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In planta fitness-cost of the Atu4232-regulon encoding for a selective GABA-binding sensor in *Agrobacterium*

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GABA (gamma-aminobutyric acid) mediates cell-to-cell communication in eukaryotes and interspecies communication in host-microbe interactions. *Agrobacterium tumefaciens* induces the development of plant tumor in which GABA accumulates. Two periplasmic binding proteins Atu2422 and Atu4243 and their appropriate ABC-transporter are involved in the binding and importation of GABA. The structure of the selective GABA-binding Atu4243 reveals a GABA conformation similar to a proposed model of GABA bound to the mammalian GABAC receptor. The *A. tumefaciens atu4243* mutant is affected for GABA uptake, aggressiveness on plant host and GABA-induced degradation of the quorum-sensing signal, hence for horizontal transfer of the tumor-inducing plasmid. Here, we report that a de-repression of *atu4243* and its co-regulated neighbor genes affect the fitness of *A. tumefaciens* during tumor colonization. Atu4243-orthologs are present in several species of the *Agrobacterium* genus. This addendum highlights the recent data on the GABA transport in the *A. tumefaciens* plant-pathogen.

The plant pathogen *Agrobacterium tumefaciens* is able to transfer a DNA fragment (T-DNA) from its tumor-inducing (Ti) plasmid to the nuclear genome of the plant host.[1] In the transformed plant cells, T-DNA encodes for synthesis of plant hormones, auxin and cytokinins, resulting in cell proliferation and development of a plant tumor. In addition, T-DNA redirects plant metabolism for the production of tumor-specific compounds, called opines, among which some stimulate the synthesis of a quorum-sensing (QS) signal, 3-oxo-octanoylhomoserine lactone (OC8HSL), in *A. tumefaciens*. Accumulation of OC8HSL promotes horizontal transfer of the Ti plasmid by conjugation, hence dissemination and maintenance of the virulence genes among the *A. tumefaciens* population which colonizes the plant tumor. Moreover, the OC8HSL accumulation amplifies aggressiveness of *A. tumefaciens*.[2]

In addition to opines, the *Agrobacterium*-induced plant tumors accumulate a wide variety of sugars and amino-acids, including GABA,[3] which may be also used as nutrients or signals to trigger gene expression in *A. tumefaciens*. GABA plays two roles in the *A. tumefaciens*-host interaction: first, GABA moderates gravity of the symptoms provoked by *A. tumefaciens*;[4] second, GABA induces the expression of an OC8HSL-cleaving lactonase BlcC (also named AttM), therefore may slightly delay the quorum-sensing signal accumulation and the conjugation of the Ti plasmid.[5,6] Uptake of GABA into the *Agrobacterium* cells is required for enhancing the expression of the lactonase BlcC.[4,7,8] The transport of GABA involves two periplasmic binding proteins (PBPs) Atu2422 and Atu4243, which bring GABA to their cognate ABC-transporters and then to the cytoplasm (**Fig. 1**). This addendum summarizes recent data[8,9] on these two PBPs and their role in the GABA-uptake and GABA-mediated gene regulation in *A. tumefaciens*.

These two PBPs display a similar affinity for GABA (μM range of the K_d values), however, their sequence, binding site composition and selectivity, as well as the conformation of the liganded GABA strongly differ.[8,9] Atu2422 can bind a large spectrum of amino acids with a short lateral chain, such as Alanine, Valine, Proline, which are competitive inhibitors of the transport of GABA, hence antagonists of the GABA-induced degradation of the quorum-sensing signal OC8HSL. In contrast, the Atu4243-mediated transport of GABA is only altered by a synthetic analog *trans*-4-aminocrotonic acid (TACA), in which a double bond confers rigidity and mimics GABA in a planar conformation. GABA is a flexible molecule which can adopt various conformations in aqueous solution.[10] GABA bound to Atu4243 exhibits so far a unique planar and extended conformation (PDB code 4EUO), while the Atu2422-interacting GABA adopts a non-planar and curved

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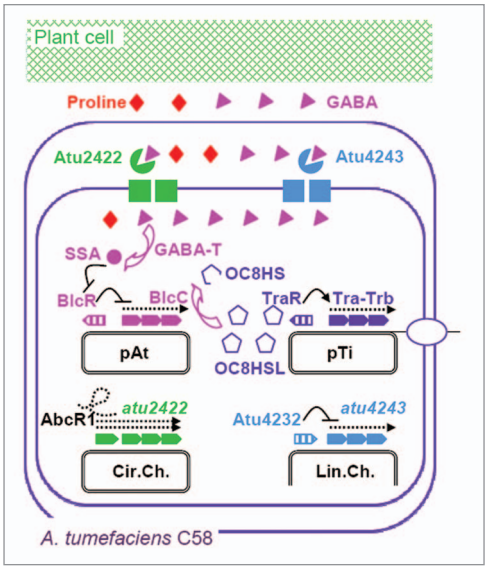

Figure 1. Working model of GABA-uptake and GABA-signaling in *A. tumefaciens*. The PBP Atu4243 is selective for GABA, while the Atu2422-mediated transport of GABA may be competed by free Proline. In *A. tumefaciens* cells, GABA is converted into semialdehyde succinic (SSA) by a still uncharacterized GABA-transaminase (GABA-T), and then SSA interacts with the transcriptional repressor BlcR which controls the expression of the *blcABC* operon encoded by the plasmid At (pAt). The lactonase BlcC converts the QS-signal OC8HSL into the inactive product (OC8HS), hence affects the level of the accumulated OC8HSL. In complex with the transcriptional regulator TraR, OC8HSL acts as a positive signal for the expression of the *tra* and *trb* operons which promote the horizontal transfer of the virulence plasmid Ti (pTi). The *atu2422* gene harbored by the circular chromosome (Cir. Ch.) is constitutively expressed, but is susceptible to the activity of the non-coding RNA AbcR1. The *atu4243* gene belongs to a large cluster on the linear chromosome (Lin. Ch.) of which expression is controlled by the transcriptional repressor Atu4232. Additional details are described in the text.

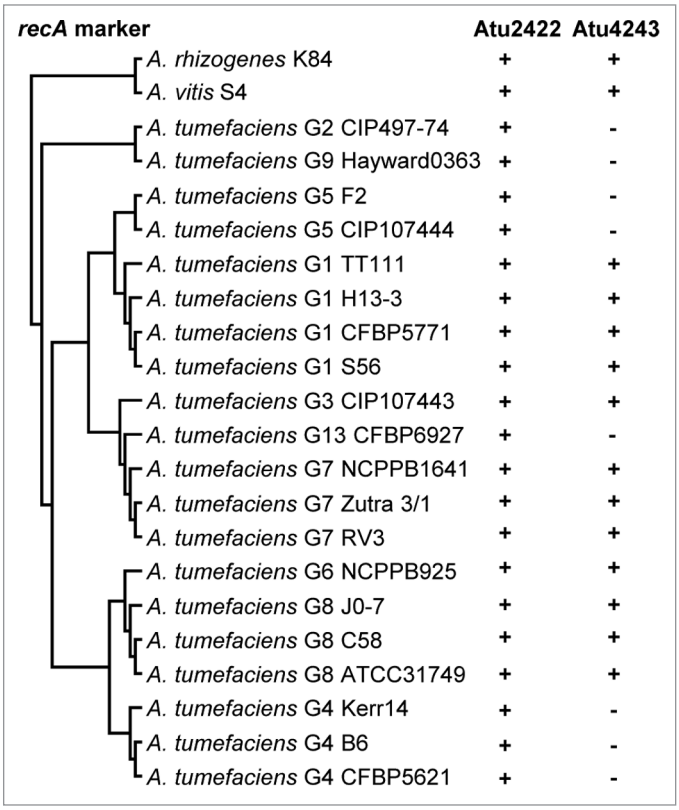

Figure 2. Occurrence of Atu4243 and Atu2422 in Agrobacterial genomes. Among the available *Agrobacterium* genomes (AgrobacterScope project at www.genoscope.cns.fr), the presence (+) and absence (-) of PBPs Atu2422 and Atu4243 were indicated. Phylogenetic tree of *Agrobacterium* strains was constructed using *recA* gene.

conformation (PDB code 3IP9), as observed in few liganded GABA-protein structures in the Protein Data Bank (PDB codes 2OKK, 2OKJ, 6JDW). No structures of the mammal GABA-receptors are available. The ionotropic GABA-receptors $GABA_A$ and $GABA_C$ belong to the Cys-loop receptor family in which the ionic channel exhibits a ligand-binding domain.[11] The metabotropic receptor belongs to the class C G-Protein Coupled Receptor (GPCR) family,[12] in which the extracellular ligand-binding domain exhibits similarities with the general fold of bacterial PBPs. Modeling the $GABA_B$ PBP-like domain using Atu2422 or Atu4243 as a matrix was unsuccessful. In contrast, our work highlights four characteristics of the GABA binding mode that Atu4243 and the $GABA_C$-receptor model proposed by Harrison and coworkers[13] would share: the planar and extended conformation of GABA, the sensitivity to TACA, the stacking of GABA between aromatic residues and the interaction between its carboxylate and an Arg residue. Our data suggest that the global fold of the GABA binding proteins would be less informative than the conformation of GABA to predict its molecular interactions with the protein residues of the binding site.

Phylogenetic analysis revealed that Atu2422 and Atu4243 PBPs belong to different protein clusters. Their occurrence differs among Proteobacteria, the *atu2422*-orthologs were more frequently encountered than the *atu4243*-orthologs.[8] Their co-occurrence was observed in some strains belonging to the α-proteobacteria *Agrobacterium*, *Azospirillum, Mesorhizobium, Rhizobium* and *Sinorhizobium,* and gamma-proteobacteria *Pseudomonas* and *Marinomonas*. In the *Agrobacterium* genus (**Fig. 2**), the *atu2422* gene is present in all available genomes, while the *atu4243* gene was identified in strains of the *A. rhizogenes* and *A. vitis* species, as well as *A. tumefaciens* genomic species G1, G7, G6 and G8, but not in strains of the *A. tumefaciens* genomic species G2, G4, G5 and G13. The *Agrobacterium* genome encompasses more than a hundred of PBPs, of which some of them were already described as key-loci for species identification.[14] Hence, the gene encoding the selective GABA-binding PBP Atu4243 would also be used for discriminating the *A. tumefaciens* genomic species.

In *A. tumefaciens* C58, Atu4243 and Atu2422 also differ by the regulation of their expression (**Fig. 1**). The *atu2422* gene is constitutively transcribed[15] and post-transcriptionally controlled by small RNA AbcR1.[16] In contrast, the Atu4243-system is strongly controlled by the transcriptional repressor Atu4232 belonging to the GntR family.[8] The environmental conditions

or compounds which could release the repressing activity of Atu4232 are still unknown. To evaluate whether a tight control of the Atu4232 regulon would confer a selective advantage in the course of the plant-tumor colonization, we performed competition experiments associating different *A. tumefaciens* C58 derivatives:[8] the Gm^R-strain C58-107 which harbors the *atu2422* and *atu4232* wild-type alleles, in which expression of Atu4243 is efficiently repressed (WT Atu2422 and WT Atu4243); the constructed *atu2422::acc1* Gm^R-mutant which is defective for Atu2422 only (KO-Atu2422 and WT Atu4243); and the *gaba+* mutant, which is an *atu2422::acc1* derivative harboring a punctual mutation in the repressor Atu4232, hence constitutively expresses the Atu4232-regulon including Atu4243 and adjacent genes (KO-Atu2422 and $Atu4243^C$). Each of the strain was able to colonize the plant tumor at a similar level. By contrast, competition assays associating *gaba*$^+$ (KO-Atu2422 and $Atu4243^C$) and the C58-107 (WT Atu2422 and WT Atu4243) or KO-*atu2422* (KO-Atu2422 and WT Atu4243) derivatives revealed fitness lost of the constitutive expression of the Atu4232-regulon in the course of plant-tumor colonization whatever the expression of the *atu2422* gene (**Fig. 3**). Fitness lost probably results from toxicity effects. Indeed, the GABA imported via the selective Atu4243-mediated system is converted to the toxic succinic semialdehyde[4,17] by a still unidentified GABA-transaminase in *A. tumefaciens* cells.[6] However, we could not exclude other causes as the Atu4232-regulon encompasses 12 additional genes of which the function is still unknown.[8] These competition assays highlight the importance of a tight regulation of *atu4232*-regulon in the course of the *A. tumefaciens*-plant host interaction. However, when the Atu4232 regulon is repressed, the unique pathway for GABA-uptake involves the PBP Atu2422 which is not selective for GABA, hence Atu2422-mediated GABA-uptake is moderated by natural competitors, which are abundant in the plant tumor, such as the free amino-acids Proline, Alanine, Valine.[7]

In conclusion, *A. tumefaciens* seems to mobilize two different systems for the transport of GABA that are the PBPs Atu2422 and Atu4243 and their cognate ABC-transporters. In the both systems, GABA uptake is tightly controlled at transcriptional, post-transcriptional, and/or PBP-ligand interaction levels.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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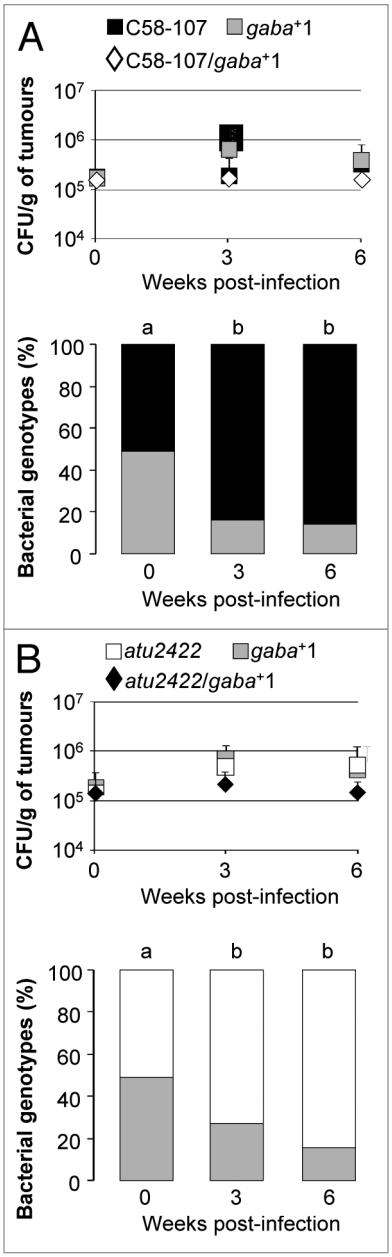

Figure 3. Competition assays in host plant. Stems of 5-week old tomato plants (*Solanum lycopersicum* L. cv Dona) were infected by approximately 10^5 CFU of monoclonal (C58-107, *gaba*$^+$1 and *atu2422*) and mixed (1:1 ratio) populations (C58-107/*gaba*$^+$1 and *atu2422*/*gaba*$^+$1) of the *A. tumefaciens* derivatives. At infection time (t = 0), and 3 and 6 weeks later, populations were numerated (CFU/g of tumor fresh weight) and identified using appropriate primers targeting the mutated loci. All the strains harbor the same *acc1* gene encoding for gentamycin resistance. Graphs at the top of the panels (**A and B**) indicate level of the monoclonal and mixed populations in the plant tumor, while lower bars represent the relative abundance (%) of individuals in mixed populations only. The experiment was done in two independent replicates (each with 10 plants). Statistical differences using the Kruskal-Wallis one-way ANOVA ($p < 0.01$) are noted by different letters.

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