Niche Construction and Exploitation by *Agrobacterium*: How to Survive and Face Competition in Soil and Plant Habitats

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Abstract *Agrobacterium* populations live in different habitats (bare soil, rhizosphere, host plants), and hence face different environmental constraints. They have evolved the capacity to exploit diverse resources and to escape plant defense and competition from other microbiota. By modifying the genome of their host, *Agrobacterium* populations exhibit the remarkable ability to construct and exploit the ecological niche of the plant tumors that they incite. This niche is characterized by the accumulation of specific, low molecular weight compounds termed opines that play a critical role in *Agrobacterium*'s lifestyle. We present and discuss the functions, advantages, and costs associated with this niche construction and exploitation.

1 Introduction

Agrobacterium is known among microbiologists, geneticists, and biotechnologists as a robust and versatile tool used to introduce foreign genes into plants or fungi (for reviews, see Vain 2007; Idnurm et al. 2017). However, most members of this genus are primarily plant pathogens that induce galls on dicotyledonous plants. Formerly, the *Agrobacterium* genus encompassed various species such as *A. rubi*, *A. larrymoorei*, *A. vitis*, *and A. tumefaciens*. The latter species is now recognized as a complex of several species including *A. fabrum* to which belongs *A. fabrum* C58, whose genome was the first sequenced in *Agrobacterium* (for more on *Agrobacterium* taxonomy see, *e.g.,* Mousavi et al. 2014; Kuzmanović et al. 2015; De Lajudie and Young 2017). In this chapter, we deal with members of the *Agrobacterium* genus and related genera, irrespective of their species designation, but the most abundant literature is associated with the *A. tumefaciens* species complex, and especially with the strain C58. For consistency, we will retain the ancient name *A. tumefaciens* to designate this strain.

Collectively, agrobacteria belong to the family Rhizobiaceae of the class alpha-proteobacteria, members of which are often found in soils of various origins and appear to be among the most common inhabitants of these environments (*e.g.*, Bouzar and Moore 1987; Nüsslein and Tiedje 1998; Texeira et al. 2010; Inceoglu et al. 2011; Lundgerg et al., 2012; Bulgarelli et al. 2012). Interestingly, agrobacteria isolated from soils, including rhizospheric soils, are most often avirulent (Bouzar and Moore 1987; Burr et al. 1987), *i.e.*, they do not harbor a Ti plasmid, the key replicon that determines virulence, unless the soil has an history of crown gall disease (Bouzar et al. 1993; Krimi et al. 2002). These findings suggest that agrobacteria are soil- and rhizosphere-adapted bacteria. As expected, agrobacteria exhibit several traits to exploit soil and rhizosphere resources and to survive under competition with other micro- and macro-organisms. Aside from these adaptive traits, the acquisition of a Ti plasmid that confers pathogenicity can be considered as a process leading to the construction of a more specific and less competitive ecological niche on plant hosts. Data to support these views on niche exploitation and construction by agrobacteria in the soil and plant habitats are presented below.

2 *Agrobacterium*: A Soil-Adapted Bacterium

Depending on the soil type, agrobacteria members can be either rare or relatively abundant among cultivatable bacteria, with concentrations ranging from 10^3 to 10^7 CFU/g (Bouzar and Moore 1987; Bouzar et al. 1993; Krimi et al. 2002). Agrobacterial traits that favor the adaptation to the soil environment remain largely unidentified, as they do for most soil bacteria. However, analysis of the metabolic properties of the bacteria and recent genomic data revealed several interesting features that may allow *Agrobacterium* to colonize the highly competitive soil environment.

2.1 Exploiting Soil Resources

Agrobacteria may survive for weeks and months under oligotrophic conditions, including pure water (Iacobellis and Devay 1986). Surface waters and aerosols could therefore contribute to dissemination of *Agrobacterium* populations. Members of this genus are also resistant to osmotic stress, both by taking up osmoprotectants (Nobile and Deshusses 1986; Boncompagni et al. 1999) or by synthesizing them (Smith et al. 1990).

However, bare soils are rare. Most often they are covered by plants that decompose in fall and winter to form humic acids in which agrobacteria can survive for months (Süle 1978). Plants also release at their root system a mixture of carbon compounds known as rhizodeposits. The rhizodeposits consist mainly of root cell debris and exudates, these later originating from plant photosynthesis and metabolism (for reviews, see Hinsinger et al. 2009; Jones et al. 2009; Sánchez-Cañizares et al. 2017). In possible relation with the supply of diverse carbon sources in the rhizosphere, agrobacteria have evolved a wide metabolic capability. For instance and with some variations from one strain to another, agrobacteria can degrade a large range of oses, polyols, and sugar derivatives often from plant origin, including cellobiose, trehalose, maltitol (Marasco et al. 1995; Ampomah et al. 2013), altritol and galactitol (Wichelecki et al. 2015), xylose and glucosamine (Zhao and Binns 2014), melezibiose, raffinose, gentobiose, turanose, lyxose, tagatose, D- and L-fucose, aldonitol, D- and L-arabitol, dulcitol, inositol, sorbitol, xylitol, gluconic acid, keto-gluconic acid, arbutin, esculin, and salicin (Dessaux, unpublished). Agrobacteria can also utilize a wide range of nitrogen-containing compounds as nitrogen sources such as urea (Riley and Weaver 1977), amino-valerate, amino benzoate, ethanolamine, tryptamine (Dessaux, unpublished), and gamma amino-butyrate (Chevrot et al. 2006). In relation to these potential nutrients, agrobacteria exhibit potent urease (Dessaux et al. 1986a, b) and transaminase activities (Sukanya and Vaidyanathan 1964) and a putative nitrilase that permits the scavenging of nitrogen from the plant glycoside amygdalin (Dessaux et al. 1989) and possibly from other cyanogenic compounds. In agreement with the above

catabolites, agrobacteria also encode a large number of diverse transporters likely used to take up various potential nutrients.

2.2 *Facing and Sustaining Competition*

In the soil, agrobacteria are armed to face microbial competitors. Indeed, agrobacteria benefit from a set of potent siderophores that permit an efficient recovery of iron in iron-deprived environments. Several types of siderophores have been identified. The first of these discovered is agrobactin, a derivative of 2,3-dihydroxybenzoic acid, spermidine, and threonine (Ong et al. 1979). The second one is a hydroxamate (Penyalver et al. 2001). The third one, detected in strain C58, remains unidentified (Rondon et al. 2004) but may be specific for this strain (Baude et al. 2016). In addition, with respect to microbial competitors, agrobacteria appear to be partly resistant to antibiotics such as chloramphenicol (Tennigkeit and Matzura 1991), penicillin, erythromycin, streptomycin, and moderately to tetracycline (Khanaka et al. 1981). Aside from these traits, some agrobacteria also express a type VI secretion system (T6SS; for review, see Ryu 2015) that drives the injection of at least three effectors with enzymatic activities (DNase and putative peptidoglycan amidase) into neighboring, competing bacteria (Wu et al. 2008; Ma et al. 2014).

When *Agrobacterium* colonizes a plant habitat, it can resist adverse antimicrobial compounds such as phenolics produced by plants upon wounding or biotic stress (reviews: Kefeli et al. 2003; Bhattacharya et al. 2010; Caretto et al. 2015). Phenolics play multiple roles in plant protection. With respect to the microflora, phenolics can be potent growth inhibitors of fungi and antibacterial agents (for reviews, see Cushnie and Lamb 2005; Lattanzio et al. 2008). However, nonpathogenic *Agrobacterium* strains possess an efflux pump active on a group of phenolics, the isoflavonoids that include medicarpin and coumestrol (Palumbo et al. 1998). Other phenolics such as vanillyl alcohol, vanillin, coniferyl alcohol, coniferyl aldehyde, sinapyl alcohol, sinapinaldehyde, and syringaldehyde can also be degraded by nonpathogenic agrobacteria (Brencic et al. 2004). Recently, ferulic acid was also shown to be degraded by *Agrobacterium* strain C58 (Baude et al. 2016). In addition, pathogenic agrobacteria can detoxify other phenolics via the products of two Ti plasmid genes, *virH1* and *virH2*, located in the virulence region. The VirH1 and VirH2 proteins share sequence homology with cytochrome P450-like enzymes (Kanemoto et al. 1989), and VirH2 appears to be an O-demethylase that is active on over 15 phenolic substrates such as sinapinic acid and acetosyringone. VirH2 can also convert vanillic acid to protocatechuate, which can be further metabolized via the β-ketoadipate pathway (Brencic et al. 2004). Taken together, these data indicate that pathogenic agrobacteria are more resistant to phenolics than are nonpathogenic ones, a result confirmed by the analysis of a *virH2* mutant (Brencic et al. 2004). Remarkably, many of the above-mentioned phenolics are inducers of the virulence genes of *Agrobacterium* (Bolton et al. 1986;

Engström et al. 1987) and a few may also be chemoattractant (Parke et al. 1987), a feature that could allow agrobacteria to move upward the concentration gradient toward the wounded plant cells (for review, see Shaw 1991). The route to the plant is also traced by root exudates that are also chemoattractant for agrobacteria (Hawes and Pueppke 1987; Hawes and Smith 1989).

3 The Plant Tumor: A Niche Extension for Agrobacteria

The above data indicate that *Agrobacterium* is well-equipped to survive in the soil and the plant rhizosphere. However, these environments remain quite competitive. The ability of *Agrobacterium* to generate a plant tumor can therefore be seen as a "coup de génie" that permits these bacteria to benefit from a much more private habitat, *i.e.,* a quasi-specific niche (Fig. 1). *Agrobacterium* takes a triple ecological advantage from tumor-niche construction: (i) an increase of resources supporting its proliferation to a high population level; (ii) a decrease of plant defense response in

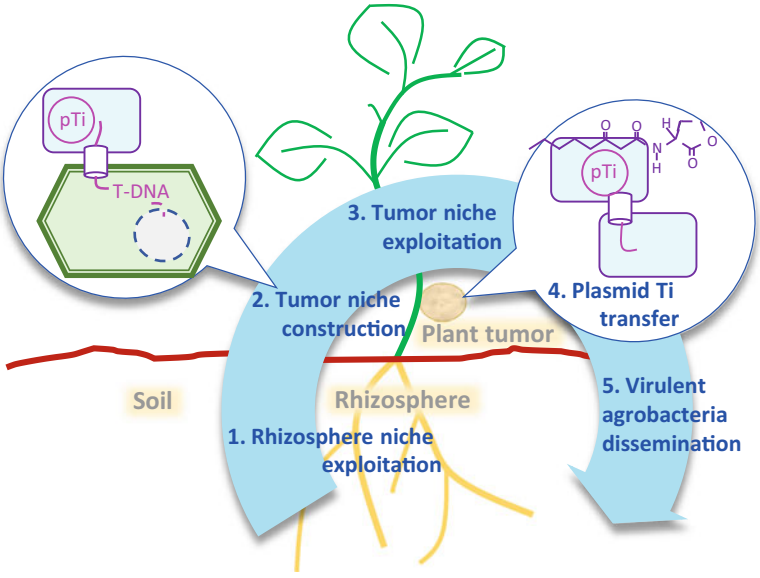
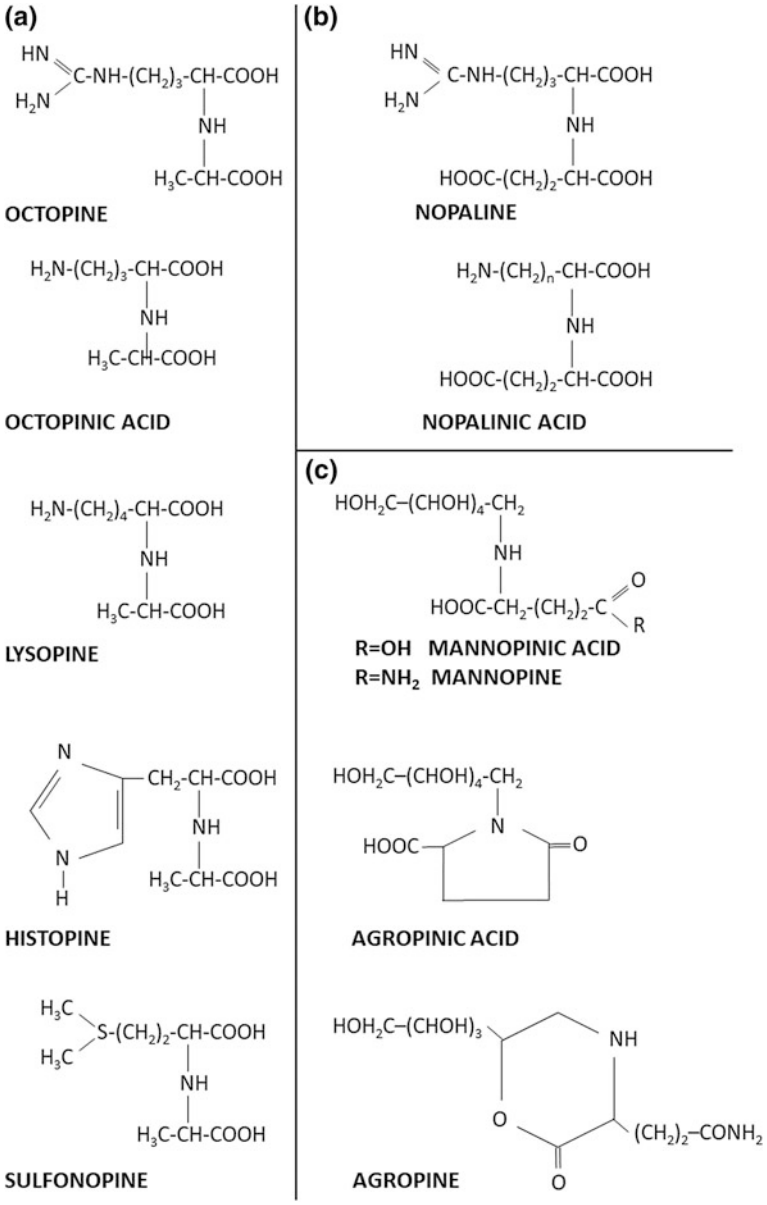


Fig. 1 Ecological niches of *Agrobacterium.* Saprophytic and pathogenic (carrying Ti plasmid) *Agrobacterium* populations efficiently colonize the rhizosphere of host and non-host plants. Upon permissive conditions (wounding), virulent agrobacteria construct a novel ecological niche that is the plant tumor, as the result of the transfer and expression of the T-DNA in plant host genome. *Agrobacterium* populations exploit the tumor resources, including opines which confer a selective advantage to *Agrobacterium* pathogens. Opines also activate quorum-sensing pathways that promote Ti plasmid conjugative transfer, hence contributing to the maintenance and propagation of the virulence genes. The high abundance of virulent *Agrobacterium* in plant tumor facilitates the dissemination to a new host as well as the maintenance of populations in the rhizosphere and soil



plant tumor tissues; and, (iii) a decrease of competition with resident microbiota, especially through the exploitation of specific growth substrates known as opines. The first point is still poorly understood but could be hypothesized from the high abundance of organic and mineral nutrients that accumulate in plant tumors (Deeken et al. 2006; Lang et al. 2016), whereas tumor development represents a

◂**Fig. 2** Structural formulas of opines **a** octopine family, **b** nopaline family, **c** agropine family, **d** agrocinopines family, **e** cucumopine family, **f** succinamopine and leucinopine families, **g** chrysopine family, **h** ridéopine and heliopine families. Octopine family opines are all synthesized by the enzyme octopine synthase and derive from various proteinous and nonproteinous amino acids, and pyruvate. They include the most recently discovered opine sulfonopine (Flores-Mireles et al. 2012). Nopaline and nopalinic acid synthesized by nopaline synthase derive from alpha-ketoglutarate and, respectively, arginine and ornithine. Succinamopine, leucinopine, cucumopine (and its diastereomer mikimopine) are also alpha-ketoglutarate condensates and exhibit asparagine, leucine, and histidine moieties, respectively. Heliopine (also termed vitopine) is a condensation product of pyruvate and glutamine. The mannityl opines are sugar and glutamate or glutamine-containing compounds as are the closely related opines of the chrysopine family. Other sugar opines include the agrocinopines A and B that are the only phosphorus-containing opines

metabolic sink from the plant host; this process makes diversified and abundant resources available to the pathogen. The second point was revealed by transcriptomic and genetic analyses of plant defense pathways (Gohlke and Deeken 2014). Tumor tissue development not only results in abnormally proliferating cells, but also causes differentiation and serves as a mechanism to balance pathogen defense, thereby contributing to the long-term coexistence of agrobacteria and the host plant. The third point, *i.e.*, the opine contribution to *Agrobacterium* lifestyle in plant tumors, is detailed below.

3.1 An Instance of Natural Genetic Engineering

Agrobacterium'*s* ability to incite a plant tumor, known as crown gall, depends upon the presence in the bacteria of a large plasmid termed the tumor-inducing (Ti) plasmid. During the infection process, a portion of this plasmid, T-DNA, is transferred via a type IV secretion system ($T4SS_{T\text{-}DNA}$) as a single-stranded DNA linked with proteins with plant nuclear localization signals. These proteins and T-DNA localize to the nucleus of the plant where T-DNA is eventually integrated into the genome and expressed. These proteins and the $T4SS_{T\text{-}DNA}$ are encoded by the non-transferred virulence (*vir*) genes also located on the Ti plasmid (for reviews and more details on the transfer machinery and genetic transformation formation process, see Pitzschke and Hirt 2010; Gelvin 2012; Lacroix and Citovsky 2013; Subramoni et al. 2014; Nester 2015; Christie 2016; Gelvin 2017). Two major sets of genes are borne on T-DNA. The first set, the oncogenes, is responsible for the synthesis of the plant hormones auxin and cytokinin by the transformed host cells, a feature that triggers their proliferation to form a tumor (Ooms et al. 1981; Akiyoshi et al. 1983; Ream et al. 1983). The second set is responsible for the synthesis, of low molecular weight compounds collectively termed opines (see Fig. 2) at the expense of the metabolite pool of the plant. Opines play key ecological roles in the *Agrobacterium*/plant interaction (for reviews, see Dessaux et al. 1998; Subramoni et al. 2014).

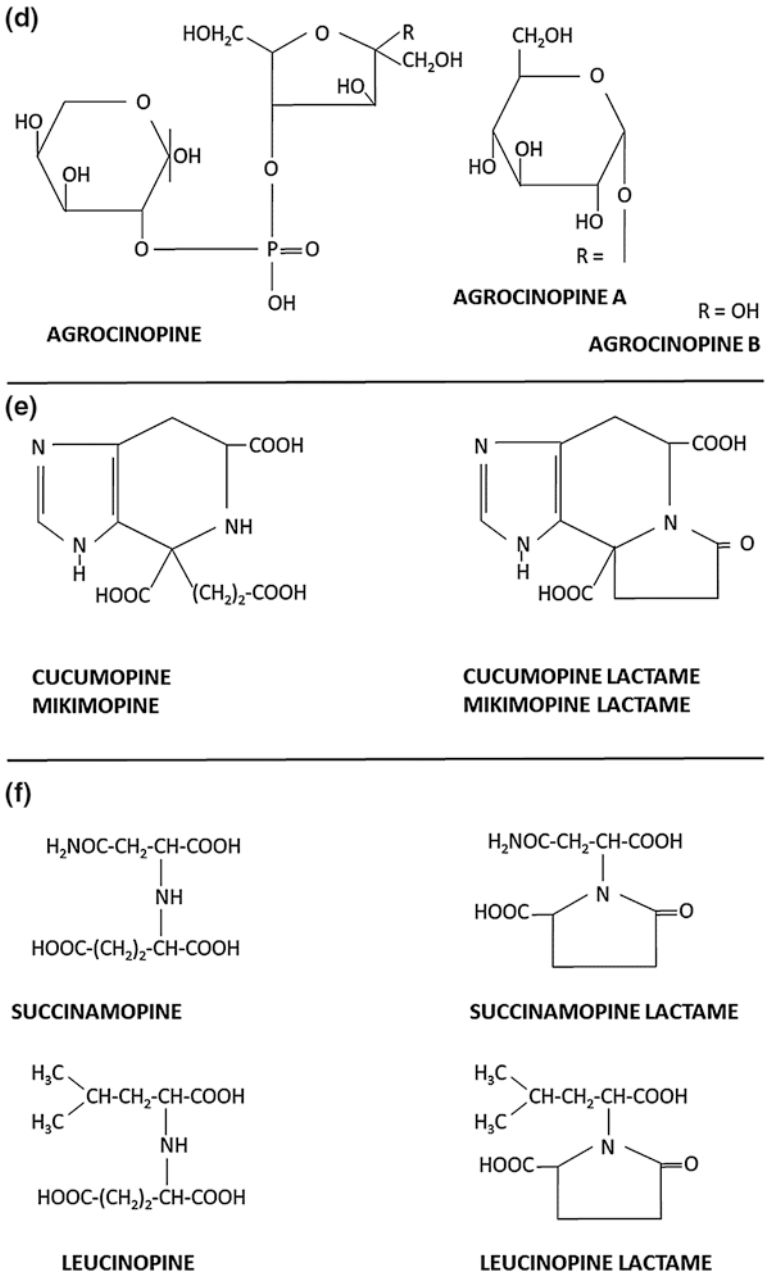


Fig. 2 (continued)

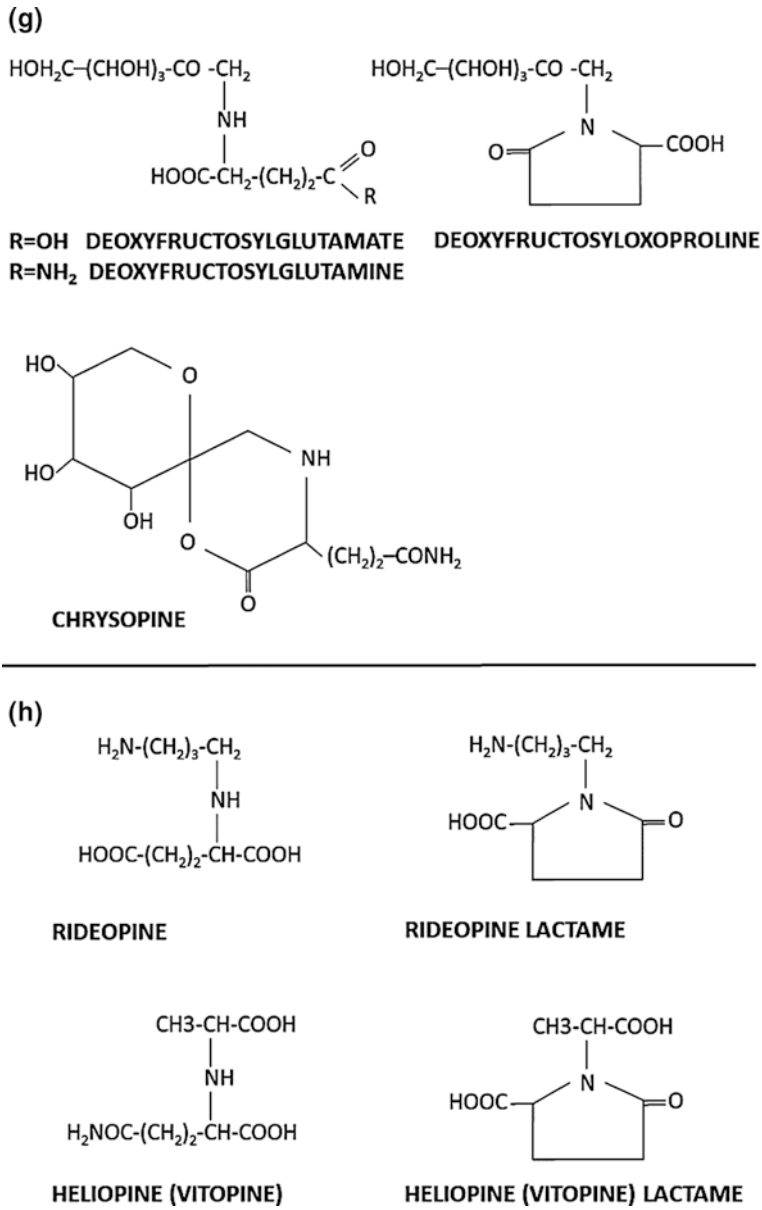


Fig. 2 (continued)

3.2 The Opine Concept

Opine synthesis by crown gall tumors and their assimilation by agrobacteria represents an archetype of ecological niche construction and exploitation processes by a pathogen. Opines are secreted by transformed plant cells into the intercellular spaces in the tumor, and to a lesser extent the whole plant (Savka and Farrand 1992; Savka et al. 1996). Opines play two major roles in niche construction for agrobacteria. First, they serve as growth substrates for the tumor-inciting strain and, second, they stimulate the conjugative transfer of the Ti plasmid from pathogenic *Agrobacterium* to other *Agrobacterium* cells (for a review, see Dessaux et al. 1998). These features are at the origin of the opine concept that describes opines as chemical mediators of parasitism. Synthesis of opines is induced by the pathogen, thus providing an environment favorable to the growth of the bacteria and dissemination of its pathogenicity (Schell et al. 1979; Tempé and Petit 1983).

The opine concept was formulated years before the discovery of plants that naturally harbor in their genomes DNA regions highly homologous to *Agrobacterium* T-DNA. Among these species are members of the genera *Nicotiana*, *Linaria,* and *Ipomoea* (White et al. 1983, Aoki et al. 1994; Suzuki et al. 2002; Matveeva et al. 2012; Kyndt et al. 2015; Quispe-Huamanquispe et al. 2017). Interestingly, some of these plants produce detectable amounts of opines (Chen and Otten 2017). The opine concept could therefore incorporate both the tumorous temporary niche and the permanent niche that naturally genetically modified plants and their offspring represent. However, no clear demonstration of a stimulation of the community of opine-degrading bacteria at the root system of these naturally transformed plants has yet been reported.

3.3 Opine Metabolism Genes

Opines are most often synthesized from common molecules such as amino acids, alpha-ketoacids, and sugars. Over 20 opine molecules are known (Fig. 2a–h). They are not all present at the same time in a tumor and some opines are specific for a given agrobacteria species. Indeed, the type opine synthesized by plant cells and degraded by agrobacteria depends upon the type of Ti plasmid, a feature that has been used to classify agrobacterial Ti plasmids (for a review, see Dessaux et al. 1998). The current list of agrobacterial opines is likely to be near complete. Indeed, over the last 15 years no novel opine has been discovered except sulfonopine, a sulfur-containing molecule detected in tumors induced by a single octopine-type *Agrobacterium* strain (Flores-Mireles et al. 2012).

Genes involved in the biosynthesis and catabolism of opines are known for several opine systems. Generally, opines derived from amino acids and alpha-ketoacids (such as octopine or nopaline; Fig. 2a, b) are synthesized in one step by a protein encoded by a single gene located on T-DNA (De Greve et al.

1982; Koncz et al. 1983). The same is true for phospho-sugar opines of the agrocinopine family (Joos et al. 1983; Fig. 2d). On the contrary, opines derived from condensation of sugars and amino acids, the mannityl opines or the chrysopine family opines (Fig. 2c, g), are synthesized in one, two, or three steps by the corresponding number of enzymes encoded by one, two, or three genes. These are most often located on a T-DNA separate from that which carries the oncogenes (Hood et al. 1986; Palanichelvam et al. 2000).

Opine catabolic genes are generally clustered in operons and regulons in delineated regions of the Ti plasmids, and their expression is inducible by the degraded opines (Klapwik et al. 1977; Klapwik et al. 1978; Chilton and Chilton 1984; Dessaux et al. 1988). Two sets of genes are present in the catabolic region. The first encodes the transport system (*e.g.,* Klapwik et al. 1977; Zanker et al. 1992) that often consists of an ABC transporter and its cognate, high affinity (nM–μM range) periplasmic-binding protein (Lang et al. 2014; El Sahili et al. 2015; Marty et al. 2016; Vigouroux et al. 2017). The second encodes the enzymes involved in the degradation of the opines to molecules that belong to central bacterial metabolism. For example, octopine and nopaline are degraded into arginine, ornithine, and glutamate, and pyruvate or alpha-ketoglutarate, respectively (Montoya et al. 1977; Ellis et al. 1979; Dessaux et al. 1986a, b). Remarkably, for some opines such as the mannityl opines, genes, and functions involved in the synthesis and degradation are closely related, suggesting that duplication events occurred in the course of the evolution of the Ti plasmids (Kim et al. 1996; Hong et al. 1997; Kim and Farrand 1996). A similar duplication also occurred with respect to genes involved in the synthesis and degradation of the phospho-sugar opines agrocinopines A and B (Kim and Farrand 1997).

3.4 Opines as Growth Substrates

The opine concept has been elaborated from the observation that all crown gall tumors, including those initially reported not to contain any opine (*i.e.,* the so-called null type tumors), indeed contain such compounds (Guyon et al. 1980). The opine hypothesis later received experimental validation. The first support for the opine concept came from comparison of the growth of two closely related *Agrobacterium* strains, one capable of degrading opines, the other not, at the root system of transformed plants producing opines. The experiment revealed that plants producing opines preferentially promote the growth of opine-degrading agrobacteria (Guyon et al. 1993). A second set of experiments involved transformed plants producing opines and two closely related *Pseudomonas* strains, one engineered—via the introduction of an opine catabolic plasmid—to degrade opines, the other not. The experiment demonstrated that the growth of the opine-degrading pseudomonad was favored at the root and leaf surface of opine-producing plants (Wilson et al. 1995; Savka and Farrand 1997). A recent experiment (Lang et al.

2014) involved the wild-type (WT) *Agrobacterium* strain C58 and a mutant unable to degrade nopaline, the major opine found in the tumors incited by this strain. When both strains were inoculated separately onto a plant, they multiplied in the tumor to reach a similar bacterial concentration. However, when co-inoculated the WT opine-degrading bacteria outcompeted the mutant. This observation formally demonstrated that the presence of the opine does not increase the carrying capacity of the tumor habitat for *Agrobacterium* but "*selects for those able to assimilate it*" (Lang et al. 2014). A similar study extended this paradigm to the octopine-niche (submitted by Vigouroux et al. 2017).

3.5 *Opines as Inducers of Ti Plasmid Horizontal Transfer*

The discovery of the Ti plasmid as key pathogenic element for *Agrobacterium* (Van Larebeke et al. 1974; Watson et al. 1975) was rapidly followed by the demonstration that these plasmids can be transferred by conjugation between bacteria; a phenomenon also regulated by opines (Kerr et al. 1977; Genetello et al. 1977). The nature of the opines that induce conjugation varied as a function of the opine-type of the plasmid. Thus, octopine induces the transfer of octopine-type plasmids, whereas agrocinopines A and B induce transfer of nopaline-type plasmids, and agrocinopines C and D the conjugation of agropine-type plasmids (Klapwijk et al. 1978; Petit et al. 1978; Ellis et al. 1982).

Ti plasmid transfer is also regulated by quorum sensing (QS; Piper et al. 1993; Zhang et al. 1993). QS is a widely occurring regulatory process that couples gene expression (in a positive or negative way) with bacterial cell concentration. It relies upon the production and sensing by a bacterial population of diffusible signal(s), the concentration of which indicates that of the microbial cells. Once a threshold concentration of signal is reached in the environment, the presence of the signal is sensed by receptors and translated into activation or repression of the expression of the genes regulated by QS (for recent reviews on QS, see Garg et al. 2014; Grandclément et al. 2016; Papenfort and Bassler 2016).

In the reference *Agrobacterium* strain C58, the presence of agrocinopines A and B triggers the expression of the *acc* operon of the Ti plasmid that encodes agrocinopine degradation, and that of the adjacent *arc* operon by releasing the repression exerted by the master regulator AccR (Beck von Bodman et al. 1992). Agrocinopine A can be cleaved into arabinose-2-P and sucrose by AccF, because only arabinose-2-phosphate (and not agrocinopine A) interacts with AccR (El Sahili et al. 2015). One of the genes of the *arc* operon is *traR*. It encodes the regulatory protein TraR that, once bound to the QS signal, dimerizes and activates the transcription of the *traAFB*, *traCDG*, and *trb* operons (Piper et al. 1999). The *tra* operons encode components of the DNA transfer and replication (DTR) system that recognizes and cleaves plasmid DNA at the origin of transfer (oriT) located between the two *tra* operons (Farrand et al. 1996; Zechner et al. 2001). The *trb*

operon encodes components of a type IV secretion system ($T4SS_{pTi}$) that permits the transfer of the plasmid DNA and associated proteins to recipient bacteria (Li et al. 1999). Interestingly, the first gene of the *trb* operon is *traI*. The eponym protein TraI is responsible for the synthesis of a diffusible QS signal that belongs to the widely distributed *N*-acyl homoserine lactone (AHL) class of signals (Hwang et al. 1994). In the presence of agrocinopines but at low cell concentration, the *trb* operon—hence, the TrbI QS signal synthase—is very weakly expressed and only low amounts of QS signals accumulate in the environment. In the presence of agrocinopines and at high cell concentrations, the QS signal concentration increases and its presence is sensed by TraR that becomes activated and induces the full expression of the $T4SS_{pTi}$ and DTR system, stimulating the transfer of the Ti plasmid (Li et al. 1999; Li and Farrand 2000).

3.6 *Cost and Control of Opine-Niche Construction and Exploitation*

As indicated above, the key step of opine-niche construction is the transfer of T-DNA to plant cells via a dedicated T4SS ($T4SS_{T\text{-}DNA}$) that imposes a fitness cost to agrobacteria (Platt et al. 2012). In a competitive arena, individuals expressing the $T4SS_{T\text{-}DNA}$ are disadvantaged compared to those impaired for $T4SS_{T\text{-}DNA}$ or defective for a Ti plasmid. Indeed, in short-term experimental evolution cultures in the presence of acetosyringone (an inducer of $T4SS_{T\text{-}DNA}$ expression) and in plant tumors, spontaneous mutants arose in the progeny of a virulent *Agrobacterium* ancestor. These mutants were altered in virulence because of alteration or loss of the Ti plasmid (Bélanger et al. 1995; Fortin et al. 1992, 1993; Llop et al. 2009). Virulent agrobacteria exhibit three potential mechanisms to balance the fitness cost and damage imposed by T-DNA transfer: (i) a tight control of *vir* gene expression by phenolics, acidic pH, and sugars contributes to optimize the cost/benefit of $T4SS_{T\text{-}DNA}$ expression, hence the success of T-DNA transfer into plant cells (Nair et al. 2011; He et al. 2009); (ii) Ti plasmid horizontal transfer that may re-introduce the Ti plasmid into those cells which have lost it (Lang et al. 2013); (iii) a fitness gain to agrobacteria—that have kept or acquired a Ti plasmid—because of opine-niche exploitation (Lang et al. 2014). Conditioning the transfer of the Ti plasmid to the tumor environment (opine as ecological proxy) ensures that the Ti plasmid-carrying *Agrobacterium* individuals will gain a selective advantage in the most compatible ecological niche.

In nature, the Ti plasmid may be transferred to other agrobacteria (other species or clonal lineages) or non-agrobacteria that is free of a Ti plasmid, whereas this transfer could be considered as advantageous for the Ti plasmid per se (selfish gene and reservoir hypotheses), and it could be disadvantageous for the Ti plasmid donor lineage because potential bacterial competitors could acquire the opine-niche exploitation trait. Another important consideration in Ti plasmid transfer is its cost

as the process uses a second $T4SS_{pTi}$. An experimental evolution experiment conducted with an *A. tumefaciens* C58 derivative expressing QS and $T4SS_{pTi}$ revealed the emergence of mutants defective for QS signal synthesis (mutations in *traR*) or exhibiting a QS-hijacking behavior or defective for the presence of a Ti plasmid (Tannières et al. 2017). *Agrobacterium* Ti plasmid donors exhibit several mechanisms to finely control QS, and hence Ti plasmid transfer. QS relates Ti plasmid transfer to a high population level of donors. This major requirement allows a virulent population to become dominant in a plant tumor habitat before activating Ti plasmid transfer, which is costly (growth slowdown) and hazardous (increase of opine-assimilating competitors). Additional mechanisms which are not present in all agrobacteria also contribute to delay QS signaling, therefore leaving time for donors to proliferate before transferring a Ti plasmid. First, the TraM protein encoded by *traM* on the Ti plasmid interacts with TraR and blocks the formation of an active TraR homodimer at low QS signal concentrations (Khan et al. 2008; Qin et al. 2007). Second, the lactonases BlcC and AiiB open the gamma-butyrolactone ring of AHLs (Haudecoeur et al. 2009). The *traM* and *aiiB* genes are encoded by the Ti plasmid and are expressed in the presence of agrocinopines in strain C58. The *blcC* gene (formerly *attM*) belongs to the *blcABC* (formerly the *attKLM*) operon located on pAt plasmids. *BlcC* is activated in the presence of gamma-butyrolactone (GBL), gamma-hydroxybutyric acid (GHB), and succinic semialdehyde (SSA), which are activated and repressed by a high and low gamma-aminobutyric acid (GABA)/proline ratio, respectively (Carlier et al. 2004; Lang et al. 2016), whereas TraM titrates TraR and prevents its early production, intracellular lactonases constrain the level of AHLs in the intra- and extracellular environments, hence their binding to TraR. Both these QS-delaying mechanisms are bypassed when TraR and AHLs are produced at a high level (Khan et al. 2008; Haudecoeur et al. 2009).

3.7 Competition for the Opine Niche by the Plant Microbiota

Although engineered by *Agrobacterium* as a niche, the tumor can be colonized by other opine-degrading microorganisms, including bacteria such as pseudomonads, *Sinorhizobium meliloti*, *Arthrobacter* sp., coryneform isolates (Tremblay et al. 1987; Nautiyal and Dion 1990; Nautiyal et al. 1991; Moore et al. 1997; Faist et al. 2016), or by fungal strains (*Cylindrocarpon heteronema* and *Fusarium solani*; Beauchamp et al. 1990). These microorganisms are naturally present in soils of diverse origins, and their growth can be stimulated by opines produced by the tumor and released at the root system of the plant independently of the soil and plant considered (Oger et al. 1997; Mansouri et al. 2002, Mondy et al. 2014; Faist et al. 2016). Interestingly, as indicated earlier, opines are chemoattractants for *Agrobacterium* (Kim and Farrand 1988). This feature may provide a way for

agrobacteria that migrate from the tumor to return to the opine-rich niche of the crown gall. A possibility exists that opines could also attract non-agrobacterial organisms, but to the best of our knowledge, this has not yet been investigated.

3.8 Exploitation of Other Plant Tumor Resources

Besides opines, a wide variety of organic (amino acids, organic acids, oses, polyols, etc.) and mineral compounds, which are potential resources for agrobacteria, accumulate in plant tumors (Deeken et al. 2006; Lang et al. 2016). Unlike opines, these compounds are not specific to tumor tissues and Ti plasmid type. To be considered part of the niche construction process, these compounds should not only accumulate in plant tumors, and their exploitation should also confer a selective advantage to agrobacteria for colonizing this habitat. Numerous traits are potentially consistent with this definition but experimental evidence is missing. A chromosomal locus *picA*, which may be involved in the degradation of plant polymers and whose expression is induced in the presence of plant tissues, may be such a candidate (Rong et al. 1991).

Numerous *Agrobacterium* isolates (carrying or lacking a Ti plasmid) harbor larger plasmids known as pAt plasmids (Merlo and Nester 1977; Rosenberg and Huguet 1984; Hynes et al. 1985). pAt plasmids can be very different from one strain to another, whereas they may comprise up to 10% of the agrobacterial genome, only a limited number of pAt functions are known. In *A. tumefaciens* C58, aside from utilization of GBL, GHB, and SSA (a by-product of GABA) as nutrients (Carlier et al. 2004), the plasmid pAtC58 encodes degradation of the Amadori compound deoxy-fructosyl-glutamine (Vaudequin-Dransart et al. 1995; Baek et al. 2003). Exploitation of these plant compounds could contribute to tumor colonization by virulent (carrying Ti plasmid) and avirulent (free of Ti plasmid) agrobacteria.

The question about the cost associated with At plasmid maintenance has been investigated in *Agrobacterium* strain C58 by comparing different derivatives carrying two, only one, or none of the plasmids pAtC58 or pTiC58. In culture medium when the $T4SS_{T\text{-}DNA}$ and $T4SS_{pTi}$ are not expressed, the cost of carrying the pAt plasmid was higher than that of the Ti plasmid (Morton et al. 2014). This may be related to the large size of the pAt plasmid as well as to the constitutive expression of the $T4SS_{pAt}$ that promotes its conjugative transfer (Chen et al. 2002). A fitness gain associated with the pAt plasmid was reported in the rhizosphere of *Helianthus annuus* (Morton et al. 2014), but this question remains unsolved in plant tumors. Interestingly, in *Agrobacterium* strain C58 the transfer of the pAt plasmid is co-regulated with that of the Ti plasmid and strongly depends upon the activity of the master regulatory protein AccR encoded by a Ti plasmid gene, the transcription of which is induced in the presence of agrocinopines A and B (Lang et al. 2013). This observation suggests that the tumor habitat stimulates a simultaneous propagation of both the pAt and pTi plasmids, probably meaning that a selective

advantage could be conferred by the acquisition of the two plasmids. In some *Agrobacterium* strains, pTi and pAt plasmids can cointegrate and cooperate for opine degradation (Vaudequin-Dransart et al. 1998). This cooperation has also been observed for some Ri plasmids (Costantino et al. 1980; Petit et al. 1983). In strains devoid of a Ti plasmid, the transfer of the pAt plasmid may also be regulated, by QS, by-products of genes located on this plasmid. In this case, the existence of one or more possible inducers of conjugation has not been demonstrated (Mhedbi-Hajri et al. 2016).

4 Niche Construction and Exploitation by *Agrobacterium*-Related Genera

All the findings described above paved the path to investigate whether the opine concept can be extended outside the *Agrobacterium* clade. Experiments performed with closely related Rhizobiaceae revealed that transformed plant roots induced by *Rhizobium rhizogenes* (formerly *Agrobacterium rhizogenes*) also contain opines (Petit et al. 1983). These two pathogens are closely related. Indeed, as in *Agrobacterium* spp., pathogenic strains of *R. rhizogenes* harbor large plasmids known as Ri plasmids (Moore et al. 1979). A portion of these plasmids, T-DNA, is transferred to the nucleus of the plant cells where it integrates into the genome upon infection (Chilton et al. 1982; Willmitzer et al. 1982; White et al. 1982). *R. rhizogenes* T-DNA harbors oncogenes that for the most part differ from those of *A. tumefaciens* and trigger the formation of transformed roots (*e.g.,* Durand-Tardif et al. 1985; Slightom et al. 1986; Cardarelli et al. 1987; Spena et al. 1987). However, the genes involved in opine biosynthesis are often highly related to those of *Agrobacterium* Ti plasmids, and several of them direct the synthesis of opines, such as cucumopine or mannityl opines (Fig. 2d, e), that are also found in crown gall tumors (Tepfer and Tempé 1981; Jouanin 1984; De Paolis et al. 1985; Petit and Tempé 1985).

A further extension dealt with nitrogen-fixing nodules incited by *Sinorhizobium meliloti* and *Rhizobium leguminosarum* strains on leguminous plants. Some of these nodules contain opine-like molecules, identified as *scyllo*-inosamine (SI) and 3-*O*-methyl-*scyllo*-inosamine (3OSI; Murphy et al. 1987; Saint et al. 1993) and collectively termed rhizopines (Fig. 3). However, only a limited number of strains of these species (ca. 11–12% of assayed clones) were able to produce and degrade rhizopines, independent of their geographical origin (Rossbach et al. 1995; Wexler et al. 1995). Genes involved in both the synthesis and degradation of SI and 3OSI have been identified. They are adjacent on the symbiotic plasmid of the bacteria (Murphy et al. 1987). In contrast to the *Agrobacterium* system, these biosynthetic genes are not transferred to plant cells but are expressed by the bacteria itself in the nodule context only. As with *Agrobacterium* opines, rhizopines provide a selective advantage for rhizopine utilizers in the plant environment, possibly by providing a

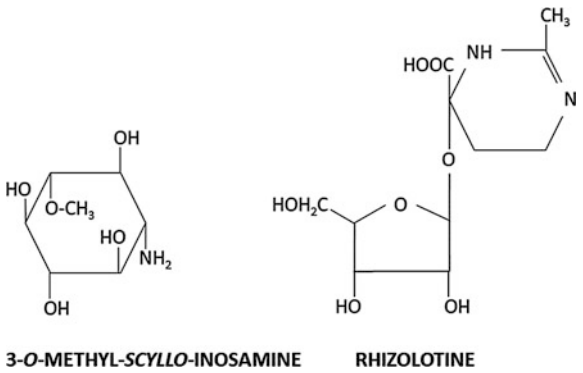


Fig. 3 Structural formulas of opine-like molecules found in nodules. The opine-like compound 3-*O*-methyl-*scyllo*-inosamine (as well as *scyllo*-inosamine, not shown) is opine-like molecules detected in nodules incited in alfalfa (*Medicago sativa*) by some strains of *Rhizobium meliloti*. Rhizolotine is an opine-like compound found in *Lotus* spp. nodules incited by *Mesorhizobium loti* strain NZP2037. This riboside molecule exhibits a tetrahydropyrimidine ring

selective nutrient to members of the population living around the nodules. This selective advantage has been demonstrated by competition experiments that involved a wild-type *S. meliloti* strain and a mutant unable to degrade rhizopines (Gordon et al. 1996; Heinrich et al. 1999). For a recent review on rhizopines and more data on genes involved in biosynthesis and degradation, the reader can refer to Savka et al. (2013).

Two other opine-like molecules have been detected in the nitrogen-fixing nodules induced on *Lotus* spp. by *Mesorhizobium loti*. One was identified as the riboside of an alpha-hydroxy-imino acid and named rhizolotine (Fig. 3). The second is an unidentified ninhydrin-positive compound (Shaw et al. 1986; Scott et al. 1987). No indication of the competitive advantage given to the rhizolotine-degrading strains in nature is available.

Aside from the above-described interactions, other interactions between bacteria and their hosts involve a trophic link. This is the case, for instance, for rhizobia that induce nodules on mimosa (*Acacia dealbata*) or *Leucaena* spp. plants. Plants of both genera produce large amount of mimosine, a toxic amino acid that only rhizobia nodulating these plants can degrade (Soerdajo et al. 1994), providing them with a selective advantage (Soedarjoa et al. 1998). Also the alkaloids calystegins present in the roots and exudates of morning glory (*Convolvulus arvensis*), hedge bindweed (*Calystegia sepium*), and belladonna (*Atropa belladonna*) can be efficiently degraded by *Sinorhizobium meliloti* strain Rm 41, a strain that is frequently detected in the root system of these plants, though they are not members of the legume clade and not hosts for symbiotic nitrogen fixation (Tepfer et al. 1988). None of these interactions, however, fits the description of the opine concept that remains limited to agrobacteria and—to a certain extent—to some rhizobia. As most rhizobia are symbionts, the opine concept should therefore be reformulated as "opines are chemical mediators of plant-microbe interactions, the synthesis of

which is induced by the micro-organism, thus providing an environment favorable to its growth and dissemination of its plant-interacting capacity."

All the above data prompted scientists to propose that the growth of beneficial microbial populations in the rhizosphere could be engineered and favored by establishing an opine-based, trophic link between the plant to protect and selected microbial population (Savka et al. 2002: Dessaux et al. 2016). Though elegant, and in spite of encouraging preliminary results obtained for some plant growth promoting rhizobacteria (Dessaux et al. 1987; Guyon et al. 1993; Wilson et al. 1995; Savka and Farrand 1997; Oger et al. 1997), this concept has not yet received definitive experimental validation.

5 Unsolved Mysteries in *Agrobacterium* Ecology

5.1 Where Do Pathogenic Agrobacteria Hide in Nature?

Though some pathogenic *Agrobacterium* strains can be isolated from uncultivated pasture soil (Schroth et al. 1971), natural soil, and plant rhizospheres, agrobacteria isolates are most often nonpathogenic unless the place of isolation has a history of crown gall contamination (Bouzar et al. 1993; Krimi et al. 2002; Dessaux, unpublished). This feature led scientists to wonder whether pathogenic agrobacteria can be isolated from some nursery soils because plants are contaminated and therefore provide the source of bacteria, or whether the plants are contaminated because virulent agrobacteria are present in these soils. This "chicken or egg" causal dilemma cannot be definitively resolved at this time, but some factual and speculative elements can be proposed. First, it is clear that exchange of contaminated plant material between various locations and countries could be at the origin of crown gall outbursts (Pionnat et al. 1999). Once contaminated, and in spite of seasonal fluctuations, soils can host agrobacterial populations and maintain them for years (Bouzar et al. 1993; Krimi et al. 2002). Second, it cannot be excluded that pathogenic agrobacteria can "hide" in the rhizosphere of non-host plants (*i.e.,* plants that do not develop crown gall symptoms) and, consequently, in places where they will not be searched for. In agreement with this proposal, agrobacteria have been detected at the root system of maize (Gomes et al. 2001) and wheat (Bednárová et al. 1979).

An alternative or complementary hypothesis is that *Agrobacterium* do not hide, but Ti plasmids do. It could be speculated that Ti plasmids could conjugate in tumors to other, non-agrobacterial isolates where they could replicate. In support of this model, Ti plasmids could conjugate to *E. coli* under laboratory conditions but they do not replicate in this host (Holsters et al. 1978). They can also be transferred to rhizobia that possess genetic backgrounds in which Ti plasmids can replicate but do not always express their tumorigenic functions (Hooykaas et al. 1977; van Veen et al. 1989; Teyssier-Cuvelle et al. 1999). Though attractive, this later hypothesis is

not really supported by probabilistic elements. First, the conjugative transfer frequency of the Ti plasmid in vitro reaches at best 10^3 per donor (Lang et al. 2013). Second, to generate a pathogenic *Agrobacterium*, the Ti plasmid would have to conjugate from the replicative bacteria back to an agrobacterial isolate in environments deprived of opines that are precisely the inducers of this conjugative transfer.

Clearly, the question of the reservoirs of Ti plasmids in nature remains an open but critical one. Further investigations are necessary to identify such reservoirs and complement our understanding of the ecology of both agrobacteria and Ti plasmid. Studies that aim to elucidate the genes and functions that contribute to bacterial fitness in tumors, the rhizosphere, bare soil, and possibly surface waters could contribute to reach these objectives.

5.2 *Origin of T-DNA, Origin of Opines*

Analyses of Ti plasmids revealed that they exhibit a chimeric structure (Otten et al. 1993; Otten and De Ruffray 1994) composed mostly of four key clusters of genes: the T-DNA, the virulence region that encodes the $T4SS_{T\text{-}DNA}$ involved in T-DNA transfer, the opine catabolic region, and the conjugative transfer regions that includes the $T4SS_{pTi}$. Interestingly, in *A. tumefaciens* Ti plasmids, T-DNA, the $T4SS_{T\text{-}DNA}$, and the conjugative region(s) are highly related, whereas the opine catabolic regions differ from one plasmid type to another. The homology of several genes that encode the $T4SS_{T\text{-}DNA}$ and the $T4SS_{pTi}$ (Chen et al. 2002) clearly suggests that both may derive from a common ancestral protein secretion system. Similarly, T-DNA genes responsible for the production of the plant hormone auxin, namely *iaaM* and *iaaH*, are orthologues of genes found in members of the *Pseudomonas savastanoi* species (Yamada et al. 1985). The cytokinin biosynthetic gene, *ipt* or *tmr*, is also related to the cytokinin biosynthetic gene *ptz* of *P. savastanoi* (Powell and Morris 1986).

As indicated above, *A. tumefaciens* T-DNAs differ from one another mostly by the nature of the opine anabolic genes. A parsimonious hypothesis therefore implies that T-DNA and the $T4SS_{T\text{-}DNA}$ have been acquired before the genes involved in opine metabolism in the evolutionary history of the plasmids, possibly as a way to reduce plant defense (Dunoyer et al. 2006; Gohlke and Deeken 2014). Furthermore, opine metabolic genes could have different origins. Some of these opine metabolic genes have evolved by duplication from common ancestor(s). This is the case of the genes involved in the synthesis and degradation of the mannityl opines. The synthesis of mannopine and mannopinic acid proceeds in two steps: (i) the condensation of glucose with glutamine or glutamate, respectively, to Schiff bases and their Amadori rearrangement compounds to form deoxy-fructosyl-glutamine (dfgln) and deoxy-fructosyl-glutamate (dfglu; Fig. 2g); (ii) the reduction of dfgln and dfglu to mannopine or mannopinic acid, respectively (Ellis et al. 1984). Mannopine can be dehydrated to yield the cognate lactone agropine, another mannityl opine (Dessaux

et al. 1986a, b). Degradation proceeds almost in the reverse way. Agropine undergoes a lactonolysis to mannopine that is in turn degraded to dfgln and mannose and glutamine. Dfglu is degraded to mannose and glutamate. In this scheme, dfgln appears to play a central role. First, it is also an opine found in the tumors induced by strains of *Agrobacterium* that harbor a chrysopine-type Ti plasmid; second, and in contrast to most opines, dfgln can be degraded by numerous strains of *Agrobacterium* irrespective of their virulence (Bouzar et al. 1995; Chilton et al. 1995; Vaudequin-Dransart et al. 1995). Accordingly, the Ti plasmid-free derivative of the reference strain C58 can metabolize dfgln via the product of genes located on the At plasmid that are highly homologous to genes found on the Ti plasmids (Baek et al. 2003). Remarkably, contrary to the situation with other opines, dfgln and dfglu occur widely in nature, *i.e.,* outside *Agrobacterium*-induced tumors. As with numerous Amadori compounds, dfgln and dfglu form spontaneously in decaying plant material (Anet 1957; Anet and Reynolds 1957). It is tempting to speculate that their common occurrence in nature provides a sufficient selective pressure to account for the emergence and selection of degradative functions, as seen in nonpathogenic strains of *Agrobacterium*. The duplication of the degradative opine genes and their incorporation into a "proto T-DNA" could have provided *Agrobacterium* with a way to force living plant cells to produce dfgln and dfglu. A further step in the evolution of the Ti plasmid could be the acquisition of opine anabolic and catabolic functions to allow the conversion of the two Amadori compounds to mannopine and mannopinic acid and later agropine (and vice versa) that are less accessible to competing organisms. Though entirely speculative, this model is nevertheless consistent with the physiological, biochemical, and molecular data available today.

The origins of other opine metabolic functions are even more speculative than those of the dfgln and mannityl opines. Octopine is synthesized in the muscle of marine animals such as octopus and squid during anaerobic muscle contraction (Thoai and Robin 1959) as a way to re-oxidize NADH, regenerate ATP, and reduce the concentrations of both arginine and pyruvate that accumulate under this condition (Grieshaber and Gäde 1976). Because marine agrobacterial isolates have been obtained (Rüger and Höfle 1992), a possibility exists that octopine degradation in these bacteria arose in relation with the presence of octopine in marine animals. In agreement, octopine-degrading bacteria have been isolated from mussels and oysters (Dion 1986). The related structures and sequence homologies of both the catabolic and anabolic genes for octopine and nopaline (Zanker et al. 1992, 1994) also suggest that these two opine systems may have evolved from a common ancestor.

The origin of sugar opines, such as the agrocinopines, is also unclear. Agrocinopine A is composed of sucrose linked to L-arabinose by a phosphodiester bond, whereas in Agrocinopine C, a D-glucose is present instead of the L-arabinose in agrocinopine A. Agrocinopines B and D differ from A and C, respectively, by lacking one sugar from the sucrose moiety (Ellis and Murphy 1981). In *Agrobacterium* strain C58, agrocinopine A is cleaved into arabinose-2-phosphate that is able to interact with AccR for activating quorum-sensing and Ti plasmid conjugation (El Sahili et al. 2015). Noticeably, arabinose-2-phosphate is uncommon

(unique until now) in the living world due to the unusual phosphate linkage on the C2 atom of the pyranose. This exemplifies the capacity of *Agrobacterium* to innovate by the use of a signal that is discriminable among the diverse sugars in plant hosts.

The occurrence of various opine anabolic and degradative systems may appear puzzling at first glance. However, the occurrence of multiple opine systems could indeed allow the diversification and coexistence of various agrobacterial populations in the same plant environment. These populations can therefore be considered as sympatric, and may eventually evolve novel species in further evolutionary steps (Lassalle et al. 2015). In agreement, whereas octopine or heliopine can be found in tumors incited by numerous *Agrobacterium* species, a number of opines such as cucumopine or ridéopine have been found only in grapevine tumors induced by members of the *A. vitis* species (Chilton et al. 2001). Similarly, cucumopine (or mikimopine) are detected only in hairy roots induced by *R. rhizogenes* (Davioud et al. 1988).

5.3 *Is* Agrobacterium*'s Ability to Transfer DNA to Organisms Belonging to Other Kingdoms Unique?*

Agrobacterium spp. and *R. rhizogenes*, due to the presence of Ti and highly related Ri plasmids, are to the best of our knowledge rare examples of bacteria naturally capable of transferring DNA to members of the eukaryote kingdom (Lacroix and Citovsky 2016). However, *Ensifer adhaerens*, a related bacterium, has recently been reported to transform plant cells when equipped with an appropriate plant transformation plasmid vector of the pCAMBIA series (Wendt et al. 2012). Aside from *Agrobacterium*, the only transkingdom DNA transfer that has been reported under laboratory conditions is between the pathogen *Bartonella henselae* and a human endothelial cell line (Schröder et al. 2011). *B. henselae* is not a major human pathogen except in immune-compromised patients where it may trigger a disease known as bacillary angiomatosis (Dehio 2005). A mobilisable cryptic plasmid from another *Bartonella* species was tagged with a fluorescent protein gene expressed only in eukaryotic backgrounds and introduced into a *B. henselae* strain that was used to infect endothelial cells. Post-infection, a low numbers of cells were fluorescent, indicating a T4SS-mediated transfer frequency of the plasmid of $\sim 2 \times 10^{-4}$. There is, however, no direct evidence that such a transfer may occur in animals, and no indication that such a transfer may lead to a permanent transformation of the recipient eukaryotic cells.

A recurring question is why no other bacteria have evolved comparable host transformation systems? First, to inquire whether other systems comparable to the Ti and Ri plasmids exist, 21 bp DNA border sequences have been compared to sequences of bacterial genomes or soil microbial metagenomes in data banks. The only hits identified were members of the two former genera (*Agrobacterium* and *Rhizobium*; Dessaux, unpublished). Second, the uniqueness of the *Agrobacterium*

spp. and *R. rhizogenes* transformation machinery could be explained by some of the evolutionary elements presented above which indicate that the occurrence of Ti and Ri plasmids proceeded in several steps, under selective pressures that may have rarely encountered in the living world. In other words, acquisition of Ti and Ri plasmids was quite an exceptional event.

In addition, once Ti and Ri plasmids evolved, it appears that their propagation in other bacteria was restricted by various factors. For instance, Ti plasmids do not replicate in firmicutes and actinobacteria, nor do they in beta- and gamma-proteobacteria such as *E. coli* or pseudomonads (Holsters et al. 1978; Dessaux, unpublished). Also, cloned Ti plasmid genes such as the opine catabolic genes are generally not expressed in other bacteria, including proteobacteria (Dessaux et al. 1987). Even in the related *Rhizobium* group where Ti plasmids replicate, tumor-inducing functions may or may not be expressed (Klein and Klein 1953; Hooykaas et al. 1977; van Veen et al. 1989) possibly because chromosomal genes involved in this process (see for instance Douglas et al. 1985; Close et al. 1985; Thomashow et al. 1987) may be missing. These data imply that transfer of the Ti plasmid outside the *Agrobacterium* genera, the *R. rhizogenes* species, and some *Rhizobium* species may be an evolutionary cul-de-sac either because the plasmid does not replicate or because the plasmid functions are not expressed. To a certain extent, and from an anthropomorphic point of view, *Agrobacterium* drastically protects the invention of the Ti plasmids that allow it to shift from a generalist behavior in the soil and the rhizosphere to a specialist behavior in the tumor where it escapes most microbial competitors and a part of plant defense.

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