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Subtitle: Plant host exploitation by the *Agrobacterium* biotroph

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Word count: 8100 words (excluding references)

Tables: 0

Figures: 7

Supplementary: 4 figures S1 to S4 and 4 excel tables

Abstract (about 200 words)

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

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

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

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

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

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

Introduction

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

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

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

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

Materials and methods

Bacterial strains and culture conditions

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

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

A. tumefaciens was cultivated at 28°C in TY medium (Bacto tryptone, 5 g/L; yeast extract, 3 g/L; agar, 15 g/L) or *Agrobacterium* broth (AB) minimal medium (K_2HPO_4,3g/L; NaH_2PO_4,1g/L; $MgSO_4.7H_2O$, 0.3 g/L; KCl, 0.15 g/L; CaCl2, 0.01 g/L; $FeSO_4.7H_2O$, 2.5 mg/L; pH7) (Chilton *et al.*, 1974) supplemented with sucrose or gamma-hydroxybutyric acid (GHB) at 10 mM as carbon source, and ammonium chloride (NH_4Cl) or gamma-aminobutyric acid (GABA) at 20 mM as nitrogen source. *Escherichia coli* MFD*pir* harboring the pSAM_DGm plasmid (Skurnit *et al.*, 2013), auxotroph for diaminopimelic acid, was used as transposon donor for mutagenesis. *E. coli* DH5α was the routine host for cloning. *E. coli* strains were cultivated at 37ºC in Lysogenic Broth modified medium (LBm; 10 g/L peptone, 5 g/L yeast extract, NaCl 5 g/L). Media were supplemented when appropriate with gentamycin (25 µg/mL), ampicillin (50 µg/mL), rifampicin (100 µg/mL) and diaminopimelic acid (300 µg/mL).

Plant culture, metabolomics and infection assays

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

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

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

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

Transposon library construction and use

A. tumefaciens C58 was mutagenized using a Himar1 mariner transposon carrying a gentamicin (Gm) resistance cassette. The pSAM_DGm plasmid donor *E. coli* MFDpir and *A. tumefaciens* C58 rifampicin-resistant recipient were cultivated separately: *A. tumefaciens* C58 Rif^R overnight in TY medium and *E. coli* MFDpir (pSAM_DGm) for 4 hours in LBm supplemented with 300 µg/mL diaminopimelic acid. Both cultures were centrifuged and adjusted to 1 unit of OD_{600}. Equivalent volumes (0.4 mL) of cell suspensions were mixed, centrifuged and suspended in TY with diaminopimelic acid. 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 28ºC. 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 OD_{600} of 0.05. AB
197 medium was supplemented with three combinations of carbon and nitrogen sources:
198 sucrose-NH_4, GHB-NH_4 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 37ºC. Digested DNA was incubated for 1
205 hour at 37ºC with FastAP Thermosensitive Alkaline phosphatase (ThermoScientific)
206 followed by enzyme inactivation by heating at 75ºC 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-adaptors 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

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

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

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

insertions between the initial TY culture condition and each of the three AB medium conditions. In Con-ARTIST, 0.01 is the p-value cutoff in Mann–Whitney *U* test for defining genes with significantly different read numbers. This allowed the identification of *A. tumefaciens* genes and pathways that were required for efficient proliferation in the presence of sucrose and NH_4Cl, GHB and NH_4Cl and sucrose and GABA as nutrients.

Transcriptomics and DESeq2 analysis

An overnight culture of *A. tumefaciens* C58 was grown in AB medium with sucrose and ammonium as sources of carbon and nitrogen respectively. This culture was washed twice with NaCl 0.8% and served to inoculate AB medium supplemented with the three different combinations of carbon and nitrogen sources as above for the Tn-seq experiments: sucrose-NH_4, GHB-NH_4 and sucrose-GABA. Inoculations (at OD_{600}=0.05) were performed in triplicate. At exponential phase (at OD_{600}=0.30), bacterial cultures were centrifuged and RNA extracted with the MasterPure™ Complete DNA and RNA Purification Kit according to the supplier's instructions. RNA-Seq libraries were constructed using the Ribo-Zero and ScriptSeq-V2 kits (Illumina). Libraries were sequenced on a NextSeq 500 instrument (Illumina, CA, USA) at the I2BC platform (Gif-sur-Yvette, France) using the 75-cycles NextSeq 500 High Output Kit. Count tables have been filtered to retain only genes with a gene count over 1 count per million in half of the samples of the dataset. Normalization and differential analyses were performed using generalized linear models as described in the DESeq2 package (version 1.12.4) (Love *et al.*, 2014). The cutoff chosen for differentially expressed genes (DEG) are a False Discovery Rate (FDR) < 0.01 and a log2 fold change (LFC) > 2.

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

Chevreuses, France) following the manufacturer's instructions. The qPCRs were performed with a Lightcycler 96 (Roche) apparatus. The data were processed using the 2−ΔΔCT method (Livak & Schmittgen, 2001) and compared with the expression profile acquired from the RNA-seq transcriptome. The internal controls used were the *atu1789* (for the GHB condition) and *nocT* (*atu6027,* for GABA condition*)* genes. All the data are available in the supplementary files.

Results

Metabolic resources in the plant tumor niche

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

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

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

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

Genome-wide mutant library of *A. tumefaciens*

The *A. tumefaciens* C58 genome contained 115 525 TA dinucleotides that are potential insertion sites of the *Himar1* mariner transposon. They are positioned along the circular chromosome (55 348 TA within 2 841 580 bp), linear chromosome (41 503 TA within 2 075 577 bp) and the two plasmids, the pAt (13 084 TA within 542 868 bp) and pTi (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 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 Tn*5* 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 Tn*5*-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

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

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

The transposon mutant population was cultivated in a minimal medium for searching the genes associated with exploitation of either sucrose or GHB as a carbon source (with ammonium as a nitrogen source) and GABA as a nitrogen source (with sucrose as a carbon source). For each of the bacterial culture replicates, between 3 and 6 million filtered reads were obtained and analyzed by ARTIST. Among replicates of a same condition, transposon distribution was highly correlated (r^2> 0,93), hence reads of a same condition were pooled. By comparing the transposon distribution between the initial growth condition in TY rich medium and the 3 culture conditions in minimal media, Con-ARTIST revealed 69, 37 and 47 genes of which mutants were impaired for growth in the presence of sucrose-NH_4, GHB-NH_4 and sucrose-GABA, respectively (Figure 3, supplementary Table S3.2 to S3.4). Most of them are involved in the amino acid and nucleobase biosynthesis and were shared between the tested conditions. This was expected because cultures in AB minimal medium were compared with an initial

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

In the presence of sucrose and ammonium as nutrients, the noticeable genes were *pgi* (*atu0404*) coding for glucose-6-phosphate isomerase, *pycA* (*atu2726*) allowing conversion of pyruvate into oxaloacetate, *cisY* (*atu1392*) for conversion of oxaloacetate into citrate and the *sdhCDA* (*atu2643-2644-2645*) genes for malate conversion and energy production (Table S3.2). These genes are pivotal for the entry of carbon compounds into the Entner-Doudoroff pathway and the TCA cycle. When *A. tumefaciens* grew on GHB-NH_4, key-fitness genes were *blcAB* (*atu5137-5138*) coding for the conversion of GHB into succinate, as well as *sdhDC*, *pckA* (*atu0035*) and *eno* (*atu1426*) for connecting the TCA cycle and gluconeogenesis, and *gpsA* (*atu2650*) which links gluconeogenesis and lipid biosynthesis (Table S3.3). In the presence of GABA as a nitrogen source, we did not identify the expected GABA-transaminase key-gene that could convert GABA into succinic semialdehyde, probably because of functional redundancy.

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

Using the same minimal media supplemented with sucrose and ammonium, GHB and ammonium, and sucrose and GABA, we produced RNA-seq transcriptomes of *A. tumefaciens* C58 under exponential growth culture condition. RNA-seq trancriptomic data were validated by quantitative PCR assays on a set of 9 genes (Supplementary Figure S3). In the GHB-NH_4 vs sucrose-NH_4 transcriptome comparison (Figure 4; Table S4.1), 203 genes were differentially expressed (Log2 fold change >2; p-value < 0.05). Among them, 109 genes were up-regulated and 94 were down-regulated in the GHB

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

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

When the sucrose-GABA vs sucrose-NH_4 transcriptomes were compared, 163 genes were differentially expressed (Log2 fold change >2; p-value < 0.05). Most of them (109) were up-regulated in GABA-sucrose condition. In the top five of the highest up-regulated genes (Log2 fold change between 5.4 and 4.8) were the above-mentioned *blcABC* genes (Figure 4; Table S4.2). Among the up-regulated genes, we searched for putative GABA-transaminase genes coding for the conversion of GABA to SSA. We

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

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

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

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

Discussion

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

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

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

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

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

When *A. tumefaciens* was grown on GABA as a nitrogen source, the Tn-seq approach failed to identify any genes coding for a putative GABA transaminase, nor a SSA dehydrogenase that would be involved in the degradation of GABA and the 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 (Carlier *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

A. tumefaciens to extracellular SSA induces an oxidative stress response and increases resistance of *A. tumefaciens* to H_2O_2. Noticeably, this effect was lost in a *blcABC* KO-mutant (Wang *et al.*, 2016). Our study showed that a *blc* KO-mutant was impaired for inducing tumors on tomato stems, as well as colonizing plant tumors and competing a wild-type strain in the plant tumors. Two nonexclusive explanations of this selective advantage could be proposed: an impaired assimilation of GHB or GABA as nutrients and an impaired SSA-mediated activation of the oxidative stress response to face plant defense.

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

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

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

Acknowledgments

We thank Julien Lang (I2BC) for performing plant metabolome assays, Anthony Kwasiborski (I2BC) for performing a part of the transcriptome experiments, and Erwan Gueguen (MAP, Université Lyon) for kindly providing plasmid pSAM_DGm. This work has benefited from the facilities and expertise of the high throughput sequencing platform and the plant culture facilities of the institute for integrative biology of cell (I2BC) and the metabolomics platform of the Plant Observatory-Chemistry and Metabolomics (Versailles, France). This work was supported by CNRS (SE2016-2017), University Paris-Sud (PhD grant to AGM) and LabEx Saclay Plant Sciences-SPS (ANR-10-LABX-0040-SPS).

The authors declare no conflict of interest

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

Data accessibility: all the data are available in supplementary files

Supplementary files:

- **Figure S1. The *A. tumefaciens* C58 essential genes identified in the Tn*5* and *Himar1* transposon libraries.**
- **Figure S2. Classification of essential genes by COG categories.**
- **Figure S3. RT-qPCR and transcriptome comparative gene expression.**
- **Figure S4. Growth on GABA as a sole nitrogen source.**
- **Table S1. Primer list.**
- **Table S2. Plant metabolomics.**
- **Table S3. Tn-seq data.**
- **Table S4. Transcriptomics.**

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Figure legends

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

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

Figure 3. *A. tumefaciens* C58 fitness genes for growth with different carbon and nitrogen sources. From the outside to the inside, the tracks represent: forward and reverse CDS (in blue) and fitness genes ("essential genes" according to Con-ARTIST analysis) when *A. tumefaciens* grew in the presence of sucrose and NH_4 (in orange), GHB and NH_4 (in green) and sucrose and GABA (in purple). The Venn diagram represents a comparison of fitness genes in the three conditions. Some examples of fitness genes are indicated.

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-NH_4 and sucrose-NH_4 growth conditions. The fitness genes
882 (identified by Tn-seq) in GHB-NH_4 growth condition are colored in green, while those in
883 sucrose-NH_4 growth condition are represented in orange color. **b** Differential expressed
884 genes between sucrose-GABA and sucrose-NH_4 growth conditions. The essential
885 genes (identified by Tn-seq) in sucrose-GABA condition are in purple color, while those
886 in sucrose-NH_4 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 (OD_{600}<0.05). **b,** Fresh weight of tomato tumors induced with KO-
900 mutants and control strains. **c,** Colonization efficiency (bacterial numeration, log_{10}
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 $p < 0.001$).