1 **Dissecting the co-transcriptome landscape of** 2 **plants and microbiota members**

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14 Abstract

15 Interactions between plants and each neighboring microbial species are
 16 fundamental building blocks that collectively determine the structure and function
 17 of the plant microbiota, but the molecular basis of such interactions is poorly
 18 characterized. Here, we monocolonized *Arabidopsis* leaves with nine
 19 plant-associated bacteria from all major phyla of the plant microbiota and profiled
 20 co-transcriptomes of plants and bacteria. These strains elicited quantitatively
 21 different plant transcriptional responses including typical pattern-triggered
 22 immunity responses. Genes of non-pathogenic bacteria involved in general
 23 metabolism and energy production were commonly suppressed *in planta* in
 24 contrast to a virulent pathogen. Various nutrient acquisition pathways that are
 25 frequently encoded in the genomes of plant-associated bacteria were induced *in*
 26 *planta* in a strain-specific manner, shedding light on bacterial adaptation to the
 27 plant environment and identifying a potential driving force of niche separation.
 28 Integrative analyses of plant and bacterial transcriptomes suggested that the
 29 transcriptional reprogramming of plants is largely uncoupled from that of bacteria
 30 at an early stage of interactions. This study provides insights into how plants
 31 discriminate among bacterial strains and sets the foundation for in-depth
 32 mechanistic dissection of plant-microbiota interactions.

33 Introduction

34 In nature, plants can reproducibly assemble bacterial communities with
 35 well-defined taxonomic structures (the plant microbiota) (Hacquard et al., 2015),
 36 which can be harnessed for plant health and survival (Carrión et al., 2019; Durán
 37 et al., 2018; Kwak et al., 2018). How plants discriminate among various bacterial
 38 strains and establish strain-specific associations in a community context remains
 39 an open question in basic plant microbiota research and is key to facilitate the
 40 application of microbiota-based strategies to improve plant health in agricultural
 41 settings. Answering this question requires a comprehensive and unified
 42 understanding of plant and bacterial responses during their interactions.

43 Plant responses to microorganisms are controlled by the plant innate immune
 44 system, which contributes to the assembly and maintenance of healthy bacterial
 45 communities (Chen et al., 2020; Lebeis et al., 2015). A crucial part of the plant
 46 immune system is the perception of environmental microbes using cell surface
 47 receptors that detect conserved microbial epitopes, termed microbe-associated
 48 molecular patterns (MAMPs) (Boller and Felix, 2009). Recognition of MAMPs
 49 triggers defense responses collectively called pattern-triggered immunity (PTI),
 50 which can inhibit pathogen growth (Jones and Dangl, 2006). MAMPs such as the
 51 bacterial flagellin peptide flg22 are widely conserved in non-pathogenic bacteria
 52 as well as pathogenic bacteria (Hacquard et al., 2017), and some
 53 non-pathogenic Proteobacteria strains were shown to trigger defense responses
 54 in plant leaves likely via PTI pathways (Vogel et al., 2016). On the other hand,
 55 diverse microbiota members can suppress PTI triggered by flg22 in roots
 56 (Garrido-Oter et al., 2018; Teixeira et al., 2021; Yu et al., 2019), which can
 57 facilitate colonization by the root microbiota (Teixeira et al., 2021; Yu et al., 2019).
 58 Thus, PTI activation by divergent MAMPs and subsequent PTI modulation by
 59 plant-associated bacteria might steer plant responses in a bacterial
 60 strain-specific manner, contributing to microbiota assembly in roots. In leaves,

61 however, our current understanding of plant responses to individual microbiota
62 members is largely limited to a specific phylum of bacteria, Proteobacteria, and
63 little is known about how plants respond to bacterial strains belonging to other
64 major phyla such as Actinobacteria, Bacteroidetes, and Firmicutes.

65 When colonized densely and heterogeneously by various bacterial species,
66 plants might not be able to tailor their responses to individual bacterial strains.
67 Yet, it might be possible that different plant-associated bacterial species respond
68 differently to the same microenvironments created by plants. If so, analyzing
69 plant responses alone does not wholly explain bacterial responses during
70 interactions with hosts. The explanation requires directly interrogating bacterial
71 responses *in planta* at the genome-wide scale. *In planta* bacterial omics
72 approaches, such as transcriptomics, are powerful in understanding bacterial
73 gene functions in the plant microbiome and how plants influence bacterial
74 activities (Levy et al., 2018). To date, however, there is a handful of *in planta*
75 bacterial transcriptome studies, all focusing on pathogenic Proteobacteria strains
76 (Chapelle et al., 2015; Lovelace et al., 2018; Nobori et al., 2018, 2020; Yu et al.,
77 2013, 2014). It is, therefore, unknown whether plant-associated bacteria have
78 any common or phylum-specific signatures in the usage of their genomes and
79 what kind of functions are important for their non-pathogenic and sometimes
80 beneficial traits in plants. Furthermore, to our knowledge, no study has analyzed
81 plant and bacterial transcriptome responses at the same time, limiting our ability
82 to build hypotheses about the molecular dialogue between plants and microbiota
83 members.

84 Here, in monoassociation conditions, we co-profiled the transcriptomes of the
85 model plant *Arabidopsis thaliana* and various bacterial strains isolated from
86 healthy (asymptomatic) plants in nature (hereafter commensal strains),
87 representing all major phyla of the plant microbiota residing in leaves.
88 Commensal strains commonly induced PTI responses in plants, but these
89 differed in intensity. We found examples of both common and strain-specific

90 regulation of commensal gene expression in plants. Bacterial genes enriched in
91 plant-associated strains tended to be induced *in planta*. These included genes
92 involved in sulfur, nitrogen, and carbon transport and metabolism, which were
93 induced *in planta* in a strain-specific manner. This suggests that nutrient status
94 differs for different strains in plants, which may affect bacterial fitness and niche
95 separation. We also observed that plants could elicit different transcriptional
96 responses from different bacterial strains without tailoring their own
97 transcriptional reprogramming. This study provides a framework for dissecting
98 plant-microbiota interactions at the strain level using co-transcriptomics and
99 unravels diverse modes of interactions between plants and commensal bacteria.

100 Results

101 Co-transcriptome analysis of plants and plant microbiota 102 members

103 We developed a pipeline to simultaneously investigate the transcriptomes of both
104 plants and bacteria during plant colonization with a single bacterial strain. We
105 monocolonized *A. thaliana* wild-type Col-0 leaves with individual commensal
106 strains by hand-infiltration and profiled transcriptomes of plants and bacteria at 6
107 hours post-inoculation (hpi) by RNA-seq (Fig. 1A). For *in planta* bacterial
108 RNA-seq, we used a previously developed method with some modifications (see
109 Methods). Briefly, bacterial cells are isolated from plant leaves before extracting
110 RNA, followed by rRNA depletion and RNA-seq (Nobori et al., 2018). For plant
111 and bacterial RNA-seq, respectively, 18 and nine commensal strains were
112 selected covering all major phyla of the plant microbiota (Fig. 1B and Table 1).
113 Three biological replicates from independent experiments were taken for each
114 condition. We used the same strain IDs as in the original study where these
115 bacterial strains were isolated from wild *A. thaliana* plants (leaves and roots) or

116 soil (Bai et al., 2015). A strain ID indicates the original compartment from which
117 the strain was isolated, but many root/soil isolates can also colonize the shoot,
118 indicating extensive niche overlap (Bai et al., 2015).

119 Nine commensal strains were used for co-transcriptome analysis (**Fig. 1C**).
120 These strains could colonize to various degrees in the leaf endosphere when
121 inoculated on the leaf surface (**Fig. S1**). For plants, we compared gene
122 expression changes between bacteria-inoculated plants and water-inoculated
123 plants (**Fig. 1C, left**). For bacteria, we compared expression changes between *in*
124 *planta* and *in vitro* (rich media) conditions (**Fig. 1C, right; Fig. S2A**). We also
125 included previously generated *in planta/in vitro* transcriptome data of the virulent
126 pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto*) and its avirulent
127 mutant D36E (36 type III effectors are depleted) (Nobori et al., 2018) in the
128 analysis. To directly compare bacterial gene expression patterns among
129 phylogenetically diverse bacterial strains, genes of different strains were grouped
130 based on sequence homology, resulting in 6,823 orthologous groups (OGs) (**Fig.**
131 **S2B**). Of these OGs, only 454 OGs were shared among all strains (**Data S1**),
132 indicating that the commensal strains used in this study possess highly diverse
133 gene sets. Principal component analysis using the shared OGs showed that all
134 commensal strains except for Leaf1, showed relatively similar gene expression
135 patterns both *in planta* and under *in vitro* conditions (**Fig. 1D**). Interestingly, while
136 the gene expression pattern of the avirulent pathogen D36E *in planta* was similar
137 to those of commensals, the virulent *Pto* showed a distinct pattern, i.e., *Pto in*
138 *planta* resembled commensals *in vitro* (**Fig. 1D**). Thus, the commensal strains
139 used in this study have highly different genomes, but *in planta* expression of
140 shared genes is similar to each other and highly distinct from that of a virulent
141 pathogen.

Fig. 1: Co-transcriptomics of plants and bacteria (A) Experimental scheme. Individual bacterial strains were syringe-infiltrated into leaves of *A. thaliana*. Leaves were sampled at 6 h post-inoculation. Total RNA was extracted for plant RNA-seq. For bacterial RNA-seq, bacterial cells were isolated from plant leaves before extracting RNA using a method previously reported (Nobori et al., 2018). (B) Bacterial strains used in this study. Stars indicate the strains used for co-transcriptome analysis. Detailed taxonomic information is shown in Table 1. (C) The number of genes differentially regulated (|log₂FC| > 1; FDR < 0.01; two-tailed Student's t test followed by Storey's q-value) in plant or bacterial

152 RNA-seq. Plant: bacteria-inoculated vs. water-inoculated. Bacteria: *in planta* 6 h
153 vs. *in vitro* (rich media). Bacterial strains used for co-transcriptomics are shown.
154 **(D)** Principal component analysis of bacterial gene (orthologous group)
155 expression *in planta* and *in vitro*. **(E)** Gene expression fold changes (*in planta* vs.
156 *in vitro*) of bacteria. The data of *Pto* and D36E are from a previous study (Nobori
157 et al., 2018). Genes not detected or missing in the genome are shown in gray.
158 See **Data S2** for gene expression data. **(A, B, D, and E)** The taxonomic affiliation
159 (phylum/class level) of each strain is indicated with different colors.

ID	Phylum	Class	Order	Family	Genus	RNA-seq
Leaf1	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	P, B
Root563	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Janibacter	P
Soil763	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	P, B
Leaf176	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	P, B
Leaf404	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	P, B
Root935	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	P, B
Leaf187	Firmicutes	Bacilli	Bacillales	Exiguobacterium	Exiguobacterium	P, B
Root147	Firmicutes	Bacilli	Bacillales	Bacillaceae		P
Leaf155	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium	P, B
Leaf177	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	P, B
Leaf53	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia	P
Leaf70	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xanthomonas	P
Leaf148	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xanthomonas	P
Root604	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae		P
Leaf48	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	P
Leaf58	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	P
Leaf127	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	P
Leaf130	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	P, B
<i>Pto</i>	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	P, B
D36E	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	P, B

160 **Table 1: List of bacterial strains used in this study** RNA-seq data (P, plant; B,
161 bacteria) obtained in this study are indicated.

162 Conserved and strain-specific regulation of commensal 163 functions *in planta*

164 The high variability in bacterial genomes complicates a genome-wide comparison
165 of bacterial gene expression among phylogenetically diverse strains (**Fig. 1E**). To
166 overcome this problem, we sought to compare the regulation of bacterial
167 functions rather than of individual genes. For each strain, we performed

functional enrichment analysis on genes significantly up- or down-regulated *in* *planta* using KEGG ontologies (KOs) assigned to individual OGs (see Method for details). Then, enrichment scores (*p* values) for individual KOs were summarized for all the strains (Fig. 2A and Fig. S3A). Data from *Pto* grown in a minimal medium were included to determine the effect of nutrient availability on gene expression changes in a pathogen. A clear pattern distinguishing virulent and avirulent strains was seen in the process “ribosome” (Fig. 2A and 2B). Genes encoding ribosomal subunits were significantly suppressed *in planta* in all the commensal strains tested and the avirulent pathogen D36E, while these genes were induced in the virulent pathogen *Pto* (Fig. 2A and 2B). The population density of *Pto* remains unchanged at this time point (Nobori et al., 2018), indicating that the gene expression changes are not the consequence of bacterial growth *in planta*. This process was also suppressed in *Pto* grown in a minimal medium compared with a rich medium (Fig. 2A and 2B). Similarly, genes encoding proton ATPases, which are involved in energy production, were induced in *Pto in planta*, but suppressed or not altered in the commensal strains (Fig. 2A and 2B). Together, these results suggest that commensal strains are metabolically less active *in planta* compared with a virulent pathogen, which is consistent with the notion that commensal strains do not proliferate in plants to the same extent as virulent pathogens. Since D36E is a mutant of *Pto* lacking PTI-suppressing effector molecules, PTI is likely responsible for suppressing bacterial metabolism *in planta*, at least in this strain. Catalase genes were commonly induced in most commensals and D36E at varying degrees but not in *Pto* (Fig. 2C), suggesting that commensals are responding to plant ROS burst, a characteristic PTI response.

Fig. 2: Conserved and strain-specific regulation of bacterial functions in plants (A) KEGG ontology (KO) terms enriched in genes that are significantly up- (orange) or down (purple)-regulated *in planta* compared with *in vitro* (rich media). The heatmaps indicate $-\log_{10}$ p-value (FDR corrected by Benjamini–Hochberg method). A KO can be both significantly up and down-regulated in the same strain. The left green panel shows the number of genes involved in each KO term. The top color bars indicate the taxonomic affiliation (phylum/class level) of each strain. See Fig. S2 for a more comprehensive list of KOs. (B–G) Expression fold changes (*in planta* vs. *in vitro*) of genes involved in (B) general metabolism, (C) catalase metabolic pathway, (D) motility, (E) secretion systems, (F) siderophore biosynthesis, and (G) unknown functions. T3SS, type III secretion system. T4SS, type IV secretion system. T6SS, type VI secretion system. MM, *Pto* grown in a minimal medium. Results are shown as box plots with boxes displaying the 25th–75th percentiles, the centerline indicating the median, whiskers extending to the minimum, and maximum values no further than 1.5 inter-quartile range. All individual data points (genes) are overlaid with colors for DEGs (red: upregulated, blue: downregulated, black: non-DEG).

Genes involved in bacterial motility were differentially regulated among bacteria in plants. Many of these genes were suppressed in *Pto in planta* but induced in D36E (Fig. 2A and 2D). Leaf177, a *Burkholderia* (Betaproteobacteria) strain, showed a similar pattern to D36E (Fig. 2A and 2D). However, the *Rhizobia* (Alphaproteobacteria) Leaf155 more closely resembled virulent *Pto* – a majority of the genes were suppressed *in planta*, whereas some genes were significantly induced (Fig. 2D). Motility-related genes can be classed into two major functional categories, flagellar assembly, and chemotaxis. Genes encoding flagellar assembly proteins were globally suppressed *in planta* in Leaf155 as in *Pto*, and many Leaf177 chemotaxis-related genes were induced *in planta* in contrast to *Pto* (Fig. 2D). Thus, physiological processes are differentially regulated among different plant-associated commensal bacteria strains, with some species even exhibiting similarity to a virulent pathogen.

The type III secretion system, an essential component of the virulence of bacterial pathogens, including *Pto* (Toruño et al., 2016), was strongly induced in *Pto* and D36E, but these genes were absent in the commensals (Fig. 2E). The

227 type IV secretion system is involved in multiple processes such as translocating
 228 proteins and DNA into other cells and bacterial motility (Costa et al., 2015). This
 229 process was globally suppressed in Leaf130 and Leaf177, but not in Leaf155
 230 (Fig. 2E). The type VI secretion system is an injection machine involved in
 231 bacteria-host and bacteria-bacteria interactions (Russell et al., 2014). This
 232 machinery was globally induced in Leaf155 and Leaf404 and partially induced in
 233 Leaf177 and *Pto* (Fig. 2E). Lastly, preprotein translocase subunits, which are
 234 involved in the bacterial general secretory pathway (Osborne et al., 2005),
 235 tended to be suppressed in all commensals and D36E, but not in *Pto* (Fig. 2E).

236 It has been shown that genes encoding iron-chelating siderophores are
 237 strongly induced in *Pto* upon plant infection, and the induction of these genes is
 238 blocked by plant immunity to suppress bacterial growth (Nobori et al., 2018) (Fig.
 239 2F). Commensal strains either harbor only a few or completely lack genes
 240 encoding siderophores, and most of these genes were not induced *in planta* (Fig.
 241 2F). We speculate that siderophores are not actively used by commensals in
 242 leaves and/or that plant immunity suppresses the production of siderophores to
 243 control commensal growth. Notably, many genes annotated as “Function
 244 unknown” were significantly induced *in planta* in various commensals (Fig. 2G).
 245 These functionally unannotated genes induced *in planta* may have unique roles
 246 in plant-bacterial interactions.

247 **Phylum and strain-specific gene expression**

248 To compare expression of individual genes between different strains, we
 249 conducted comparative transcriptome analysis focusing on specific phyla (Fig.
 250 S4A and S4C). This approach allows more comprehensive comparative
 251 transcriptome analysis as more genes are shared among closely related strains.
 252 We focused on Bacteroidetes and Proteobacteria, in which 1,422 and 1,122 OGs
 253 were shared, respectively (far more than the 454 OGs shared among the nine

commensals) (Fig. S2B, S4A, and S4C). Overall, many genes were differentially regulated in a single strain (Fig. S2B, S4A, and S4C). In both of these phyla, a larger number of genes were commonly suppressed among the three strains in plants than commonly induced (Fig. S4B, S4D, and S5). Clusters of genes commonly suppressed *in planta* (clusters 7 & 8 in Bacteroidetes and clusters 1 & 4 in Proteobacteria) were enriched with “ribosome”-related genes (Fig. S4E). “Transporters” were enriched in multiple clusters with various expression patterns (Fig. S4F and S6A), suggesting that transporters can be separated into sub-groups based on regulation in plants. Also, genes annotated as part of a “two-component system” showed strain-specific expression patterns (Fig. S4G and S6B). Taken together, our intraphylum analysis reveals that even relatively closely related commensal strains respond differently *in planta* at the transcriptional level.

***In planta* bacterial transcriptomics illuminates bacterial adaptation to the leaf environment**

Various bacterial functions were differentially regulated in plants in a strain-specific manner. Is such functional regulation relevant for bacterial fitness in plants? Comparative genomics is one way to infer bacterial functions associated with adaptation to the plant environment. A previous study compared the genomes of nearly 4,000 plant-associated and non-plant-associated bacterial strains and defined “plant-associated (PA) genes” that are significantly enriched in plant-associated strains (Levy et al., 2017). We analyzed how PA genes are regulated in plants in our transcriptome data. When analyzing the genes shared among nine commensal strains, we observed that genes induced *in planta* tended to be enriched with PA genes, whereas genes suppressed *in planta* tended to be enriched with nonPA genes (Fig. 3A). Remarkably, PA and nonPA genes were significantly enriched with plant-induced and plant-suppressed

281 genes, respectively, for all the commensals, except for the *Firmicutes* strain
 282 Leaf187 (Fig. 3B). Therefore, our data suggest that bacterial genes associated
 283 with adaptation to the plant environment were indeed activated *in planta*.

284 **Fig. 3: Genes enriched in plant-associated bacteria are induced *in planta***
 285 **(A)** (Right panel) Bacterial gene expression fold changes (FC) in plants
 286 compared with *in vitro* (rich media). (Middle panel) Genes differentially expressed
 287 *in planta* compared with *in vitro* ($|\log_2 \text{FC}| > 1$; FDR < 0.01; two-tailed Student's t
 288 test followed by Storey's q-value). (Left panel) Genes previously shown to be
 289 "plant-associated" (Levy et al., 2017) are shown as black. The bar and dots
 290 indicate the taxonomic affiliation (phylum/class level) of each strain. **(B)** Boxplots
 291 showing expression changes of plant-associated (PA) and non-plant-associated
 292 (nonPA) genes between *in planta* and *in vitro*. Each dot represents a gene.
 293 Genes significantly up- or downregulated *in planta* are colored in red and blue,
 294 respectively. Asterisks indicate statistically significant enrichment (FDR < 0.05;
 295 Hypergeometric test corrected by Benjamini-Hochberg method) of up or
 296 down-regulated genes in the PA or nonPA category. The proportion of genes up-
 297 or downregulated are shown. For the full expression data with the orthologous
 298 group, KEGG annotation, DEG, and PA information, see **Data S3**.

299 We then performed KO enrichment analysis for PA genes induced in plants
 300 and nonPA genes suppressed in plants. Ribosome-related genes were
 301 conserved among all strains (and are thus nonPA genes) and were generally
 302 suppressed in plants (**Fig. 4A**), which may be a strategy by which plants control
 303 bacterial growth. Glycan degradation genes were highly plant-associated and
 304 induced in Bacteroidetes strains Leaf176 and Root935 (**Fig. 4A and 4B**). Among
 305 such genes were homologs of beta-galactosidase, alpha-L-fucosidase, and
 306 glucosylceramidase, which can degrade plant cell wall components. Thus,
 307 Leaf176 and Root935 may have evolved the ability to degrade the plant cell wall
 308 enabling the establishment of favorable niches during plant colonization.

309 "Sulfur metabolism"-related genes were classified as PA genes and were
 310 induced *in planta* in the three *Proteobacteria* strains (**Fig. 4A and 4C**). "ABC
 311 transporters" was another PA function that was induced in *Proteobacteria* strains
 312 *in planta* (**Fig. 4A and 4C**). Genes involved in both categories were sulfur uptake
 313 transporters (**Fig. 4C**). A previous proteomics study showed that the expression
 314 of proteins involved in sulfur metabolism and uptake was induced on the leaf
 315 surface in two commensal *Proteobacteria*, *Sphingomonas melonis* and

316 *Methylobacterium extorquens* (Müller et al., 2016). These results suggest that
 317 sulfate acquisition is important for the adaptation of commensal Proteobacteria to
 318 the plant environment. On the other hand, sulfur metabolism-related genes were
 319 not found to be PA genes and were suppressed *in planta* in the Bacteroidetes
 320 strains Leaf176 and Leaf404 (Fig. 4A and 4C). The number of genes predicted
 321 to be involved in sulfur metabolism was lower in Bacteroidetes strains than in
 322 Proteobacteria strains (Fig. 4A). This may indicate that Bacteroidetes strains are
 323 less reliant on sulfur acquisition during plant colonization.

324 Urea transporters are other ABC transporters that are PA genes and were
 325 induced specifically in some Proteobacteria strains *in planta* (Fig. 4D). Also,
 326 genes encoding ureases, which hydrolyze urea in the bacterial cytoplasm, were
 327 induced in some Proteobacteria strains (Fig. 4D). It has been shown that
 328 *Yersinia enterocolitica*, a Gammaproteobacteria strain, can use urea as a
 329 nitrogen source (Young et al., 1996). These results suggest that Proteobacteria
 330 (especially Leaf177 and D36E) might require urea as a nitrogen source in the
 331 plant apoplast. Also, genes involved in the nitrate transport system were induced
 332 in Leaf130, Leaf155, and D36E *in planta*, but not in *Pto* (Fig. S7), suggesting that
 333 some commensal Proteobacteria strains activate nitrogen acquisition systems in
 334 plants. Similarly, ribose transporters and glycerol transporters are PA genes and
 335 were commonly induced *in planta* in commensal or avirulent Proteobacteria
 336 strains (Leaf155, Leaf177, and D36E) but not in the virulent *Pto* (Fig. 4F).
 337 Moreover, arabinose and xylose transporters (both monosaccharide transporters)
 338 were induced in Leaf177 and D36E *in planta*, but not in *Pto* (Fig. S7). Thus,
 339 these Proteobacteria strains may use various types of sugars as carbon sources
 340 in plants. The induction of urea and sugar acquisition systems may indicate that
 341 commensal bacteria activate nutrient starvation responses in the leaf apoplast.
 342 We speculate that plants may sequester nitrogen and carbon sources from the
 343 apoplast to limit the growth of commensal and avirulent pathogenic bacteria. In
 344 line with this hypothesis, the high-affinity plant urea transporter *AtDUR3* was

345 induced upon inoculation with many commensal strains while suppressed by the
 346 virulent *Pto* (Fig. 4E). A previous study showed that plants sequester
 347 extracellular sugars by activating the sugar influx transporter *AtSTP13* via the
 348 PTI pathway (Yamada et al., 2016). Indeed, our plant transcriptome data showed
 349 that *AtSTP13* is induced by the commensals as well as D36E and *Pto* (Fig. 4G).
 350 On the other hand, *Pto* can induce plant sugar efflux transporters (Chen et al.,
 351 2010), which might increase sugar availability in the apoplast and explain why
 352 *Pto* did not activate its sugar transporters in plants. A non-mutually exclusive
 353 possibility is that the virulent *Pto* switches its metabolic preference to other
 354 substrates during successful infection in plants. In summary, we revealed
 355 bacterial phylum/strain-specific gene repertoires and gene regulation, which may
 356 be actively controlled by plants and drive bacterial niche separation *in planta*.

Fig. 4: Nutrient acquisition systems are associated with bacterial adaptation to the plant environment in a strain-specific manner (A) KEGG enrichment analysis of genes that are plant-associated (PA) and significantly induced *in planta* compared with *in vitro* (rich media) (orange) and genes that are nonPA and significantly suppressed *in planta* compared with *in vitro* (purple). The left panel shows the number of genes involved in each KEGG term. (B-D, F) Expression fold changes (*in planta* vs. *in vitro*) of genes with different functions.

365 The top bars indicate the ratio of PA genes in each strain. All individual data
 366 points (genes) are overlaid to the box plots with colors for DEGs (red:
 367 upregulated, blue: downregulated, black: non-DEG). **(E and G)** Expression of the
 368 plant gene **(E)** *AtDUR3* (urea transporter) and **(G)** *AtSTP13* (sugar transporter)
 369 based on the RNA-seq data. Asterisks indicate statistically significant difference
 370 ($|\log_2FC| > 1$; FDR < 0.01; two-tailed Student's t test followed by Storey's
 371 q-value) compared with the mock (water-inoculated) condition. In the box plots,
 372 boxes display the 25th–75th percentiles, the centerline indicating the median,
 373 whiskers extending to the minimum, and maximum values no further than 1.5
 374 inter-quartile range. For the full expression data with the orthologous group,
 375 KEGG annotation, DEG, and PA information, see **Data S3**.

376 **Commensals activate plant PTI in a strain-specific manner**

377 As commensal strains showed differing responses in plants, indicating strain
 378 specificity in the interactions of plants with bacteria, we investigated
 379 genome-wide plant responses to individual commensals by RNA-seq. In addition
 380 to the nine strains used for the bacterial transcriptome analysis, we added nine
 381 more commensal strains to enrich our dataset. Global gene expression changes
 382 (bacteria-inoculated vs. water-inoculated) were similar among all commensal
 383 strains as well as D36E at 6 hpi (**Fig. 5A**). Interestingly, plant gene expression
 384 changes triggered by commensals overlapped markedly with responses to flg22
 385 (Hillmer et al., 2017), a potent PTI inducer. PTI-inducible genes accounted for
 386 clusters of genes commonly and strongly induced by most of the commensals
 387 (clusters 3 & 5 in **Fig. 5A**). GO enrichment analysis showed that these clusters
 388 are enriched with genes related to defense responses (**Fig. 5B**). Thus,
 389 commensal strains, when infiltrated into plant leaves, can induce MTI.

390 The degree of PTI induction varied among strains in a manner that is partly
 391 determined by phylogeny: Gammaproteobacteria and Actinobacteria strains
 392 induced stronger PTI than Bacteroidetes strains (**Fig. 5C**). We then investigated
 393 the amino acid sequences of the major MAMPs flg22 and elf18 across the
 394 different strains. Intriguingly, strains with flg22 and elf18 sequences similar to
 395 those known to be particularly potent PTI inducers (Felix et al., 1999; Kunze et
 396 al., 2004) tended to elicit strong PTI induction (gene expression fold changes in

clusters 3 or 5) (Fig. 5D). Thus, sequence variation in these MAMPs may partly determine the degree of PTI induced by some of these commensal strains.

Fig. 5: Plant transcriptome responses to phylogenetically diverse commensals (A) (Green/purple heatmap) Gene expression fold changes (FCs) between bacteria-inoculated plants and water-inoculated plants. (Red/blue heatmap) Plant genes significantly induced or suppressed upon flg22 treatment at different time points (Hillmer et al., 2017). The number of strains causing differential gene expression ($|\log_2 \text{FC}| > 1$; FDR < 0.01; two-tailed Student's t test followed by Storey's q-value) are indicated in the sidebar (# of DEG strains). DEG, differentially expressed gene. Genes were clustered by k-mean clustering ($k = 8$). The bars on the heatmaps indicate the taxonomic affiliation (phylum/class level) of each strain. See **Data S4** for gene expression data. **(B)** Gene ontology enrichment analysis for genes in clusters 3, 4, 5, 7, and 8 of **(A)**. $-\log_{10}$ p-values (FDR corrected by Benjamini-Hochberg method) were shown. **(C)** Expression fold changes (FC; z-score) of genes in clusters 3 and 5. Results are shown as box plots with boxes displaying the 25th–75th percentiles, the centerline indicating the median, whiskers extending to the minimum, and maximum values no further than 1.5 inter-quartile range. **(D)** Relationships between amino acid (AA) sequence conservation of flg22 or elf18 and normalized expression FCs of genes in clusters 3 and 5. AA sequence conservation of flg22 and elf18 compared with the canonical sequences known to induce strong defense responses in plants (Elf18: SKEKFERTKPHVNVGTIG. Flg22: QRLSTGSRINSAKDDAAGLQIA). The Pearson correlation coefficients are shown. **(C-D)** The same color code was used for the taxonomic affiliation.

Plant responses do not correlate with bacterial responses in plants

Principal component analysis showed that plant and bacterial transcriptome patterns were incongruent with each other (**Fig. 6A**). For instance, we observed similarity between the plant transcriptome changes elicited by different Actinobacteria strains (Leaf1 and Soil763), but the strains themselves respond highly differently *in planta* (**Fig. 6A**). Also, *Bacteroidetes* strains (Leaf176, Leaf404, and Root935) showed similar transcriptional changes in plants, but plant transcriptome changes triggered by these strains differed more (**Fig. 6A**). To get deeper insights into the relationships between plant and bacterial gene expression, we measured the correlation between gene expression changes of individual plant genes and shared bacterial OGs using co-transcriptome data of nine commensal strains. To prevent a single outlier strain from impacting correlation scores, we took a bootstrapping approach in which correlations were calculated using all the combinations of eight strains as well as all the nine

435 strains and then combined (see Methods in detail) (Fig. 6B and Fig. S8). This
 436 analysis revealed that the expression of a majority of plant and bacterial genes is
 437 not correlated, further indicating that the plant and bacterial responses are largely
 438 uncoupled in our dataset (Fig. 6C). For instance, in many cases, commensal
 439 strains that triggered similar plant transcriptional responses (e.g., Soil763 and
 440 Leaf1; Fig. 6C) showed distinct gene expression in plants (Fig. 6C). However, a
 441 subset of plant and bacterial genes showed a stronger correlation (Fig. 6C). KO
 442 enrichment analysis showed that expression of genes annotated as belonging to
 443 the bacterial processes “proton ATPases” and “purine metabolism” positively
 444 correlates with plant defense-related genes (Fig. 6C). More specifically, the
 445 expression of such bacterial genes was higher when plants showed stronger PTI
 446 activation. The biological relevance of this observation remains to be tested.
 447 Together, our data indicate that plant and bacterial gene expression can be
 448 largely uncoupled at an early stage of interaction.

Fig. 6: Plant and bacterial transcriptomes are largely uncoupled (A)
Principal component analysis of gene expression fold changes (FCs) of plants (left: bacteria-inoculated vs. water-inoculated) and bacteria (right: *in planta* vs. *in vitro*). Orthologous groups (OGs) of bacterial genes shared among all strains are used for the analysis. **(B)** Schematic diagram of the integration of plant and bacterial RNA-seq data. For each interaction condition, the correlation coefficients between individual plant genes and bacterial OGs were calculated. The correlation coefficient data was corrected by bootstrapping (see Methods and **Fig. S8**) **(C)** A map of correlation coefficients between plant genes and bacterial OGs calculated as described in **(B)**. Rows and columns are bacterial OGs and plant genes, respectively. The top and left heatmaps indicate gene expression FCs of plants and bacteria, respectively. See **Data S5** for the full correlation data. KEGG enrichment analysis was performed for the clusters of plant and bacterial genes with strong correlation.

Discussion

Previous studies of the plant microbiota have suggested that plants assemble bacterial communities and regulate their functions by interacting with commensals in a strain-specific manner. However, there are a limited number of studies that have interrogated the responses of plants and commensal bacteria at a genome-wide scale, and we thus have no comprehensive understanding of the two-way molecular dialogue between plants and microbiota members. Here, we profiled, for the first time, co-transcriptomes of plants and commensal bacteria under monoassociation conditions using diverse strains covering all major phyla of the plant microbiota. Our dataset demonstrated that different commensal strains 1) trigger qualitatively similar yet quantitatively different immune responses in plants and 2) show both common and highly strain-specific responses in plants.

We found that suppression of genes related to general metabolic activity and energy production *in planta* is a common trait among phylogenetically diverse commensals, in marked contrast to a virulent pathogen, which elicited the opposite response (**Fig. 2**). PTI was commonly induced by the commensal strains (**Fig. 5A**), suggesting that plant immunity might act to keep commensal

481 metabolic activity in check to avoid overgrowth. This notion is in line with a
482 previous finding that commensals can proliferate in an unrestrained manner in
483 the leaf apoplast of plant mutants lacking key immune components under high
484 humidity, which further compromises plant immunity (Chen et al., 2020; Xin et al.,
485 2016). Further transcriptome analysis of commensals in immunocompromised
486 plants and environmental conditions will unravel how different immune pathways
487 tailor their responses to effectively control commensal growth and function.

488 We provide evidence that bacterial genes enriched in the genomes of
489 plant-adapted strains are induced *in planta* (Fig. 3A and 3B), suggesting that
490 those genes which enable bacteria to thrive in the plant environment are indeed
491 activated in plants. This finding is somewhat in contrast to a previous study,
492 which showed that gene expression of a bacterial pathogen *in planta* does not
493 correlate with fitness scores determined by transposon insertion mutagenesis
494 (Helmann et al., 2019). Importantly, loss-of-function screening with single
495 mutants has limitations in assigning gene function owing to functional
496 redundancy. In this case, a gain-of-function assay is a complementary, albeit also
497 limited, approach. For instance, we previously showed that *in planta* bacterial
498 transcriptome data can predict bacterial genes that contribute to bacterial growth
499 *in planta* when overexpressed (Nobori et al., 2018, 2019). It will be necessary, in
500 the future, to rigorously validate the biological relevance of bacterial
501 transcriptional regulation in plants at the mechanistic level.

502 We found that processes involved in the uptake of nutrients, such as sulfur,
503 urea, and sugars, were enriched in plant-associated Alpha- and
504 Betaproteobacteria (L155 and L177) and induced *in planta* (Fig. 4). We observed
505 that plants induce transporters that could sequester urea and sugars from the
506 apoplast during interactions with commensals (Fig. 4E and 4G), which potentially
507 affects bacterial nutrient acquisition processes and eventually bacterial fitness in
508 plants. Notably, we also found many other bacterial nutrient transporters to be
509 regulated *in planta* in a strain-specific manner (Fig. S7). The results imply that

510 different commensals experience distinct nutrient status in the plant apoplast,
511 which might affect bacterial fitness *in planta*. Our co-transcriptome data sets the
512 stage for investigating whether plants control nutrient availability for particular
513 strains to drive bacterial niche separation in plants and shape the plant
514 microbiota.

515 We did not observe a strong association between gene expression changes in
516 plants and commensals (**Fig. 6A and 6C**), implying that different commensals
517 could respond divergently to similar programs of plant gene expression. This
518 seems plausible, given that plants have to deal with complex bacterial
519 communities residing in an area smaller than the plant cell. However, our single
520 time point experiment does not allow us to draw a firm conclusion regarding this
521 idea, as there may be a time lag between plant responses and bacterial
522 responses. In the future, time-course analysis of a larger number of strains
523 combined with plant and bacterial genetics will facilitate the prediction of
524 biologically meaningful links between plant and bacterial responses.

525 In this study, bacterial cells were syringe-infiltrated into leaves to bypass
526 stomatal entry as different commensals might have different abilities to access
527 the apoplast. Transcriptomes were profiled at 6 hpi, where the population density
528 of even the virulent pathogen *Pto* has not yet increased (Nobori et al., 2018), and
529 thus we assumed that the population density of commensals remained the same
530 at this time point. Therefore, our experimental setup allowed us to characterize
531 strain-specific co-transcriptomes under controlled conditions without the influence
532 of stomatal immunity and differences in the sizes of bacterial populations. It is,
533 however, important to note that transcriptome analysis under more natural
534 conditions would reveal additional layers of plant-microbiota interactions. This
535 requires technological innovations that enable *in planta* transcriptome analysis of
536 bacteria with much smaller populations.

537 Since plants used in this study were not grown in a strictly sterile condition,
538 we do not exclude the possibility that the pre-existing plant microbiota influenced

539 on plant and bacterial responses. However, influence of the pre-existing
540 microbiota on data interpretation was minimized by including the mock control
541 (for plant transcriptomics) and randomizing sampling and taking three
542 independent replicates (for plant and bacterial transcriptomics). We
543 demonstrated that an *in planta* bacterial transcriptome approach can be applied
544 to all major phyla of the plant microbiota, opening a new avenue for *in planta*
545 transcriptome analysis of synthetic communities that are generated by mixing
546 bacterial strains in a desired manner. This *in planta* bacterial metatranscriptome
547 approach can capture more complex traits such as microbe-microbe interactions,
548 which are important to understand the functions of the microbiota as a
549 community.

550 This study has provided a wealth of information regarding gene regulation of
551 both plants and commensals during monoassociations. In [Fig. S9-12](#), we
552 provided additional insights on the regulation of genes related to diverse
553 functions, including biosynthesis/metabolism of various compounds, transporters,
554 and nucleic acid regulation. Notably, we found many genes with unknown
555 functions to be dynamically regulated in commensals during interactions with
556 plants ([Fig. 2G](#)). To explore these commensal functions, it will be critical in the
557 future to link bacterial transcriptome responses to bacterial niche preference and
558 reproductive fitness in plants. Our co-transcriptome dataset will provide a robust
559 platform for hypothesis-driven functional investigation of plant and bacterial
560 genes that play critical roles in plant-microbiota interactions.

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574 **Author contributions:**

575 T.N. and K.T. designed the research. T.N., Y.C., F.E., and Y.T. performed
576 experiments. T.N., E.D., and R.G.O. performed analysis. T.N. and K.T. wrote the
577 paper with input from all authors.

578 **Data availability:**

579 The RNA sequencing data used in this study are deposited in the National
580 Center for Biotechnology Information Gene Expression Omnibus database
581 (accession no. GSE150422). Key data and scripts are available at
582 <https://github.com/tnobori/co-transcriptomics>.

583 **Conflict of interest:**

584 The authors declare no conflict of interest.

585 **Methods**

586 Plant materials and growth conditions

587 The *Arabidopsis thaliana* accession Col-0 plants were grown in a chamber at
588 22°C with a 10-h light period and 60% relative humidity for 24 days and then in
589 another chamber at 22°C with a 12-h light period and 60% relative humidity. For
590 all experiments, 31- to 33-day-old plants were used.

591 Bacterial strains

592 Commensal strains were previously isolated from wild *A. thaliana* plants (Bai et
593 al., 2015) (<http://www.at-sphere.com/>) (**Table 1**). The *Pto* mutant D36E was
594 previously described (Wei et al., 2015). Bacterial strains were cultured at 20°C
595 (commensal strains) or 28°C (*Pto* and D36E) at 200 rpm in liquid 50% TSB
596 medium (Sigma-Aldrich, USA).

597 Sampling of bacteria *in vitro*

598 Commensal strains were grown in liquid 50% TSB medium and harvested at the
599 late log phase. 0.1 volume of the stop buffer (95% EtOH, 5% Phenol) was added
600 to bacterial cultures before centrifuging to collect bacterial cells. Target OD₆₀₀ for
601 each strain (the late log phase): Leaf1 = 0.7, Leaf130 = 1.2, Leaf155 = 0.5,
602 Leaf176 = 0.9, Leaf177 = 0.6, Leaf187 = 0.8, Leaf404 = 0.6, Root935 = 0.8,
603 Soil763 = 1.8.

604 Bacterial inoculation to plant leaves and sampling

605 Commensal strains were grown in the liquid 50% TSB medium. For each strain,
606 multiple cultures were prepared with different bacterial densities to ensure that
607 unsaturated cultures were used for experiments. Bacterial cells were harvested
608 by centrifugation, washed twice with sterile water, and resuspended in sterile

609 water to OD₆₀₀ of 0.5. Plants grown in pots were randomized before bacterial
 610 inoculation. For bacterial RNA-seq, 80–100 *A. thaliana* leaves (four fully
 611 expanded leaves per plant) were syringe-inoculated with bacterial suspensions
 612 using a needleless syringe. For plant RNA-seq, approximately six leaves (two
 613 fully expanded leaves per plant) were treated. Mock control (water infiltration)
 614 was included in every plant RNA-seq experiment. Leaves were harvested at 6
 615 hours after inoculation. Sampling was conducted for one plant genotype at a
 616 time, and approximately 5 min were needed per genotype. Leaves were
 617 immediately frozen in liquid nitrogen and stored at -80°C. Three biological
 618 replicates from independent experiments were taken for each condition of plant
 619 and bacterial RNA-seq.

620 Sequencing library preparation and RNA sequencing

621 *In planta* bacterial transcriptome analysis was conducted as described previously
 622 (Nobori et al., 2018) with slight modifications. Briefly, bacteria-infected leaves
 623 were coarsely pulverized and released into bacterial isolation buffer (9.5%
 624 ethanol, 0.5% phenol, 25 mM TCEP (tris(2-carboxyethyl)phosphine) pH 4.5
 625 adjusted with NaOH) at 4°C, filtered, and centrifuged to isolate bacterial cells
 626 from plant cells. The original RNA extraction method based on chemical lysis of
 627 bacterial cells by TriFast (Nobori et al., 2018) did not work for some bacterial
 628 strains, thus we used FastRNA PRO™ BLUE KIT (MP Biomedicals), which
 629 involves mechanical cell lysis. rRNA was depleted to enrich mRNA, and the
 630 cDNA libraries were prepared using Ovation Complete Prokaryotic RNA-seq kit
 631 1-8 (NuGEN).

632 For plant RNA-seq, RNA was extracted with FastRNA PRO™ KIT with Lysing
 633 Matrix E (MP Biomedicals), and DNA was digested with TURBO DNase
 634 (Ambion). RNA quality was determined using a 2100 Bioanalyzer (Agilent
 635 Technologies, USA). Initially, 500 ng total RNA was used for polyA enrichment
 636 with the NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England

637 Biolabs). Subsequent library preparation was performed with NEBNext® Ultra™
638 II Directional RNA Library Prep Kit for Illumina® (New England Biolabs)
639 according to the manufacturer's instructions.

640 Libraries were immobilized and processed onto a flow cell with cBot (Illumina)
641 and subsequently sequenced on the HiSeq3000 system (Illumina) with 1 x 150
642 bp single reads. Primary data analysis (incl. image analysis, cluster identification,
643 base calling, assignment of quality scores) has been performed with RTA
644 (real-time analysis software; Illumina) installed on the sequencing platform.

645 For bacterial and plant samples, approximately 10 and 30 million reads,
646 respectively, were obtained. Bacterial reads were mapped onto the
647 corresponding bacterial genomes (Bai et al., 2015) using Bowtie2 (Langmead
648 and Salzberg, 2012). Plant reads were mapped onto the *Arabidopsis* genome
649 (TAIR10) using HISAT2 (Kim et al., 2015). Mapped reads were counted with the
650 Python package HTSeq (Anders et al., 2015). The RNA-seq data used in this
651 study are deposited in NCBI Gene Expression Omnibus database (accession no.
652 GSE150422).

653 Raw data

654 Raw RNA-seq count and bacterial gene annotation files are available at
655 <https://github.com/tnobori/co-transcriptomics>.

656 Data analysis – plant RNA-seq

657 The statistical analysis of the RNA-seq data was performed in the R
658 environment. Genes with average counts < 5 were excluded from the analysis.
659 The count data were TMM-normalized and log-transformed using the function
660 calcNormFactors in the package edgeR (Robinson et al., 2010) and the function
661 voomWithQualityWeights in the package limma (Ritchie et al., 2015),
662 respectively. To each gene, a linear model was fitted by using the function lmFit
663 in the limma package (Ritchie et al., 2015). The eBayes function in the limma

664 package was used for variance shrinkage during the calculation of the p-values.
 665 The false discovery rate (FDR; the Storey's q-values) was calculated using the
 666 qvalue function in the qvalue package (Storey and Tibshirani, 2003). Genes with
 667 q-value < 0.01 and $|\log_2 \text{fold change}| > 1$ were defined as differentially expressed
 668 genes. The prcomp function was used for principal component analysis.
 669 Heatmaps were created with the pheatmap function in the R environment.
 670 Enriched GO terms were identified using the BiNGO plugin for Cytoscape (Maere
 671 et al., 2005). Scatter plots and box plots were generated using the R-package
 672 ggplot2.

673 Data analysis – bacterial RNA-seq

674 **Bacterial phylogenetic analysis**

675 The bacterial genomes were searched for the bacterial small ribosomal subunit
 676 16S rRNA gene using RNAmmer (Lagesen et al., 2007). Next, a multiple
 677 sequence alignment was performed using Clustal Omega (Sievers et al., 2011)
 678 with default parameters. Finally, we employed FastTree (Price et al., 2010) to
 679 build a maximum-likelihood phylogeny based on the gamma time reversible
 680 substitution model (GTR). This tree was visualized (Fig. 1B) using the interactive
 681 Tree of Life (Letunic and Bork, 2019).

682 **Orthologous gene prediction and KEGG annotation**

683 De novo orthology prediction was performed by using OrthoFinder (Emms and
 684 Kelly, 2015) with default parameters on the predicted protein coding sequences
 685 from the bacterial genomes. Individual genes were annotated with the KEGG
 686 database as a reference (Kanehisa et al., 2014) using the blastkoala webserver
 687 (Prokaryotes group) (Kanehisa et al., 2016). Subsequently, orthologous genes
 688 were assigned a single KO annotation by majority vote of individually annotated
 689 sequences in each group. The genomes of the commensal strains were

690 previously reported (Bai et al., 2015) and are available at our GitHub repository
691 (<https://github.com/tnobori/co-transcriptomics>).

692 **Data normalization and visualization**

693 RNA-seq data were normalized for each strain. After omitting genes with average
694 count < 5, count data was TMM-normalized and log-transformed as described
695 above. Genes with FDR < 0.01 (corrected by Benjamini-Hochberg method) and
696 $|\log_2 \text{ fold change}| > 1$ were defined as differentially expressed genes.
697 Commensal genes were annotated with OGs to integrate gene expression data
698 of different strains. When multiple genes are annotated with the same OG, the
699 mean expression value was taken. Data visualization was performed as
700 described above. UpSet plots were generated in the R environment using the
701 package UpSetR (Conway et al., 2017).

702 KO enrichment analysis (related to [Fig. 2A](#) and [Fig. S3A](#))

703 A custom KEGG ontology (KO) database was created by taking only functional
704 terms encoded in at least one bacterial genome (downloaded in January 2019).
705 For each strain, a list of KOs was generated by subsetting the corresponding
706 KEGG IDs from the custom KO database (**Data S6**). KO enrichment test was
707 performed using a hypergeometric test (FDR corrected by Benjamini-Hochberg
708 method). KOs with FDR < 0.01 and containing more than three genes were
709 defined as significantly enriched KOs. An R script and KO databases are
710 available at <https://github.com/tnobori/co-transcriptomics>.

711 Generating plots of genes with various functions (related to [Fig. 2B-G](#), [Fig. 4B-D](#) 712 [and F](#), [Fig. S3B and C](#), [Fig. S6](#), [Fig. S7](#), [Fig. S9-12](#))

713 Bacterial genes were selected by KEGG pathway annotations or keyword
714 searches from KEGG BRITE annotations. R scripts for this analysis are available
715 at <https://github.com/tnobori/co-transcriptomics>.

716 Intersecting plant-associated bacterial genes and differentially regulated genes in 717 *planta* (related to Fig. 3)

718 In a previous study (Levy et al., 2017), comparative genomics analyses defined
719 “plant-associated (PA) genes” for each phylum/class using multiple statistical
720 tests. The study defined two groups of Actinobacteria (Actinobacteria1 and
721 Actinobacteria2). The Actinobacteria strains used in the present study are all
722 Actinobacteria1). We defined genes that passed at least one statistical test as
723 “PA genes” and the others were defined as nonPA genes. An R script and
724 PA-gene datasets for this analysis are available at
725 <https://github.com/tnobori/co-transcriptomics>.

726 MAMP conservation analysis (related to Fig. 5D)

727 Canonical flg22 and elf18 sequences were blasted against the bacterial
728 genomes using blastp (Camacho et al., 2009) with standard settings. The results
729 of these homology searches were filtered by retaining hits covering at least 90%
730 of the length of the MAMP sequence in the alignment and subsequently
731 retrieving the alignment with the highest percentage identity.

732 Integration of plant and bacterial RNA-seq data (related to Fig. 6)

733 Co-transcriptome fold change data (bacteria: *in planta* vs. *in vitro*; plants: bacteria
734 vs. mock) of nine strains were used for this analysis. Plant genes whose
735 expression was significantly changed by at least one strain were used. Pearson’s
736 correlation coefficients between individual plant genes and bacterial OGs were
737 calculated. The same analysis was performed for all the combinations of eight
738 strains (bootstrapping). Among these 8-strain and 9-strain datasets, the weakest
739 correlation coefficient value was used for each combination of a bacterial OG and
740 a plant gene (Fig. S8). An R script and plant/bacterial gene expression datasets
741 for this analysis are available at <https://github.com/tnobori/co-transcriptomics>.

742 **Determination of bacterial colony forming units (related to Fig. S1)**

743 Bacterial colonization of the leaves was determined following a previous study
 744 (Chen et al., 2020) with slight modifications. The Cl₂-gas-sterilized seeds were
 745 stratified for 2 days at 4°C, sown on half Murashige & Skoogs (MS,
 746 Duchefa-Biochemie, MO255.0050) agar medium with 1% sucrose, and allowed
 747 to germinate for 5 days. Seedlings of the same physiological state were
 748 transplanted on half MS agar medium and were grown for another 9 days (a total
 749 of 2 weeks) prior to inoculation with bacteria. One day before inoculation,
 750 bacterial cultures were grown on half TSB for 24 hours at 22°C with 200 rpm
 751 shaking. On the day of inoculation, bacterial cells were harvested by
 752 centrifugation at 3000 rpm for 15 min, washed twice with sterile water, and then
 753 finally suspended in 10 mM MgCl₂. The resulting bacterial suspensions were
 754 diluted to a final OD₆₀₀ of 0.5 with sterile water and with this, each plate of
 755 2-week-old seedlings was flood-inoculated for 1 min, drained, and allowed to dry
 756 for 15 min. Plants were then grown for 3 days and 2-3 leaves of the same
 757 physiological state were harvested aseptically and weighed. To quantify bacteria
 758 in the endophytic compartment, leaves were surface-sterilized with 75% ethanol
 759 for 30 seconds and washed twice with sterile water, and the leaves were
 760 homogenized on 10 mM MgCl₂ buffer using TissueLyserII (Qiagen) with the
 761 frequency of 30 s⁻¹ for 5 min. The samples were then serially diluted (10⁰ to 10⁵)
 762 and spread-plated on 0.5x TSB agar medium. Plates were incubated at ambient
 763 temperature, colonies were observed and counted for 1-3 d and colony forming
 764 units were expressed per mg FW. The total compartment was assayed similarly
 765 but without surface sterilization.

766 Data S1 OG distribution

767 Data S2 Expression of all OGs

768 Data S3 List of PA and nonPA KOs

769 Data S4 Plant RNA-seq data

770 Data S5 Correlation matrix of plant and bacterial transcriptomes

771 Data S6 KO database for each strain

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931 Supplementary figures

932 **Fig. S1 Bacterial growth in plant leaves** Bacteria were flood-inoculated to
 933 three-week-old *A. thaliana* Col-0 at OD₆₀₀ = 0.5. Bacterial growth was measured
 934 three days after inoculation. Endophytic bacteria were counted after washing and
 935 sterilizing the leaf surface, whereas total bacteria were counted without any
 936 surface washing and sterilization (see Methods).

Fig. S2 Transcriptome analysis of bacteria (A) The correlation plot of each replicate of bacterial RNA-seq data for individual strains. vivo: bacteria in plants. vitro: bacteria in rich media. **(B)** Genes that exist are colored in red. The

941 taxonomic affiliation (phylum/class level) of each strain is indicated with different
942 colors. See **Data S1** for the gene presence-absence table.

Fig. S4: Intrapylum comparative transcriptomics of commensals (A and C)
Gene expression fold changes between *in planta* and *in vitro* (rich media) of (A) Bacteroidetes and (C) Proteobacteria strains. Orthologous groups shared among three strains were used for the analysis. Differentially expressed genes (DEGs) (*in planta* vs. *in vitro*; |log₂FC| > 1; FDR < 0.01; two-tailed Student's t test followed by Storey's q-value) are indicated in the sidebars. Gene clusters defined by k-mean clustering are shown (k = 8). (B and D) UpSet intersection plots of

965 DEGs either up- (red) or down (blue)-regulated *in planta* in the **(B)** Bacteroidetes
 966 and **(D)** Proteobacteria strains. Intersection size and set size indicate the number
 967 of shared DEGs and the number of DEGs in each strain, respectively. **(E-G)**
 968 Enrichment analysis of genes with the KEGG ontology terms **(E)** “Ribosome”, **(F)**
 969 “Transporters”, and **(G)** “Two-component system”.

Fig. S5: Bacterial genes differentially regulated in plants UpSet intersection plots of differentially expressed genes (DEGs; $|\log_2FC| > 1$; FDR < 0.01; two-tailed Student's t test followed by Storey's q-value) either (A) up- or (B) downregulated *in planta*. Intersection size and set size indicate the number of shared DEGs and the number of DEGs in each strain, respectively. Combinations of more than one strain with intersection size > 5 are shown. The color sidebars indicate the taxonomic affiliation.

Fig. S6: Expression of various processes of commensals in planta (A and B) Expression fold changes (*in planta* vs. *in vitro*) of genes related to (A) transporters and (B) two-component system. Results are shown as box plots with boxes displaying the 25th–75th percentiles, the centerline indicating the median, whiskers extending to the minimum, and maximum values no further than 1.5 inter-quartile range. Box color indicates the taxonomic affiliation (phylum/class level) of each strain. All individual data points (genes) are overlaid with colors for DEGs (red: upregulated, blue: downregulated, black: non-DEG).

987 **Fig. S7: Expression of genes related to nutrient acquisition processes in**
 988 **commensals** Expression fold changes (*in planta* vs. *in vitro*) of genes related to

989 nutrient transporters. Results are shown as box plots with boxes displaying the
 990 25th–75th percentiles, the centerline indicating the median, whiskers extending
 991 to the minimum, and maximum values no further than 1.5 inter-quartile range.
 992 Box color indicates the taxonomic affiliation (phylum/class level) of each strain
 993 (see Fig. S6 for the color code). All individual data points (genes) are overlaid
 994 with colors for DEGs (red: upregulated, blue: downregulated, black: non-DEG).

995 **Fig. S8: Integration of plant and bacterial transcriptomes** Schematic diagram
 996 showing a bootstrapping approach to evaluate correlations between individual
 997 plant and bacterial genes. To obtain robust correlation scores, Pearson's
 998 correlation coefficients were calculated using all the combinations of eight strains
 999 as well as using all the nine strains. Among these 8-strain and 9-strain datasets,
 1000 the weakest correlation coefficient value was used for each combination of a
 1001 bacterial OG and a plant gene ("Combining data").

1002 **Fig. S9: Expression of commensals genes related to various physiological**
 1003 **processes *in planta*** Expression fold changes (*in planta* vs. *in vitro*) of genes
 1004 related to various functions. Results are shown as box plots with boxes
 1005 displaying the 25th–75th percentiles, the centerline indicating the median,
 1006 whiskers extending to the minimum, and maximum values no further than 1.5
 1007 inter-quartile range. Box color indicates the taxonomic affiliation (phylum/class
 1008 level) of each strain. All individual data points (genes) are overlaid with colors for
 1009 DEGs (red: upregulated, blue: downregulated, black: non-DEG).

Fig. S10: Expression of commensal genes related to various physiological processes in planta Expression fold changes (*in planta* vs. *in vitro*) of genes related to various functions. Results are shown as box plots with boxes

1014 displaying the 25th–75th percentiles, the centerline indicating the median,
 1015 whiskers extending to the minimum, and maximum values no further than 1.5
 1016 inter-quartile range. Box color indicates the taxonomic affiliation (phylum/class
 1017 level) of each strain. All individual data points (genes) are overlaid with colors for
 1018 DEGs (red: upregulated, blue: downregulated, black: non-DEG).

1019 **Fig. S11: Expression of commensal genes related to various physiological**
 1020 **processes *in planta*** Expression fold changes (*in planta* vs. *in vitro*) of genes
 1021 related to various functions. Results are shown as box plots with boxes
 1022 displaying the 25th–75th percentiles, the centerline indicating the median,
 1023 whiskers extending to the minimum, and maximum values no further than 1.5
 1024 inter-quartile range. Box color indicates the taxonomic affiliation (phylum/class
 1025 level) of each strain. All individual data points (genes) are overlaid with colors for
 1026 DEGs (red: upregulated, blue: downregulated, black: non-DEG).

1027 **Fig. S12: Expression of commensal genes related to various physiological**
 1028 **processes *in planta*** Expression fold changes (*in planta* vs. *in vitro*) of genes
 1029 related to various functions. Results are shown as box plots with boxes
 1030 displaying the 25th–75th percentiles, the centerline indicating the median,
 1031 whiskers extending to the minimum, and maximum values no further than 1.5
 1032 inter-quartile range. Box color indicates the taxonomic affiliation (phylum/class
 1033 level) of each strain. All individual data points (genes) are overlaid with colors for
 1034 DEGs (red: upregulated, blue: downregulated, black: non-DEG).