Import pathways of the mannityl-opines into the bacterial pathogen Agrobacterium tumefaciens: structural, affinity and in vivo approaches

Armelle Vigouroux1#, Jeanne Dore2#, Loïc Marty1, Magali Aumont-Nicaise1, Pierre Legrand3, Yves Dessaux1, Ludovic Vial2 & Solange Moréra1*

1Université Paris-Saclay, CEA, CNRS, Institute for Integrative Biology of the Cell (I2BC), 91198, Gif-sur-Yvette, France

2Ecologie Microbienne, CNRS, UMR 5557, INRA, UMR 1418, Université Lyon 1, Université de Lyon, F-69622, Villeurbanne, Lyon, France

3Synchrotron SOLEIL, L’Orme des Merisiers, Saint Aubin BP48, Gif-sur-Yvette 91192, France

Short title: Mannopinic acid bound to the SBPs MoaA and MotA

*Equal contribution

*Corresponding author: solange.morera@i2bc.paris-saclay.fr

Abstract

Agrobacterium tumefaciens pathogens use specific compounds denoted opines as nutrients in their plant tumor niche. These opines are produced by the host plant cells genetically modified by agrobacteria. They are imported into bacteria via solute-binding proteins (SBPs) in association with ABC transporters. The mannityl-opine family encompasses mannopine, mannopinic acid, agropine and agropinic acid. Structural and affinity data on mannopinic acid bound to SBPs are currently lacking while those of the three others mannityl opines are available. We investigated the molecular basis of two pathways for mannopinic acid uptake. MoaA was proposed as the specific SBP for mannopinic acid import in mannityl opines-assimilating agrobacteria, which was validated here using genetic studies and affinity measurements. We structurally characterized the mannopinic acid binding mode of MoaA in two crystal forms at 2.05 and 1.57 Å resolution. We demonstrated that the non-specific SBP MotA, so far characterized as mannopine and Amadori compound importer, was also able to transport mannopinic acid. The structure of MotA bound to mannopinic acid at 2.2 Å resolution defines a different mannopinic acid binding signature, similar to that of mannopine. Combining in vitro and in vivo approaches, this work allowed us to complete the characterization of the mannityl-opines assimilation pathways, highlighting the important role of two dual imports of agropinic and mannopinic acids. Our data shed new light on how the mannityl-opines contribute to the establishment of the ecological niche of agrobacteria from the early to the late stages of tumor development.

Keywords mannopinic acid, mannityl-opine, periplasmic binding protein, solute binding protein, Agrobacterium
Introduction

Pathogenic agrobacteria harbor a Tumor-inducing (Ti) plasmid [1, 2], and transfer a portion of this, namely the T-DNA, to the plant cells upon infection [3, 4]. In a wide range of mostly dicotyledoneous plants, this allows the creation of an ecological niche (called tumor niche or opine niche) for the agrobacteria, characterized by the formation of a tumor [1]. The expression of T-DNA genes in plants drives the synthesis of (i), plant hormones responsible for the tumor formation (crown-gall disease); and (ii) novel organic compounds called opines. Opines are either sugar-phosphodiesters or the products of condensed amino acids with ketoacids or sugars [2, 5, 6]. They are specifically utilized as nutrients by the inciting agrobacteria and confer upon these pathogens an advantage when in competition with other members of the soil microflora for the colonization of opine-rich environments [7-12]. Some opines, designated conjugative opines, such as octopine or agrocinopine (actually only the arabinose-2-phosphate moiety of the agrocinopine is recognized) [13], stimulate the horizontal transfer (conjugative transfer) of the Ti-plasmid from pathogenic Agrobacterium to non-pathogenic Agrobacterium [6].

Over 20 opines have been identified in Agrobacterium-induced plant tumors. They fall into distinct families according to their precursors, which are sugars, amino acids and ketoacids. They are not all present at the same time in a tumor, and opine contents as well as opine degradation properties are specific for a given Ti-plasmid. For example, A. tumefaciens strains B6 and R10 possess Ti plasmid genes that code for enzymes responsible for the synthesis of the mannityl-opines, a group composed of four compounds containing mannose conjugated with either glutamate or glutamine. Mannopine (1-deoxy-mannosyl-glutamine) and mannopinic acid (1-deoxy-mannosyl-glutamate) are unmodified conjugates while agropine (1-deoxy-mannitosyl-glutamine,1',2'-lactone) and agroinic acid (1-deoxy-mannosyl-glutamate lactam) are cyclized derivatives of mannopine [14] (Figure 1). Agropine is formed from mannozinopine [15, 16], while mannozinopine and mannopinic acid are formed from a precursor from the chrysopine opine family, named 1-deoxy-fructosyl-glutamine (DFG) and 1-deoxy-fructosyl-glutamate (DFGA), respectively [17]. DFG and DFGA are known as Amadori compounds (a name used for any derivative of aminodeoxy sugars) present in decaying plant materials. They differ only in their amino-acid moiety (glutamine versus glutamate). Notably, mannozinopine and agropine can quickly degrade into agroinic acid (mannityl-glutamic acid lactam) through a spontaneous lactamization whereas mannopinic acid does it only slowly [6, 14].

Opine catabolic genes (import and degradation) are located in segments outside the T-DNA region on the Ti-plasmid. They are generally clustered in operons and regulons, and their expression is inducible by the opine itself or a catabolized/degraded form of it [6, 13, 18-20]. Two sets of genes are present in the catabolic region. The first one encodes the transport system that often consists in an ATP-binding cassette (ABC) transporter and its cognate periplasmic-binding protein, commonly named solute-binding protein (SBP). The second encodes the enzymes involved in the degradation of the opines to molecules that belong to central bacterial metabolism. To date, we have characterized three mannityl-opine binding-SBPs: MotA (mannopine opine import), AgtB (agropine and agroinic acid opines import) and AgaA (agroinic acid opine import) (Figure 1) [21, 22]. Only one mannityl-opine compound i.e. mannozic acid, remained structurally unstudied with SBPs so far.

To finalize our work on mannityl-opine import into pathogenic agrobacteria, we investigated the transport system of mannozic acid and the structural and biochemical properties of the SBP MoaA, which was predicted as a mannozic acid binding protein. This prediction results from genetic investigations (mapping of gene regions) and opine uptake/transport experiments [23-27]. We first focused on the genetic and molecular role of the SBP MoaA through an integrative approach using a defective mutant in vivo, crystallography and affinity measurements. We showed that MoaA very
efficiently binds mannopinic acid in *A. tumefaciens* R10. Nonetheless, MoaA was not the sole transporter of this opine as shown by data obtained using the defective non-polar mutant R10ΔmoaA. Using genetic approaches through single and double non-polar defective mutants (R10ΔmotA and R10ΔmoaAΔmotA) and affinity measurements, we demonstrated that the non-specific SBP MotA, so far characterized as mannopine and Amadori compound importer, was also capable to transport mannopinic acid. We also performed the *in vivo* study of agropinic acid transport using single and double non-polar defective mutants (R10ΔagtB, R10ΔagaA and R10ΔagtBΔagaA).

We structurally characterized the mannopinic acid binding mode of both MoaA and MotA and identified two mannopinic acid binding signatures (a selective and a non-selective). We also used the synthetic opine analogue glucopinic acid, which constitutes the sugar part-C2 epimer of mannopinic acid to probe ligand specificity. Overall, our data on import and specificity on mannityl-opine family are now complete and provide new insights into their utilization in host-interacting agrobacteria.

**Experimental**

**Synthesis and purification of opines.** The used agropine and agropinic acid were synthesized from mannotripe as previously described [14, 22]. The used mannopine was the same as in Marty *et al* [21]. Mannopinic acid and the synthetic glucopinic acid were laboratory products also checked by mass spectrometry.

**Construction of the single and double non-polar defective mutants in *A. tumefaciens* R10.** The octopine-mannityl-type *A. tumefaciens* R10 strain was from our laboratory collection. The octopine-mannityl-type R10ΔmoaA, R10ΔmotA and R10ΔagaA, single defective mutants were constructed according to a strategy previously described for R10ΔagtB mutant [22, 28]. Briefly, approximately 1000 pb of the recombinant region containing the upstream and downstream region flanking the *moaA* or *motA* or *agaA* gene were generated by PCR using primers listed in Table S1. These fragments were designed to delete only the coding sequence of each target gene without affected neighboring genes. Each recombinant fragment was inserted into a *Apal* and *SpeI* digested suicide vector pJQ200sk [29], by infusion technology (Takara clonetech, Kyoto, Japan). The resulting plasmid (pJQ200sk-ΔmoaA, pJQ200sk-ΔmotA or pJQ200sk-ΔagaA) was introduced into *A. tumefaciens* R10 by electroporation. The colonies with plasmid integration were selected on YPG (yeast extract, 5 g per liter, peptone, 5 g per liter, glucose, 10 g per liter, and pH adjusted to 7.2) medium plates supplemented with gentamicin (20 μg/mL) and verified by PCR with primers UpF-Dwr. Double-crossover events were searched by cultivating overnight the gentamicine resistant colonies into YPG medium without antibiotic before plating on YPG plates containing 5% of sucrose. Gene deletion in the sucrose resistant colonies were verified by PCR (with primers UpF-Dwr, Table S1). The double non-polar R10ΔmoaAΔmotA and R10ΔagaAΔagtB mutants were obtained following the same procedure, pJQ200sk-ΔmoaA or pJQ200sk-ΔagaA plasmid was introduced into *A. tumefaciens* R10ΔmotA and R10ΔagtB.

**Bacterial culture conditions.** The octopine-mannityl-type *A. tumefaciens* R10 and all defective mutant strains were cultivated overnight at 28°C in AB medium supplemented with mannitol (2 g/l) instead of glucose. After washing the pellet with 0.8% NaCl, strains were inoculated in AB minimal medium supplemented with 2 mM of carbon sources (mannopine, mannotripe acid, agropinic acid and agropine) or in absence of any carbon source, in 24-well sterile plates incubated at 28°C during 50 hours. Cells growth was measured every 20 minutes by optical density at 600 nm using Tecan Infinite M1000 (Tecan, Männedorf, Switzerland). Analyses were performed in four technical replicates and in two biological replicates.
Cloning, expression and purification of mature MoaA and MotA. The synthetic gene (Genscript, Piscataway, NJ) coding for the mature MoaA was inserted into pET-29b. E. coli BL21 pLysS were transformed by pET-29b-moaA. Cells were grown at 37°C in LB broth then at 16°C supplemented with 0.1 mM isopropyl β-D-1-thiogalactopyranoside to induce protein production during 4 h. Cells were centrifuged, resuspended in a buffer of 50 mM Tris-HCl, pH 8.0, 20 mM imidazole and 300 mM NaCl, and disrupted by sonication. After centrifugation at 25,000 g for 30 min at 4°C, the supernatant was loaded onto a 5 mL His-Trap column (GE Healthcare). Protein elution was performed with 50 mM Tris-HCl pH 8.0, 300 mM imidazole and 300 mM NaCl. Protein fractions were loaded onto a gel filtration column (HiLoad 26/60 Superdex 200 prep grade, GE Healthcare) equilibrated with 50 mM Tris-HCl pH 8.0 and 150 mM NaCl. The protein fractions were pooled, concentrated at 14 mg/ml and stored at -80°C. MotA was expressed and purified as previously described [21].

Expression and purification of mature seleniated MoaA. The E. coli BL21 cell transformed with the plasmid pET-29b-moaA were grown overnight at 28°C in M9 media supplemented with 0.4% glucose, 2 mM MgSO4, 1 mM CaCl2, 100 mg/L of lysine, threonine, and phenylalanine, 50 mg/L of leucine, valine, isoleucine and methionine. The pelleted cells were resuspended in fresh M9 media (same as above) with 100 mg/L of selenomethionine instead of methionine for 1 h at 37°C before inducing the expression with 0.1 mM IPTG 4h at 16°C. Cells were centrifuged at 4000 g for 15 min at 4°C. The purification protocol was the same as described above for the wild-type protein.

Crystallization and structure determination. MotA was co-crystallized with mannopinic acid and glucopinic acid instead of mannopine or glucopine as previously described [21]. Crystallization conditions (Table 1) for liganded seleniated MoaA (molar ratio of protein:ligand used was 1:5) were screened using QIAGEN kits (Valencia, CA) with a Mosquito nanodrop robot (TTP Labtech) and were manually optimized. Crystals were transferred to a cryo-protectant solution (mother liquor supplemented with 25% PEG 400 for PEG conditions or 30% sucrose for AS conditions) and flash-frozen in liquid nitrogen. Diffraction data were collected at 100 K on the PROXIMA 1 and 2 beamlines equipped with a PILATUS 6M and an EIGER 9M as detectors, respectively, at SOLEIL synchrotron (Saint-Aubin, France). Data processing was performed using the XDS package [30] (Table 1). The crystal structure of MoaA-mannopinic acid complex was determined by single anomalous dispersion (SAD) method from selenomethionine-labelled protein and further refined at 2.05 Å resolution using a higher resolution dataset. Solvent content analysis using CCP4 (Collaborative Computational Project, Number 4) indicated the presence of two monomers in the asymmetric unit. The positions of 4 over 5 selenium atoms per monomer were found using SHELX suite program [31]. The SAD phases were calculated using PHASER [32] and further refined by density modification performed using PARROT (CCP4 suite). An iterative process of manual building in COOT combined with phase calculation where a partial model was used as input, allowed modelling of the complete polypeptide chain. Other structure determinations of MoaA and those of MotA were performed by molecular replacement with PHASER [32]. The coordinates of MotA (PDB 5L9G and 5L9L) [21] were used as search models for MotA-mannopinic acid and MotA-agropinic acid structures, respectively. Refinement of each structure was performed with BUSTER-2.10 [33] using TLS group and NCS restraints. Inspection of the density maps and manual rebuilding were performed using COOT [34]. The three-dimensional models of mannopinic acid and glucopinic acid were generated with the ProDRG webserver [35]. Refinement details of each structure are shown in Table 1. Molecular graphic images were generated using PyMOL (http://www.pymol.org).

Fluorescence titration measurements. Each ligand bound to MoaA or MotA was monitored by autofluorescence by exciting the protein at a wavelength of 295 nm and monitoring the quenching of fluorescence emission of tryptophan at 335 nm. All experiments were performed at 22°C in 96 well
plates (1/2 Area Plate-96F, Perkin Elmer) using Tecan Infinite M1000 (Tecan, Männedorf, Switzerland) in 25 mM Tris-HCl pH 8.0 and 150 mM NaCl with a fixed amount of proteins (1 µM) and increasing concentrations of ligand. Each ligand has no emission signal at 335 nm. The data were analysed using Origin® 7 software and fitted to the equation $f=ΔFluorescence_{max} \cdot \text{abs}(x)/(K_D+\text{abs}(x))$.

**Isothermal titration microcalorimetry measurements.** Isothermal titration microcalorimetry experiments were performed with an ITC200 isothermal titration calorimeter from MicroCal (Malvern). The experiments were carried out at 20°C. Protein concentration in the microcalorimeter cell (0.2 mL) varied from 50 to 100 µM. Nineteen injections of 2 µl ligand solution (mannopine, mannopinic acid agropine and agropinic acid) at concentrations ranging from 580 to 1000 µM were performed at intervals of 180 s while stirring at 500 rpm. The experimental data were fitted to theoretical titration curves with software supplied by MicroCal (ORIGIN®). This software uses the relationship between the heat generated by each injection and $ΔH$ (enthalpy change in Kcal.Mol$^{-1}$), $K_d$ (the association binding constant in M$^{-1}$), $n$ (the number of binding sites), total protein concentration and free and total ligand concentrations [36].

**Distance tree.** Sequences were analysed using BlastP from NCBI (https://blast.ncbi.nlm.nih.gov/) and MicrosScope (https://www.genoscope.cns.fr/). Alignments of MoaA and related sequences were conducted using MUSCLE software. The distance tree was built using Mega software, version 7. Topology was inferred using the neighbor-joining method [37]. The bootstrap consensus tree inferred from 1000 replicates was generated to represent the sequence relatedness of the proteins analysed. The sequence distances were computed using the Poisson correction method and are in units of the number of amino acid substitutions per site.

**Results**

**Each SBP MoaA and MotA can import mannopinic acid and only MotA can import mannopine.** The non-polar R10ΔmoaA and R10ΔmotA mutants correspond to a mutant defective for MoaA and MotA, respectively whereas the non-polar R10ΔmoaAΔmotA to a mutant defective for the two SBPs MoaA and MotA. In each strain, one gene (either moaA or motA) is deleted but this deletion has no effect on the expression of the downstream genes, when compared to the wild type (WT) R10 strain. The growth profiles of WT, R10ΔmoaA, R10ΔmotA and R10ΔmoaAΔmotA were compared in minimal medium containing mannopinic acid (Figure 2A) or mannopine (Figure 2B) as the sole source of carbon. Under these conditions, each single deficient mutant (MoaA and MotA) and the WT strains grew on mannopinic acid. In contrast, the R10ΔmoaAΔmotA double mutant did not grow on mannopinic acid or mannopine, neither did the R10ΔmotA mutant on mannopine. Therefore, MotA associated with its ABC transporter is the sole transport system responsible for the uptake and assimilation of mannopine in pure culture while both MotA and MoaA with their respective ABC transporter can import mannopinic acid used as nutrient.

**Each SBP AgaA and AgtB can import agropinic acid and only AgtB can import agropine.** We have previously showed that AgtB associated to its ABC transporter is the sole transport system responsible for the uptake and assimilation of agropine in pure culture [22]. In this study, we constructed the non-polar R10ΔagaA and R10ΔagtBΔagaA defective mutants. The growth profiles of WT, R10ΔagtB, R10ΔagaA and R10ΔagtBΔagaA were compared in minimal medium containing agropinic acid (Figure 2C) or agropine (Figure 2D) as the sole source of carbon. Under these conditions, the R10ΔagaA mutant and WT strains grew on both agropinic acid and mannopinic acid. In contrast, the R10ΔagtBΔagaA double mutant did not grow on agropinic acid, proving that both
SBPs AgaA and AgtB were responsible for agropinic acid uptake and assimilation in pure culture in line with their affinity for agropinic acid [22].

**MoaA exhibits a high affinity for mannopinic acid and MotA can bind mannopinic acid.** Binding of mannopinic acid to the purified recombinant mature proteins MoaA and MotA was explored using tryptophan fluorescence spectroscopy (MoaA and MotA possess 8 and 11 tryptophan residues, respectively) and isothermal titration microcalorimetry. Intrinsic protein fluorescence titration experiments for MoaA and MotA yielded apparent dissociation constant $K_D$ values of $2.1 \pm 0.2 \text{ nM}$ and $3.9 \pm 0.2 \mu\text{M}$ respectively (Figure 3). Therefore, MoaA is highly efficient for mannopinic acid binding. No interaction could be measured between MoaA and mannopine. This result is in agreement with the results of the growth assays showing that MotA is the sole SBP for mannopine uptake. MotA displays an apparent dissociation constant $K_D$ value of $4 \pm 0.5 \text{ nM}$ for mannopine [21]. As expected, no interaction could be measured between MoaA or MotA and agropinic acid or agropine. No interaction could be either measured between MotA and glutamate.

Both MoaA and MotA can bind the synthetic glucopinic acid with lower affinities than those for mannopinic acid (Figure 3). Using isothermal titration microcalorimetry, similar $K_D$ values were obtained (Figure 3). The microcalorimetry data confirmed the 1:1 binding stoichiometry for both MoaA and MotA and revealed a negative enthalpy change for the ligand, meaning that the binding was enthalpy-driven, involving polar interactions mainly.

**Ligand binding site of MoaA, a SBP from cluster C.** The expressed mature SBP MoaA lacks the first 29 signal sequence residues that serve for the localization to the bacterial periplasm and contains a C-terminal sequence His-tag. The X-ray structure of the seleniated liganded MoaA was determined at 2.5 Å resolution in $P3_212$ space group by SAD method and further refined at 2.05 Å resolution using a dataset from a different crystal grown in ammonium sulphate conditions (Table 1). MoaA with glucopinic acid crystallized in the same condition and the complexed structure was determined at 2 Å resolution. The MoaA-mannopinic acid complex also crystallized in PEG conditions leading to $P2_1$ space group crystals diffracting up to 1.57 Å resolution. Both crystal forms contain two very similar MoaA structures within the asymmetric unit, as indicated by the average root mean square deviation (root mean square deviation (RMSD) of 0.4 Å for all Ca atoms). The mature monomeric MoaA of 494 amino acids possesses a typical fold of cluster C within the SBP structural classification [38] (Figure 4A). The N-terminal lobe consists of residues (30-273 and 492-523) and the C-terminal lobe comprises the residues 279-488. Two short segments define the hinge region connecting the two lobes. SSM-EBI (http://www.ebi.ac.uk/msd-srv/ssm) reports RMSD between MoaA and similar SBPs range from 2 to 2.2 Å over 448-463 Ca atoms corresponding to 19-24% sequence identity. Thus, a detailed structural comparison is irrelevant because MoaA presents a distinct ligand-binding site.

The mannopinic acid bound between the two lobes of MoaA is well defined in the electron density maps. It is surrounded by Thr54, Arg57, Gln254, Glu277, Asn279, Arg312, Gly369, His395, His399, Tyr412, Gly413, Arg416 and Asn488 and makes numerous polar interactions with MoaA (Figure 4B). The glutamate moiety forms two salt bridges with Arg57 and Arg416 and a H-bond with the side chain of Tyr412. Its carboxylate group interacts with the side chains of His395 and His399 and the amino group of Thr54 while its amino group makes hydrogen bonds with the carboxyl group of Glu277 and Thr54 side chain. The sugar moiety interacts with seven amino acids: Gln254, Asn279, Glu277, Arg312, Gly369, Gly413 and Asn488. The synthetic analog glucopinic acid binds to MoaA very similarly than does mannopinic acid although its C2 hydroxyl in the sugar part is shifted by 1.9 Å compared to mannopinic acid (Figure 4C).

**Mannopinic acid bound to MotA.** We previously reported the structures of the mature MotA (a SBP from cluster D) in complex with mannopine, DFG and the synthetic glucopine [21]. The X-ray
structures of MotA liganded with mannopinic acid and glucopinic acid were determined at 2.2 Å and 1.85 Å resolution, respectively (Table 1, Figure 5) by molecular replacement using the coordinates of MotA-mannopine structure (PDB 5L9G) and MotA-glucopine structure (PDB 5L9L) as a search model, respectively. Both liganded crystals contained four very similar molecules in the asymmetric unit (RMSD of 0.28 Å). They adopt the same closed conformation as previously observed (RMSD between 0.35 Å and 0.42 Å for all Cα molecules). Mannopinic acid and mannopine fully overlap, as do glucopinic acid and glucopine (Figures 5B and 5C). Thus, mannopinic acid interactions with MotA are identical to those of mannopine with MotA previously described [21]. This is also the case between glucopinic acid and glucopine. The glutamate moiety of mannopinic acid interacts with the protein side chains of Ser128, Asp261 and Thr297. The carboxylic moiety of each ligand superimpose making similar protein contacts whereas their sugar moiety adopt a different conformation that induces the rearrangement of Asp92 side chain and slightly different protein interactions.

**MoaA is present in a very few bacteria only.** Searching for MoaA conservation in the bacterial kingdom (protein database at NCBI), and subsequent topology analysis revealed the existence of 10 SBPs with sequence at least 70% identical to that of MoaA from strain R10, and the occurrence of clades (Figure S1). One of these, termed the MoaA clade, contains MoaA R10 and 7 highly similar orthologues sharing over 88% sequence identity. It encompasses octopine/mannityl-opine type (R10, B6, Ach5, 15955 and TT111) and agrocinopine/mannityl-opine type (LBA4404, Bo542, CFBP1873) *A. tumefaciens* strains. These bacteria possess the mannopinic acid-binding signature of 13 amino acids: Thr54, Arg57, Gln254, Glu277, Asn279, Arg312, Gly369, His395, His399, Tyr412, Gly413, Arg416 and Asn488. Outside the MoaA-clade, more distantly related (71-72% sequence identity), MoaA orthologues were detected in other plant-associated bacteria such as *Rhizobiales*, *Bosea* and *Methyllobacterium*. In these bacteria, the signature degenerates with two mutated residues (Gln254 versus Ala and Gly369 versus Ser) and modelling indicates that the binding may be slightly affected due to the presence of the serine side chain at position 369.

**Discussion**

This work allowed us to finalize our study on the molecular and affinity characterization of the transport system of all members of the mannityl-opine family, which encompasses four opines: mannopine, mannopinic acid, agropine and agropinic acid. In contrast to all others opines from pathogenic agrobacteria such as the well-known members of octopine or nopaline families, the mannityl-opines do not result from a one-step formation in the transformed plant cells (Figure 6).
Three enzymes namely Mas2, Mas1 and Ags, encoded by genes located in the T-DNA region and integrated into the plant genome, are responsible for the production of the mannityl opines. More precisely, the Amadori compounds DFG/DFGA are synthesized from fructose and glutamine/glutamate by Mas2, mannopine/mannopinic acid from DFG/DFGA by Mas1 and agropine from mannopine by Ags [14] (Figure 6). Agropinic acid originates from the rapid spontaneous lactamization of mannopine and agropine, and also of mannopinic acid but slower [6] (Dessaux, unpublished results). Once produced in the plant cells, these opines are released and imported into pathogenic agrobacteria to be assimilated as nutrients.

Combining in vitro approaches (affinity and structural data with phylogeny analyses) and in vivo approaches with bacterial growth assays using defective mutants, we characterized mannopinic acid assimilation, provided molecular and structural data of the SBP MoaA and new insights about specificity and selectivity of the SBP MotA [21]. Together with the reported study of the two SBPs AgtB and AgaA [22], the four MoaA-, AgaA-, MotA- and AgtB-mediated mannityl-opines transport systems in pathogenic agrobacteria are now well characterized.

The SBPs MoaA and AgaA are encoded by genes belonging to the same Ti-plasmid region that is distinct from that harboring the genes coding for the SBPs MotA and AgtB (Figure 7). Previous genetic experiments suggested that MoaA-mediated transport system should import mannopinic acid into agrobacteria [23, 26, 27]. Here, we demonstrated that the SBP MoaA recognizes mannopinic acid as a unique ligand. In parallel, AgaA binds agropinic acid as the unique mannityl-opine [22]. It should be noted that MoaA and AgaA belong to the same cluster C of SBP classification, and each possesses a specific and selective molecular binding signature for its single mannityl-opine. In contrast, MotA and AgtB, which do not belong to cluster C and do not share a similar cluster fold, appear not selective for a mannityl-opine. Indeed, MotA described as binding mannopine and DFG (the precursor of mannopine) [21] is also capable of binding mannopinic acid (Figure 6) and AgtB can take up both agropine and agropinic acid. Therefore, two binding modes of mannopinic acid import and two binding modes of agropinic acid import coexist in agrobacteria. From growth assays with single and double non-polar defective mutants of MotA, AgtB, MoaA and AgaA, we proved that A. tumefaciens R10/B6 strains use these two double uptake/degradation systems of mannityl-opines. This is in agreement with previous genetic approaches suggesting the occurrence of two pathways/regions for mannopinic acid and agropinic acid catabolism in the octopine/mannityl-opines type Ti plasmid [23, 27, 39].

The above data questions the evolutionary interest of agrobacteria to have acquired and maintained two double uptake systems, characterized by two agropinic acid-binding SBPs and two mannopinic acid-binding SBPs with their own molecular signature (selective versus non-selective). The most probable value of the occurrence of two import systems for agropinic and mannopinic acid might be the gain of a trophic advantage for agrobacteria all along the plant tumor construction. Indeed, even before the appearance of the outgrowth characteristic for the tumor, mannityl-opines are detected at the bacterial inoculation site on plants, the most abundant being mannopinic acid, mannopine and agropine, whereas agropinic acid is present only in trace amounts. In this scheme, we propose that MoaA efficiently import mannopinic acid (K_D of 2.1 nM) as soon as the opine is produced. In agreement, the phylogenetic analysis of the conservation of its molecular binding signatures indicate that MoaA is present only in octopine/mannityl-opines-type or agrocinopine/mannityl-opine type Agrobacterium strains. This is almost the same observation than that made for the PBP MotA, which transports mannopine very efficiently (K_D of 4 nM) compared to its precursor DFG (K_D of 104.5 nM) and the related compound mannopinic acid (K_D of 3.9 μM) [21]. The SBP AgtB is also mostly present in pathogenic strains of Agrobacterium unlike the SBP AgaA specific for agropinic acid uptake, which
is found in a number of nonpathogenic Rhizobiaceae [22]. The ability of A. tumefaciens B6/R10 strains to import with high affinity and to degrade mannopinic acid and mannopine from the early stage of plant infection or tumor formation would allow the bacteria to settle immediately in the emerging tumor environment, gaining an immediate advantage over competing non agrobacterial strains. In the course of tumor maturation, the formation of agropine, which involves the transformation of mannopine, would also reduce the amount of available mannopine and modulate the ratio of opines over time. Therefore, this could limit the competition between agrobacteria and non-agrobacterial isolates associated with plants, some of these later being indeed naturally capable of assimilating (transport and degradation) mannopine, while the degradation of agropine is almost limited to only agrobacteria and members of closely related clades (Dessaux, unpublished results). In the case that mannopine is all transformed into agropine or is present at very low concentration, the PBP MotA would serve as a second transporter of mannopinic acid allowing a larger import of this specific nutrient. Notably, mannopinic acid is the most stable opine in time, in contrast to agopine and mannopine, which become agropinic acid. In aging tumors, the existence of the double transport systems of import of mannopinic acid and agropinic acid is likely crucial, especially since agropinic acid accumulates in these tumors [40], (Dessaux, unpublished results). Overall, our data shed new light on how the four manityl-opines contribute to the establishment of the ecological niche of agrobacteria from the early to the late stages of tumor development.

**Database depositions.** The atomic coordinates and structure factors have been deposited at the Protein Data Bank under PDB 6TFQ and 6TFX for MoaA in complex with mannopinic acid in P3\_12 and P2\_1 space groups, respectively, PDB 6TFS for MoaA with glucopinic acid and PDB 6TG2 and 6TG3 for MotA with mannopinic acid and glucopinic acid, respectively.

**Abbreviations:** SBP, solute binding protein; RMSD, root mean square deviation; PDB, protein data bank; ITC, isothermal titration microcalorimetry.

**Author contribution.** YD provided the agropine, agropinic acid, mannopine, mannopinic acid and glucopinic acid compounds and information on their metabolism and related determinants. ID and LV performed the non-polar mutants and the growth assays. AV, LM, PL and SM performed all the crystallography work. LM and AV performed the fluorescence assays. MAN performed the microcalorimetry experiments. AV and SM performed the phylogenetic analysis. SM wrote the manuscript. All the authors discussed the results and contributed to the writing of the manuscript.

**Funding.** AV, LM and SM were supported by CNRS and ANR-Blanc SENSOR (ANR-12-BSV8-0003-01/02/03). This work benefited from the I2BC crystallization and microcalorimetry platforms supported by FRISBI ANR-10-INSB-05-01.

**Acknowledgements.** We acknowledge SOLEIL for provision of synchrotron radiation facilities (proposals ID 20140774 and 20170872) in using PROXIMA beamlines.

**Competing interests.** The authors declare no competing interests associated with the manuscript.

**References**

1. Nester, E. W. (2014) Agrobacterium: nature’s genetic engineer. Front Plant Sci. 5, 730.
2. Dessaux, Y. & Faure, D. (2018) Niche Construction and Exploitation by Agrobacterium: How to Survive and Face Competition in Soil and Plant Habitats. Curr Top Microbiol Immunol.
3. Pitzschke, A. & Hirt, H. (2010) New insights into an old story: Agrobacterium-induced tumour formation in plants by plant transformation, EMBO J. 29, 1021-32.
4. Gelvin, S. B. (2003) Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool, Microbiol Mol Biol Rev. 67, 16-37, table of contents.

5. Tempé, J. & Petit, A. (1983) La piste des opines. In: Pühler A (ed), Molecular Genetics of the Bacteria–Plant Interaction Springer-Verlag: Berlin-Heidelberg, 14–32.

6. Dessaux, Y., Petit, A., Farrand, S. K. & Murphy, P. J. (1998) Opines and opine-like molecules involved in Plant-Rhizobacteria Interactions, Spink HP et al (eds) The Rhizobacteria, Molecular Biology of Model Plant-associated Bacteria Klauer Academic Publisher: Dordrecht, The Netherlands, 173–197.

7. Schell, J., Van Montagu, M., De Beuckeleer, M., De Block, M., Depicker, A., De Wilde, M., Engler, G., Genetello, C., Hernalsteens, J. P., Holsters, M., Seurinck, J., Silva, B., Van Vliet, F. & Villarroel, R. (1979) Interactions and DNA transfer between Agrobacterium tumefaciens, the Ti-plasmid and the plant host, Proc R Soc Lond B Biol Sci. 204, 251-66.

8. Wilson, M., Savka, M. A., Hwang, I., Farrand, S. K. & Lindow, S. E. (1995) Altered Epiphytic Colonization of Mannityl Opine-Producing Transgenic Tobacco Plants by a Mannityl Opine-Catabolizing Strain of Pseudomonas syringae, Appl Environ Microbiol. 61, 2151-8.

9. Savka, M. A. & Farrand, S. K. (1997) Modification of rhizobacterial populations by engineering bacterium utilization of a novel plant-produced resource, Nat Biotechnol. 15, 363-8.

10. Oger, P., Petit, A. & Dessaux, Y. (1997) Genetically engineered plants producing opines alter their biological environment, Nat Biotechnol. 15, 369-72.

11. Guyon, P., Petit, A., Tempé, J. & Dessaux, Y. (1993) Transformed plants producing opines specifically promote growth of opine-degrading agrobacteria, Molecular Plant-Microbe Interactions. 6, 92–98.

12. Lang, J., Vigouroux, A., Planamente, S., El Sahili, A., Blin, P., Aumont-Nicaise, M., Dessaux, Y., Morera, S. & Faure, D. (2014) Agrobacterium uses a unique ligand-binding mode for trapping opines and acquiring a competitive advantage in the niche construction on plant host, PLoS Pathog. 10, e1004444.

13. El Sahili, A., Li, S. Z., Lang, J., Virus, C., Planamente, S., Ahmar, M., Guimaraes, B. G., Aumont-Nicaise, M., Vigouroux, A., Soulere, L., Reader, J., Queneau, Y., Faure, D. & Morera, S. (2015) A Pyranose-2-Phosphate Motif Is Responsible for Both Antibiotic Import and Quorum-Sensing Regulation in Agrobacterium tumefaciens, PLoS Pathog. 11, e1005071.

14. Tate, M. E., Ellis, J. G., Kerr, A., Tempé, J., Murray, K. E., and Shaw, K. J. (1982) Agropine: a revised structure, Carbohydr Res. 104, 105-120.

15. Dessaux, Y., Guyon, P., Farrand, S. K., Petit, A. & Tempé, J. (1986) Agrobacterium Ti and Ri plasmids specify enzymic lactonization of mannopine to agropine, J Gen Microbiol. 132, 2549-59.

16. Hong, S. B., Hwang, I., Dessaux, Y., Guyon, P., Kim, K. S. & Farrand, S. K. (1997) A T-DNA gene required for agropine biosynthesis by transformed plants is functionally and evolutionarily related to a Ti plasmid gene required for catabolism of agropine by Agrobacterium strains, J Bacteriol. 179, 4831-40.

17. Hong, S. B. & Farrand, S. K. (1994) Functional role of the Ti plasmid-encoded catabolic mannopine cyclase in mannopine catabolism by Agrobacterium spp, J Bacteriol. 176, 3576-83.

18. Klapwijk, P. M., Oudshorn, M. & Schilperoort, R. A. (1977) Undicuble permease involved in the uptake of octopine, lypoip and octopinic acid by Agrobacterium tumefaciens strains carrying virulence-associated plasmids, J Gen Micobiol. 102, 1-11.

19. Klapwijk, P. M., Scheulderman, T. & Schilperoort, R. A. (1978) Coordinated regulation of octopine degradation and conjugative transfer of Ti plasmids in Agrobacterium tumefaciens: evidence for a common regulatory gene and separate operons, J Bacteriol. 136, 775-85.

20. Chilton, W. S. & Chilton, M. D. (1984) Mannityl opine analogs allow isolation of catabolic pathway regulatory mutants, J Bacteriol. 158, 650-8.

21. Marty, L., Vigouroux, A., Aumont-Nicaise, M., Dessaux, Y., Faure, D. & Morera, S. (2016) Structural Basis for High Specificity of Amadori Compound and Mannopine Opine Binding in Bacterial Pathogens, J Biol Chem. 291, 22638-22649.

22. Marty, L., Vigouroux, A., Aumont-Nicaise, M., Pelissier, F., Meyer, T., Lavire, C., Dessaux, Y. & Morera, S. (2019) Structural basis for two efficient modes of agropinic acid opine import into the bacterial pathogen Agrobacterium tumefaciens, Biochem J. 476, 165-178.
23. Dessaux, Y., Tempe, J. & Farrand, S. K. (1987) Genetic analysis of mannityl opine catabolism in octopine-type Agrobacterium tumefaciens strain 15955, *Mol Gen Genet.* **208**, 301-8.
24. Farrand, S. K., Tempe, J. & Dessaux, Y. (1990) Localization and characterization of the region encoding catabolism of mannopinic acid from the octopine-type Ti plasmid pTi15955, *Mol Plant Microbe Interact.* **3**, 259-67.
25. Kim, K. S., Chilton, W. S. & Farrand, S. K. (1996) A Ti plasmid-encoded enzyme required for degradation of mannopine is functionally homologous to the T-region-encoded enzyme required for synthesis of this opine in crown gall tumors, *J Bacteriol.* **178**, 3285-92.
26. Oger, P., Kim, K. S., Sackett, R. L., Piper, K. R. & Farrand, S. K. (1998) Octopine-type Ti plasmids code for a mannopine-inducible dominant-negative allele of traR, the quorum-sensing activator that regulates Ti plasmid conjugal transfer, *Mol Microbiol.* **27**, 277-88.
27. Lyi, S. M., Jafri, S. & Winans, S. C. (1999) Mannopinic acid and agropinic acid catabolism region of the octopine-type Ti plasmid pTi15955, *Mol Microbiol.* **31**, 339-47.
28. Meyer, T., Vigouroux, A., Aumont-Nicaise, M., Comte, G., Vial, L., Lavire, C. & Morera, S. (2018) The plant defense signal galactinol is specifically used as a nutrient by the bacterial pathogen Agrobacterium fabrum, *J Biol Chem.*
29. Quandt, J. & Hynes, M. F. (1993) Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria, *Gene.* **127**, 15-21.
30. Kablsch, W. (2010) Xds, *Acta Crystallogr D Biol Crystallogr.* **66**, 125-32.
31. Sheldrick, G. M. (2008) A short history of SHELX, *Acta Crystallogr A.* **64**, 112-22.
32. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007) Phaser crystallographic software, *J Appl Crystallogr.* **40**, 658-674.
33. Blanc, E., Roversi, P., Vonrhein, C., Flensburg, C., Lea, S. M. & Bricogne, G. (2004) Refinement of severely incomplete structures with maximum likelihood in BUSTER-TNT, *Acta Crystallogr D Biol Crystallogr.* **60**, 2210-21.
34. Emsley, P. & Cowtan, K. (2004) Coot: model-building tools for molecular graphics, *Acta Crystallogr D Biol Crystallogr.* **60**, 2126-32.
35. Schuttelkopf, A. W. & van Aalten, D. M. (2004) PRODRG: a tool for high-throughput crystallography of protein-ligand complexes, *Acta Crystallogr D Biol Crystallogr.* **60**, 1355-63.
36. Wiseman, T., Williston, S., Brandts, J. F. & Lin, L. N. (1989) Rapid measurement of binding constants and heats of binding using a new titration calorimeter, *Anal Biochem.* **179**, 131-7.
37. Saitou, N. & Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees, *Mol Biol Evol.* **4**, 406-25.
38. Berntsson, R. P., Smits, S. H., Schmitth, L., Slotboom, D. J. & Poolman, B. (2010) A structural classification of substrate-binding proteins, *FEBS Lett.* **584**, 2606-17.
39. Dessaux, Y., Guyon, P., Petit, A., Tempe, J., Demarez, M., Legrain, C., Tate, M. E. & Farrand, S. K. (1988) Opine utilization by Agrobacterium spp.: octopine-type Ti plasmids encode two pathways for mannopinic acid degradation, *J Bacteriol.* **170**, 2939-46.
40. Scott, I. M., Firmin, J. L., Butcher, D. N., Searle, L. M., Sogeke, A. K., Eagles, J., March, J. F., Self, R. & Fenwick, G. R. (1979) Analysis of a range of crown gall and normal plant tissues for Ti plasmid-determined compounds, *Molec gen genet.* **176**, 57-65.
41. Karplus, P. A. & Diederichs, K. (2012) Linking crystallographic model and data quality, *Science.* **336**, 1030-3.
42. Chen, V. B., Arendall, W. B., 3rd, Head, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S. & Richardson, D. C. (2010) MolProbity: all-atom structure validation for macromolecular crystallography, *Acta Crystallogr D Biol Crystallogr.* **66**, 12-21.
Figure legends

**Figure 1.** Transport of mannopine, agropine (both grey and green), agropinic acid (dark orange and green) and mannopinic acid (blue and green) from transformed plant cells to *A. tumefaciens* octopine/mannityl-type R10/B6 strain. In plant tumor cells, all the genes allowing the synthesis of mannopine, mannopinic acid and agropine are located on the T-DNA incorporated into the plant genome. Agropinic acid results from the spontaneous lactamization of the three other mannityl opines: agropine, mannopine and mannopinic acid. In *A. tumefaciens* R10/B6, the (*motA*-D) genes located on the Ti plasmid, shown in pink code for DFG and mannopine import [21]. The (*agtABC*) and (*agaA*-D) genes coding for agropine/agaropinic acid and agropinic acid import [22], respectively, are shown in blue and magenta. The (*moaA*-D) genes located on the Ti plasmid are proposed as encoding mannopinic acid import in cyan.

**Figure 2.** Mannopinic acid (A), mannopine (B), agropinic acid (C), and agropine (D) uptake in wild-type and single/double mutants of *A. tumefaciens* R10 strains. 3000 minutes growth (OD at 600 nm) of *A. tumefaciens* R10 wild-type strain (grey) and the R10ΔmoaA (cyan), R10ΔmotA (pink), R10ΔmoaAΔmotA (orange), R10ΔagaA (magenta), R10ΔagitB (blue) and R10ΔagitBΔagaA (brown) mutants in AB minimal medium supplemented with each mannityl-opine as a carbon source.

**Figure 3.** ITC and fluorescence $K_D$ measurements of MoaA and MotA towards mannopinic acid and mannopine, and fluorescence $K_D$ measurements of MoaA and MotA towards glucopinic acid. Protein concentration was at 1 μM for fluorescence experiments. The top graphs show fluorescence monitoring of each protein upon titration with each ligand and fit (solid line) to a single binding model using Origin 7. Measures were done in triplicate. The lower graphs correspond to microcalorimetry experiments. The top panel shows heat differences upon injection of ligand and low panel shows integrated heats of injection with the best fit (solid line) to a single binding model using Microcal ORIGIN. ITC experiments were performed twice. Calculated parameters for each experiment are indicated in a table and the previous data for MotA with mannopine are indicated [21].

**Figure 4.** Ribbon representation of MoaA structure and its ligand-binding site. (A) Mannopinic acid in green is located in the cleft of MoaA between the lobes 1 and 2 shown in cyan and in blue, respectively, and the hinge region is in red. (B) Mannopinic acid (green) and (C) glucopinic acid (orange) bound to the binding site of MoaA in the same code colour as in (A). Each ligand is shown in its annealing Fo-Fc omit map contoured at 4σ. Hydrogen bonds between MoaA and each ligand are shown as dashed lines in black (distances are up to 3.2 Å).

**Figure 5.** Ribbon representation of MotA structure and its ligand-binding site. (A) Mannopinic acid in green is located in the cleft of MotA between the lobes 1 and 2 shown in purple and in pink, respectively, and the hinge region is in red. (B) Superposition of mannopinic acid in green and mannopine in grey (PDB: 5L9G) in the binding site of MotA. (C) Superposition of glucopinic acid in orange and glucopine in yellow (PDB: 5L9L) in the binding site of MotA. (D) Mannopinic acid in green and (E) glucopinic acid in orange bound to the binding site of MotA in the same code color as in (A), and their annealing Fo-Fc omit map contoured at 4σ. Hydrogens bonds between MotA and each ligand are shown as dashed lines in black (distance below 3.2 Å).

**Figure 6.** Metabolism of mannityl-opines in transformed plant cells and in *A. tumefaciens* R10/B6 strains. In modified plant cell, the *mas1* and *mas2* genes responsible for the biosynthesis of mannopinic acid and mannopine are located on the T-DNA as well as *ags* gene product, which catalyzes the lactonization of mannopine to agropine. Agropinic acid results from a spontaneous lactamization of the three mannityl-opines: agropine, mannopine and mannopinic acid. In *A.
*tumefaciens* B6/R10 strain, two transport pathways for agropinic acid and mannopinic acid co-exist. The *(moaACDB)* genes (cyan) and the *(motABDC)* genes (pink) located on the Ti plasmid code for the selective and non-selective transport of mannopinic acid, respectively. The *(agaACDB)* genes (magenta) and *(agtABC)* genes (blue) code for the selective and non-selective transport of agropinic acid, respectively. The structure of the four SBPs MoaA-, AgaA-, AgtB- and MotA-mediated transport bound to their preferred mannityl-opine, which are mannopinic acid, agropinic acid, agropine and mannopine, respectively are shown. Affinity values are indicated. The *(agaE)*, *(agaFG)*, *(agcA)*, *(mocC)* and *(mocDE)* genes products are involved in mannityl-opines degradation. The *(moaR)* and *(mocR)* genes code for a corresponding transcriptional regulator of each genes clusters.
Table 1. Crystallographic data and refinement parameters

|                  | MoaA  | SeMet | MoaA  | MoaA  | MotA  | MotA  |
|------------------|-------|-------|-------|-------|-------|-------|
| PDB code        | SAD phasing | 6TFQ  | 6TFX  | 6TF5  | 6TG2  | 6TG3  |
| Wavelength (Å)  | 0.978 | 1     | 1     | 1     | 0.978 | 0.978 |
| Crystallization conditions | 2M AS, 100 mM Tris-HCl pH 8 | 2M AS, 100 mM Tris-MES pH 6.5, 200 mM MgCl₂ | 2M AS, 100 mM Tris-HCl pH 8 | 2M AS, 100 mM Tris-HCl pH 8, 250 mM MgCl₂ | 25% PEG 4000, 250 mM Tris pH 8.5, 200 mM CaCl₂ |
| Number of molecules in asymmetric unit | 2 | 2 | 2 | 2 | 4 | 4 |
| Opine           | Mannopinic acid | Mannopinic acid | Mannopinic acid | Glucopinic acid | Mannopinic acid | Glucopinic acid |
| Space group     | P3212 | P3212 | P21   | P3212 | P21   | P21   |
| Cell parameters (Å, °) | a = 155.8 b = 155.8 c = 182.1 β = 120 | a = 156.0 b = 156.0 c = 181.7 β = 107.46 | a = 156.6 b = 156.6 c = 183.0 β = 120 | a = 156.6 b = 156.6 c = 183.0 β = 120 | a = 67.9 b = 132.1 c = 92.1 β = 91.7 |
| Resolution (Å)  | 50-2.5 (2.65-2.5) | 50-2.05 (2.17-2.05) | 50-1.57 (1.66-1.57) | 50-2 (2.12-2) | 50-2.2 (2.34-2.2) | 50-1.85 (1.96-1.85) |
| No. of observed reflections | 1833447 (293177) | 2267911 (371179) | 894940 (140376) | 2987025 (453519) | 182317 (28269) | 106626 (16773) |
| No. of unique reflections | 87534 (13939) | 157907 (25177) | 134434 (21895) | 171659 (26916) | 60300 (9388) | 106626 (16773) |
| Rsym (%)        | 15.1 (84.9) | 10.6 (161.3) | 9.4 (94.7) | 14 (247) | 13.5 (90.7) | 8.4 (114.3) |
| Rpim (%)        | 3.4 (18.7) | 2.9 (42.6) | 4 (40.2) | 3.5 (60.7) | 9.3 (61.9) | 3.4 (46.2) |
| Completeness (%) | 99.8 (98.9) | 99.9 (99) | 99.5 (97.6) | 99.5 (96.9) | 97.8 (94.4) | 99.5 (96.9) |
| R/σ             | 17.36 (3.1) | 15.01 (1.27) | 11.8 (1.7) | 13.61 (0.88) | 6.31 (1.27) | 12.42 (1.42) |
| CC1/2           | 99.8 (88.8) | 99.9 (54.6) | 99.8 (64.6) | 99.9 (51.8) | 99 (51) | 99.9 (69.9) |
| Rcryst (%)      | 18.5 | 16.3 | 18.3 | 18.6 | 17.8 |
| Rfree (%)       | 19.9 | 18.2 | 19.4 | 21.9 | 19.8 |
| rms bond deviation (Å) | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| rms angle deviation (%) | 1.11 | 1.07 | 1.06 | 1.06 | 1.1 |
| Average B (Å²)  | Protein A/B/C/D Ligand solvent | 50.6/56 42.3/44.4 58.6 | 23.3/24.1 18.9/18.8 52.1 | 47.4/52.3 40/41.8 57.1 | 41.6/42.9/44.7/43 32/35.1/32.7/31.3 41.3 | 42.7/43.4/48.1/48.2 36/36/35/36 41.1 |
| Clashscored MolProbity scored | 1.54 | 1.31 | 1.34 | 0.86 | 1.09 | 1.18 |
| Ramachandran plot (%)   | Favoured Outliers | 97.25 98 97.15 97.99 | 0 0 | 98.92 |

Values for the highest resolution shell are in parentheses.
CC$_{1/2}$ = percentage of correlation between intensities from random half-dataset [41].

$^d$ Calculated with MolProbity [42].
| MoaA-glucopinic acid | MoaA-mannopinic acid |
|----------------------|----------------------|
| 4 ± 0.5              | 7.1 ± 0.2            |
| 0.99                 | 0.97                 |
| 198 ± 3              | 215 ± 3              |
| 0.84                 | 0.86                 |
| -2660 ± 479          | -266 ± 266           |
| -1037                | -6282 ± 2767         |
| 5857.89              | 7797.79              |
| -1037                | -592 ± 16            |

**Fluorescence**

| Kd (nM) | R² |
|---------|----|
| 2.1 ± 0.2 | 0.99 |
| 390 ± 194 | 0.98 |
| 420 ± 2150 | 0.93 |

**ITC**

| ΔH (cal/mol) | ΔS (cal/mol/K) | ΔG (cal/mol) |
|--------------|---------------|--------------|
| -1872 ± 316  | -13.3         | -1995 ± 13   |
| -3688.89     | -7797.79      | -5925.89     |
| -1872 ± 316  | -13.3         | -1995 ± 13   |
| -3688.89     | -7797.79      | -5925.89     |
Supplementary material

Import pathways of the mannityl-opines into the bacterial pathogen Agrobacterium tumefaciens: structural, affinity and in vivo approaches
Armelle Vigouroux1#, Jeanne Dore2#, Loïc Marty1, Magali Aumont-Nicaise1, Pierre Legrand3, Yves Dessaux1, Ludovic Vial2 & Solange Moréra1*

1Université Paris-Saclay, CEA, CNRS, Institute for Integrative Biology of the Cell (I2BC), 91198, Gif-sur-Yvette, France
2Ecologie Microbienne, CNRS, UMR 5557, INRA, UMR 1418, Université Lyon 1, Université de Lyon, F-69622, Villeurbanne, Lyon, France
3Synchrotron SOLEIL, L’Orme des Merisiers, Saint Aubin BP48, Gif-sur-Yvette 91192, France

Short title: Mannopinic acid bound to the SBPs MoaA and MotA

*Equal contribution

*Corresponding author: solange.morera@i2bc.paris-saclay.fr

Supplementary Material, Supplementary Figure S1 and Table S1.
**Figure S1.** Distance tree and MoaA binding signature. For each protein clade, the residues, which are identical to (black) and different from (red) those involved in the mannopinic acid binding of *A. tumefaciens* R10/B6 MoaA are indicated (glutamate part in blue box and sugar part in green box). The sequence distances were computed using the Poisson correction method and are in units of the number of amino acid substitutions per site.
| Target                   | Primer name | Primer sequence (5'→3')                                      |
|-------------------------|-------------|---------------------------------------------------------------|
| *agaA* upstream region  | **UpagaAF** | ATAGGGCGAATTGGGTACCGTCCAGGGAAGTATCCTGACGATCG                 |
|                         | **UpagaAR** | GTCAGGAAGCCATGATGTTCCTTGCCTTGCTGC                           |
| *agaB* downstream region| **DwagaAF** | GAACATCATGGCTTCTGACTTCCAGTCTGGG                              |
|                         | **DwagaAR** | GGCGGCCGCTCTAGAATAGTCCAGGACTGAGATGGACAGGC                   |
| *motA* upstream region  | **UpmotAF** | ATAGGGCGAATTGGGTACCGATGCAATCTCCAAGATCCGTATCG                |
|                         | **UpmotAR** | GATCGACCGACAAGTTTGCTTCGCGG                                  |
| *motA* downstream region| **DwmotAF** | AACGAAGTATGGGTACCGATCCCAACTTCTGG                           |
|                         | **DwmotAR** | GCGGCCGCTCTAGAATAGCCTTCCGATCAGGAAAAGC                       |
| *moaA* upstream region  | **UpmoaAF** | ATAGGGCGAATTGGGTACCGTCTGACTTCCGACCTGGG                     |
|                         | **UpmoaAR** | AGATTTCATGCGACCATCCTGCGAATTTC                             |
| *moaA* downstream region| **DwmoaAF** | GGATGGTCCACTGCAAATCTCGGACCTGGG                             |
|                         | **DwmoaAR** | GGCGGCCGCTCTAGAATAGTCTCAGAGAAGGATCCTCAAGG                 |