *abcg30* to cultivate microbes related to heavy metal remediation and detoxification of toxic chemicals: (1) these microbes are especially adept at utilizing and/or neutralizing the phenolic compounds present in the *abcg30* root exudates, and/or (2) the increase in phenolics in the exudates have antimicrobial activity against many of the other bacterial species found in wild-type-grown soil. Both possible explanations are evident in our experimental results. Many bacterial species involved in heavy metal remediation and toxic chemical(s) decontamination can readily utilize phenolic compounds as substrates (Grech-Mora et al., 1996; Kaplan and Kittis, 2004; Vainberg et al., 2006; Kane et al., 2007; Kim et al., 2008), such as those secreted by *abcg30*. It is also evident based on the in vitro soil dilution plating results that *abcg30* root exudates support a less abundant total microbial community than wild-type root exudates, potentially reducing antagonistic microbe-microbe interactions, which act to suppress some of the OTUs in the wild-type soils. Some of the microbial changes might not be the result of direct interactions with the plant but due to its direct influence on other members of the soil community. Further studies are needed to know how the native soil microbe-induced root exudate profiles of the wild type and *abcg30* alter the total soil microbes at the community level.

We also reported that *abcg30* appears to culture *Xyllela* sp. and *Mycobacterium* sp. compared to the wild type. Both of these microbes could be related to important plant or human pathogenic bacteria, respectively, and thus deserve further identification. It is likely that the wild type does not support the growth of these bacteria because of no particular interactions with the plant; however, the alteration of the ratio of root exudates might inadvertently promote the growth of these bacteria in the soil cultured by the mutant. Thus, putative compounds in the exudates of the wild type could potentially be used as antimicrobials specific for these otherwise pathogenic bacteria.

Overall, our results show that *abcg30* and the resultant ratio and composition of both phenolics and sugars in the root exudates have a profound effect on natural soil microbial composition. This is the first study that explicitly shows how changing the blend of rhizosphere chemicals can lead to changes in microbial composition. In addition, this study shows that specific mutations in genes involved in root exudation of

phytochemicals, such as ABC transporters, present in otherwise functional plants can have significant effects on soil microbial composition, thus strengthening the notion that the function of certain genes might not be restricted to intrinsic plant physiology but to interactions with the environment. As such, we believe that this work provides a strong foundation for the development of new technologies that exploit ABC transporters' control of root exudation to modify the soil microbial community composition for beneficial purposes.

MATERIALS AND METHODS

Soil Experiment

For performing the soil microbe diversity experiment, we followed the method described by Broeckling et al. (2008) with slight modifications. We used Illinois soil collected from under Arabidopsis (*Arabidopsis thaliana*) plants for the soil microbe diversity experiment. The soil was collected in 2007 (collected by Joy Bergelson, University of Chicago, and described in detail in Broeckling et al., 2008) at 42° 05′ 34″ N, 86° 21′ 19″ W, elevation 630 feet. The collected field soil was shipped to Fort Collins, Colorado, in air-tight coolers and stored in a cold room (4°C) until further use. The soil was air-dried, cleaned of plant debris, homogenized by hand, and transferred into pots (9 × 9 × 12-cm pots). The bottom of the pots were lined with Whatmann 3MM filter paper to avoid soil loss. The pots were moved to a greenhouse bench and watered sufficiently (two or three times a week) for 3 weeks, during which the soil's existing seed bank seedlings were continuously removed. After the complete removal of the existing seed bank seedlings, we sowed surface-sterilized Arabidopsis seeds. Pots were maintained in a greenhouse under ambient conditions for optimum plant growth. For each mutant (and Col-0), nine replicate pots were maintained. The pots without plants served as negative controls to see the environmental effects contributing to the changes in soil microbial community structure. The aerial portions of the plants were harvested after 10 weeks for each generation, the tissue was dried for 3 d at 70°C, and the dry weight biomass was recorded. A 2- to 3-week dormancy period (no watering) was applied between each generation to allow the root systems of previous plants to die. The first generation was seeded in August 2007 and harvested in October 2007, and the second generation was seeded in December 2007 and harvested in February 2008. A list of Arabidopsis mutant lines and their parental background used in this experiment is presented in Supplemental Table S6.

Soil Sampling

The top 2.0 cm of soil within a 0.7-cm radius around the crown of the plant was sampled using a cork borer sterilized with bleach and rinsed thoroughly with distilled water. This sampling procedure was adopted to allow for multigenerational sampling without significantly disturbing the soil texture between the generations. Soil samples were transferred into scintillation vials and stored at −20°C until processing. For the ARISA analyses, we pooled the soil samples collected from three different pots of each Arabidopsis line for a total of three replicates per treatment.

Soil DNA Extraction

To characterize the soil microbial community, total DNA was extracted from soil and amplified by PCR using internal-transcribed spacer (ITS)-specific primers, and the amplified products were sequenced to identify taxa level. Briefly, DNA was extracted from the soils using a MoBio ultraclean soil DNA kit (Mo Bio) according to the manufacturer's instructions except for the addition of an extra ethanol wash. The DNA was quantified using a Nanodrop spectrophotometer (Nanodrop Technologies) and all DNA had a $A_{260}:A_{280}$ ratio between 1.7 and 1.9.

ARISA

PCR amplification was performed with the fungal-specific 2234C and 3126T primer set (Sequerra et al., 1997) or the bacterial-specific ITSF and

ITSReub primer set (Cardinale et al., 2004). PCR reactions contained 5 µL (10 ng µL^{-1}) soil DNA, 10 µL 2× jumpstart reaction mix (Sigma-Aldrich), 2.4 µL 25 mM MgCl$_2$, 0.2 µL 1 mM fluorescein, and 0.4 µL 10 mM forward and reverse primers and were brought to 20 µL with deionized water. The PCR products were amplified for 30 cycles (at 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s). PCR reactions were diluted with 80 µL of distilled water, and a 2-µL aliquot was added to 10 µL loading buffer (1,250 µL formamide and 50 µL Genescan 2500 [TAMRA] size standard) and analyzed directly by capillary electrophoresis (ABI Prism 310; Applied Biosystems) without further modification, i.e. denaturation heating. Electrophoresis conditions were as follows: 47-cm capillary, Genescan POP4 polymer, 15-s injection for 15 kV, and 45-min electrophoresis at 15 kV. Scoring of amplicons into unique bins was performed using Genemapper software (version 4). Species richness of each sample was determined as the number of amplicons above a threshold of 50 relative fluorescence units with the understanding that any given peak may contain amplicons from multiple species (Manter and Vivanco, 2007).

Pyrosequencing Analyses

Amplification of the fungal ITS or bacterial (16S) rRNA genes were performed with the following primers:

fungi, 2234C, 5′-gcctccctcgcgccatcagGTTTCCGTAGGTGAACCTGC-3′ and 3126T, 5′-gccttgccagcccgctcagATATGCTTAAGTTCAGCGGGT-3′; bacteria, 27F, 5′-gcctccctcgcgccatcagAGAGTTTGATCMTGGCTCAG-3′ and 388R, 5′-gccttgccagcccgctcagTCTGCTGCCTCCCGTAGGAGT-3′.

The lowercase and underlined regions of the above primers are necessary adapters for binding and amplification using the pyrosequencing process, and the uppercase regions are primers targeted to conserved regions of the rRNA genes. The fungal primers listed here were previously employed in several fungal diversity studies (Sequerra et al., 1997; Ranjard et al., 2001; Lejon et al., 2005; Broz et al., 2007) and have clearly demonstrated an ability to amplify fungi belonging to Ascomycota, Basidiomycota, Zygomycota, Oomycota, Chytridiomycota, and Plasmodiophoromycota, while not amplifying bacteria or plant DNA (Ranjard et al., 2001). The bacterial primers were demonstrated to amplify a wide variety of bacteria (Lane, 1991; Marchesi et al., 1998); however, we must include the caveat that no single combination of rRNA primers can amplify all fungal or bacterial isolates (Brunk et al., 1996). PCR reaction conditions were the same as outlined above for the ARISA analysis. Following PCR, the products were visually checked on an agarose gel, and each successful reaction was purified using AMPure beads (Agencourt).

Purified PCR amplicons, from the three replicate soil samples per treatment, were pooled at a ratio of 4:1 v/v (bacteria:fungi). We chose this pooling procedure, as opposed to combining all reactions on an equimolar ratio, because an accurate determination of the number of molecules in the fungal amplicons is impossible due to length heterogeneity in the ITS1 region. The pyrosequencing was performed under contract with the University of Florida Genomics Facility using 1/16 of a PicoTiter-Plate (454 Life Sciences) yielding a total of approximately 8,000 sequence reads.

In order to minimize the effects of random sequence errors, we removed all sequences with multiple undetermined residues ($n > 3$) or a single primer nucleotide mismatch. The resulting sequences, after removal of the unique barcode, averaged 275 bp in length. Because sampling effort (i.e. number of sequence reads) can influence estimates of total species richness, a subset of 160 fungi and 2,489 bacterial sequences were randomly selected and used to create a single library for each of the *Atabcg30* and wild-type soils. A multiple sequence alignment was performed individually for each of the fungal ($n = 320$) and bacterial ($n = 4978$) libraries using MUSCLE (parameters set to -maxiters 2; Edgar, 2004). From this alignment, a distance matrix was constructed using DNADIST (Jukes-Cantor correction) from PHYLIP version 3.68 (Felsenstein, 1989, 2005). The distance matrix was then input into DOTUR (Schloss and Handelsman, 2005) for clustering the sequences into OTUs based on the genetic distance between sequences and the generation of rarefaction curves for making estimates of the total OTU richness (ACE and Chao1; Chao and Lee, 1992; Chao et al., 1993) and diversity (Shannon-Weaver Index; Magurran, 1988) indices. Community similarity was determined using ∫-libshuff (Schloss and Handelsman, 2006), which uses a Monte Carlo testing procedure to evaluate differences between each of the communities (Schloss, 2008).

A more detailed analysis of the difference between the DOTUR assigned microbial OTUs (1% dissimilarity) in the *abcg30* and wild-type soils was conducted as follows: for each OTU, the probability that the OTU relative abundance differed between the *abcg30* and wild-type soils was estimated

using the statistical test developed by Audic and Claverie (1997). For the bacterial OTUs, taxonomic assignments were made with the ribosomal database project's naïve Bayesian classifier (80% confidence threshold) using a single representative sequence from each OTU (Wang et al., 2007). For the fungal OTUs, taxonomic assignments were made based on the nearest neighbor in the GenBank nonredundant database (homology ≥80%) using a single representative sequence from each OTU. All reported taxonomic assignments are the lowest identified taxonomic level with a confidence threshold (bacteria) or homology (fungi) ≥80%. Homology (*H*) was calculated using the following equation:

$$H = \frac{I - G}{L} \times 100$$

where *L* = length of query sequence, *I* = identities, and *G* = gaps from the default BLAST output.

Plant Material and Growth Conditions

Arabidopsis seeds were surface-sterilized with bleach for 1 min followed by five rinses in sterile distilled water and plated on Murashige and Skoog (MS; Murashige and Skoog, 1962) salts supplemented with 3% Suc and 0.8% Bacto agar 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 six-well culture plates (VWR Scientific), with each well containing 5 mL of liquid MS (MS 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 previously published methods (Loyola-Vargas et al., 2007; Badri et al., 2008), when plants were 18 d old, they were washed with sterile water to remove the surface-adhering exudates and transferred to new six-well plates containing 5 mL 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 d after transfer. For each replicate analysis, we collected 1500 mL exudates from 300 individually grown Arabidopsis plants. Root exudates were collected for the wild type and mutants from two independent experiments in triplicate.

Extraction of Phytochemicals

Three days after the transfer described above, the collected liquid media were filtered through nylon filters of 0.45-μm pore size (Millipore) prior to freeze drying (Labconco). The freeze-dried powder was dissolved in 10 mL of distilled water and partitioned three times with an equal volume of ethyl acetate (EtOAc; Fisher Scientific). All three EtOAc fractions were pooled, and the remaining water residues were removed using sodium sulfate as a drying agent. The dried concentrate was dissolved in 800 μL of methanol for subsequent NMR analysis.

NMR Experiments

EtOAc extracts of Arabidopsis root exudates were dissolved in 1 mL of 50% MeOH-d_4 in buffer (90 mM KH$_2$PO$_4$, pH 6) containing 0.05% w/v trimethyl silyl propionic acid sodium salt (TMSP). The mixture was vortexed at room temperature for 30 s, ultrasonicated for 1 min, and centrifuged at 30,000 rpm at 4°C for 5 min. NMR spectra were acquired at 25°C on a 600-MHz Bruker AV-600 spectrometer equipped with a cryoprobe operating at proton frequency of 600.13 MHz. MeOH-d_4 was used as the internal lock. Each ^{1}H-NMR spectrum consisted of 256 scans requiring 8 min and 30 s acquisition time with the following parameters: 0.12 Hz/point, pulse width of 30° (11.3 μs), and relaxation delay of 2 s. A presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay. Free induction decay was Fourier transformed with a line-broadening factor of 0.3 Hz. The resulting spectra were manually phased, baseline corrected, and calibrated to the internal standard TMSP at 0.0 μL L^{-1} using Topspin (version 2.1; Bruker). Two dimensional *J*-resolved NMR spectra were acquired using 16 scans per 64 increments for F1 and 1,638.4 k for F2 using spectral widths of 7239.4 Hz in F2 (chemical shift axis) and 50 Hz in F1 (spin-spin coupling constant axis). A 1.5-s relaxation delay was employed. Data sets were zero-filled to 512 points in F1, and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. *J*-resolved spectra were tilted by 45°, aligned about F1,

and then calibrated to TMSP using Topspin. In order to obtain projected spectra, the signals of two-dimensional *J*-resolved spectra were summed up to the F2 direction by Topspin. The COSY spectra were acquired with a 1.0-s relaxation delay and 6,009.6 Hz spectral width in both dimensions. The window function for the COSY spectra was Qsine (SSB = 2.0). The HSQC spectra were obtained with a 1.0-s relaxation delay and 6,009.15-Hz spectral width in F2 and 164 Hz in F1. The HMBC spectra were recorded with the same parameters as the HSQC spectrum except for 31,692.7 Hz of the spectral width in F2.

NMR Data Analysis

Spectral intensities of ^{1}H-NMR spectra were scaled to the total intensity and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.3 to δ 10.5 using AMIX (version 3.8; Bruker). The regions of δ 4.7 to δ 5.0 and δ 3.28 to δ 3.40 were excluded from the analysis because of the residual signal of water and methanol. PCA was performed with the SIMCA-P software (version 11.0; Umetrics). The scaling method for PCA was Pareto. Matching of two-dimensional *J*-resolved signals was performed by AMIX using automatic noise level.

Soil Microbe Screening

Viable soil microbe screening was performed by supplementing microbe cultures with plant root exudates to check whether root exudates have any effect on microbes in vitro. Arabidopsis-adapted soil planted with wild-type Arabidopsis was serially diluted with sterile distilled water and spread onto soil extract agar (SEA) plates (Alef and Nannapieri, 1995) for bacterial enumeration or SEA plates amended with streptomycin (50 μg/mL) and ampicillin ((50 μg/mL) for fungal enumeration. Before spreading onto SEA plates, root exudates (1:5, v/v) collected from the wild type (Col-0), *abcg30*, or a control (MS liquid media) were added to the serial dilutions and incubated for 16 h at 26°C. Colony-forming units of fungal and bacterial colonies were counted after 24- and 48-h incubations at 26°C. Root exudates were collected by growing wild-type and *abcg30* plants in MS liquid media for 30 d 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, filtered through nylon filters of 0.45-μm pore size (Millipore; Durapore membrane filters) prior to freeze drying (Labconco). The freeze-dried powder was dissolved in distilled water and filter-sterilized through syringe filters before supplementing into soil serial dilutions.

Statistical Analyses

We used nonparametric multidimensional scaling (NMS) to examine bacterial and fungal communities in the rhizospheric soil samples of Arabidopsis wild-type and ABC transporter mutants. NMS is an interactive best-fit ordination technique that arranges samples so that the distance between soil samples in ordination space is in rank order with their similarities in community structure (Clarke, 1993). The Sorenson distance metric was used as a measure of dissimilarity in all NMS ordinations (Faith et al., 1987; McCune and Grace, 2002). We also used MRPP to test the null hypotheses of no difference in the microbial communities of all soil samples included in this study. MRPP is a nonparametric, multivariate method used to make statistical comparisons among two or more groups (Zimmerman et al., 1985). The *P* value associated with the MRPP test statistic describes how likely an observed difference is between the groups.

Microarray Analyses

We used 20-d-old plants raised in liquid culture as described in this study for total RNA extraction from root tissues of both the wild type and *abcg30* by using Trizol reagent following the manufacturer's instructions. cRNA was prepared following the manufacturer's instructions (www.affymetrix.com/support/technical/manual/expression-manual.affx). The Affymetrix microarrays (Arabidopsis ATH1 genome array) containing 22,810 probe sets representing approximately 80% of the gene sequences on a single array was used in this study. Labeling and hybridization on the ATH1 microarrays (one sample per chip) was performed according to the manufacturer's instructions (www.affymetrix.com/support/technical/manual/expression-manual.affx). The labeling and hybridization analyses were performed at Yale University Microarray facility center (Keck Biotechnology Resources

Laboratory). The probe arrays were scanned and further analyzed with GENESPRING software (version 5.0; Silicon Genetics). Normalization per gene and per chip of the $\log_2$ values was performed to allow the comparison of two independent biological replicates. In addition, normalization was performed separately for each biological replicate using the flags ("present," "marginal," or "absent") assigned by Affymetrix treatment of the arrays. Such a procedure allows eliminating the transcripts with very low signals in both treatments followed by multiple hypotheses testing to generate P value. Data were analyzed for each of the *Atabcg30* replicates with corresponding wild-type replicates. The genes that reveal significant changes ($P = 0.01$) in their expression were considered. Moreover a cutoff value of 1.5-fold change was adopted to discriminate expression of genes that were differentially altered in *Atabcg30* compared with the wild type. Annotation of the genes represented on the microarray to genomic open reading frames was done with "gene description" and "gene ontology" programs of GENESPRING based on the information from the International Arabidopsis Genome Initiative sequencing project in collaboration with The Institute for Genomic Research.

Supplemental Data

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

Supplemental Figure S1. Comparative analysis of relative abundance (peak height) of fungal (A) and bacterial (B) phylotypes significantly differed between wild type (Col-0) and *abcg30*.

Supplemental Figure S2. ^{1}H NMR (A), 2D *J*-resolved (B), and projected 1D *J*-resolved (C) spectra of control Arabidopsis root exudates (Col 0) in the range of δ 6.0 to δ 8.7.

Supplemental Figure S3. Loading plot of PC1 (A) and loading plot of PC2 (B) of principal component analysis of Arabidopsis root exudates using projected one-dimensional J-resolved spectrum.

Supplemental Figure S4. Two-dimensional *J*-resolved spectrum of wild-type root exudates with compound assignments.

Supplemental Figure S5. Two-dimensional *J*-resolved spectrum of wild-type root exudates with compound assignments.

Supplemental Figure S6. Two-dimensional *J*-resolved spectrum of wild-type root exudates with compound assignments.

Supplemental Figure S7. Comparison of one-dimensional projected *J*-resolved spectra of Arabidopsis wild-type and mutant root exudates in the range of δ 8.6 to δ 5.7.

Supplemental Figure S8. Comparison of one-dimensional projected *J*-resolved spectra of Arabidopsis wild-type and mutant root exudates in the range of δ 5.6 to δ 4.3.

Supplemental Figure S9. Comparison of one-dimensional projected *J*-resolved spectra of Arabidopsis wild-type and mutant root exudates in the range of δ 4.3 to δ 3.0.

Supplemental Figure S10. Comparison of one-dimensional projected *J*-resolved spectra of Arabidopsis wild-type and mutant root exudates in the range of δ 3.0 to δ 0.5.

Supplemental Figure S11. Percentages of genes belonging to different functional categories were significantly up-regulated and down-regulated in *abcg30* root tissues compared with wild type (Col-0) by whole-genome expression analyses.

Supplemental Figure S12. Phylogenetic tree showing the evolutionary relationships of 66 taxa of the group α-proteobacteria based on rDNA sequences.

Supplemental Table S1. Multiresponse permutation procedures (MRPP) analysis comparing the microbial community associated with each Arabidopsis ABC transporter mutant.

Supplemental Table S2. Pair-wise comparisons of the wild type (Col-0) from MRPP analysis comparing the microbial community associated with each Arabidopsis ABC transporter mutant.

Supplemental Table S3. Relative NMR intensity of changed metabolites in Col-0 and *abcg30* using projected *J*-resolved spectra (signals are normalized to 1,000 of internal standard [TMSP] at δ 0.0).

Supplemental Table S4. Select list of genes differentially expressed in *abcg30* roots compared with the wild type.

Supplemental Table S5. In vitro analysis of the Arabidopsis wild type (Col-0), *abcg30* root exudates, and MS liquid media (control) effect on Arabidopsis soil microbes by plate counting assay

Supplemental Table S6. List of ABC transporters and their T-DNA knockout mutants used in this study.

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