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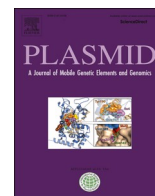
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Original Research Article

Characterization of the *Agrobacterium* octopine-cucumopine catabolic plasmid pAtAg67

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ABSTRACT

In addition to tumor-inducing agrobacteria, non-pathogenic strains are often isolated from crown gall tumors. Such non-pathogenic strains sometimes contain catabolic plasmids that allow them to take advantage of the opines produced in the tumors. Here we characterize for the first time an octopine catabolic plasmid, pAtAg67, which is derived from an *Agrobacterium* strain isolated from a grapevine tumor in Crete. By sequence analysis, we deduce that pAtAg67 enables its host to catabolize not only octopine, but also cucumopine and agrocinopine-like compounds. We found that a highly similar set of catabolic genes was present in the Ti plasmids of tumorigenic octopine-cucumopine grapevine strains such as pTiAg57. However, the catabolic genes in octopine-cucumopine Ti plasmids were interrupted by a T-DNA segment. As no T-DNA remnants, virulence genes or border repeats were found in pAtAg67, catabolic plasmid pAtAg67 does not appear to be a degenerate octopine-cucumopine Ti plasmid. In line, plasmid pAtAg67 was found to be compatible with *incRh1* octopine Ti plasmids, but to be incompatible with the *incRh2* agropine Ti plasmid pTiBo542, forming cointegrates by recombination in the homologous *trb* genes.

1. Introduction

The bacterium *Agrobacterium tumefaciens* is well known for its ability to induce crown gall tumors on plants and is in use nowadays as a vector for the genetic modification of plants. Since its discovery many different *Agrobacterium* strains have been isolated from soil and crown galls. They all belong to the bacterial family of the Rhizobiaceae, but they can be classified in three biotypes with different physiological traits (Kerr and Panagopoulos, 1977), which have now been reclassified in the genera *Agrobacterium, Rhizobium* and *Allorhizobium* (Mousavi et al., 2015; Ormeño-Orrillo et al., 2015). Tumor-inducing bacteria have a tumor-inducing (Ti) plasmid, which carries the virulence genes that allow the bacteria to transfer a tumor-inducing segment of the Ti plasmid, called the T-DNA, into plant cells (Gelvin, 2003; Hooykaas and Beijersbergen, 1994). Within the T-DNA, genes are located which stimulate plant cells to proliferate and thus eventually cause an overgrowth, a crown gall tumor (Otten, 2021). In crown galls specific metabolites, called opines, are formed by the condensation of an amino acid and a keto acid (pyruvate or α-ketoglutarate) or a sugar (mannose), which can be specifically used for growth by the infecting *Agrobacterium* strain

(Dessaux et al., 1993). Another class of opines is formed by the agrocinopines, which are sugar phosphodiesters (Ryder et al., 1984). Opines are formed by opine synthases which are encoded by genes in the T-DNA; catabolic genes for degradation of the corresponding opines are generally located in the Ti plasmid in a region close to the T-DNA.

Several different tumor-inducing bacteria are often isolated from a crown gall tumor in nature or from the soil from around the infected plants (Burr and Otten, 1999; Kuzmanović and Puławska, 2019). In addition, avirulent bacteria are often also isolated, sometimes including bacteria that can still catabolize certain opines via genes that are plasmid localized (Kuzmanović and Puławska, 2019). What the relationship is between these catabolic plasmids and the Ti plasmids is not yet clear and only three of such catabolic plasmids have been characterized in detail, viz. the nopaline catabolic plasmids from strains K84 (Slater et al., 2009) and Colt5.8 (Kuzmanović and Puławska, 2019) and the mannopine catabolic plasmid pAoF64/95 (Wetzel et al., 2014). Here we characterize the first octopine catabolic plasmid pAtAg67, which was found in an *Agrobacterium* strain, which was isolated from grapevine in Crete by Panagopoulos and Psallidas (1973). Previously, we transferred pAtAg67 to a new host strain and found that the transconjugants could

catabolize octopine, but remained avirulent on all host plants tested. Plasmid pAtAg67 was shown to be compatible with octopine Ti plasmids, which like octopine-cucumopine Ti plasmids belong to the *incRh1* group (Hooykaas et al., 1980; Szegedi and Otten, 1998). Here we show that pAtAg67 is incompatible with the agropine Ti plasmid pTiBo542, which belongs to *incRh2* (Hooykaas, 1983; Yamamoto et al., 2017), and can form a cointegrate with pTiBo542. Bioinformatics analysis of the complete sequence of pAtAg67 showed that pAtAg67 is an opine catabolic plasmid, lacking all the virulence genes present on Ti plasmids, but with a set of opine catabolic genes almost identical to that present in octopine-cucumopine Ti plasmids.

2. Materials and methods

2.1. Bacterial strains used and conjugation experiments

The bacterial strains LBA645 and LBA649 were previously described derivatives of *Agrobacterium* strain LBA285 (C58 Ti cured StrR NalR) with the octopine plasmids pAtAg67 and pTiAg57, respectively (Hooykaas, 1979; Hooykaas et al., 1980; Hoekema et al., 1984). Strain LBA645 was used as a donor in conjugation experiments with strains LBA288 (C58 Ti cured RifR; Hooykaas et al., 1980), and with derivatives of LBA288 containing pTiB6S3::Tn1Δ*occ* (*incRh1*), pTiBo542 (*incRh2*), and pRi1855::Tn5 (*incRh3*). Conjugation was performed on minimal medium plates with octopine and transconjugants were selected on octopine containing BTB medium as previously described (Hooykaas et al., 1979). The plasmid content of transconjugants was analyzed by gel electrophoresis (Eckhardt, 1978). Strains LBA1190 and LBA1191 contain a putative cointegrate of pAtAg67::pTiBo542.

2.2. DNA sequencing and genome assembly

The genome of LBA645 was sequenced on an Illumina HiSeq 2000 machine at the Leiden Genome Technology Center (LGTC) of the Leiden University Medical Center (Leiden, The Netherlands). The 4.8 million reads were quality filtered with fastp (Chen et al., 2018). The filtered reads were assembled with a number of de-novo assemblers, of which SKESA (Souvorov et al., 2018) yielded the best result. The resulting assembly, consisting of 39 contigs, was aligned to the C58 genome (Slater et al., 2013). The contigs corresponding to both chromosomes and the pAt plasmid revealed only very few differences with C58, as expected. A few of these difference must be responsible for the resistance to streptomycin and nalidixic acid of the strain used, but were not further investigated. To obtain full length sequences based on the C58 genome but including these few mutations, we used our reads to polish C58 chromosome and pAtC58 sequences with Pilon. These sequences were then combined with the contig from our de novo assembly which corresponded to the pAtAg67 plasmid.

The genomes of LBA649, and two strains carrying a putative pTiBo542 - pAtAg67 co-integrated plasmid: LBA1190 and LBA1191, were sequenced on a MinION flow cell (version R9.4.1) in house. Oxford Nanopore sequencing libraries were generated with high molecular weight genomic DNA isolated with QIAGEN Genomic tip gravity flow columns using the SQK-RBK004 Rapid Barcoding Kit. Barcoded samples were pooled with other samples before sequencing. A separate barcode was used to sequence LBA649. In contrast, DNA of strains LBA1190 and LBA1191 were each tagged with the same barcode as a sample from an unrelated project (a yeast DNA sample). This allowed sequencing of more than 12 samples on a single flow cell (only 12 barcodes were available in the kit). Since reference sequences were available and Agrobacterium and yeast genomes are not similar to each other, we could bin the reads per sample even without a barcode to distinguish the reads. After basecalling with Guppy (version 3.2.10, fast mode) LBA649, LBA1190 and LBA1191 reads were demultiplexed (per barcode), filtered by size (>500 bp) and trimmed (100 bp head cropped). LBA1190 and LBA1191 reads were then mapped to reference sequences (both yeast and Agrobacterium genomes and plasmids) with

minimap2 (Li, 2018). Reads mapping to Agrobacterium sequences were extracted. For LBA1190 the yield was 238 Mbp ($\sim 41 \times$ coverage), while the yield for LBA1191 was 129 Mbp ($\sim 22 \times$ coverage), with an average base quality of 10.4 and a median read length of 7674 bp and 4808 bp, respectively. The reads were mapped to the C58 genome and pTiBo542 and pAtAg67 sequences with NGMLR (CoNvex Gap-cost alignMents for Long Reads), followed by variant calling with Sniffles (Sedlazeck et al., 2018). For both LBA1190 and LBA1191 a "translocation" was detected between both plasmids in their *trb* regions, indicating the area where recombination had occurred between both plasmids. The reads were also filtered by size (>5 kb) and then used for genome assembly with Flye (Kolmogorov et al., 2019). The assemblies were polished with Medaka (https://github.com/nanoporetech/medaka). For LBA1190 the resulting genome assembly consisted of four contigs: two chromosomes, a large (pAtC58) plasmid and the co-integrated plasmid (aligning to both pTiBo542 and pAtAg67). The LBA1191 genome assembly was very similar, but consisted of five contigs, since the linear chromosome sequence was divided over two contigs. Alignments between LBA1190 and LBA1191 co-integrated plasmid sequences and pTiBo542 and pAtAg67 confirmed that co-integration had taken place in the *trb* regions. The (long) LBA1190 reads were also used to confirm the correctness of the pAtAg67 sequence assembled from Illumina reads at some difficult (repetitive) regions.

LBA649 reads (yield 443 Mbp, $\sim 78 \times$ coverage, median read length 7076 bp) were filtered by size at different size cut-offs (0.5 kb, 5 kb or 10 kb). Subsequently multiple assembly tools, Flye (2.7.1), Miniasm (0.3-r179) (Li, 2018) and Raven (1.1.10) (Vaser and Šikić, 2021), were ran to generate in total seven assemblies. Trycycler (0.3.1) (Wick et al., 2021) was used to obtain a consensus sequence and this sequence was polished with Medaka. Previously, the restriction maps of several octopine-cucumopine Ti plasmids were published (Otten and De Ruffray, 1994). The *Hind*III map of our pTiAg57 sequence was fully consistent with the earlier published HindIII map (Otten and De Ruffray, 1994). BLASTn comparisons between plasmids were performed and visualized with BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011). The R package genoplotR (Guy et al., 2010) was used to draw the opine catabolic regions and show tBLASTx comparisons.

3. Results and discussion

3.1. Conjugative transfer of plasmid pAtAg67 and incompatibility with pTiBo542

In order to characterize the octopine plasmid of grapevine *Agrobacterium* strain Ag67 we previously transferred this plasmid to strain LBA280, a Ti cured, streptomycin and nalidixic acid resistant derivative of the common *A. tumefaciens* laboratory strain C58 (Hooykaas et al., 1980). The transconjugants, including strain LBA645, were characterized and turned out to confer the ability to catabolize octopine on the new host, but the transconjugants remained avirulent on plants, indicating that the transferred plasmid was a catabolic plasmid rather than a Ti plasmid (Hooykaas, 1979). Subsequently, it was shown that the plasmid, now called pAtAg67, was compatible with octopine and nopaline Ti plasmids, which belong to *incRh1* (Hooykaas et al., 1980). Here we used LBA645 in crosses with LBA288, a Ti cured strain, and derivatives of LBA288 carrying either pTiB6::Tn1Δ*occ* (*incRh1*), an octopine Ti plasmid with a Tn1 insertion conferring carbenicillin resistance and an *occ* deletion, pTiBo542 (*incRh2*), or pRi1855::Tn5 (*incRh3*), respectively. Transconjugants were obtained with a frequency of between 10^{-2} and 10^{-3}, but with a 100-fold lower frequency in case pTiBo542 was present in the recipient. The recipients with pTiB6::Tn1Δ*occ* remained carbenicillin resistant upon receipt of pAtAg67 in agreement with the compatibility of pAtAg67 with octopine Ti plasmid pTiB6. Also the recipients with pRi1855::Tn5 remained kanamycin resistant after introduction of pAtAg67, suggesting a lack of incompatibility, which was confirmed by analysis of the plasmid content

by gel electrophoresis. However, when recipients with pTiBo542 were analyzed for plasmid content they turned out not to have a plasmid of the size of pAtAg67, but rather a plasmid of a size compatible with the size of a putative cointegrate of pAtAg67 and pTiBo542 (Fig. S1). These results suggest that plasmid pAtAg67 belongs to incompatibility group *incRh2* like pTiBo542. In order to obtain more insight into the structure of pAtAg67 we have sequenced this plasmid and analyzed it in silico. We have subsequently used Nanopore sequencing to obtain evidence that pAtAg67 and pTiBo542 form cointegrates when introduced into the same cell.

3.2. General features of pAtAg67 and pTiBo542::pAtAg67 cointegrates

Using Illumina sequencing we obtained the full length sequence of pAtAg67, which revealed that the plasmid had a length of 132,297 bp and a GC content of 59%. In total 120 protein coding sequences were found with an average size of 995 bp (Fig. 1). Plasmid pAtAg67 has a *repABC* replicator like most plasmids in the *Rhizobiaceae* family (Cevallos et al., 2008; Pinto et al., 2012). A *rinQ* gene for a resolvase involved in plasmid dimer resolution is located next to *repC* in an inverted orientation like in many other *Agrobacterium/Rhizobium* plasmids (Quintero et al., 2002). On the other side, adjacent to *repA* of the *repABC* operon a *traI* gene (the encoded enzyme catalyzes the synthesis of an N-acyl-homoserine lactone) as part of a *trbB-trbI* operon for conjugative mate pair formation is located (Lang and Faure, 2014). The *traAFBH* and *traCDG* Dtr (DNA transfer and replication) conjugation

genes necessary for DNA metabolism are located about 60 kbp away in pAtAg67 (Fig. 1). Plasmid pAtAg67 thus belongs to Group I of the large *repABC* plasmids with putative Class I quorum-regulated conjugative transfer systems like the Ti plasmids (Wetzel et al., 2015). No virulence genes or T-DNA sequences were found in pAtAg67, corroborating that pAtAg67 is a catabolic plasmid.

Sequencing of two of the strains (LBA1190, LBA1191) containing putative pAtAg67::pTiBo542 cointegrates, showed that they indeed harboured such cointegrates consisting of both complete plasmids, which had recombined within their homologous *trb* genes (Fig. 2). In both cases co-integration apparently occurred by homologous recombination in/near the *trbE* gene, but led in LBA1191 to a longer conversion tract so that a large part of *trbE* and *trbJ* in both chimeric operons now have the pTiBo542 signature.

3.3. Gene content of pAtAg67 and comparison to other plasmids

We analyzed the gene content of pAtAg67 and compared its genes to those present in other opine catabolic plasmids, and a variety of Ti and Ri plasmids. We have included in this comparative analysis the sequences from *A. vitis* octopine-cucumopine Ti plasmids pTiCFBP2407 (from France) and pTiK306 (from Australia; genome announced in Xi et al., 2020), which have not yet been described in all details, but which are available in the NCBI database and also that of pTiAg57 (from Crete like pAtAg67), which we sequenced ourselves for this purpose. For genes of pAtAg67 for which we did not find homologous sequences in any of
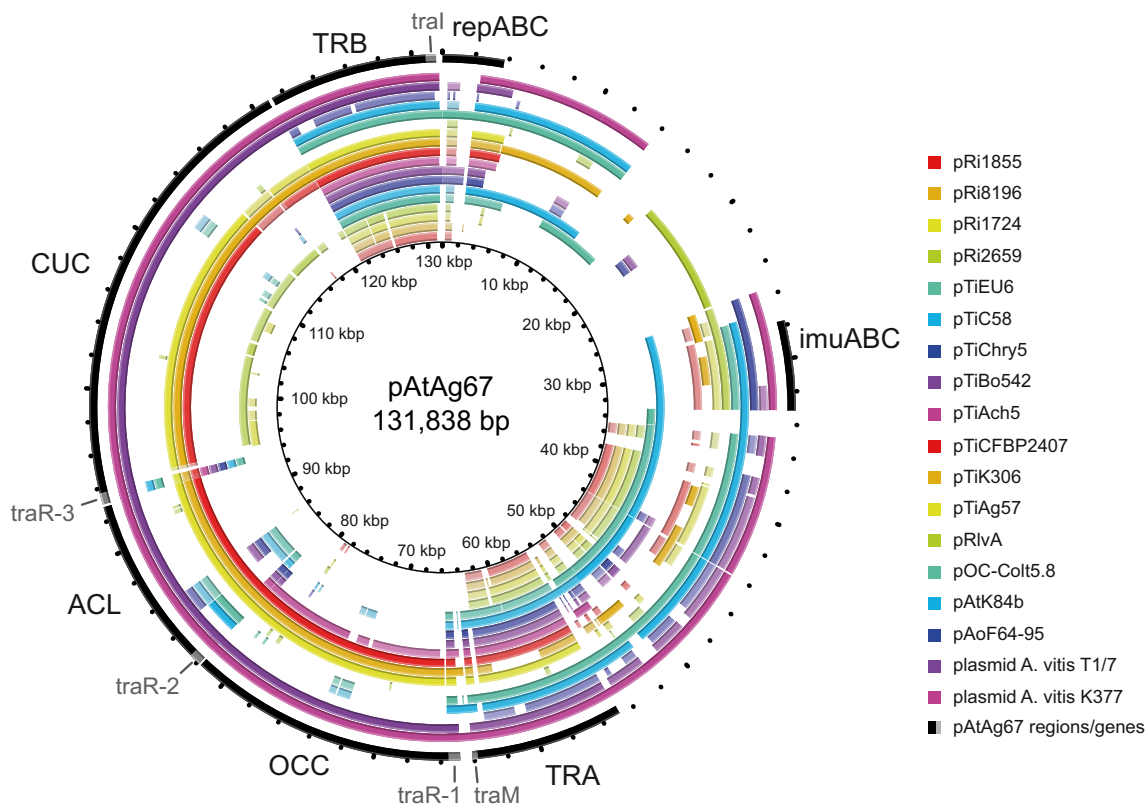

Fig. 1. Map of pAtAg67.

The outermost ring shows the locations of a number of gene regions and individual genes. The other rings, from inner to outer ring, show BLASTn comparisons to the following plasmids: agropine Ri plasmid pRi1855 (accession CP044124), mannopine Ri plasmid pRi8196 (NZ_JAAMDI010000041.1), cucumopine Ri plasmid pRi2659 (NZ_CP019703), mikimopine Ri plasmid pRi1724 (NC_002575), succinamopine Ti plasmid pTiEU6 (KX388535), nopaline Ti plasmid pTiC58 (NC_003065), chrysopine Ti plasmid pTiChry5 (KX388536), agropine Ti plasmid pTiBo542 (NC_010929), octopine Ti plasmid pTiAch5 (NZ_CP007228), octopine-cucumopine Ti plasmid pTiCFBP2407 (NZ_KY000060), octopine-cucumopine Ti plasmid pTiK306 (NZ_JABFNP010000003), octopine-cucumopine Ti plasmid pTiAg57 (MZ773647), pRlvA from *Rhizobium leguminosarum* bv. viciae strain UPM791 (NZ_CP025507), nopaline catabolic plasmid pOC-Colt5.8 (NZ_MK318973), nopaline catabolic plasmid pAtK84b (NC_011990), mannopine catabolic plasmid pAoF64–95 (NC_019555), contig "NODE9" from whole genome shotgun sequencing assembly of *A. vitis* T1/7 (NZ_MBFE02000009), plasmid unnamed1 of *A. vitis* K377 (NZ_JACXXJ020000001). Color intensity indicates the degree of sequence similarity.
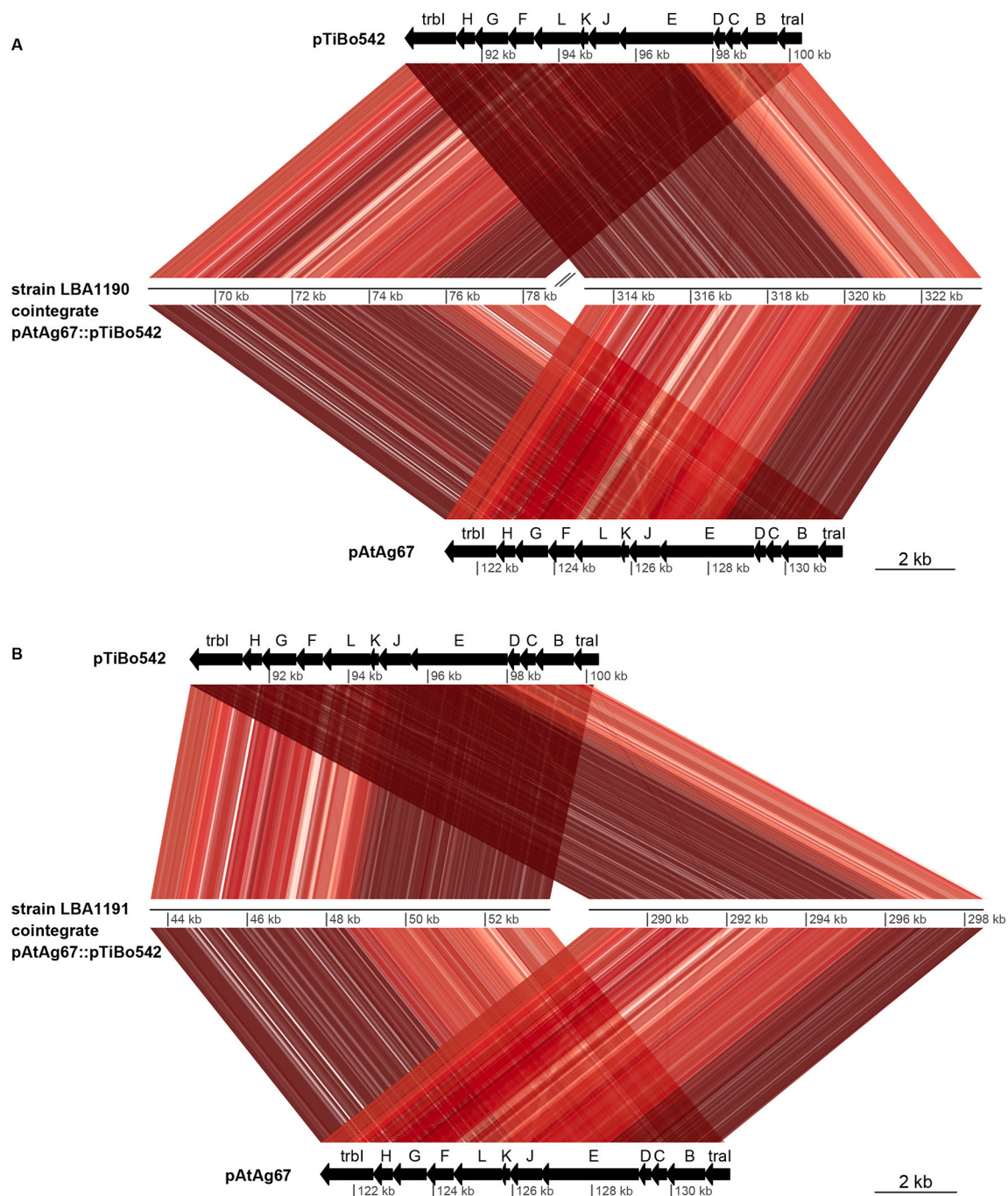
Fig. 2. Chimeric *trb* operons of pAtAg67::pTiBo542 cointegrates.
The pAtAg67::pTiBo542 cointegrates of strains LBA1190 (A) and LBA1191 (B) harbour two *trb* operons. tBLASTx (e < 0.001) comparisons shows which part of each operon shows higher similarity to which parental plasmid (pAtAg67 and pTiBo542). Darker red color indicates a higher degree of sequence identity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

these plasmids, we searched in the NCBI database for the presence of such homologous sequences in yet other *Rhzobium* plasmids.

Between *repC* and *traG* (map position from 4 to 55 kbp) a large region is present in pAtAg67 with genes of largely unknown function (Fig. 1). These genes can also be found in a variety of other plasmids from different rhizobia and agrobacteria. For instance the nopaline catabolic plasmids pAtK84b and pOC-Colt5.8 as well as the nopaline Ti plasmid pTiC58 share most of these genes with the exception of the area between map position 14–26 kbp, which contains genes with similarity to genes present in the pTiEU6 plasmid (left part) and genes present in *Rhizobium* plasmids pPR5 and pRlvA (right part), respectively (Fig. 1). It is worthwhile to mention

that in the section from map position 26–36 kbp several recombination genes are located. Besides a gene with similarity to *ligC*, involved in non-homologous end-joining, and a gene with a Toprim signature, known from topoisomerases and primase, an *imuABC* operon is present with error prone DNA polymerase genes. Such genes have been shown to be involved in spontaneous and damage-induced mutagenesis in *Caulobacter crescentus* (Alves et al., 2017), but have also been found responsible for hyper-mutagenesis and the evolution and adaptation of the genes on a symbiotic plasmid in a new host (Remigi et al., 2014). They may allow co-evolution of the pAtAg67 catabolic plasmid and new hosts in particular ecological niches.

The about 60 kbp region between *trbI* and *traH* (map position from 64 to 121 kbp) contains genes that are probably all involved in catabolism of opines: for degradation of octopine (Fig. 1 OCC), for degradation of cucumopine (Fig. 1 CUC), and for degradation of an agrocinopine-like compound (Fig. 1 ACL), which are described in more detail in the following paragraph. As plasmid pAtAg67 confers the ability to degrade octopine on its host, a set of octopine catabolic genes was expected, but the discovery of cucumopine catabolic genes in pAtAg67 suggests that agrobacteria containing pAtAg67 can optimally profit from the opines produced in tumors induced by agrobacteria with octopine-cucumopine Ti plasmids.
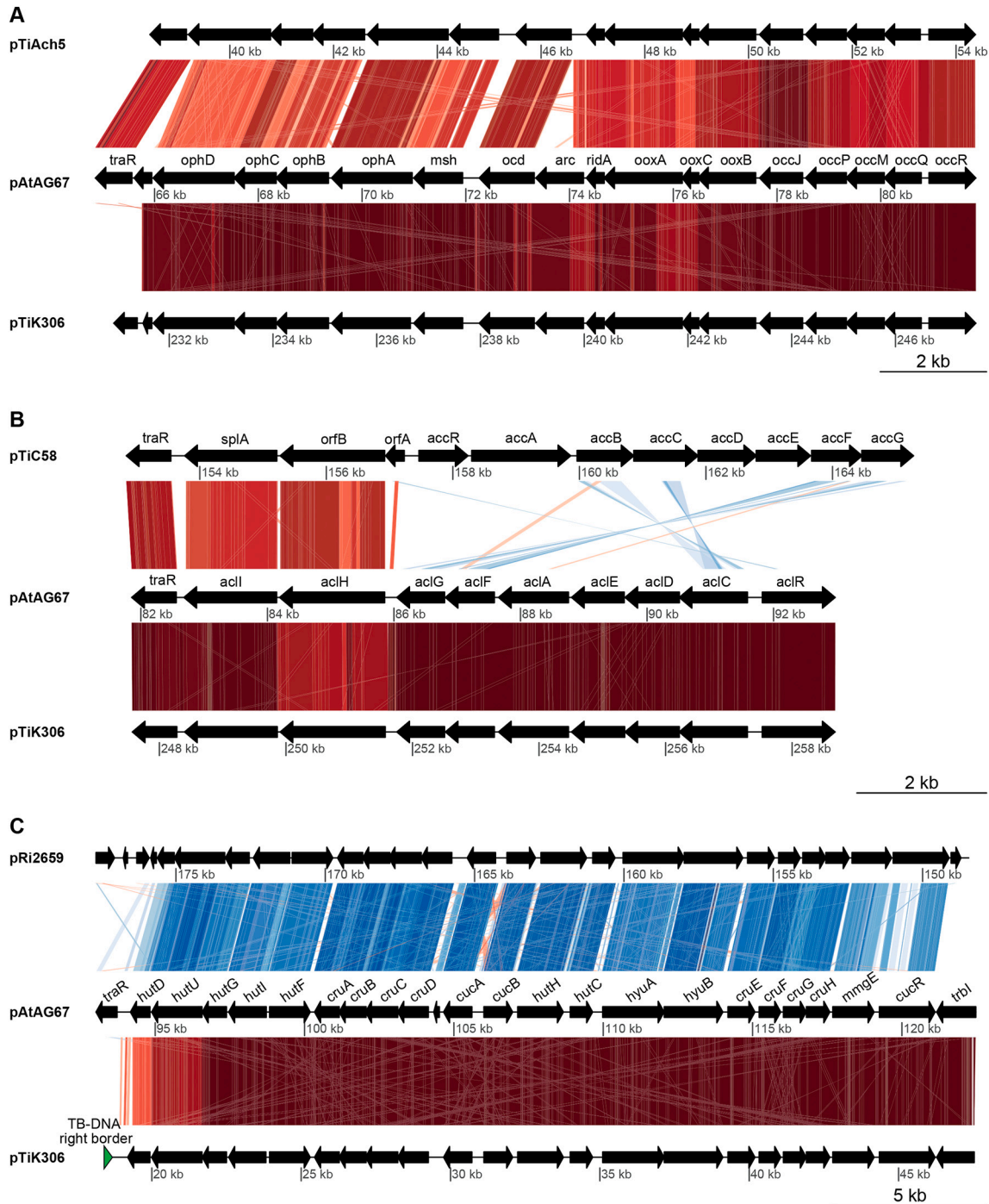

Fig. 3. pAtAg67 opine catabolic regions.

The octopine (A), agrocinopine-like (B) and cucumopine (C) catabolic regions of pAtAg67 were compared to those in other plasmids. tBLASTx hits show the degree of similarity (darker color indicating higher sequence similarity). All three opine catabolic regions were very similar to those in octopine-cucumopine Ti plasmids such as pTiK306 (of *A. vitis* strain K306). The octopine catabolic region had almost the same gene content as that of octopine Ti plasmid pTiAch5. The first part of the second catabolic region showed similarity to the *arc* (agrocinopine regulation of the conjugation) operon of pTiC58, while the second part had some sequence similarity to the pTiC58 *acc* operon, containing genes for agrocinopine transport and catabolism. Finally, the pAtAg67 cucumopine region showed strong similarity to that of cucumopine plasmid pRi2659.

3.4. Gene clusters for opine catabolism

The octopine catabolic genes share strong similarity with the octopine genes from the well characterized octopine Ti plasmid pTiAch5 (Zhu et al., 2000). They are almost identical (97% nucleotide identity) to the octopine genes of the *A. vitis* octopine-cucumopine Ti plasmids such as pTiK306 (Fig. 1; Fig. 3). In pAtAg67 the cluster of genes involved in the degradation of octopine is located adjacent to the *tra* genes ending with *traM* (Fig. 1). The order of the genes in the different octopine operons is identical and starts at the 5′ end with the octopine permease genes *occQMPJ* followed by the octopine dehydrogenase genes *ooxBCA*. Then in pAtAg67 and the octopine-cucumopine Ti plasmids an extra open reading frame is present encoding an arginase, which is absent from the operon in octopine Ti plasmids such as pTiAch5 as can be seen in the comparison in Fig. 3. It was also interesting to note that the octopine operon of pAtAg67 seems to end with a copy of *traR* like that of the pTiAch5 octopine operon, but that *traR* is not present at the end of the octopine operon of octopine-cucumopine Ti plasmids and there ends with the *ophABCD* permease genes. The octopine operon of octopine Ti plasmids is controlled by the OccR regulator, which is encoded by a gene that is located adjacent to the octopine operon, but oriented divergently (Habeeb et al., 1991). In pAtAg67 and the *A. vitis* octopine-cucumopine Ti plasmids a very similar *occR* gene is located in a similar position (Fig. 3).

Adjacent to *trbI* is a region with genes with high similarity to the cucumopine catabolic genes of the cucumopine Ri plasmid and the *A. vitis* octopine-cucumopine Ti plasmids (Fig. 1; Fig. 3). These genes are distributed over several operons, but have so far not been characterized in detail. Two genes with similarity to the cucumopine synthase (*cus*) gene, which is present in the T-DNA of octopine-cucumopine Ti and cucumopine Ri plasmids (Otten, 2021), are present, one of which (CucA) encodes a protein similar to the Cus protein over its entire length, while the other (CucB) only shares the C-terminal part with Cus. Either of these proteins or both together may be the dehydrogenase carrying out the reverse reaction, degrading cucumopine into histidine and α-keto-glutaric acid. Genes for histidine utilization (*hut*-genes) are present in this area in pAtAg67, which may be controlled by a *hutC* gene encoding a GntR-like regulator, which is conserved in many proteobacteria for the control of histidine utilization genes (Aravind and Anantharaman, 2003). In the octopine-cucumopine Ti plasmids and in the cucumopine Ri plasmid similar *hut*-genes are present, which were previously already identified in the mikimopine Ri plasmid pRi1724 (Moriguchi et al., 2001). Mikimopine is a condensate of histidine and α-keto-glutaric acid and a diastereoisomer of cucumopine (Dessaux and Faure, 2018). Cucumopine may form a lactam (Dessaux and Faure, 2018) and genes encoding hydantoinase A and B for opening the lactam ring are also present in this region. There are two sets of putative opine uptake genes in this area, probably for uptake of cucumopine and its lactam, respectively, which we have indicated as "cucumopine related uptake" (*cru*) genes *cruA-cruD* and *cruE-cruH* in Fig. 3. Lastly, this area contains a *mmgE/prpD*-like gene encoding a putative dehydratase of unknown function, which can also be found in some other opine catabolic regions, and a *lysR*-like regulator, which we have indicated as *cucR* in Fig. 3.

In between the octopine and cucumopine catabolic areas of pAtAg67 a set of genes is located, probably forming an operon, with at its 3′ end genes with similarity to three of the four genes of the *arc* (agrocinopine regulation of the conjugation) operon of nopaline Ti plasmids (Piper et al., 1999) encoding a protein with some similarity to a phosphate transporter, a putative sucrose phosphorylase (SplA) and a TraR transcriptional activator (Fig. 2). In nopaline Ti plasmids the *arc* operon is located adjacent to the *acc* operon for agrocinopine catabolism and both operons are inducible by agrocinopine A via AccR (Piper et al., 1999). At the 5′ end the operon of pAtAg67 is enlarged by "agrocinopine-like" (*acl*) genes *aclA-aclE* encoding a putative transport system and two genes encoding an AccF-like phosphodiesterase (*aclF*) and a AccG-like phosphatase (*aclG*), respectively (Kim and Farrand, 1997). The prototype AccF enzyme from nopaline strain C58,

which has similarity with agrocinopine synthase Acs (Kim and Farrand, 1997), is involved in the catabolism of agrocinopine A releasing glucose-2-phosphate (El Sahili et al., 2015) which is probably dephosphorylated by the phosphatase AccG. A largely identical *acl* operon is present in octopine-cucumopine Ti plasmids pTiK306, pTiCFBP2407 and pTiAg57 (Figs. 1, 3). These Ti plasmids have an *acs* gene encoding agrocinopine synthase in either or both of their T-DNAs, and evidence was provided for the presence of agrocinopine A in the tumors induced by octopine-cucumopine strains Tm4 and AB3 (Paulus and Otten, 1993; Otten, 2021). However, while agrocinopine A was taken up from the medium by a strain carrying pTiTm4, this was not the case for strains harboring pTiAB3 or pTiAg57 (Paulus and Otten, 1993). Therefore, we speculate that the *acl* operon of pAtAg67 and the octopine-cucumopine Ti plasmids such as pTiAg57 is involved in the catabolism of a still unknown agrocinopine-like compound that is produced in the tumors induced by the strains with these octopine-cucumopine Ti plasmids. The sugars that are liberated from agrocinopines may be broken down by enzymes encoded elsewhere in the genome. However, it is striking that the *arc* and *acl* operons contains a gene *splA* for a putative sucrose phosphorylase (Piper et al., 1999), suggesting that sucrose may be one of the sugars present in this agrocinopine. Sucrose phosphorylases catalyze the conversion of sucrose to D-fructose and α-D-glucose-1-phosphate. It can also be speculated that the putative phosphate transporter encoded by the operon may provide some of the phosphate required for this reaction. An AccR-type regulator is not present, but instead a "repressor, open reading frame, kinase" (ROK)-family repressor with in the C-terminus a carbohydrate sensing domain shared with sugar kinases (Titgemeyer et al., 1994) is encoded by a gene which we have called *aclR* in Fig. 3. It is located adjacent to the 5′ end of the operon, but in a divergent direction. This may be the agrocinopine-responsive regulator controlling this operon. Neither in pAtAg67, nor in pTiAg57 or pTiCFBP2407 an *acc* operon is present, but such *acc* operon with high similarity to that of nopaline catabolic plasmids pAtK84b and pOC-Colt5.8 is located in the octopine-cucumopine Ti plasmid pTiK306. It may be that presence of this operon allows certain octopine-cucumopine Ti plasmids to take up agrocinopine A in contrast to strains lacking it. Plasmid pTiK306 also contains an *acl* operon as above described for pAtAg67. Octopine-cucumopine Ti plasmids often have two, sometimes degenerated, T-DNAs called TA-region and TB-region with in each an *acs* gene (Otten, 2021), suggesting that each may be responsible for synthesis of a different agrocinopine that may in turn require a specific set of genes for uptake and catabolism. It was shown recently that the agrocinopine synthases from nopaline strains producing agrocinopines A and B and those from agropine and chrysopine strains, which produce agrocinopines C and D form separate groups in a phylogenetic tree (Tanaka et al., 2022). Nopaline strains in addition have a second *acs*-like gene of unknown function; the encoded proteins, called ACS2, form yet another separate group in the phylogenetic tree (Tanaka et al., 2022). One of the agrocinopine synthases encoded by octopine-cucumopine strain K306 and also the single Acs of strain CFBP2407 does not clearly belong to either of these groups, in line with the possibility that they produce yet another agrocinopine (Figs. S2, S3). The second Acs encoded by strain K306 belongs to the nopaline Ti Acs group (Fig. S3), which explains why tumors induced by certain octopine-cucumopine strains may contain agrocinopine A.

3.5. Plasmid pAtAg67 contains three traR copies

Conjugative transfer of Ti plasmids and opine catabolic plasmids is under regulatory control. Transfer is seen in the presence of specific opines and dependent on quorum sensing (Lang and Faure, 2014). The TraR protein acts as an activator of the conjugation genes; it binds as a dimer to *tra*-boxes in the promoters of the conjugation genes. Dimerization is promoted by its acyl-homoserine lactone ligand produced by TraI (Qin et al., 2000; Zhu and Winans, 2001). The *traR* gene itself may be part of an opine catabolic operon and thus its expression may be under control of the "conjugative opine" acting as an inducer of this operon (Fuqua and Winans, 1996; Piper et al., 1999). A protein called TraM is an anti-activator

and is present in these systems to inhibit activation of the conjugative transfer genes in the absence of the opine inducers (Luo et al., 2000). Plasmid pAtAg67 is a conjugative plasmid, transfer of which was found on media containing octopine (Hooykaas et al., 1980). We found a copy of *traM* and three complete copies of very similar *traR* genes in pAtAg67 (Fig. S4). Each of the three opine catabolic operons is associated with one of these *traR* genes, and it can be speculated that each of them is under control of a different opine: octopine, an agrocinopine-like opine and cucumopine, respectively. There is precedent for plasmids under control of multiple conjugative opines. In the catabolic plasmid pAtK84b two *traR* genes are present, one under control of nopaline, the other under control of agrocinopine (Oger and Farrand, 2002).

3.6. Relationship between pAtAg67 and octopine-cucumopine Ti plasmids

In the hope to obtain some insight in the evolutionary relationship between the pAtAg67 catabolic plasmid and the octopine-cucumopine Ti plasmids, we sequenced pTiAg57, as this Ti plasmid was obtained from an *Agrobacterium* strain from Crete like pAgAt67 (Panagopoulos and Psallidas, 1973). A circular representation of this 223,232 bp large plasmid can be seen in Fig. 4. The current sequence corroborated the restriction map of this plasmid and the T-DNA segments which were described before (Otten et al., 1992; Otten and De Ruffray, 1994; Otten, 2021). Almost over its entire length plasmid pTiAg57 shares high similarity with pTiCFBP2407, but these plasmids had a large, about 50 kbp section (map position from 100 to 150 kbp) which is absent from another sequenced octopine-cucumopine plasmid pTiK306 and from catabolic plasmid pAtAg67. This section of pTiAg57 includes genes that are involved in the catabolism of

tartrate, a trait which is common for *A.vitis*, but not necessarily determined by its Ti plasmid (Salomone and Otten, 1999). Octopine-cucumopine Ti plasmids pTiAg57 and pTiCFBP2407 share with the pAtAg67 the three clusters of opine catabolic genes. When the order of genes is compared it can be seen that the opine catabolic gene clusters have the same order in pAtAg67 as in the octopine-cucumopine Ti plasmids such as pTiAg57 and pTiCFBP2407 (Figs. 1, 4). However, in the octopine-cucumopine Ti plasmids the TB-DNA is located in between the *acl* genes and the *cuc/cru* genes, while the TA-DNA and the virulence genes are present in between the *tra* genes and the *occ* genes (Fig. 4). Interestingly, octopine-cucumopine Ti plasmid pTiAg57 has only one copy of *traR*, that associated with the *acl* genes. In contrast to those of pAtAg67 the octopine and cucumopine gene clusters in octopine-cucumopine Ti plasmids do not have a *traR* copy, suggesting that this was lost during evolution of the Ti plasmids or that reversely each of these catabolic clusters in the catabolic plasmid pAtAg67 gained such *traR* copy during evolution. The catabolic plasmid might have been a precursor progenitor of the octopine-cucumopine Ti plasmids by acquiring virulence genes and T-DNA segments over time, or reversely be derived from these Ti plasmids by loss of its virulence genes and T-DNAs. The latter scenario has been observed before in a *Rhizobium* plasmid called p42a, which still had virulence genes, but only degenerated T-DNAs (Hooykaas and Hooykaas, 2021; Otten, 2021). However, neither any virulence genes nor remnants of T-DNA regions or border repeats were found in pAtAg67, indicating that pAtAg67 is not a (degenerated) Ti plasmid. Also, while the octopine-cucumopine Ti plasmids from grapevine seem to evolve rapidly by the action of transposable elements resulting in insertion mutations and deletions (Otten et al., 1992; Otten, 2021), it is striking that pAtAg67 is lacking transposable elements altogether, another
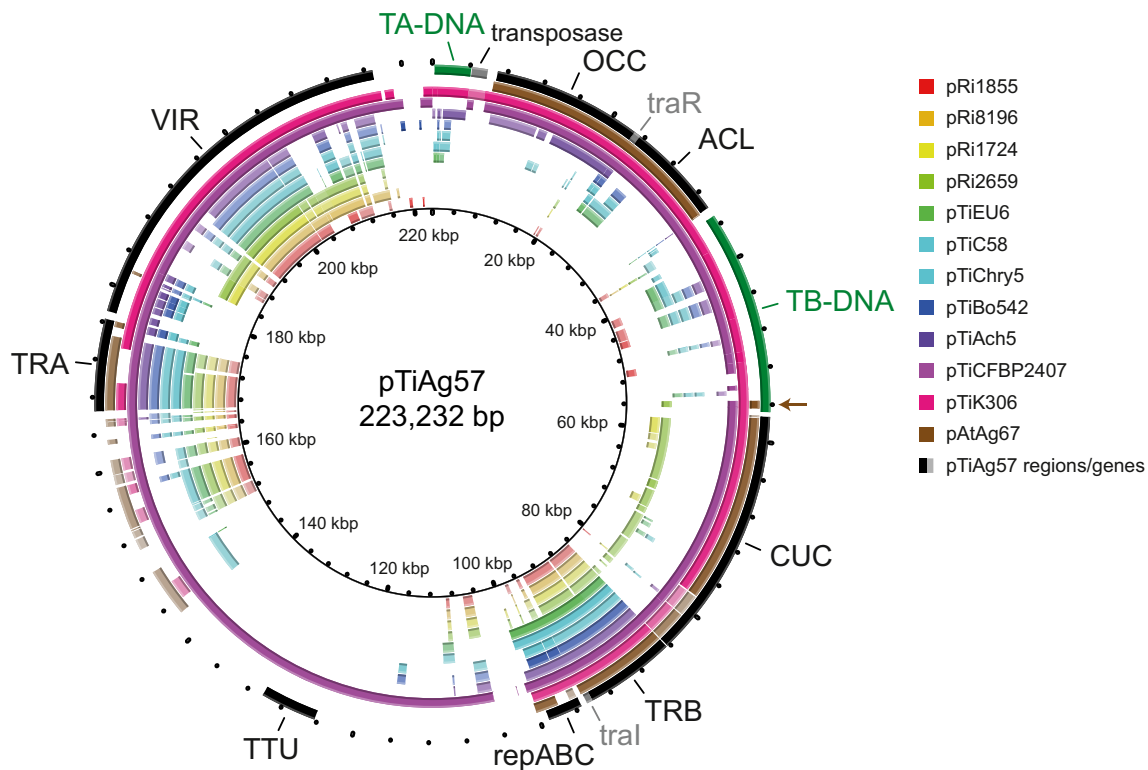

Fig. 4. Map of pTiAg57.

The sequence of octopine-cucumopine Ti plasmid pTiAg57 was compared to that of other Ti plasmids and to pAtAg67. From inner to outer ring, BLASTn comparisons to the following plasmids are shown: agropine Ri plasmid pRi1855 (accession CP044124), mannopine Ri plasmid pRi8196 (NZ_JAAMDI010000041.1), mikimopine Ri plasmid pRi1724 (NC_002575), cucumopine Ri plasmid pRi2659 (NZ_CP019703), succinamopine Ti plasmid pTiEU6 (KX388535), nopaline Ti plasmid pTiC58 (NC_003065), chrysopine Ti plasmid pTiChry5 (KX388536), agropine Ti plasmid pTiBo542 (NC_010929), octopine Ti plasmid pTiAch5 (NZ_CP007228), octopine-cucumopine Ti plasmid pTiCFBP2407 (NZ_KY000060), octopine-cucumopine Ti plasmid pTiK306 (NZ_JABFNP010000003), and pAtAg67 (CP079858). The outermost ring shows both T-DNAs, TRA, TRB, VIR, TTU (*ttu* genes for tartrate catabolism) and opine catabolic regions, as well as a number of individual genes. The brown arrow indicates the only part of the pTiAg57 TB-DNA which shows similarity to pAtAg67. This is the *cus* gene, of which two homologs are located in the cucumopine catabolic area of pAtAg67 (Fig. 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

indication that this plasmid has not been derived from a Ti plasmid.

When searching for plasmids in Genbank with similarity to pAtAg67 we found some other catabolic plasmids containing all the catabolic gene clusters present in pAtAg67, such as the catabolic plasmid from *A. vitis* strain K377 with a size of 187,327 bp, and a whole genome shotgun sequencing contig of 243,478 bp from *A. vitis* strain T1/7, which is likely a catabolic plasmid as well (Fig. 1). These even larger catabolic plasmids in addition have a set of *acc* genes with high similarity to those from the nopaline catabolic plasmids pAtAg84b and pOC-Colt5.8 and a fourth *traR* gene, which are located between the *tra* genes and the *occ* genes, and thus have four continuous opine catabolic clusters. The octopine-cucumopine Ti plasmid pTiK306 also contains these genes, but here they are present between the *trb* genes and the cucumopine catabolic genes, and surrounded by a *trbI* gene copy. Insertion in pTiK306 thus seems to have been accompanied by a duplication of the *trbI* gene. Both the *acc* and the *acl* gene cluster in pTiK306 have an associated *traR* gene. It has previously been shown that homologous repeated sequences shared by these clusters may cause plasmid instability and deletion (Fournier et al., 1994).

4. Conclusions

Sequence analysis of the pAtAg67 octopine plasmid showed it to be a catabolic plasmid lacking virulence genes and T-DNAs. On the basis of sequence similarity three sets of genes involved in catabolism of three different opines were identified located adjacent to each other in an about 60 kbp region between the *trb* genes (*trbI*) and the *tra* genes (*traH*) in this plasmid. It can therefore be predicted that pAtAg67 allows its host cells to catabolize not only octopine, but also cucumopine and an agrocinopine-like compound. Almost identical sets of opine catabolic genes were found by us in octopine-cucumopine Ti plasmids. These octopine-cucumopine Ti plasmids allow their host bacteria to induce crown gall tumors on plants in which octopine and cucumopine have been detected (Paulus et al., 1989). The presence of genes with similarity to octopine synthase, cucumopine synthase, vitopine synthase and agrocinopine synthase in their T-DNAs (Otten, 2021) suggests that in addition to octopine and cucumopine (Paulus et al., 1989), also vitopine and agrocinopine-like compounds may be produced in the tumors. Agrocinopine A was indeed discovered in some tumors induced by strain Tm4, but not in tumors induced by a number of other octopine-cucumopine strains (Paulus and Otten, 1993), which nevertheless possess an agrocinopine synthase gene in the T-DNA suggesting that another agrocinopine-like compound may be produced in these tumors. Indeed, the octopine-cucumopine strains encode an agrocinopine synthase that differs from the agrocinopine synthases from nopaline strains producing agrocinopines A and B as well as from agrocinopine synthases from agropine and chrysopine strains producing agrocinopines C and D (Figs. S2, S3). Some octopine-cucumopine strains have a second *acs* gene encoding an agrocinopine synthase with high similarity to that of nopaline strains (Figs. S2, S3), explaining why agrocinopine A was discovered in the tumors which they induce.

A few opine catabolic plasmids have been described before. The best known example is the catabolic pAtK84b plasmid present in the biocontrol strain K84. This avirulent strain contains an agrocin plasmid by which it produces agrocin84. This agrocin selectively inhibits and kills nopaline strains, while its nopaline catabolic plasmid pAtK84b allows it to profit from the nopaline (and agrocinopines) produced in the tumors at the cost of the tumorigenic nopaline strains (Kim and Farrand, 1997). Another, similar nopaline catabolic plasmid was recently described in the nonpathogenic strain Colt5.8, which was isolated from a crown gall tumor together with some tumorigenic nopaline strains (Kuzmanović and Puławska, 2019). A mannopine catabolic plasmid (pAoF64/95) has also been described (Wetzel et al., 2014), which belongs to Group II of the large *repABC* plasmids with putative Class I quorum-regulated conjugative transfer systems like the Ri plasmids (Wetzel et al., 2015). This plasmid is similar to pArA4, a catabolic

plasmid present in *Rhizobium rhizogenes* strain A4, which complements the opine catabolic properties of this strain as pRiA4 lacks genes for the catabolism of a number of opines formed in the hairy roots which it induces (Wetzel et al., 2014). Plasmid pAtAg67 is the first example of a catabolic plasmid with a virtually identical set of catabolic gene clusters as in a class of Ti plasmids isolated from the same infection site. The catabolic properties conferred by pAtAg67 thus allow its host bacteria to profit optimally from the opines formed in tumors that are induced by the virulent octopine-cucumopine strains on grapevine (Figs. 1, 4). We found that in the octopine-cucumopine plasmid pTiAg57 the *occ* genes and the *acl* genes are located between the TA-DNA and the TB-DNA, while the *cuc/cru* genes are located between the TB-DNA and the *trb* genes (*trbI*). If pAtAg67 would have been formed by deletion from such Ti plasmid, some remnants of the TB-DNA might still be present in pAtAg67 between the *acl* genes and the *cuc* genes and possibly also some remnants of either the TA-DNA and/or the *vir* genes in the region between the *occ* genes and *traM* on the other side of the plasmid. However, we have not detected any border repeats or any other remnants of the T-DNAs or virulence genes in pAtAg67, which indicates that pAtAg67 is not directly derived from an octopine-cucumopine Ti plasmid. This is also in line with the finding that about 40% of pAtAg67 (region between 4 and 55 kbp. in Fig. 1) contains genes that are shared with nopaline plasmids rather than with octopine-cucumopine Ti plasmids. Our finding that pAtAg67 does not belong to *incRh1* like these octopine-cucumopine Ti plasmids (Szegedi and Otten, 1998), but rather belongs to *incRh2* together with the agropine Ti plasmid pTiBo542 also suggests a different evolutionary origin. Plasmids of different origin such as pAtAg67 and pTiAg57 may still have acquired highly similar DNA segments by various forms of DNA recombination including cointegrate formation with another plasmid followed by deletion or resolution of the cointegrate via recombination at another site than where co-integration occurred.

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Declaration of Competing Interest

None.

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