Functional and Mutated Agrocinopine Synthase Genes on Octopine T-DNAs

François Paulus and Léon Otten

CNRS Plant Molecular Biology Laboratory 12, Rue du Général Zimmer, 67084, Strasbourg, France
Received 10 April 1992. Accepted 19 November 1992.

Agrocinopine synthase genes have so far been detected only on the *Agrobacterium tumefaciens* nopaline Ti plasmid pTiC58 and on the *A. rhizogenes* Ri plasmid pRiA4. The TA region of the octopine/cucumopine (o/c) *A. vitis* Ti plasmid pTiTm4 strongly resembles the TL DNA of biotype I octopine Ti plasmids. In addition, it carries an intact and functional agrocinopine synthase gene close to its left border. TA/TL sequence comparison shows that the biotype I TL region still carries a 5′-deleted *acs* gene, strongly indicating that this gene was originally part of the TL region and belongs to the "common DNA" region of octopine and nopaline Ti plasmids. Tm4 and C58C1 (pTiTm4) remove agrocinopine A from the medium indicating that pTiTm4 also carries agrocinopine uptake genes. In spite of this, Tm4 and related strains are resistant against agrocin 84. Two o/c Ti plasmids that are closely related to pTiTm4, pTiHm1, and pTiAB3, have a mutated *acs* gene; whereas Hm1 can still take up agrocinopine A, AB3 cannot. These results yield new insights in the evolution of octopine, nopaline, and o/c strains.

Additional keywords: Agrobacterium, agrocin 84 sensitivity, evolution, Ti plasmids, *Vitis vinifera.*

Agrobacterium tumefaciens and *A. rhizogenes* cause crown gall tumors or hairy root disease on susceptible plant species (reviewed in Kado 1991 and Winans 1992). Virulent strains contain large tumor-inducing Ti plasmids or root-inducing Ri plasmids and transfer part of the plasmid, called T-DNA, to the plant cell during infection. The T-DNA is integrated and expressed and leads to tumor or hairy root induction and the production of low molecular weight compounds, collectively called opines, which can be degraded by the inciting bacterium. Often, various genes for opine utilization are located on the Ti/Ri plasmid. Some opines have been found to stimulate the conjugative transfer of the Ti plasmid and are therefore called "conjugative opines" (reviewed in Petit and Tempé 1985).

Agrocinopines constitute a family of phosphorylated sugars found in tumors induced by *A. tumefaciens* biotype I nopaline strains and agropine strain Bo542, and in roots induced by agropine and mannopine strains of *A. rhizogenes* (Ellis and Murphy 1981). Agrocinopine A consists of sucrose and L-arabinose linked by a phosphodiester bond between the 4-OH of fructose and the 2-OH of arabinose; agrocinopine B can be derived from agrocinopine A by removal of glucose (Ryder *et at.* 1984). Synthesis of agrocinopine A is encoded by the agrocinopine synthase (*acs*) gene located on the T-DNA of nopaline Ti plasmids and on the TL DNA of agropine/mannopine *A. rhizogenes* Ri plasmids (Joos *et al.* 1983; Willmitzer *et al.* 1983; Slightom *et al.* 1986). It has been proposed that the agrocinopine catabolism (*acc*) genes of strains A4, K84, and C58 differ (Hayman and Farrand 1990).

The agrocinopine permease genes of pTiC58 play an important role in the sensitivity of nopaline strains towards agrocin 84, a highly specific antibiotic produced by the avirulent biotype II *A. radiobacter* strain K84. Agrocin 84 is a fraudulent nucleotide (Tate *et al.* 1979; Murphy *et al.* 1981) which is taken up by the agrocinopine permease of nopaline strains and blocks DNA synthesis (Kerr and Htay 1974; Das *et al.* 1978; Ellis *et al.* 1979). Agrocinopine A stimulates the uptake of agrocin 84 (Ellis and Murphy 1981).

Agrocinopine A induces the transfer of nopaline-type Ti plasmids such as pTiC58 (Ellis *et al.* 1982a, 1982b). In octopine strains, octopine is the conjugal inducer (Petit *et al.* 1978).

The classical octopine and nopaline strains belong to the biotype I group. However, octopine and nopaline strains are also found in the biotype III group. This group has recently been renamed *A. vitis,* because of its specific association with grapevine (*Vitis vinifera*) (Ophel and Kerr 1990). The o/c Ti plasmids carry two separate T regions, TA and TB (Buchholz and Thomashow 1984; Knauf *et al.* 1984; Yanofsky *et al.* 1985; Huss *et al.* 1989; Paulus *et al.* 1989a). The TA region is very similar to the TL region of the biotype I octopine strains, whereas the TB region is specific for the o/c strains and carries auxin biosynthesis (*iaa*) genes and the cucumopine synthase (*cus*) gene (Paulus *et al.* 1989a). O/c Ti plasmids can be divided into "large TA" and "small TA" plasmids based on the structure of their TA regions (Paulus *et al.* 1989a, 1991a, 1991b). "Small TA" regions result from the insertion of two copies of the bacterial insertion sequence IS*868* in the large ancestral TA region and loss of the internal region by recombination between the two IS copies. The sequences that are common between "large TA" and "small TA" strains are 99.7% homologous; because of this, the different forms must be of recent evolutionary origin. The high sequence conservation and the availability of many independent isolates have enabled us to reconstruct the evolutionary history of the TA region (Paulus *et al.* 1991b). In the course of these studies it was noticed that the large TA region of

MPMI, Vol. 6, No. 3, pp. 393-402

pTiTm4 carried a 1.3-kb region of unknown origin, not found in the TL region of biotype I octopine Ti plasmids. Here we show that this region corresponds to a functional *acs* gene which was probably part of the ancestor octopine T-region which gave rise to the TL region of biotype I octopine Ti plasmids and the TA region of biotype III o/c Ti plasmids.

RESULTS

Sequence of the Tm4 *acs* region.

In the course of a sequence project to determine the left end structure of the pTiAB3 TA region it was noted (F. Paulus, unpublished) that this region showed nucleotide homology with the agrocinopine synthase (*acs*) gene of the *A. rhizogenes* Ri plasmid pRiA4 (Slightom *et al.* 1986). Since the putative pTiAB3 *acs* gene was expected to be truncated due to the large internal TA deletion characteristic for "small TA" strains (Paulus *et al.* 1991b), we first determined the sequence of the corresponding area of the intact TA region of the "large TA" plasmid pTiTm4. The restriction map of the TA region of the Ti plasmid of the wide host range o/c strain Tm4 has been described (Paulus *et al.* 1989a; 1991b). Its organization and that of the related TL region from octopine-type Ti plasmids present in biotype I strains (Gielen *et al.* 1984) are presented in Figure 1. The pTiTm4 TA region differs from the TL region by the absence of genes 6a and 7, the presence of the insertion element IS*866* in the TA-*iaaH* gene and a pTiTm4-specific fragment close to the left TA border.

The sequence of this specific fragment was determined (Fig. 2). It is 1,336 bp long and contains a 1,257-bp open reading frame with 59.4% DNA homology to the *acs* gene of the TL region of the Ri plasmid from *A. rhizogenes* strain A4 (Slightom *et al.* 1986, Fig. 3). This ORF is preceded by the sequence CCACTACATTATT resembling the upstream activator element of the Ach5 *ipt* gene (de Pater 1987) and a TATA box (TATGAAT) 89 bp in front of the ATG start codon. Five possible polyadenylation sites and five transcription termination enhancement sites

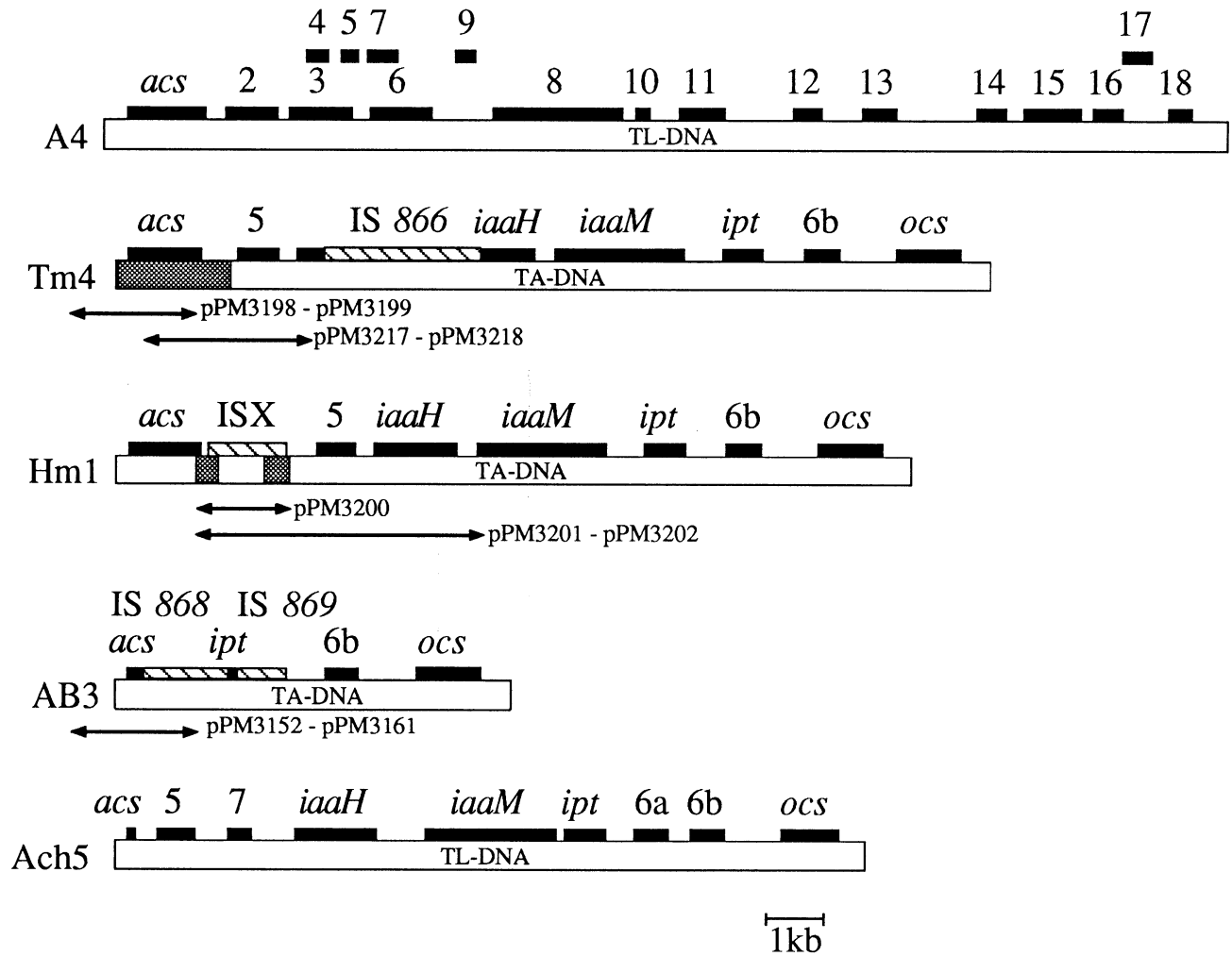

Fig. 1. Maps of T-DNAs used in this work. pRiA4: Slightom *et al.* (1986). pTiTm4, pTiHm1, and pTiAB3: Paulus *et al.* (1991b) and this work. pTiAch5: Gielen *et al.* (1984). Fragments shown are delimited by T-DNA borders. Numbers on pRiA4 TL DNA indicate ORFs. *acs:* agrocinopine synthase gene; *iaaM*: tryptophan monooxygenase gene; *iaaH*: indole acetamide hydrolase gene; *ipt*: isopentenyl transferase gene; *ocs:* octopine synthase gene. Black boxes: ORFs; hatched boxes: insertion elements; gray boxes: areas sequenced in this work. Positions of pPM clones used in this work are indicated.

(YGTGTTYY) are found after the stop codon, TAA, at the positions indicated in Figure 2. The predicted pTiTm4 *acs* amino acid sequence is compared with the corresponding pRiA4 sequence in Figure 4. The predicted sizes of the proteins are 419 and 443 amino acid residues, respectively; amino acid homology is 61%.

Biotype I octopine Ti plasmid contains a truncated *acs* gene.

The presence of an apparently intact *acs* gene on the pTiTm4 TA region prompted us to search for similar sequences in the homologous *A. tumefaciens* biotype I octopine-type TL region of pTiAch5 (Gielen *et al.* 1984). A

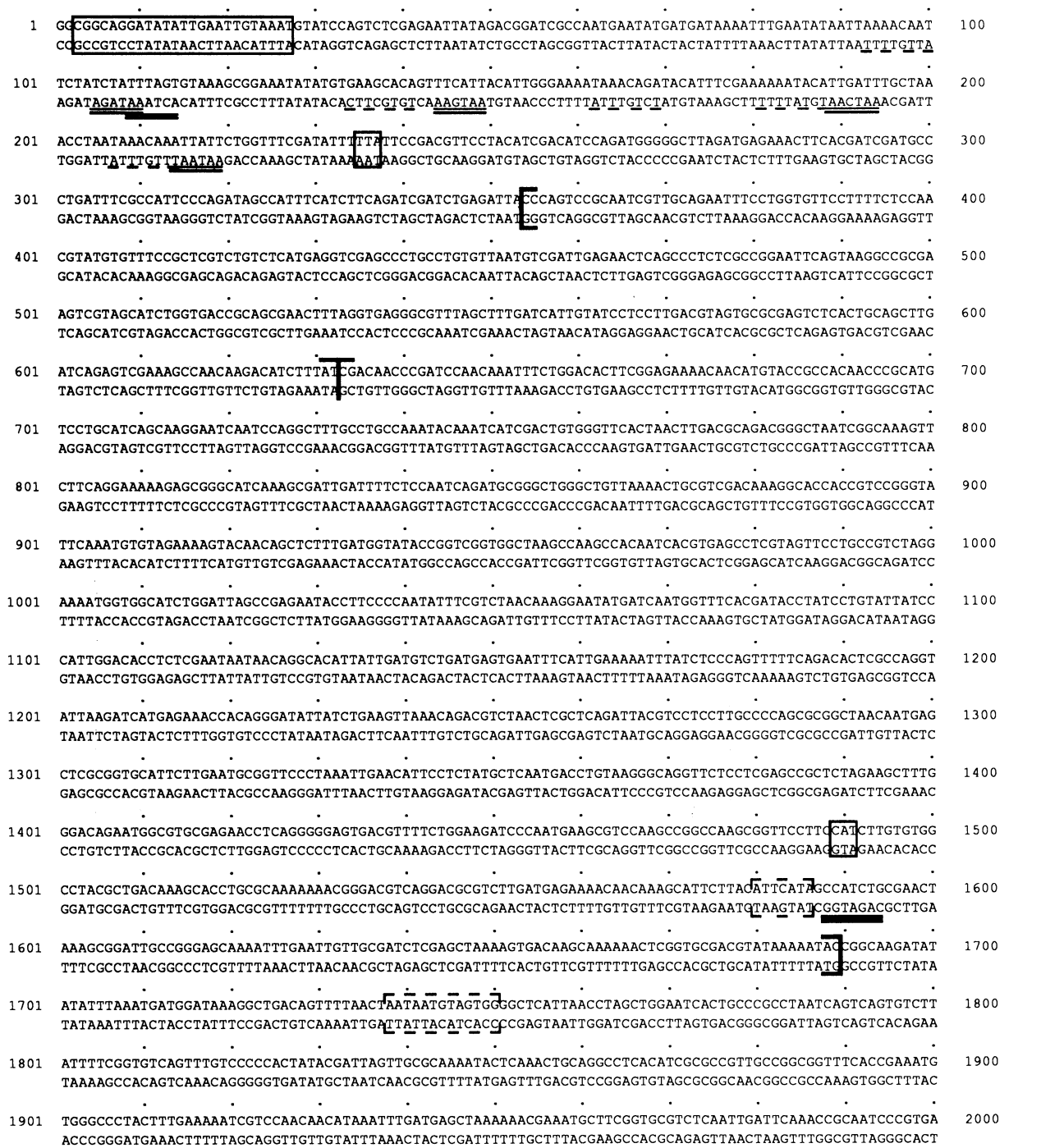

Fig. 2. Sequence of pTiTm4 *acs* gene. Large boxed region: left TA border; small boxed regions: start and stop codon. Hatched boxes: putative upstream activator element (large box) and putative TATA box (small box). Doubly underlined: putative polyadenylation sites. Stippled underlined: putative transcription termination sites. Brackets indicate area deleted in pTiAch5. Large T: site of IS*868* insertion in pTiAB3. Underlined: sequence duplicated in pTiHm1, ISX is inserted immediately to the right of this sequence.

119-nucleotide fragment representing the 3′ part of the *acs* coding sequence, with 90.8% DNA homology to the pTiTm4 T-*acs* gene was found at coordinates 547–665 of pTiAch5. In Figure 3 the truncated pTiAch5 *acs* fragment is aligned with the corresponding pTiTm4 sequence. The first possible polyadenylation site after the stop codon (Fig. 2) is conserved in both sequences (not shown). The region between the 3′ end of the *acs* gene and the left border

Fig. 3. Sequence comparison between *acs* genes from three different Ti/Ri plasmids. Sequences of pRiA4 (Slightom *et al.* 1986), pTiTm4 (this work), and pTiAch5 (Gielen *et al.* 1984) *acs* genes are compared. Boxed areas: start and stop codon.

of the T-DNA shows only 60% homology. The 25-bp-long left borders are found at 208 bp (pTiTm4) and 216 bp (pTiAch5) from the *acs* stop codon and show only one mismatch.

acs genes in other o/c strains.

The TA region of the Ti plasmid of strain Hml was mapped earlier (Paulus *et al.* 1989a): it differs from that of pTiTm4 at two positions: it lacks IS*866* (inserted in the TA-*iaaH* gene) and has an additional, pTiHml-specific 1.3-kb DNA element located to the left of the TA-*iaaH* gene (Paulus *et al.* 1991b; L. Otten, unpublished). More precise restriction mapping of the cloned pTiHml TA region and comparison with pTiTm4 maps enabled us to locate and sequence the ends of the element: it is situated 104 bp upstream of the *acs* start codon, 9 bp 5′ of the putative TATA box, and about 150 bp downstream of an activatorlike sequence (Fig. 2). Comparison of the pTiHml and pTiTm4 TA sequences shows that the extremities of the element form an imperfect palindrome of 15 bp (left inverted repeat GACATCTCTAAAAACC right inverted repeat GGTATTTCGAGGTGT, mismatches underlined); the element is flanked by a direct 7 bp duplication (CCATCTG). These features strongly indicate that the element is a bacterial insertion sequence, provisionally called ISX.

The sequence of the *acs* region in pTiAB3 was also determined (Fig. 2). The pTiAB3 *acs* gene was found to be partially deleted. A fragment of 398 nucleotides of the 3′ end of the coding sequence of the *acs* gene (corresponding to pTiTm4 TA coordinates 235–632) has been conserved. The sequence of this fragment is identical to the corresponding pTiTm4 sequence.

Agrocinopine A synthesis in different tumors.

Sterile Tm4-, Hml-, AB3-, and C58-induced tumor tissues were tested for agrocinopine A synthesis by incubation of liquid cultures with $^{32}PO_4^-$ and analysis of the culture supernatants by paper electrophoresis (Messens *et al.* 1985). Agrocinopine A was identified by its electrophoretic properties in citrate buffer and by virtue of its specific removal from culture medium by *Agrobacterium* strain C58 but not by the Ti plasmid-cured C58 strain GV3101. After 1 and 2 wk of incubation, tumors induced by C58 and Tm4, but not by AB3 or Hml were found to release labeled agrocinopine A into the medium (results not shown). In only two out of 10 Tm4-induced tumor lines and two out of 10 C58-induced tumor lines agrocinopine A could be demonstrated. Variability in agrocinopine A synthesis has also been noted by others (Messens *et al.* 1985). Incorporation of ^{32}P into agrocinopine A was low: even after 2 wk less than 1% of the radioisotope was incorporated.

Agrocinopine A uptake.

^{32}P-labeled agrocinopine A was purified from a C58-induced tumor line incubated with $^{32}PO_4^-$ by paper electrophoresis and used to measure removal from culture medium by various *Agrobacterium* strains. Silver nitrate staining and quantification with phloroglucinol showed

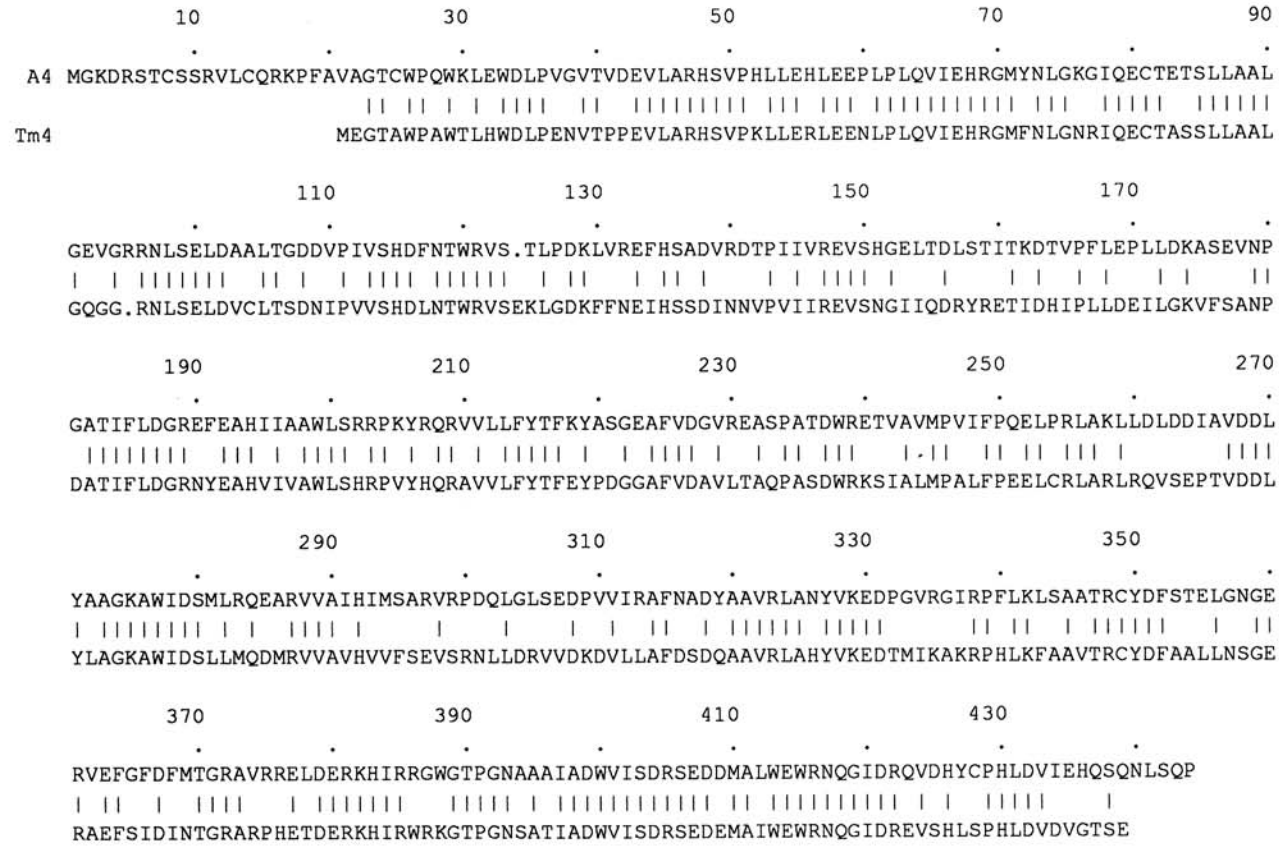

Fig. 4. Deduced amino acid sequences of *acs* gene of pRiA4 (upper line) and pTiTm4 (lower line).

that the amounts of labeled agrocinopine A per incubation experiment were less than 70 pmoles/μl. In initial experiments, samples of the bacterial culture supernatants were spotted onto paper. These experiments showed that *Agrobacterium* strains GV3101(pTiC58), GV3101(pTiTm4), Tm4, 2608, 2641, and K305 readily removed agrocinopine A from the medium (more than 90% within 2 hr), whereas strains AB3, LBA649, Ag57, and AB4 did not (not shown). Hm1 only removed small amounts (less than 10%). Since both Tm4 and GV3101 (pTiTm4) removed agrocinopine A, pTiTm4 probably encodes agrocinopine A uptake genes. After 2 hr of incubation, radioactivity in supernatants of cultures of strains Tm4, 2608, 2641, and K305 increased again. Electrophoretic analysis of these supernatants showed the presence of a new compound migrating twice as fast as agrocinopine A and representing about 30–40% of the initial radioactivity after 25 hr of incubation (Fig. 4). This compound may represent an agrocinopine A degradation product. It was not found in supernatants from strains GV3101(pTiC58) or GV3101(pTiTm4), showing that its appearance does not depend on the Ti plasmid but probably on the chromosomal background. Supernatants from cultures of strain AB3 did not contain this product, supernatants from strain Hm1 showed traces of it (Fig. 5). The kinetics for agrocinopine A removal demonstrate that strains GV3101 and AB3 only remove traces of agrocinopine A and that removal by strain GV3101 (pTiTm4) is at least as efficient as removal by strain GV3101 (pTiC58) (Fig. 6). Since in the case of AB3 and Hm1 agrocinopine A uptake may become measurable only after preinduction by agrocinopine A, strains GV3101 (pTiC58), GV3101, GV3101(pTiTm4), Tm4, Hm1, and AB3 were grown in precultures containing unlabeled purified agrocinopine A as described in Materials and Methods. Agrocinopine A removal was analyzed by paper electrophoresis. Whereas GV3101(pTiC58), GV3101(pTiTm4), Tm4, and Hm1 had removed agrocinopine A after 24 hr, GV3101 and AB3 did not remove these compounds, even after 48 hr. These experiments were carried out in duplicate (results not shown).

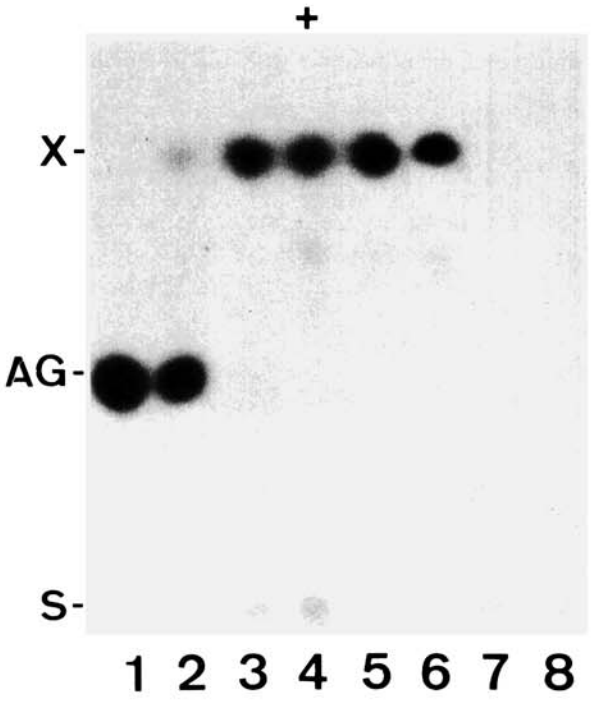

Fig. 5. Removal of radiolabeled agrocinopine A from the culture medium by different *Agrobacterium* strains. Electrophoretogram of supernatants of bacterial cultures after 25 hr of incubation with ^{32}P-labeled agrocinopine A. 1, AB3; 2, Hm1; 3, 2641; 4, 2608; 5, K305; 6, Tm4; 7, GV3101(pTiTm4); 8, GV3101(pTiC58). S, start. AG, agrocinopine. X, unknown compound. +, anode. Whereas strains AB3 and Hm1 remove very little agrocinopine A from the medium, the other strains do so efficiently. The *A. vitis* strains 2641, 2608, K305, and Tm4 (and to a much lesser extent Hm1) release an unknown compound (X) after prolonged incubation, GV3101(pTiTm4) and GV3101(pTiC58) do not.

Search for homology between pTiTm4 and agrocinopine uptake genes of pTiC58.

The results of the studies described above indicate that pTiTm4 harbors agrocinopine A uptake genes. We therefore searched for homology with the corresponding pTiC58 genes. The *Eco*RI-26 fragment and the *Hin*dIII-11 fragment of pTiC58 clone pGV0347 containing the agrocinopine catabolism (*acc*) genes (Holsters *et al.* 1980; Hayman and Farrand 1988) were hybridized against *Eco*RI- and *Hin*dIII-digested pTiTm4 DNA and against total DNA from the following strains: C58, Tm4, K305, 2608, 2641, NW244, Hm1, AB3, Ag57, and AB4 (not shown). C58 DNA hybridized strongly as expected. In the case of the *A. vitis* strains, only a weakly hybridizing *Eco*RI band of 6.8-kb was found for Hm1, and weakly hybridizing *Hin*dIII fragments for Hm1 (9 kb), 2608, and 2641 (10.5 kb). These bands were not further investigated.

Sensitivity of *acs* strains towards K84.

Sensitivity of *Agrobacterium* strains GV3101, GV3101 (pTiTm4), GV3101(pTiC58), Tm4, 2641, 2608, K305, Hm1, AB3, Ag57, LBA649, and AB4 to agrocin 84 was tested with the inhibition assay described by Hayman and Farrand (1988). Whereas K84 produced large zones of inhibi-

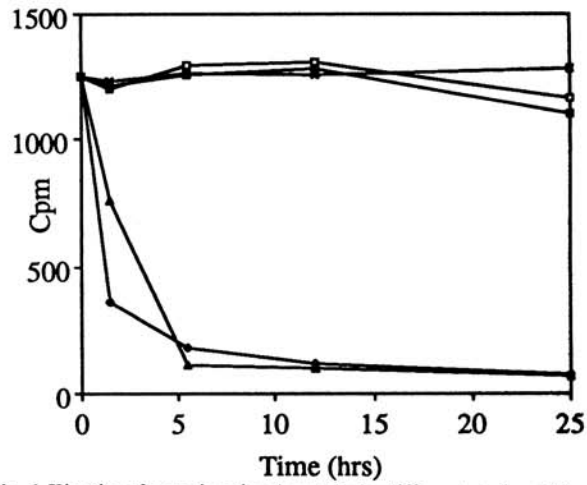

Fig. 6. Kinetics of agrocinopine A uptake by different strains. Whereas GV3101(pTiTm4) and GV3101(pTiC58) remove agrocinopine A from the medium, AB3 and GV3101 do not. Thus, agrocinopine A removal is encoded by pTiTm4. ≈, GV3101(pTiTm4); ▲, GV3101(pTiC58); ■, AB3; □, GV3101; x, no bacteria.

tion (mean radius of 15 mm) in the case of GV3101 (pTiC58), no inhibition was observed for the other strains. In the presence of 30 nmol purified agrocinopine A, the radius of the inhibition zones for GV3101(pTiC58) increased by 4–5 mm; GV3101 or GV3101(pTiTm4) remained insensitive. Thus, although pTiTm4 confers agrocinopine A uptake, it does not render cells sensitive to agrocin 84. It has been reported (Dhanvantari 1983) that some *Agrobacterium* strains isolated from grapevine are sensitive to agrocin 84 when grown on a modified Stonier medium. We therefore also tested the following strains on this medium: GV3101, GV3101(pTiTm4), Tm4, K305, 2608, 2641, AB3, Ag57, Hm1, and AB4. All except GV3101(pTiC58) (inhibition radius of 27 mm) were insensitive to agrocin 84. Growth on the same medium in the presence of 0.4 mM agrocinopine A was tested for GV3101, GV3101 (pTiTm4), Tm4, AB3, Hm1, and GV3101(pTiC58). Whereas GV3101(pTiC58) showed increased sensitivity in the presence of agrocinopine A (inhibition radius of 33 mm), the other strains remained insensitive.

DISCUSSION

The TA region of the o/c *Agrobacterium* Ti plasmid pTiTm4 carries an intact agrocinopine synthase gene that encodes agrocinopine A production in Tm4-induced tumors. This *acs* gene has 59.4% DNA homology with the pRiA4 *acs* gene, the predicted amino acid sequences are 61% homologous. The Tm4 *acs* gene has 89.5% DNA homology and 88.6% predicted amino acid homology with the recently sequenced *acs* gene from the classical nopaline Ti plasmid pTiC58 (M. Sormann, personal communication). We propose to name the pTiTm4 *acs* gene T-*acs;* the A4 and C58 gene could be called R-*acs* and C-*acs,* respectively. The Tm4 Ti plasmid most probably also carries one or several genes for agrocinopine A uptake as shown by the disappearance of unlabeled and radiolabeled agrocinopine A from culture supernatants of GV3101 (pTiTm4) but not of GV3101. It should be noted that binding of agrocinopine A to *Agrobacterium* cells without uptake cannot yet be excluded. Three other o/c strains with Ti plasmids with pTiTm4-like TA regions (2608, 2641, and K305) likewise remove radiolabeled agrocinopine A from the medium. After longer incubation, Tm4, K305, 2608, and 2641 release an as yet unidentified ^{32}P-labeled compound which represents up to 40% of the initial label and may represent an agrocinopine A breakdown product. The appearance of this compound is dependent on the chromosomal background and not on the Ti plasmid, and is not seen in culture supernatants of biotype I strains.

In spite of an agrocinopine A uptake system, K305 and other biotype III strains have been reported to be resistant against K84 (Kerr and Panagopoulos 1977; Loubser 1978; Panagopoulos *et al.* 1978; Burr and Hurwitz 1981; van Zyl *et al.* 1986). The biotype III strains used in this study are not sensitive to agrocin 84 under conditions in which strain C58 is sensitive, even in the presence of agrocinopine A, which increases the sensitivity of C58 to agrocin 84. Possibly, the uptake of agrocinopine A by Tm4-like strains does not allow uptake of agrocin 84 or the antibiotic is inactivated upon entry into the cell. Alternatively, o/c strains may become sensitive under certain conditions. *Agrobacterium* grapevine isolates of unknown opine type were reported to be sensitive to agrocin 84 (Dhanvantari 1983). Using the conditions described by this author, we were unable to obtain inhibition, with or without agrocinopine A. Possibly, the discrepancy is due to differences between the strains used.

Although pTiTm4 most likely encodes an agrocinopine A uptake system, it does not hybridize to the pTiC58 *acc* genes. A similar situation has been found for pAtK84, which encodes agrocinopine A uptake (Ryder *et al.* 1987) but has only little homology with the pTiC58 *acc* region (Hayman and Farrand 1988). This has led Hayman and Farrand to propose that the pAtK84-encoded agrocinopine permease differs from the agrocin 84-sensitive pTiC58 permease. The same may hold for the pTiTm4 permease.

The only *A. vitis* nopaline strain studied here (AB4) is insensitive to agrocin 84 and does not take up agrocinopine A. Its DNA does not hybridize to *acc* genes from pTiC58. Further studies should establish whether pTiAB4 differs in structure from pTiC58 and whether the T-DNA of pTiAB4 contains an *acs* gene.

Strains AB3 and Hm1 have lost the capacity to induce agrocinopine synthesis: A large part of the pTiAB3 *acs* gene was deleted, whereas the 5′ noncoding region of the pTiHm1 *acs* gene underwent insertion by an Hm1-specific bacterial insertion sequence which remains to be characterized. Strain AB3 also lost the capacity to remove agrocinopine A from the culture medium; at low agrocinopine A concentrations the uptake by Hm1 seems to differ from the uptake properties of other "large TA" strains.

Most probably, the as yet unidentified agrocinopine utilization genes were part of the pTiTm4/pTiAB3/pTiHm1 ancestor Ti plasmid. The reason for the loss of a functional agrocinopine A synthesis and utilization system within the o/c Ti plasmid family is unknown. The loss of both functions in the case of pTiAB3 (and their loss and possible modification in the case of pTiHm1) may have occurred in several ways. Agrocinopine A uptake could have become a handicap in the presence of agrocin 84 producers. In that case, the loss of the uptake function would have preceded the loss of the synthesis gene. Alternatively, agrocinopine A synthesis may be deleterious to tumor growth or to the production of other opines. In that case, synthesis genes may have been lost first. Both possibilities seem somewhat unlikely since Tm4 and similar strains that induce agrocinopine A synthesis and take up agrocinopine A are among the most common o/c isolates (Paulus *et al.* 1989a, 1989b).

Possibly, agrocinopine A uptake by *A. vitis* strains like Tm4 is not followed by efficient utilization. The secretion of large amounts of an agrocinopine A-derived compound by Tm4, but not by C58, points in this direction. If utilization is inefficient, both synthesis and uptake genes may have little selective value and could be lost without severe consequences. Identification and sequence analysis of the uptake genes encoded by the Ti plasmids from Tm4, Hm1, and AB3 may indicate in which order the different functions were lost.

An *acs*-like sequence is also found in the TL region of the biotype I octopine Ti plasmid pTiAch5: This se-

quence most probably represents a 5′-deleted *acs* gene and would have been part of the "common DNA" found on octopine and nopaline T-DNAs (Engler *et al.* 1981). The truncated pTiAch5 *acs* gene has recently also been noticed by comparison with the unpublished *acs* sequence of the biotype I nopaline T region (M. Sormann, personal communication). TL/TA sequence comparisons did not provide a clue to how this deletion occurred. If biotype I octopine Ti plasmids originally induced agrocinopine A synthesis, they also may have carried an agrocinopine A uptake and degradation system. Identification of the pTiTm4 *acc* sequences may lead to the detection of *acc*-like sequences on biotype I octopine Ti plasmids.

In biotype I nopaline bacteria like C58, agrocinopine A induces the conjugal transfer of the Ti plasmid. It would be interesting to test whether the transfer of pTiTm4, pTiHm1, and pTiAB3 can likewise be induced by agrocinopine A and/or octopine. Such studies may also shed light on the differences and similarities between agrocinopine A- and octopine-induced transfer systems and on their respective evolutionary origins.

MATERIALS AND METHODS

Strains and plasmids.

Bacterial strains and plasmids used are listed in Table 1.

Cloning and sequencing procedures.

pTiTm4 TA region. The 2.9-kb *Eco*RI-*Bam*HI fragment from the pTiTm4 TA region was isolated, filled in by T4 DNA polymerase and cloned in both orientations in *Sma*I-digested pKS(−) (Stratagene, La Jolla, CA), yielding pPM3217 and pPM3218. Deletions were made with exonuclease III and resulting clones were sequenced. The 2.0 *Hin*dIII TA fragment was cloned in pKS(−) (pPM3198) and pKS(+) (pPM3199) in one orientation. The remaining part of the TA region (including the left border sequence) was sequenced with oligonucleotides derived from the overlapping sequences of pPM3217 and pPM3218 and from the sequences of pPM3152 and pPM3161 (Paulus *et al.* 1991a) (Fig. 1).

pTiHm1 TA region. The 5.0-kb *Hin*dIII fragment of pTiHm1 (from pPM1040, Paulus *et al.* 1991b) was cloned in pKS(−) in both orientations, yielding pPM3202 and pPM3201. pPM3200 was derived from pPM3201 by *Xho*I digestion and religation, leaving the *Hin*dIII-*Xho*I TA fragment (Fig. 1). Sequences were established starting from the *Hin*dIII site of pPM3202 and the *Xho*I site of pPM3200.

Sequences were determined as described by Sambrook *et al.* (1989) and analyzed with a microVAX using GCG software (Devereux *et al.* 1987).

DNA hybridization.

DNA hybridization was performed as described by Sambrook *et al.* (1989).

Labeling of agrocinopine A.

^{32}P-labeled agrocinopine A was prepared according to Messens *et al.* (1985). Tumors were induced on *Nicotiana rustica* in the greenhouse. One-month-old tumor tissue was excised, placed on solid Linsmaier and Skoog (LS) medium (Linsmaier and Skoog 1965) supplemented with 500 mg/L cefotaxim (Claforan, Hoechst), and subcultured monthly.

Table 1. Strains and plasmids used in this study

Name	Description	Reference
E. coli strains		
NM522	*sup*E *thi* Δ(*lac-proAB*) *hsd*Δ5 F′ *proAB lacI*^{q} *lacZ*ΔM15	Murray, University of Edinburgh, U.K.
Agrobacterium strains		
C58	*A. tumefaciens* nopaline strain	Zimmerer *et al.* 1966
GV3101	Cured C58, rif resistant	Depicker *et al.* 1980
GV3101(pTiC58)	rif resistant C58 derivative	Depicker *et al.* 1980
Tm4	*A. vitis*, "large TA" o/c strain	Szegedi 1985
GV3101(pTiTm4)	GV3101 carrying pTiTm4	Huss *et al.* 1989
Hm1	*A. vitis*, "large TA" o/c strain	Szegedi 1985
K305	idem	Brisbane and Kerr 1983
2608	idem	Paulus *et al.* 1989a
2641	idem	Paulus *et al.* 1989a
NW244	idem	Paulus *et al.* 1989a
AB3	*A. vitis*, "small TA" o/c strain	Szegedi 1985
Ag57	idem	Panagopoulos and Psallidas 1973
LBA649	C58C9(pTiAg57)	Hoekema *et al.* 1984
AB4	*A. vitis* nopaline strain	Szegedi 1985
pKS(−) and pKS(+)	pBluescript vectors	Stratagene, La Jolla, CA
pPM1040	5.0 kb *Hin*dIII (TA Hm1) in pUC18	Paulus *et al.* 1991b
pPM3152	2.2 kb *Hin*dIII (TA AB3) in pKS(−), one orientation	Paulus *et al.* 1991a
pPM3161	2.2 kb *Hin*dIII (TA AB3) in pKS(−), opposite orientation	Paulus *et al.* 1991a
PPM3198	2.0 kb *Hin*dIII (TA Tm4) in pKS(−)	This study
pPM3199	2.0 kb *Hin*dIII (TA Tm4) in pKS(+)	This study
pPM3200	1.6 kb *Hin*dIII-*Xho*I(TA Hm1) in pKS(−)	This study
pPM3201	5.0 kb *Hin*dIII (TA Hm1) in pKS(−), *acs* away from *Kpn*I polylinker site	This study
pPM3202	5.0 kb *Hin*dIII (TA Hm1) in pKS(−), opposite orientation	This study
pPM3217	2.9 kb *Eco*RI-*Bam*HI (TA Tm4) in pKS(−), *Eco*RI site close to *Kpn*I polylinker site	This study
pPM3218	2.9 kb *Eco*RI-*Bam*HI(TA Tm4) in pKS(−), opposite orientation	This study

Small tissue fragments (about 100 mg) were tested for agrocinopine A synthesis by incubation in 0.2 ml of liquid K3 medium (Kao *et al.* 1974) with Claforan, supplemented with $^{32}PO_4^-$ (8 μCi/ml, 8,500 Ci/mmole) and buffered with 50 mM MES, pH 5.8. After 1 or 2 wk, the medium was analyzed by a modified paper electrophoresis method: 2 μl of medium was spotted on Whatman 3MM paper, and the paper was carefully wetted with 50 mM citrate buffer (pH 7.0), without touching the sample positions. The paper was fitted in a standard DNA electrophoresis tank in such a way that the part on which the samples were migrating did not touch the bottom of the tank. Electrophoresis was carried out at 10 V/cm for 2 hr. After electrophoresis, the paper was dried and exposed in a cassette with intensifying screens to Kodak X-Omat film for varying periods of time. Culture supernatants from tumors induced by C58 yielded only two spots: one heavy spot (PO_4^-) and a weaker spot corresponding to agrocinopine A (as also noted by Messens *et al.* 1985). Agrocinopine A was eluted with water and stored at −20° C.

Purification of unlabeled agrocinopine A.

Kalanchoe daigremontiana stems were infected with the wild-type *A. tumefaciens* strain C58. Agrocinopine A was extracted from 2-mo-old tumors according to Ellis and Murphy (1981). Tumor tissue was crushed and macerated in 70% ethanol (1–2 ml/g fresh weight). The extract was filtered and cleared by centrifugation, dried by rotatory evaporation, and dissolved in 70% ethanol (one tenth of the original volume). Agrocinopine A was purified by preparative paper electrophoresis as follows: 1 ml of concentrated extract was applied onto Whatman 3MM paper in a narrow band over a length of 12 cm. Electrophoresis in formic acid/acetic acid/water (2:8:90, v/v/v), pH 1.7, was carried out at 25 mA in a DNA electrophoresis tank as described above. Xylene cyanol was used as a marker. Electrophoresis was stopped when the marker had reached a distance of 6 cm. At the position of the marker, the paper was cut into four 1-cm-wide strips. The liquid was recovered by centrifugation in Eppendorf tubes, lyophilized with a Speedvac, and dissolved in 100 μl of water. The different fractions were analyzed by paper electrophoresis in 0.1 M NH_4HCO_3 buffer (pH 9.2) at 25 mA. The paper was dried at 80° C and agrocinopine A was stained by alcaline silver nitrate (Dahl *et al.* 1983). Fractions containing agrocinopine A from different preparative electrophoresis experiments were combined, and in some cases further purified by preparative paper electrophoresis in 0.1 M NH_4HCO_3 buffer (pH 9.2). Agrocinopine A was quantified and expressed in arabinose equivalents by the phloroglucinol method according to Dische and Borenfreund (1957) and Ellis and Murphy (1981).

Uptake of ^{32}P-agrocinopine A.

Uptake of agrocinopine A by different strains was tested by adding 40 μl of tenfold concentrated bacteria (harvested at early stationary phase) to 360 μl of ^{32}P-agrocinopine A-containing YEB (Leemans *et al.* 1983) medium. After incubation at 28° C, the cultures were centrifuged in an Eppendorf centrifuge, and a portion of the supernatant was electrophoresed as described above or its radioactivity was determined in a Beckman scintillation counter after addition of 5 ml of Ready Gel (Beckman).

Uptake of unlabeled agrocinopine A.

Bacteria were pregrown in liquid YEB for 24 hr. These cultures served to start liquid cultures in 100 μl of AB glucose medium (Lichtenstein and Draper 1985) with or without 0.4 mM agrocinopine A. After growth for 24 hr, optical density was measured, bacteria were concentrated by centrifugation, and resuspended in AB medium at a concentration of 10^8 cells/ml. Three microliter bacterial suspensions were added to 90 μl AB medium with 0.4 mM agrocinopine A. After 24 and 48 hr, agrocinopine A content was measured by paper electrophoresis as described above.

Agrocin 84 inhibition assay.

The inhibition of bacterial growth by agrocin 84, both in the absence and in the presence of agrocinopine, was tested according to Hayman and Farrand (1988), using FMS medium plates (Formica 1990) or plates with the modified Stonier medium used by Dhanvantari (1983).

GenBank accession numbers.

GenBank accession numbers of sequences determined in this study are M91189 (pTiTm4 sequences) and M91188 (pTiAB3 sequences).

ACKNOWLEDGMENTS

We thank J. Schell for pGV347 and M. Sormann, J. De Lafonteyne, and M. van Montagu for communicating results prior to publication. We thank the Hoechst Company for a free sample of Claforan.

LITERATURE CITED

Brisbane, P. G., and Kerr, A. 1983. Selective media for three biovars of *Agrobacterium*. J. Appl. Bacteriol. 54:425-431.

Buchholz, W. G., and Thomashow, M. F. 1984. Comparison of T-DNA oncogene complements of *Agrobacterium tumefaciens* tumor-inducing plasmids with limited and wide host ranges. J. Bacteriol. 160:319-326.

Burr, T. J., and Hurwitz, B. 1981. Occurrence of *Agrobacterium radiobacter* pv. *tumefaciens* (Smith and Townsend) Conn biotype 3 on grapevines in New York State. (Abstr.) Phytopathology 71:206.

Dahl, G. A., Guyon, P., Petit, A., and Tempé, J. 1983. Silver nitrate-positive opines in Crown gall tumors. Plant Sci. Lett. 32:193-203.

Das, P. K., Basu, M., and Chatterjee, G. C. 1978. Studies on the mode of action of agrocin 84. J. Antibiot. 31:490-492.

de Pater, S. 1987. Plant expression signals of the *Agrobacterium* T-cyt gene. Ph.D. thesis. University of Leiden, The Netherlands.

Depicker, A., De Wilde, M., De Vos, G., De Vos, R., Van Montagu, M., and Schell, J. 1980. Molecular cloning of overlapping segments of the nopaline Ti-plasmid pTiC58 as a means to restriction endonuclease mapping. Plasmid 3:193-211.

Devereux, J., Haeberli, P., and Marquess, P. 1987. The program manual for the sequence analysis software package of the Genetics Computer Group. Nucleic Acids Res. 12:387-395.

Dhanvantari, B. N. 1983. Etiology of grape crown gall in Ontario. Can. J. Bot. 61:2641-2646.

Dische, Z., and Borenfreund, E. 1957. A new color reaction for the determination of aldopentose in presence of other saccharides. Biochim. Biophys. Acta 23:639-642.

Ellis, J. G., and Murphy, P. J. 1981. Four new opines from crown gall tumors—Their detection and properties. Mol. Gen. Genet. 181:36-43.

Ellis, J. G., Kerr, A., Van Montagu, M., and Schell, J. 1979. *Agrobac-*

terium: Genetic studies on agrocin 84 production and the biological control of crown gall. Physiol. Plant Pathol. 15:311-319.

Ellis, J. G., Kerr, A., Petit, A., and Tempé, J. 1982a. Conjugal transfer of nopaline and agropine Ti plasmids: The role of agrocinopines. Mol. Gen. Genet. 186:269-274.

Ellis, J. G., Murphy, P. J., and Kerr, A. 1982b. Isolation and properties of transfer regulatory mutants of the nopaline Ti plasmid pTiC58. Mol. Gen. Genet. 186:275-281.

Engler, G., Depicker, A., Maenhaut, R., Villarroel, R., Van Montagu, M., and Schell, J. 1981. Physical mapping of DNA base sequence homologies between an octopine and a nopaline Ti plasmid of *Agrobacterium tumefaciens*. J. Mol. Biol. 152:183-208.

Formica, J. 1990. Conditions for the production of agrocin 84 by *Agrobacterium radiobacter* K84. J. Ind. Microbiol. 5:33-44.

Gielen, J., De Beuckeleer, M., Seurinck, K., Deboeck, F., De Greve, H., Lemmers, M., Van Montagu, M., and Schell, J. 1984. The complete nucleotide sequence of the TL DNA of the *Agrobacterium tumefaciens* plasmid pTi Ach5. EMBO J. 3:835-846.

Hayman, G. T., and Farrand, S. K. 1988. Characterization and mapping of the agrocinopine-agrocin 84 locus on the nopaline Ti plasmid pTiC58. J. Bacteriol. 170:1759-1767.

Hayman, G. T., and Farrand, S. K. 1990. *Agrobacterium* plasmids encode structurally and functionally different loci for catabolism of agrocinopine-type opines. Mol. Gen. Genet. 223:465-473.

Hoekema, A., De Pater, B. S., Fellinger, A. J., Hooykaas, P. J. J., and Schilperoort, R. A. 1984. The limited host range of an *Agrobacterium tumefaciens* strain extended by a cytokinin gene from a wide host range T-region. EMBO J. 3:3043-3047.

Holsters, M., Silva, B., Van Vliet, F., Genetello, C., De Block, M., Dhaese, P., Depicker, A. Inzé, D., Engler, G., Villaroel, R., Van Montagu, M., and Schell, J. 1980. The functional organization of the nopaline *A. tumefaciens* plasmid pTiC58. Plasmid 3:212-230.

Huss, B., Bonnard, G., and Otten, L. 1989. Isolation and functional analysis of a set of auxin genes with low root-inducing activity from an *Agrobacterium tumefaciens* biotype III strain. Plant Mol. Biol. 12:271-283.

Joos, H., Inzé, D., Caplan, A., Sormann, M., Van Montagu, M., and Schell, J. 1983. Genetic analysis of T-DNA transcripts in nopaline crown galls. Cell 32:1057-1067.

Kado, C. I. 1991. Molecular mechanisms of Crown Gall tumorigenesis. Crit. Rev. Plant Sci. 10:1-32.

Kao, K. N., Constable, F., Michayluk, M. R., and Gamborg, O. L. 1974. Plant protoplast fusion and growth of intergeneric hybrid cells. Planta 120:215-227.

Kerr, A., and Htay, K. 1974. Biological control of crown gall through bacteriocin production. Physiol. Plant Pathol. 4:37-44.

Kerr, A., and Panagopoulos, C. G. 1977. Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. Phytopathol. Z. 90:172-179.

Knauf, V. C., Yanofsky, M., Montoya, A., and Nester, E. W. 1984. Physical and functional map of an *Agrobacterium tumefaciens* plasmid that confers a narrow host range. J. Bacteriol. 160:564-568.

Leemans, J., Hernalsteens, J-P., Deblaere, R., De Greve, H., Thia-Toong, L., van Montagu, M., and Schell, J. 1983. Genetic analysis of T-DNA and regeneration of transformed plants. Pages 322-330 in: Molecular Genetics of Bacteria-Plant Interaction. A. Pühler, ed. Springer-Verlag, Berlin.

Lichtenstein, C., and Draper, J. 1985. Genetic engineering of plants. Pages 67-119 in: DNA Cloning, Vol. II. A Practical Approach. D. M. Gloor, ed. IRL Press, Oxford.

Linsmaier, E. M., and Skoog, F. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18:100-127.

Loubser, J. T. 1978. Identification of *Agrobacterium tumefaciens* biotype 3 on grapevine in South Africa. Plant Dis. Rep. 62:730-731.

Messens, E., Lenaerts, A., Hedges, R. W., and Van Montagu, M. 1985. Agrocinopine A, a phosphorylated opine is secreted from crown gall cells. EMBO J. 4:571-577.

Murphy, P. J., Tate, M. E., and Kerr, A. 1981. Substituents at N^6 and C-5′ control selective uptake and toxicity of the adenine-nucleotide bacteriocin, agrocin 84, in *Agrobacteria*. Eur. J. Biochem. 115:539-543.

Ophel, K., and Kerr, A. 1990. *Agrobacterium vitis* sp. nov. strains of *Agrobacterium* biovar 3 from grapevines. Int. J. Syst. Bacteriol. 40:236-241.

Panagopoulos, C. G., and Psallidas, P. G. 1973. Characteristics of greek isolates of *Agrobacterium tumefaciens*. J. Appl. Bacteriol. 36:233-240.

Panagopoulos, C. G., Psallidas, P. B., and Alivizatos, A. S. 1978. Studies on biotype 3 of *Agrobacterium radiobacter* var. *tumefaciens*. Pages 221-228 in: Proc. Int. Conf. Plant Pathol. Bacteriol., Angers, 4th.

Paulus, F., Huss, B., Bonnard, G., Ridé, M., Szegedi, E., Tempé, J., Petit, A., and Otten, L. 1989a. Molecular systematics of biotype III Ti plasmids of *Agrobacterium tumefaciens*. Mol. Plant Microbe-Interact. 2:64-74.

Paulus, F., Ridé, M., and Otten, L. 1989b. Distribution of two *Agrobacterium tumefaciens* insertion elements in natural isolates: Evidence for stable association between Ti plasmids and their bacterial hosts. Mol. Gen. Genet. 219:145-152.

Paulus, F., Canaday, J., and Otten, L. 1991a. Limited host range Ti plasmids: Recent origin from wide host range Ti plasmids and involvement of a novel IS element, IS*868*. Mol. Plant Microbe-Interact. 4:190-197.

Paulus, F., Canaday, J., Vincent, F., Bonnard, G., Kares, C., and Otten, L. 1991b. Sequence of the *iaa* and *ipt* region of different *Agrobacterium tumefaciens* biotype III octopine strains: Reconstruction of octopine Ti plasmid evolution. Plant Mol. Biol. 16:601-614.

Petit, A., and Tempé, J. 1985. The function of T-DNA in nature. Pages 625-636 in: Molecular Form and Function of the Plant Genome. L. Van Vloten-Doting, G. S. P. Groot, and T. G. Hall, eds. Plenum, New York.

Petit, A., Tempé, J., Kerr, A., Holsters, M., Van Montagu, M., and Schell, J. 1978. Substrate induction of conjugative activity of *Agrobacterium tumefaciens* Ti plasmids. Nature 271:570-571.

Ryder, M. H., Tate, M. E., and Jones, G. P. 1984. Agrocinopine A, a tumor-inducing plasmid-coded enzyme product, is a phosphodiester of sucrose and L-arabinose. J. Biol. Chem. 259:9704-9710.

Ryder, M. H., Slota, J. E., Scarim, A., and Farrand, S. K. 1987. Genetic analysis of agrocin 84 production and immunity in *Agrobacterium* spp. J. Bacteriol. 169:4184-4189.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Slightom, J. L., Tardif, M. D., Jouanin, L., and Tepfer, D. 1986. Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. J. Biol. Chem. 261:108-121.

Szegedi, E. 1985. Host range and specific L(+)-tartrate utilization of biotype 3 *Agrobacterium tumefaciens*. Acta Phytopathol. Acad. Sci. Hungary 20:17-22.

Tate, M. E., Murphy, P. J., Roberts, W. P., and Kerr, A. 1979. Adenine N^6-substituent of agrocin 84 determines its bacteriocin-like specificity. Nature 280:255-264.

van Zyl, F. G. H., Strijdom, B. W., and Staphorst, J. L. 1986. Susceptibility of *Agrobacterium tumefaciens* strains to two agrocin-producing *Agrobacterium* strains. Appl. Environ. Microbiol. 52:234-238.

Willmitzer, L., Dhaese, P., Schreier, P. H., Schmalenbach, W., Van Montagu, M., and Schell, J. 1983. Size, location and polarity of T-DNA-encoded transcripts in nopaline crown gall tumors; common transcripts in octopine and nopaline tumors. Cell 32:1045-1056.

Winans, S. C. 1992. Two-way chemical signaling in *Agrobacterium*-plant interactions. Microbiol. Rev. 56:12-31.

Yanofsky, M., Montoya, A., Knauf, V., Lowe, B., Gordon, M., and Nester, E. W. 1985. Limited-host-range plasmids of *Agrobacterium tumefaciens*: Molecular and genetic analysis of transferred DNA. J. Bacteriol. 163:341-348.

Zimmerer, R. P., Hamilton, R. H., and Pootjes, C. 1966. Isolation and morphology of temperate *Agrobacterium tumefaciens* bacteriophage. J. Bacteriol. 92:746-750.