

Lignin engineering in field-grown poplar trees affects the endosphere bacterial microbiome

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Cinnamoyl-CoA reductase (CCR), an enzyme central to the lignin biosynthetic pathway, represents a promising biotechnological target to reduce lignin levels and to improve the commercial viability of lignocellulosic biomass. However, silencing of the CCR gene results in considerable flux changes of the general and monolignol-specific lignin pathways, ultimately leading to the accumulation of various extractable phenolic compounds in the xylem. Here, we evaluated host genotype-dependent effects of field-grown, CCR-down-regulated poplar trees (*Populus tremula* × *Populus alba*) on the bacterial rhizosphere microbiome and the endosphere microbiome, namely the microbiota present in roots, stems, and leaves. Plant-associated bacteria were isolated from all plant compartments by selective isolation and enrichment techniques with specific phenolic carbon sources (such as ferulic acid) that are up-regulated in CCR-deficient poplar trees. The bacterial microbiomes present in the endosphere were highly responsive to the CCR-deficient poplar genotype with remarkably different metabolic capacities and associated community structures compared with the WT trees. In contrast, the rhizosphere microbiome of CCR-deficient and WT poplar trees featured highly overlapping bacterial community structures and metabolic capacities. We demonstrate the host genotype modulation of the plant microbiome by minute genetic variations in the plant genome. Hence, these interactions need to be taken into consideration to understand the full consequences of plant metabolic pathway engineering and its relation with the environment and the intended genetic improvement.

host genotype modulation | CCR gene silencing | plant-associated bacteria

The plant bacterial microbiome epitomizes the mutualistic coexistence of eukaryotic and prokaryotic life providing a plethora of reciprocal advantages and represents one of the key determinants of plant health and productivity (1, 2). However, the extent to which variation in the plant host genotype influences the associated bacterial microbiota remains virtually unexplored. In contrast, the host genotype-dependent associations that shape the human gut microbiome have been extensively characterized, whereby even variations in single host genes strongly affected the human gut microbiota (3–5). The interactions between a plant and its microbiome are highly complex and dynamic, involving multiple reciprocal signaling mechanisms and an intricate interplay between the bacteria and the plant's innate immune system (6). Therefore, even small changes in the host genome (ecotypes, cultivars, genetically modified genotypes, etc.) may influence the plant microbiome and may even feed back to modulate the behavior and the productivity of the host plant (2, 7–9). Only a few studies have explored the host genotype modulation of bacterial microbiota. Recently, the host genotype-dependent effects of several *Arabidopsis thaliana* ecotypes have been evaluated and have revealed a significant, but weak, host genotype-dependent impact on the selection of the *Arabidopsis* root-inhabiting bacterial communities (10, 11). Furthermore, the crucial importance of the plant immune system (with a main role for salicylic acid) in the successful endophytic colonization and assemblage of a normal root microbiome has been reported (12).

Here, we examine the host genotype-dependent effects of field-grown poplar (*Populus tremula* × *Populus alba*) trees modified in their lignin biosynthesis, on the bacterial rhizosphere and endosphere microbiome, namely the microbiota present in the roots, stems, and leaves. Transgenic poplar trees were produced by silencing of the gene encoding cinnamoyl-CoA reductase (CCR), the first enzyme in the monolignol-specific branch of lignin biosynthesis (13, 14). In this manner, feedstocks with reduced recalcitrance due to decreased amounts of lignin polymers can be generated for the production of end-use products, such as second-generation biofuels (15, 16). CCR-down-regulated poplar trees grown in the greenhouse and in field trials in Belgium (Ghent) and France (Orléans) repeatedly displayed reduced lignin levels (17, 18). Simultaneously, CCR gene silencing in poplar led to the accumulation of various extractable phenolic compounds in the xylem (17). Hence, the carbon sources available for the associated microbiota differed profoundly between WT and CCR-deficient genotypes. Moreover, phenolic-related compounds, such as those accumulating in CCR-deficient trees, have been implicated in the modulation of the rhizosphere microbiome in *Arabidopsis* (19), underlining their potential to affect bacterial communities. Furthermore, perturbations in the lignin biosynthesis via CCR-down-regulation resulted in compositional alterations of the cell wall. Cell wall features play important roles during endophytic colonization that regulate endophytic

Significance

The composition of bacterial populations in the human digestive tract is known to affect our well-being, to influence our ability to overcome diseases, and to be tightly linked with the host genotype. In contrast, the extent to which variation in the plant genotype affects the associated bacteria and, hence, plant health and productivity remains unknown, particularly of field-grown plants. Genetically modified poplars, down-regulated for cinnamoyl-CoA reductase, substantially influence the microbiome of the plant endosphere without perceptible impact on the rhizosphere microbiota. Unraveling the host genotype-dependent plant-microbe associations is crucial to comprehend the effects of engineering the plant metabolic pathway and possibly to exploit the eukaryote-prokaryote associations in phytoremediation applications, sustainable crop production, and the production of secondary metabolites.

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competence (7, 8) and have been reported to serve as assembly (colonization) cues in root microbiota of *Arabidopsis* (10). Therefore, compositional alterations in the cell wall may cause changes in the bacterial colonization of the *CCR*-downregulated trees.

We hypothesized that the poplar-associated bacteria depend on the host genotype and that the differential accumulation of compounds in the xylem of *CCR*-deficient poplar trees influences the metabolic capacities of the bacterial microbiome. *CCR*-deficient poplar trees are, except for the T-DNA construct, isogenic with the WT poplar trees, making them prime candidates to investigate the host genotype impact and the direct causality of the *CCR* gene silencing on the plant-associated bacterial communities.

Results and Discussion

Collection and Processing of Samples. WT and *CCR*-deficient (*CCR*⁻) field-grown poplar (*P. tremula* × *P. alba* cv. ‘717-1-B4’) trees (15) were sampled in October 2011. The sampled poplar trees were part of a field trial planted (May 2009) in a randomized block design (density = 15,000 trees per ha, interplant distance = 0.75 m) (18, 20). The samples were collected from four compartments (number of individual trees sampled: $n_{WT} = 12$ and $n_{CCR} = 12$): rhizosphere soil (strictly defined as soil particles attached to the roots) and root, stem, and leaf compartments defined as plant tissues depleted of soil particles and epiphytic bacteria by sequential washing.

Nonselective and Selective Isolation of Bacterial Cells. First, to compare the total number of bacterial cells, we isolated them from the rhizosphere soil and surface-sterilized plant compartments of WT and *CCR*⁻ poplar trees with a nonselective approach on nutrient medium (Fig. 1A). Bacterial cell counts (cfus per g) were highly comparable between WT and *CCR*⁻ poplars across all compartments, indicating that the *CCR* gene silencing had no major effect on the rhizospheric and endophytic colonizations and on the stable establishment of bacterial communities. Furthermore, within each genotype, the abundance of the bacterial cells (cfus per g) consistently decreased from the rhizosphere soil over the root to the stem, implying a normal colonization pattern of plant-associated bacteria (7). Soil-residing bacteria initially colonize the rhizosphere and rhizoplane, largely driven by chemoattraction to rhizodeposits (carbohydrates, amino acids, root cap border cells, etc.) that are released into the root zone by the host plant (8, 21–23). Following rhizosphere and rhizoplane colonization, only certain soil-borne bacteria can, through passive or active mechanisms, cross physical barriers (such as endodermis and pericycle) to reach the xylem vessels and further colonize the roots, stems, and leaves (8, 23). The bacterial cell count was slightly (not significantly) higher in the leaves than in the stems, most probably attributable to endophytic colonization via the stomata (Fig. 1A) (23, 24).

To link the influence of the *CCR* gene silencing and resulting changes in the poplar xylem composition with modifications in the metabolic capacities of the bacterial communities, we selectively isolated bacterial cells from all four compartments (WT and *CCR*⁻ trees) by using specific phenylpropanoids (ferulic acid, sinapic acid, and *p*-coumaric acid) as sole carbon sources in the nutrient medium (Fig. 1B–D). Ferulic and sinapic acids, and derivatives thereof, had previously been shown to be up-regulated in the *CCR*⁻ poplar (17) and ferulic acid to be even incorporated into the lignin polymer (17, 18, 25). In contrast to the nonselective approach, the bacterial cell counts were higher in *CCR*⁻ poplar than those in the WT. Differences in bacterial cell counts between genotypes were exclusively found inside the plant (root, stem, and leaf). In the rhizosphere soil, pairwise comparison of bacterial cell counts between genotypes revealed highly comparable bacterial abundances across the various

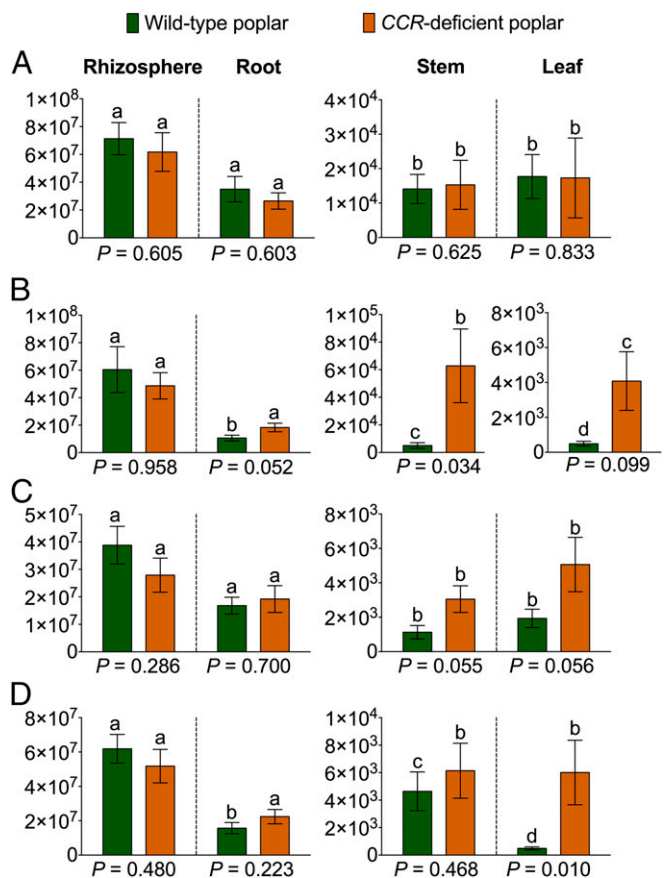


Fig. 1. Bacterial cell counts (abundance) expressed as cfus per gram of soil (rhizosphere soil) or per gram of fresh plant tissue (root, stem, and leaf). (A) Nonselective isolation of bacterial cells from different compartments for both genotypes. (B–D) Selective isolation of bacterial cells with ferulic acid (B), sinapic acid (C), and *p*-coumaric acid (D) as sole carbon source. cfus are averages of at least eight replicates ± SE. Significant differences ($P < 0.05$) between the different plant compartments within each genotype are indicated with lowercase letters. P values of pairwise comparisons between WT and *CCR*⁻ poplars within each compartment are given below each graph.

phenolic carbon sources, indicating that the host genotype has a profound effect on the metabolic capacities present in the endophytic communities. The capacity to degrade specific phenolic carbon sources was clearly enhanced in the endosphere of the *CCR*⁻ poplar. Interestingly, the most pronounced differences occurred with ferulic acid as sole carbon source, of which increased levels had previously been identified in *CCR*-deficient genotypes (17).

Selective Enrichment of Bacterial Cells: Cell Counts. Further, we selectively enriched bacterial cells (ferulic acid as carbon source, 36 d of incubation) from the rhizosphere soil and surface-sterilized plant tissues of WT and *CCR*⁻ poplar. We determined bacterial cell counts (cfus per g) (Fig. 2A) as well as the bacterial community structures (16S rRNA Sanger sequencing) (Fig. S1). In accordance with the selective isolation from the rhizosphere soil, highly similar numbers of bacterial cells (cfus per g ± SD) were obtained from the *CCR*⁻ poplars ($8.33 \times 10^7 \pm 1.44 \times 10^7$) compared with the WT ($9.16 \times 10^7 \pm 1.93 \times 10^7$) ($P = 0.613$), but bacterial abundances inside roots ($P = 0.0011$), stems ($P < 0.001$), and leaves ($P < 0.001$) of *CCR*⁻ trees were consistently higher (Fig. 2A). Again, this result indicates that *CCR* gene silencing and the ensuing changes in xylem composition drive the

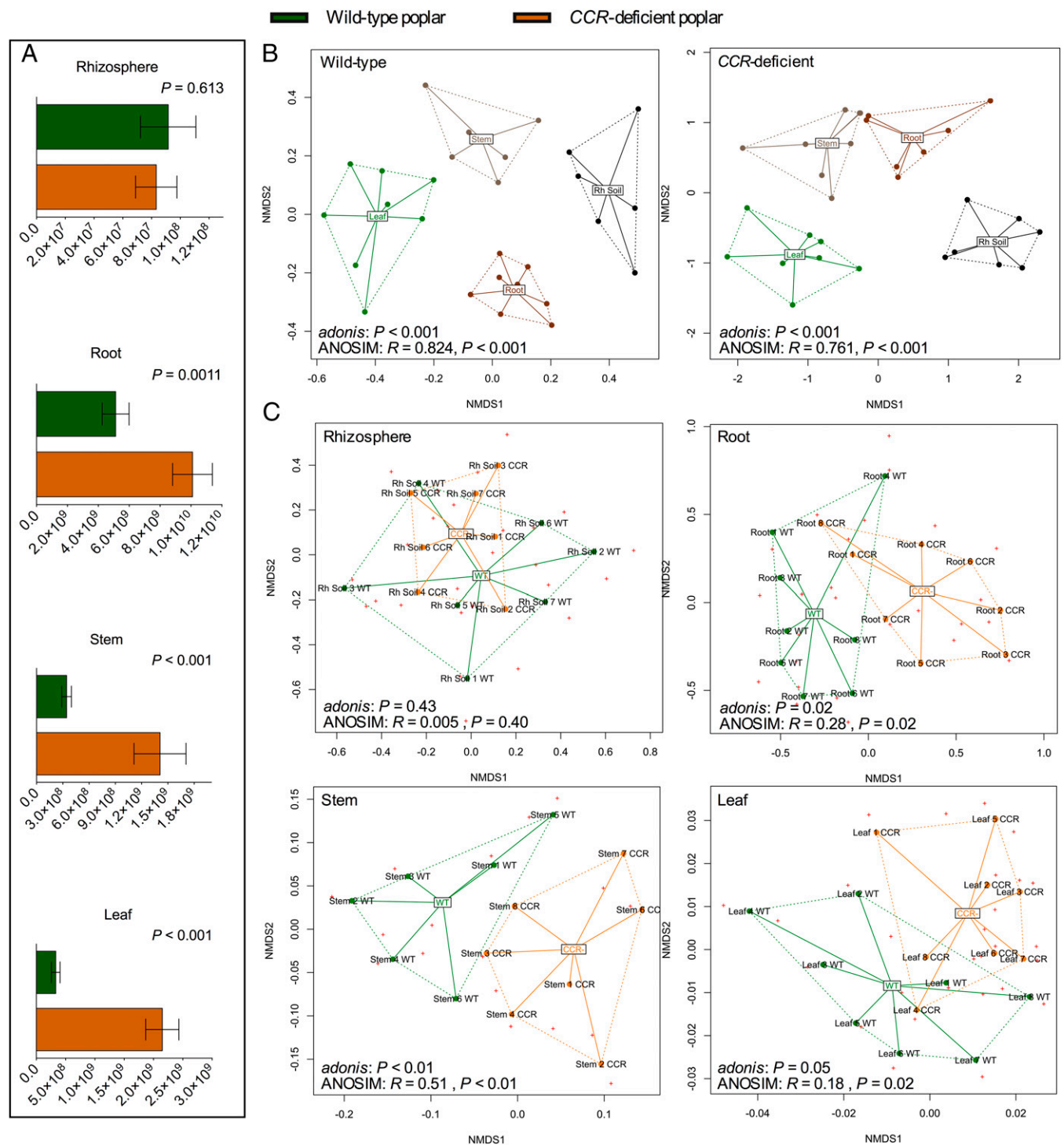


Fig. 2. Selective enrichment of bacterial cells from each compartment and genotype with ferulic acid as sole carbon source. (A) Bacterial cell counts expressed as cfu per gram of soil (rhizosphere soil) or per gram of fresh plant tissue (root, stem, and leaf). *P* values of pairwise comparisons between WT and CCR⁻ poplars within each plant compartment are indicated on the graphs. (B) NMDS with Bray–Curtis distances of square-root-transformed OTU abundance data of bacterial communities isolated from each plant compartment within both genotypes. (C) NMDS analyses of pairwise comparisons between WT and CCR⁻ poplar trees within each compartment (rhizosphere soil, root, stem, and leaf). NMDS analyses contain at least six replicates per plant compartment. Statistical support for the NMDS clustering is provided by the permutation-based hypothesis test analysis of similarities (ANOSIM) and permutational multivariate analysis of variance (*adonis*). Results from both hypothesis tests are indicated below each graph.

metabolic abilities present in the endosphere toward an increased degradation potential for specific phenolics. Further, we observed considerably more variation in the bacterial cell counts from the CCR⁻ trees than in those from the WT (Fig. 2A). This

discrepancy can be attributed to variation among the genotypes in the levels of gene silencing that are reflected by different intensities of the red-brown xylem coloration associated with CCR down-regulation and the related variation in the xylem

composition (17, 18), thereby potentially influencing the plant-associated bacterial community.

Selective Enrichment of Bacterial Cells: Species Composition. To compare species composition within the different sampled compartments and genotypes, we grouped all isolated bacterial strains based on their morphology to create provisional operational taxonomic units (OTUs) that were validated with full-length 16S-rRNA Sanger sequencing and we determined abundance data (cell counts) for each bacterial OTU. The OTU abundance data were square-root-transformed and similarities were displayed with nonmetric multidimensional scaling (NMDS) with Bray–Curtis distances (26, 27) (*SI Materials and Methods*). For both genotypes, NMDS analyses revealed strong clustering of the bacterial communities according to the different compartments (rhizosphere soil, root, stem, and leaf) (Fig. 2B). To statistically support the visual clustering of the bacterial communities in the NMDS analyses, we compared the different compartments by means of permutation-based hypothesis tests: analysis of similarities (ANOSIM), an analog of univariate ANOVA, and permutational multivariate analysis of variance (*adonis*) (*SI Materials and Methods*). All compartments rendered microbiota significantly dissimilar from each other ($P < 0.001$) (Fig. 2B and Fig. S2). Each plant compartment represents a unique ecological niche with specific available nutrients (10), an active systemic colonization originating from the rhizosphere, and a resulting endophytic competence limited to specific strains (7). Microbiome niche differentiation at the rhizosphere soil–root interface has been reported previously (10, 28–30).

To determine the influence of the host genotype on the bacterial communities, we compared the genotypes pairwise within each compartment by means of NMDS with Bray–Curtis distances (Fig. 2C). In the rhizosphere soil, bacterial communities showed no relevant clustering according to the genotype as visually apparent by the NMDS analysis (Fig. 2C). Both ANOSIM and *adonis* statistically confirmed that the bacterial communities of both genotypes were highly comparable within the rhizosphere (ANOSIM: $R < 0.01$, $P = 0.40$; *adonis*: $P = 0.43$) (Fig. S3A). These data are in agreement with the report in which transgenic poplar lines modified in lignin biosynthesis normally formed ectomycorrhiza (EM) and in which variations in the EM community structure between the different transgenic poplar lines were similar to variations in commercial poplar hybrids (31). Furthermore, colonization by arbuscular mycorrhiza or other fungi was not altered in barley (*Hordeum vulgare*) mutants at a lignin production-influencing locus *rob1* (32). However, in contrast to the rhizosphere soil, the poplar genotypes differed significantly in the endosphere. Moreover, these differences were highly consistent throughout the various plant compartments. NMDS analyses and permutation-based hypothesis tests of the bacterial communities in the roots, stems, and leaves revealed strong clustering according to the genotype (Fig. 2C and Fig. S3B–D). The clustering according to genotype was the most pronounced in the stems, where no visual overlap between the bacterial communities was seen (ANOSIM: $R = 0.51$, $P < 0.01$; *adonis*: $P < 0.01$) (Fig. S3C). Because the lignification process is the highest in the stem xylem (13), the most distinct effects of *CCR* gene silencing and changes in xylem composition are expected to occur in the stems (17). Furthermore, lumen colonization of xylem vessels by bacterial endophytes has been reported as a route for bacterial dispersal to vegetative plant parts, ensuring direct contact between endophytes and nutrients available in xylem cells (7, 24). In roots and leaves, the clustering according to genotype persisted with small visual overlaps between WT and *CCR*[−] poplar trees. Pairwise statistical analyses of the genotypes in roots and leaves confirmed the significance of the visually observed differences with NMDS at the 95% significance level (Fig. S3B and D).

Selective Enrichment of Bacterial Cells: Univariate Ecological Measures and Species–Genotype Association Analysis. Furthermore, we calculated OTU richness (Margaleff), evenness (Shannon–Wiener), and diversity (inverse Simpson) within each compartment and genotype based on OTU abundance (Table S1). For all ecological indices, the average values were highly similar between WT and *CCR*[−] poplar trees in the rhizosphere soil. However, in the endosphere, OTU richness, evenness, and diversity were consistently lower in *CCR*[−] trees than those in the WT, except for the OTU richness in the leaves. High variation in the indices reduced the significance of the results, but borderline significant differences were found for the OTU evenness in roots ($P = 0.095$) and stems ($P = 0.075$). Pairwise comparison of the total endophytic evenness (calculated as the average of roots, stems, and leaves) between WT and *CCR*[−] poplar trees revealed a significant decrease in the total endophytic evenness ($P = 0.023$) of the *CCR*-deficient trees.

To ascertain the bacterial species responsible for the observed community differentiation in the endosphere of poplar, we used a species–genotype association analysis. Associations were calculated with the Dufrene–Legendre indicator species analysis routine (Indval, indicator value) in R (Table S2) (27). We identified a significant association in roots ($R = 0.85$, $P < 0.01$) and stems ($R = 0.98$, $P < 0.01$) of the *CCR*[−] trees for *Pseudomonas putida*, which is known for its diverse metabolic capacities and adaptation to various ecological niches, including the ability to thrive in soils and sediments with high concentrations of toxic metals and complex organic contaminants (33). *P. putida* strains are routinely found as plant growth-promoting rhizospheric and endophytic bacteria (34, 35) and several degradation pathways have been elucidated in *P. putida* for lignin-derived low-molecular-weight compounds (such as the ferulate catabolic pathway) (36). The relative abundance of *P. putida* in roots and stems was 26.3% and 75.9%, respectively (Fig. S1B and C). The selective pressure exerted by the host genotype, specifically changes in the accessible carbon and energy sources (up to 2.8-fold increase in soluble phenolics) in the xylem as a consequence of *CCR* gene silencing (17) yielded endophytic bacteria more adapted to degrade complex phenolic compounds, such as *P. putida*.

Metabolic Analyses: Ferulic Acid Degradation Capacity of Isolated Bacterial Strains. Finally, to quantify the ferulic acid degradation capacity of each isolated bacterial OTU and thereby evaluate the effects of the *CCR* gene silencing on the individual bacterial metabolisms, we evaluated the bacterial strains of all identified OTUs respirometrically with Biolog MT2 plates (in triplicate) (Fig. 3A). Interestingly, the results of the respirometric analysis supported the results at the population level. In the rhizosphere soil, the average ferulic acid degradation capacity of all bacterial OTUs from WT and *CCR*[−] poplars was highly similar ($P = 0.560$). In contrast, bacterial metabolisms within the endophytic communities were significantly dissimilar between both genotypes. The respirometric response was significantly higher in bacterial endophytes isolated from *CCR*[−] poplars in the roots ($P < 0.001$), stems ($P < 0.01$), and leaves ($P < 0.05$) than that from the WT, indicating a higher ferulic acid degradation capacity of the bacterial endophytes present in the *CCR*[−] trees.

To examine whether the significant differences observed in the average respirometric responses were attributable to a limited number of bacterial strains, we also assessed the metabolism evenness (Shannon) of the bacterial OTUs within each compartment and genotype (Fig. 3C and Fig. S4). For all compartments, the evenness values were highly comparable, except in the stem, where the community metabolic evenness of the *CCR*[−] poplar was significantly lower than that of the WT ($P < 0.05$), indicating that the higher respirometric response in *CCR*[−] was limited to specific strains. Further, we evaluated the individual respirometric response of each identified OTU (Fig. 3B and Fig. S5) within each compartment and genotype, to relate the OTU

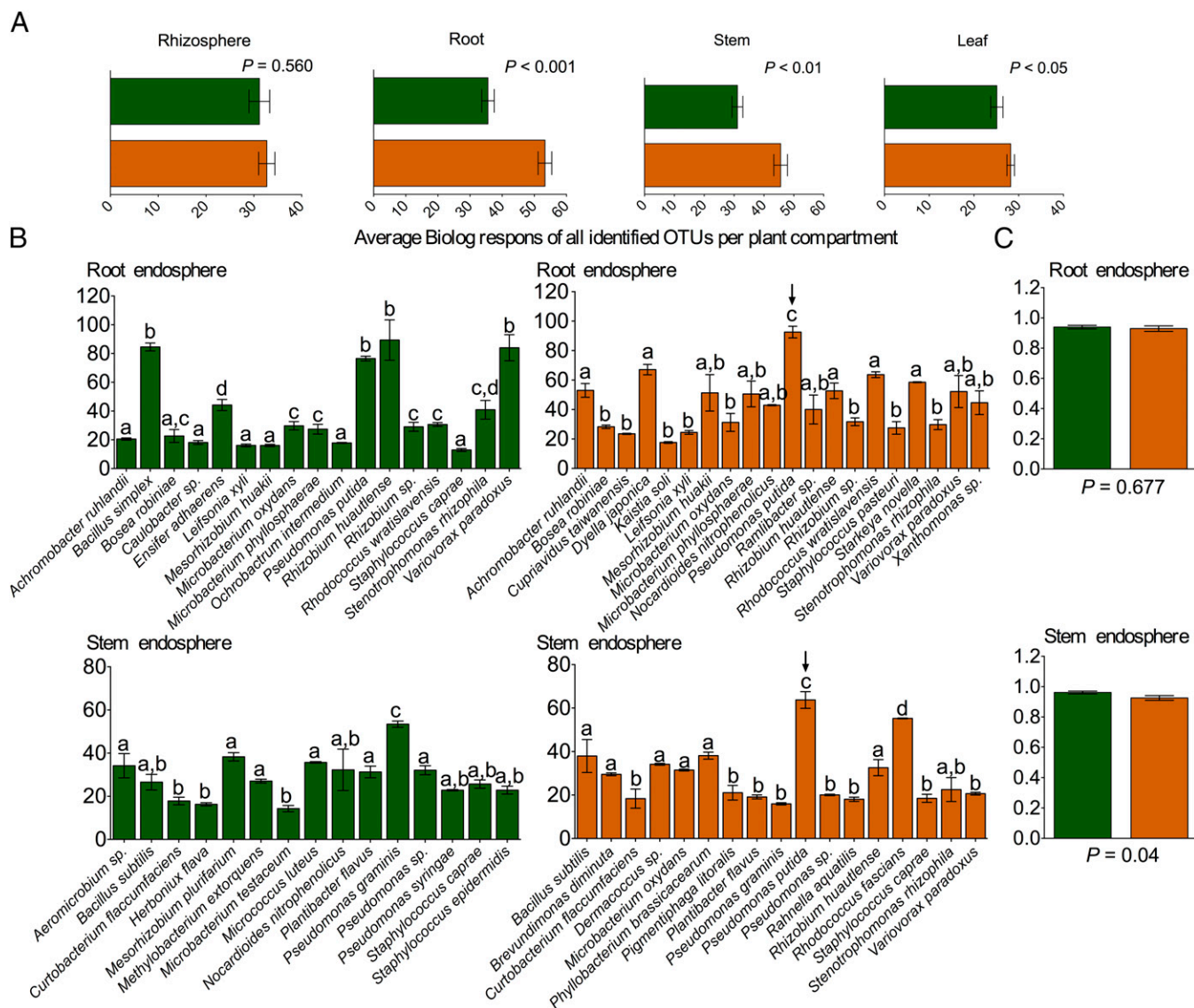


Fig. 3. Respirometric metabolomics analyses by Biolog with 1 mM ferulic acid. (A) Average respirometric responses of all bacterial strains isolated from the different plant compartments per genotype. P values of pairwise comparisons between WT and CCR^- poplars within each plant compartment are indicated on the graphs. (B) Species-level OTU breakdown of respirometric responses for the root and stem compartments. Significant differences in variance of parameters at the 95% significance level are indicated with lowercase letters ($P < 0.05$). Arrows indicate highest responses in the CCR^- poplar trees of *P. putida*. (C) Metabolic evenness of respirometric responses in the roots and stems of WT and CCR^- poplar trees.

abundance to the respirometric response to ferulic acid. *P. putida* from roots and stems of the CCR^- poplar clearly possessed the highest degradation capacity for ferulic acid. Therefore, the high relative OTU abundance of *P. putida* in roots (26.3%) and stems (75.9%) (Fig. S1 B and C) of the CCR^- trees can be attributed to their efficient ferulic acid degradation (Fig. 3B), whereas in the WT trees no such relation was observed.

Conclusion

Host genotype effects that are reminiscent of the host genotype-dependent associations that shape the human microbiome (4, 5) have been identified in plants. The host genotype had a profound effect on the metabolic capacities and bacterial species in the endosphere of CCR^- poplar trees, without perceptible effects on the bacterial communities of the rhizosphere. Our data show that the effects of CCR down-regulation go beyond those that could have been expected from perturbation of the lignin biosynthesis as described in textbooks. Indeed, compounds

that accumulate because of perturbations in the CCR gene expression are apparently further metabolized by the endophytic community. These new metabolites may interfere with the intended phenotype caused by the perturbation. Clearly, these interactions need to be taken into account when engineering the plant metabolic pathways and to understand their relation to the environment. For this study, we chose a targeted methodology to isolate cultivable bacteria that predominantly focused on the differential accumulation of phenolic carbon sources in the WT and CCR^- poplar trees. Implementing high-resolution 16S rDNA sequencing technologies could further elucidate the host genotype-dependent modulation of the plant microbiome by CCR -down-regulation and possibly contribute to the exploitation of the eukaryote–prokaryote associations.

Materials and Methods

A full description of the materials and methods is provided in *SI Materials and Methods*.

Field Trial. The study site was a field trial established in May 2009 by the VIB with 120 CCR-deficient clonally replicated trees of two independent transgenic lines and 120 WT poplar trees (*P. tremula* × *P. alba*). Both CCR⁻ and WT poplar trees were simultaneously micropropagated in vitro, acclimatized in a greenhouse, and 6-mo-old greenhouse-grown poplars (2 m high) were pruned and transferred to the field 10 d later (18, 20).

Collection of Samples. Samples from four compartments (rhizosphere soil, root, stem, and leaf) were collected from the poplar trees in October 2011 ($n_{WT} = 12$, $n_{CCR^-} = 12$). Samples were spread, as much as possible, between different randomized blocks, taking into account tree health, general appearance, and CCR down-regulation level visualized by the red wood coloration. All sampled trees corresponded with redness class 5 (18).

Processing of Samples. Plant tissues were depleted of soil particles and epiphytic bacteria by surface sterilization with 70% (vol/vol) ethanol and 0.1% (vol/vol) NaClO. Bacterial cells were isolated from all four compartments via (i) direct nonselective and selective isolation techniques with a standard carbon source mix and specific phenolic compounds (ferulic acid, sinapic acid, and *p*-coumaric acid) as sole carbon sources and (ii) a selective enrichment technique with ferulic acid.

Identification of Bacterial Strains. Genomic DNA was extracted from all purified morphologically different bacterial strains in triplicate with the DNeasy Blood and Tissue kit (Qiagen). PCR amplification of bacterial small-subunit ribosomal RNA genes (16S) was done with the 26F/1392R (*Escherichia coli* numbering) primer pair. All sequences were queried against Greengenes (37) and the National Center for Biotechnology Information (NCBI BLAST).

Metabolic Analyses. To quantify the ferulic acid degradation capacity, all isolated bacterial strains were semiquantitatively evaluated with MT2 plates (Biolog).

Statistical Analyses. Statistical analysis of the multivariate ecological species data included (i) robust unconstrained ordination (NMDS), (ii) rigorous statistical testing of the hypothesis (ANOSIM and *adonis*), and (iii) indicator species analysis (Dufrene–Legendre). Statistical analyses were performed with packages and scripts developed in R (27).

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