

New Phytologist Supporting Information

Article title: *Agrobacterium tumefaciens* fitness genes involved in the colonization of plant tumors and roots

Authors: Marta Torres, Audren Jiquel, Etienne Jeanne, Delphine Naquin, Yves Dessaux and Denis Faure.

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Table S1. Average bacterial population and percentage of TA-containing CDS with transposon insertions in Tn-mutant populations recovered from plant assays.

Table S2. Agrobacterium tumefaciens C58 fitness genes involved in plant colonization.

This is a large Excel file submitted separately.



Fig. S1 A. tumefaciens pathways involved in colonization of tomato tumors and roots.

Fitness genes are related to three general processes: carbon and nitrogen metabolism (in blue and yellow boxes), synthesis and repair of DNA, RNA and proteins (in green boxes) and cell envelope and surface functions (along bacterial envelope representation). Genes required for tomato tumor colonization are indicated in red, for root colonization in dark orange and for both tomato tumors and roots, in blue. Dashed arrows indicate that several enzymatic steps exist between the two connected metabolites. Not all the fitness genes are represented, those coding for unknown functions are omitted (see Table **S2** for a complete list).





Fig. S2 In vitro inhibition growth of Agrobacterium tumefaciens C58 using antagonists targeting fitness genes.

In vitro inhibition growth was conducted in AB medium containing 10 mM sucrose, supplemented with levulinic acid (2 mg ml⁻¹), 3-indoleacrylic acid (0.1 mg ml⁻¹), tannic acid (0.05 mg ml⁻¹) and aminoethylcysteine (0.5 mg ml⁻¹) using *A. tumefaciens* C58 at OD₆₀₀ 0.05 as the inoculum. In order to assess the impact of treatments on *A. tumefaciens*, growth was measured as OD₆₀₀ after 24, 48 and 120 h, normalized to the OD of each strain in the absence of compounds and expressed as a percentage. Error bars show standard deviations. Dashed vertical lines represent the dose chosen for the plant assays.





Fig. S3 Effect of antagonists on tomato plant growth and development.

Tomato roots were infected by *A. tumefaciens* C58 derivative C107-Km^R Rif^R and treated weekly by chemicals, either levulinic acid (LA), 3-indoleacrylic acid (IA), tannic acid (TA), aminoethylcysteine (AE) or a mix of them, or biocontrol agents, either *Pseudomonas protegens* CHA0 or *P. protegens* CHA400 (a CHA0 derivative that is defective for siderophore synthesis). A combination of biological and chemical (TA) treatments was also tested. After 4 four weeks, dry weight (**a**) and development (**b**) of aerial parts were assessed. Error bars show standard deviations. Significant differences with the untreated control were calculated using a one-way ANOVA test coupled with a post-hoc Dunnett test and are shown with asterisks: ***, *P*<0.001.



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Fig. S4 *In vitro* inhibition assay of *Agrobacterium tumefaciens* C58 by *Pseudomonas protegens*

Antagonism by *P. protegens* CHA0 (**a**) and the siderophore pyoverdine-negative *P. protegens* CHA400 (**b**) was verified in TY (tryptone yeast medium) in an agar-overlay assay in the absence or presence of 0.05 mg ml⁻¹ of tannic acid (TA) with or without 1 mM iron citrate, and it was measured as an inhibition halo. Error bars show standard deviations. Significative differences were assessed by post hoc Tukey analysis and are shown with different lowercase letters.





Notes S1. DNA isolation, transposon library sequencing and analysis.

Genomic DNA of mutant populations was extracted (Gonzalez-Mula et al., 2019) and then the amplified DNA of Tn-insertion sites were sequenced on an Illumina NextSeq 500 instrument (Illumina, San Diego, USA) in a paired-end 2 × 75 run at the I2BC-sequencing platform (Gif-sur-Yvette, France). Each population generated more than 4.5 million reads after trimming (Bolger et al., 2014). The mapping results (.bam files) were analyzed by the ARTIST pipeline (Pritchard et al., 2014) using MATLAB software (MathWorks, Natick, USA). ARTIST compares the observed (data from reads) and predicted numbers of transposons at each of the 115,525 insertion sites (TA dinucleotides) along the A. tumefaciens C58 genome. We focused on the 89,112 TA sites that are present in coding regions. Only six coding regions (atu0520, atu1968, atu4643, atu8120, atu8173, atu8197) do not contain TA sites. Under TY culture condition, 97.6% of the analyzed coding regions (those containing TA sites) contained at least one Tn insertion, revealing good coverage of the Tn-library (Gonzalez-Mula et al., 2019). This percentage was 91.5% and 92.2% for tomato tumors and roots, 92.6% for maize roots, and 94.4% for poplar tumors. These values were of the same order as those obtained (93.4%) when the Tn-mutant populations were cultivated in a minimal medium with a single source of carbon and energy (sucrose) and a single source of nitrogen (ammonium) (Gonzalez-Mula et al., 2019).

The CON-ARTIST pipeline (Pritchard et al., 2014) was used to compare the distribution of transposon insertions between the initial transposon library culture in TY medium (Gonzalez-Mula et al., 2019) and each replicate of the four in planta conditions to identify the genes with significantly different read numbers using a *P*-value cutoff of *P*<0.01 in the Mann-Whitney U test. CON-ARTIST categorized each gene in five classes: genes with a conditionally essential domain (class 1), conditionally essential genes (class 2), genes with a conditionally enriched domain (class 3), conditionally enriched genes (class 4) and genes conditionally non-essential, not enriched (class 5). To elaborate a unique list of fitness genes for each condition (tomato and poplar tumors and tomato and maize roots), we did not merge sequencing data obtained for the different replicates as we previously did for the growth of A. tumefaciens Himar1-mutants in synthetic media (Gonzalez-Mula et al., 2019). To take into account a potential heterogeneity across replicates, which could be caused by the complexity of the growth condition in planta, we used a two-step approach. First, the CON-ARTIST pipeline allowed identification of fitness genes (class 1 and class 2 genes using ARTIST nomenclature) in each replicate. Then, we retained in a final unique list of fitness genes those which had been assigned to classes 1 or 2 in at least half of the replicates of a considered condition.



Notes S2. Mutant construction by reverse genetics.

The Gm-resistance cassette (Dennis & Zylstra, 1998) was used for construction of knockout (KO) mutants. The *atu0018* (*=trpB*), *atu0022* (*=trxA*), *atu0041* (*=hisH*), *atu1445* (*=ntrB*), *atu1733* (*=nrdJ*), *atu2155* (*=metH*), *atu2304* (*=sbmA*) *atu2555* (*=kamA*), *atu2804* (*=cobN*), *atu3325* (*=exoQ*), *atu3687* (*=fecA*), *atu4614* (*=wbbL*) and *atu6019* (*=noxA*) genes were cloned into the pGEMT Easy vector (Promega, Madison, USA), and the mutated alleles were created by inserting a Gm-resistance cassette in a unique restriction site of the open reading frame. The constructed plasmids were electroporated in a Rif^R derivative of *A. tumefaciens* C58. Double recombination was verified amongst Gm resistant transformants by PCR. Previously constructed *A. tumefaciens* C58 mutants were also used. They were the strain C58 derivatives C107-Gm^R and C107-Km^R in which the Gm and Km cassettes were cloned in a non-coding region of the Ti plasmid (Haudecoeur *et al.*, 2009), and which were made Rif^R for this study. As controls for the effect of the two antibiotic-resistance cassettes, competition *in vitro* and *in planta* between C107-Km^R Rif^R and C107-Gm^R Rif^R was performed.

Competition between *A. tumefaciens* C107-Km^R Rif^R and each of the constructed KO mutants (Gm^R Rif^R) was performed in TY liquid medium and *in planta*. Each of the bacterial genotypes were cultivated in TY medium separately and centrifuged (3,000 × *g* for 15 minutes). Then the bacterial cells were washed with a 0.8% NaCl solution, centrifuged and resuspended in 0.8% NaCl to an OD₆₀₀ of 1. An equal volume of each washed culture was mixed individually with an equal volume of the washed culture of strain C107-Km^R Rif^R before inoculation. Competition assays were carried out in tomato and maize roots and tomato and poplar stem tumors. Five weeks after infection, tumors and roots were recovered, weighed and crushed in 0.8% NaCl to enumerate colony forming units (CFU) of agrobacteria.

Notes S3. Root colonization and tumorigenesis assays.

Plant assays were performed in a greenhouse under non-gnotobiotic conditions as Tn-Seq to test whether these compounds and *P. protegens* could impair root colonization. We also combined tannic acid and aminoethylcysteine to target simultaneously two pathways (i.e. iron assimilation and β -lysine conversion), and P. protegens and tannic acid to target iron availability in two ways. *A. tumefaciens* was introduced at 10⁵ CFU g⁻¹ soil in the rhizosphere of unwounded plants, mimicking an epidemic outbreak in a nursery (Krimi et al., 2002). After three weeks of treatment,



A. tumefaciens, fluorescent pseudomonads and total culturable bacteria were counted on the sampled roots. Plant growth was also inspected.

Root colonization assays were conducted on 4-week-old tomato plants in 1-liter pots filled with non-sterile Floradur B horticultural substrate. Two independent replicates were conducted for each condition, each consisting in a series of 6 replicates on tomato plants. All the plants were infected with *ca.* 10^8 *A. tumefaciens* (C107-Km^R Rif^R) cells. For chemical assays, levulinic acid (2 mg cm⁻³ in the pots), 3-indoleacyrlic acid (0.1 mg cm⁻³), tannic acid (0.05 mg cm⁻³) and aminoethylcysteine (0.5 mg cm⁻³) were added every 8 days to the soil. In the biological treatment, *ca.* 10^8 *P. protegens* cells were inoculated in each pot every 10 days. In the combined assay, both *P. protegens* and tannic acid were added to the plants at the concentration and number indicated above. After 30 days, plant roots were recovered, weighed and crushed in a 0.8% NaCl solution. In order to enumerate CFU per g of root FW, serial dilutions were prepared and spotted onto selective agar media for *A. tumefaciens*, on King's B (KB) (King *et al.*, 1954) medium for *Pseudomonas*, and on TY agar to enumerate total population. Plant aerial parts were photographed and weighed. One-way ANOVA tests (*P*<0.05) and post-hoc Dunnett tests (*P*<0.05) were performed to evaluate bacterial abundance differences.

Root tumorigenesis assays were conducted on 10-day tomato plantlets grown on halfstrenght Murashige and Skoog (pH 5.7) media (MS) solidified with agar at 7 g l⁻¹. Roots were cut and submerged in a *A. tumefaciens* cell suspension (10⁹ UFC ml⁻¹) in NaCl 0.8% for 5 minutes, and then placed onto MS/2 agar plates containing the tested chemical agents (tannic acid at 0.05 mg ml⁻¹ and aminoethylcysteine at 0.5 mg ml⁻¹) or spread by 10⁹ UFC per plate of *P. protegens* CHA0 (wt) and CHA400. Plates with infected plants (30 plantlets per condition) were incubated at 22-24°C under long day (16 h) period and emerging galls were counted and weighed after 4 weeks. Treatments were compared to the untreated condition (control) using a Fisher's exact test.

Notes S4. Validation of the fitness genes by reverse genetics.

To validate candidate fitness genes identified by Tn-Seq, we constructed KO mutants for 12 of them. As a control, we constructed a KO mutant of the gene *sbmA* (*=atu2304*) that did not contribute to fitness *in planta* according to our Tn-Seq data. The 12 candidate fitness genes were chosen because they belong to pathways of the three general processes presented above, i.e. carbon and nitrogen metabolism (*trpB=atu0018, hisH=atu0041, ntrB=atu1445, metH=atu2155,*



cobN=atu2804, noxA=atu6019), synthesis and repair of DNA, RNA and proteins (*trxA=atu0022, nrdJ=atu1733, kamA=atu2555*), and envelope functions (*exoQ=atu3325, fecA=atu3687, wbbL=atu4614*). In addition, these genes represent some potential tumor-specific fitness genes (*fecA* and *noxA*), while the others were identified as fitness genes under all conditions. Then, we experimentally evaluated the relative fitness of these KO mutants and calculated a competitive index (CI) in TY culture medium and after plant inoculations.

In the TY rich medium that was used to construct the Tn-library and in which the 12 genes were not considered as conditionally essential, no significant fitness difference (CI=1) could be observed when bacteria carrying the defective and wild-type alleles competed (Fig. 2). In competition assays *in planta* (tomato tumors, tomato roots, poplar tumors, maize roots), the *sbmA* KO mutant was not impaired in fitness, in agreement with the Tn-Seq data. In contrast, fitness of the other *A. tumefaciens* constructed mutants was affected under almost all plant conditions (Fig. 2). Overall, considering all the 65 dual competitions (13 mutants including *sbmA* in four plant conditions and one condition in TY medium) only five discrepancies with Tn-Seq data were observed. From these data, we conclude that our Tn-Seq experimental data represent quite a solid overview of the main fitness genes of *A. tumefaciens* living in plants.

Notes S5. Proline, nopaline and spermidine pathways.

Proline accumulates in tomato tumors at 0.16 nmol mg⁻¹ FW, nopaline at 1.1 10⁻³ nmol mg⁻¹ FW and spermidine at 5.8 10⁻⁴ nmol mg⁻¹ FW (Gonzalez-Mula *et al.*, 2019). They are metabolically connected in *A. tumefaciens* (Fig. **3**). As an osmoprotectant, proline was proposed to be synthesized by the host plant in response to desiccation of tumor tissues (Wächter *et al.*, 2003). Proline is exploited as a nutrient by *A. tumefaciens* (*putA=atu4157* as fitness gene) and acts as a competitive inhibitor of GABA-induced expression of the quorum sensing signal-degrading lactonase BlcC (Dessaux *et al.*, 1986; Haudecoeur *et al.*, 2009).

Opine nopaline synthesis is encoded by the T-DNA transferred from *A. tumefaciens* C58 to the plant cell. Tn-Seq revealed *noxAB* (=*atu6019-atu6021*), *arc* (=*atu6018*) and *ocd* (=*atu6016*) as fitness genes, being all involved in the degradation of nopaline into proline (Zanker *et al.*, 1994). The nopaline degradative pathway generates amino acids (arginine, ornithine, proline), urea and ammonium. Nopaline had been proposed as the main source of urea in tumors that were incited by *A. tumefaciens* C58 on *A. thaliana* (Deeken *et al.*, 2006). Our competition assays



in tomato tumors with a *noxA* mutant confirmed that assimilation of nopaline was important for *A. tumefaciens* fitness in plant tumors. Our Tn-Seq data revealed nopaline assimilation as the main Ti plasmid-encoded function involved in the exploitation of the tumor ecological niche, confirming the key-role of this opine in *Agrobacterium* lifestyle in tumors (Guyon, 1993; Savka & Farrand, 1997; Lang *et al.*, 2014). None of the genes involved in the assimilation of the opine agrocinopine were identified by Tn-Seq. Agrocinopine accumulates in tomato tumors at 8.0 10⁻³ nmol mg⁻¹ FW. However, its degradation releases nutrients that are already abundant in tomato tumors: sucrose (2.5 nmol mg⁻¹ FW), arabinose (4.3 10⁻² nmol mg⁻¹ FW) and phosphate (4.2 nmol mg⁻¹ FW) (Gonzalez-Mula *et al.*, 2019). Hence, contribution of agrocinopine to the fitness of *A. tumefaciens* appears secondary in tomato tumors. In contrast, as a conjugative opine, agrocinopine plays a key-role in the dissemination of virulence genes (Beck von Bodman *et al.*, 1992). Degradation of agrocinopine provides arabinose-2-phosphate that in turn activates quorum-sensing and Ti plasmid conjugation (El Sahili *et al.*, 2015). In *A. tumefaciens* C58, opines nopaline and agrocinopine clearly play distinctive roles.

Spermidine, either synthesized by *A. tumefaciens* or imported from its surrounding environment, is essential for the survival of this bacterial species (Kim *et al.*, 2016). Tn-Seq revealed that spermidine synthesis (*atu4169* and *atu4170* coding for carboxyspermidine decarboxylase and carboxyspermidine dehydrogenase) is important for colonization of tomato tumors, strongly suggesting that spermidine from plant tumors was not enough to support *A. tumefaciens* growth, hence that spermidine remains a limiting resource in that environment.



Table S1. Average bacterial population and percentage of TA-containing CDS with transposon insertions in Tn-mutant

populations recovered from plant assays. Averages values ± standard deviation are shown.

	Average A. tumefaciens mutants		Average total culturable bacteria		TA-containing CDS
	Per g (CFU g-1 FW)	Total (CFU)	Per g (CFU g-1 FW)	Total (CFU)	with insertions
Tomato tumors - average	$3.8 \times 10^6 \pm 0.5 \times 10^6$	$3.8 \times 10^7 \pm 0.8 \times 10^7$	$3.8 \times 10^6 \pm 0.2 \times 10^6$	$3.6 \times 10^7 \pm 0.3 \times 10^7$	91.5%
Tomato roots - average	$4.6 \times 10^4 \pm 0.3 \times 10^4$	$4.5 \times 10^5 \pm 0.6 \times 10^5$	$4.1 \times 10^6 \pm 0.3 \times 10^6$	$8.1 \times 10^7 \pm 0.4 \times 10^7$	92.2%
Maize roots - average	$8.5 \times 10^3 \pm 3.6 \times 10^3$	$1.1 \times 10^5 \pm 0.5 \times 10^5$	$2.1 \times 10^7 \pm 0.1 \times 10^7$	$3.6 \times 10^8 \pm 0.5 \times 10^8$	92.6%
Poplar tumors - average	$7.9 \times 10^5 \pm 4.1 \times 10^5$	$7.3 \times 10^{6} \pm 3.3 \times 10^{6}$	$1.1 \times 10^6 \pm 0.3 \times 10^6$	$9.9 \times 10^6 \pm 0.3 \times 10^6$	94.4%



References Notes

- **Beck von Bodman S, Hayman T, Farrand S**. **1992**. Opine catabolism and conjugal transfer Qf the nopaline Ti plasmid pTiC58 are coordinately regulated by a single repressor. *Proceedings of the National Academy of Sciences* **89**: 643–647.
- **Bolger AM, Lohse M, Usadel B. 2014**. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**: 2114–2120.
- Deeken R, Engelmann JC, Efetova M, Czirjak T, Müller T, Kaiser WM, Tietz O, Krischke M, Mueller MJ, Palme K, et al. 2006. An integrated view of gene expression and solute profiles of Arabidopsis tumors: a genome-wide approach. The Plant Cell 18: 3617–3634.
- Dennis J, Zylstra G. 1998. Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. *Applied and environmental microbiology* 64: 2710–2715.
- **Dessaux Y, Petit A, Tempé J, Demarez M, Legrain C, Wiame JM**. **1986**. Arginine catabolism in *Agrobacterium* strains: role of the Ti plasmid. *Journal of Bacteriology* **166**: 44–50.
- El Sahili A, Li SZ, Lang J, Virus C, Planamente S, Ahmar M, Guimaraes BG, Aumont-Nicaise M, Vigouroux A, Soulère L, *et al.* 2015. A pyranose-2-phosphate motif is responsible for both antibiotic import and quorum-sensing regulation in *Agrobacterium tumefaciens*. *PLOS Pathogens* 11: e1005071.
- **Gonzalez-Mula A, Lachat J, Mathias L, Naquin D, Lamouche F, Mergaert P, Faure D**. 2019. The biotroph *Agrobacterium tumefaciens* thrives in tumors by exploiting a wide spectrum of plant host metabolites. *New Phytologist* 222: 455–467.
- **Guyon P. 1993**. Transformed plants producing opines specifically promote growth of opinedegrading agrobacteria. *Molecular Plant-Microbe Interactions* **6**: 92.
- Haudecoeur E, Tannières M, Cirou A, Raffoux A, Dessaux Y, Faure D. 2009. Different regulation and roles of lactonases AiiB and AttM in Agrobacterium tumefaciens C58. Molecular Plant-Microbe Interactions 22: 529–537.
- **Kim SH, Wang Y, Khomutov M, Khomutov A, Fuqua C, Michael AJ**. **2016**. The essential role of spermidine in growth of *Agrobacterium tumefaciens* is determined by the 1,3-diaminopropane Moiety. *ACS Chemical Biology* **11**: 491–499.
- **King E, Ward M, Raney D**. **1954**. Two simple media for the demonstration of pyocyanin and fluorescin. *The Journal of Laboratory and Clinical Medicine* **44**: 301–307.
- Krimi Z, Petit A, Mougel C, Dessaux Y, Nesme X. 2002. Seasonal fluctuations and long-term persistence of pathogenic populations of *Agrobacterium* spp. in soils. *Applied and Environmental Microbiology* 68: 3358–3365.



- Lang J, Vigouroux A, Planamente S, El Sahili A, Blin P, Aumont-Nicaise M, Dessaux Y, Moréra S, Faure D. 2014. Agrobacterium uses a unique ligand-binding mode for trapping opines and acquiring a competitive advantage in the niche construction on plant host. PLoS Pathogens 10: e1004444.
- Pritchard JR, Chao MC, Abel S, Davis BM, Baranowski C, Zhang YJ, Rubin EJ, Waldor MK. 2014. ARTIST: high-resolution genome-wide assessment of fitness using transposon-insertion sequencing. *PLoS Genetics* 10: e1004782.
- Savka MA, Farrand SK. 1997. Modification of rhizobacterial populations by engineering bacterium utilization of a novel plant-produced resource. *Nature Biotechnology* **15**: 363–368.
- Wächter R, Langhans M, Aloni R, Götz S, Weilmünster A, Koops A, Temguia L, Mistrik I, Pavlovkin J, Rascher U, et al. 2003. Vascularization, high-volume solution flow, and localized roles for enzymes of sucrose metabolism during tumorigenesis by *Agrobacterium tumefaciens*. *Plant Physiology* **133**: 1024–1037.
- Zanker H, Lurz G, Langridge U, Langridge P, Kreusch D, Schröder J. 1994. Octopine and nopaline oxidases from Ti plasmids of *Agrobacterium tumefaciens*: molecular analysis, relationship, and functional characterization. *Journal of Bacteriology* **176**: 4511–4517.