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REVIEWS AND THEORETICAL ARTICLES

Opine Biosynthesis and Catabolism Genes of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*

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Abstract—Agrobacterium is a genus of soil bacteria with the ability to transform plant cells by a T-DNA-sequence located on the pTi/pRi-plasmid containing a set of genes expressed in plant cells. Expression of these genes leads to a proliferation of transformed cells, with the subsequent formation of tumors or growths of roots and the synthesis of opines—products of the condensation of amino acids with ketoacids or sugars used by agrobacteria as a source of carbon and nitrogen. In this review, we systematized the information about most common opines in plant–*Agrobacterium* systems and their biosynthesis and catabolism genes, as well as the role of opines in the interaction of pathogenic *Agrobacterium* with plants and with other *Agrobacterium* strains, including the genetic consequences of such interactions.

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INTRODUCTION

Agrobacterium is a genus of soil bacteria of the family Rhizobiaceae. The most famous feature of agrobacteria is their ability to transfer genetic information (T-DNA) into plant cells and to integrate it sustainably in the plant genome. Genetic expression of T-DNA leads to a proliferation of transformed cells and the formation of root, root galls, or hairy roots, which synthesize opines digestible by *Agrobacterium*. Pathogenic agrobacteria are traditionally divided into three types: *Agrobacterium tumefaciens*, *A. rhizogenes*, and *A. vitis*, which are also known as *Agrobacterium* of biotypes 1, 2, and 3, respectively. There is substantial complexity with the systematics (the three allocated species do not fully comply with the biotypes) [1]. In this review we used the traditional division into *A. tumefaciens*, *A. rhizogenes*, and *A. vitis*. All three species, upon infecting plants and transforming them with their T-DNA, induce a proliferation of transformed host cells, causing the formation of tumors of various types. *Agrobacterium tumefaciens* causes the formation of crown galls—tumors of various degrees of differentiation, which are usually located on the roots of the plants on the root crown or in the lower part of the stem. Similar tumors induced by *A. vitis*. *A. rhizogenes* induce the formation of so-called "bearded root" mass of fast growing, highly branched ageotrophic roots [2–4].

A lot of plants are affected by agrobacterial diseases. Different strains vary by their target hosts; pathogenic Agrobacterium can cause diseases in more than 1000 species of dicotyledonous plants of 61 different families, and transformation of monocots is also possible. A key factor in *Agrobacterium*–plant interactions are opines, and this review is dedicated to the genetic control of opine synthesis and degradation.

GENES OF OPINE BIOSYNTHESIS. OPINE ROLE IN *AGROBACTERIUM* AND PLANT INTERACTIONS. OPINE CONCEPT.

Opines are specific compounds, the products of condensed amino acids with ketoacids or sugars. *N*-Carboxyl acyl amino acids are classic opines. Opines also include a number of other compounds that perform functions similar to classic opines [5]. *N*-Carboxyl acyl amino acids are formed by reductive condensation of amino acid and ketoacid; a reaction occurs between the amino group of an amino acid and a ketone group of the ketoacid (Fig. 1).

The first evidence of the existence of opines was obtained in the 1950s as the result of an investigation into arginine metabolism in tumors induced by *A. tumefciciens*. The following observations played a key role in understanding the role of opines and the nature of tumor formation [6].

(1) *Agrobacterium* can metabolize opines.

(2) *Agrobacterium* metabolize only opines that are synthetized in the tumor and induced by a particular strain.

(3) The set of opines produced by a tumor depends on the strain of *Agrobacterium*, not on the host plant.

The second and third observations, along with the surprising ability of tumors to continue growth after the destruction of the *Agrobacterium* by antibiotics, were the basis for the assumption about horizontal gene transfer from *Agrobacterium* to the plant [7],

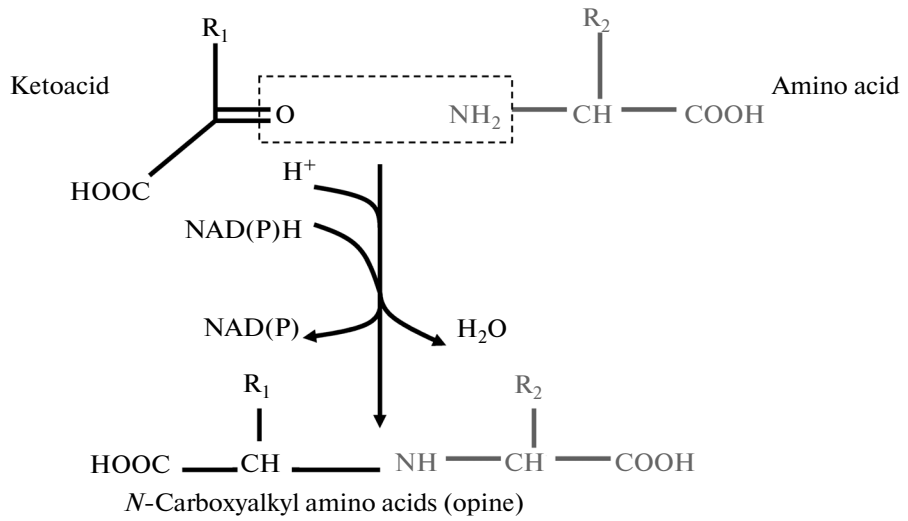

Fig. 1. Opine biosynthesis.

which was later confirmed [8]. Pathogenic strains of *Agrobacterium* contain large (about 200 kb) conjugative and replicative plasmids (pTi or pRi) [8] possessing all of the major genes of pathogenicity. These genes include a special segment called T-DNA. T-DNA is transferred into plant cells and incorporated into the plant DNA. T-DNA possesses a set of genes that are functionally divided into two groups: oncogenes, which provide malignant transformation of the host cells, and opine biosynthesis genes. In some strains (e.g. octopine and agropine strains), T-DNA is divided into two separate fragments: T_L-DNA and T_R-DNA (Fig. 2).

In 1979, the opine concept, which brings together all the facts about the *Agrobacterium*-mediated transformation, was formed. This concept postulates that the synthesis of opines for the nutrition of *Agrobacterium* has an adaptively significant effect on DNA transfer, transformation, and the formation or hairy roots [9]. Final approval of the opine concept was prevented by the existence of "null" plasmids, which were thought to induce tumors not accompanied by the synthesis of opines. However, previously unknown opines (agropine [10], succine amopine [11]), were found in such tumors in the early 1980s, and the opine concept was finally approved.

pTi and pRi plasmids are divided into groups (octopine, nopaline, agropine, mannopine, etc.) based on the type of opines produced in the tissues transformed by the T-DNA of these plasmids. The opine type is defined by the opine synthesis genes of T-DNA.

Agropine plasmids possess the *mas1* and *mas2* genes and the *ags* gene (agropine synthase). All three genes form a single pathway providing agropine synthesis from glutamine and glucose. Mannopine plasmids (for example, pRi8196) possess only two opine synthesis genes: *mas1* and *mas2*. Accordingly, in this case, the final product is mannopine.

However, the overall situation with the formation of multistage biosynthesis pathways is not typical for the biosynthesis of opines. Others opines are synthesized in a single stage, and one enzyme, encoded by a single gene of T-DNA, is required for their synthesis. Despite this, pTi/pRi plasmids usually possess several opine genes, and they provide synthesis of several different opines [5, 6, 12]. The main types of plasmids of *A. tumefaciens* and *A. rhizogenes*, classified based on opine synthase sets and opine biosyntheis genes located in their T-DNA, are presented in Tables 1 and 2, and the position of opine biosynthesis genes is shown on the genetic T-DNA in Fig. 2.

Chrysopine strains, known as plasmid pTiChry5, induce the synthesis of deoxy-fructosyl-glutamate (DFG) and chrysopine in the tumor. DFG is an intermediate product of mannopine synthesis, and chrysopine is DFG lactone. The fact that T_r-DNA controls the biosynthesis of these opines was demonstrated [13]. It contains a common set of *mas2-mas1-ags* genes. It is assumed that chrysopine is synthesized from DFG; however, a gene encoding such an enzyme with such activity is unknown [13].

Minimal functional T-DNA is known for the *A. tumefaciens* strain AB2/73 isolated from *Lippia canescens* [14]. This strain has a very limited and quite specific host range (out of model plants, it infects only *Nicotiana glauca*; it does not infect those highly sensitive to *Agrobacterium*-mediated transformation—*Kalanchoe daigremontiana*, *Nicotiana tabacum*, and *Solanum lycopersicum*). Its T-DNA has a length of 3.5 kb and contains only two genes, the *lso* oncogene (Lippia strain oncogene), with weak homology with oncogenes of normal *A. tumefaciens*, and *A. rhizogenes* strains, which are also similar to the nopaline synthase

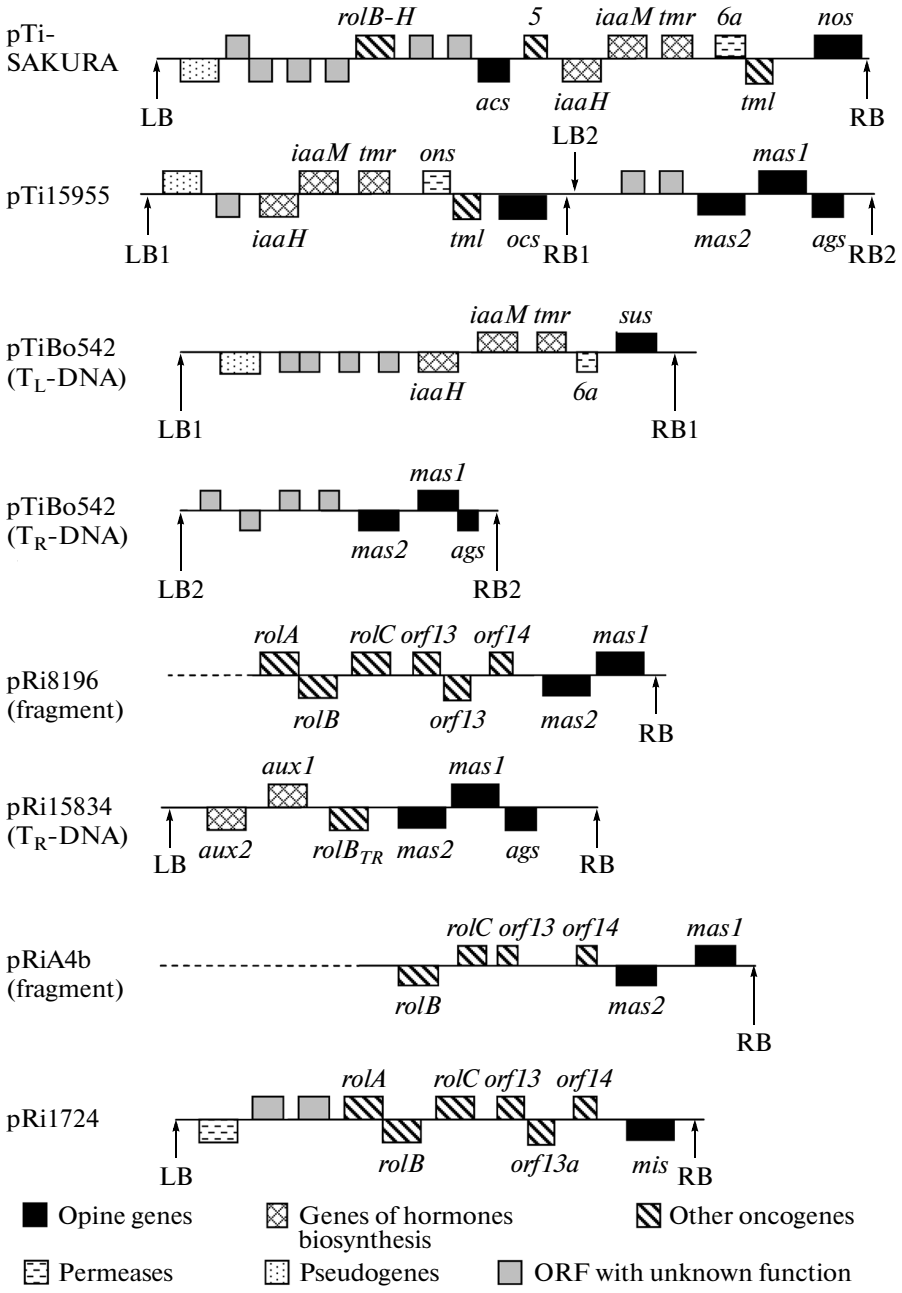

Fig. 2. Physical genetic maps of T-DNA of different *Agrobacterium* strains. Names of pTi/pRi-plasmids are shown on the left of maps. Length of genes and intergenic gaps are shown not according to scale. The dotted line indicates unsequenced regions of T-DNA of strains for which complete T-DNA sequences are not available at the present.

lsn gene. Despite the similarity of this enzyme to nopaline synthase, the type of opines synthesized by this gene product is unknown, since this strain does not metabolize nopaline [14].

Some very interesting groups of opines are the agrocinopines A, B, C, and D.

Chemically, agrocinopines are not classic opines; they are phosphoesters of sugars. However, they have the same function and are therefore traditionally considered together with opines. The widespread occurrence of the agrocinopine biosynthesis gene is of particular interest. The *acs* gene is present in almost all pTi/pRi plasmids in the form of the 5'-deleted pseudogene at the beginning of the T-DNA; the gene was probably located on the ancestor plasmid. Agrocinopine plasmids are retained intact in the *acs* gene; the nopaline plasmids contain the *acs* pseudogene and an additional working copy of the gene. It is interesting that only these strains retained the ability to metabolize agrocinopine; strains with an inactive or deleted *acs* gene lost this ability [15].

Table 1. Opine genes of pTi-plasmids [5, 6, 12]

Plasmid type	Opine biosynthesis genes	Opines, synthesized by the tumor
Nopaline	*nos* (nopaline synthase), *acs* (agrocinopine synthase)	Nopaline, nopaline acid, agrocinopines A and B*
Octopine	T_L: *ocs* (octopine synthase), T_R: *mas1*, *mas2* (mannopine synthases), *ags* (agropine synthase)	Mannopine, mannopinoc acid; agropine, agropinic acid; octopine*, octopinic acid*, lysopine*, histopine, lysopinic acid
Succcinamopine	T_L: *sus* (succcinamopine synthase), T_R: *mas1*, *mas2* (mannopine synthases), *ags* (agropine synthase)	Mannopine, mannopinoc acid; agropine, agropinic acid; succinamopine, succinamopine-lactam
Chrysopine	T_L: *mas1*, *mas2* (mannopine synthases), *ags* (agropine synthase)	DFG (deoxy-fructosyl glutamate), chrysopine (DFG lacton)

* Known conjugative opines.

Table 2. Opine genes of pRi-plasmids [5, 6, 12]

Plasmid type	Opine biosynthesis genes	Opines, synthesized by the tumer
Agropine	T_L: *acs* (agrocinopine synthase), T_R: *mas1*, *mas2* (mannopine synthases), *ags* (agropine synthase)	Mannopine, mannopinoc acid; agropine, agropinic acid; agrocinopines C and D
Mannopine	*mas1*, *mas2* (mannopine synthases)	Mannopine, mannopinoc acid
Mikimopine	*mis* (mikimopine synthase)	Mikimopine, mikimopine-lactam
Cucumopine	*cus* (cucumopine synthase)	Cucumopine, cucumopine-lactam

Strains capable of metabolizing agrocinopine are sensitive to nucleotide bacteriocin agrocine-84, which inhibits RNA and DNA synthesis. This substance is released by the nonpathogenic *Agrobacterium radiobacter* strain K84. After mimicking agrocinopine, it is selectively absorbed by *Agrobacterium* cells if they are able to metabolize agrocinopine. Strain K84 is very similar to the pathogenic nopaline strains of *Agrobacterium*, but it lost T-DNA. There is an assumption that the K84 strain can displace pathogenic Agrobacterium strains from soil around tumors induced by this strain because of the synthesis of agrocine-84 and it can absorb opines secreted by plants [16–18].

Mikimopine plasmids are also of great interest. The T-DNA of these types of plasmids were fixed in the plant genome with their subsequent vertical inheritance in many known cases of this type (the phenomenon is known in *Nicotiana* and *Linaria* genera [19–21]). At the moment, it is unclear whether this is an accidental coincidence. Only one such "wild" *A. rhizogenes* strain, 1724, is known [22].

As can be seen in Tables 1 and 2, the number of opine biosynthesis genes does not coincide with the number of opines synthesized by tumors. This is due to the fact that many opine synthases have low substrate specificity and may use a number of different amino acids as their substrate. For example, with the octopine strain, the products of four opine biosynthesis genes provide the synthesis of nine different opines, five of them produced by octopine synthase by using different amino acids; agropinic acid is spontaneously formed from agropine (Table 3).

In fact, the situation is even more complicated, since not all possible substrates are known. Experiments in vitro [23] demonstrated that octopine synthase can use at least 12 different amino acids (proteinogenic and nonproteinogenic) as a substrate with an activity not lower than 20% (assuming that 100% activity is exhibited in the reaction with arginine), or about 8 amino acids with lower activity, as well as other ketoacids. Optionally, all of these reactions in vivo may have the same value; however, the actual number of opines can be considerably higher than that with other types of strains. The authors pay special attention to the fact that octopine synthase can use sulfur containing the amino acids methionine and *S*-methylmethionine to form sulfur opines. Sulfonopine, produced from *S*-methylmethionine utilized by octopine strains and octopine catabolism operon, contains the *msh* gene, which is most likely involved in the catabolism of sulfonopine [23].

Opine biosynthesis genes are well known for the high-efficiency transcription promoters and terminators used in the genetically engineered constructions. Promoters of the majority of opine genes, for example, agropine synthase (*ags*), octopine synthase (*ocs*), nopaline synthase (*nos*), both mannopine synthases (*mas1*, *mas2*), and possibly all others genes, contain

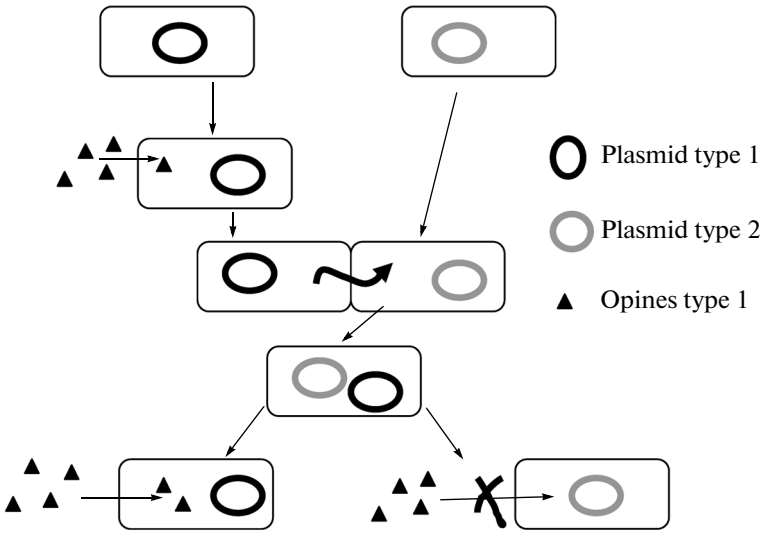

Fig. 3. Change in digestible opines initiated by conjugation opines, transfer of pTi/pRi-plasmids, and the loss of one plasmid due to incompatibility.

ocs-elements: 16-nucleotides palindromic sequences and binding transcription factor OCSTF. The ocs-elements of opine genes are very similar to the same elements of the p35S promoter of cabbage mosaic virus. Due to this, a high level of opine synthesis is maintained [24].

Some opines, the so-called "conjugative opines," in addition to the nutritional functions, also perform a signaling role, initiating conjugation with the transfer of pTi/pRi-plasmids together with the auto-inductor of quorum sensing. pTi/pRi-plasmids can be transferred not only to a bacterium that has lost pTi/pRi-plasmids but also between two bacteria with plasmids, i.e., the presence of pTi/pRi-plasmids does not make such a transfer impossible. After such a transfer, there is an unstable situation when two pTi/pRi-plasmids exist in the cell. If the plasmids belong to the same incompatibility group, for example if they are octopine and nopaline plasmids, one of them will be lost, and the preservation of a new plasmid and loss of the original plasmid is possible (Fig. 3). If the plasmids are different, then the strain type changes with respect to the opine set [25]. The possibility of the simultaneous existence of two different plasmids in one cell allows for exchanges between different plasmids blocks. Hooykaas [25] directly demonstrated the formation of cointegrates from octopine and nopaline pTi-plasmids. This eventually leads to a mosaic structure of these plasmids. For example, pRi-plasmids by certain regions (replication unit) are similar to the symbiotic plasmids of rhizobia more than to pTi-plasmids [26]. According to a phylogenetic tree based on sequences of *vir*-genes, nopaline strains are more closely related to agropine strains than to octopine strains [27]; if the construction of the same tree is based on sequences of *trbB* genes, the situation is opposite [26]. This does not allow reconstruction of the history of the evolution of these plasmids.

Transfer of pTi/pRi-plasmids to other genera of bacteria with the formation of new pathogenic strains is also possible [28].

OPINE CATABOLISM GENES

There are a few opine biosynthesis genes, and they provide for the synthesis of large quantities of opines due to the low substrate specificity, which are then released from plant cells.

Opine catabolism genes control the transport of opines in bacterial cells and their utilization. These genes are organized in the form of operons located on pTi/pRi-plasmids (Table 4). These genes can be divided into three groups:

(1) Permeases genes encoding ABC-type transporters, which pump opines into bacterial cells.

(2) Actual opine catabolism genes. These genes encode enzymes that convert opines in digestible compounds.

(3) Other genes encoding the regulatory proteins that control the operons of opine catabolism and are involved in the initiation of opine-inducible conjugative transfer of pTi/pRi-plasmids, and a number of other genes with unknown functions or unclear relations with opines.

The fact that the set of opine catabolism genes on the plasmid of pathogenic *Agrobacterium* always corresponds to the set of biosynthesis genes is very interesting. The presence of catabolism genes for their "own" opines is clear according to the opine concept. The reason why the unknown pathogenic strains assimilating "foreign" opines, i.e., opines they cannot synthe-

Table 3. Enzymes of opine biosynthesis: substrates and products [5, 6, 12]

Enzyme	Substrate—amino acid	Substrate—sugar or ketoacid	Products
Acs (agrocinopine synthase)	–	Sucrose, arabinose	Agrocinopines A and B
Ocs (octopine synthase)	Arginine, lysine, histidine ornithine	Pyruvic acid	Octopine, octopinic acid, lysopine, histopine, lysopinic acid
Nos (nopaline synthase)	Arginine, ornithine	2-Oxoglutarate	Nopaline, nopaline acid
Mas1, 2 (mannopine synthases)	Glutamine, glutamic acid	Glucose	Mannopine, mannopinoc acid, deoxy-fructosyl-glutamine (intermediate product)
Ags (agropine synthase)	Mannopine	–	Agropine

Table 4. Opine catabolism genes

Opine	Transporters genes	Opine degradation genes	Other genes
Agrocinopine [29]	*accA, B, C, D, E*	*accF*—agrocinopine phosphodiesterase, *accG*—arabinose-phosphate phosphatase	*accR*—regulator of operon
Nopaline [30, 31]	*nocP, T, Q, M*	*noxA, B*—nopaline oxidase, *Arc*—arginase	*nocR*—regulator of operon
Octopine [31, 32]	*occP, M, Q, T*	*ooxA*—octopine oxidase A, *ooxB*—octopine oxidase B, *ocd*—ornithine cyclodeaminase	*occR*—regulator of operon (*lysR*-type)
Agropine, mannopine [33, 34]	*agtA, B, C, D*—agropine transporter, *agaD, B, C, A*—agropinic acid transported, *moaA, B, C, D*—mannopinic acid and mannopine transporter	*agaE*—egradation of agropinic acid, *agcA*—degradation of agropine, *agaF, G*—degradation of mannopinic acid, *mocC, D*—degradation of mannopine	*moaR, mocR*—regulators of operon, *mocA*—6-phosphohexose dehydratase, *mocB*—6-phosphohexose dehydrogenase

size, is less clear, especially the fact that many nonpathogenic *Agrobacterium* strains metabolize opines, sometimes in unusual combinations thereof [35–38]. They do not acquire alien catabolism genes, but rather the loss of biosynthetic genes causes mutations or the deletion of catabolism genes. For example, none of the currently known strains that do not contain a working copy of the *acs* gene can any longer metabolize agrocinopine [15].

The genes for the biosynthesis and catabolism of opines usually are not homologous, even if they catalyze the same reaction in the opposite directions. Neither octopine nor nopaline oxidase is homologous for the respective biosynthetic genes (*ocs* and *nos*) [39]. However, a different situation is observed for agropine and mannopine plasmids; the biosynthesis and catabolism of agropine is performed in opposite directions by the same pathway (Fig. 4), and genes of the biosynthesis and catabolism enzymes, which work at each stage, are pairwise homologous. The *ags* gene is homologous to the catabolic *agcA* gene [34], *mas1* is homologous to *mocC*, and *mas2* is homologous to *mocD* [40].

This situation is also interesting because deoxyfructosyl glutamate (DFG) is an intermediate of the biosynthetic chain and, unlike other opines, it is widely present in the environment where it formed as the product of the decomposition of plant residues. Based on the homology of genes of agropine and mannopine biosynthesis and catabolism, a hypothesis about the origin of pTi-plasmids was proposed. According to the hypothesis, these plasmids originally appeared as catabolic and allowed bacteria to use DFG as a carbon and nitrogen source, metabolizing it into mannopine and agropine, which were less available to other soil bacteria. Then, after acquiring the ability of incorporating DNA into the plant genome, these activities were used for the production of opines in plant cells [12]. A similar hypothesis exists for the appearance of octopine plasmids as catabolic plasmids for assimilation of octopine produced by mollusks [12]. None of these hypotheses have been proven yet.

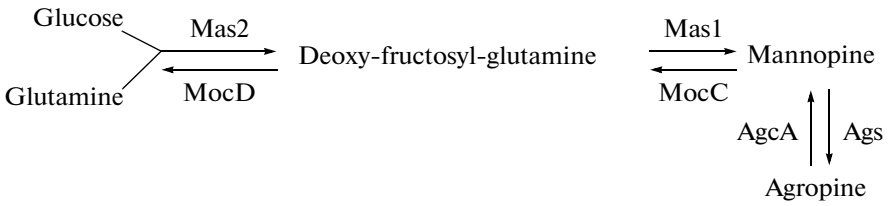

Fig. 4. Biosynthesis and catabolism of agropine and mannopine.

OPINE CATABOLISM IN OTHER MICROORGANISMS

Another interesting question concerns the presence of the ability to catabolize opines in other organisms. The answer may explain some features of the *Agrobacterium*–plant interaction, such as the great variety of opines and their biosynthetic genes (including individual plasmids).

Previously, it was thought that the occurrence of opines in nature is limited to *Agrobacterium*–plant systems [5], except for octopine, which was found in the tissues of marine invertebrates. Octopine is formed in the muscle tissue of marine mollusks (e.g. bivalves *Pecten* sp., cephalopods *Loligo* sp.) during long and intensive work leading to anaerobic conditions in the muscles. Octopine in this case is the end product of glycolysis; NADH is used for its production [41], and large quantities of octopine are accumulated. It is interesting that the ability to utilize octopine was demonstrated for some marine bacteria associated with these mollusks. Thus, three types of pseudomonads, which are able to grow on media containing octopine as the sole source of carbon and nitrogen, were isolated from mussels and oysters and were allocated [42].

In soil, as it turned out, opine-utilizing microorganisms are also widespread. Among Gram-negative opine metabolizing bacteria, the most important bacteria belong to the genus *Pseudomonas*, as they are widespread. Among soil isolates a high proportion assimilate opine, and the number of different opines metabolized by strains in total covers almost the entire set of opines produced by *Agrobacterium* tumors. Thus, in the study by Canfield and Moore [35], of the 183 strains of *Pseudomonas* sp., isolated from *Malus* roots infected with *A. tumefaciens*, 90 were able to metabolize opine (nopaline, octopine or mannopine, and in some cases, two of these three opines). In the study by Nautiyal and Dion [36] where the number of tested opines was higher, the ability of pseudomonads to metabolize all opines used in this work (nopaline, octopine, succinamopine, and mannopine) was demonstrated. Atropine and chryzopine were also metabolized by pseudomonads [37].

Of the Gram-positive bacteria, the ability to metabolize opine was detected for coryneformic bacteria (phylum Actinobacteria). Their ability to metabolize nopaline, succinamopine, and mannopine was demonstrated [36–38]. A catabolic enzyme with broad substrate specificity towards opines was isolated from *Arthrobacter* sp. strain 1C [43].

The ability to metabolize opines was also found in fungi. The ability of fungi to consume mannopine (specific types were not identified [37]) was demonstrated. Also the ability to metabolize octopine, octopinic acid, succinamopine, and mannopine in various combinations was shown for one strain of *Fusarium solani*, two strains of *Cylindrocarpon destructans*, and six strains of *Cylindrocarpon heteronema* [44]. Strains capable of metabolizing nopaline were not detected [44].

Another competitor for opines are nonpathogenic strains of *Agrobacterium* that are unable to induce the formation of tumors in plants but are capable of metabolizing various opines. Such nonpathogenic strains, unlike pathogenic strains, occur more frequently in the soil [45] and constitute a significant portion of the soil bacteria capable of catabolizing opines [36–38].

Thus, the *Agrobacterium* infecting plants and thereby producing sources of opines have to compete for opines with other bacteria. Moreover, some of them, such as *Pseudomonas fluorescens* or the K84 *Agrobacterium radiobacter* strain described above, also antagonize the *Agrobacterium* by mechanisms that effectively allow suppressiong of their growth [17, 19, 46]. It is not excluded that the observed diversity of opine biosynthesis genes in the T-DNA of Ti/Ri-plasmids, including the diversity within the same T-DNA, is a method for avoiding competition within these substrates.

CONCLUSIONS

The volume of knowledge differs greatly for various aspects of the biology of the *Agrobacterium*. A lot of attention is focused on the *Agrobacterium* as a tool of genetic engineering for the transformation of plants, and often the opine genes not involved in these applications are not investigated.

Meanwhile, opines and the respective genes of biosynthesis and catabolism play a key role in the interaction of pathogenic wild-type *Agrobacterium* strains with the plants infected by them. Opine genes are associated with a large number of unsolved problems. For example, the reasons for the large variety of opines

and the respective genes of their biosynthesis and "strange" compliance of induced and digestible opines are not known. Little data are available for the transformation of monocot by wild-type *Agrobacterium*, although the fact that opine synthesis can occur in cases of tumor formation was shown [47]. Solving these problems may shed light on the problem of the occurrence of the unique capacity of *Agrobacterium* for the "genetic colonization" of plants.

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