# Distinct within-host bacterial populations ensure function, colonization and transmission in leaf symbiosis

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## Abstract

Hereditary symbioses have the potential to drive transgenerational effects, yet the mechanisms responsible for transmission of heritable plant symbionts are still poorly understood. The leaf symbiosis between *Dioscorea sansibarensis* and the bacterium *Orrella dioscoreae* offers an appealing model system to study how heritable bacteria are transmitted to the next generation. Here, we demonstrate that inoculation of apical buds with a bacterial suspension is sufficient to colonize newly-formed leaves and propagules, and to ensure transmission to the next plant generation. Flagellar motility is not required for movement inside the plant, but is important for the colonization of new hosts. Further, stringent tissue-specific regulation of putative symbiotic functions highlight

the presence of two distinct subpopulations of bacteria in the leaf gland and at the shoot meristem. We propose that bacteria in the leaf gland dedicate resources to symbiotic functions, while dividing bacteria in the shoot tip ensure successful colonization of meristematic tissue, glands and propagules. Compartmentalization of intra-host populations, together with tissue-specific regulation may serve as a robust mechanism for the maintenance of mutualism in leaf symbiosis.

## Importance

Several plant species form associations with bacteria in their leaves, called leaf symbiosis. These associations are highly specific, but the mechanisms responsible for symbiont transmission are poorly understood. Using the association between the yam species *Dioscorea sansibarensis* and *Orrella dioscoreae* as a model leaf symbiosis, we provide experimental evidence that bacteria are transmitted vertically and distributed to specific leaf structures via association with shoot meristems. Flagellar motility is required for initial infection, but does not contribute to spread within host tissue. We also provide evidence that bacterial subpopulations at the meristem or in the symbiotic leaf gland differentially express key symbiotic genes. We argue that this separation of functional symbiont populations, coupled to tight control over bacterial infection and transmission, explain the evolutionary robustness of leaf symbiosis. These findings may provide insights into how plants may recruit and maintain beneficial symbionts at the leaf surface.

47

48 # Introduction

49 Heritable symbioses are common in animals, with many examples in invertebrates. For example,
50 aphids (Hemiptera) harbor *Buchnera* bacteria, and 16% of all known insect species interact with
51 *Wolbachia* bacteria (1–3). These model systems have provided tremendous insights into the cellular
52 mechanisms underlying heritable symbiont transmission (4–6). In contrast to animal symbioses,
53 most well-described plant-microbe symbioses rely on horizontally-transmitted symbionts, such as
54 the interactions involving rhizobia or mycorrhizal fungi (7). Heritable transmission of symbionts has
55 been demonstrated for only a handful of plant taxa, and the mechanisms governing symbiont
56 transmission are still poorly understood (8, 9). However, recent evidence suggests that vertically-
57 transmitted symbionts may also account for important transgenerational phenotypes (10–12). In
58 addition, mode of transmission has important implications for the evolution of host-microbe
59 associations. Indeed, while horizontal transmitted symbionts are usually vetted through a
60 combination of partner choice and sanctions and rewards, vertical transmission is thought to be an
61 efficient mechanism to establish successful cooperation through partner fidelity feedback (13, 14).

62 Three Angiosperm families include species that harbor possible vertically-transmitted bacterial
63 symbionts (15). In the Primulaceae family, 30 out of 35 species of *Ardisia* display small glands at the
64 leaf margin, colonized by *Burkholderia* bacteria (16). Congruent phylogenies of host and symbiotic
65 bacteria suggest co-speciation and a strictly vertical mode of transmission (17). In the Rubiaceae
66 family, nearly 500 plant species engage in leaf nodule symbiosis, with about 350 species in the
67 *Pavetta* genus, 85 in *Psychotria* and 12 in *Sericanthe* (18–20). Unlike the Primulaceae family, the
68 structures housing the bacteria have variable morphologies and may be distributed throughout the
69 leaf lamina or along the midvein (19). Similar to *Ardisia*, the symbiosis involves specific associations
70 with *Burkholderia* bacteria. Here, phylogenetic patterns suggest a mixed mode of transmission, with
71 vertical transmission and occasional events of host-switching (16, 21, 22). In addition, the symbionts
72 of *Psychotria punctata* are present in all life stages of the plant, including flower buds, anthers,
73 gynoecium and embryos, providing strong evidence of vertical transmission in this taxon (23). Leaf
74 nodule bacteria lack the genetic ability to fix nitrogen or metabolize phytohormones. Instead, the
75 symbionts provide secondary metabolites that may protect the host against phytophagous insects or
76 competitors (15, 24, 25). Because of their mutual dependence, the study of the molecular
77 mechanisms underlying the associations between heritable leaf nodule bacteria and their hosts is
78 challenging. For example, aposymbiotic seeds of *Psychotria* sp. and *Ardisia crenata* germinate
79 normally, but fail to develop more than a few leaves and do not reach maturity (26). Moreover,

genomes of leaf nodule bacteria do not encode known signalling pathways such as Nod factors, type III secreted effectors or plant hormones (15), and the molecular functions enabling colonization and transmission are unknown.

More than thirty years ago, Miller and Reporter used microscopy techniques and described the presence of a bacterial symbiont in the leaf acumen of *Dioscoreae sansibarensis*, but did not identify the bacterium (Miller & Reporter, 1987). Interestingly, symbiont-free plants could reportedly be obtained by surface-sterilization of bulbils, although these aposymbiotic plants readily became colonized by bacteria upon transfer to a non-sterile environment. We recently isolated and described these symbiotic bacteria as *Orrella dioscoreae* (*Alcaligenaceae*) (28, 29). Leaves of *D. sansibarensis* are heart-shaped and end with a distal acumen or forerunner-tip which exclusively harbours *O. dioscoreae*. In contrast to symbionts of Rubiaceae and Primulaceae, *O. dioscoreae* can be cultured outside of the host plant and is amenable to genetic manipulation (29). *O. dioscoreae* can be isolated from vegetative propagules and recent data indicates that the association with *D. sansibarensis* is ubiquitous throughout the range of the host plant. This suggests a vertical mode of transmission, but low phylogenetic congruence between plant and symbiont genetic markers indicates possible horizontal or host-switching transmission (30). Moreover, genomes of *O. dioscoreae* strains do not display any of the hallmarks of genome reductive evolution, a common phenomenon in vertically transmitted leaf symbioses (15).

In this work, we show that the association between *D. sansibarensis* and *O. dioscoreae* is tissue-specific. Using a newly developed gnotobiotic system, we demonstrate that bacteria are transmitted vertically, with possible horizontal transmission relying on bacterial motility and infection of developing apical buds. Our results provide insights into the transmission of a heritable bacterial symbiont in land plants and some of the molecular mechanisms that shape the evolution of leaf-bacteria symbioses.

# Material and Methods

## Plant culture and propagation

Plants were maintained in the greenhouse of the Laboratory of interactions Plant-Microbe-Environment (LIPME) in Castanet-Tolosan, France. Unless otherwise indicated plants were grown in climate chambers at 28°C, 70% humidity and a light cycle of 16h light (210 µmol/m$^2$/s), 8h dark. Chemicals and reagents were purchase from Merck, France unless otherwise indicated. Micropropagation of *Dioscorea sansibarensis* was done using a protocol adapted from Alizedah et al. (31). Node cuttings were collected from greenhouse-grown plants after 2-4 months of growth.

112 Explants were surface sterilized by submerging them in a 5% solution of Plant Preservative Mixture
113 (PPM, Plant Cell Technology, USA) with shaking at 100 rpm for 8hrs at 28°C, in the dark. After 8
114 hours, the bleached extremities of the explants were removed with a sterile scalpel. Explants were
115 placed in sterilized growth medium (Murashige and Skoog basal salts (MS) : 4.4g/L, 2% sucrose,
116 vitamins: glycine (2mg/L), myo-inositol (100 mg/L), nicotinic acid (0.5mg/L), pyridoxine-HCl
117 (0.5mg/L), thiamine.Cl (0.1mg/L) and L-cystein (20mg/L), pH=5.7), supplemented with 200 µg/ml
118 carbenicillin (Meridis, France), 200 µg/ml cefotaxime (Meridis, France) and 0.2% v/v plant
119 preservative mixture (PPM, Plant Cell Technology, USA). Explants were incubated at 28°C, 16h/8h of
120 light cycle for 10 days. The medium was refreshed after 10 days, including supplements and
121 antibiotics. After 21 days of incubation, the medium was replaced with growth medium containing
122 MS, sucrose, PPM and vitamins as described above but without the antibiotics. Cuttings were
123 transferred in magenta GA-7 vessels (Merck), incubated at 28°C, 16h of light until rooting.

124 ## Detection and identification of bacteria

125 The tip of the leaf was dissected with tweezers and a scalpel, and the tissue was homogenized using
126 100 µl 0.4% NaCl and 3 sterile glass beads for 1 minute at 30 Hz in a ball mill (Retsch MM 400). The
127 homogenized suspension was centrifuged briefly to pellet debris. One hundred µL of supernatant
128 was directly plated out on TSA (Sigma) plates and incubated for 2 days at 28°C. If the plate showed
129 growth, one isolate per colony type was picked and identified using colony PCR with primers specific
130 for *O. dioscoreae* (nrdA-01-F, nrdA-02-R, Table S2), or with universal 16S rRNA primers (pA and pH,
131 Table S2) followed by Sanger sequencing.

132 ## Inoculation of *D. sansibarensis* with bacteria

133 Node cuttings were grown in axenic conditions (25ml MS + 2% sucrose + 0.2% PPM in Magenta
134 vessel, 28°C, 16h/8h light cycle) until a new shoot appeared (after 6 weeks approximately). Verified
135 aposymbiotic plants (tested as stated above) were inoculated with a strain of interest as followed:
136 bacterial cultures in the exponential phase of growth were centrifuged (5000 rpm, 10 min) and
137 washed twice with sterile 0.4% NaCl. Cell suspensions were normalized to $OD_{600nm}$ = 0.2. The biggest
138 leaf at the apical bud was gently pushed aside and 2 µl of the bacterial suspension ($OD_{600nm}$ = 0.2)
139 was gently deposited onto the apical bud (Suppl Figure 1). Plants were transferred to sterile
140 microboxes (50ml MS + 2% sucrose + 0.2% PPM) at 28°C, 16h of light until new leaves emerged.
141 Colonization was evaluated by dissecting a leaf tip and spreading the contents on suitable
142 microbiological medium as described above. Plants were transferred to pots with soil and incubated
143 in growth chamber. Shortly before senescence, plants develop bulbils. These bulbils were harvested

144 and stored in a dark, dry place at room temperature for about 6 months or until dormancy broke.
145 Sprouting bulbils were planted in soil and pots were left at 25°C, 16h of light.

146 Bacterial genetics

147 *O. dioscoreae* strain R-71412 is a spontaneous nalidixic acid-resistant strain derived from *O.*
148 *dioscoreae* LMG 29303$^T$ (29). To obtain strain R-71417, a mini-Tn7 cassette containing the mCherry
149 reporter gene was introduced into *O. dioscoreae* R-71412 by tri-parental mating as in Choi and
150 Schweizer (32). Briefly, overnight cultures of recipient (*O. dioscoreae* R-71412), donor (*E. coli* S17-1
151 mini-Tn7::*mCherry* ) and helper strain (*E. coli* S17-1 pUX-BF13) were diluted 1:100 in fresh medium
152 without antibiotics (LB for *E. coli* and TSB for *O. dioscoreae*) and grown to $OD_{600nm}$ = 0.5 while shaking
153 at 37°C or 28°C. Cells were washed once in sterile 0.9% sodium chloride and re-suspended in sterile
154 LB medium to $OD_{600nm}$ ~ 1. About 100 µL of each suspension was spotted on LB agar without
155 antibiotics and incubated overnight at 37°C. Cells were suspended in 500 µl of 0.9% NaCl solution
156 and plated on selective medium (TSA supplemented with nalidixic acid (30 µg/mL) and gentamycin
157 (20 µg/mL)) and incubated at 30 °C for 48h. Fluorescent colonies were visualized with a
158 stereomicroscope (Leica DFC 7000T). The insertion of the transposon downstream of the *glmS* gene
159 was confirmed by PCR using primers "Mini Tn7 primer forward" and "Mini Tn7 primer reverse"
160 (Table S2).

161 To create a motility impaired *O. dioscoreae* mutant, a mutant allele of a *motB* homolog (locus tag
162 ODI_R2122) was created by PCR amplification of three overlapping DNA fragments, containing the
163 flanking regions of the gene of interest and the kanamycin resistance cassette from pKD4 (33). The
164 upstream flanking region of the *motB* gene was amplified by using primers motB-UpF-GW and motB-
165 UpR-kan, and the downstream flanking region of the *motB* gene was amplified by using primers
166 motB-DnF-kan and motB-DnR-GW (Table S2). The up- and downstream fragments were fused
167 together and amplified by using primers GW-*attB*1 and GW-*attB*2 (Table S2) by overlap extension
168 PCR (SOE PCR) to generate the *motB* mutant allele. Once the fragments were verified, the PCR
169 constructs were ligated into pDONRPEX18Tp-SceI-pheS (34) using the Invitrogen BP ligation kit and
170 transformed by electroporation into *E. coli* Top 10. Suicide plasmids were introduced in *O.*
171 *dioscoreae* (R71417) by triparental mating as above, using *E. coli* harbouring plasmid pRK600 as
172 helper. Transconjugants were selected on TSA medium supplemented with kanamycin 50 µg/ml and
173 nalidixic acid 30 µg/ml and incubated for 2 days at 28°C. Counter-selection of merodiploid clones
174 was done by spreading on AB minimal medium supplemented with 0.2% citrate, 0.1% yeast extract
175 and 0.1% (wt/vol) *p*-chlorophenylalanine (cPhe) (DL-4-chlorophenylalanine; Sigma-Aldrich). Colonies
176 were screened for loss of trimethoprim resistance on TSA medium supplemented with nalidixic acid
177 30 µg/ml and kanamycin 30 µg/ml. Selected clones were validated by PCR and whole genome

178 sequencing to rule out ectopic mutations using Illumina paired-end libraries as described previously.
179 Sequences were deposited in the European Nucleotide Archive with accession number ERR7179810.

180 For genetic complementation, the *motAB* locus (locus tags ODI_R2121 and ODI_R2122, including the
181 promoter region) was amplified by PCR using primers motAB-Fwd-KpnI and motAB-rev-SacI (Table
182 S2) and ligated into plasmid pBBR1MCS-3 after restriction with enzymes SacI and KpnI (NEB).
183 Ligation products were transformed into *E. coli* Top10 by electroporation. Constructs were verified
184 using PCR and Sanger sequencing. Plasmids were introduced into *O. dioscoreae* by electroporation.
185 Briefly, 1 mL overnight cultures of *O. dioscoreae* were washed 3 times in sterile ultrapure water and
186 resuspended in 40 μL. About 0.5 μg of plasmid DNA were mixed with the cell suspension and
187 transferred to ice-cold 1mm gap cuvettes (Bio-Rad). Cells were electroporated in a Bio-Rad Gene
188 Pulser Xcell system using settings: 1.8 kV voltage, 25μF, 200 Ω. Transformants were selected on TSA
189 medium supplemented with tetracycline (20 μg/L).

190 Transmission electron microscopy

191 Samples were fixed in 2% (v/v) glutaraldehyde (EMS) + 0.5% (v/v) paraformaldehyde (EMS) in a 50
192 mM sodium buffer, pH 7.2 at room temperature and under vacuum. After four hours, the fixative
193 solution was refreshed and samples were kept at 4°C for 26 days. Samples were rinsed twice in 50
194 mM cacodylate sodium buffer (pH 7.2) and postfixed in 2% (v/v) osmium tetroxide in water for 1.5
195 hours at room temperature in darkness. Samples were rinsed three times in water and dehydrated
196 using a graded ethanol series (10%-100%, 10% increments). Samples were then incubated in
197 propylene oxide (PO) (EMS)) for 2 times 1 hour and infiltrated in Epon using a PO/Epon series over
198 multiple days at 4°C. Samples were embedded in flat embedding molds and polymerized for 48
199 hours at 60°C. Thin sections of 1 µm were cut using a Leica Ultracut E Reichert and contrasted using
200 Uranyless and lead citrate (Delta Microscopies, France). Samples were viewed using a Hitachi
201 HT7700 electron microscope.

202 Scanning electron microscopy

203 Samples were fixed in 2.5% (v/v) glutaraldehyde in 50 mM cacodylate sodium buffer (pH 7.2) for 3
204 hours at RT and transferred to 4°C for 2 days. Samples were dehydrated using a graded ethanol
205 series. The samples were dried using a critical point drier (Leica EM CPD 300) using $CO_2$ as
206 transitional medium. A platinum coating was applied and samples were examined using a FEG FEI
207 Quanta 250 electron microscope.

208 Light Microscopy

209 Samples were fixed in 4 % (v/v) formaldehyde in PEM buffer (100 mM 1,4-
210 piperazinediethanesulfonic acid, 10 mM $MgSO_4$, and 10 mM ethylene glycol tetra-acetic acid, pH 6.9)

211 and rinsed in water. Samples were washed in PBS ($Na_2HPO_4$ 0.148 g, $KH_2PO_4$ 0.043 g, NaCl 0.72 g,
212 $NaN_3$ 0.9 g in 100 mL distilled water, pH 7.1) and dehydrated using a graded ethanol series (30, 50,
213 70, 85, 100 % (v/v)). Samples were polymerised in LR White acrylic resin (medium grade, London
214 Resin Company, UK) using polypropylene capsules at 37 °C for three days. Semi-thin sections of 350
215 nm were cut using Leica UC6 ultramicrotome (Leica Microsystems, Vienna) equipped with a diamond
216 knife. Sections were collected on polylysine-adhesion slides (Carl Roth, Germany). Sections were
217 stained with 1% (w/v) toluidine blue O (Merck, Germany) in 1% $Na_2B_4O_7$ for 20 seconds at 50°C,
218 rinsed with $dH_2O$ and mounted in DePeX.

219 Samples stained with Calcofluor and Auramine O were processed as followed: Wild-type acumens
220 were fixed in 4% paraformaldehyde in PBS at 4°C overnight, washed twice in PBS and cleared by
221 subsequently incubating samples in clearing solutions for one week at 37°C. The first solution
222 contained 5% v/v glycerol + 10% v/v sodium deoxycholate + 10% v/v urea + 10% v/v xylitol and urea
223 and xylitol concentrations increased to 20% and 30% in week 2 and week 3, respectively. Cleared
224 samples were stained overnight at 4°C in 0.01% Calcofluor and 0.01% Auramine O.

225 For vibratome sectioning, samples were enclosed in 8 % agarose, glued upon the cutting stage using
226 superglue (Roticoll 1, Carl Roth, Karlsruhe, Germany) and cut into 30 µm thick sections with a
227 vibrating microtome (HM650V, Thermo Fisher Scientific, Waltham, MA, USA). Sections were stained
228 with 0.5% (w/v) astra blue, 0.5% (w/v) chrysoidine and 0.5% (w/v) acridine red for 3 minutes, rinsed
229 in water, dehydrated with isopropyl alcohol and mounted in Euparal (Carl Roth, Karlsruhe,
230 Germany). All sections were observed using a Nikon Eclipse Ni-U bright field microscope equipped
231 with a Nikon DS-Fi1c camera.
232 To visualize mCherry tagged *O. dioscoreae* (R71417) in the shoot tips, fresh plant samples were
233 sectioned with a razor blade and imaged using a laser scanning confocal microscope (Leica TCS SP2).
234 LAS X software was used to process the images.

235 ## Estimation of infection bottleneck

236 Bacterial strains (R-67170 and R-71416) were cultured in TSB medium. Bacterial cultures in the
237 exponential phase of growth were centrifuged (5000 rpm, 10 min) and washed twice with 0.4% NaCl.
238 Cell suspensions were normalized to $OD_{600nm}$ = 0.2. Suspensions of R-71416 (GFP-tagged and
239 resistant to gentamycin) were serially diluted with suspensions of the non-tagged strain to yield
240 different concentrations of target strain (1:1, 1:10, 1:100, 1:1000, 1:10 000, 1:100 000) at a constant
241 OD. These suspensions were used to inoculate aposymbiotic plants as described above. Per
242 condition, 5 plants were inoculated. Plants were left at 28°C, 16h of light. After 5 weeks, acumens of
243 young leaves were ground in 100µl sterile 0.4% NaCl as described above and serial dilutions were

244 plated out on selective (TSA medium supplemented with nalidixic acid 30 µg/ml and gentamycin 50
245 µg/ml) and non-selective (TSA medium supplemented with nalidixic acid 30 µg/ml) medium as
246 described above.

247 ## In vitro motility test

248 Bacteria of interest were grown in liquid culture in TSB medium and 5 µl of overnight cultures were
249 spotted on motility agar medium: pancreatic digest of casein Bacto peptone (10g/L), meat extract
250 (3g/L), sodium chloride (5g/L) and agar: (4g/L), triphenyltetrazolium chloride (TTC) 0.05g/L. Plates
251 were incubated at 28°C and the bacterial halo was measured after 48 hours.
252

253 ## Measurement of gene expression

254 Apical buds and leaf acumens were ground in liquid nitrogen. RNA samples (four biological replicates
255 per sample) were isolated using the RNeasy Plant Mini Kit (Invitrogen) with DNAse treatment
256 following the manufacturer's recommendations. Ribonucleic acid was quantified using a NanoDrop
257 Spectrophotometer ND-100 (NanoDrop Technologies, Wilmington, DE, USA) and integrity was
258 evaluated with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Reverse
259 transcription was performed with 2 µg of total RNA using the Reverse transcriptase Superscript II
260 (Invitrogen) and random hexamer primers (Eurofins Genomics, Germany) for bacterial transcript
261 quantification. Quantitative PCRs were conducted with SybrGreen (Roche) on 384-well plates using a
262 LightCycler 480 (Roche) following manufacturer recommendations and the primers shown in Table
263 S2. The *gyrB* encoding gene was used as an internal standard for sample comparisons. The specificity
264 and efficiency of the amplification were verified by analyses of melting curves and standard curves,
265 respectively. The $2^{-\Delta\Delta Ct}$ method was used for the calculation of relative expression (35).

266

267

268 # Results

269 **Anatomy of the *D. sansibarensis* leaf gland and relationship with the symbiotic bacteria**

270 To investigate the distribution of the symbiotic bacteria in *D. sansibarensis*, we dissected various
271 surface-sterilized organs and tissues and counted colonies of *O. dioscoreae* after maceration and
272 serial dilution plated on TSA medium (Table 1). The acumens on *D. sansibarensis* leaves contain the
273 highest number of viable bacteria, with 2.31 x $10^{11}$ cfu/g on average (Figure 1, Table 1). Cross-
274 sections of the forerunner tip showed from 2 kidney-shaped glands, and up to 6 glands per acumen
275 in large leaves (Figure 1B). Glands run along the entire length of the acumen and are lined by a
276 cuticle (Miller & Reporter, 1987). Glands are closed at the adaxial side, where a remaining suture is
277 apparent (Figure 1B). A thick outer layer, which stains intensely with Auramine O, lines the inside of
278 the glands. Auramine O is a lipophilic fluorescent dye with affinity for regions containing acidic and
279 unsaturated cuticle waxes (36). This cuticle layer forms a physical barrier between the mesophyll and
280 the lumen of the gland (Figure 1C). Long vermiform trichomes project into the lumen of the gland
281 (Figure 1C), which also contains a high density of bacteria (Figure 1B, Figure 2A). Trichome cells
282 contain multiple vacuoles and vesicles, indicating intense cytotic activity (Figure 2A). In addition,
283 bacteria display a thick, electron-lucent capsule with visible membranous projections (Figure 2B).
284 Trichome cells close to the bacteria-filled lumen contain Golgi, endoplasmatic reticula and numerous
285 vesicles (Figure 2C). Some vesicles are seen merging with the plasma membrane indicating cytotic
286 activity (Figure 2D). At this interface between trichome head cells and bacteria, the electron-dense
287 cuticle presents small gaps (Figure 2C). We did not observe structures resembling bacteria inside
288 plant cells, suggesting a strict extracellular lifestyle. Apical and lateral buds also showed high levels
289 of endophyte colonization. However, leaf lamina and stems contain nearly undetectable quantities
290 of bacteria (Table 1). This suggests that, outside of the leaf gland, bacteria only associate with
291 organogenic tissues. We also detected bacteria inside bulbils, the main form of propagule of *D.*
292 *sansibarensis*. Although difficult to detect by fluorescence microscopy, bacteria may be present in
293 the intercellular spaces of the bulbil growth center, from which new shoots emerge after
294 germination (Table 1, Figure S2).

295 ## Development of the symbiotic gland

296 These observations indicate that glands are an important site of exchange between the symbiotic
297 partners. To understand how the symbiotic bacteria colonize the newly formed leaf glands, we
298 studied the development of the gland in the apical bud (Figure 3). The leaf acumen, sometimes
299 called a forerunner tip (37), is the first leaf structure formed as leaf primordia emerge. At later
300 stages, the tip of the leaf folds, with margins meeting in the center to form a chamber (Figure 3B-C).

301 Each apical bud contains 5-6 primordial leaves, of which only the three oldest develop a primordial
302 forerunner tip (Figure 3D-F). The adaxial side of leaves displays high densities of glandular trichomes
303 (Figure S3). The abaxial side also presents glandular trichomes, albeit in fewer numbers (Figure S3).
304 Few visible bacteria are embedded in mucus associated with glandular trichomes at the adaxial side
305 of young developing leaves (Figure S4), in an enclosed space delineated by the youngest leaf pair
306 and the apical meristem which is reminiscent of the leaf enclosed chamber of *Psychotria punctata*
307 (23)(Figure 3A). We did not observe the same shape of glandular trichomes in the closed acumens.
308 Instead, vermiform glandular trichomes fill the gland together with mucus and the bacterial
309 symbiont. Together, this indicates that bacteria originate from diffuse colonies near shoot meristems
310 and colonize the symbiotic acumens as soon as the structure emerges.

311 ## The shoot tip is a symbiotic hub

312 We developed a symbiont-replacement assay to visualize the journey of *O. dioscoreae* from the
313 apical bud to the leaf gland. To inoculate the plant with exogenous *O. dioscoreae*, we first designed a
314 method to generate aposymbiotic plants to eliminate spatial competition. Initial attempts to obtain
315 aposymbiotic plants by growing surface-sterilized bulbils in sterile medium as in Miller and Reporter
316 (Miller & Reporter, 1987) consistently resulted in plants colonized by wild-type *O. dioscoreae* (data
317 not shown). Instead, we obtained aposymbiotic plants by submerging node cuttings in a mixture of
318 antibiotics in plant growth medium. Aposymbiotic plantlets were then inoculated by depositing cell
319 suspensions of *O. dioscoreae* strains expressing GFP or mCherry (strains R-71416 and R-71417,
320 respectively) onto the apical bud in otherwise sterile conditions (Figure S1). Glands of new leaves,
321 which emerged above the point of inoculation, exclusively contained tagged bacteria, while older
322 leaves below did not. This indicates that bacteria colonize symbiotic tissue during early development
323 near the shoot meristem, but do not spread to older tissue via apoplastic or symplastic routes. At
324 the apical bud, bacteria seem to adhere to the trichomes on primordial leaves (Figure 4).

325 To investigate if bacteria are transferred to the next generation, we inoculated aposymbiotic plants
326 with mCherry-tagged *O. dioscoreae* R-71417. After 5 weeks of growth in gnotobiotic conditions, we
327 transferred the plants to open pots filled with soil. We harvested bulbils of the plants that survived
328 the transfer to open pots at the end of the growing season and planted the bulbils in soil. Plants
329 germinated from bulbils all contained fluorescent *O. dioscoreae* in their forerunner tips,
330 demonstrating that presence of *O. dioscoreae* at the apical bud is sufficient for the colonization of
331 plant tissues, including reproductive structures such as bulbils. These results show that the bacterial
332 symbiont is transmitted through the bulbils. Unfortunately, we were not able to establish if sexual
333 reproductive structures also contain symbiotic bacteria because *D. sansibarensis* rarely flowers in
334 the wild, and never in cultivation (38).

335 Although artificial, our symbiont-replacement assay also shows that horizontal acquisition of *O.*
336 *dioscoreae* is possible. To gain a better understanding of how likely exogenous bacteria are to enter
337 the apical bud, we infected aposymbiotic plants with mixed cell suspensions of GFP-tagged *O.*
338 *dioscoreae* R-71416 and a wild type *O. dioscoreae* R-71412 in ratios ranging from 1:1 to $1:10^5$, for a
339 total number of approximately $2 \times 10^5$ cells per inoculum. Leaf glands were harvested from plants
340 grown in gnotobiotic conditions at 5 weeks post-infection, macerated and the contents plated on
341 selective medium and non-selective medium to count colonies of tagged and total bacteria,
342 respectively. We detected GFP-tagged bacteria in only 20% of plants inoculated with a dilution factor
343 of 1:100, and none with dilution factors above 1:1000 (Figure S5). This suggests that the number of
344 bacteria establishing in the plant is in the low hundreds. Together with the fact that all our attempts
345 to force ingress of exogenous bacteria in already symbiotic plants failed (data not shown), we
346 conclude that horizontal symbiont transmission is probably a rare event, in accordance with our
347 previous phylogenetic analyses (30).

## 348 Populations of *O. dioscoreae* in the leaf enclosed chambers and leaf glands are physiologically 349 distinct

350 As bulbils and tubers grow from modified shoot buds, we propose that the small colony of *O.*
351 *dioscoreae* in leaf-enclosed chambers provides the initial inoculum for the developing leaf glands, as
352 well as lateral meristems and the reproductive organs. We hypothesized that bacteria occurring in
353 leaf glands may dedicate their metabolism to symbiotic functions, whereas bacteria in buds may
354 allocate resources for multiplication and transmission. We have previously identified a set of genes
355 in three putative operons that were highly upregulated in the leaf gland compared to axenic cultures
356 (29). The *smp1*, *smp2* and *opk* genes are related to non-ribosomal peptide and polyketide synthesis,
357 respectively, and were upregulated >150-fold in the leaf gland vs. culture (29). To test if bacterial
358 populations at the leaf glands and at the apical buds have distinct metabolic characteristics, we
359 measured the expression of select *smp* and *opk* genes by RT-qPCR. Expression levels of *smp* and *opk*
360 transcripts were at least 10-fold lower in apical bud bacteria compared to leaf gland (Table 2). This is
361 likely an underestimation, since transcript levels of target genes were below detection levels in some
362 apical bud samples (Table S3).

## 363 Motility is dispensable for host colonization, but necessary for horizontal transmission

364 Motility is often required by plant pathogens and symbionts to colonize their hosts (39–43). Our
365 observation that *O. dioscoreae* do not colonize leaf glands below the inoculation point however
366 suggests that movement of bacteria within the plant is limited. Moreover, obligate *Burkholderia* leaf
367 symbionts of Rubiaceae and Primulaceae lack flagella, suggesting that motility is not essential for

368 within-host spread or vertical transmission in leaf symbiosis (16). To test whether flagellar motility is
369 required for colonization of *D. sansibarensis*, we generated strain *O. dioscoreae* TA01 by allelic
370 exchange with a copy of a *motB* homolog (locus tag ODI_R2122) interrupted by a kanamycin
371 resistance cassette. MotB is a component of the flagellar motor complex and is essential for motility
372 (44). We confirmed that MotB is involved in motility in *O. dioscoreae* by measuring the halo of
373 colonies spotted onto soft motility agar. The colony diameter of strain R-71417 (WT) on motility agar
374 was 6.03 ± 1.11 cm (95% confidence interval) while strain TA01 (*ΔmotB*) was unable to move beyond
375 the initial spot on the agar (colony diameter of 0.93 ± 0.05 cm (95% C.I.). The complemented strain
376 TA01 $motB^{+}$, showed intermediate levels of motility with a colony diameter of 2.17 ± 1.23 cm (95%
377 C.I.). Importantly, we did not notice a difference in growth rates between strains TA01 and R-71417
378 (data not shown). To test the effect of impaired motility *in planta*, we introduced strains R-71417 or
379 TA01 into aposymbiotic plants. After inoculation and incubation for five weeks, leaf glands were
380 macerated, and the contents plated out on selective media to allow for selective counting of strain
381 R-71417 or TA01. Colonization rates were high across all conditions, with bacteria in the leaf glands
382 of 59 out of 68 plants (one to four leaves checked per plant). The success rate of inoculations with R-
383 71417, TA01 or TA01 *motB+* did not differ significantly in single inoculations, with 75%, 66.67%, and
384 80% of plants successfully colonized, respectively. Furthermore, bacterial densities inside leaf glands
385 did not differ significantly between plants inoculated with parental strain R-71417, TA01, or TA01
386 $motB^{+}$ (Student T-test $p$-value > 0.05, Figure 5A). Altogether, this indicates that flagellar motility is
387 not required for host colonization. However, the inoculum used in our assays contained a large
388 excess of bacteria and these results may not reveal subtle differences in colonization fitness
389 between the strains. To test whether non-motile strains are outcompeted by motile strains in our
390 assay, we performed co-inoculations of aposymbiotic plants with strain TA01 and R-71417 in 1:1
391 ratio. Bacterial densities of strain TA01 inside leaf glands were between 10 to $10^{10}$ times lower than
392 R-71417, with a median competitive index of $1.0 \times 10^{-5}$ (Figure 5B). Moreover, we performed a
393 complementation experiment by co-inoculating strains TA01 $motB^{+}$ and R-71417 pBBR1MCS. The
394 expression of a functional copy of *motB in trans* significantly raised the competitive index of strain
395 TA01 $motB^{+}$, bringing it to a median value of $1.0 \times 10^{-3}$ (Two-sided Wilcoxon rank sum test $p < 0.05$).
396 These data thus show that flagellar motility is not required for plant colonization, but may facilitate
397 horizontal transmission and host-switching.

398

399 # Discussion

400 The unusual tractability of the *D. sansibarensis*/*O. dioscoreae* symbiosis makes this association a
401 valuable model system to study the determinants of vertical transmission of plant microbiota, as
402 well as the molecular mechanisms governing the specificity of association of plants with bacteria at
403 the leaf surface. In this work, we show that *O. dioscoreae* symbiotic bacteria are housed in
404 specialized structures at the tip of the leaves, formed by the folding of the leaf margins. These glands
405 hold high densities of bacteria (up to $10^{11}$ CFU/g) which are separated from the epidermis by a
406 cuticle layer. Several lines of evidence indicate that the large numbers of trichomes which project
407 inside the leaf gland may play an essential role in the interaction with the bacterial symbionts. The
408 cuticle layer and cell wall appear thinner in the area directly in contact with the bacteria, with zones
409 of discontinuity in the electron-dense layer (Figure 2). The plant cuticle acts as a diffusion barrier for
410 water and hydrophilic compounds (45), and gaps in the cuticle layer may enable the diffusion of
411 water-soluble and ionic solutes (46). The presence of numerous vesicles in trichome head cells
412 supports the hypothesis that trichomes act as a major interface between the symbiotic partners.
413 These specialized trichomes are possibly involved in the delivery of nutrients to the bacterial
414 symbiont as well as the uptake of metabolites of bacterial origin. The genome of *O. dioscoreae* does
415 not contain genes coding for secreted polysaccharide-degrading enzymes, and lacks a functional
416 glycolysis, Entner-Doudorroff pathway or oxidative branch of the pentose phosphate pathway.
417 However, *O. dioscoreae* isolates display leucine arylamidase activity (28), indicating that the bacteria
418 have the ability to mineralize organic nitrogen in peptide bonds (47). Trichome secretions may thus
419 be at least partly responsible for the mucous substance surrounding the bacteria in the leaf gland.
420 Although of unknown chemical composition, this mucus may play a direct, possibly dual role as
421 biofilm matrix component and source of complex nutrients.

422 Although the leaf gland is the most striking feature of the symbiosis, *O. dioscoreae* inhabits other
423 aerial tissues. The distribution of bacteria within the host is however not random. Somatic tissues
424 like stems or leaf lamina contain very few bacteria, but shoot organogenic tissues such as apical and
425 lateral buds, as well as vegetative propagules (bulbils), consistently contained symbiotic bacteria.
426 Similar to within the leaf glands, bacteria are found in a mucus, which surrounds putative secretory
427 trichomes. Although the bacterial colonies near the shoot apical meristem are more diffuse, bacteria
428 grow within a structure analogous to the leaf-enclosed chamber, previously described in leaf
429 nodulating *Psychotria* species (20, 23). This leaf-enclosed chamber lacks the striking
430 compartmentalization seen in the leaf gland. *D. sansibarensis* thus seems to tolerate bacteria in
431 contact with shoot meristematic tissue, although bacterial densities in buds and growth centers of
432 bulbils are several orders of magnitude lower than in the leaf gland (Table 1). This close proximity of

433 bacteria at the shoot tip is surprising, since shoot meristems are often thought to be sterile (48).
434 However, recent studies indicate that some species host specific bud-associated microbiota (49).
435 Because shoot meristems are the hub of aerial organogenesis, tolerance of bacteria near shoot
436 meristematic tissue may be a key feature of the *D. sansibarensis*/*O. dioscoreae* symbiosis that
437 enables a permanent symbiotic association.

438 Inoculation of fluorescent-tagged *O. dioscoreae* at the shoot tip resulted in plants that contained
439 bacteria in all leaves formed above the point of inoculation. Strikingly, we never found evidence of
440 bacteria in the glands of leaves which had emerged prior to the time of inoculation. Microscopic
441 observation also indicates that *O. dioscoreae* attaches to the trichomes of new leaves as they
442 emerge from primordia, before the folding of the tip takes place (Figure 4). Together, this supports
443 the view that growth and distribution of the symbiont is concomitant with leaf development, and
444 supported by elongation after gland formation. The fact that we were able to inoculate aposymbiotic
445 plants artificially also suggests that access to the leaf-enclosed chamber remains open after
446 germination. However, we show that out of 2 x $10^5$ *O. dioscoreae* cells, only a few hundred
447 successfully establish in the host after inoculation. This is indicative of stringent barriers to
448 inoculation, similar to some symbiotic systems with horizontal transmission, for example that
449 between the bean bug *Riptortus pedestris* and *Burkholderia* (50, 51), or between *Vibrio* and the
450 bobtail squid (52). Whether the potential infection barriers in *D. sansibarensis* are selective to *O.*
451 *dioscoreae* or if they allow ingress of other bacteria remains to be tested.

452 Similar to symbiotic systems with horizontal transmission, flagellar motility contributes to the
453 colonization of new hosts (53–55). The infectivity of *O. dioscoreae motB* mutants was several orders
454 of magnitude lower than that of reference strains (Figure 5). This suggests that flagellar motility
455 facilitates crossing of host barriers to reach the leaf-enclosed chamber and propagate within the
456 host. Despite this competitive disadvantage, *O. dioscoreae motB* mutants were still capable of
457 infecting aposymbiotic plants and grew to normal densities *in planta.* Moreover, the genome of *O.*
458 *dioscoreae* lacks genes for alternative types of motility, such as twitching motility (28). Interestingly,
459 genes linked to chemotaxis or motility functions are entirely lacking from the genomes of some
460 *Burkholderia* leaf nodule symbionts of Rubiaceae and Primulaceae (16, 56, 57). These data strongly
461 suggest that bacterial motility is not required for within-host colonization and trans-generational
462 transmission, but may instead facilitate horizontal transmission and host switching. Indeed,
463 phylogenetic analysis indicate a strict vertical mode of transmission for leaf symbionts entirely
464 lacking a flagellar apparatus (17, 30, 56). Evidence that motility appears dispensable *in planta*
465 indicates that spread of the symbiotic bacteria from the leaf-enclosed chamber to the leaf glands
466 perhaps relies on attachment to specific host structures within the plant. Similar modes of growth

467 and transmission has been hypothesized for vertically-transmitted fungal endophytes of grasses:
468 *Epichloë* hyphae attach to host cells at the shoot apical meristem and elongate simultaneously with
469 leaf tissue, allowing asymptomatic colonization of leaves (58). The number of hyphae remains
470 constant in tissue as leaves mature, and may be an adaptation to avoid uncontrolled proliferation
471 and triggering of plant defenses (59). In *D. sansibarensis*, the number of bacteria remains constant in
472 apical and lateral buds, as well as bulbil growth centers with approximately 1-7 x $10^6$ cfu/g of tissue
473 (Table 1). Reciprocal signaling events between host and symbiont presumably control attachment
474 and growth of the bacteria in leaf tissue, a key feature of this leaf symbiosis.

475 In addition to controlling bacterial proliferation, specific signals may also control expression of
476 bacterial symbiotic functions in target tissue (60, 61). The *smp* and *opk* genes of *O. dioscoreae*
477 encode putative enzymes of the secondary metabolism, which we hypothesized to play a central
478 role in the leaf symbiosis (29, 30). Genes of the *smp* and *opk* putative biosynthetic gene clusters are
479 highly expressed in the leaf gland, representing nearly 30% of all mRNA (29). However, our data
480 reveal that bacteria in the apical buds express key *smp* and *opk* genes in much lower levels than in
481 the leaf gland (Table 2). This difference in expression may reflect a strategy by the bacteria to
482 maximize use of limited resources in the apical buds towards growth. We propose a model whereby
483 two distinct populations of *O. dioscoreae* are maintained in the plant (Figure 6): bacteria in
484 organogenic structures (e.g. apical or lateral buds) maintain synchronous growth with host tissue to
485 serve as a mother colony. Bacteria of this "reproductive" pool have two distinct fates: serve as an
486 inoculum for the leaf gland, and transmit bacteria to the next generation via propagules. Bacteria in
487 the leaf gland provide the main symbiotic services to the plant via secretion of metabolites, but are
488 at a reproductive dead-end. *D. sansibarensis* is an annual plant, with leaves senescing at the end of
489 the season and bacteria presumably dying or at least excluded from the reproductive pool in the
490 plant. We postulate that this division of labor between reproductive and productive symbionts may
491 have important consequences for the evolution of leaf symbiosis, and would ensure that "cheater"
492 bacteria, which do not provide symbiotic services to the host, do not outcompete mutualistic
493 bacteria for access to plant reproductive structures.

494 In conclusion, we provide direct experimental evidence of vertical transmission of symbiotic bacteria
495 in *Dioscorea sansibarensis*. Our work thus provides fundamental insights into the mechanisms
496 governing host colonization and transmission of vertically-transmitted bacteria in plants. The unique
497 tractability of the *Dioscorea*/*Orrella* association makes this an appealing model to understand
498 mechanisms of non-pathogenic plant-bacteria interactions in the phyllosphere.

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## AUTHOR CONTRIBUTIONS

TA and AC designed the research; TA, SM,FDM, OC, and MB carried out the experiments. TA, AC, OC, OD and PW analyzed data; TA and AC wrote the manuscript with input from all authors.

## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

## DATA AVAILABILITY

The datasets generated and/or analyzed during the current study are available in the European Nucleotide Archive repository, with the following accession number: ERR7179810.

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667 # Tables

668 **Table 1**. **Bacterial symbiont colonization in different tissues.** Dissected and homogenized *D.*
669 *sansibarensis* meristematic tissues were plated out and quantified. Of each tissue, 5 samples were
670 taken
671 and
672 plated
673 out,
674 95%
675 confiden
676 ce
677 intervals
678 are
679 given.

| Tissue | CFU | Weight (mg) |
|---|---|---|
| Leaf gland | $3.20 \times 10^{10} \pm 3.94 \times 10^{7}$ | 138.4 |
| Apical bud | $6.27 \times 10^{4} \pm 4.23 \times 10^{2}$ | 9.3 |
| 1 $cm^2$ leaf surface | 0 | 26.1 |
| Bulbil growth centre | $7.83 \times 10^{4} \pm 6.61 \times 10^{2}$ | 43.2 |
| Lateral bud | $3.53 \times 10^{4} \pm 1.53 \times 10^{2}$ | 24.8 |
| Stem under apical bud | $4.87 \times 10^{1} \pm 7.2 \times 10^{1}$ | 7.65 |

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689 **Table 2. Differential regulation of *O. dioscoreae* putative secondary metabolism in leaf gland vs**
690 **shoot tip by quantitative RT-PCR.** Acumen and shoot tip RNA samples were collected during the
691 day. Primers nrp 88-89, pqqc 90-91 and KASII 82-83 correspond to a representative gene from the
692 *smp1* (ODI_R1490), *smp2* (ODI_R1505) and *opk* (ODI_R2249) gene clusters, respectively. Fold
693 changes ($2^{-\Delta\Delta Ct}$) in transcript abundance are given in the leaf gland *vs.* the apical shoot tip.

| | *smp1* | *smp2* | *opk* |
|---|---|---|---|
| *Plant 1* | 58.08 | 290.01* | 35.50* |
| *Plant 2* | 118.60 | 296.11* | 10.62* |
| *Plant 3* | 390.72 | 1478.58* | 64.00* |
| *Plant 4* | 16.33 | 29.04 | 10.48* |

694 * Values marked with an asterisk are lower-bound estimates, due to a lack of detection in the
695 reference sample (shoot tip, see Table S3).

698 ## Figure legends

699 **Figure 1: Anatomy of the *Dioscorea sansibarensis* leaf and acumen**. A: Juvenile leaf from *D.*
700 *sansibarensis.* Juvenile leaves are lobed and evolve to heart-shaped leaves in adult plants. Adult
701 leaves can measure up to 46 centimeters long by 58 wide with an acumen at the distal side
702 measuring up to 6 cm. B: An acumen cross-section. Leaves in all developmental stages contain *O.*
703 *dioscoreae* in the acumen. In the acumen, two glands (G) that are filled with trichomes (T) and
704 bacteria (stained by acridine orange) residing in mucus can be distinguished. The glands are closed at
705 the adaxial side with a seam (S) running along the long axis of the acumen. Around the glands,
706 several vascular bundles can be found (V). C: Auramine staining of the acumen shows the thick
707 cuticle (yellow) surrounding the gland (G) that closes up at the adaxial side into a seam (S) and forms
708 a physical barrier to the symbiont (not visible). Plant cell walls are stained with calcofluor white.

709

710 **Figure 2. Structure of the trichome-bacteria interface in the symbiotic leaf gland.** A. Light
711 microscopic image of a cross section of the fore-runner tip of *D. sansibarensis.* Trichome cells (T) are
712 densely stained, and multiple vesicles are visible (arrows). Around trichomes, many bacteria (B)
713 reside in a mucilage layer. B. TEM photograph showing the cavity in a symbiotic gland. Trichomes (T)
714 are surrounded by encapsulated bacteria (B) that fill the lumen. C. TEM image of the interface
715 between the bacteria (B) and a trichome (T) shows the presence of multiple vesicles (white arrows),
716 endoplasmatic reticulum (ER) and Golgi (G). Multiple gaps in the electron dense layer are apparent
717 (hollow arrows). D. Close-up of the trichome cuticle shows a vesicle (V) merging with the
718 plasmolemma, suggesting cytotic activity.

719

720 **Figure 3. Early development of the *D. sansibarensis* leaf and fore-runner tip in the apical bud.** A.
721 Apical meristem surrounded by a leaf primordium. B. Second youngest leaf of the shoot tip, with
722 many trichomes visible at the adaxial side and an open distal tip. C. Third youngest leaf of the shoot
723 tip, where the acumen is starting to form, yet remains open. An abundance of trichomes and mucus
724 can be seen at the adaxial surface. D-E-F: the third to last, second to last and last leaf of the shoot
725 tip, respectively. The acumen progressively closes along the long axis and the leaf lamina starts to
726 unfold slowly at the proximal side. Trichomes and mucus are abundant throughout development.

727 **Figure 4. *O. dioscoreae's* habitat in the shoot tip.** Scanning electron (left) and confocal (right)
728 microscopy pictures of leaf primordia in the shoot tip show *O. dioscoreae* colonizing the glandular
729 trichomes. A. Trichomes in the apical bud consist of one stalk cell (SC) and 5 or 6 glandular cells (GC).

730 Bacteria (arrow) and mucus (M) surround the trichomes. B. Confocal microscopy of glandular
731 trichomes in the shoot tip, showing association with mCherry-tagged *O. dioscoreae*.

732 **Figure 5. Quantification and competitive index of *O. dioscoreae* strains *in planta*.** A. colonization
733 quantification of acumens after single inoculation on the apical bud, one leaf per biological sample.
734 Newly grown acumens were macerated and plated out, growth was quantified by colony counting.
735 Single inoculation with *O. dioscoreae*, either mCherry-tagged strain (R-71417), the motility impaired
736 mutant (TA01) or the complemented strain (TA01 pBBR1MCS-3::*motAB*). There is no significant
737 difference in average bacterial densities between the 3 conditions (pairwise two-sided Student's T-
738 test $p > 0.05$). B. Competitive index of O*. dioscoreae* in co-inoculations of motility-impaired mutant
739 vs. parental strain. Aposymbiotic plants were co-inoculated with a 1:1 mix of motility impaired
740 mutants (strain TA01) and the parental strain (R-71417). As a control, a 1:1 mix of a complemented
741 motility mutant (TA01 pBBR1MCS-3::*motAB*) with the parental strain containing the empty plasmid
742 used for complementation (R-71417 pBBR1MCS) was inoculated into aposymbiotic plants. Lastly, to
743 control for the effect of the empty plasmid, strain TA01 pBBR1MCS was co-inoculated with strain R-
744 71417 pBBR1MCS.

745 **Figure 6. Schematic of *O. dioscoreae* transmission and functional predictions.** *D.*
746 *sansibarensis* harbors symbiotic bacteria (*O. dioscoreae*) which are contained within leaf glands,
747 bulbils, and shoot apical or axillary buds. The apical bud acts as a reservoir for the symbiont to
748 ensure colonization of newly formed aerial organs, such as the forerunner tip, lateral buds and
749 propagules. Allocation of *O. dioscoreae* cells to bulbils ensures transmission to the next plant
750 generation. Flagellar motility is hypothesized not being required for vertical transmission, but
751 occasional horizontal transmission seem to at least partly rely on bacterial motility (Left panel). The
752 main function of *O. dioscoreae* of this "reproductive" pool residing in the shoot tip may be to serve
753 as an inoculum for the leaf gland and transmit bacteria to the next generation via bulbils.
754 Accordingly, biosynthesis of bacterial secondary metabolites is down-regulated in the apical bud. *O.*
755 *dioscoreae* attaches to trichomes (T) in the apical bud and grows synchronously with the plant (top
756 right panel). In contrast, the fate of *O. dioscoreae* in the leaf gland may be to provide secondary
757 metabolites to the plant, via exchange of metabolites through the permeable cell wall of specialized
758 trichomes (lower right panel). Leaf gland bacteria are at a reproductive dead-end and are not
759 transmitted to the next plant generation. P, leaf primordium; SAM, shoot apical meristem; V,
760 vesicles; T, trichome.

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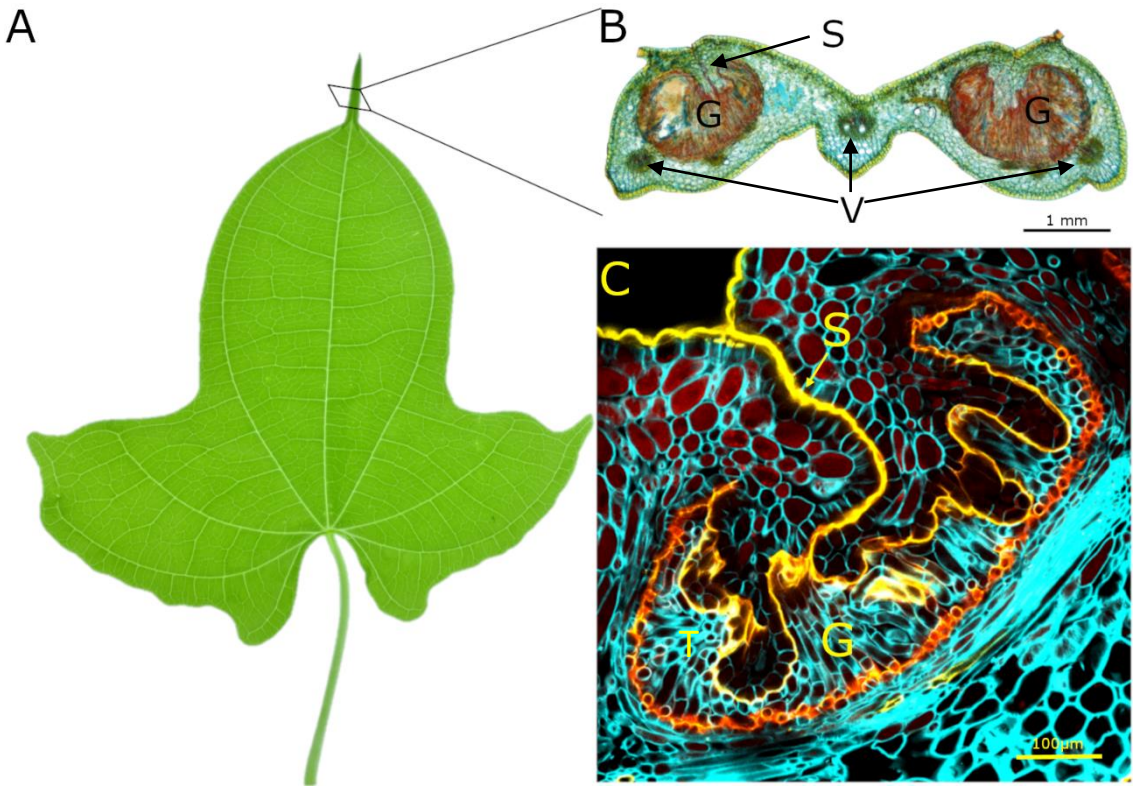


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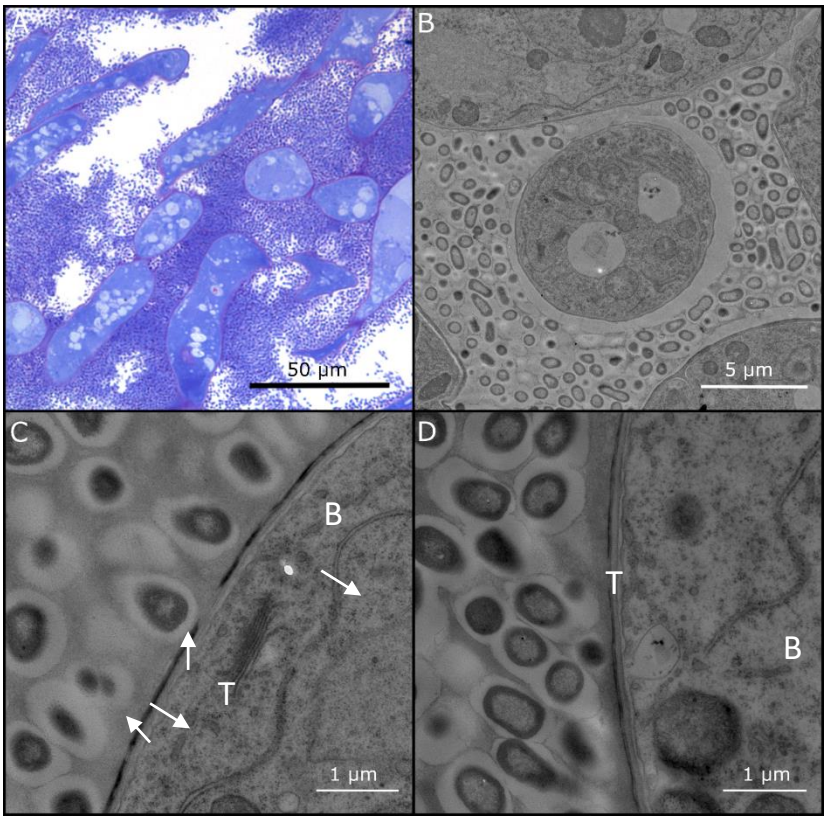


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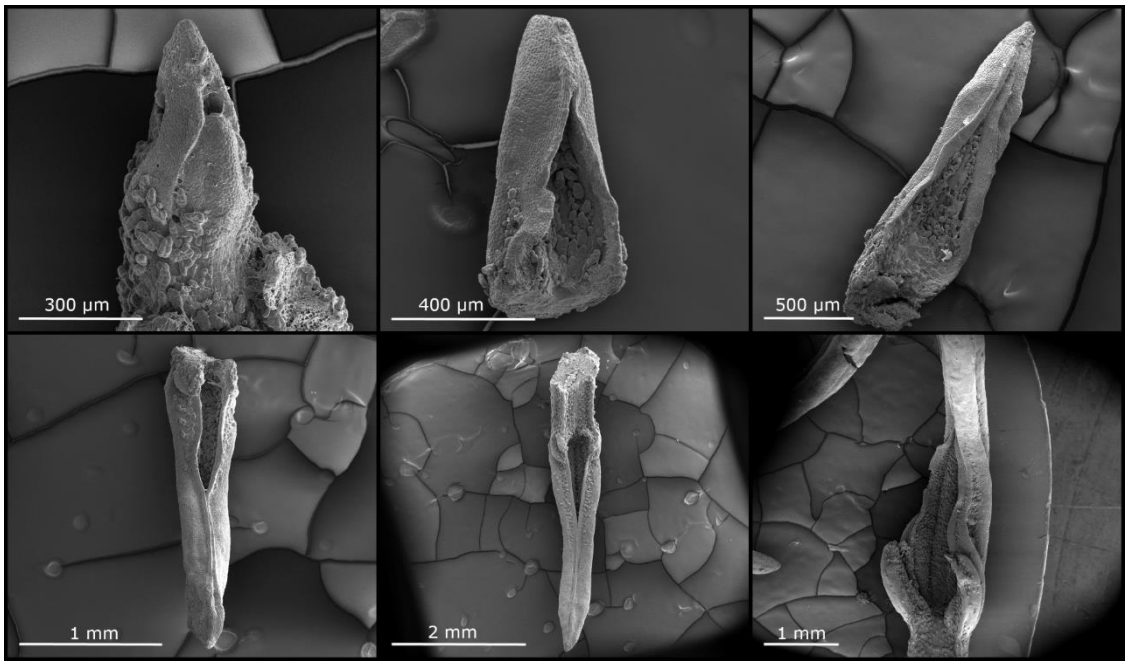


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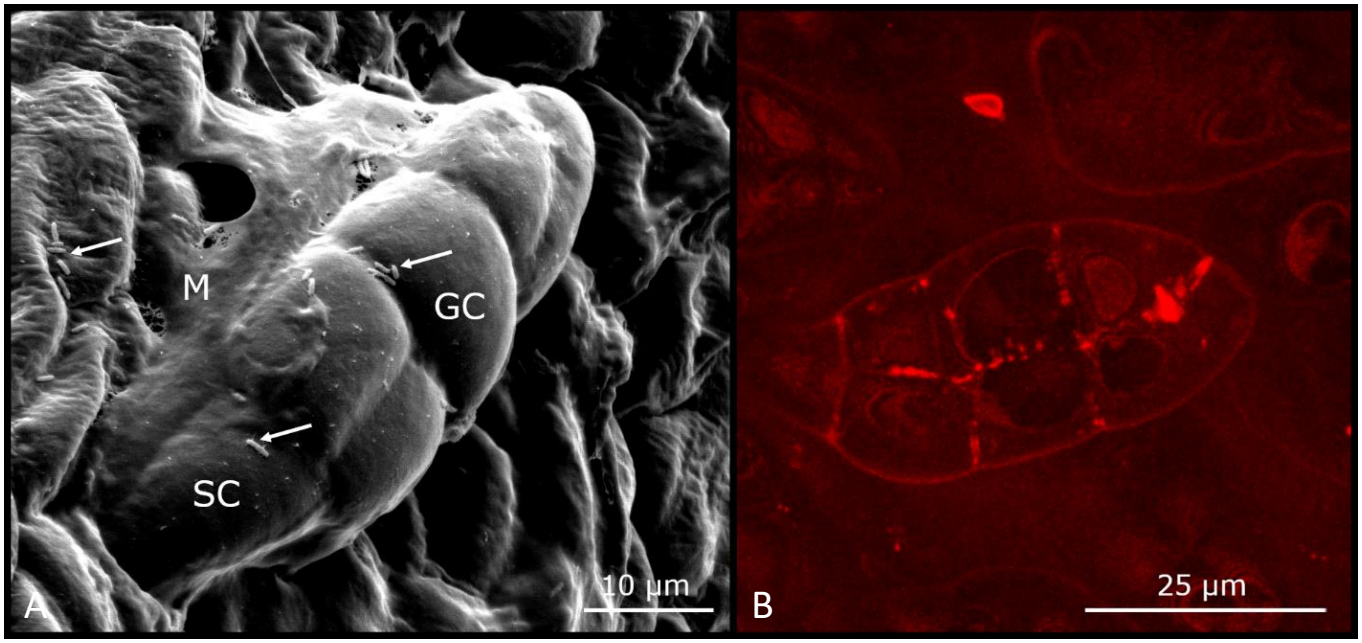


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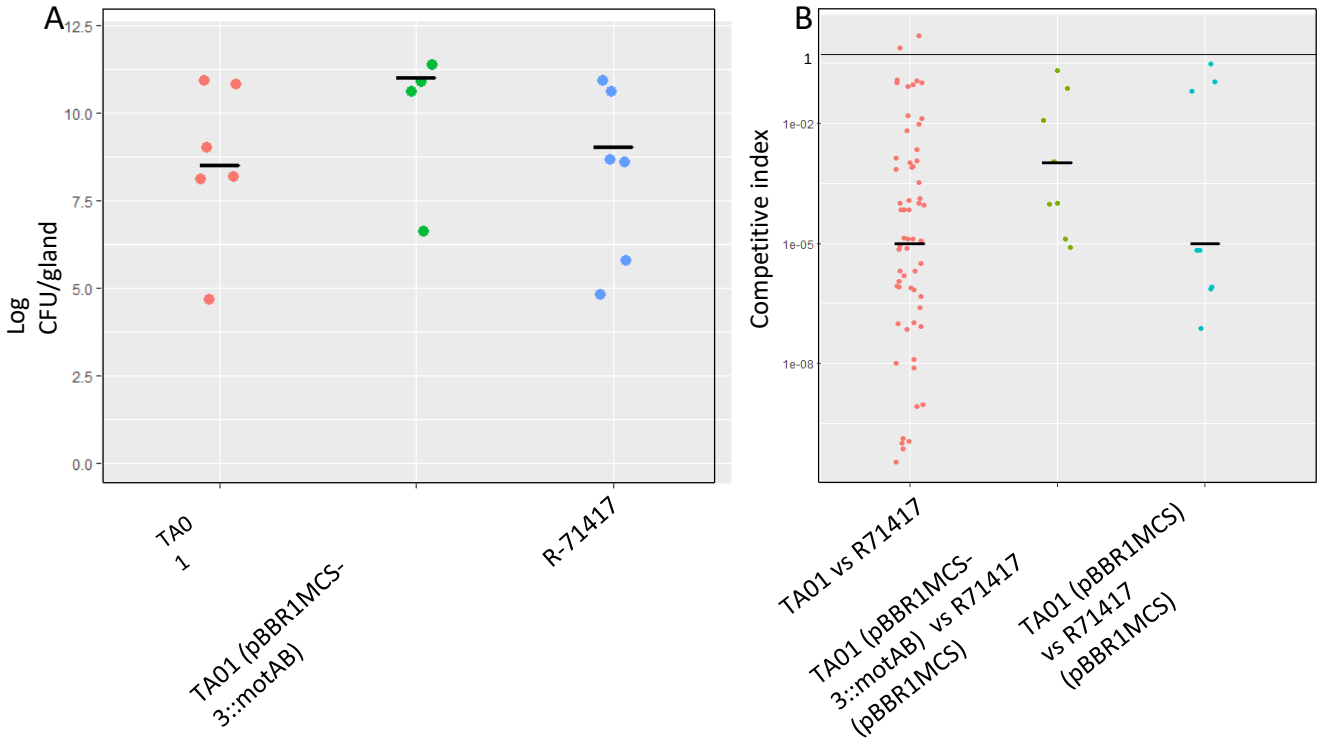


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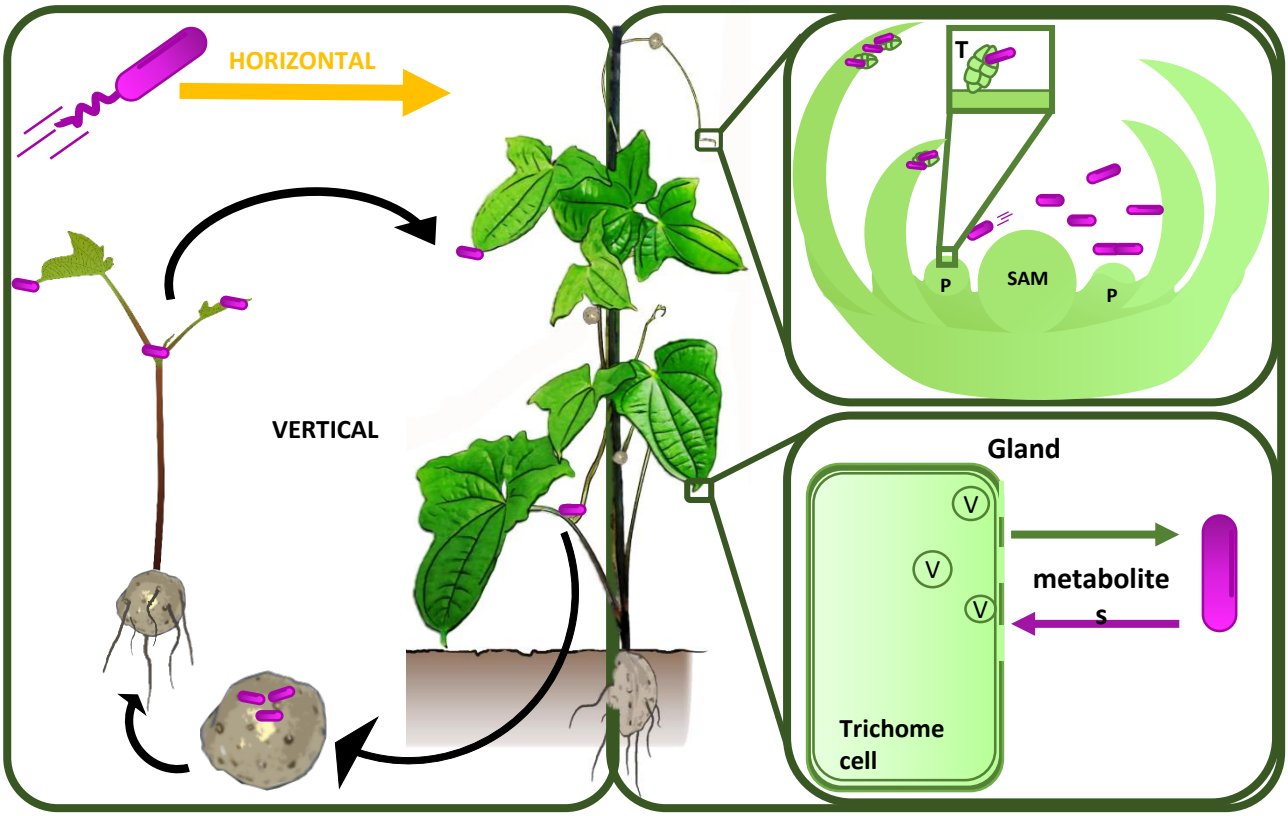


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