MYB72-dependent coumarin exudation shapes root microbiome assembly to promote plant health

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Plant roots nurture a tremendous diversity of microbes via exudation of photosynthetically fixed carbon sources. In turn, probiotic members of the root microbiome promote plant growth and protect the host plant against pathogens and pests. In the Arabidopsis thaliana–Pseudomonas simiae WCS417 model system the root-specific transcription factor MYB72 and the MYB72-controlled β-glucosidase BGLU42 emerged as important regulators of beneficial rhizobacteria-induced systemic resistance (ISR) and iron-uptake responses. MYB72 regulates the biosynthesis of iron-mobilizing fluorescent phenolic compounds, after which BGLU42 activity is required for their excretion into the rhizosphere. Metabolite fingerprinting revealed the antimicrobial coumarin scopoletin as a dominant metabolite that is produced in the roots and excreted into the rhizosphere in a MYB72- and BGLU42-dependent manner. Shotgun-metagenome sequencing of root-associated microbiota of Col-0, myb72, and the scopolin biosynthesis mutant f6’h1 showed that scopoletin selectively impacts the assembly of the microbial community in the rhizosphere. We show that scopoletin selectively inhibits the soil-borne fungal pathogens Fusarium oxysporum and Verticillium dahliae, while the growth-promoting and ISR-inducing rhizobacteria P. simiae WCS417 and Pseudomonas caperellum WCS358 are highly tolerant of the antimicrobial effect of scopoletin. Collectively, our results demonstrate a role for coumarins in microbiome assembly and point to a scenario in which plants and probiotic rhizobacteria join forces to trigger MYB72/BGLU42-dependent scopoletin production and scopoletin excretion, resulting in improved niche establishment for the microbial partner and growth and immunity benefits for the host plant.

Significance
Plant roots nurture a large diversity of soil microbes via exudation of chemical compounds into the rhizosphere. In turn, beneficial root microbiota promote plant growth and immunity. The root-specific transcription factor MYB72 has emerged as a central regulator in this process. Here, we show that MYB72 regulates the excretion of the coumarin scopoletin, an iron-mobilizing phenolic compound with selective antimicrobial activity that shapes the root-associated microbial community. Selected soil-borne fungal pathogens appeared to be highly sensitive to the antimicrobial activity of scopoletin, while two MYB72-inducing beneficial rhizobacteria were tolerant. Our results suggest that probiotic root-associated microbes that activate the iron-deficiency response during colonization stimulate MYB72-dependent excretion of scopoletin, thereby potentially improving their niche establishment and enhancing plant growth and protection.

Supporting Information includes additional figures, tables, and references.

Author contributions: I.A.S., K.Y., and K.F. contributed equally to this work.

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Besides being essential for the onset of ISR, MYB72 emerged as an integral part of the plant’s adaptive strategy to iron deficiency. Together with its closest paralog, MYB10, MYB72 is essential for plant survival in alkaline soils where iron availability is largely restricted (17). Under iron-limiting conditions, MYB72 is strongly induced in the roots of Arabidopsis as part of a set of coordinated responses that boost iron mobilization and uptake from the soil environment (collectively referred to as the “strategy I iron-deficiency response”) (18, 19). The core of this response consists of rhizosphere acidification by the activity of the H^+-ATPase AHA2 (20), which increases the concentration of soluble ferric iron (Fe^{3+}). Fe^{3+} is subsequently reduced to ferrous iron (Fe^{2+}) by FERRIC REDUCTION OXIDASE2 (FRO2), after which it is transported from the soil environment into root cells via IRON-REGULATED TRANSPORTER1 (IRT1) (21, 22). The basic helix-loop-helix (bHLH) transcription factor FIT (FER-LIKE IRON DEFICIENCY TRANSCRIPTION FACTOR) is a central regulator of the iron-uptake response (19) and also regulates the expression of MYB72 (25).

During iron deprivation, plant roots exude a whole suite of secondary metabolites into the rhizosphere which aid in the mobilization and uptake of iron. These metabolites include phenolics, organic acids, flavins, and flavonoids (24–26). In Arabidopsis roots, MYB72 is required for the biosynthesis of iron-mobilizing fluorescent phenolic compounds, while BGLU42 is important for their subsequent excretion into the rhizosphere (12). These so-called “coumarins” are synthesized in the phenylpropanoid pathway via FERULOYL-COA 6-HYDROXYLASE1 (F6H11) and are excreted into the rhizosphere by the iron deficiency-regulated ABC transporter PLEIOTROPIC DRUG RESISTANCE9 (PDR9) (24–27). Arabidopsis knockout mutants myb72 and bglu42 are impaired in the excretion of iron-mobilizing coumarins and in the ability to mount rhizobacteria-mediated ISR, suggesting a mechanistic link between the iron-deficiency response and plant immunity (12, 28).

Interplay between plant immunity and adaptive plant responses to nutrient deficiencies is also reported for the phosphate-starvation response (29). Interestingly, phosphate-starved plants release metabolites into the rhizosphere that are also exuded during conditions of iron deficiency (30, 31). Plants responding to changes in their biotic or abiotic environment show changes in root exudation (32, 33), which in turn affect the composition of the root microbiome (1). Likewise, mutations in defense and phosphate-starvation signaling pathways significantly impact the composition of the root-associated microbial community (29, 34, 35). Collectively, the picture is emerging that components of the plant immune system and the plant’s adaptive response to nutrient starvation are intertwined in influencing the assembly of the root-associated microbiome, and vice versa (36). However, the molecular mechanisms and the ecological and evolutionary advantages of this apparent relationship are largely unknown.

Because MYB72 is an essential regulator of (i) the onset of ISR by beneficial members of the root microbiome and (ii) the excretion of iron-mobilizing coumarins into the rhizosphere, we set out to investigate the identity of the MYB72-dependent metabolites that are excreted into the rhizosphere and their impact on the root-associated microbiome. We performed metabolite fingerprinting analysis of root extracts and exudates of wild-type Col-0 and mutant myb72 and bglu42 plants. We identified the coumarin scopoletin as a major metabolite that is produced and excreted into the rhizosphere in a MYB72–BGLU42-dependent manner. Scopoletin possesses antimicrobial activity (37) and was previously linked to disease resistance in different plant species (38–40). Metagenome analysis of microbiota associated with roots of Col-0 plants and roots of the scopoletin biosynthesis mutant f6h1 shows that this antimicrobial and iron-mobilizing coumarin impacts the assembly of the root-associated microbiome. We further show that scopoletin selectively inhibits the growth of two soil-borne fungal pathogens in vitro but has little or no effect on the growth of two beneficial ISR-inducing rhizobacteria. Collectively, our results suggest that the MYB72–BGLU42-mediated production and excretion of scopoletin favors the interaction between plant roots and pro-biotic members of the root microbiome.

**Results**

**Metabolite Fingerprinting of Root Exudates.** In Arabidopsis roots MYB72 and BGLU42 are both required for the onset of rhizobacteria-mediated ISR. Moreover, MYB72 is required for the biosynthesis of iron-mobilizing fluorescent phenolic compounds, while BGLU42 has a role in their subsequent excretion into the rhizosphere (12). Under iron-starvation conditions, Col-0 roots exuded high quantities of fluorescent phenolic compounds (Fig. 1A) and activated the iron-deficiency marker genes FRO2, IRT1, MYB72, and F6H1 (Fig. 1B). The mutant myb72 did not exude detectable levels of the fluorescent compounds, and in bglu42 plants their exudation was significantly impaired, confirming previous findings (12). To identify the metabolites that were synthesized and secreted from Arabidopsis roots in a MYB72- and BGLU42-dependent manner, we analyzed the metabolome of root exudates of wild-type Col-0 and mutant myb72 and bglu42 plants. The three genotypes were grown under iron-sufficient and iron-deficient conditions, and the root exudates were analyzed by metabolite fingerprinting using ultra performance liquid chromatography (UPLC)-electrospray ionization (ESI)-TOF-MS (Dataset S1). Filtering the data resulted in a set of 722 metabolite features with a false-discovery rate (FDR) below 0.001. Principal component analysis (PCA) of the profiles of these 722 metabolite features indicates that the exudates of the different genotypes grown under iron-sufficient conditions are similar (Fig. 1C and Fig. S1). In contrast, the root exudates of iron-starved plants were clearly different from those of iron-sufficient plants. The root exudates of iron-starved myb72 plants clearly separated from those of iron-starved Col-0 and bglu42 plants, indicating that the metabolite profiles of myb72 root exudates are markedly different from those of Col-0 and bglu42 plants.

To obtain a global overview of the metabolite features and their relative abundances (RAs) in the root exudates, the profiles of the selected 722 metabolite features were clustered by means of one-dimensional self-organizing maps (1D-SOMs) (41). 1D-SOMs organized the intensity profiles in 10 clusters (prototypes) (Fig. 1D). Analogous to the PCA plot, the intensity profiles of the metabolite features of Col-0, myb72, and bglu42 plants grown under iron-sufficient conditions are highly similar. For all three genotypes, the iron-starvation treatment changed the metabolome of root exudates, which resulted mainly in increased signal intensities, most strikingly visible in clusters 4–7 and 9–10. Clusters 1–3 and 8 represent features with rather unaffected intensity profiles or with only a weak accumulation in the bglu42 mutant. Cluster 4 represents features that accumulated highly in exudates of Col-0 plants, while clusters 6 and 10 show features mainly enriched in myb72 or bglu42 exudates, respectively. The metabolite features of clusters 5 and 9 show lower intensities for iron-starved myb72 than for iron-starved Col-0 and bglu42. Collectively, these results indicate that MYB72 activity affects the composition of the root exudate metabolite profile under conditions in which the iron-deficiency response is activated.

**Exudation of MYB72- and BGLU42-Dependent Metabolites.** To determine the identity of the MYB72- and BGLU42-dependent metabolites in the root exudates of iron-starved Arabidopsis plants, we generated a data subset of the metabolome data shown in Fig. 1 containing only the metabolite fingerprinting analyses of the root exudates of iron-deprived Col-0, myb72, and bglu42 plants. Filtering the data of this subset resulted in 311 metabolite features with an FDR below 0.001 (Dataset S2).
myb72

in root exudates of iron-starved Col-0 plants, plants. Besides coumarins, the majority matched plants, respectively. Fig. 2 E5215 MYB72 0.001, * plants grown under iron-sufficient and iron-starvation conditions. (PNAS 0.05, Student’s t test. (IRT1 myb72 bglu42 no. 22 SEE COMMENTARY | plants grown in Hoagland medium with (IRT1) μ (encircled in red), and (IRT1) μ (encircled in blue) plants grown in Hoagland medium with (+Fe) or without (−Fe) iron. Data were obtained by metabolite fingerprinting (UPLC-ESI-TOF-MS analysis in positive and negative ionization mode), and the PCA represents the data subset after the filtering. PC1, principal component 1; PC2, principal component 2. (D) 1D-SOM clustering and prototype assignment of 722 high-quality metabolite features (FDR <0.01) derived from the positive as well as the negative ionization mode of the metabolite fingerprinting analysis. The number of features assigned to one prototype determines its width. The raw intensity of individual features (Upper) and prototypes (Lower) mostly affected by iron deficiency are highlighted in the red boxes. Heatmaps correspond to the intensity of each individual feature (Upper) and the average intensity of each cluster/prototype (Lower). The color key shows the range of signal intensities. Three biological replicates per treatment were used for analysis.

The intensity profiles of these 311 features were clustered by means of 1D-SOMs, resulting in five prototypes (Fig. 2A). Metabolite features in clusters 1, 2, and 5 showed clearly lower intensities in myb72 than in Col-0 exudates, indicating that the abundance of the corresponding metabolites increased in Col-0 root exudates in a MYB72-dependent manner. The metabolite features of prototype 1 showed in addition a depletion in root exudates in a MYB72-dependent manner. The metabolite features in clusters 1, 2, and 5 showed clearly lower intensities in myb72 than in Col-0 exudates, indicating that the abundance of the corresponding metabolites increased in Col-0 root exudates in a MYB72-dependent manner. The metabolite features of prototype 1 showed in addition a depletion in bglu42 exudates, suggesting that the corresponding MYB72-dependent metabolites are excreted in a BGLU42-dependent manner.

Among the metabolite features that showed depletion in the root exudates of iron-starved myb72 plants, the majority matched to coumarins by a database search on the basis of exact masses comparison. Coumarins play a role in iron mobilization and uptake from alkaline substrates (24–26). The intensity profiles of the coumarins scopolin, scopoletin, esculin, esculentin, and iso-fraxidin show a strong increase in root exudates of iron-starved Col-0 plants (with scopoletin being the most abundant), while no increase is observed in root exudates of iron-starved myb72 plants (Fig. S2). Most of the features summarized in cluster 1 (Fig. 2A) are related to scopoletin. The diversity of the scopoletin-related features is caused by the intense adduct formation of the highly abundant coumarin scopoletin during ESI. The scopoletin-related features in cluster 1 showed very low signal intensities and inconclusive database matches. High-resolution tandem mass spectrometry (MS²) analysis confirmed the identity of the detected coumarins (Table S1). Of these excreted MYB72-dependent coumarins, only scopoletin levels were reduced in the root exudates of iron-starved bglu42 plants. Quantification of scopoletin and its aglycone scopoletin by HPLC with diode array UV detection (HPLC-DAD) (Fig. 2B) confirmed their patterns observed in the nontargeted metabolite fingerprinting approach (Fig. S2). Scopoletin accumulated to over 3 μg·mL⁻¹ in root exudates of iron-starved Col-0 plants, while it was undetectable or strongly reduced in root exudates of the myb72 and bglu42 plants, respectively. Fig. 2B shows that the glycosylated form of scopoletin, scopolin, also accumulated in a MYB72-dependent manner in the exudates of iron-starved roots, albeit only to about 10% of the concentration of scopoletin (compare the scales of the y axes). However, the bglu42 mutation had no effect on the iron-starvation-induced levels of scopolin. Collectively, these results show that scopoletin is the major MYB72- and BGLU42-dependent metabolite that is secreted by roots of iron-starved Arabidopsis plants. Besides coumarins, the coumarin precursor phenylalanine and the citric acid cycle components citrate, malate, and succinate also increased in abundance in root exudates of iron-starved Col-0 plants (Fig. S2 and Table S1), confirming previous findings (24). However, their excretion was not dependent on MYB72 and BGLU42.
Role of BGLU42 in the Excretion of Scopoletin. BGLU42 encodes a β-glucosidase that belongs to glycoside hydrolase (GH) family 1, and it was previously shown that the GH family 1 members BGLU21, BGLU22 and BGLU23 can hydrolyze scopolin, resulting in the production of scopoletin (42). Glycosylation and deglycosylation can change phenylpropanoid solubility, stability, and toxic potential and can influence their compartmentalization and biological activity (43). While myb72 plants are blocked in the ability to produce fluorescent phenolic compounds in their roots, iron-starved bglu42 roots still accumulate significant amounts (Fig. 2C). Hence, we hypothesized that if BGLU42 activity facilitates the excretion of scopoletin, then iron-starved bglu42 mutant plants should still accumulate the glycosylated form scopolin within their roots. To test this, we analyzed the metabolite profiles of the polar and nonpolar extractions of the roots of Col-0, myb72, and bglu42 plants grown under iron-sufficient and iron-starved conditions by UPLC-ESI-TOF-MS (Datasets S3 and S4). Similar to the observations for the root exudates, the root metabolome showed different metabolite profiles in terms of iron treatment and plant genotype (Figs. S1 and S3 A and B). Metabolite features related to coumarins were abundantly present in the polar extracts of the root, with scopoletin, scopolin, and esculin showing highly abundant signal intensities (Fig. S3C and Table S2). Scopolin, scopolin, and esculin were produced within the roots in a MYB72-dependent manner, and BGLU42 did not negatively impact their abundance in the roots (Fig. 2D and Fig. S3C). Scopolin even increased to higher levels in the roots of iron-starved bglu42 plants than in the roots of Col-0 plants, supporting the notion that BGLU42-mediated deglycosylation of scopolin is impaired in this mutant. As opposed to root exudates of iron-starved Col-0 plants, where scopoletin levels were about 10-fold higher than those of scopolin (Fig. 2B), within the roots of iron-starved Col-0 plants the scopoletin levels were over 25-fold lower than those of scopolin (Fig. 2D). Collectively, these results indicate that roots of iron-starved Col-0 plants produce scopoletin and scopolin in a MYB72-dependent manner and that BGLU42 activity is important for the deglycosylation of scopoletin and the subsequent excretion of the aglycone scopoletin into the rhizosphere (Fig. 2E).

In addition to the polar fraction, we also analyzed the nonpolar fraction of the root extracts (Fig. S3B and Dataset S4).
Metabolite fingerprinting yielded a rich source of metabolite features with either MYB72- or BGLU42-dependent patterns. However, because none were impaired in both the myb72 and the bglu42 mutant background, they are not described further in this study.

Effect of Scopoletin on Rhizosphere Microbiome Assembly. Previously, coumarin derivatives were shown to possess antimicrobial activity (37). Because beneficial ISR-inducing members of the root microbiome induce MYB72 and coumarin biosynthesis genes upon colonization of the roots (12), we hypothesized that coumarins may play a role in shaping the microbial community in the rhizosphere. To investigate the effect of MYB72-dependent coumarins, in particular scopoletin, on the composition of the root-associated microbial community, we analyzed the root-associated microbiomes of Col-0 and myb72 and the scopoletin biosynthesis mutant f6′h1 (26, 44). Arabidopsis seedlings were grown in vitro under iron-sufficient or iron-starved conditions to verify their coumarin-exudation status at the start of the experiment. As expected, iron-starved Col-0 seedlings abundantly exuded fluorescent phenolic compounds, while myb72 and f6′h1 seedlings did not (Fig. 3A). Subsequently, the plants were transplanted into natural Reijerscamp soil (45) that was limed to maintain iron limitation and differential coumarin excretion patterns. At the moment of transplantation, MYB72, FRO2, and IRT1 were strongly induced in the roots of iron-starved Col-0 seedlings (Fig. 3B). Their induced expression leveled off over time but was still detectable at day 3 after transplantation. In this experimental setup, differences in iron availability of the Col-0 seedlings after transplantation to the natural Reijerscamp soil had no major effect on the expression of the root immunity marker gene CYP71A12, the glucosinolate biosynthesis genes CYP79B2 and CYP79F2, and the pathogenesis-related protein gene PR3 (Fig. S4). Hence, potential differences in microbiome composition are not likely the result of differences in basal root defenses. Next, we analyzed the microbiota associated with the roots of iron-starved Col-0, myb72, and f6′h1 plants that were grown in limed soil for 3 d. As a control, we characterized the root microbiota of Col-0 plants that were pregrown under iron-sufficient conditions. Day 3 after transplantation was chosen

Fig. 3. Metagenome analysis of bulk soil and root-associated microbiomes of Col-0, myb72, and f6′h1 plants. (A) Excretion of fluorescent phenolic compounds by 26-d-old Col-0, myb72, and f6′h1 plants grown in Hoagland medium with (+Fe) or without (−Fe) iron. Visualization of fluorescence was achieved under UV light (365 nm). (B) Gene-expression profiles of MYB72 and the iron-deficiency marker genes FRO2 and IRT1 in roots of Col-0 plants pregrown for 14 d in Hoagland medium with (+Fe) or without (−Fe) iron and transplanted on day 0 to limed Reijerscamp soil. Gene expression was quantified by qRT-PCR. Transcript levels were normalized to that of reference gene PP2AA3 (At1g13320). Data are means of three biological replicates. Error bars represent the SEM. Asterisks indicate significant differences between treatments: ****P < 0.0001, **P < 0.01, *P < 0.05, two-way ANOVA, Sidak’s test. (C) PCoA using Bray-Curtis metrics displays the dissimilarity of microbial communities in soil (triangles) and root samples (circles) of Col-0 plants pregrown under iron-sufficient (+Fe) conditions and Col-0, myb72, and f6′h1 plants pregrown under iron-starved conditions (−Fe). (D) Shannon diversity (effective number of species) displaying the within-sample diversity of different treatments. Horizontal bars correspond to the median and interquartile range of values. Different letters indicate significant differences: P < 0.05, one-way ANOVA, Tukey’s test.
because enhanced expression levels of MYB72, FRO2, and IRT1 were still detectable at that time point (Fig. 3B), and phenolics excreted by the roots can reside in the soil for several days (46). To analyze the effect of MYB72 and F6/H1 activity on the composition of the root microbiome, genomic DNA of the root-associated microbiota was subjected to shotgun metagenome sequencing. Per sample, Illumina NextSeq 500 sequencing yielded between 52.4 and 108.7 million paired-end reads with a length of 150 bp (Table S3). For the analysis of the metagenomes, we followed a classification-first approach using Kaiju, a program that estimates the sequence similarity of metagenomic reads with reference prokaryotic and eukaryotic microbial protein databases (47). For the root samples, Kaiju classified 28–32% of the reads at the genus level to Arabidopsis and 21–24% of the reads to microbiota in the reference database (Table S3). For the bulk soil samples, a very small number of reads compared with the total sample reads were classified to Arabidopsis (sample B1: 10.995, 0.03%; sample B2: 3.534, 0.01%; sample B3: 2.802, 0.01%), while 37% matched with microbiota in the reference database. For all samples, 45–60% of the reads could not be classified at the genus level.

Genus-level classification using publicly available taxonomy databases as in ref. 48 allowed us to track shifts in higher taxonomic ranking between the root-associated microbiota and those assembled in the unplanted soil (Fig. S5). Taxonomic classification at the genus level resulted in the assignment of reads to 4,046 genera belonging to the domains of Bacteria, Eukaryota, and Archaea (Dataset S5). Bacteria were the most dominant domain in terms of RA, with Eukaryota being the second and Archaea representing only a small fraction of the microbial communities (Fig. S5A). Bacteria displayed a significant decrease in the root samples of all genotypes compared with bulk soil. In contrast, Eukaryota showed a significant increase in the root samples, while the RA of Archaea remained unaffected in both compartments (Fig. S5A). At the phylum level, we focused on phyla with an RA of over 0.5% (Fig. S5B). Proteobacteria were the most abundant (around 50% RA) with Actinobacteria, Firmicutes, and Bacteroidetes being overrepresented as well with lower RA. The phyla Acidobacteria, Actinobacteria, Bacteroidetes, and Firmicutes showed statistically significant changes in RA between soil and all root/rhizosphere samples. For the phyla Chlorofyta, Mucoromycota, Planctomycetes, and Nanobacteria only a single phylum combination showed significant changes in the RA in root/rhizosphere versus bulk soil samples, while the phyla Verrucomicrobia, Cyanobacteria, Chloroflexi, Basidiomycota, and Ascomycota remained unaffected (Fig. S5B).

To gain further insight into the effect of plant genotype and treatment on root microbial diversity, we performed β- and α-diversity analyses. In the principal coordinate analysis (PCoA) of Bray–Curtis similarities (β-diversity), the microbial diversity of bulk soil clearly separates from those of the different plant genotypes (Fig. 3C), indicating a significant effect of the plant on root microbiome assembly. Along the second principal coordinate, the root-associated microbial communities of iron-sufficient (+Fe) and iron-starved (−Fe) Col-0 plants differentiate (Fig. 3C), suggesting that root exudates produced during iron starvation affected microbiome assembly. The microbial communities of iron-starved Col-0 and myb72 plants both displayed intermediate separation from that of iron-sufficient Col-0 plants, while the microbial community on the scopoletin biosynthesis mutant f6/h1 diverged most distinctly. To pinpoint genotype-mediated differences within the root-associated microbiota, the microbial diversity within each sample (α-diversity) was calculated as Shannon diversity (Fig. 3D). This calculation showed that microbial communities in unplanted bulk soil are significantly more diverse and complex than the root-associated microbial communities and that the Shannon diversity for the f6/h1 root-associated microbiota was significantly lower than that for the other genotypes. The observation that the scopoletin-biosynthesis mutant f6/h1 clearly separates from the other genotypes in both between- and within-samples diversity estimations suggests that F6/H1 activity affects root microbiome assembly.

To dissect the plant genotype-mediated differences in root microbiome structure on lower taxonomic ranking, we performed pairwise comparisons using DESeq2. Pairwise comparison between the root-associated microbiota of iron-starved and iron-sufficient Col-0 plants revealed 21 genera with differential abundance, predominantly from the Proteobacteria and Firmicutes phyla (Fig. S6A). Psychrobacillus, Stenotrophomonas, Paenibacillus, and Dyella were the genera with the highest differential abundance in the root microbiomes of iron-starved Col-0 plants, indicating that these genera grow better in the rhizosphere of iron-starved Col-0 plants than in that of iron-sufficient Col-0 plants. Conversely, Adhaeribacter, Niastella, and Hymenobacter were most highly enriched on roots of iron-sufficient Col-0 plants, indicating that they grow better in the rhizosphere of iron-sufficient Col-0 plants than in that of iron-starved Col-0 plants. Comparison between the root microbiomes of iron-starved Col-0 and myb72 plants yielded only three genera above the limit of statistical significance (Fig. S6B). However, these genera were also detected in the comparison between iron-starved Col-0 and f6/h1 plants, which revealed the largest number (35) of genera with differential abundance (Fig. 4). The 22 genera significantly enriched on Col-0 over f6/h1 roots (growth positively affected by F6/H1/scopoletin activity) represent seven phyla, with Psychrobacillus and Paraclostridium from the Firmicutes phylum and Rhizophagus, a genus of mycorrhizal fungi from the phylum Mucoromycota, being among the most strongly stimulated genera. The 13 genera significantly enriched on f6/h1 over Col-0 roots (growth negatively affected by F6/H1/scopoletin activity) represent four phyla, with Pontibacter, Rufibacter, and Adhaeribacter, all belonging to the Hymenobacteraeaceae family, being among the most strongly affected genera. Interestingly, Adhaeribacter and Hymenobacter were both significantly less abundant on the roots of iron-starved Col-0 plants than on the roots of iron-starved f6/h1 and iron-sufficient Col-0 plants, suggesting that these genera are particularly sensitive to F6/H1-dependent coumarins that are secreted under conditions of iron starvation. Together, these results demonstrate that the F6/H1-dependent root exudation patterns, with the coumarin scopoletin as a major compound, can positively and negatively influence the abundance of specific genera in the root-associated microbiome of Arabidopsis, thereby impacting the assembly of the root microbiome.

Differential Antimicrobial Effect of Scopoletin. MYB72 and BGLU42 are both important for the onset of rhizobacteria-mediated ISR and the excretion of iron-mobilizing coumarins into the rhizosphere (12, 23). Metabotive fingerprinting using UPLC-ESI-TOF-MS confirmed that at 48 h after colonization by P. simiae WCS417, Col-0 roots already showed increased accumulation of the coumarins scopolin, scopoletin, and esculin and the tricarboxylic acid (TCA) cycle intermediates citrate, malate, and succinate (Fig. S4, Fig. S7, and Dataset S6). Production of coumarins in response to colonization of Arabidopsis roots by ISR-inducing P. simiae WCS417 bacteria occurs in a F6/H1-dependent manner (Fig. 5A), which raises the question how and to what extent such plant-beneficial rhizobacteria benefit from the excretion of antimicrobial compounds into the rhizosphere. To assess the effect of coumarin production on root colonization by WCS417, we monitored the growth of WCS417 on roots of iron-starved and iron-sufficient Col-0 plants over a time period of 7 d after the plants were transplanted into limed Reijerscamp soil (Fig. 5B; same experimental setup as used for the microbiome analysis). Within 1 d, the density of WCS417 bacteria had increased by 10-fold in the rhizosphere of Col-0 plants in comparison with the density in unplanted bulk soil. This increase in WCS417 density was maintained for the 7-d period.
Pseudomonas capeferrum

The rhizosphere microbiome is highly diverse, and its interaction with other microbes (51, 52), indicating that scopoletin potentially exerts its effect on multiple determinants of fungal performance. To study the effect of scopoletin in root exudates on F. oxysporum f. sp. raphani hynhal growth, we performed a chemotropism assay developed by Turrà et al. (53). We used root exudates of iron-starved Col-0 and scopoletin biosynthesis-mutant f6/h1 plants, increasing concentrations of scopoletin, and pectin as a positive control. Fig. S8B shows that fungal hyphae from germinating conidia are chemotropically attracted toward pectin, confirming previous findings (53). Conversely, scopoletin deterred the fungal hyphae, which preferentially grew away from this antimicrobial coumarin. Root exudates of iron-starved Col-0 plants, which exude large amounts of scopoletin (Figs. 1 and 2), similarly deterred the fungal hyphae, while root exudates of iron-starved f6/h1 plants significantly attracted the fungal hyphae. Together, these results indicate that scopoletin in root exudates can have a multifaceted negative effect on the performance of scopoletin-sensitive microbes in the rhizosphere.

Discussion

MYB72 and BGLU42 Are Required for the Production and Secretion of Scopoletin. The rhizosphere microbiome is highly diverse, and its interplay with plant roots has a marked influence on plant fitness (2). Specific root microbiome members can trigger ISR through the activity of the root-specific transcription factor MYB72 and the MYB72-regulated β-glucosidase BGLU42 (6, 14). MYB72 and BGLU42 also have a role in the production and excretion of fluorescent phenolic compounds when plants grow under conditions of iron starvation (12). We used this knowledge to identify the metabolites that are produced and excreted by Arabidopsis roots in a MYB72- and BGLU42-dependent manner and investigated their possible role in plant-microbiome interactions. Untargeted UPLC-ESI-TOF-MS metabolomics of root exudates and root extracts revealed coumarins as a major group of compounds whose production relies on MYB72 activity (Fig. 2 and Figs. S2 and S3). Coumarins, such as scopolin, scopoletin, esculin, esculin, and isofraxidin, are produced via the phenylpropanoid pathway (54) and accumulate abundantly in roots and exudates of iron-starved plants (24–26) where they play a role in the mobilization and uptake of iron (26, 27). Mutant bglu42 roots produced wild-type levels of coumarins but were specifically impaired in the excretion of scopoletin, the most abundant coumarin in the root exudates (Fig. 2 and Figs. S2 and S3). However, the glycosylated form of scopoletin, scopolin, accumulated to high levels in iron-starved bglu42 roots, which suggests that BGLU42 functions in the hydrolysis of scopolin and that this activity is required for the excretion of scopoletin into the rhizosphere. This is in line with previous findings in which the Arabidopsis β-glucosidases BGLU21, BGLU22, and BGLU23 were shown to specifically hydrolyze scopolin into scopoletin in vitro (42). β-Glucosidases can hydrolyze glycosidic bonds either between two carbohydrates or between a carbohydrate and a noncarbohydrate (55), often resulting in the release of bioactive derivatives (55, 56). The activity of BGLU42 seems to be specific for scopolin/scopoletin, since other coumarin pairs such as esculin/esculetin were not affected by bglu42 (Figs. S2 and S3). We thus conclude that BGLU42 activity in iron-starved Arabidopsis roots plays an important role in the processing of scopolin of monitoring. No difference was observed in the level of colonization on iron-starved and iron-sufficient Col-0 roots, suggesting that WCS417 is insensitive to possible antimicrobial effects of root exudates that are excreted in the rhizosphere during conditions of iron starvation.

To confirm that P. simiae WCS417 is insensitive to coumarins, we tested the effect of scopoletin on the growth of WCS417 in vitro, using the antibiotic tetracycline as a positive control (Fig. 5C (40)). While tetracycline prevented the growth of WCS417, scopoletin concentrations of up to 2 mM had no effect on the growth of this PGPR. Additionally, we tested the effect of scopoletin on another well-characterized ISR- and MYB72-inducing PGPR, Pseudomonas capeferrum WCS358 (7, 23), WCS358 was also highly insensitive to scopoletin (Fig. 5D). We then reasoned that the excretion of coumarins would aid the rhizobacteria that induce their production in selectively outcompeting other microbes in the rhizosphere that compete for the same niche. To provide proof of concept for this hypothesis, we tested the effect of scopoletin on the growth of two soil-borne pathogens of Arabidopsis, Fusarium oxysporum f. sp. raphani and Verticillium dahliae JR2 (Fig. 5 E and F) (49, 50). Scopoletin concentrations of 500 μM and higher significantly inhibited the growth of both fungi in vitro and inhibited the growth of V. dahliae to the same extent as the fungicide Delvocid. Since we detected scopoletin concentrations of over 3 μg·mL⁻¹ (∼15 μM) in the growth medium of iron-starved Col-0 plants (Fig. 2B), it is likely that in the narrow region of soil directly surrounding the roots much higher concentrations of scopoletin can occur. Together, these results indicate that scopoletin has a differential antimicrobial effect to
Scopoletin Affects Root Microbiome Assembly. Scopoletin possesses antimicrobial activity (37). Hence, its excretion likely influences the composition of the microbial community in the rhizosphere. In our search for scopoletin-mediated effects on root microbiome assembly, shotgun sequencing of the microbial communities in our search for scopoletin-mediated effects on root microbiome assembly, shotgun sequencing of the microbial communities in the rhizosphere. In comparison with the large differences between root-associated and bulk soil microbial communities, the plant genotype-mediated differences in root microbiome composition were more subtle (Fig. 3C), which is in line with previous findings (29, 35, 63). In comparison with the microbiomes assembled on Col-0 roots, those assembled on myb72 roots showed fewer differences than those assembled on f6h1 roots, possibly because in iron-starved soil MYB72 functions redundantly with MYB10 (17). The microbiomes assembled on the roots of Col-0 and f6h1 plants displayed the largest microbial communities that were formed within 3 d after transplantation into the soil. The fact that general concepts of microbiome assembly were already detectable within this short time frame highlights the speed with which root-associated microbial communities are established in response to chemical and structural cues in the rhizosphere.

In comparison with the large differences between root-associated and bulk soil microbial communities, the plant genotype-mediated differences in root microbiome composition were more subtle (Fig. 3C), which is in line with previous findings (29, 35, 63). In comparison with the microbiomes assembled on Col-0 roots, those assembled on myb72 roots showed fewer differences than those assembled on f6h1 roots, possibly because in iron-starved soil MYB72 functions redundantly with MYB10 (17). The microbiomes assembled on the roots of Col-0 and f6h1 plants displayed the largest differences, indicating that excretion of scopoletin affects the composition of the root microbiome. Genus-level analysis showed that the abundance of genera such as 

Stenotrophomonas, Lactobacillus, Psychrobacillus, Elizabethkingia, Chlamydomonas, and Geobacillus was enhanced on scopoletin-producing roots (Fig. 4). Interestingly, these genera were previously found to be able to hydrolyze different coumarins (64-68). Among the most strongly promoted genera on
Differential Antimicrobial Activity of Scopoletin: Selecting Friends from Foes? Exudation of coumarins is a response that is shared by the iron- and the phosphate-starvation response of plants (28, 30, 31). In analogy to our findings with iron uptake-deficient mutants, Castrillo et al. (29) observed that phosphate uptake-deficient and phosphate-hyperaccumulating Arabidopsis genotypes assembled significantly different root-associated microorganisms than wild-type plants. Additionally, amendment of root exudates rich in phenolic compounds into the soil showed more pronounced effects on the microbial community than other classes of compounds (78). Hence, phenolic compounds in root exudates can be potent modulators of root microbiome assembly. This raises the question how ISR-inducing and plant growth-promoting microorganisms in the rhizosphere benefit from the induction of the MYB72- and BGLU42-dependent exudation of scopolin in the rhizosphere. Scopolin and other coumarins play a role in the mobilization and uptake of Fe\textsuperscript{3+} from the soil environment (26, 27) and may thereby contribute to the mutually beneficial growth-promoting effect that such PGPRs have on the plant. Besides its role in the iron-uptake response, scopolitin possesses antimicrobial activities. In Arabidopsis and tobacco, scopolitin has been shown to accumulate around infection sites, where it inhibited the growth of the foliar pathogens B. cinerea, Alternaria alternata, and P. syringae (38, 40, 79). Other studies have shown that coumarins can negatively affect biofilm formation and the virulence of pathogenic bacteria in different systems (80, 81). In our experimental setup, scopolitin had no growth-inhibiting effect on the ISR-inducing PGPR P. simiae WCS417, either in vivo on Arabidopsis roots or in vitro, and mildly reduced growth of ISR-inducer P. cepaerum WCS358 only at high concentrations (Fig. 5). By contrast, scopolitin strongly inhibited the growth of the soil-borne fungal pathogens F. oxysporum and V. dahliae (Fig. 5). We found that, in addition to reducing the growth of these fungi, scopolitin alters the germination of F. oxysporum spores (Fig. S8B) and inhibits pigmentation of F. oxysporum mycelium (Fig. S8A). Such mycelial pigments can have protective activities against environmental stresses (52, 82). Hence, the inhibition of their formation by scopolitin could render this fungus less viable in the rhizosphere.

Collectively, our microbiome and targeted microbial growth analyses show that some soil-borne microbes can be sensitive to the antimicrobial activity of scopolitin, while others are tolerant. Beneficial ISR-inducing rhizobacteria, such as P. simiae WCS417 and P. cepaerum WCS358, induce MYB72 and BGLU42, which leads to the onset of ISR and the excretion of scopolitin in the rhizosphere. Like P. simiae WCS417 (Fig. S4A), the ISR inducers Pseudomonas fluorescens SS101 and Paenibacillus polymyxa BFKC01 (83, 84) also stimulate the production of coumarins upon colonization of Arabidopsis roots. It is tempting to speculate that in these mutualistic plant-microbe interactions, the rhizobacteria benefit because scopolitin outcompetes scopolitin-sensitive microbes in the same root niche, while the plant benefits because the population of plant-beneficial microbes increases while scopolitin-sensitive soil-borne pathogens are suppressed.

Materials and Methods
Cultivation of Plants and Microbes. A detailed description of the growth conditions of wild-type and mutant A. thaliana plants for the metabolite fingerprinting and root microbiome analyses and of the cultivation conditions of P. simiae WCS417, P. cepaerum WCS358, F. oxysporum, and V. dahliae in the different assays used in this study are provided in SI Materials and Methods.

Metabolite Fingerprinting Analysis. The protocol used for the UPLC-Q-TOF-MS metabolite fingerprinting analysis of the root extracts and root exudates is described in detail in SI Materials and Methods.

Root Microbiome Analysis. The experimental setup used to study the root microbiomes and the metagenome analysis pipeline is described in detail in SI Materials and Methods.

Quantification, Visualization, and Determination of Biological Effects of Coumarins. Quantification and visualization of fluorescent coumarins in root extracts and root exudates and assessment of the effect of scopolitin on the performance of P. simiae WCS417, P. cepaerum WCS358, F. oxysporum, and V. dahliae are described in detail in SI Materials and Methods.

All other methods are described in SI Materials and Methods.

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