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## Leaf-associated microbiomes of grafted tomato plants

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Bacteria and fungi form complex communities (microbiomes) in above- and below-ground organs of plants, contributing to hosts' growth and survival in various ways. Recent studies have suggested that host plant genotypes control, at least partly, plant-associated microbiome compositions. However, we still have limited knowledge of how microbiome structures are determined in/on grafted crop plants, whose above-ground (scion) and below-ground (rootstock) genotypes are different with each other. By using eight varieties of grafted tomato plants, we examined how rootstock genotypes could determine the assembly of leaf endophytic microbes in field conditions. An Illumina sequencing analysis showed that both bacterial and fungal community structures did not significantly differ among tomato plants with different rootstock genotypes: rather, sampling positions in the farmland contributed to microbiome variation in a major way. Nonetheless, a further analysis targeting respective microbial taxa suggested that some bacteria and fungi could be preferentially associated with particular rootstock treatments. Specifically, a bacterium in the genus *Deinococcus* was found disproportionately from ungrafted tomato individuals. In addition, yeasts in the genus *Hannaella* occurred frequently on the tomato individuals whose rootstock genotype was "Ganbarune". Overall, this study suggests to what extent leaf microbiome structures can be affected/unaffected by rootstock genotypes in grafted crop plants.

In both natural and agricultural ecosystems, bacteria and fungi in diverse taxonomic groups are associated with plants, positively and/or negatively influencing the survival and growth of their hosts<sup>1-4</sup>. An increasing number of studies have shown that plant-associated microbes not only improve nutritional conditions of host plants but also increase plants' resistance to abiotic stresses (e.g., high temperature, drought, and soil pollution) and that to pathogens and pests<sup>5-8</sup>. In contrast, bacterial and fungal communities associated with plants can be regarded as serious risk factors in agriculture and forestry because they are occasionally dominated by plant pathogenic species or strains<sup>9,10</sup>. Therefore, controlling plant-associated microbiomes has been recognized as a major challenge towards the development of stable and sustainable management of crop fields and plantations<sup>11-14</sup>.

Host plant genotypes, along with external environmental conditions, are important factors determining microbiome structures<sup>15-18</sup>. Developing disease-resistant crop plant varieties has been one of the major goals in breeding science<sup>19-21</sup>. Moreover, recent studies have explored genes and mutations influencing whole microbiome structures<sup>22,23</sup>, providing a basis for optimizing communities of plant-growth-promoting bacteria and/or fungi. Meanwhile, to gain more insights into mechanisms by which plant microbiomes are controlled, studies using plant individuals with complex genetic backgrounds have been awaited. Specifically, by using grafted plants, whose above- and below-ground genotypes are different with each other, we will be able to examine, for instance, how below-ground genetic factors control above-ground microbiome structures. Because root genotypes can control not only uptake of water and nutrients but also transport of phytohormones or signaling molecules<sup>24-26</sup>, their effects on leaf physiology potentially influence community compositions of endophytic and epiphytic microbes in above-ground plant tissue. Although studies focusing on such mechanisms interlinking above- and below-ground processes can provide essential insights into plants' microbiome control, few attempts<sup>27,28</sup>, to our knowledge, have been made to conduct experiments using grafted plants.

Grafting *per se* is a classic technique but it has been increasingly considered as a promising method for increasing yield, crop quality, abiotic stress resistance, and pathogen resistance of various plants (e.g., tomato, melon, grapevine, apple, and citrus) in agriculture<sup>29-33</sup>. In general, performance of grafted plants depends greatly on

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compatibility between scion and rootstock genotypes<sup>34–36</sup>. However, we still have limited knowledge of how scion–rootstock genotypic combinations determine microbiome structures in leaves, roots, and other plant organs<sup>27</sup>. Moreover, although some pioneering studies have investigated microbial community compositions of grafted plants<sup>37–39</sup>, most of them focused on subsets of microbiomes (i.e., either bacteria or fungi but not both). Therefore, new lines of studies examining relationships between scion/rootstock genotypes and whole microbiome structures in roots/leaves have been awaited.

In this study, we evaluated how below-ground genotypes of plants determine bacterial and fungal community structures in/on leaves under field conditions. After growing grafted tomato [*Solanum lycopersicum* (= *Lycopersicon lycopersicum*)] individuals in a field experiment, we analyzed the leaf microbial community compositions of the sampled tomatoes based on Illumina sequencing. The contributions of below-ground genotypes on the leaf microbiome structures were then evaluated by comparing the microbial community datasets of eight tomato rootstock varieties. We also performed randomization-based statistical analyses to explore bacterial and fungal taxa that had strong signs of preferences for specific tomato rootstock varieties. Overall, this study suggests to what extent below-ground genotypes of plants influence plant–microbe associations in leaves, providing a basis for managing microbiomes of grafted plants in agriculture and forestry.

## Methods

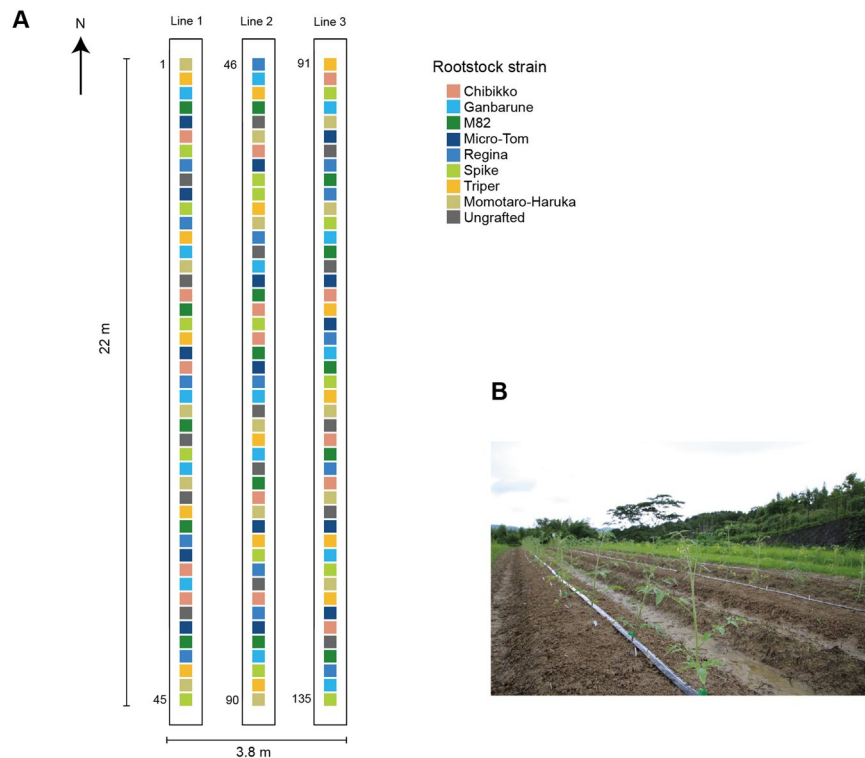
**Grafted tomato seedlings.** To prepare rootstocks, seeds of eight tomato varieties (“Chibikko”, “Ganbarune”, “M82”, “Micro-Tom”, “Regina”, “Spike”, “Triper”, and “Momotaro-Haruka”; see Supplementary Table 1 for characteristics of respective varieties) were sown on June 7, 2017 for “Momotaro-Haruka” and June 1, 2017 for the others, and then the pots were grown in a greenhouse of Togo Field, Nagoya University, Nagoya, Japan (35.112°N; 137.083°E). To reproduce conventional agricultural conditions for raising seedlings, the seeds were sown in 6-cm pots filled with commercially available potting soil [Hanachan-baiyodo, (Hanagokoro Co., Ltd., Nagoya): Vermiculite GS (NITTAI Co., Ltd., Osaka) = 1:1]. On June 22–23, seedlings for the field experiment detailed below were produced by grafting “Momotaro-Haruka” scions on each of the eight varieties of rootstocks: i.e., above-ground parts of the grafted seedlings were all Momotaro-Haruka, while below-ground parts differed among seedling individuals. Ungrafted “Momotaro-Haruka” seedlings were also prepared as control samples. The grafted (including Momotaro-Haruka/Momotaro-Haruka self-grafted seedlings) and ungrafted seedlings (in total, nine treatments) were grown in a greenhouse of Togo Field and, on July 7, they were transported to Center for Ecological Research, Kyoto University, Kyoto, Japan (34.972°N; 135.958°E). Each seedling was then transferred to a 9-cm pot filled with commercially-available culture soil (Rakuyo Co., Ltd., Kyoto) on the day and they were kept on the field nursery shelf of Center for Ecological Research until the field experiment.

**Field transplantation.** On July 13, base fertilizer was provided to the soil in the experimental field of Center for Ecological Research (N = 13.6 g/m<sup>2</sup>; P<sub>2</sub>O<sub>5</sub> = 13.6 g/m<sup>2</sup>; K<sub>2</sub>O = 13.6 g/m<sup>2</sup>). On July 25, the abovementioned seedlings (ca. 50 cm high) were transplanted to the open field at 50 cm horizontal intervals in three lines in a randomized order (9 seedling treatment × 5 replicates per line × 3 lines (sets) = 135 individuals; Fig. 1). The tomato individuals were watered twice (morning and evening) every day. On September 13, a ca. 1-cm<sup>2</sup> disc of a mature leaf was sampled from each tomato individual and placed in a 2-mL microtube. The leaf samples were transferred to a laboratory of Center for Ecological Research using a cool box and they were then preserved at –80 °C in a freezer until DNA extraction.

**DNA extraction, PCR, and sequencing.** Each leaf disc was immersed in ×1/100 NaClO (Nacalai Tesque; 31518-35) for 1 min and it was subsequently washed in 70% ethanol. DNA extraction was extracted with a cetyltrimethylammonium bromide (CTAB) method after pulverizing the leaves with 4 mm zirconium balls at 25 Hz for 3 min using a TissueLyser II (Qiagen).

For each leaf disc sample, the 16S rRNA V4 region of the prokaryotes and the internal transcribed spacer 1 (ITS1) region of fungi were PCR-amplified. The PCR of the 16S rRNA region was performed with the forward primer 515f<sup>40</sup> fused with 3–6-mer Ns for improved Illumina sequencing quality<sup>41</sup> and the forward Illumina sequencing primer (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG- [3–6-mer Ns] - [515f] -3') and the reverse primer 806rB<sup>42</sup> fused with 3–6-mer Ns and the reverse sequencing primer (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G [3–6-mer Ns] - [806rB] -3') (0.2 μM each). To inhibit the PCR-amplification of mitochondrial and chloroplast 16S rRNA sequences of host plants, specific peptide nucleic acids [mPNA and pPNA<sup>41</sup>] (0.25 μM each) were added to the reaction mix of KOD FX Neo (Toyobo). To reduce the proportion of host mitochondrial/chloroplast reads to prokaryote sequencing reads through selective amplification, the number of PCR cycles was set to 35. The temperature profile of the PCR was 94 °C for 2 min, followed by 35 cycles at 98 °C for 10 s, 78 °C for 10 s, 60 °C for 30 s, 68 °C for 50 s, and a final extension at 68 °C for 5 min. To prevent generation of chimeric sequences, the ramp rate through the thermal cycles was set to 1 °C/sec.<sup>43</sup> Illumina sequencing adaptors were then added to respective samples in the supplemental PCR using the forward fusion primers consisting of the P5 Illumina adaptor, 8-mer indexes for sample identification<sup>44</sup> and a partial sequence of the sequencing primer (5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC AC - [8-mer index] - TCG TCG GCA GCG TC-3') and the reverse fusion primers consisting of the P7 adaptor, 8-mer indexes, and a partial sequence of the sequencing primer (5'-CAA GCA GAA GAC GGC ATA CGA GAT - [8-mer index] - GTC TCG TGG GCT CGG-3'). KOD FX Neo was used with a temperature profile of 94 °C for 2 min, followed by 8 cycles at 98 °C for 10 s, 55 °C for 30 s, 68 °C for 50 s (ramp rate = 1 °C/s), and a final extension at 68 °C for 5 min.

The PCR amplicons of the 135 tomato individuals (and negative control samples) were then pooled after a purification/equalization process with the AMPureXP Kit (Beckman Coulter). Primer dimers were removed from the pooled library by a supplemental AMPureXP purification process, in which the ratio of AMPureXP reagent to the pooled library was set to 0.6 (v/v).



**Figure 1.** Field site. **(A)** Nine tomato rootstock varieties (treatments) in the field. For each rootstock variety, 15 replicate samples were transplanted to the field site (15 replicates  $\times$  9 varieties = 135 tomato individuals). The above-ground parts of all the 135 tomato individuals had the genotype of the tomato variety “Momotaro-Haruka”. **(B)** Transplanted tomato individuals.

The PCR of the fungal ITS1 region was performed with the forward primer ITS1F\_KYO1<sup>45</sup> fused with 3–6-mer Ns for improved Illumina sequencing quality<sup>41</sup> and the forward Illumina sequencing primer (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG- [3–6-mer Ns] - [ITS1F\_KYO1] -3′) and the reverse primer ITS2\_KYO2<sup>45</sup> fused with 3–6-mer Ns and the reverse sequencing primer (5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G [3–6-mer Ns] - [ITS2\_KYO2] -3′). The PCR was performed based on the buffer and polymerase system of KOD FX Neo with a temperature profile of 94 °C for 2 min, followed by 35 cycles at 98 °C for 10 s, 58 °C for 30 s, 68 °C for 50 s, and a final extension at 68 °C for 5 min. Illumina sequencing adaptors and 8-mer index sequences were added in the additional PCR and then the amplicons were purified and pooled as described above.

The sequencing libraries of the prokaryote 16S and fungal ITS regions were processed in an Illumina MiSeq sequencer (run center: KYOTO-HE; 15% PhiX spike-in). In general, quality of forward sequence data is generally higher than that of reverse sequence data in Illumina sequencing. Therefore, we optimized the settings of the Illumina sequencing run by targeting only forward sequences. Specifically, the numbers of the forward and reverse cycles were set 271 and 31, respectively: the reverse sequences were used only for discriminating between 16S and ITS1 sequences *in silico* based on the sequences of primer positions. Note that similar results of molecular taxonomic assignment have been obtained for 200 bp 16S/ITS sequences and for longer 16S/ITS sequences in a comprehensive benchmark analysis with the bioinformatic pipeline detailed below<sup>46</sup>.

**Bioinformatics.** The raw sequencing data were converted into FASTQ files using the Illumina’s program bcl2fastq 1.8.4. The obtained FASTQ files were demultiplexed with the program Claident v0.2.2018.05.29<sup>46,47</sup>, by which sequencing reads whose 8-mer index positions included nucleotides with low (<30) quality scores were removed. The sequencing data were deposited to DNA Data Bank of Japan (DDBJ) (DDBJ Sequence Read Archive accession: DRA007061). Only forward sequences were used in the following analyses after trimming low-quality 3′-end sequences using Claident: each sequencing read was trimmed to the point at which the quality values of three consecutive nucleotides were 30 or higher. Noisy reads<sup>47</sup> were subsequently discarded and then denoised dataset consisting of 1,201,840 16S and 1,730,457 ITS1 reads were obtained.

For each region (16S or ITS1), filtered reads were clustered with a cut-off sequencing similarity of 97% using the program VSEARCH<sup>48</sup> as implemented in Claident. The operational taxonomic units (OTUs) representing less than 10 sequencing reads were discarded and then the molecular identification of the remaining OTUs was performed based on the combination of the query-centric auto-*k*-nearest neighbor (QCauto) algorithm of reference database search<sup>46</sup> and the lowest common ancestor (LCA) algorithm of taxonomic assignment<sup>49</sup> as implemented in Claident. Note that taxonomic identification results based on the QCauto-LCA pipeline are comparable to, or sometimes more accurate than, those with alternative approaches<sup>46,50,51</sup>. In total, 143 prokaryote (bacterial or

archaeal) OTUs and 529 fungal OTUs were obtained for the 16S and ITS1 regions, respectively (Supplementary Data 1). The UNIX codes used in the above bioinformatic pipeline are provided as Supplementary Data 2.

For each target region (16S or ITS1), we obtained a sample  $\times$  OTU matrix, in which a cell entry depicted the number of sequencing reads of an OTU in a sample (Supplementary Data 3). To minimize effects of PCR/sequencing errors, cell entries whose read counts represented less than 0.1% of the total read count of each sample were removed (cf. ref.<sup>52</sup>). The filtered matrix was then rarefied to 500 reads per sample using the “rarefy” function of the vegan 2.4–5 package<sup>53</sup> of R 3.4.3<sup>54</sup>. Samples with less than 500 reads were discarded in this process. In total, the rarefied matrices of the 16S and ITS1 regions included 125 and 132 samples, respectively: at least 13 replicate samples per treatment were retained in both datasets (Supplementary Data 4).

**Microbiome structure.** Relationship between the number of sequencing reads and that of prokaryote/fungal OTUs was examined for each dataset (16S or ITS1) with the vegan “rarecurve” function of R. Likewise, relationship between the number of samples and that of OTUs was examined with the vegan “specaccum” function. For each dataset, difference in order- or genus-level community compositions among seedling treatments (rootstock varieties) was examined by the permutational analysis of variance (PERMANOVA<sup>55</sup>) with the vegan “adonis” function (10,000 permutations). To control spatial effects in the field experiment data, the information of replicate sample sets (Fig. 1) was included as an explanatory variable in the PERMANOVA. The “Raup-Crick” metric<sup>56</sup> was used to calculate  $\beta$ -diversity based on the order- or genus-level data matrices (Supplementary Data 5).

To explore prokaryote/fungal taxa whose occurrences on tomato individuals were associated with rootstock varieties, a series of analysis of variance (ANOVA) was performed. Specifically, based on the genus-level matrix of the 16S or ITS1 dataset (Supplementary Data 5), an ANOVA model was constructed for each prokaryote/fungal genus by including the proportion of the sequencing reads of the target genus and the rootstock variety information of host tomatoes as response and explanatory variables, respectively. The information of replicate samples (i.e., location information) was included as an additional explanatory variable. Genera that occurred in less than 30 tomato individuals were excluded from the analysis.

**Host-genotype preferences.** We further explored prokaryote/fungal taxa showing preferences for specific rootstock varieties based on a randomization analysis. In the sample  $\times$  genus matrix of the 16S or ITS1 dataset (Supplementary Data 5), the labels of rootstock varieties were shuffled (100,000 permutations) and then preference of a prokaryote/fungal genus ( $i$ ) for a rootstock variety ( $j$ ) was evaluated as follows:

$$Preference(i, j) = [N_{\text{observed}}(i, j) - \text{Mean}(N_{\text{randomized}}(i, j))]/SD(N_{\text{randomized}}(i, j)),$$

where  $N_{\text{observed}}(i, j)$  denoted the mean number of the sequencing reads of genus  $i$  across rootstock variety  $j$  tomato samples in the original data, and the Mean ( $N_{\text{randomized}}(i, j)$ ) and SD ( $N_{\text{randomized}}(i, j)$ ) were the mean and standard deviation of the number of sequencing reads for the focal genus–rootstock combination across randomized matrices. Genera that occurred in 30 or more tomato individuals were subjected to the randomization analysis.

For the genera that showed preferences for specific tomato rootstock varieties, we performed an additional analysis to evaluate which bacterial/fungal OTUs in each genus had strong host-variety preferences. Specifically, the randomization analysis of the above preference index (100,000 permutations) was applied to rarefied sample  $\times$  OTU matrix of the 16S or ITS1 dataset (Supplementary Data 4). OTUs that occurred in less than 30 tomato individuals were excluded from the analysis.

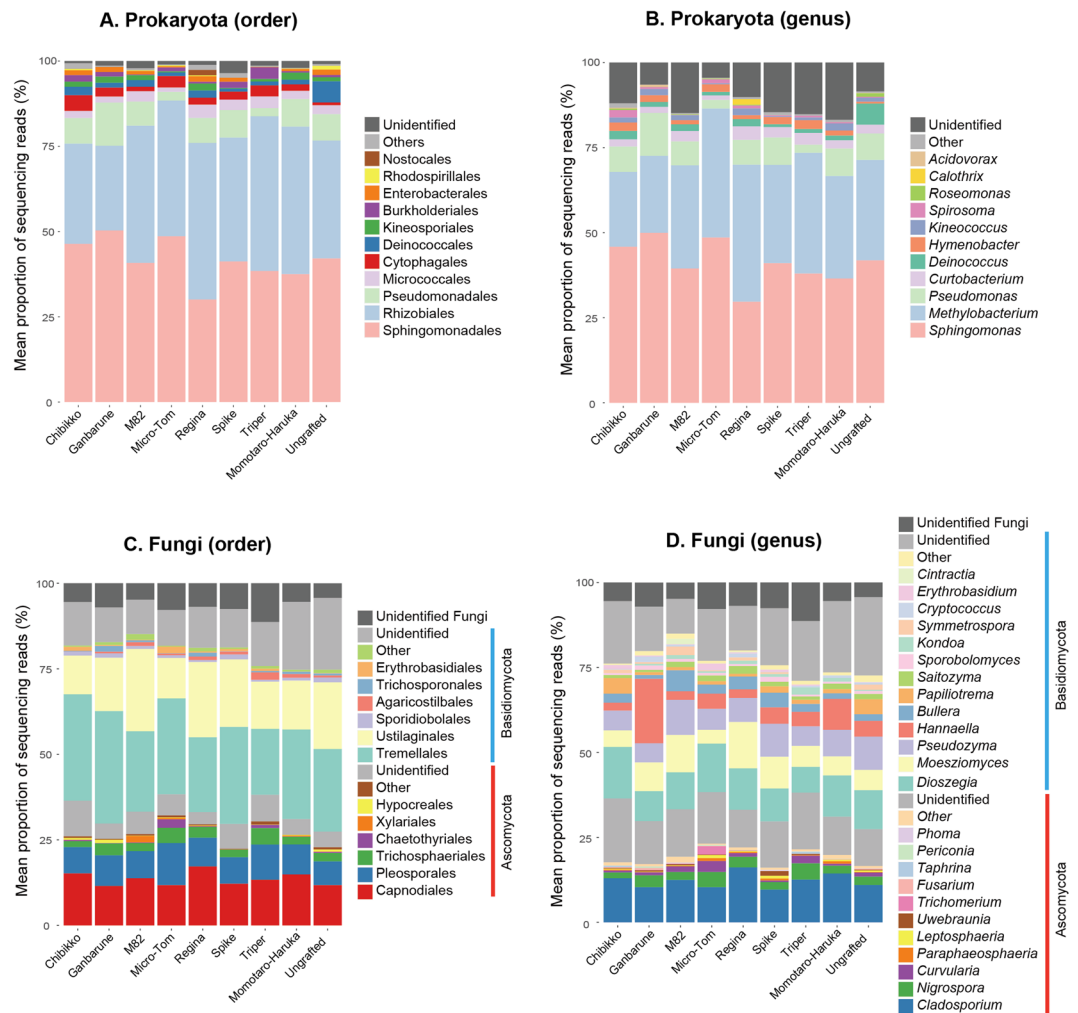
## Results

**Microbiome properties.** On average, 13.6 (SD = 4.2) prokaryote and 26.3 (SD = 9.4) fungal OTUs per sample were observed in the rarefied data matrices (Supplementary Fig. 1). The total numbers of prokaryote and fungal OTUs included in the rarefied datasets were 116 and 413, respectively (Supplementary Data 4). All the prokaryote OTUs belonged to Bacteria: no archaeal OTUs were observed.

In the bacterial community of the tomato leaves, bacteria in the orders Sphingomonadales and Rhizobiales were dominant (Fig. 2A). Bacteria in the order Pseudomonadales were frequently observed, too, across the tomato varieties examined. Meanwhile, bacteria in the order Deinococcales were abundant only in the ungrafted tomato individuals (Fig. 2A). At the genus-level, the genera *Sphingomonas*, *Methylobacterium*, and *Pseudomonas* were frequently observed across the rootstock varieties examined, while *Deinococcus* bacteria were abundant only in the ungrafted tomatoes (Fig. 2B).

In the leaf-associated fungal community, ascomycete fungi in the orders Capnodiales and Pleosporales and the basidiomycete fungi in the orders Tremellales and Ustilaginales were abundant (Fig. 2C). At the genus-level, *Cladosporium*, *Dioszegia*, *Moesziomyces* (anamorph = *Pseudozyma*), and *Hannaella* were frequently observed (Fig. 2D). Among them, *Hannaella* fungi dominated the leaf-associated fungal community of the tomato rootstock variety “Ganbarune” (the proportion of *Hannaella* reads = 19.0%), while their proportion was relatively low on other host varieties (2.3–9.1%; Fig. 2D).

A statistical test based on PERMANOVA showed that replicate sampling positions, but not tomato rootstock varieties, significantly explained variation in the whole structure of the bacterial/fungal community (Table 1). However, further analyses targeting respective genera (Tables 2 and 3) indicated that the proportion of the fungal genus *Hannaella* varied among tomato rootstock varieties, although the pattern was non-significant after a Bonferroni correction of  $P$  values. Meanwhile, the proportion of some taxa such as the bacterial genus *Sphingomonas* and the fungal genus *Cladosporium* varied significantly among replicates (Tables 2 and 3), suggesting that spatial positions in the experimental field affected the formation of the leaf-associated microbiomes of the tomato plants.



**Figure 2.** Structure of the leaf-associated microbial communities. The leaf-associated microbial community compositions were compared among tomato individuals with different rootstock genotypes. **(A)** Order-level community structure of prokaryotes. **(B)** Genus-level community structure of prokaryotes. **(C)** Order-level community structure of fungi. **(D)** Genus-level community structure of fungi.

Taxon	Taxonomic level	Variable	df	$F_{\text{model}}$	$R^2$	$P$
Prokaryotes	Order	Variety	8	1.0	0.061	0.4731
		Location	14	1.6	0.173	0.0379
	Genus	Variety	8	1.1	0.064	0.3733
		Location	14	2.1	0.207	0.0035
Fungi	Order	Variety	8	0.6	0.033	0.7509
		Location	14	2.2	0.213	0.0119
	Genus	Variety	8	0.9	0.050	0.5586
		Location	14	1.9	0.185	0.0350

**Table 1.** Effects of rootstock varieties and spatial positions on the entire microbial community structure. A PERMANOVA was conducted for each target community (prokaryotes or fungi) at each taxonomic level (order or genus). The rootstock varieties of host tomato and spatial positions in the field (location; Fig. 1A) were considered as explanatory variables.

**Host-genotype preferences.** A randomization analysis showed that the bacterial genus *Deinococcus* occurred preferentially on the ungrafted tomato individuals (Fig. 3A; Table 4). Likewise, the fungal genus *Hannaella* showed preferences for the rootstock variety “Ganbarune” (Fig. 3B). In an additional randomization analysis, a bacterial OTU phylogenetically allied to *Deinococcus citri* (P\_040) and fungal OTUs allied to

Genus	Variety			Location		
	df	F	P	df	F	P
<i>Curtobacterium</i>	8	0.3	0.9710	14	1.1	0.3260
<i>Deinococcus</i>	8	1.8	0.0944	14	1.3	0.2386
<i>Hymenobacter</i>	8	0.5	0.8730	14	1.1	0.3900
<i>Kineococcus</i>	8	0.7	0.6710	14	0.6	0.8970
<i>Methylobacterium</i>	8	1.7	0.0986	14	2.0	0.0229
<i>Pseudomonas</i>	8	1.7	0.1060	14	0.6	0.8490
<i>Sphingomonas</i>	8	2.0	0.0538	14	3.2	0.0004
<i>Spirosoma</i>	8	1.0	0.4230	14	1.0	0.4310

**Table 2.** Effects of rootstock varieties and spatial positions on the proportion of each prokaryote genus in the community data. For each prokaryote genus, an ANOVA model of the mean proportion of sequencing reads was constructed by including the rootstock varieties of host tomato and spatial positions in the field (location; Fig. 1A) as explanatory variables. Genera that occurred in 30 or more tomato individuals were subjected to the analysis.

Genus	Variety			Location		
	df	F	P	df	F	P
<i>Bullera</i>	8	0.8	0.5740	14	1.0	0.4570
<i>Cladosporium</i>	8	0.7	0.6752	14	2.4	0.0051
<i>Cryptococcus</i>	8	1.1	0.3830	14	1.0	0.4620
<i>Curvularia</i>	8	1.3	0.2640	14	0.8	0.6470
<i>Dioszegia</i>	8	0.4	0.9390	14	1.1	0.3670
<i>Hannaella</i>	8	2.3	0.0281	14	0.8	0.7046
<i>Kondoa</i>	8	1.0	0.4730	14	0.8	0.6720
<i>Leptosphaeria</i>	8	1.1	0.3660	14	1.4	0.1820
<i>Moesziomyces</i>	8	1.5	0.1507	14	1.6	0.0833
<i>Nigrospora</i>	8	0.7	0.7050	14	1.2	0.3240
<i>Papiliotrema</i>	8	1.5	0.1720	14	0.7	0.7450
<i>Paraphaeosphaeria</i>	8	0.7	0.6570	14	1.0	0.4990
<i>Pseudozyma</i>	8	0.5	0.8690	14	0.5	0.9500
<i>Saitozyma</i>	8	0.2	0.9800	14	1.1	0.3890
<i>Sporobolomyces</i>	8	0.5	0.8504	14	1.8	0.0475

**Table 3.** Effects of rootstock varieties and spatial positions on the proportion of each fungal genus in the community data. For each fungal genus, an ANOVA model of the mean proportion of sequencing reads was constructed by including the rootstock varieties of host tomato and spatial positions in the field (location; Fig. 1A) as explanatory variables. Genera that occurred in 30 or more tomato individuals were subjected to the analysis.

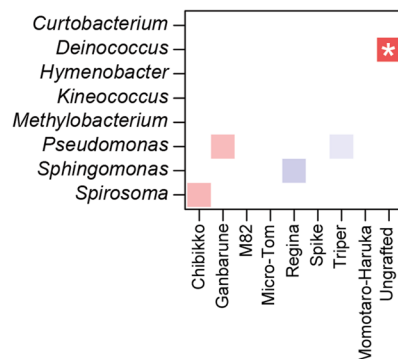
*Hannaella oryzae* (F\_427 and F\_428) displayed preferences for ungrafted and “Ganbarune” tomato plants, respectively (Table 4).

## Discussion

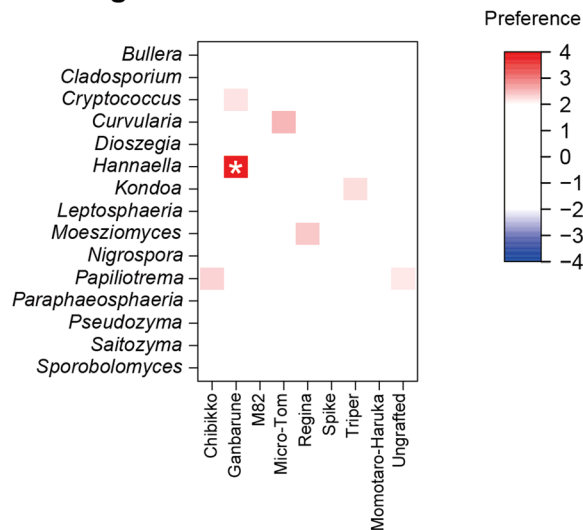
The field experiment using eight tomato rootstock varieties suggested that below-ground plant genotypes did not significantly affect the entire structures of the leaf-associated microbiomes (Table 1). However, detailed analyses suggested the existence of leaf microbial taxa whose associations with host plants might be affected by below-ground plant genotypes (Figs 2 and 3; Tables 2–4). Thus, along with recent studies on tomato-associated microbiomes<sup>28,57</sup>, this study provides a starting point for evaluating to what extent leaf-endophytic microbiome structures of grafted crop plants are affected/unaffected by rootstock varieties.

The leaf-associated bacterial communities of the tomato individuals analyzed in this study were dominated by Alphaproteobacteria (e.g., *Sphingomonas* and *Methylobacterium*) as well as Gammaproteobacteria (e.g., *Pseudomonas*) as has been reported in previous studies on crop and non-crop plants<sup>1,2,58</sup> (Fig. 2). Among the dominant bacteria, *Pseudomonas* is recognized mainly as plant pathogenic taxon<sup>59,60</sup>, although some *Pseudomonas* species are known to suppress leaf fungal pathogens by producing antibiotics<sup>61,62</sup>. The genus *Sphingomonas* is known to involve species that protect host plants against *Pseudomonas* pathogens<sup>63,64</sup> or promote plant growth by producing phytohormones such as gibberellins and indole acetic acid<sup>65</sup>. Bacteria in the genus *Methylobacterium* are often localized around stomatal pores on leaves<sup>66</sup>, using plant-derived methanol as principal carbon source<sup>67–70</sup>. Genomic studies have shown that *Methylobacterium* genomes involve genes of metabolic pathways that potentially contribute to host plant growth (e.g., auxin biosynthesis, cytokine biosynthesis, and vitamin B<sub>12</sub>

## A. Prokaryotes



## B. Fungi



**Figure 3.** Randomization analysis of preferences for rootstock varieties. An asterisk indicates significant preference index score in a combination of a microbial genus and a host rootstock variety (Bonferroni correction for each OTU compared across nine rootstock conditions;  $\alpha = 0.05$ ). (A) Prokaryote genera. (B) Fungal genera.

biosynthesis<sup>71</sup>. *Methylobacterium* is also known to induce resistance of plants against fungal pathogens, nominated as prospective a biocontrol agent<sup>72</sup>. Thus, these dominant bacteria, whose associations with hosts are likely irrespective of host below-ground genotypes (Fig. 2), may affect physiological conditions of tomato plants both positively and negatively.

Our data also indicated that fungi in the ascomycete genus *Cladosporium* and the basidiomycete genera *Dioszegia* and *Moesziomyces* (anamorph = *Pseudozyma*) were abundant within the leaf-associated microbiome of tomato plants (Fig. 2). Among them, *Cladosporium* involves a well-characterized pathogenic species, *C. fulvum*, which causes tomato leaf mold<sup>73–76</sup>. The basidiomycete taxa listed above are characterized by their anamorphic yeast forms and they have been observed in leaves of various plant species<sup>77–80</sup>. For example, *Dioszegia*, a basidiomycete taxon in the order Tremellales, has been reported from cereal and *Arabidopsis*<sup>80,81</sup>, potentially playing key roles within microbe–microbe interaction webs in leaf-associated microbiomes<sup>11</sup>. The genus *Moesziomyces* is represented by plant-pathogenic smut fungi<sup>82</sup>. However, a recent phylogenetic study of teleomorph ( *Moesziomyces*) and anamorphic (*Pseudozyma*) specimens<sup>77</sup> suggested that this Ustilaginaceae taxon could involve not only phytopathogenic species but also species with antifungal properties against the causal agent of cucumber powdery mildew (*Podosphaera fuliginea*)<sup>83</sup> or species that can induce resistance of host plants against fungal pathogens such as *Botrytis cinerea*<sup>84</sup>. Thus, the community data, as a whole, suggest that not only dominant bacterial taxa but also various fungal taxa potentially play complex physiological roles in tomato leaves.

While there were bacterial and fungal taxa commonly associated with tomato plants irrespective of host below-ground genotypes, fungi in the genus *Hannaella* displayed signs of preferences for rootstock genotypes (Fig. 3; Tables 3 and 4). Specifically, *Hannaella* was the most abundant fungal taxon in the tomato individuals whose rootstock genotype was “Ganbarune” (Fig. 2). Like other yeast taxa in Tremellaceae (e.g., *Dexomyces* and *Dioszegia*)<sup>85</sup>, *Hannaella* yeasts are frequently observed in the phyllosphere of various plant species<sup>86–89</sup>. Some *Hannaella* species are known to produce indol acetic acid<sup>87,90</sup>, although a study has suggested that the yeasts do not necessarily promote plant growth<sup>90</sup>. Therefore, it remains a challenge to understand how *Hannaella* yeasts interact with other yeasts and bacterial/fungal species in/on plant leaves and how they influence plant performance host-genotype specifically.

The randomization analysis performed in this study also implied that a bacterial OTU, which are phylogenetically allied to the *Deinococcus* species isolated from leaf canker lesions of citrus trees<sup>91</sup>, was preferentially associated with ungrafted tomato individuals (Fig. 3; Tables 2 and 4). Given that this bacterial OTU was rarely observed in self-grafted tomato individuals (Fig. 2), grafting treatment *per se*, rather than plant genotypes, could be responsible for the biased distribution of the bacterium. This finding is of particular interest because *Deinococcus* is famous for its high tolerance to desiccation<sup>92,93</sup>. Potential physiological effects of this bacteria on host plants deserve some inoculation experiments.

Although this study provides some implications for how leaf-associated microbiomes of grafted plants can be influenced by rootstock genotypes, potential pitfalls of the present results should be taken into account. First, as our data were based on snapshot sampling in the late growing season of tomato, we are unable to infer the timing at which the observed bacteria and fungi colonized the tomato leaves. Therefore, some of the detected bacterial and fungal OTUs might colonize the tomato individuals before they were transplanted into the experimental field. However, given that spatial positions within the field had significant effects on the microbial community structures (Table 1), colonization of indigenous (resident) microbes in the field could be a major factor determining

OTU	Preferred variety	Phylum	Class	Order	Family	Genus	NCBI Blast top hit	Accession	Cover	Identity
<b>Prokaryotes</b>										
P_040	Ungrafted (P = 0.00321*)	Deinococcus-Thermus	Deinococci	Deinococcales	Deinococcaceae	<i>Deinococcus</i>	<i>Deinococcus citri</i>	LT602922	100%	100%
<b>Fungi</b>										
F_427	Ganbarune (P = 0.00078*)	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	<i>Hannaella</i>	<i>Hannaella oryzae</i>	KY103504	89%	99%
F_428	Ganbarune (P = 0.00099*)	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	<i>Hannaella</i>	<i>Hannaella oryzae</i>	KY103504	89%	99%

**Table 4.** Prokaryote and fungal OTUs showing statistically significant preferences for tomato rootstock varieties. An asterisk indicates a significant preference for a rootstock condition (Bonferroni correction for each OTU compared across nine rootstock conditions;  $\alpha = 0.05$ ).

the observed microbiome pattern. For more comprehensive understanding, time-series sampling throughout the growing season of tomato should be conducted in multiple field sites differing in biotic/abiotic environmental conditions. Second, we need to acknowledge that microbiome profiling with high-throughput DNA sequencing *per se* does not reveal the fine-scale distribution of the detected microbial OTUs in/on plant leaves. Although we tried to surface-sterilize the leaf samples, the microbiome data involved not only possibly endophytic taxa but also bacteria and fungi that have been regarded as epiphytes (e.g., *Methylobacterium*)<sup>66,94</sup> (but see ref.<sup>95</sup>). Microscopic analyses with taxon-specific fluorescent probes, for example, will provide essential insights into the localization of the observed microbes in/on leaves. Third, while this study was designed to examine effects of below-ground genotypes on above-ground parts of grafted plants, recent studies have shown that genetic materials (i.e., DNA) can be transported between scion and rootstock tissue, at least at graft junction region, in a grafted plant<sup>96</sup>. Thus, contributions of above-/below-ground genotypes to root/leaf microbiomes may be much more complex than had been assumed in this study.

Overall, this study suggested that majority of leaf-associated microbes can colonize grafted tomato plants irrespective of rootstock genotypes of their hosts. Meanwhile, leaf-associated microbial taxa may display preferences for grafted/ungrafted plants or specific host rootstock varieties. Both grafting and the use of plant-beneficial microbes have been regarded as prospective options for securing agricultural/forestry production in the era of increasing biotic and abiotic environmental stresses<sup>12,13,29,34</sup>. Further integrative studies will help us explore best conditions in which grafting and microbiome technologies are merged into a solid basis of stable and sustainable agricultural practices.

## Data Availability

The raw DNA sequencing data are deposited on the DDBJ Sequence Read Archive (accession: DRA007061). The data matrices used in the statistical analyses are provided as Supplementary Data 1–5.

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## Author Contributions

H.T. and M.N. designed the work. H.T., K.O., and M.N. performed experiments. H.T. analyzed data. H.T. wrote the paper with M.N. All authors read and approved the manuscript.

## Additional Information

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