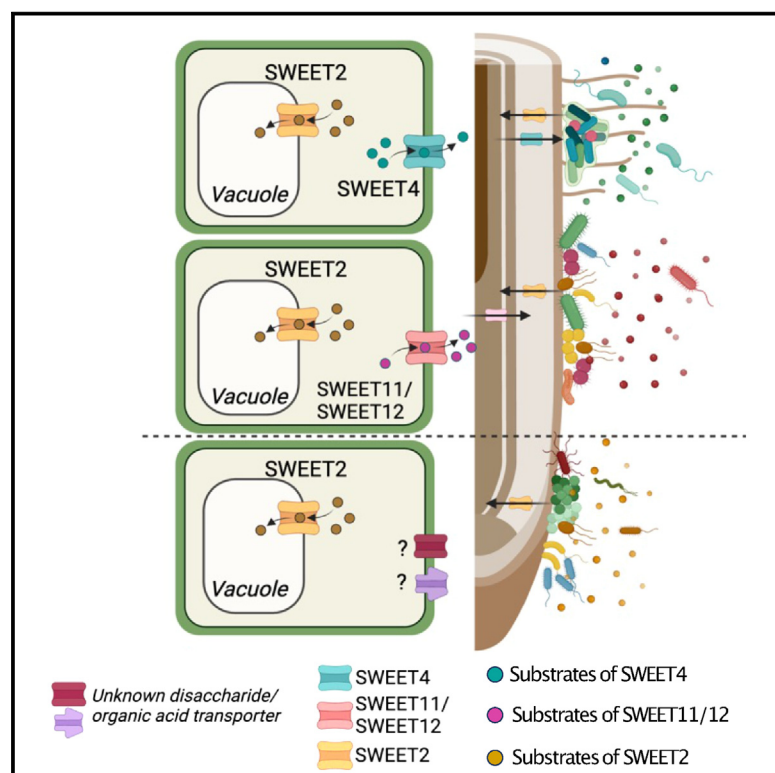


# Cell Host & Microbe

## Sugar transporters spatially organize microbiota colonization along the longitudinal root axis of *Arabidopsis*

### Graphical abstract



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### In brief

Loo et al. develop two growth systems to define the spatial organization of microbiota and metabolites along the longitudinal axis of the *Arabidopsis thaliana* root. SWEET sugar transporters contribute to the distribution of sugar (and other metabolites) along the root, which is required for spatial colonization of root bacteria.

### Highlights

- Root microbiota, metabolites, and SWEET uniporters are patterned along the root axis
- SWEET sugar uniporter organization correlates with sugar metabolism pathways in the root
- Root metabolites and microbiota are spatially reorganized in *sweet*-deficient plants
- SWEET2, -4, and -11;12 play a functional role in spatial colonization of microbiota



## Article

# Sugar transporters spatially organize microbiota colonization along the longitudinal root axis of *Arabidopsis*

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## SUMMARY

Plant roots are functionally heterogeneous in cellular architecture, transcriptome profile, metabolic state, and microbial immunity. We hypothesized that axial differentiation may also impact spatial colonization by root microbiota along the root axis. We developed two growth systems, ArtSoil and CD-Rhizotron, to grow and then dissect *Arabidopsis thaliana* roots into three segments. We demonstrate that distinct endospheric and rhizosphere bacterial communities colonize the segments, supporting the hypothesis of microbiota differentiation along the axis. Root metabolite profiling of each segment reveals differential metabolite enrichment and specificity. Bioinformatic analyses and GUS histochemistry indicate microbe-induced accumulation of SWEET2, 4, and 12 sugar uniporters. Profiling of root segments from *sweet* mutants shows altered spatial metabolic profiles and reorganization of endospheric root microbiota. This work reveals the interdependency between root metabolites and microbial colonization and the contribution of SWEETs to spatial diversity and stability of microbial ecosystem.

## INTRODUCTION

In human and other model animal systems, the gut microbiota is differentially distributed along both the longitudinal and transverse axes due to physiological variations, including nutrient and chemical gradients, intestinal architecture, and host immunity.<sup>1–3</sup> The midgut of *Drosophila* consists of subregions, where cells in subregions perform specific functions and have different immune responses.<sup>4</sup> Accordingly, the host senses and balances the populations of harmful and beneficial microbes across the different regions in the gut.<sup>5</sup>

Similar to the guts of animals, plants have evolved specialized organs, i.e., roots, for nutrient acquisition.<sup>6,7</sup> Roots also harbor

specific bacterial communities that extend the host's metabolic repertoire.<sup>8</sup> The core principles of microbiota establishment within the gut and plant roots are similarly driven by nutrition, host genotype, immune system, and microbe-microbe interactions.<sup>6,9,10</sup> Since the root shows functional heterogeneity at the levels of cell differentiation, transcriptome, metabolic states, and immune responses,<sup>11–16</sup> it is likely that the variability of these factors in the root gives rise to regionalization of the root microbiota. However, current literature lacks information<sup>17,18</sup> on spatial differentiation and its drivers, which could help to better understand the colonization strategies of root-associated microbes.

Plant roots are responsible for water and nutrient uptake from the soil, and roots release up to 20% of the photosynthetically



fixed carbon into the soil via rhizodeposition through processes such as root exudation.<sup>19,20</sup> Since the late 1950s, researchers have reported active exudation sites in the root and shown that different compounds are released from different parts of the root.<sup>21</sup> Recent metabolite mapping of root cells showed differences in levels and localization of metabolites in different cell types.<sup>15</sup> A recent metabolite atlas for *Medicago* root tissues revealed a differential accumulation of metabolites between root cell types.<sup>22</sup> Extensive studies have been performed to compare metabolic changes within the root as a result of biotic and/or abiotic stimuli that alter the composition of root exudates. It is well established that the metabolite composition of the root exudate affects root microbiota.<sup>23–25</sup> However, it remains to be investigated whether and how the spatial distribution of metabolites in the root affects spatial colonization by root microbiota.

Among the numerous components exuded from roots, the three most abundant classes of organic substances with low molecular weight are sugars, carboxylic acids, and amino acids.<sup>19</sup> It remains unclear why plants invest high amounts of photosynthetically fixed carbon into root exudates. The rates of monosaccharide uptake by microorganisms range from seconds to minutes,<sup>26</sup> rendering sugar uptake by soil microbes a strong sugar sink in the soil. Hence, ecological theories pose that root exudation is a mechanism plants acquired for survival advantages via the recruitment of beneficial microorganisms for the provision of other essential nutrients or for strengthening immune responses.<sup>27</sup>

SWEETs are sugar uniporters found in many pro- and most eukaryotes.<sup>28</sup> SWEETs transport monosaccharides and/or disaccharides along the substrate concentration gradient. Most diploid plant genomes contain about 20 SWEET genes.<sup>29</sup> SWEETs play important roles across multiple plant species in various physiological processes, including phloem loading and unloading, seed filling, pollen nutrition, and nectar secretion. SWEETs have also been implicated in several symbiotic interactions, including those of arbuscular mycorrhizal fungi with potato, soybean, and *Medicago*, and between nitrogen-fixing rhizobia and lotus.<sup>30–33</sup> In *Arabidopsis thaliana* (*A. thaliana*), there are 17 SWEET uniporter homologs, classified as AtSWEET1 to AtSWEET17 into 4 clades, whereby clade I members (SWEET1–SWEET3) mainly transport hexoses, clade II transporters (SWEET4–SWEET8) preferentially transport glucose, clade III members (SWEET9–SWEET15) mainly utilize sucrose as a substrate, and members of the clade IV (SWEET16 and SWEET17) mediate fructose transport.

There is evidence for the manipulation of SWEET transporter transcript abundance by pathogens and an impact of SWEET activities on virulence.<sup>28</sup> For instance, *sweet2* knock-out lines showed increased susceptibility to a pathogenic oomycete, intimating a role for AtSWEET2 in sequestering glucose into the vacuole to limit pathogen feeding.<sup>34</sup> Similarly, in rice, cassava, and cotton, *Xanthomonas* species hijack SWEET transporter activity, which is crucial for the growth and proliferation of the pathogens, since the inability to induce SWEETs results in disease resistance.<sup>35–38</sup> Root-secreted sucrose triggers the synthesis of levans in the soil by the bacterium *Bacillus subtilis*, which in turn promotes solid-surface motility and root colonization.<sup>39</sup> Collectively, these studies intimate that microbes have built-in mechanisms for manipulating SWEETs for sugar efflux to facilitate colo-

nization and growth. Beyond plant-microbe mono-association studies,<sup>34</sup> SWEETs had not been demonstrated to play a role in plant-root microbiota interactions, i.e., a possibility for SWEETs to serve as a directory for root microbes to facilitate their proliferation along the root axis.

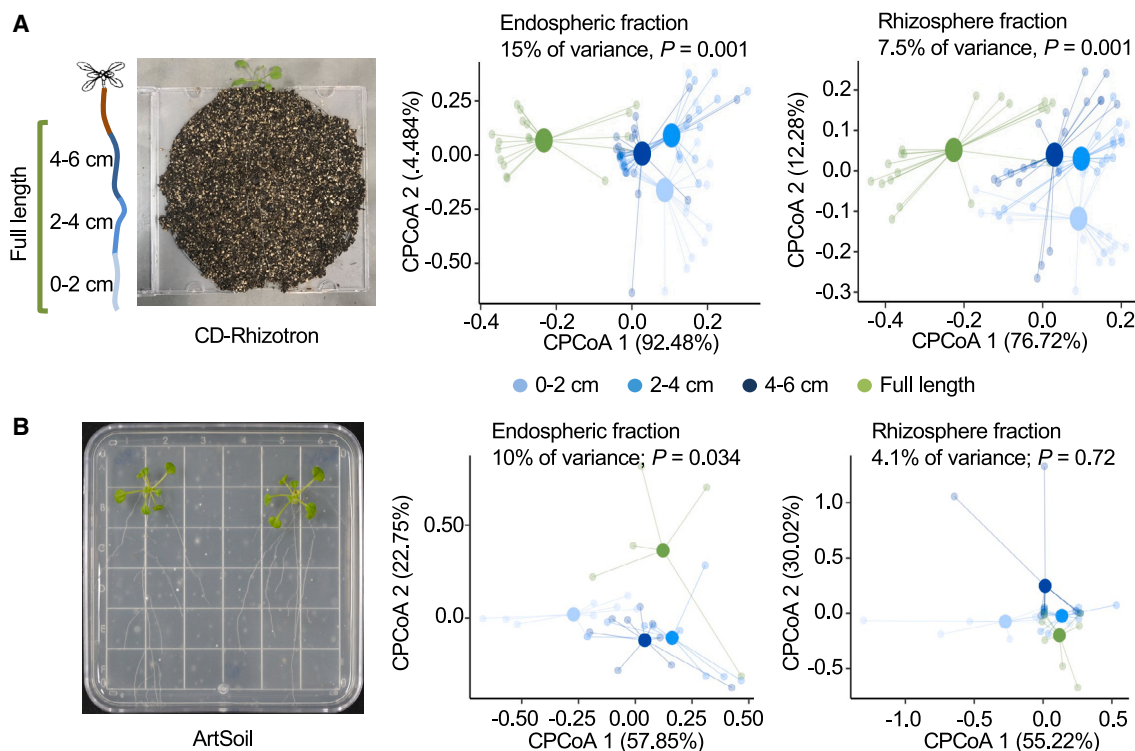
Here, we developed two plant growth systems to enable the dissection of roots into 2-cm segments for spatial microbiota and metabolite profiling. Amplicon sequencing revealed distinctive community structures along the length of the root that correlated with differences in metabolic profiles of the adjacent root segments. Since sugars play a more prominent role compared with organic acids in bacterial community assembly,<sup>40,41</sup> we used public transcriptome repositories and bioinformatics to examine potential spatial differentiation of candidate sugar efflux transporter (SWEET) accumulation along the longitudinal root axis. *In silico* and histochemical analyses supported spatial divergence of accumulation of SWEET2, SWEET4, and SWEET12 sugar transporters along the root in a root microbe-dependent manner. Microbiota and metabolite profiling along the root segments of *sweet2*, *sweet4*, and *sweet11;12* knock-out lines provided evidence for the loss of spatial organization of both root microbiota and various metabolites. This work illustrates the involvement of SWEETs in maintaining the spatial distribution of the root microbial communities.

## RESULTS

### Development of CD-Rhizotron and artificial soil (ArtSoil) for testing differential microbiota colonization along the longitudinal root axis

We hypothesized that spatial differentiation of the root transcriptome<sup>11</sup> could lead to differential local bacterial colonization. To test this hypothesis, a growth system based on CD cases, which we named CD-Rhizotron, was developed to enable the dissection of individual root strands of plants grown in soil into segments (Figures 1A and S1A–S1E). To evaluate the microbiota community of roots grown in the CD-Rhizotron, full-length roots of *A. thaliana* grown in the CD-Rhizotron were compared with data from soil-grown plants that had previously been reported.<sup>42,43</sup> Alpha and beta-diversities of the root microbiota were consistent with the root microbiota community observed in soil-grown plants (Figures S1F and S1G), validating the reliability of the CD-Rhizotron system to simulate soil growth conditions. Distinct clustering of 16S rRNA amplicon samples along the x-axis of the constrained principal coordinates analysis (CPCoA) using Bray-Curtis dissimilarities indicated that rhizospheric and endospheric root communities derived from the full-length root were significantly different from bulk soil, i.e., soil without plant growth. Further, decreased microbial alpha diversity was observed between the soil and the root endophytic compartments (Kruskal-Wallis, Dunn's test post hoc,  $p = 0.001$ ; Figures S1F and S1G). The relative microbial abundances and alpha diversity of the soil and the rhizospheric and endospheric microbiota successfully reproduced phylum-level plant-enrichment patterns seen in soil-grown *A. thaliana*<sup>42–44</sup> (Figure S1H).

The CD-Rhizotron system was then used to analyze the colonization of bacteria along the longitudinal root axis by examining the community structure of discrete root segments. Individual strands of both main and lateral roots were used for subsequent analyses



**Figure 1. Differential spatial colonization by root microbiota along the longitudinal root axis**

(A) Phenotype of wild-type *A. thaliana* Col-0 grown on CD-Rhizotron, with root segments marked. CPCoA of the Bray-Curtis dissimilarities of endospheric ( $n = 63$ ) or rhizosphere samples ( $n = 65$ ), constrained by the distance to the tip from which they were harvested. Different colors represent respective longitudinal segments or whole root samples, with colors of clusters corresponding to the root segments depicted in the plant cartoon. Centroids are indicated for each cluster of root region.

(B) Phenotype of wild-type *A. thaliana* Col-0 grown on ArtSoil. CPCoA of the Bray-Curtis dissimilarities of root endosphere ( $n = 39$ ) or rhizosphere samples ( $n = 40$ ), constrained by the distance to the tip from which they were harvested. Colors of clusters correspond to segments of the root depicted in the plant cartoon. Centroids are indicated for each cluster of root region.

See also Figures S1 and S2.

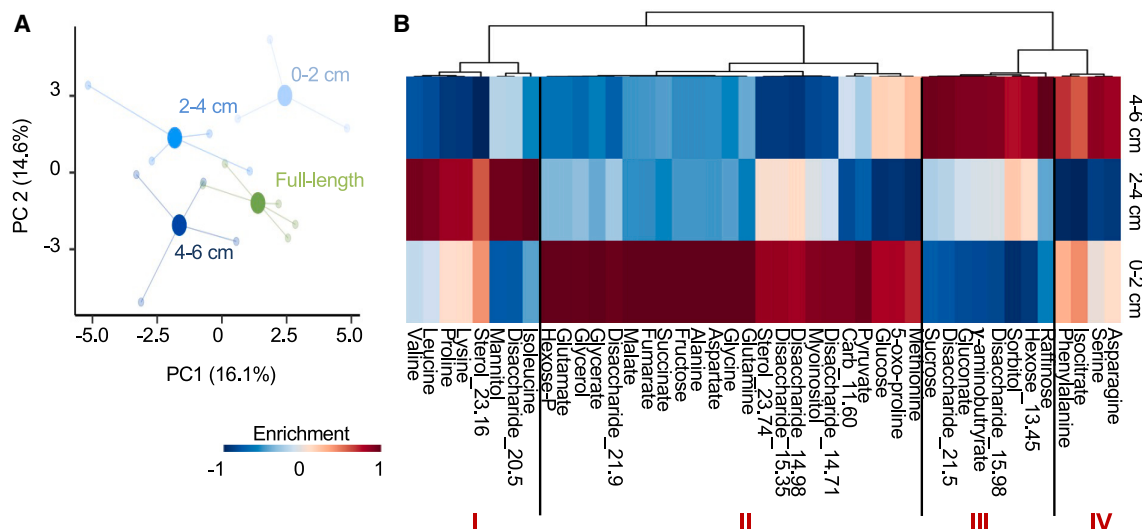
since the bacterial community profiles of main and lateral roots did not show significant differences (Figure S1I). Roots of 4-week-old plants grown in CD-Rhizotrons were sectioned into 2 cm segments, measuring 0–2, 2–4, and 4–6 cm from the root tip (here on designated as 2, 4, and 6 cm segments, respectively), for bacterial profiling using 16S amplicon sequencing. PCoA of Bray-Curtis dissimilarities constrained by root segment explained 15% of the variation in the endospheric fraction and 7.5% of the variation in the rhizospheric fraction ( $p = 0.001$ ; Figure 1A; Table S1). Thus, we identified a significant separation between samples from the three root segments, indicating dissimilarities between bacterial community structures.

To be able to monitor root growth and simplify root sectioning, an agar-based plant growth system was developed. Typically, plant growth media are supplemented with sucrose or glucose to facilitate seed germination and plant growth. For many plants, including *Arabidopsis*, the sugars are necessary for efficient growth; however, they also impact root physiology and alter microbiota dynamics.<sup>40,45–47</sup> To enable growth without the unphysiological supplementation with sugars, an ArtSoil system was developed. In this system, plant growth was supported on an agar matrix inoculated with an aqueous soil extract. The phenotype and growth of plants grown on ArtSoil were comparable

to that of plants grown on half-strength Murashige-Skoog ( $\frac{1}{2}$  MS-salt) media supplemented with 1% sucrose (Figure S2A).

To determine the adequate amount of soil inoculum for ArtSoil that would recapitulate natural soil bacterial diversity and root colonization, dilution-to-extinction assays were performed by inoculating ArtSoil with  $10^{-2}$ ,  $10^{-4}$ , or  $10^{-6}$  dilutions of soil-derived live bacterial suspension (Figure S2B). Rhizobiota analysis using 16S rRNA amplicon sequencing was performed on 3-week-old *A. thaliana* seedlings grown on ArtSoil. ArtSoil made from all three dilutions recapitulated the relative abundance and diversity of the core root microbiome of *A. thaliana* grown on soil,<sup>44</sup> and the differences in community structures between the three soil dilutions were not significant (Figure S2C). Furthermore, PCoA analysis of Bray-Curtis dissimilarities revealed distinct rhizospheric, endospheric, and bulk agar clusters, similar to that found in roots of soil-grown plants<sup>43</sup> (Figure S2D).

To investigate whether ArtSoil could reconstitute the spatial colonization of rhizobiota as found using the CD-Rhizotron system (Figure 1A), dissimilarity matrix ordination analysis was performed on bacterial communities in each segment and for full-length roots. Constrained PCoA of the Bray-Curtis dissimilarities revealed that root segments explained 10% of the variation in the



**Figure 2. Diversity and region-specificity of metabolites along the root axis**

(A) Principal-component analysis (PCA) of metabolites extracted from the full-length root, and segments 0–2, 2–4, and 4–6 cm from the root tip. The colors of clusters correspond to regions of the root depicted in the plant cartoon in Figure 1A. Centroids are indicated for each cluster of root region.

(B) Heatmap depicting the enrichment or depletion of indicated metabolites in respective root segments. Clustering performed based on average linkage Pearson hierarchical clustering of metabolites. Clusters are indicated in red roman numbers. Colors on the scale bar indicate the level of enrichment or depletion of each metabolite across the root segments.

See also Figure S3.

endospheric fraction ( $p = 0.034$ , Figure 1B; Table S1). Four distinct clusters observed in the endospheric fractions indicated distinct rhizobacteria community structures for each root segment, as observed using the CD-Rhizotron system. However, ArtSoil did not fully reconstitute the spatial colonization pattern of microbiota-derived from the rhizospheric fraction (4.1% of the variation with  $p = 0.72$ , Figure 1B; Table S1), most likely due to loss of microbiota diversity. The roots of *A. thaliana* grown in gnotobiotic CD-Rhizotron inoculated with a 60-member synthetic microbiota community<sup>48</sup> (SynCom) exhibited spatial root organization in the endospheric fraction but not the rhizospheric fraction (Figure S2E). Failure to reconstitute spatial rhizospheric colonization from plants grown in ArtSoil and gnotobiotic systems hinted toward a need for high microbiota diversity to establish spatial organization in the “rhizosphere.” Taken together, differential patterning of root-associated microbiota colonization along the longitudinal root axis of *A. thaliana* was identified using two growth systems with natural soil microbiota and a synthetic microbiota community.

### Differential enrichment of metabolites along the root longitudinal axis

Since exudation of root metabolites is one of the key processes by which plants interact with soil microbes,<sup>20</sup> we hypothesized that spatial differentiation in root colonization could be caused at least in part by differential root metabolism along the root axis. To test this hypothesis, metabolites from full-length roots and segments 2, 4, and 6 cm of seedlings grown on ArtSoil were extracted for untargeted gas chromatography-mass spectrometry analyses. 46 metabolites that had >70% database matches to the National Institute of Standards and Technology (NIST) reference library or a custom in-house library could be identified (Table S4). Metabo-

lites were categorized into four superclasses based on chemical structures in the RefMet reference library.

Principal-component analysis (PCA) of metabolites from the root segments revealed that the 1<sup>st</sup> and 2<sup>nd</sup> principal components represented 30.7% of the total variation in the metabolite profiles (Figure 2A). Distinct clusters were observed, with metabolites derived from the same root segment clustering tightly, relative to metabolites derived from other segments. Average linkage hierarchical clustering based on Pearson correlation was performed to visualize the enrichment or depletion of metabolites in each root segment (Figure 2B). The metabolites followed four enrichment patterns: metabolites in cluster II showed a gradient from the 6 cm segment toward the 2 cm segment, while cluster III metabolites showed the reverse pattern. Clusters I and IV did not follow a gradient along the root but rather a segment-specific enrichment, i.e., cluster I metabolites were enriched in the 4 cm segment, while cluster IV metabolites were enriched in segment 6 cm.

Cluster II contained 23 metabolites that were enriched in the root tip (2 cm segment). Of the 23 metabolites, 11 were carbohydrates, 11 were organic acids, and 1 was a sterol lipid. The root tip (2 cm segment) showed the most diverse metabolite pattern, i.e., the highest number of enriched metabolites with even distribution between carbohydrates and organic acids. The 4 cm segment was the least diverse profile with a higher representation of organic acids (cluster I: 7 metabolites, of which 4 were organic acids, 2 carbohydrates, and 1 sterol lipid). The root segment furthest from the tip, i.e., at 6 cm, was enriched for carbohydrates (7 out of 12 metabolites fell into the carbohydrate superclass; clusters III and IV). In sum, metabolite profiling of root segments provided evidence for differentiation in the spatial accumulation of root metabolites, specifically, the presence of



**Table 1. SWEETs accumulation along the longitudinal region of roots from *A. thaliana* grown on ½ Murashige-Skoog (½ MS) media supplemented with or without sucrose, and ArtSoil**

SWEET	½ MS – sucrose	½ MS + sucrose	ArtSoil
1	regions below 2 cm	regions below 2 cm	regions below 2 cm
2	regions below 0.05 cm and above 2 cm	regions below 0.05 cm and above 2 cm	entire root
3	not tested	not tested	not tested
4	not detected	regions above 2 cm (patchy)	not detected
5	not tested	not tested	not tested
6	not tested	not tested	not tested
7	not detected	not detected	not detected
8	not detected	not detected	not detected
9	not tested	not tested	not tested
10	not tested	not tested	not tested
11	regions below 2 cm	at 2.5–3 cm region	regions below 2 cm
12	regions below 2 cm	regions 2 cm and above	regions 2 cm and above
13	regions below 2.5 cm	regions below 2.5 cm	regions below 2.5 cm
14	not tested	not tested	not tested
15	not tested	not tested	not tested
16	not tested	not tested	not tested
17	entire root	entire root	entire root

metabolite concentration gradients, and segment-specific metabolite enrichment along the longitudinal root axis.

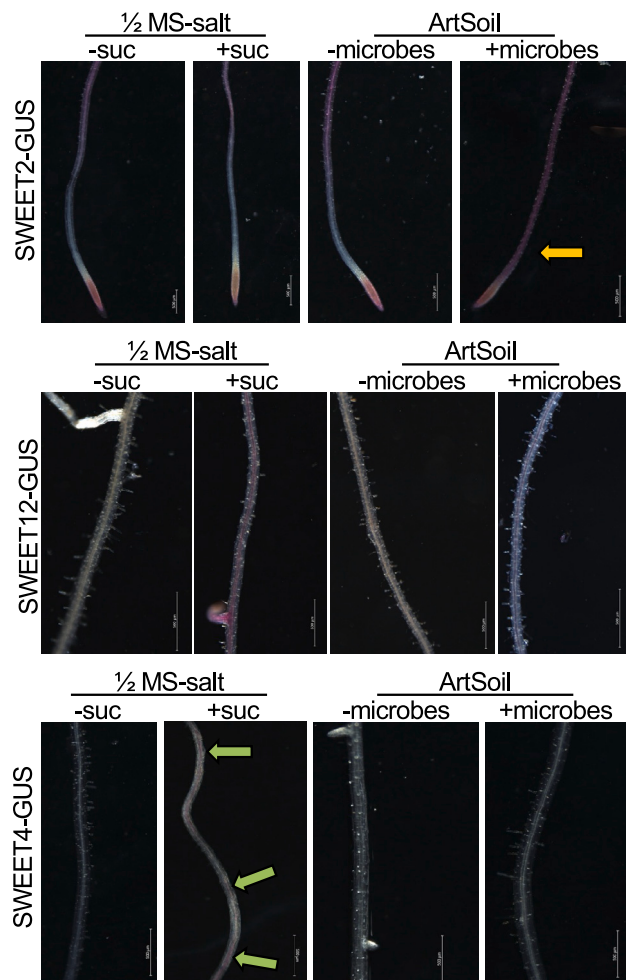
### Differential accumulation of SWEET sugar uniporters along the root axis

*In vitro* studies using bacteria derived from soil and plants, coupled with machine learning, indicated that carbon sources govern community assembly.<sup>40</sup> Notably, bacterial communities were found to assemble in a similar manner when grown in media with sugar and sugar/organic acid, but more distinct when only organic acids were supplied.<sup>40</sup> Consistently, a large-scale screening of *A. thaliana* leaf microbiota on 45 carbon sources revealed that sugars are the most commonly used carbon source in the phyllosphere.<sup>41</sup> We therefore hypothesized that interference with sugar sequestration from root cells could provide insight into the mechanism behind spatial colonization. Plants use three main sugar transporter families: SWEETs, STPs (sugar transport proteins), and SUT/SUCs (sucrose transporter/sucrose carriers). SUTs and STPs are H<sup>+</sup>-coupled symporters that take up sucrose or hexose, respectively, from the apoplast into the cytosol, while SWEETs as uniporters enable the release of sugars. The pathway activity scores (PASs) for all sugar-related metabolic pathways in the root were calculated to examine the potential involvement of SWEETs in sugar-related metabolic pathways. Differentiation of sugar metabolism and transport along the root axis was explored by modeling the conversion of sucrose into disaccharides and monosaccharides. The PAS of sugar-related metabolic pathways along the longitudinal axis of *Arabidopsis* roots was computed using publicly available microarray data<sup>11</sup> and plant metabolic pathway inventories (AraCyc: <https://plantcyc.org>). The correlation between the transcript level of each SWEET and the activity of each sugar-related metabolic pathway in the root was calculated using Spearman correlation (Figure S3A). Transcript levels of all

SWEETs except SWEET11 were significantly correlated with at least one sugar-related metabolic pathway in the root. Hence, we posited that SWEETs may play a role in root sugar flux. The average RNA levels of each SWEET in each segment of the root indicated that the accumulation of SWEETs in the roots of plants grown on full-strength MS-salt media supplemented with 1% sucrose follows a spatially differentiated pattern (Figure S3B). The mRNA transcripts of SWEET1, 2, 4, 5, 11, and 14 accumulated in the meristematic zone, whereas mRNAs of SWEET3, 8, and 15 accumulated in the elongation zone. mRNA transcripts for most of the SWEETs, i.e., SWEET6, 7, 9, 10, 11, 12, 14, 16, and 17, accumulated in the maturation zone. The *in silico* analysis provided a basis to investigate the roles of SWEET transporters in spatially organized bacteria root colonization.

### SWEETs accumulation patterns in roots are impacted by rhizobiota

To evaluate whether the microarray data for SWEETs (Figure S3B) are also reflected in respective protein levels, the accumulation of SWEET transporters was mapped in transgenic plants carrying translational SWEET-uidA reporter gene fusions driven by the native SWEET promoters (*pSWEET:SWEET-GUS*) grown on sterile ½ MS-salt media supplemented with sucrose. Microarray data were comparable to GUS histochemical analysis for SWEET1, 2, 11, 12, 13, and 17-GUS, but not for SWEET4, 7, and 8-GUS (Table 1; Figure S3B). Microarray data indicated accumulation of SWEET4, 7, and 8, mRNA in the meristematic, elongation, and maturation zones, while GUS histochemical analysis showed accumulation of SWEET4-GUS in regions above 2 cm. SWEET7-GUS and SWEET8-GUS proteins were not observed in the root. Notably, however, the microarray data are limited to regions of the root below 0.5 mm. The low correlation between SWEET mRNA and protein levels is consistent



**Figure 3. Accumulation of SWEETs along the root in the absence or presence of sugar and soil microbes**

Accumulation of respective SWEETs fused with a translational GUS reporter gene in 14-days-old *A. thaliana* seedlings grown on 1/2 MS-salt media supplemented with or without 1% sucrose (1/2 MS-salt –suc: 1/2 MS media without sucrose supplementation, 1/2 MS-salt +suc: 1/2 MS media with sucrose supplementation), on microbiome-inoculated or microbiome-killed ArtSoil (ArtSoil –microbes: ArtSoil inoculated with heat-killed soil microbes, ArtSoil +microbes: ArtSoil inoculated with soil microbes). The root segment depicted for SWEET2-GUS is <0.2 cm, and the root segments depicted for SWEET12-GUS and SWEET4-GUS are >2 cm. Scale bars, 500  $\mu$ m. The yellow arrow indicates the accumulation of SWEET2-GUS in the root zone of interest. Green arrows indicate the sporadic accumulation of SWEET4-GUS. Representative image for each genotype from N > 10.

See also Figure S4.

with a generally low correlation between RNA and protein levels for many genes, as, e.g., described in maize.<sup>49,50</sup>

To evaluate the potential effects of root microbes on SWEET protein levels in the different segments of the root, SWEET-GUS seedlings grown on sterile 1/2 MS-salt media ( $\pm$  sucrose) were compared with seedlings grown on ArtSoil (Figures 3 and S4). Histochemical analysis indicated that SWEET2-GUS was comparable all along the root region between 0 and 6 cm in plants grown on ArtSoil. In plants grown on sterile 1/2 MS-salt  $\pm$  sucrose and ArtSoil with heat-killed microbes, SWEET2-GUS

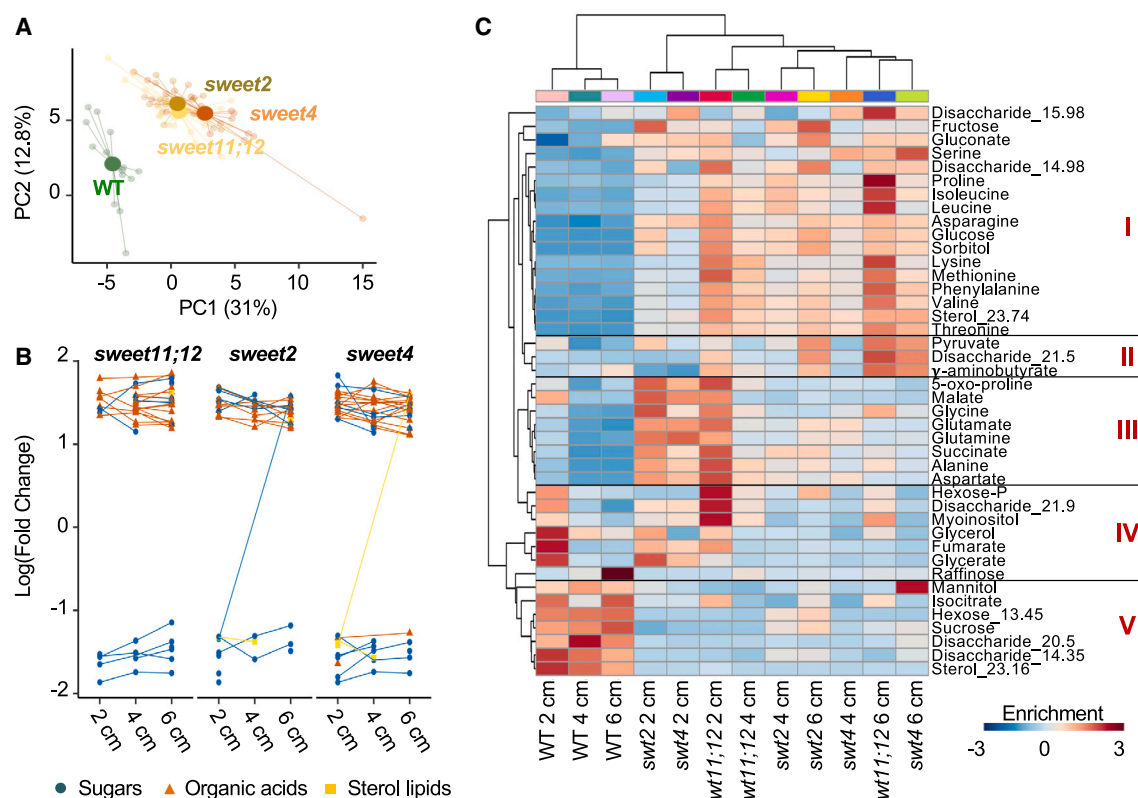
accumulated in the root tip (approximately up to 0.05 cm). In the adjacent zone (0.05–0.2 cm), GUS activity was not detectable. SWEET2-GUS was detected upward of this zone (0.2–6 cm). The observed differences in the SWEET2-GUS pattern when comparing ArtSoil and MS media-grown seedlings indicate that microbes exert an effect on SWEET2-GUS in the 0.05–0.2 cm segment. In regions above 2 cm from the root tip, SWEET12-GUS accumulated in the vasculature of plants grown on 1/2 MS-salt with sucrose, but not in roots of plants grown on 1/2 MS-salts without sucrose, nor in ArtSoil with heat-killed microbes. Low levels of SWEET12-GUS were observed in the roots of plants grown on ArtSoil, indicating that both sucrose and microbes lead to elevated SWEET12-GUS levels. Different from SWEET12, SWEET4-GUS accumulated in patches in regions above 2 cm from the root tip in plants grown on 1/2 MS-salts supplemented with sucrose. SWEET4-GUS was neither detected in the absence of sucrose, nor ArtSoil, indicating sugar-dependent accumulation of SWEET4. Taken together, histochemical analyses on SWEET-GUS point to differential effects of sucrose and root microbes on the spatial patterns of SWEET2-GUS, SWEET4-GUS, and SWEET12-GUS.

#### SWEETs affect spatial colonization of root bacteria

Since the spatial accumulation of SWEET2, SWEET4, and SWEET12 is impacted by microbes (Figure 3; Table 1), metabolite profiles and bacterial colonization of roots of *sweet2*, *sweet4*, and *sweet11;12* mutants were analyzed to investigate the physiological relevance of these SWEET transporters in the spatial patterning of metabolites and rhizobiota. SWEET11 and SWEET12 had previously been reported to serve complementary roles<sup>51–53</sup>; hence, a *sweet11;12* double knock-out mutant was used for all analyses. Under the conditions used here, *sweet2* and *sweet11;12* mutants grown on ArtSoil did not show growth penalties compared with the wild type (WT) (Figure S5A). PCA on total metabolites from WT and *sweet2*, *sweet4*, and *sweet11;12* roots revealed distinct clusters between WT and *sweet* mutants, but less distinction between the *sweet* mutants (Figure 4A). Despite the tight clustering of metabolite profiles among the three *sweet* mutants, the profiles of individual mutants were distinct (Data S1). Further dissection into metabolites in each segment of the root revealed that only three super-classes, namely organic acids, carbohydrates, and sterol lipids, were significantly altered compared with WT (Figure 4B). A global increase in organic acid levels was observed in all *sweet* mutants.

Pearson average linkage hierarchical clustering revealed five main clusters enriched or depleted depending on the genotype and/or root segment (Figure 4C). Metabolites in clusters I and V exhibited genotype-specific enrichment. Metabolites in cluster V were enriched in WT plants, whereas metabolites in cluster I were enriched in all *sweet* mutants, regardless of the root segment. Clusters II and III showed root segment-specific enrichment across all *sweet* mutants, whereas metabolites in cluster IV were enriched only in the 2 cm segment regardless of plant genotype.

Two-way ANOVA was performed to determine whether the differences in metabolite abundance between WT and *sweet* mutants could be attributed to spatial (2 vs. 4 vs. 6 cm vs. full-length root) or genotypic differences (WT vs. *sweet*). Compared



**Figure 4. Metabolite profiles of root segments from *sweet2*, *sweet4*, and *sweet11;12* are distinct from WT**

(A) Principal-component analysis (PCA) of total metabolites extracted from the whole root and root segments 2, 4, and 6 cm above the root tip of *sweet2*, *sweet4*, *sweet11;12*, and WT plants. WT: N = 16, *sweet2*: N = 19, *sweet4*: N = 20, *sweet11;12*: N = 21.

(B) Relative abundance of metabolite superclasses significantly (p value < 0.05) enriched and depleted in each root segment of *sweet* mutants compared with corresponding segments of WT.

(C) Heatmap depicting the enrichment (or depletion) of indicated metabolites in respective root segments for WT and *sweet* plants (abbreviated as *swt*). Heatmap generated based on average linkage Pearson hierarchical clustering of metabolites and root segments. Clusters are indicated in red roman numbers. Colors indicate the level of enrichment or depletion of each metabolite across the plant genotypes and root segments.

See also Figure S5.

with WT plants, the abundance of disaccharide 14.35 (unidentified) in *sweet2*, mannitol in *sweet4*, and disaccharide 14.35, gluconate, and hexose-P in *sweet11;12* was not caused by spatial or genotype differences (statistically insignificant,  $p > 0.05$ ; Table 2). The abundance of 6–9 carbohydrates (in *sweet2*: 9 metabolites, *sweet4*: 6 metabolites, and *sweet11;12*: 7 metabolites) and 7–8 organic acids (in *sweet2* and *sweet4*: 7 metabolites and *sweet11;12*: 8 metabolites) was caused by spatial differentiation rather than genotypic differences. By comparison, the abundance of 8–14 carbohydrates, 13–14 organic acids, and 1 sterol lipid were attributed to differences in plant genotype. Taken together, the loss of SWEET2, SWEET4, and SWEET11;12 functions led to alterations in the spatial abundance of root metabolites.

Since the ArtSoil system successfully captured endospheric, but not rhizospheric bacterial spatial colonization (Figure 1B), community profiling using 16S amplicon sequencing of *sweet* mutants grown *via* ArtSoil was focused on the endospheric fraction. Community profiling of the root of *sweet2*, *sweet4*, and *sweet11;12* mutants revealed significant differences between *sweet* mutants and WT plants (Kruskal-Wallis, Dunn's test post

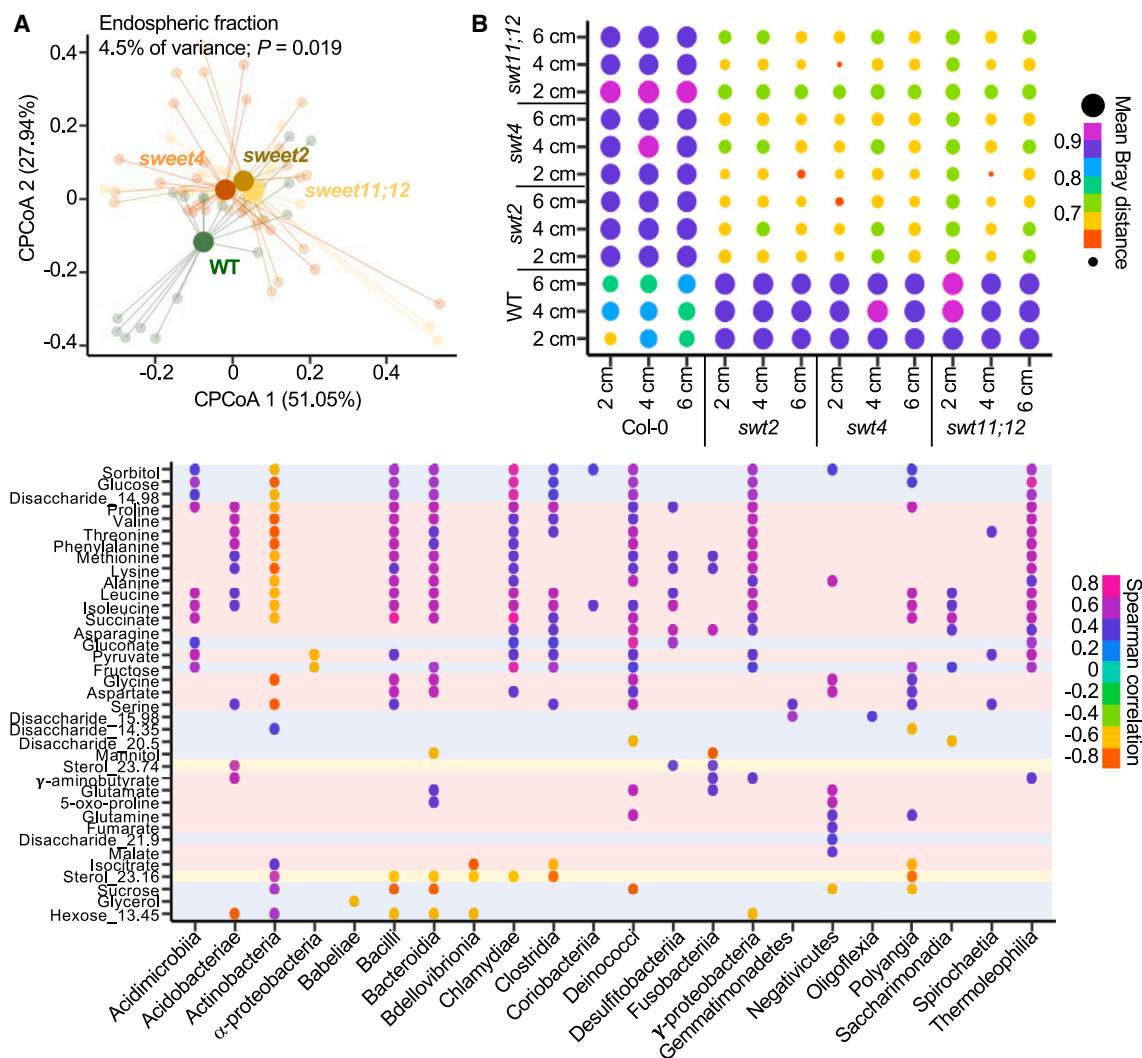
hoc,  $p = 0.019$ ; Figure 5A). To determine whether the spatial organization of the endospheric microbiota was retained in the *sweet* mutants, community profiling was performed on segments of root from the mutants. Strikingly, compositional dissimilarities measured using Bray-Curtis distance indicated that bacterial communities from root segments of *sweets* had lower dissimilarity compared with each other (Figure 5B; Table S1). Bacterial communities from the 2 cm segment of the WT root retained lower (but still distinct, Table S1) Bray-Curtis distance scores compared with 4 and 6 cm segments, indicating differences in the bacterial community between root segments of WT plants. The Bray-Curtis distance scores were higher for root segments of WT compared with corresponding segments in *sweet* mutants (Figures 5B and S5B; Tables S2 and S3), indicating high dissimilarity of bacterial communities between WT and *sweet* mutants. Notably, root segments from *sweet2* and *sweet4* showed insignificant differences in the Bray-Curtis score (Table S1). Spatial organization was retained in *sweet11;12*, but the bacterial communities were different compared with WT plants (Figures S5B; Tables S1–S3). Taken together, the organization of root microbiota along the longitudinal root axis was



**Table 2. Contribution of spatial distribution and/or plant genotype to the abundance of root metabolites**

Metabolite	Superclass	<i>sweet2</i>	<i>sweet4</i>	<i>sweet11;12</i>
Sterol 23.74	sterol lipid	N/A	N/A	N/A
Sterol 23.16	sterol lipid	genotype	genotype	genotype
Fructose	carbohydrate	genotype	genotype	genotype
Glucose	carbohydrate	genotype	genotype	genotype
Disaccharide 14.98	carbohydrate	genotype	genotype	genotype
Sorbitol	carbohydrate	genotype	genotype	genotype
Sucrose	carbohydrate	genotype	genotype	genotype
Hexose 13.45	carbohydrate	genotype	genotype	genotype
Disaccharide 15.98	carbohydrate	genotype	genotype	genotype
Mannitol	carbohydrate	genotype	N/A	genotype
Disaccharide 14.35	carbohydrate	N/A	genotype	N/A
Gluconate	carbohydrate	genotype, spatial	genotype	N/A
Hexose-P	carbohydrate	spatial	genotype	N/A
Glycerol	carbohydrate	genotype, spatial	genotype	spatial
Carb 11.60	carbohydrate	spatial	spatial	spatial
Disaccharide 21.9	carbohydrate	spatial	genotype	genotype, spatial
Glycerate	carbohydrate	spatial	genotype, spatial	genotype, spatial
5-oxo-Proline	carbohydrate	genotype, spatial	genotype, spatial	genotype, spatial
Disaccharide 21.5	carbohydrate	genotype, spatial	genotype, spatial	genotype, spatial
Raffinose	carbohydrate	genotype, spatial	genotype, spatial	N/A
Disaccharide 14.71	carbohydrate	N/A	N/A	N/A
Disaccharide 20.5	carbohydrate	N/A	N/A	N/A
Myoinositol	carbohydrate	N/A	N/A	N/A
Shikimate	organic oxygen compound	N/A	N/A	N/A
$\gamma$ -Aminobutyrate	organic acid	N/A	N/A	N/A
Malate	organic acid	spatial	spatial	spatial
Ketoglutarate	organic acid	spatial	spatial	spatial
Fumarate	organic acid	spatial	spatial	spatial
Aspartate	organic acid	genotype, spatial	genotype, spatial	genotype, spatial
Isocitrate	organic acid	genotype, spatial	genotype, spatial	genotype, spatial
Glutamine	organic acid	genotype, spatial	genotype, spatial	genotype, spatial
Alanine	organic acid	genotype	genotype, spatial	genotype, spatial
Glycine	organic acid	genotype, spatial	genotype	genotype
Succinate	organic acid	genotype	genotype	genotype, spatial
Isoleucine	organic acid	genotype	genotype	genotype
Leucine	organic acid	genotype	genotype	genotype
Lysine	organic acid	genotype	genotype	genotype
Asparagine	organic acid	genotype	genotype	genotype
Methionine	organic acid	genotype	genotype	genotype
Phenylalanine	organic acid	genotype	genotype	genotype
Proline	organic acid	genotype	genotype	genotype
Pyruvate	organic acid	genotype	genotype	genotype
Serine	organic acid	genotype	genotype	genotype
Threonine	organic acid	genotype	genotype	genotype
Valine	organic acid	genotype	genotype	genotype
Glutamate	organic acid	genotype	genotype	genotype

N/A: not applicable. The abundance of indicated metabolites is not significantly different from WT plants (two-way ANOVA,  $p > 0.05$ ).



**Figure 5. Loss of spatial microbiota colonization in roots of *sweet2*, *sweet4*, and *sweet11;12***

(A) CPCoA of the Bray-Curtis dissimilarities of endospheric bacteria derived from full-length roots of WT, *sweet2*, *sweet4*, and *sweet11;12* plants, constrained by plant genotype. Different colors represent plant genotypes with centroids indicated for each cluster of genotype. WT: N = 60, *sweet2*: N = 40, *sweet4*: N = 41, *sweet11;12*: N = 42.

(B) Bray-Curtis dissimilarities distances of endospheric bacteria derived from the 0–2, 2–4, and 4–6 cm segments from the root tip of WT, *sweet2*, *sweet4*, and *sweet11;12* plants (*sweet* abbreviated as “*swt*”) compared with each other. The colors and sizes of the dots correspond to the mean of the Bray-Curtis distance.

(C) Spearman correlation for metabolite-microbe (class level) pairs. Metabolites ranked based on Spearman average hierarchical clustering. The color of each point indicates the degree of correlation. Highlights indicate the class of metabolite: blue, sugar/carbohydrate; red, organic acid; and yellow, lipid. Spearman correlations significance,  $p < 0.05$ .

See also Figure S5.

different in *sweet2*, *sweet4*, and *sweet11;12* compared with WT plants. As a control, the root microbiota of *sweet7*, for which SWEET7-GUS accumulation was not detected in the roots, retained its spatial organization along the longitudinal axis of the root (Table 1; Figures S5C and S5D). Collectively, metabolite and microbiota profiling of the *sweet* mutants intimate a role of sugar efflux by SWEET2, 4, and 11;12 along the root as necessary for the spatial organization of root bacteria.

To visualize the relationship between the root microbes and metabolites, correlations between the abundance of microbes and metabolites were computed (Figure 5C). The majority of

the relationships between microbes and metabolites were positive, i.e., an increase in the abundance of metabolite correlated with an increase in the abundance of a specific class of microbes. Most of the metabolites (22 out of 37 metabolites) showed positive and negative relationships with the microbes, 12 metabolites (asparagine, gluconate, aspartate, disaccharide\_15.98, sterol\_23.74,  $\gamma$ -aminobutyrate, glutamate, 5-oxo-proline, glutamine, fumarate, malate, and disaccharide\_21.9) were positively correlated with the microbes only, whereas three metabolites (disaccharide\_20.05, glycerol, and mannitol) exclusively had negative relationships with the root microbes. A large

subset of the amino acids had similar relationship patterns with several sugars, i.e., sorbitol, glucose, and disaccharide 14.98, indicating possible interdependence between classes of metabolites (Figures 5C and S5E). Interdependence between sugars and organic acids was also apparent for fructose and pyruvate in which the two metabolites exhibit similar relationship patterns with seven out of nine classes of microbes. In sum, metabolite-microbe correlation analysis unveiled similarities in interaction patterns between metabolite clusters, intimating possible interdependence between metabolites.

## DISCUSSION

Here, we developed two plant growth systems that allow tracing of root growth and easy dissection of individual root strands into three longitudinal segments. Bacterial profiling of the three root segments revealed spatial organization (biogeography) of microbiota, i.e., each segment of the root harbors distinct bacterial communities (Figure 1). Metabolite profiling of root segments revealed differential enrichment of metabolites among the three segments (Figure 2). Bioinformatic analysis using publicly available microarray data indicated a high correlation between SWEETs and root sugar metabolic pathways (Figure S3). Accumulation of SWEETs in the root was tested using SWEET-GUS translational fusion plants. SWEET2, SWEET4, and SWEET12 demonstrated spatial differences in the accumulation of proteins in dependence on the presence of soil microbes (Figure 3). Metabolite and microbiota profiling of the roots of *sweet2*, *sweet4*, and *sweet11;12* loss of function mutants showed altered spatial metabolite profiles and disordered spatial bacterial colonization. Collectively, we demonstrated a role for SWEET2, SWEET4, and SWEET11;12 in the overall provision of carbohydrate supply to maintain the spatial organization of root microbiota (Figures 4 and 5).

We surmise that the apparent loss of community structure in the three *sweet* knock-out mutants could be caused by one or several mechanisms: (1) carbon starvation could impact key hub microbes and thus lead to a reduced ability to assemble the three-dimensional biogeography as found in WT roots; (2) the microbes may switch from altruistic community status to egoistic strategies of individuals as described in the “game theory”<sup>54</sup>; or (3) the shift in carbon supply away from carbohydrates to organic and amino acids may affect the overall community assembly. Modeling of resource competition between phyllospheric microbes of *A. thaliana* predicted that competition for sugar between microbes can be alleviated with organic acids supplementation.<sup>41</sup> The roots of *sweet2*, *sweet4*, and *sweet11;12* showed decreased carbohydrate levels concomitant with increased organic acid levels (Figure 4). Alteration in the abundance of organic acids that have been described to play a role in reshaping the root microbiota<sup>55,56</sup> was observed in the *sweet* mutants. Hence, in *sweet* mutants, organic acids may still support the colonization of root bacteria but result in spatial reorganization. Note however that the communities in the three mutants are not identical, consistent with differences in substrate specificity and expression pattern.

### The role of metabolites in shaping community structure

Physiological observations support the function of SWEETs in facilitating sugar influx or efflux in a case-dependent manner.

SWEET12-GUS accumulated in the vasculature in the presence of microbes, indicating phloem unloading to carbon sink caused by bacteria.<sup>28,57</sup> SWEET2 is a vacuolar transporter, and the GUS histochemistry may indicate a role for sugar sequestration in or transport from the vacuole in the presence of soil microbes.<sup>34</sup> The absence of SWEET4-GUS induction in the presence of soil microbes intimates that hexose export into the apoplast is not required in the presence of microbes.<sup>58</sup> Of note, *sweet4* exhibited a slight growth penalty<sup>59</sup> (Figure S5A), which could contribute to altered spatial root metabolite and microbiota profiles. In rice and several crop plants, pathogens in the *Xanthomonas* genus inject transcription-activation like (TAL) effectors into host cells to trigger the transcription of SWEETs for SWEET-mediated secretion of sucrose. The secreted sucrose presumably facilitates pathogen feeding and proliferation, leading to disease development.<sup>28,36,38</sup> Interference with SWEET gene activation confers pathogen resistance.<sup>37,60–63</sup> In *Arabidopsis*, SWEETs are also involved in various pathogen interactions.<sup>28,34,58</sup> The spatial distribution of SWEETs along the root could allow plants to control root colonization patterns by nutrient mobilization and/or selective feeding.

The amino acids leucine, arginine, histidine, valine, isoleucine, and tryptophan are required by *Pseudomonas fluorescens* WCS365 for the initial colonization of tomato root tip.<sup>64</sup> The requirement for multiple amino acids to be present simultaneously (as also seen in Figure 5C) implies a dependency on multiple amino acids to enable successful microbe colonization of the root. Malate, pyroglutamic acid, citric acid, succinate, and fumarate have been shown to be secreted from tomato roots as chemoattractants.<sup>65</sup> Citric acid, pyruvate, succinate, and fumarate have been implied to play a role in the enrichment of microbes from the *Comamonadaceae* family.<sup>66</sup> Malate, succinate, and fumarate also serve as energy sources for the establishment of symbiosis.<sup>67</sup> Metabolite-microbe correlation revealed that pyruvate and fructose exhibit similar relationship patterns, whereas fumarate and malate closely cluster with disaccharide 21.9 (Figures 5C and S5E). The similarity in the relationship patterns among the metabolites hints at the potential role of sugars together with organic acids to affect colonization patterns. Taken together, interdependency exists between sugars and organic acids in shaping the rhizobial colonization patterns. Further inspection into the interdependence among metabolites may provide insights into how metabolite cocktails (e.g., root exudate) shape microbial patterning.

### Potential role of immunity-related processes in the host microbiota interaction

The root of *A. thaliana* exhibits spatial differentiation of immune responses. Microbe-associated molecular pattern (MAMP)-trigger immunity (MTI) is confined to the root cap and the transition/elongation zone, whereas immune responses are apparently weaker in differentiated root parts.<sup>16</sup> Screening of 627 *A. thaliana* root commensals from diverse taxonomic origins found diversity in the response to the immunogenic flg22, which elicits antagonistic immune responses.<sup>68</sup> Flg22 induces enhanced sugar uptake activity from the apoplast via the sugar uniporter STP13.<sup>69</sup> Microbes also reduce the environmental pH using organic acids to inhibit the flg22-activated immune response.<sup>70</sup> Taken together, the selective recruitment of microbes along the root could be a strategy to select compatible

bacteria that will co-modulate host susceptibility to pathogens by either eliciting or dampening MTI responses.<sup>71</sup> MTI activation-inactivation buffers the plant immune system against pathogen perturbation and defense-associated growth inhibition, leading to commensal-host homeostasis. Our data indicate that the *sweet* mutants, although with spatial microbiota reorganization, still maintain their overall root microbiota homeostasis, as evident by the absence of growth inhibition compared with WT plants (Figure S5A).

### Other host developmental actors

The three root segments selected in this work encompass all stages of root development, i.e., meristematic, elongation, and maturation zones are within 0.05 cm from the root tip.<sup>72–74</sup> Thus, the differences in metabolite and microbiota profiles among the root segments may not correspond to the developmental stages but possibly to root suberization stages. In the presence of root microbes, full suberization may extend up to 3 cm from the root tip.<sup>75</sup> The transition from Casparian strip formation to full suberization of the endodermis starts sporadically in a patch-like manner.<sup>76</sup> Hence, it is plausible that the 2 cm segment represents non-suberized and patchy zones, the 4 cm represents patchy and fully suberized zones, and the 6 cm represents fully suberized root.<sup>77</sup> The suberization status of the endodermis influences the root colonization of microbes through the secretion of specific root metabolites. In a bidirectional feedback manner, root microbes influence diffusion barrier formation, in turn affecting the balance of mineral nutrients in the plant.<sup>75</sup>

### Conclusion and outlook

Similar to animal systems, the microbiota of plant roots are spatially differentiated.<sup>78</sup> The 2 cm segments used here indicated complex biogeography, however, there is likely a much finer spatial differentiation within these regions, both in the axial and radial axes. This work also highlights the interdependency of host and microbiota regarding metabolic activities. The rhizosphere is defined as the soil typically >2 mm from the root,<sup>79</sup> whose properties (including microbial activities) are affected by root exudations. Since the rhizosphere of media-grown plants has so far not been determined, we here defined the rhizosphere for plants grown on ArtSoil as the 5 mm region surrounding the root. We show that the endogenous microbes derived from the roots of plants grown on ArtSoil could reconstitute the endogenous microbiota present in soil-grown plants. It will be interesting to also analyze the agar fraction that extends 5 mm from the root surface of plants (the so-called rhizosphere) grown on ArtSoil, and to test whether the communities are affected by root exudation. Three-dimensional (3D)-localization of community members by using fluorescence *in situ* hybridization (FISH) or similar approaches may help to get more detailed information on this zone. At present, microbiota within the segments is treated as a general community; however, in mammalian systems, it has been established that compounds produced by one microbe are used by another, and as a result the existence of complex metabolic pathways across multiple microbiotas. It will be interesting to map out individual interdependencies of microbiota in their respective microdomains in future studies. Further dissection will help to untangle the complex microbiota and advance options to generate functional SynComs. Moreover, the work in-

dicates that we still lack many of the key transporters from the host, such as predicted sugar and organic acid effluxes in the rhizodermis.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
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  - Materials availability
  - Data and code availability
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- **METHOD DETAILS**
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  - Microbiota profiling
  - DNA extraction and library preparation
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
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  - Quantify metabolic activities using PAS
  - Flux balance analysis (FBA) to define pathways
  - Correlation between microbes and metabolites

### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.chom.2024.02.014>.

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### AUTHOR CONTRIBUTIONS

Conception: E.P.-I.L. and W.B.F. writing: E.P.-I.L. and W.B.F. experiments and data analyses: microbiota: R.G.-O, P.D., C. Durán, and E.P.-I.L.; bioinformatics: T.Y.P., M.L., and E.P.-I.L.; localization: E.P.-I.L. and C. Deng; metabolomics: P.W. and E.P.-I.L.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Biological Samples</b>		
<i>Arabidopsis thaliana</i> Col-0 P <sub>SWEET17</sub> :SWEET17-GUS	Guo et al. <sup>80</sup>	N/A
<i>Arabidopsis thaliana</i> Col-0 P <sub>SWEET2</sub> :SWEET2-GUS	Chen et al. <sup>34</sup>	N/A
<i>Arabidopsis thaliana</i> Col-0 P <sub>SWEET12</sub> :SWEET12-GUS	Chen et al. <sup>28</sup>	N/A
<i>Arabidopsis thaliana</i> Col-0 P <sub>SWEET11</sub> :SWEET11-GUS	Chen et al. <sup>28</sup>	N/A
<i>Arabidopsis thaliana</i> Col-0 sweet2c	SIGnAL <a href="http://signal.salk.edu/">http://signal.salk.edu/</a>	SALK_048430.36.85.x
<i>Arabidopsis thaliana</i> Col-0 sweet11;12	Arabidopsis Biological Resource Center <a href="https://abrc.osu.edu">https://abrc.osu.edu</a>	TAIR Germplasm: CS68845
<i>Arabidopsis thaliana</i> Col-0 sweet4a	SIGnAL <a href="http://signal.salk.edu/">http://signal.salk.edu/</a>	SALK_072225.23.65.x
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
DFS-Taq DNA polymerase	Bioron	Cat # 101005
Antarctic phosphatase	New England BioLabs	Cat # M0289
Exonuclease I	New England BioLabs	Cat # M0293
Ribitol	Sigma-Aldrich	Cat # PHR3526
Murashige&Skoog media including MES buffer	Duchefa Biochemie	Cat # M0254
Triton™ X-100, for molecular biology	Sigma-Aldrich	Cat # T8787
Agar	Sigma-Aldrich	Cat # 05040
Hygromycin B	Carl Roth	Cat # CP12.2
Thermo Scientific X Gluc	Thermo Fisher Scientific	Cat # R0851
<b>Critical Commercial Assays</b>		
FastDNA™ SPIN Kit for Soil	MP Biomedicals	Cat # SKU116560200-CF
NuceloSpin Soil Mini kit for DNA from soil	Macherey-Nagel	Cat # 740780
QIAquick gel extraction kit	Qiagen	Cat # 28706X4
Quant-iT™ PicoGreen™	Invitrogen	Cat # P7581
Gateway™ LR Clonase™ Enzyme mix	Invitrogen	Cat # 11791019
<b>Deposited Data</b>		
16S sequencing data	This paper	<a href="https://www.ebi.ac.uk/ena/browser/home">https://www.ebi.ac.uk/ena/browser/home</a> (Accession number: PRJEB63568)
Metabolomics data	This paper	<a href="https://www.metabolomicsworkbench.org/">https://www.metabolomicsworkbench.org/</a> (Study ID: ST002779)
<i>Arabidopsis</i> root spatial microarray	[ <a href="https://doi.org/10.1126/science.1146265">https://doi.org/10.1126/science.1146265</a> ]	GEO dataset: GSE8934 ID: 200008934
NIST14 Mass Spectral Library	<a href="https://www.nist.gov/srd/nist-standard-reference-database-1a-v14">https://www.nist.gov/srd/nist-standard-reference-database-1a-v14</a>	N/A
<i>Arabidopsis thaliana</i> Col-0 metabolic pathways	<a href="https://pmn.plantcyc.org/organism-summary?object=ARA">https://pmn.plantcyc.org/organism-summary?object=ARA</a>	N/A
<b>Experimental Models: Organisms/Strains</b>		
<i>Arabidopsis thaliana</i> Col-0	TAIR	TAIR accession Germplasm:1008804532

(Continued on next page)



**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Oligonucleotides</b>		
AtSWEET4-Xbal-F	IDT	GCAGGTCGACTCTAGAAGTGGTTCCACG GAGATGACG
AtSWEET4-BamHI-R	IDT	CGGTACCCGGGGATCCAGCTGAAACTCG TTTAGCTTGTCC
AtSWEET5-Xbal-F	IDT	GCAGGTCGACTCTAGATTAGGACTGACAC CAGCGATGC
AtSWEET5-BamHI-R	IDT	CGGTACCCGGGGATCCAGCCTGGCCAAG TTCGATTC
AtSWEET7-Xbal-F	IDT	GCAGGTCGACTCTAGAATTGAGGCTTGGC GTAACCTTG
AtSWEET7-BamHI-R	IDT	CGGTACCCGGGGATCCAACATTGTTAGGT TCTTGTTGG
AtSWEET8-Xbal-F	IDT	GCAGGTCGACTCTAGAACCATGACAATTT GGCTCCGAG
AtSWEET8-BamHI-R	IDT	CGGTACCCGGGGATCCAACCCTCTCCGT AGCAGAAATC
<b>Recombinant DNA</b>		
pMDC163 vector	<i>Arabidopsis</i> Biological Resource Center <a href="https://abrc.osu.edu">https://abrc.osu.edu</a>	TAIR accession Vector:1009003758
promoterless GUSplus vector	Yang et al. <sup>81</sup>	N/A
<b>Software and Algorithms</b>		
iGraph	<a href="https://CRAN.R-project.org/package=igraph">https://CRAN.R-project.org/package=igraph</a>	RRID:SCR_019225
COBRAPy	<a href="http://opencobra.sourceforge.net">http://opencobra.sourceforge.net</a>	RRID:SCR_012096
GUROBI	<a href="https://www.gurobi.com/">https://www.gurobi.com/</a>	N/A
statsmodels	<a href="https://www.statsmodels.org/stable/index.html">https://www.statsmodels.org/stable/index.html</a>	RRID:SCR_016074
Vegan	<a href="https://cran.r-project.org/web/packages/vegan/index.html">https://cran.r-project.org/web/packages/vegan/index.html</a>	RRID:SCR_011950
Qiime2	<a href="https://qiime2.org/">https://qiime2.org/</a>	RRID:SCR_008249
Flash2	<a href="https://github.com/dstreett/FLASH2">https://github.com/dstreett/FLASH2</a>	RRID:SCR_005531
Dada2	<a href="https://benjineb.github.io/dada2">https://benjineb.github.io/dada2</a>	RRID:SCR_008205
<b>Others</b>		
16S data analyses	This work	GitHub <a href="https://github.com/duranpa/sweet_collaboration">https://github.com/duranpa/sweet_collaboration</a>

**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Eliza Loo ([loo@hhu.de](mailto:loo@hhu.de))

**Materials availability**

This study did not generate new unique reagents.

**Data and code availability**

Raw 16S sequencing data have been deposited into the European Nucleotide Archive (ENA) under the accession number PRJEB63568. In addition, the scripts used for the computational analyses described in this study are available at GitHub [https://github.com/duranpa/sweet\\_collaboration](https://github.com/duranpa/sweet_collaboration). Raw metabolomics file is deposited at <https://www.metabolomicsworkbench.org> under the data track Study ID ST002779. Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

## EXPERIMENTAL MODEL

*Arabidopsis thaliana* Col-0 seeds were sterilized in a 1.5-mL microcentrifuge tube with 1 mL of 30% chlorine + 0.1% Triton X-100 for 15–20 min at room temperature with agitation. The seeds were rinsed once with 1 mL of 80% ethanol, followed by five rinses with autoclaved ddH<sub>2</sub>O. The microcentrifuge tube was wrapped in aluminum foil and cold-treated at 4 °C for 2–5 days before sowing. *A. thaliana* Col-0 seeds were incubated under standard growth conditions, i.e., 22 °C, 10 h light/ 14 h dark, 60% humidity, 180–230  $\mu\text{mol/s/m}^2$  light intensity.

### CD-Rhizotron

To assemble a CD-Rhizotron, a small opening, measuring approximately 1.5–2 cm, was cut on the top of a slimline jewel CD case (14.3 cm x 12.4 cm x 0.52 cm; [Figure S1A](#)). The teeth of the tray were snipped off and sanded down to produce a smooth surface. The resulting hole was sealed on both sides with packing tape. CD-Rhizotrons were filled with soil (Cologne Agricultural Soil, CAS<sup>42</sup>) and wrapped with aluminum foil to provide a dark environment for proper root growth ([Figures S1A–S1D](#)). Up to four sterilized *A. thaliana* Col-0 seeds were sown directly onto each CD-Rhizotron from the top aperture of the CD-Rhizotron. CD-Rhizotrons were incubated under standard growth conditions. After 5–7 days, germinated seedlings were removed to leave each CD-Rhizotron with a single seedling. Plants were grown for another 3 weeks before they were harvested for microbiota profiling. Preparation of SynCom was adapted from (Durán et al.<sup>82</sup>). The 60-member SynCom was curated based on differences in 16S rRNA sequence with a 97% threshold. Briefly, bacterial strains ([Table S5](#)) were cultivated in 50% Tryptic Soy Broth (Sigma) for one week at 28 °C with shaking at 200 rpm. The bacterial cultures were pooled in equal ratio and centrifuged at 4000x g for 10 mins. The bacterial pellet was re-suspended in 10 mM MgCl<sub>2</sub> to remove residual media and bacteria-derived metabolites. The washing step was repeated three times. The washed bacterial suspension was adjusted to an OD<sub>600</sub> of 0.02 (10<sup>7</sup> cells/mL) prior to inoculation onto sterile peat soil.

### ArtSoil preparation

For the preparation of 10% autoclaved soil suspension, sieved CAS (to remove pebbles) was dissolved in deionized water and autoclaved three times before use. Live bacterial suspension was prepared by inoculating fresh CAS into 10% autoclaved soil suspension to achieve desired bacterial dilution.<sup>83</sup> Autoclaved half-strength Murashige-Skoog (½ MS) media including MES (Duchefa Biochemie) and supplemented with 1% agar was cooled to 40–45 °C before it was inoculated with live bacterial suspension. Approximately 50 mL of ArtSoil media was used for each 12 cm x 12 cm culture plate ([Figure S2B](#)). Sterilized *A. thaliana* Col-0 seeds were sown and germinated on ArtSoil, and incubated in a plant growth chamber for 4 weeks under standard growth conditions.

## METHOD DETAILS

### Construction of translational GUS reporter lines

Genomic fragments comprising the 5'-upstream region and the entire coding region of *SWEET1*, *SWEET4*, *SWEET5*, *SWEET7*, *SWEET8*, *SWEET10*, and *SWEET13* were amplified by PCR using wild-type *A. thaliana* Col-0 genomic DNA as a template (primers for each gene are listed in Key Resource Table). The amplified products of *SWEET4*, *SWEET5*, *SWEET7*, *SWEET8*, and *SWEET10* were cloned into promoterless GUSplus vector<sup>81</sup> while *SWEET1* and *SWEET13* were cloned into pMDC163 using the Gateway cloning system. All constructs were confirmed by sequencing. Wild-type *A. thaliana* Col-0 were transformed by *Agrobacterium tumefaciens* harboring SWEET-GUS constructs using the floral dip method. Transgenic seedlings were selected on ½ MS media supplemented with 1% sucrose and 25  $\mu\text{g/mL}$  hygromycin. Transgenic *SWEET2-GUS*, *SWEET11-GUS*, *SWEET12-GUS* and *SWEET17-GUS* were previously reported in Chen et al.,<sup>28</sup> Chen et al.,<sup>34</sup> and Guo et al.<sup>80</sup>

### GUS histochemical staining

Three-week-old plants were carefully removed from ArtSoil and rinsed with 70% ethanol. Each plant was inserted into one well in a 12-well plate before 1 mL of GUS staining solution (10 mM EDTA, 50 mM phosphate buffer, 0.1% Triton X-100, 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 2 mM X-Gluc, 20% methanol) was added into each well. The plate was subsequently incubated in the dark at 37 °C for 1 h (*SWEET1*, 2, 17) or 4 h (*SWEET4*, 7, 8, 11, 12, 13). Prior to imaging, plants were removed from GUS staining buffer and rinsed with 70% ethanol.

### Root metabolite profiling

Approximately 25–35 sterilized *A. thaliana* Col-0 seeds were sown in a row on ArtSoil and grown for 3 weeks. After 3 weeks, both primary and lateral roots longer than 8 cm were harvested by slicing with a scalpel. The roots were segmented into 2-cm segments measuring 0–2, 2–4, and 4–6 cm from the root tip. Lateral roots that emerged from primary roots were removed, if present. The roots were rinsed in distilled water, and blotted dry on Whatman filter papers before they were weighed and kept in 1.5-ml microcentrifuge tubes, each containing 2 metal beads. The samples were flash-frozen in liquid nitrogen and stored at -80 °C until the extraction process. For metabolite extraction, 0.5 mL of chilled extraction buffer (2:5:2 ratio of ddH<sub>2</sub>O: methanol: chloroform containing 5  $\mu\text{M}$  ribitol as internal standard) was added into each root sample and mixed by vortex for 20 sec. Metal beads in the tubes were removed. Sample tubes were shaken on a rotary shaker for 30 mins at 4 °C. The samples were then centrifuged at 20,000 x g for 5 min at 4 °C. After

centrifugation, 0.5 ml of supernatant was carefully aspirated and transferred to a clean 1.5-ml microcentrifuge tube. Samples were stored at -80 °C until they were subjected to GC-MS. For GC-MS analysis 30 µl of sample were dried by vacuum centrifugation in glass inlet tubes. Dried samples were derivatized according to Gu<sup>84</sup> and Shim et al.<sup>85</sup> Raw data files were converted to the mzXML format using ProteoWizard<sup>86</sup> and to the NetCDF format via MetAlign<sup>87</sup> using default parameters. Deconvolution of mass spectra was conducted using the free deconvolution software AMDIS (Automated Mass Spectral Deconvolution and Identification System from NIST). Deconvoluted mass spectra were matched against the NIST14 Mass Spectral Library (<https://www.nist.gov/srd/nist-standard-reference-database-1a-v14>). Database matches with more than 70% were further compared with an in-house chemical standard library for compound annotation. Compounds, that could not be verified by the in-house library are named according to the matched compound class and the retention time. Extracted ion peaks were integrated using MassHunter Quantitative (v b08.00, Agilent Technologies). For relative quantification, all metabolite peak areas were normalized to fresh weight and the peak area of the internal standard ribitol (Sigma-Aldrich) to correct for technical error. Here, a GC-based system was employed for metabolomics, which may detect only the fraction of metabolites that can be vaporized. It may be useful for a more in-depth analysis to use LC-based systems to identify more metabolites and improve coverage.

### Microbiota profiling

Roots of 3-weeks-old plants grown *via* the CD-Rhizotrons were cut into 2-cm segments as described in “Root metabolite profiling” (Figures S1E and S1F). Each root segment was manually separated from the surrounding soil leaving only tightly adhered soil particles. The roots were then placed in 15 mL centrifuge tubes filled with 10 mL of deionized sterile water and inverted ten times to further displace residual soil from the roots. To harvest the root (endospheric) fraction, the roots were transferred to clean centrifuge tubes filled with 10 mL of detergent (0.1% Triton X-100 diluted in 1x TE) and shaken for two minutes. The roots were then transferred into clean centrifuge tubes filled with 10 mL of 80% ethanol and shaken for 30 seconds. The same step was repeated but replacing 80% ethanol with 2% bleach. Finally, roots were rinsed three times with deionized sterile water before they were dried on sterile filter papers and flash-frozen in liquid nitrogen and stored at -80 °C until further processing. To harvest the rhizospheric fraction, the soil wash-offs were centrifuged at 3000 × g for 10 min. The supernatants were discarded and the pellets were resuspended and transferred to clean 2 mL screwcap tubes. The tubes were centrifuged at 3000 × g for 10 min, after which the supernatant was discarded, and the pellets were flash-frozen in liquid nitrogen and stored in -80 °C for further processing. The similar protocol was applied for harvesting the root fraction of plants grown on ArtSoil. For rhizospheric fraction of plants grown on ArtSoil, a clean scalpel was used to scrape approximately 0.5 cm (wide) of agar along the surface where the roots grew. The agar scraps in clean microcentrifuge tubes were flash-frozen and stored at -80 °C for further processing.

### DNA extraction and library preparation

Total DNA was extracted from the aforementioned samples using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Solon, USA) or NucleoSpin Soil Mini kit for DNA from soil (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following instructions from the manufacturers. DNA samples were eluted in 50 µL nuclease-free water and used for microbial community profiling. DNA samples were used in a two-step PCR amplification protocol. In the first step, V4-V7 (799F: AACMGGATTAGATACCKG; 1192R: ACGTCATCCCCACCTTCC) of the bacterial 16S rRNA, was amplified. Under a sterile hood, each sample was amplified in triplicate in a 25 µL reaction volume containing 2 U DFS-Taq DNA polymerase, 1 × incomplete buffer (Bioron GmbH, Ludwigshafen, Germany), 2 mM MgCl<sub>2</sub>, 0.3% BSA, 0.2 mM dNTPs (Life technologies GmbH, Darmstadt, Germany) and 0.3 µM forward and reverse primers. PCR was performed using the following parameters: 94 °C/2 min, 94 °C/30 s, 55 °C/30 s, 72 °C/30 s, 72 °C/10 min for 25 cycles. Afterwards, single-stranded DNA and proteins were digested by adding 1 µL of Antarctic phosphatase, 1 µL Exonuclease I, and 2.44 µL Antarctic phosphatase buffer (New England BioLabs GmbH, Frankfurt, Germany) to 20 µL of the pooled PCR product. Samples were incubated at 37 °C for 30 min and enzymes were deactivated at 85 °C for 15 min. Samples were centrifuged for 10 min at 3,000 × g and 3 µL of this reaction were used for a second PCR, prepared in the same way as described above using the same protocol but with cycles reduced to 10 and with primers including barcodes and Illumina adapters.<sup>88</sup> PCR quality was controlled by loading 5 µL of each reaction on a 1% agarose gel and affirming that no band was detected within the negative control. Amplicon concentration was determined fluorescently (Quant-iT™ PicoGreen™, Invitrogen), and equivalent DNA amounts of each of the bar-coded amplicons were pooled in one library. Then, 80 µL of the pooled library was loaded in a 1.5% agarose gel and run for 2 h at 80 V. Subsequently, bands with a size of ~500 bp were cut out and purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). The final library concentration was estimated fluorescently (Quantus™ Fluorometer, Promega). Paired-end Illumina sequencing was performed in-house using the MiSeq sequencer and custom sequencing primers at the Max Planck Institute for Plant Breeding Research.

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Amplicon sequencing data analysis

Sequencing reads were demultiplexed using Qiime2 (qiime demux emp\_paired<sup>89</sup>), and merged using Flash2.<sup>90</sup> Reads were denoised and dereplicated using Dada2<sup>91</sup> and remaining individual reads were denoted as ASVs. Chimeras were removed using Qiime2 (vsearch uchime-denovo). Taxonomic classification was done via the Qiime feature classifier using the silva\_138 and sequences classified as mitochondrial or chloroplast were removed from the dataset. Remaining ASVs were included in count tables.

Bray-Curtis dissimilarities between samples were calculated normalized ASV tables and performed PCAs using the *cmdscale* function (vegan R package). To quantify the effects of genotype and root region we used a constrained PCoA using the *capscale* function (vegan R package). To quantify the contribution of different variables and their interactions to the variance in pairwise Bray-Curtis dissimilarities, we analysed the Bray-Curtis distance matrix between pairs of samples with 999 iterations of a permutation-based test (permutational multivariate analysis of variance (PERMANOVA), *adonis* function, vegan R package), and removed technical and batch effects, using the formula as follows: *Bray-curtis* ~ *VariableX* + *Condition(Technical\_replicates + Biological\_replicates)*. We further inspected the effects of the variables using a constrained PCoA using the *capscale* function (vegan R package). Sequencing data from the SynCom experiment was pre-processed similarly as natural community 16S rRNA data. Quality-filtered merged paired-end reads were aligned to a reference set of sequences extracted from the whole-genome assemblies of the 60 strains included in the SynCom using Rbec79 (v1.0.0). A count table that was employed for downstream analyses of diversity was generated in R (v4.0.3) with the R package *vegan* (v2.5–6). Scripts for microbiota analysis and data visualization can be found at [https://github.com/duranpa/sweet\\_collaboration](https://github.com/duranpa/sweet_collaboration)

### ANOVA for differential enrichment of metabolites

We applied the python package *statsmodels*<sup>92</sup> to perform two-way analysis of variance (ANOVA<sup>93</sup>). For a particular metabolite, we collected its mass spectrometry relative response in the WT or in *sweet* knockouts (line: WT vs. SWEET), and at different segments of the root (region: 2cm vs. 4cm vs. 6cm vs. whole). We fit a model that decomposes the relative response of the metabolite as a combination of line-specific and region-specific factors; this model is described by the following string in the code: '*relativeResponse* ~ *Line* + *Region*' and calculates a *P*-value for *Line* and one for *Region*. Using the *P*-value cutoff of 0.05 for *Line* (and *Region*), we determined the relative response of the metabolite to change significantly with *Line* (*Region*) if *P* < 0.05.

### Quantify metabolic activities using PAS

Pathway activity score (PAS) was introduced to quantify the activity of different metabolic pathways in single-cell transcriptomes.<sup>94,95</sup> It is designed with a permutation test along with a *P*-value to examine whether the gene expression of a pathway at a particular cell cluster is significantly higher or lower than the sample average. Since we are working with bulk RNA-seq and not scRNA-seq in the current study, this algorithm is modified, and the permutation test is no longer suitable and discarded.

There are 3 sources for pathways used in our analysis. (A) we obtained the pathway data of *Arabidopsis* from PlantCyc<sup>96</sup> (link to the tables <https://pmn.plantcyc.org/organism-summary?object=ARA>, downloaded on 18-NOV-2022). Pathways with patterns 'glucos', 'galactos', 'fructos', 'xylos', 'sucros', or 'maltos' in their name are considered relevant to sugar metabolism. (B) we are also interested in the potential to convert sucrose coming from the phloem into different sugars for secretion at different root segments, and these pathways are not clearly defined in PlantCyc. Thus, we applied flux balanced analysis to define the pathways of relevant metabolic reactions. See section 'Flux balance analysis (FBA) to define pathways' for the details of the algorithm. Table S6 shows the genes involved in each pathway. (C) we also assign the SWEET and SUC genes to their own single-gene-pathway to facilitate our analysis. This means the gene SWEET1 is assigned to a new pathway 'SWEET1', and so on.

Brady et al. performed bulk transcriptomics on different segments of *Arabidopsis* root.<sup>11</sup> The experiment involved two replicate roots. In the published dataset, the 13 segments of root 1 are labeled as: *LCOLUMELLASB*, *L1SB*, *L2SB*, ..., *L11SB*, *L12SB*; the 12 segments of root 2 are labeled as: *Slice1JW*, *Slice2JW*, ..., *Slice11JW*, *Slice12JW*. We found that the two roots are in different developmental stages, therefore we dropped root 2 and used only root 1 in our analysis.

Given the matrix of gene expression across different samples, we normalized the data using trimmed mean of M values (TMM) normalization.<sup>97</sup> In practice, this is implemented by the function *calcNormFactors* within the R package *edgeR*.<sup>98</sup> We used the function argument *method*="TMM" to call TMM and set *logratioTrim*=0.3.

Let us denote  $g_{i,j}$  to be the normalized read count of gene *i* in sample *j*. The read count of a gene is normalized to give the relative transcript level, which is 1 when averaged over different samples. Mathematically, the relative transcript level of gene *i* at sample *j* is denoted as  $r_{i,j}$ , and is defined as  $r_{i,j} = g_{i,j} / \left( \frac{1}{N} \sum_k g_{i,k} \right)$ , where *N* is the total number of samples, and the label *k* goes over all samples.

The PAS of pathway *t* at sample *j* is denoted as  $p_{t,j}$ , which is a weighted average of the relative transcript levels across the genes of the pathway:  $p_{t,j} = \sum_{i=1}^{m_t} w_i r_{i,j} / \sum_{i=1}^{m_t} w_i$ . Here  $m_t$  is the number of genes in pathway *t*, and  $w_i$  is the weight of gene *i*, defined as the reciprocal of the number of pathways that gene *i* is involved in. Because  $r_{i,j}$  is centered around 1, so do  $p_{t,j}$ . Thus, if  $p_{t,j} > 1$ , the expression of genes associated with pathway *t* in sample *j* is higher than the average over all samples, and vice versa.

### Flux balance analysis (FBA) to define pathways

As we suspect that the root may convert sucrose into other sugars before secretion, we would like to find out the genes involved in these conversion pathways for further analysis. To assign genes in the metabolic network to these pathways, we applied FBA,<sup>99</sup> which searches for the combination of genes and reactions that results in the highest yield for such conversions, assuming sucrose to be the sole input carbon source of the metabolic network.

We used the AraGEM<sup>100</sup> a curated FBA model of *Arabidopsis*, to perform our simulations and define the genes associated with each conversion pathway. The AraGEM model is published with 3 different sets of parameters that correspond to their own case of metabolism: (a) photosynthesis, (b) photorespiration, and (c) non-photosynthetic cell. Case (c) is relevant to the root condition,



as it considers the conversion of sucrose into other biomass metabolites. Hence, our simulations are developed from case (c), which uses sucrose as the input carbon source.

We switched off the default objective function of AraGEM, which includes a set of metabolites with weights found in real plants. To investigate the conversion of sucrose into different sugars, we set different monosaccharides, disaccharides and combinations of monosaccharides as the objective; these include all possible monosaccharides and disaccharides in AraGEM. In total, we analyzed 11 different objective functions: (1) maltose, (2) glucose, (3) beta-D-fructose, (4) D-galactose, (5) D-xylose, (6) glucose + beta-D-fructose, (7) glucose + D-galactose, (8) glucose + D-xylose, (9) beta-D-fructose + D-galactose, (10) beta-D-fructose + D-xylose, (11) D-galactose + D-xylose. Note that the molecules within objective functions have equal weights, as some of them have multiple sugar molecules.

We used the python package COBRApy<sup>101</sup> along with the GUROBI solver (Gurobi Optimizer Version 3.0. Houston, Texas: Gurobi Optimization, Inc., April 2010. (software program)) to perform FBA simulation. For each of the 11 objective functions, we performed parsimony-FBA simulation<sup>102</sup> to find out the most efficient reactions that perform the conversion of sucrose into the objective sugars. We also calculated the shadow price<sup>103</sup> of each metabolite involved in these reactions; a metabolite with negative shadow price is deemed critical, as an extra supply of this metabolite leads to a higher flux of the objective function. Reactions that have critical metabolites both at their input and output are also considered critical. We collected all the critical reactions of an objective function, and the genes associated with any critical reactions are assigned to the pathway (refer to [Table S6](#) for the genes in these pathways).

### Correlation between microbes and metabolites

We calculated the correlation between every pair of microbes and metabolites to reveal the potential cross feeding between the microbe species. At each combination of plant lineages (WT / SWEET2 / SWEET4 / SWEET11,22) and root segments (2cm / 4cm / 6cm / whole root), we performed 16s RNA sequencing to quantify the abundance of different microbial classes and also mass spectrometry to measure the abundance of different metabolites.

For each pair of microbe class and metabolite, we calculated their spearman correlation across different plant lineages and root locations to construct the correlation matrix; matrix elements that correspond to p-value >0.05 are deemed insignificant and replaced with 0 (i.e., not connected in a complex network). We used the iGraph package of R to display this bipartite network.<sup>104</sup> Communities within this network are detected using the label propagation algorithm.<sup>105</sup>

Reference ID: KRT64abe6afd69ae