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1 **The biotroph *Agrobacterium tumefaciens* thrives in tumors by**
2 **exploiting a wide spectrum of plant host metabolites**

3

4 **Subtitle: Plant host exploitation by the *Agrobacterium* biotroph**

5

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29

30 **Abstract (about 200 words)**

31 ● *Agrobacterium tumefaciens* is a niche-constructing biotroph that exploits host plant
32 metabolites.

33 ● We combined metabolomics, transposon-sequencing, transcriptomics and reverse
34 genetics to characterize *A. tumefaciens* pathways involved in the exploitation of
35 resources from the *Solanum lycopersicum* host plant.

36 ● Metabolomics of healthy stems and plant tumors revealed the common (e.g.,
37 sucrose, glutamate) and enriched (e.g., opines, GABA, GHB, pyruvate) metabolites
38 that *A. tumefaciens* could use as nutrients. Transposon-sequencing and
39 transcriptomics pinpointed the genes that are crucial and/or up-regulated when the
40 pathogen grew on either sucrose (*pgi*, *kdgA*, *pycA*, *cisY*) or GHB (*b1cAB*, *pckA*, *eno*,
41 *gpsA*) as a carbon source. While sucrose assimilation involved the Entner-Doudoroff
42 and tricarboxylic acid pathways, GHB degradation required the *b1c* genes, tricarboxylic
43 acid cycle and gluconeogenesis. The tumor-enriched metabolite pyruvate is at the
44 node connecting these pathways. Using reverse genetics, we showed that the *b1c*,
45 *pckA* and *pycA* loci were important for aggressiveness (tumor weight), proliferation
46 (bacterial charge) and/or fitness (competition between the constructed mutants and
47 wild-type) of *A. tumefaciens* in plant tumors.

48 ● This work highlighted how a biotroph mobilizes its central metabolism for exploiting a
49 wide diversity of resources in plant host. It further shows the complementarity of
50 functional genome-wide scans by transcriptomics and transposon-sequencing to
51 decipher the lifestyle of a plant pathogen.

52

53 Keywords (6): biotroph, ecological niche, fitness, plant pathogen, Tn-seq,
54 transcriptomics

55 Abbreviations: gamma-aminobutyrate (GABA), gamma-hydroxybutyrate (GHB),
56 succinic semialdehyde (SSA), transposon-sequencing (Tn-seq), tricarboxylic acid
57 (TCA)

58 **Introduction**

59 Hosts and microbes evolved a wide spectrum of biological interactions, ranging from
60 pathogenesis to symbiosis. To succeed in their lifestyle, host-interacting microbes are
61 able to escape host defense, overcome competition with other microbiota members
62 and exploit nutrients available in the hosts. Ecological niche construction, which
63 ensures a preferred access to host-derived resources, represents a recurrent strategy
64 in pathogens and symbionts (Kylafis & Loreau, 2011; McNally & Brown, 2015; Martin *et*
65 *al.*, 2017; Poole *et al.*, 2018). Identifying metabolic pathways involved in the
66 exploitation of resource and evaluating their involvement in the fitness of microbes
67 represent important issues in ecology and evolution for understanding adaptation of
68 microbes to the hosts, with applied perspectives in plant, animal and human health.

69 Different strategies have emerged to identify the microbial pathways involved in
70 resource exploitation. They basically employ a two-step methodology. The first step is
71 the identification of candidate genes and pathways by different genome-wide scans
72 (functional screening of individual mutants, transcriptomics, genomics, genome wide
73 association ...) in microbes that exploit a given resource using, in some instances, a
74 comparison with microbes that do not exploit it. The second step is the validation of
75 fitness trait by confronting microbes carrying allelic variation (natural or constructed
76 variants) in those candidate genes and pathways. Because of its relative simplicity,
77 transposon sequencing (Tn-seq), which combines transposon insertional mutagenesis
78 with massively parallel sequencing of the transposon insertion sites in transposon
79 mutant populations grown in control and test conditions, seemed as an attractive
80 approach to examine ecologically important genes and pathways in prokaryotic and
81 eukaryotic microbes (van Opijnen & Camilli, 2013). In this study, we combined plant
82 metabolomics and two functional genome-wide scans, transcriptomics and Tn-seq for
83 identifying genes and pathways involved in the exploitation of the *Solanum*
84 *lycopersicum* host by the biotrophic pathogen *Agrobacterium tumefaciens*.

85 *A. tumefaciens* is a niche-constructing pathogen that genetically modifies the plant host
86 genome by transferring a part (the T-DNA) of its virulence Ti plasmid (Barton *et al.*,
87 2018; Dessaux & Faure, 2018). When expressed into the plant cell nucleus, the T-DNA
88 genes divert the host hormonal and metabolic pathways to provoke the development of
89 galls or plant tumors (Deeken *et al.*, 2006). In previous work, we paid attention to
90 specific metabolites, the opines, which accumulate in the *A. tumefaciens*-infected plant
91 tumors (Lang *et al.*, 2014; El Sahili *et al.*, 2015; Marty *et al.*, 2016; Tannières *et al.*,
92 2017; Lang *et al.* 2017; Vigouroux *et al.*, 2017). Opines, such as agrocinopines,
93 mannopine, nopaline and octopine, result from the condensation of sugars, amino and
94 organic acids (Dessaux *et al.*, 1993). According to chemical and genome databases,
95 the opines synthesized by *Agrobacterium* T-DNA encoded enzymes are almost
96 exclusively produced by host plants infected by *A. tumefaciens*. To our knowledge, the
97 only reported exception is the opine octopine that is also produced in the muscle of the
98 marine animal octopus (Fields *et al.*, 1976). The biosynthesis of opines in the chimeric
99 plant cells expressing bacterial T-DNA could be considered as a biological innovation
100 resulting from the holobiont assembly (Faure *et al.*, 2018). In the cases of nopaline and
101 octopine, we showed that opine assimilation confer a selective advantage when *A.*
102 *tumefaciens* populations colonize the plant tumors (Lang *et al.*, 2014, 2017; Vigouroux
103 *et al.*, 2017). Aside opines, diverse metabolites accumulate in the tumors on
104 *Arabidopsis thaliana* and *Brassica rapa* (Deeken *et al.*, 2006; Simoh *et al.*, 2009; Lang
105 *et al.*, 2016), but their contribution to *A. tumefaciens* fitness and proliferation is poorly
106 documented. Recently, the transcriptome of *A. tumefaciens* C58 living in *A. thaliana*
107 tumors highlighted considerable changes in gene expression profile as compared to a
108 culture in a synthetic medium (Gonzalez-Mula *et al.*, 2018). In addition to the opines,
109 the transcriptomic data suggested the exploitation of a wide diversity of resources by *A.*
110 *tumefaciens*, but a direct evidence of the contribution of these different metabolites to
111 the *Agrobacterium* fitness in plant tumors was still missing.

112 In this work, metabolomics revealed indeed the presence of a wide spectrum of
113 potential resources in *S. lycopersicum* tumors, including metabolites which were
114 enriched compared to uninfected stems. We combined Tn-seq and transcriptomics to
115 investigate the *A. tumefaciens* pathways for exploiting three metabolites, sucrose,
116 gamma-hydroxybutyrate (GHB) and gamma-aminobutyrate (GABA) that accumulated
117 at different levels in plant tumors. Finally, we used reverse genetics and host plant
118 infections to measure the aggressiveness, proliferation and competitive fitness
119 conferred by assimilation of these metabolites when *A. tumefaciens* colonized the plant
120 tumor niche. We showed that the ecological success of the *A. tumefaciens* biotroph
121 resulted from its capacity to exploit a wider spectrum of host metabolites than the sole
122 opines. This work also highlighted the strength and limits of Tn-seq and transcriptomics
123 to decipher the microbial genetic determinants that are involved in ecological niche
124 exploitation.

125

126 **Materials and methods**

127 **Bacterial strains and culture conditions**

128 We used *A. tumefaciens* C58 of which the genome was sequenced in 2001 (Goodner
129 *et al.*, 2001; Wood *et al.*, 2001). The kanamycin-resistance and gentamicin-resistance
130 cassettes (Dennis & Zylstra, 1998) were used for the construction of the knock-out
131 (KO) mutants. The *atu0035* (*pckA*), *atu2726* (*pycA*), *atu3706* and *atu4761* genes were
132 cloned into the pGEM-T Easy vector (Promega), and the mutated alleles were created
133 by inserting an antibiotic-resistance cassette in a unique restriction site of the open
134 reading frame. The constructed plasmids were electroporated in *A. tumefaciens* C58.
135 Marker exchange was selected using Gm or Km resistance and verified by PCR.
136 Previously constructed *A. tumefaciens* C58 mutants were also used in this study: the

137 derivatives C107-Gm and C107-Km in which the gentamycin and kanamycin cassette
138 were cloned in a noncoding region of the Ti plasmid (Haudecoeur *et al.*, 2009a) and the
139 $\Delta blcRABC$ mutant in which the *blcRABC* operon was replaced by the kanamycin-
140 resistance cassette (Carlier *et al.*, 2004).

141 *A. tumefaciens* was cultivated at 28°C in TY medium (Bacto tryptone, 5 g/L; yeast
142 extract, 3 g/L; agar, 15 g/L) or *Agrobacterium* broth (AB) minimal medium
143 (K_2HPO_4 , 3g/L; NaH_2PO_4 , 1g/L; $MgSO_4 \cdot 7H_2O$, 0.3 g/L; KCl, 0.15 g/L; $CaCl_2$, 0.01 g/L;
144 $FeSO_4 \cdot 7H_2O$, 2.5 mg/L; pH7) (Chilton *et al.*, 1974) supplemented with sucrose or
145 gamma-hydroxybutyric acid (GHB) at 10 mM as carbon source, and ammonium
146 chloride (NH_4Cl) or gamma-aminobutyric acid (GABA) at 20 mM as nitrogen source.
147 *Escherichia coli* MFDpir harboring the pSAM_DGm plasmid (Skurnit *et al.*, 2013),
148 auxotroph for diaminopimelic acid, was used as transposon donor for mutagenesis. *E.*
149 *coli* DH5 α was the routine host for cloning. *E. coli* strains were cultivated at 37°C in
150 Lysogenic Broth modified medium (LBm; 10 g/L peptone, 5 g/L yeast extract, NaCl 5
151 g/L). Media were supplemented when appropriate with gentamycin (25 μ g/mL),
152 ampicillin (50 μ g/mL), rifampicin (100 μ g/mL) and diaminopimelic acid (300 μ g/mL).

153 **Plant culture, metabolomics and infection assays**

154 *S. lycopersicum* (Dona hybrif F1, Vilmorin, France) plants were cultivated in a
155 greenhouse under long day conditions and controlled temperature (24-26°C). Four
156 weeks old plants were incised with a scalpel between the first and second nodes and
157 infected by ca. 10^7 *A. tumefaciens* cells as described previously in Planamente *et al.*
158 (2010). Plant tumors were collected 4 weeks after infection.

159 For plant metabolomics, tumors and wounded but not infected stems were directly
160 frozen in liquid nitrogen, crushed, extracted and analyzed by gas chromatography-time
161 of flight-mass spectrometry (GC-TOF/MS) at the *Plateforme de Chimie du Végétal*

162 (Versailles, France). The method was previously described in detail by Lang *et al.*,
163 (2016). About 150 compounds were searched and 130 compounds, including the
164 opines nopaline and agrocinopine A, and GABA and GHB were detected and
165 quantified in three biological replicates of plant tumors and uninfected stems.

166 For virulence and fitness assays, eight plant tumors were crushed in a 0.8% NaCl
167 solution to recover the agrobacteria which were then spotted onto selective agar media
168 to enumerate colony forming units (CFU). In the case of mixed infections, the
169 proportions of the genotypes (wild type and KO alleles) were measured by testing
170 around 96 CFU. Using appropriate primers (Table S1), length of the PCR-products
171 distinguished wild type allele from the KO-alleles in which the resistance gene cassette
172 was inserted. This permitted to calculate competitive index (CI) values as previously
173 described (Macho *et al.*, 2010). Two independent assays (8 plants for each of the
174 assays) were carried out for each virulence and fitness assays. A Mann-Whitney test
175 was used to analyze the values from the two independent experiments (the null
176 hypothesis postulates that both experiments were comparable). If no difference was
177 detected, the values were pooled and a non-parametric Kruskal-Wallis test (p-value <
178 0.05) coupled with a post-hoc Tukey test (p-value < 0.05) was performed.

179 **Transposon library construction and use**

180 *A. tumefaciens* C58 was mutagenized using a Himar1 mariner transposon carrying a
181 gentamicin (Gm) resistance cassette. The pSAM_DGm plasmid donor *E. coli* MFDpir
182 and *A. tumefaciens* C58 rifampicin-resistant recipient were cultivated separately:
183 *A. tumefaciens* C58 Rif^R overnight in TY medium and *E. coli* MFDpir (pSAM_DGm) for
184 4 hours in LBm supplemented with 300 µg/mL diaminopimelic acid. Both cultures were
185 centrifuged and adjusted to 1 unit of OD₆₀₀. Equivalent volumes (0.4 mL) of cell
186 suspensions were mixed, centrifuged and suspended in TY with diaminopimelic acid.
187 The cell mixture (0.2 mL) was deposited on a nitrocellulose filter (0.45 µm diameter,

188 Millipore) on a TY agar plate and incubated overnight at 28°C. Bacterial cells were
189 removed from the filter, suspended in 0.8% NaCl solution and then plated on TY
190 medium supplemented with rifampicin and gentamycin. Serial dilutions and plating
191 were performed to determine the number of mutants obtained. After 72 hours of
192 incubation, mutants were collected. The mutant population was homogenized,
193 aliquoted and stored at -80°C in 25 % (v/v) glycerol.

194 Four aliquots of the *A. tumefaciens* mutant library were thawed and cultured in liquid
195 TY medium (4 hours at 28°C) to revive them. Bacteria were washed twice with 0.8%
196 NaCl solution and used to inoculate AB medium (10 mL) at an initial OD₆₀₀ of 0.05. AB
197 medium was supplemented with three combinations of carbon and nitrogen sources:
198 sucrose-NH₄, GHB-NH₄ and sucrose-GABA. After growth at 28°C for 24 hours,
199 bacterial cells were centrifuged and stored at -20°C for further DNA manipulation.

200 **Transposon library sequencing and ARTIST analysis**

201 Genomic DNA of mutant populations grown in TY medium or AB medium with the
202 tested carbon and nitrogen sources was extracted using the DNeasy Blood & Tissue
203 Kit (QIAGEN). Two µg of DNA were digested with the MmeI type II restriction-
204 modification enzyme (BioLabs) for 1 hour at 37°C. Digested DNA was incubated for 1
205 hour at 37°C with FastAP Thermosensitive Alkaline phosphatase (ThermoScientific)
206 followed by enzyme inactivation by heating at 75°C for 5 min. Digested DNA samples
207 were purified using QIAquick PCR purification kit (QIAGEN) and were ligated to the p-
208 adapters (Table S1) in the presence of Thermo Scientific T4 DNA Ligase (16 hours at
209 16°C). The p-adapters contain a five-nucleotide long barcode which is specific for each
210 experiment. The ligation products were used as templates to perform a PCR
211 amplification with Illumina-primers P7 and P5 (Table S1). The PCR products of about
212 130 base pairs, which contain the transposon insertion site, were separated on
213 agarose gel and purified with the QIAquick gel extraction kit (QIAGEN). The final

214 samples were mixed in equimolar amounts and sequenced on an Illumina NextSeq 500
215 instrument, in a paired-end 2 x 75 run at the I2BC-sequencing platform (Gif-sur-Yvette,
216 France).

217 The experiment-specific barcodes enabled the attribution of each sequence read to the
218 corresponding experiment. The generated data were demultiplexed using bcl2fastq2
219 (bcl2fastq v2.15.0; Illumina, San Diego, USA) and FASTX-Toolkit software
220 (http://hannonlab.cshl.edu/fastx_toolkit/). Only read 1 from each sequenced fragment
221 has been used. The 3' transposon sequence was trimmed using Trimmomatic (Bolger
222 *et al.*, 2014), and reads with a length of 75 nucleotides were removed (reads without
223 the transposon insertion). After the trimming step, reads with a length between 19 and
224 23 bp were reverse-complemented and only the reads starting with TA were mapped
225 using Bowtie (bowtie-1.1.2) (Langmead *et al.*, 2009) to the genome of *A. tumefaciens*
226 C58. Bam output files were sorted with Samtools (<http://www.htslib.org/>).
227 FeatureCounts (Liao *et al.*, 2014) was used to evaluate the number of reads by gene or
228 by CDS.

229 The mapping result (bam files) were analyzed by the ARTIST pipeline (Pritchard *et al.*,
230 2014) using Matlab software (The MathWorks, Natick, MA). ARTIST compares the
231 observed (reads) and predicted numbers of transposons at each of the 115 525
232 insertion sites (TA dinucleotides) along the *A. tumefaciens* C58 genome. Two different
233 analysis were carried out, EL-ARTIST (Essential Loci analysis) and Con-ARTIST
234 (Conditionally essential loci analysis). EI-ARTIST searches for non-random distribution
235 of transposon insertions in the constructed mutant library in TY medium. Hence, it
236 identifies all loci that are required for an optimal growth in the initial culture condition. A
237 gene is annotated as 'essential' when there are low number of transposon insertions
238 (reads) or no associated transposon insertions within the entire gene. In EL-ARTIST,
239 0.03 is the p-value threshold for calling a region significantly under-represented in
240 reads. Then, Con-ARTIST was applied to compare the distribution of transposon

241 insertions between the initial TY culture condition and each of the three AB medium
242 conditions. In Con-ARTIST, 0.01 is the p-value cutoff in Mann–Whitney *U* test for
243 defining genes with significantly different read numbers. This allowed the identification
244 of *A. tumefaciens* genes and pathways that were required for efficient proliferation in
245 the presence of sucrose and NH₄Cl, GHB and NH₄Cl and sucrose and GABA as
246 nutrients.

247 **Transcriptomics and DESeq2 analysis**

248 An overnight culture of *A. tumefaciens* C58 was grown in AB medium with sucrose and
249 ammonium as sources of carbon and nitrogen respectively. This culture was washed
250 twice with NaCl 0.8% and served to inoculate AB medium supplemented with the three
251 different combinations of carbon and nitrogen sources as above for the Tn-seq
252 experiments: sucrose-NH₄, GHB-NH₄ and sucrose-GABA. Inoculations (at OD₆₀₀=0.05)
253 were performed in triplicate. At exponential phase (at OD₆₀₀=0.30), bacterial cultures
254 were centrifuged and RNA extracted with the MasterPure™ Complete DNA and RNA
255 Purification Kit according to the supplier's instructions. RNA-Seq libraries were
256 constructed using the Ribo-Zero and ScriptSeq-V2 kits (Illumina). Libraries were
257 sequenced on a NextSeq 500 instrument (Illumina, CA, USA) at the I2BC platform (Gif-
258 sur-Yvette, France) using the 75-cycles NextSeq 500 High Output Kit. Count tables
259 have been filtered to retain only genes with a gene count over 1 count per million in half
260 of the samples of the dataset. Normalization and differential analyses were performed
261 using generalized linear models as described in the DESeq2 package (version 1.12.4)
262 (Love *et al.*, 2014). The cutoff chosen for differentially expressed genes (DEG) are a
263 False Discovery Rate (FDR) < 0.01 and a log₂ fold change (LFC) > 2.

264 Gene expression was also measured by quantitative PCR using dedicated primers
265 (Table S1). The cDNA was prepared from 1 µg of bacterial RNA using the
266 RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Saint-Remy-les-

267 Chevreuses, France) following the manufacturer's instructions. The qPCRs were
268 performed with a Lightcycler 96 (Roche) apparatus. The data were processed using the
269 $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) and compared with the expression profile
270 acquired from the RNA-seq transcriptome. The internal controls used were the *atu1789*
271 (for the GHB condition) and *nocT* (*atu6027*, for GABA condition) genes. All the data are
272 available in the supplementary files.

273

274 **Results**

275 **Metabolic resources in the plant tumor niche**

276 The abundance of 130 compounds was quantified in uninfected stems of *S.*
277 *lycopersicum* and tumors induced by *A. tumefaciens* C58 (Figure 1; supplementary
278 Table S2). In plant tumors, the 16 most abundant compounds accounted for 97% of the
279 relative abundance of all the quantified compounds. In decreasing order, these are
280 propanediol, glucose, malate, dehydroascorbate, fructose, phosphate, sucrose,
281 glutamine, glutamate, myo-inositol, citrate, asparagine and aspartate. They were also
282 present in healthy stems at a similar level. Using a threshold fold change value (≥ 4),
283 24 compounds were enriched in plant tumors as compared to uninfected stems (Figure
284 1). As expected, the tumor-enriched compounds encompassed the two opines nopaline
285 and agrocinopine, but some other metabolites were also remarkable. Six metabolites,
286 agrocinopine, ferulate-trans, nicotianamine, pyruvate, spermidine and succinic
287 semialdehyde (SSA) exhibited an enrichments by 4 to 6 orders in plant tumors. Their
288 accumulation reflected some well-known characteristics of the plant tumors: obviously,
289 the opine synthesis driven by T-DNA; but also the activation of plant defense as
290 revealed by accumulation of ferulate-trans phenolics as antimicrobial compounds and
291 nicotianamine as iron chelator (Deeken *et al.*, 2006; Aznar *et al.*, 2015); a response to

292 abiotic (hypoxia and drought) and biotic stresses as shown by the accumulation of SSA
293 and spermidine that are related to the GABA pathway (Lang *et al.* 2016; Podlešáková
294 *et al.*, 2018); and a shift to an anaerobic and heterotrophic metabolism, as suggested
295 by pyruvate accumulation (Deeken *et al.*, 2006).

296 GABA, SSA and GHB were all enriched metabolites in plant tumors (Figure 1). They
297 are metabolically connected and, together with proline, are involved in regulation of the
298 quorum-sensing signal-degrading lactonase BlcC in *A. tumefaciens* (Carlier *et al.*,
299 2004; Chevrot *et al.*, 2006; Chai *et al.*, 2007; Haudecoeur 2009b; Lang *et al.*, 2016).
300 Noticeably, GABA and proline were the two enriched metabolites which accumulated at
301 the highest concentrations (Figure 1). As osmoprotectants, proline and sucrose (this
302 later was not enriched but very abundant) were proposed to contribute to desiccation
303 resistance in plant tumors (Wächter *et al.*, 2003).

304 In the next part of this work, we focused on the non-opine compounds, and
305 investigated how *A. tumefaciens* could be able to use them as a resource. Because of
306 its role in tumor development, we chose sucrose as a representative of the abundant
307 class of metabolites. We also studied two structurally and functionally related
308 metabolites, GABA and GHB, which were enriched in plant tumors, either at a high
309 concentration (GABA) or at a lower concentration (GHB).

310

311 **Genome-wide mutant library of *A. tumefaciens***

312 The *A. tumefaciens* C58 genome contained 115 525 TA dinucleotides that are potential
313 insertion sites of the *Himar1* mariner transposon. They are positioned along the circular
314 chromosome (55 348 TA within 2 841 580 bp), linear chromosome (41 503 TA within
315 2 075 577 bp) and the two plasmids, the pAt (13 084 TA within 542 868 bp) and pTi
316 (5 590 TA within 214 233 bp). From 55 matings between the *E. coli* transposon donor

317 and *A. tumefaciens* C58 recipient, we collected $1.1 \cdot 10^6$ mutant colonies on TY medium
318 supplemented with rifampicin and gentamicin, hence around 10-fold more than the TA
319 site number in *A. tumefaciens*. After homogenization of all mutant colonies, around 3
320 10^{10} individuals were kept in each frozen aliquot.

321 For analyzing the constructed transposon mutant population, four mutant library
322 aliquots were cultivated for 4 hours in TY medium. Total DNA was extracted and
323 transposon insertion sites were sequenced. Between 4 to 8 million of filtered reads
324 were obtained for each replicate. When the replicates were compared, transposon
325 distribution revealed a high homogeneity ($r^2 > 0,98$), hence all the sequencing reads
326 were analyzed together by EL-ARTIST. Mutants in most genes (4730) (Figure 2a,
327 supplementary Table S3.1) were unaffected in their fitness ('non-essential gene'
328 according to the EL-ARTIST classification). Mutants in 513 genes ('essential genes'
329 according to the EL-ARTIST classification) were impaired in their fitness for growth in
330 the rich TY medium (Figure 2c, supplementary Table S3.1). Some other genes (105)
331 (Figure 2b, supplementary Table S3.1) contained a domain in which transposon
332 insertion provoked a decrease of the fitness (genes with an 'essential domain'
333 according to the EL-ARTIST classification). The 513 fitness genes in TY medium
334 represented around 10% of the total genes of *A. tumefaciens* C58, a similar percentage
335 as reported in other bacteria (Christen *et al.*, 2011; DeJesus & Ioerger, 2013). Most of
336 them (428, hence 83%) were located on the circular chromosome (supplementary
337 Table S3.1). Using a Tn5 mutant library, Curtis and Brun (2014) reported 372 essential
338 genes in *A. tumefaciens* C58. Even if the two approaches were different in the choice
339 of transposons, growth condition, library sequencing and data analysis, most of the
340 Tn5-picked essential genes (307 of 372) were also present in the list of the *Himar1*-
341 identified mutants (Table S3, Figure S1), hence consolidating the two approaches.

342 The 513 fitness genes were classified according to clusters of orthologous genes
343 (COG) (Tatusov *et al.*, 2000). The most represented functional category was that of

344 translation, ribosomal structure and biogenesis (Figure S2, supplementary Table S3.1).
345 Genes coding for some ribosomal proteins (*atu1928-atu1951*) exemplified this COG
346 category. Genes involved in the COG category energy production and conversion were
347 also found as abundant. This is the case of the *nuoABCDEFGHIJKLMN* (*atu1268-*
348 *atu1283*) operon involved in respiration. Noticeably, the Tn-seq approach revealed
349 some genes that are not essential for cell viability, but essential for the maintenance of
350 the *A. tumefaciens* C58 plasmids, such as the operons *repABC* in the At (*atu5000-*
351 *atu5002*) and pTi (*atu6043-atu6045*) plasmids. This is explained because a transposon
352 insertion in these replicative functions caused the loss of the respective plasmid,
353 hence, after growth, the disappearance of these mutants in the mutagenized
354 population.

355

356 ***A. tumefaciens* key-genes for exploiting sucrose, GHB and GABA**

357 The transposon mutant population was cultivated in a minimal medium for searching
358 the genes associated with exploitation of either sucrose or GHB as a carbon source
359 (with ammonium as a nitrogen source) and GABA as a nitrogen source (with sucrose
360 as a carbon source). For each of the bacterial culture replicates, between 3 and 6
361 million filtered reads were obtained and analyzed by ARTIST. Among replicates of a
362 same condition, transposon distribution was highly correlated ($r^2 > 0,93$), hence reads of
363 a same condition were pooled. By comparing the transposon distribution between the
364 initial growth condition in TY rich medium and the 3 culture conditions in minimal
365 media, Con-ARTIST revealed 69, 37 and 47 genes of which mutants were impaired for
366 growth in the presence of sucrose-NH₄, GHB-NH₄ and sucrose-GABA, respectively
367 (Figure 3, supplementary Table S3.2 to S3.4). Most of them are involved in the amino
368 acid and nucleobase biosynthesis and were shared between the tested conditions. This
369 was expected because cultures in AB minimal medium were compared with an initial

370 culture in TY rich medium. We focused on genes that are specific to each of the
371 growth-conditions: there were 28 genes identified in the sucrose-ammonium condition,
372 11 in the GHB-ammonium condition, and 9 in the sucrose-GABA condition.

373 In the presence of sucrose and ammonium as nutrients, the noticeable genes were *pgi*
374 (*atu0404*) coding for glucose-6-phosphate isomerase, *pycA* (*atu2726*) allowing
375 conversion of pyruvate into oxaloacetate, *cisY* (*atu1392*) for conversion of oxaloacetate
376 into citrate and the *sdhCDA* (*atu2643-2644-2645*) genes for malate conversion and
377 energy production (Table S3.2). These genes are pivotal for the entry of carbon
378 compounds into the Entner-Doudoroff pathway and the TCA cycle. When *A.*
379 *tumefaciens* grew on GHB-NH₄, key-fitness genes were *blcAB* (*atu5137-5138*) coding
380 for the conversion of GHB into succinate, as well as *sdhDC*, *pckA* (*atu0035*) and *eno*
381 (*atu1426*) for connecting the TCA cycle and gluconeogenesis, and *gpsA* (*atu2650*)
382 which links gluconeogenesis and lipid biosynthesis (Table S3.3). In the presence of
383 GABA as a nitrogen source, we did not identify the expected GABA-transaminase key-
384 gene that could convert GABA into succinic semialdehyde, probably because of
385 functional redundancy.

386

387 ***A. tumefaciens* transcriptomes during growth on sucrose, GHB and GABA**

388 Using the same minimal media supplemented with sucrose and ammonium, GHB and
389 ammonium, and sucrose and GABA, we produced RNA-seq transcriptomes of *A.*
390 *tumefaciens* C58 under exponential growth culture condition. RNA-seq transcriptomic
391 data were validated by quantitative PCR assays on a set of 9 genes (Supplementary
392 Figure S3). In the GHB-NH₄ vs sucrose-NH₄ transcriptome comparison (Figure 4; Table
393 S4.1), 203 genes were differentially expressed (Log₂ fold change >2; p-value < 0.05).
394 Among them, 109 genes were up-regulated and 94 were down-regulated in the GHB

395 condition. In the GHB condition, the top10 of the highest up-regulated genes (Log2 fold
396 change between 6.44 and 4.17) encompassed the *blcABC* operon and *pckA* gene,
397 which, except for the lactonase-encoding *blcC* gene, were all also identified by Tn-seq
398 as crucial under GHB assimilation. The *blcC* gene encodes a lactonase that is involved
399 in gamma-butyrolactone cleavage, but not in GHB degradation (Carlier *et al.*, 2004;
400 Chai *et al.*, 2007). Other remarkable up-regulated genes were *sdhCD* (also revealed by
401 Tn-seq), *atu3740* and *pfp* (*atu2115*) encoding two successive steps converting
402 glyceraldehyde-3P to fructose-6P in gluconeogenesis, and *dctA* (*atu3298*) coding for a
403 transporter of C4-dicarboxylic acids. Most of the other up-regulated genes belong to
404 the COG category of energy production and conversion, including oxidative
405 phosphorylation pathways (*cyd* and *fix* genes) and nitrate reductase (*nap* genes).

406 Considering the up-regulated genes in the sucrose condition, the most remarkable
407 gene was *kdgA* (*atu4494*) that is coding for the last step of the Entner-Doudoroff
408 pathway. Some others were involved in sugar uptake, such as the *agl* genes (*atu0590-*
409 *0594* coding for a transcriptional regulator, a sugar ABC-transporter and a glucosidase)
410 and the *rbs* genes (*atu4369-4372* coding a sugar ABC-transporter). Most of the other
411 up-regulated genes belonged to the COG category inorganic ion transport and
412 metabolism, including iron siderophore synthesis (*atu3670-3673* and *atu3675-3685*)
413 and uptake (*atu5311-5316*) and copper resistance genes (*atu3990-3992*). The
414 differentially expressed genes related to carbon conversion from GHB and sucrose
415 were positioned in a simplified scheme of metabolic pathways (Figure 5).

416 When the sucrose-GABA vs sucrose-NH₄ transcriptomes were compared, 163 genes
417 were differentially expressed (Log2 fold change >2; p-value < 0.05). Most of them (109)
418 were up-regulated in GABA-sucrose condition. In the top five of the highest up-
419 regulated genes (Log2 fold change between 5.4 and 4.8) were the above-mentioned
420 *blcABC* genes (Figure 4; Table S4.2). Among the up-regulated genes, we searched for
421 putative GABA-transaminase genes coding for the conversion of GABA to SSA. We

422 found two candidate genes *atu4761* and *atu3407*, highlighting a potential redundancy
423 of this activity in *A. tumefaciens* C58. The gene *atu4761* was co-expressed with
424 *atu4762*, a *blcA* paralogous gene coding for a SSA dehydrogenase. Most of the other
425 up-regulated genes belonged to COG category amino acid transport and metabolism,
426 including several transporters (*amtB=atu2758*; genes *atu1387-1391* and *atu3903-*
427 *3905*) and regulatory proteins (*glnK=atu2757*). Remarkably, expression of the genes
428 coding catalase KatE (*atu5491*) and superoxide dismutase SodB (*atu4726*) was also
429 enhanced, indicating a response to an oxidative stress.

430

431 **Validation of *A. tumefaciens* fitness traits when exploiting the host plant**

432 We constructed single and double mutants of the genes *atu4761* and *atu3407* coding
433 for the putative GABA transaminases. None of these mutants was impaired for growth
434 on GABA as a sole nitrogen source (Figure S4), suggesting that either they are not
435 coding for GABA-transaminase or their mutation was compensated by one (or more)
436 other genes expressing GABA-transaminase activity.

437 We pursued our investigations on carbon metabolism by comparing GHB and sucrose
438 pathways as archetypes of two major carbon entries (the TCA cycle and Entner-
439 Doudoroff pathway) in *A. tumefaciens* when it lives on the host plant. Based on the Tn-
440 seq and transcriptomics data, we constructed two *A. tumefaciens* C58 mutants,
441 *pckA::Gm* and *pycA::Gm*, which are affected in pivotal reactions connecting the TCA
442 cycle to gluconeogenesis and Entner-Doudoroff pathways. We also used an already
443 constructed mutant *blcRCAB::Km* deleted for the *blcRCAB* gene cluster (Carlier *et al.*,
444 2004). Two other *A. tumefaciens* C58 derivatives, 107-Km and 107-Gm, carrying a Km
445 or Gm-resistance cassette respectively, in the same non-coding region were used as
446 controls (Haudecoeur *et al.*, 2009a). We verified that the *pycA::Gm* was impaired for

447 growth on sucrose, fructose and glucose, and the *pckA::Gm* mutant on GHB, succinate
448 and nopaline as carbon source. Growth of both mutants was impaired on pyruvate. The
449 mutant *blcRCAB::Km* was only impaired in the GHB assimilation (Figure 6a). The
450 control strains 107-Km and 107-Gm grew on all carbon sources.

451 All these mutants were tested for aggressiveness (tumor weight), proliferation (bacterial
452 charge) and competitive fitness (against wild type allele) on the tomato host plant. For
453 single strain inoculation experiments, the weight and bacterial charge of 5-week tumors
454 were measured (Figure 6b,c). The *A. tumefaciens* derivatives 107-Km and 107-Gm
455 exhibited similar traits on the host plant and were used as control conditions. When
456 *blcRCAB::Km* and 107-Km derivatives, which harbor the same Km-resistance cassette,
457 were compared, a decrease of both tumor weight and pathogen charge were observed
458 in the *blcRCAB* mutant. When *pycA::Gm*, *pckA::Gm* and 107-Gm mutants were
459 compared, a decrease of tumor weight and bacterial charge was observed in the
460 *pycA::Gm* mutant only. A previous study reported a decreased aggressiveness of a
461 *pckA* mutant (Liu *et al.*, 2005), but the virulence assay conditions, hence resource
462 availability, could explain this discrepancy: stem of entire tomato plants (our study) vs
463 tobacco leaf disks (Liu *et al.*, 2005).

464 Dual competitions were performed for evaluating the fitness of the *blcRCAB::Km*,
465 *pycA::Gm* and *pckA::Gm* mutants as compared to the control derivatives 107-Km or
466 107-Gm. All the three mixed populations reached a bacterial charge of 10^6 CFU/tumor
467 (Figure 7a). The *blcRCAB::Km*, *pycA::Gm* and *pckA::Gm* mutants were impaired in
468 competitive fitness (Figure 7b). Finally, we performed competitions between the
469 *pycA::Gm* and *pckA::Gm* mutants to know whether one of the two pathways (Entner-
470 Doudoroff or gluconeogenesis) could be a major contributor to bacterial fitness in plant
471 tumors. The competitive index was close to 1 showing that the two pathways
472 contributed equally to the tumor niche exploitation by *A. tumefaciens*. Noticeably, the
473 mixed population composed of the *pycA::Gm* and *pckA::Gm* mutants colonized less

474 efficiently the plant tumors (bacterial charge in Figure 7a) as compared to the other
475 mixed populations, especially the *pycA::Gm* and C107-Gm mix. This result suggested
476 that *A. tumefaciens* could gain an advantage in the simultaneous expression of the
477 Entner-Doudoroff pathway and gluconeogenesis.

478 Discussion

479 The biotrophic pathogen *A. tumefaciens* diverts the plant development and metabolism
480 for constructing and exploiting a privileged ecological niche: the plant tumor. Numerous
481 works deciphered the tumor niche construction process by studying the T-DNA transfer
482 and expression in the host plant, as well as mechanisms to escape plant defense
483 (Gohlke & Deeken, 2014; Gelvin, 2017). In this work, we combined different omics
484 (metabolomics, transcriptomics and Tn-seq) to uncover the role of *A. tumefaciens*
485 genes and pathways in tumor niche exploitation.

486 Metabolomics of tumor tissues induced on *S. lycopersicum* by *A. tumefaciens* revealed
487 a wide variety of metabolites (e.g., sugars, polyols, amino acids, organic acids,
488 phenolics). They are potential nutrients supporting the proliferation of *A. tumefaciens*
489 that reached 10^6 CFU g^{-1} of fresh tumor tissues. Most of the quantified metabolites
490 (106 of 130) were accumulated at a quite similar concentration in uninfected stems and
491 tumors (Figure 1). The most abundant metabolites were also the common ones in the
492 two tissues (e.g. glucose, sucrose, malate, glutamate), as well as in tomato seeds and
493 root exudates (Kamilova *et al.*, 2006). These plant metabolites could support the
494 growth of *A. tumefaciens* when it colonizes either asymptomatic or symptomatic plants.

495 The 24 other metabolites that we quantified, such as GABA, proline, pyruvate, GHB,
496 SSA, opines were enriched at least 4 times in plant tumors as compared to healthy
497 stems. Some of them (e.g. agrocinopine, pyruvate, SSA) were increased in plant
498 tumors by several orders of magnitude. These enriched compounds are chemical
499 signatures of the tumor niche: *A. tumefaciens* was expected to have evolved pathways
500 for detoxifying and exploiting these compounds as nutrients and signals. This paradigm
501 is well exemplified by the two opines nopaline and agrocinopine and ferulic derivatives.
502 Nopaline confers a selective growth advantage to a nopaline-assimilating *A.*
503 *tumefaciens* in *S. lycopersicum* tumors (Lang *et al.*, 2014). The agrocinopine is cleaved

504 into sucrose and arabinose-2-phosphate, which plays an important signaling role:
505 arabinose-2-phosphate enhances the quorum-sensing, which in turn activates the
506 horizontal transfer of the Ti plasmid, hence the dissemination of the virulence genes (El
507 Sahili *et al.*, 2015). *Agrobacterium* detoxifies ferulic derivatives using different pathways
508 (Brencic *et al.*, 2004; Campillo *et al.*, 2014).

509 By combining transcriptomics, Tn-seq and plant infection assays, we investigated the
510 degradative pathways of one common metabolite, sucrose, and two tumor-enriched
511 metabolites, GHB as a carbon source and GABA as a nitrogen source. In the case of
512 sucrose, the combination of Tn-seq and transcriptomics led us to identify the
513 assimilative circuit that starts by the conversion of glucose and fructose into glucose-6P
514 (*pgi* as a fitness gene), then its conversion into pyruvate *via* the Entner-Doudoroff
515 pathway (*kdgA* as an up-regulated gene) before entering into the TCA cycle (*pycA*,
516 *cisY* and *sdhCDA* as fitness genes). These pathways are consistent with a previous
517 metabolic study showing the absence of glycolysis in *A. tumefaciens* and
518 demonstrating the Entner-Doudoroff pathway as a main road of sugar degradation
519 (Fuhrer *et al.*, 2005). The same work also pointed to a high carbon flux (almost 100%)
520 between the Entner-Doudoroff pathway and the TCA cycle. These two metabolic
521 characteristics are shared by Rhizobiaceae such as *A. tumefaciens* and the legume
522 symbiont *Sinorhizobium meliloti*, and contrasted to sugar assimilation in other bacteria
523 such as *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas fluorescens* (Fuhrer *et al.*,
524 2005). The importance of the TCA cycle in a complete exploitation of sugars as
525 resource by *A. tumefaciens* was also supported by our Tn-seq data, with *pycA*, *cisY*
526 and *sdhCDA* as key fitness genes in the presence of sucrose.

527 These coherent results led us to evaluate the role of the carbon flows into and from the
528 TCA cycle in the *A. tumefaciens*-host plant interaction using a reverse genetics
529 approach. In co-infection assays in plant tumors, the *pycA* and *pckA* mutants were
530 outcompeted by a wild type genotype, but they showed a similar relative fitness when

531 they were competed together. A wild-type *A. tumefaciens* could be considered as a
532 generalist for the assimilation of a wide spectrum of metabolites. In contrast, the
533 constructed *pycA* and *pckA* mutants could be considered as specialists for a restricted
534 range of metabolites that are assimilated by either the gluconeogenesis or the Entner-
535 Doudoroff pathway. A fitness decrease of each mutant in competition with the wild-type
536 highlighted the advantage that the biotrophic pathogen gained by assimilating a wide
537 spectrum of plant metabolites. Moreover, the co-existence of the two *pycA* and *pckA*
538 mutants revealed that the two types of resource were abundant enough and/or
539 differentially distributed to sustain the growth of these two specialists in plant tumors. A
540 remarkable study reported an increase of the sucrose concentration in tumors
541 according to the age of the tumors, as well as in a gradient from the center to the
542 periphery of the tumors on *Ricinus communis* (Wächter *et al.*, 2003). In further studies,
543 the spatial and temporal distribution of the metabolites should be considered as an
544 important parameter driving the resource exploitation strategy of *A. tumefaciens* when
545 it colonizes the heterogeneous environment that the plant tumors are.

546 Our data also revealed that a mixture of the two specialists (the *pycA* and *pckA*
547 mutants) was less efficient for exploiting the host in terms of bacterial charge than a
548 mixture containing the generalist (the wild-type) and one of the two specialists (Figure
549 7). This suggested that *A. tumefaciens* could take an advantage of the simultaneous
550 expression of the Entner-Doudoroff pathway and gluconeogenesis in a same individual,
551 quite simply because these two pathways are connected for allowing the recycling of
552 several metabolites such as glyceraldehyde-3-P and phosphoenolpyruvate (Figure 5).
553 As discussed above, some other explanations related to spatial and temporal
554 distribution of the resource can not be excluded. The transcriptome of *A. tumefaciens*
555 living in tumors on the *Arabidopsis thaliana* host plant is consistent with a simultaneous
556 expression of pathways to exploit of a wide diversity of carbon and nitrogen sources
557 (González-Mula *et al.*, 2018). In *A. tumefaciens* and in some other Rhizobiaceae, the

558 separation of carbon flows incoming and outgoing into and from the TCA cycle using
559 the Entner-Doudoroff pathway and gluconeogenesis (instead of a unique, reversible
560 glycolysis/gluconeogenesis pathway) could be a biological innovation contributing to an
561 optimal exploitation of the diversified resources available in the plant hosts. The
562 capacity of the microbial pathogens to activate and regulate their carbon assimilative
563 pathways is crucial for survival and invasion in plant and animal hosts (Alteri *et al.*,
564 2009; Brock, 2009; Basu *et al.*, 2018).

565 The tumor-enriched metabolites GHB, GABA and SSA are metabolically connected.
566 GABA is the highest abundant non-protein amino-acid in tumor tissues of *A. thaliana*
567 and *S. lycopersicum* (Deeken *et al.*, 2006; Lang *et al.*, 2016; this work). In the host
568 plant, GABA is mainly produced from glutamate (by GABA decarboxylase) and then
569 degraded into SSA (by GABA transaminase), which is in turn converted into succinate
570 (by SSA dehydrogenase) or GHB (by GHB reductase) (Bown & Shelp, 2016). SSA is a
571 toxic metabolite provoking an oxidative stress in plants and other organisms and micro-
572 organisms, including *A. tumefaciens* (Bouché *et al.*, 2003; Ludewig *et al.*, 2008; Wang
573 *et al.*, 2016). In plant tumors, *A. tumefaciens* may exploit plant GABA and GHB as
574 nitrogen and carbon sources, but have to face toxic SSA: either exogenous SSA
575 resulting from plant metabolism or endogenous SSA as an intermediate of the *A.*
576 *tumefaciens* GABA and GHB degradation pathways. The published transcriptome of *A.*
577 *tumefaciens* in plant tumors showed that the pathogen responded to the presence of
578 GABA and its derived metabolites SSA and GHB, since the *b1c* genes, as well as the
579 *atu4761* gene coding for a putative GABA-transaminase were up-regulated (Gonzalez-
580 Mula *et al.*, 2018).

581 When *A. tumefaciens* was grown on GABA as a nitrogen source, the Tn-seq approach
582 failed to identify any genes coding for a putative GABA transaminase, nor a SSA
583 dehydrogenase that would be involved in the degradation of GABA and the
584 detoxification of SSA. This may be explained by a redundancy of genes coding these

585 two enzymatic activities. Transcriptomics supported this hypothesis. Two genes coding
586 for putative transaminases (*atu3407* and *atu4761*) and two others for SSA
587 dehydrogenases (*atu4762* and *blcA*) were up-regulated in *A. tumefaciens* growing on
588 GABA. The role of the SSA dehydrogenases in stress response and quorum-sensing
589 signal decay was previously studied (Wang *et al.*, 2006). In this work, simple and
590 double knock out-mutants of the two transaminases still grew on GABA, suggesting the
591 presence of at least a third gene encoding a GABA transaminase in *A. tumefaciens*. In
592 the related species *Rhizobium leguminosarum*, three GABA transaminases are
593 involved in the degradation of GABA (Prell *et al.*, 2009).

594 When *A. tumefaciens* was grown in the presence of GHB as a carbon source, a
595 combination of Tn-seq and transcriptomics identified the *blcAB* genes, which are
596 required for the conversion of GHB to succinate (Carrier *et al.*, 2004; Chai *et al.*, 2007).
597 This approach also permitted to connect this particular pathway to the central
598 metabolism by highlighting genes of the TCA cycle (*sdhDC*, *pckA*, *eno*, *atu3740*,
599 *atu2115* and *gpsA*), gluconeogenesis and synthesis of lipid precursors. The *A.*
600 *tumefaciens* mutants defective in *blc* or *pckA* genes were unable to grow on GHB as a
601 nutrient, validating the data collected from Tn-seq. Tn-seq and transcriptomics
602 appeared as complementary for deciphering microbial pathways.

603 Besides a potential growth advantage related to nutrient exploitation, the GABA- and
604 GHB-transcriptomes highlighted an oxidative stress response in *A. tumefaciens*. In the
605 presence of GABA, the concerned up-regulated genes were, for instance, *katE* and
606 *sodB* coding for catalase and superoxide dismutase, respectively. In the presence of
607 GHB, the stress-response up-regulated genes were the *cyd* and *fix* genes coding for
608 oxidative phosphorylation pathways, while the down-regulated genes were involved in
609 siderophore synthesis and uptake (Figure 4a). A decrease of iron uptake would
610 contribute to reduce the production of highly deleterious hydroxyl radicals via the
611 Fenton reaction. In culture assays, Wang *et al.*, (2016) showed that a pre-exposure of

612 *A. tumefaciens* to extracellular SSA induces an oxidative stress response and
613 increases resistance of *A. tumefaciens* to H₂O₂. Noticeably, this effect was lost in a
614 *blcABC* KO-mutant (Wang *et al.*, 2016). Our study showed that a *blc* KO-mutant was
615 impaired for inducing tumors on tomato stems, as well as colonizing plant tumors and
616 competing a wild-type strain in the plant tumors. Two nonexclusive explanations of this
617 selective advantage could be proposed: an impaired assimilation of GHB or GABA as
618 nutrients and an impaired SSA-mediated activation of the oxidative stress response to
619 face plant defense.

620 Different arguments supported the impaired oxidative stress response as an important
621 cause of the decreased aggressiveness and fitness in the *blc* mutant: (i) *A.*
622 *tumefaciens* mutants of catalase and superoxide dismutase were impaired in virulence,
623 highlighting oxidative stress response as an important trait during plant infection (Xu &
624 Pan, 2000; Saenkham *et al.*, 2007); (ii) while the *blc* mutant was affected in
625 aggressiveness (tumor weight), bacterial invasion (bacterial charge) and fitness
626 (competition versus wild type allele), the *pckA* mutant was impaired in competitive
627 fitness only, suggesting that the *blc* genes conferred an advantage that could not be
628 exclusively explained by nutrition.

629 In *A. tumefaciens*, the *blc* genes are carried by the dispensable At plasmid which
630 reaches the size of 0.5 Mb in *A. tumefaciens* C58 (Goodner, 2001; Wood *et al.*, 2001).
631 Several studies have highlighted the fitness cost imposed by maintenance and
632 expression of At plasmid genes (Morton *et al.*, 2013; Platt *et al.*, 2014; González-Mula
633 *et al.*, 2018). This study showed the fitness gains conferred by *blc* genes in plant host
634 infection. The selective advantage conferred by the *blc* operon would not be restricted
635 to *Agrobacterium* pathogens, as data mining analysis revealed its presence in the
636 genome of several host-interacting bacteria, such as *Rhizobium etli*, *Burkholderia*
637 *phenoliruptrix* and *Pantoea* sp.

638 Beyond the use of opines, our study expanded the ecological traits supporting
639 exploitation of tumor niche by *A. tumefaciens*, highlighting novel targets for controlling
640 its virulence and proliferation.

641

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652

653 **The authors declare no conflict of interest**

654

655 **Author contribution:** PM, DF designed research; AGM, JL, LM, AK, DN performed
656 research; AGM, DF, JL, FL analyzed data; AGM, DN, PM, DF wrote the paper.

657

658 **Data accessibility: all the data are available in supplementary files**

659

660 **Supplementary files:**

- 661 - **Figure S1. The *A. tumefaciens* C58 essential genes identified in the Tn5**
- 662 **and *Himar1* transposon libraries.**
- 663 - **Figure S2. Classification of essential genes by COG categories.**
- 664 - **Figure S3. RT-qPCR and transcriptome comparative gene expression.**
- 665 - **Figure S4. Growth on GABA as a sole nitrogen source.**
- 666 - **Table S1. Primer list.**
- 667 - **Table S2. Plant metabolomics.**
- 668 - **Table S3. Tn-seq data.**
- 669 - **Table S4. Transcriptomics.**
- 670

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855 **Figure legends**

856 **Figure 1. Metabolome of *S. lycopersicum* healthy stems vs *A. tumefaciens* C58**
857 **tumors.** The graphic shows the relative abundance of compounds found in *A.*
858 *tumefaciens* tumors when compared with uninfected tissue.

859

860 **Figure 2. Genome of *A. tumefaciens* C58 with locations of transposon insertions**
861 **in the constructed transposon library.** From the outside to the inside, the tracks
862 represent: forward and reverse CDS (in blue), number of transposon insertions per TA
863 site for each gene expressed in log10 (in black), and EL-ARTIST analysis in which non-
864 essential genes are in green, genes with a domain essential in yellow and essential
865 genes in red. The circle chart shows the total number of non-essential genes (in
866 green), essential genes (in red) and genes with a domain essential (in yellow). Panels
867 **a, b** and **c** exemplifies these three categories of genes, showing the number of
868 transposon insertions per TA sites in each of the genes *atu3056*, *chvA*, *nuoM* and
869 *nuoN*.

870

871 **Figure 3. *A. tumefaciens* C58 fitness genes for growth with different carbon and**
872 **nitrogen sources.** From the outside to the inside, the tracks represent: forward and
873 reverse CDS (in blue) and fitness genes (“essential genes” according to Con-ARTIST
874 analysis) when *A. tumefaciens* grew in the presence of sucrose and NH₄ (in orange),
875 GHB and NH₄ (in green) and sucrose and GABA (in purple). The Venn diagram
876 represents a comparison of fitness genes in the three conditions. Some examples of
877 fitness genes are indicated.

878

879 **Figure 4. Volcano plot of transcriptomic data.** The data for all genes are plotted as
880 log₂ fold change versus the -log₁₀ of the adjusted p-value. **a** Differentially expressed
881 genes between GHB-NH₄ and sucrose-NH₄ growth conditions. The fitness genes
882 (identified by Tn-seq) in GHB-NH₄ growth condition are colored in green, while those in
883 sucrose-NH₄ growth condition are represented in orange color. **b** Differential expressed
884 genes between sucrose-GABA and sucrose-NH₄ growth conditions. The essential
885 genes (identified by Tn-seq) in sucrose-GABA condition are in purple color, while those
886 in sucrose-NH₄ condition are represented and in orange color. The identity of some
887 genes is indicated.

888

889 **Figure 5. *A. tumefaciens* key-pathways for exploiting sucrose and GHB.** This
890 scheme combines the Tn-seq and transcriptomics data. Tn-seq revealed fitness genes
891 for growing on either GHB (*blcAB*, *pckA*, *eno*, *gpsA* in red color) or sucrose (*pgi*, *pycA*,
892 *cisY* in blue color), and on both carbon sources (*sdhCDA* in purple color).
893 Transcriptomics revealed response genes when *A. tumefaciens* was grown on either
894 GHB (*blcAB*, *sdhCDA*, *pckA*, *atu3740*, *atu2115* in yellow) or sucrose (*kdgA* in blue).

895

896 **Figure 6. Metabolic capacity, aggressiveness and colonization of the constructed**
897 ***A. tumefaciens* mutants.** **a**, *A. tumefaciens* KO-mutants and control strains growing in
898 AB medium supplemented with different carbon sources. Open circles represent the
899 absence of growth (OD₆₀₀<0.05). **b**, Fresh weight of tomato tumors induced with KO-
900 mutants and control strains. **c**, Colonization efficiency (bacterial numeration, log₁₀
901 CFU/mg fresh weight) in tomato tumors. Mean values (indicated by a cross), median
902 (horizontal line) and standard deviations (SD) of two independent experiments are

903 presented. Nonparametric Kruskal-Wallis and post-hoc Tukey tests (n=16; $p < 0.05$)
904 were used and different letters indicate statistical significance.

905

906 **Figure 7. Competitive fitness of the constructed *A. tumefaciens* mutants. a,**
907 Colonization efficiency (total bacterial numeration (mutant plus control), \log_{10} CFU/mg
908 fresh weight) in tomato tumors. Mean values (indicated by a cross), median (horizontal
909 line) and standard deviations (SD) of two independent experiments are presented.
910 Nonparametric Kruskal-Wallis and post-hoc Tukey tests (n=16; $p < 0.05$) were used and
911 different letters indicate statistical significance. **b,** Relative abundance of KO-mutant
912 and control strain was compared at infection time and in tumors. A competitive index
913 value < 1 indicates a fitness loss of the mutant strain in plant tumors. Average
914 (indicated by a cross), median (horizontal line) and SD were calculated from 2
915 independent experiments (n=16). Significant fitness loss of mutants is noted by a triple
916 asterisk (Wilcoxon signed rank test $p < 0.001$).