# Natural Instability of *Agrobacterium vitis* Ti Plasmid Due to Unusual Duplication of a 2.3-kb DNA Fragment

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**The octopine/cucumopine (o/c) Ti plasmids of *Agrobacterium vitis* carry two T regions, TA and TB. The TA region resembles the octopine TL region. The TB region contains the auxin synthesis genes TB-*iaaM* and TB-*iaaH* and the cucumopine synthesis gene *cus*. Within the group of o/c isolates, strains 2608 and 2641 are closely related. However, 2641 lacks the TB region. The restriction maps of pTi2608 and pTi2641 were established and showed that the TB deletion resulted from intramolecular recombination between two directly repeated sequences separated by 66 kb in pTi2608. The 2,294-bp repeated sequence lacks inverted repeats and does not duplicate its target site, indicating that it is not a classical bacterial insertion sequence (IS element). It was therefore called an RSAv element (repeated sequence of *A. vitis*). The RSAv element carries two open reading frames: ORF234 is homologous to the *traR* gene of the *A. tumefaciens* nopaline Ti plasmid pTiC58; ORF488 is homologous to the sucrose phosphorylase gene of *Leuconostoc mesenteroides* and the glucosyl transferase A gene of *Streptococcus mutans*. The RSAv repeat starts precisely at the start codon of ORF488 and ends two base pairs 3′ of the stop codon of ORF234. The structural organization of the RSAv element suggests that the amplification event did not result from a random amplification process. A study of the distribution of the two RSAv copies (RSAv-1 and RSAv-2) in pTi2608 and other o/c isolates indicates that the ancestor o/c Ti plasmid contained only RSAv-1 and that this sequence was duplicated at one point during the divergent evolution of the o/c Ti plasmids. Repeated subculturing of strain 2608 resulted in the same deletion in the Ti plasmid as that found in strain 2641, demonstrating that the Ti plasmids of o/c *A. vitis* strains with two RSAv copies are unstable.**

*Additional keywords:* bacterial evolution, DNA amplification, plasmid instability.

*Agrobacterium tumefaciens* (Smith and Townsend) Conn causes crown gall tumors on a large number of plant species. The mechanism of tumor induction is well known (for recent reviews see Ream [1989], Kado [1991], and Zambryski [1992]) and requires the transfer of bacterial genes into the plant cell nucleus during infection. The transferred genes are located on one or several T regions, which are part of a large plasmid, the tumor-inducing or Ti plasmid. The T-DNA genes are transcribed in the transformed plant cells and lead to tumor formation and to the synthesis of compounds of low molecular weight called opines (Petit and Tempé 1983), which are metabolized by the bacterium. Generally, Ti plasmids also code for T region transfer, opine utilization, and Ti plasmid conjugation.

The related species *A. vitis* (formerly called *A. tumefaciens* biotype 3) (Panagopoulos and Psallidas 1973; Kerr and Panagopoulos 1977; Burr and Katz 1983; Burr *et al.* 1987; Ophel and Kerr 1990) is remarkably host-specific; so far, it has been isolated only from *Vitis vinifera* (grapevine). *A. vitis* strains can be divided into three subgroups, according to the opines induced in the tumors and metabolized by the tumor-inciting strain (Thomashow *et al.* 1981; Perry and Kado 1982; Knauf *et al.* 1983; Szegedi 1985; Szegedi *et al.* 1988; Paulus *et al.* 1989a): octopine/cucumopine (o/c) strains, nopaline strains, and vitopine strains. The o/c strains have been studied extensively, because of their interesting host range properties. Some (wide host range or WHR strains) induce tumors on tomato and tobacco, but others (limited host range or LHR strains) do not (Thomashow *et al.* 1980; Knauf *et al.* 1982). Molecular analysis of Ti plasmids from the o/c strains has identified two T regions, the TA and TB regions (Buchholz and Thomashow 1984; Knauf *et al.* 1984; Yanofsky *et al.* 1985; Huss *et al.* 1989). The TA region of the WHR strains

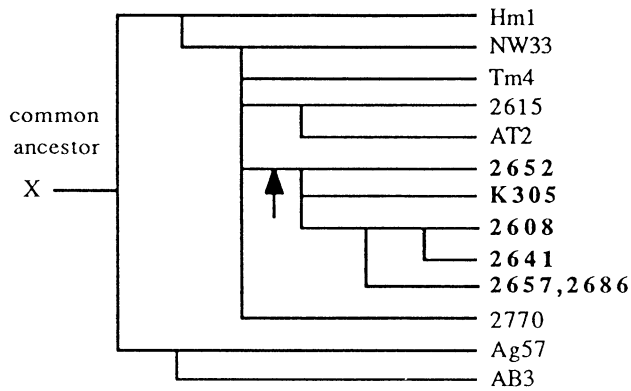


**Fig. 1.** Phylogenetic tree of *Agrobacterium* strains used in this work. The tree is based on the distribution of chromosomal IS*866* and IS*867* elements in different octopine/cucumopine (o/c) strains (redrawn from Paulus *et al.* 1989b). Only the branching order is given; the time scale is unknown. The arrow indicates the point at which RSAv-1 was most likely duplicated (see text).

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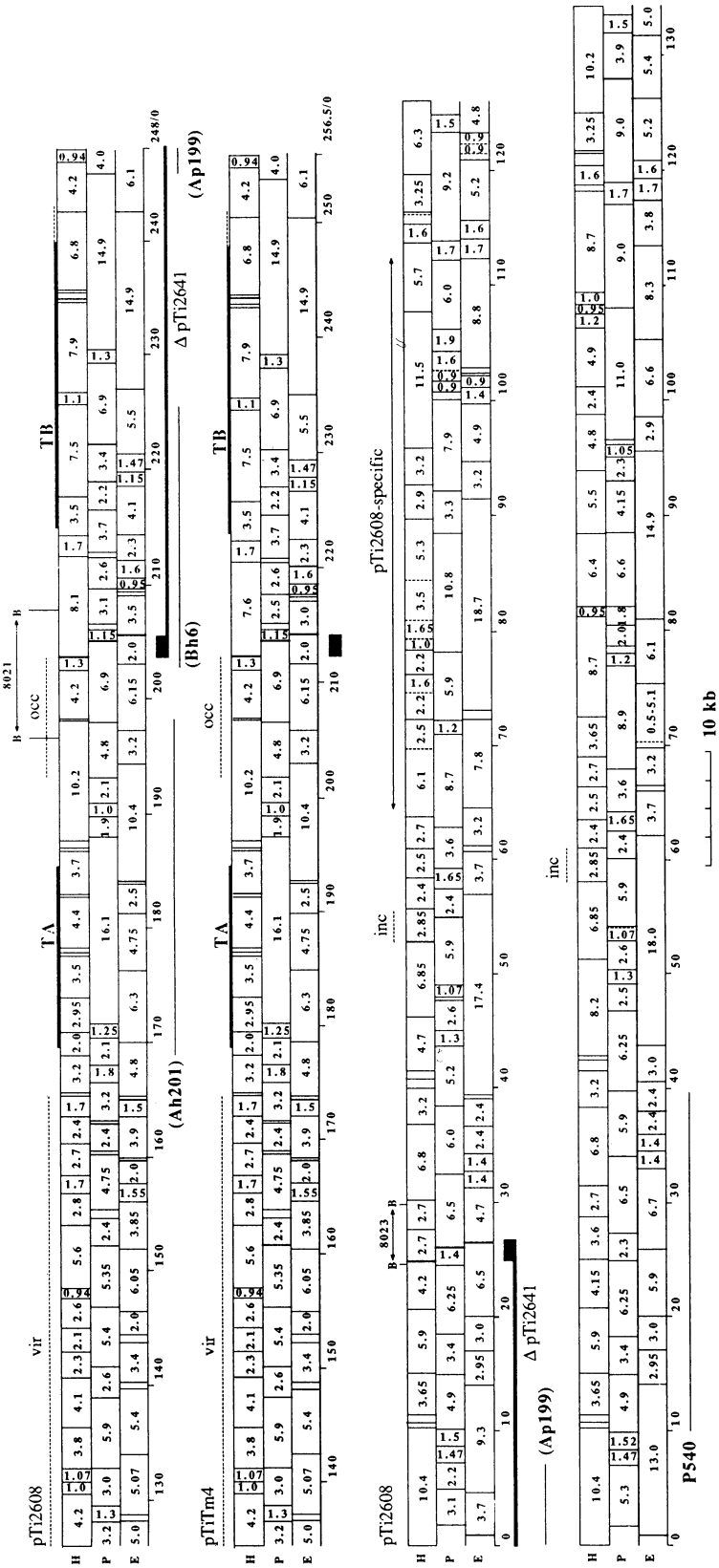


**Fig. 2.** Restriction maps of pTi2608 and pTiTm4 (the latter redrawn from Otten *et al.* 1993). Heavy line: deletion in pTi2641. Thin lines: positions of pTiTm4 clone P540 and of the heterologous clones (in parentheses) Bh6 (from pTiAB3), Ah201, and Ap199 (from pTiAg57). Black boxes: repeated sequence (RSAv) elements. Broken lines: functional regions (van Nuenen *et al.* 1993), including *occ* (octopine catabolism), *vir* (virulence region), and *inc* (incompatibility region). H: *Hin*dIII; P: *Pst*I; E: *Eco*RI; B: *Bam*HI (for this enzyme only the sites in clones pPM8021 and pPM8023 are shown). Coordinates and sizes of fragments are given in kilobases.

(Paulus *et al.* 1989a) strongly resembles the TL region of the *A. tumefaciens* octopine strains (Barker *et al.* 1983), whereas the TA region of the LHR strains is a deleted version of the WHR TA region (Paulus *et al.* 1991). The TB region of WHR and LHR strains is o/c Ti plasmid–specific and carries a set of auxin synthesis genes (TB-*iaaM* and TB-*iaaH*) (Buchholz and Thomashow 1984; Yanofsky *et al.* 1985; Huss *et al.* 1989) and the cucumopine synthase gene (Paulus *et al.* 1989a; Fournier *et al.* 1993). Studies in our laboratory have revealed the existence of different TA- and TB-region structures in different o/c strains. However, all of these T regions derived from common ancestor structures. Divergence has been shown to be caused by the insertion of various bacterial insertion sequences (ISs) and by deletions due to recombination between such elements (Otten *et al.* 1992; Otten and van Nuenen, 1993). Analysis of the restriction maps of four o/c Ti plasmids has demonstrated that other regions of these plasmids are also variable and contain several large, Ti plasmid type-specific sequences (van Nuenen *et al.* 1993). In the course of these studies, the o/c Ti plasmid pTi2641 was found to lack the TB auxin synthesis genes TB-*iaaM* and TB-*iaaH* (Paulus *et al.* 1989a). Here we present a detailed analysis of this deletion variant and demonstrate that it originated by the recombination of two copies of a 2.3-kb Ti plasmid sequence with unusual features which became duplicated in the course of the evolution of the o/c Ti plasmids.

## RESULTS

### Properties of *A. vitis* strain 2641.

*A. vitis* strain 2641 was isolated in 1985 from a tumor on *V. vinifera* cv. Cabernet Franc in Lussac, France. Hybridization analysis of total DNA of this strain with different probes showed that it has the same TA structure as the related strain Tm4, which belongs to the o/c group of *A. vitis* (Paulus *et al.* 1989a). The restriction map of pTiTm4 has been published (Otten *et al.* 1993), and its two T regions, TA and TB, have been structurally and functionally analyzed (Huss *et al.* 1989, 1990; Paulus *et al.* 1991). Preliminary studies with a TB-region probe from pTiTm4 demonstrated that, unlike 30 other strains with TA regions of the Tm4 type, pTi2641 lacks the TB *iaa* genes, TB-*iaaM* and TB-*iaaH* (Paulus *et al.* 1989a). Strain 2641 is avirulent on grapevine (L. Otten, unpublished). Although the o/c strains are highly related, most of them can nevertheless be distinguished by their chromosomal IS patterns (Paulus *et al.* 1989b). The phylogenetic tree of the o/c strains used in the present study, based on the chromosomal IS patterns, is shown in Figure 1. In the case of strain 2641 this pattern is identical to that of strain 2608, which was isolated from the same tumor as strain 2641 (M. Ridé, personal communication). Both isolates contain the chromosomal IS*866* copies IST-24, -25, and -35; the IS*867* copy IST-38 (Paulus *et al.* 1989b); and the chromosomal IS*870* copy IS*870*-3 (Fournier *et al.* 1993). None of the other *A. vitis* strains in our collection carries this combination of IS elements. Because of the close similarities between 2608 and 2641, and because of their isolation from the same tumor, we assumed as a working hypothesis that pTi2641 might be a deleted version of pTi2608.

### Extent of deletion in pTi2641.

In a preliminary experiment, the extent of the deletion of pTi2641 was determined by hybridization of Southern blots of different digests of total DNA of 2608 and 2641 to clone Bh6 from the related o/c Ti plasmid pTiAB3. Bh6 contains the left part of the pTiAB3 TB region and a further 11-kb region to the left of it (van Nuenen *et al.* 1993) (Fig. 2). The hybridization patterns showed that the deletion in pTi2641 starts to the right of the 2.0-kb *Eco*RI fragment situated at a position 9.5 kb to the left of the left border of the pTiTm4 TB region (*Eco*RI-2.0; see Fig. 2 and below). The left end of the deletion in pTi2641 is therefore close to or within *Eco*RI-2.0. After preliminary Southern analysis of 2608 and 2641 DNA digested with different enzymes and hybridized with the *Eco*RI-2.0 probe, *Bam*HI fragments of both plasmids were cloned in pUC18, and colonies carrying an 11.2- and a 5.2-kb *Bam*HI fragment from 2608 (pPM8021 and pPM8023, respectively) and a 12.3-kb *Bam*HI fragment from pTi2641 (pPM8022) were identified by hybridization to *Eco*RI-2.0. The three plasmids were mapped by *Eco*RI, *Hin*dIII, and *Pst*I digestion (Fig. 3). The restriction map of pPM8021 corresponds to the pTiTm4 map at coordinates 204–215 (Otten *et al.* 1993). The position of pPM8023 could not be identified by comparing the maps of pTiTm4 and pTi2608, but it was found to hybridize to pTiTm4 at coordinates 20–30 (not shown). The inserts of pPM8021 and pPM8023 have three restriction sites in common, suggesting a direct repeat of about 2 kb. The pTi2641 clone pPM8022 is a recombined fragment combining the left part of pPM8021 and the right part of pPM8023; it carries one copy of the repeat at the

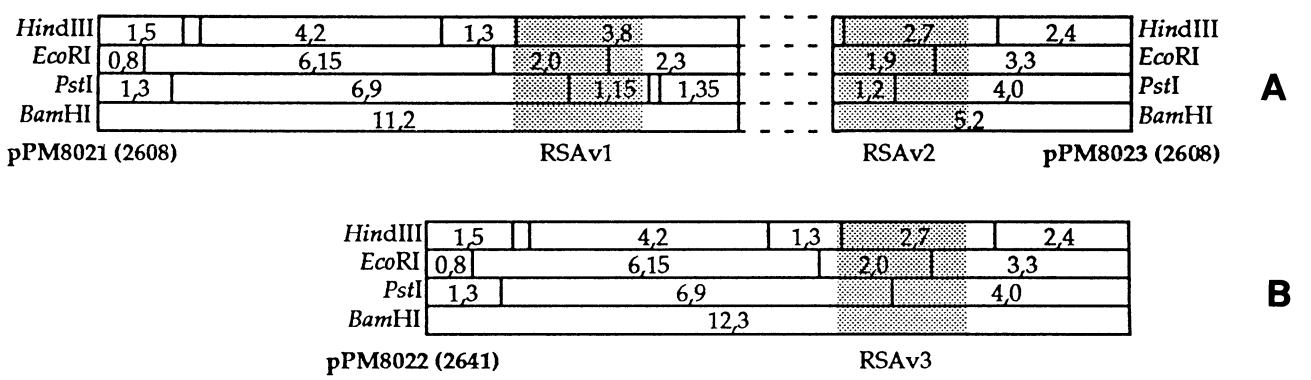


**Fig. 3.** Restriction maps of duplicated and recombined RSAv-1, RSAv-2, and RSAv-3 (in gray) and their surroundings. **A,** pTi2608. **B,** pTi2641. The plasmid numbers refer to the *Bam*HI clones used in this study. Sizes of fragments are given in kilobases.

junction (Fig. 3). This suggests that pTi2641 is derived from a pTi2608-like plasmid by intraplasmidic recombination involving the two repeats. For convenience, we will use the abbreviations RSAv-1 (for pPM8021), RSAv-2 (for pPM8023), and RSAv-3 (for pPM8022) (RSAv for repeated sequence of *A. vitis*). Hybridization of the *Eco*RI-2.0 fragment (Fig. 3) to DNA of the previously mapped representative o/c Ti plasmids pTiTm4, pTiAB3, pTiAg57, and pTiHm1 (van Nuenen *et al.* 1993) and comparison of the map of the RSAv-1 and RSAv-2 regions with the maps of the corresponding regions of the other four plasmids showed that they carry RSAv-1 but not RSAv-2 (data not shown). The RSAv repeats of pTi2608 and pTi2641 were analyzed by sequencing (see below). The restriction map of the pTi2608 RSAv-2 region showed that in this region pTi2608 differed from the four other o/c Ti plasmids. For this reason, we first determined the complete restriction maps of pTi2608 and pTi2641.

**Cloning and mapping pTi2608 and pTi2641.**

Plasmid DNA from strain 2608 was partially digested with *Pst*I, and 15- to 25-kb fragments were cloned into pUC18. Preliminary restriction analysis of over 100 clones showed that the fragments totaled more than 500 kb, although the size of pTi2608 was expected to be similar to that of pTiTm4 (256 kb). Plasmid analysis of 2608 demonstrated a single plasmid band with an approximate size of 250 kb (not shown). We therefore assumed that 2608 contained two plasmids of about the same size, called pTi2608 and pAv2608. Among the clones obtained, pTi2608 clones were identified by comparing the restriction patterns for *Pst*I, *Hin*dIII, and *Eco*RI of 180 partial *Pst*I clones to the map of pTiTm4 (Otten *et al.* 1993); this yielded three groups, which were linked by hybridizing heterologous probes (Ap199 and Ah201 from pTiAg57; Fig. 2) to Southern blots with restricted plasmid DNA of pTi2641 and pTi2608. The map of pTi2608 was aligned with the map of pTiTm4 (Otten *et al.* 1993), as shown in Figure 2. As 0 coordinate for pTi2608, the 0 coordinate of pTiTm4 was used. pTi2608 is not identical to pTiTm4; a 50-kb region between pTi2608 coordinates 64 and 112 differs from the corresponding region of pTiTm4 (coordinates 70–116). Outside this large region, several minor differences were noted. The map of pTi2641 was established by hybridizing pTi2608 clones representative of the entire Ti plasmid to *Eco*RI-, *Hin*dIII-, and *Pst*I-digested pTi2641 DNA. Except for the deleted area, the maps of the two plasmids were found to be indistinguishable. Restriction analysis of pTi2641 DNA showed that all fragments corresponded to predicted Ti plasmid fragments (results not shown). The deletion within pTi2641 extends over 66 kb; it removes the complete TB region but does not affect the virulence region, the TA region, or the octopine catabolism region.

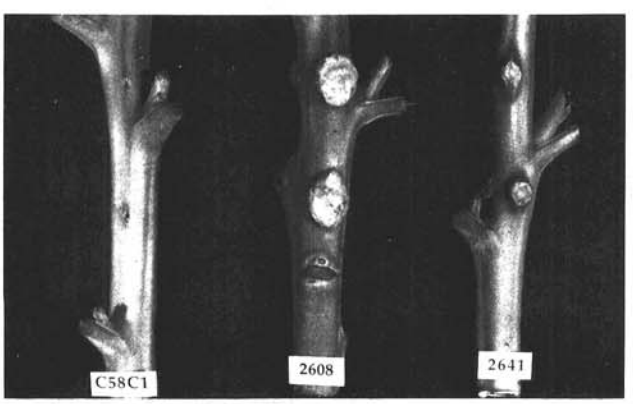


**Fig. 4.** Tumor-inducing activity of *Agrobacterium tumefaciens* strains 2608 and 2641 on *Nicotiana rustica*. Stems of *N. rustica* were infected with 25 µl of an overnight culture of strains GV3101 (C58C1), 2608, and 2641, and photographed 4 weeks after infection. Although 2641 is less virulent than 2608, it still has a distinct tumor-inducing activity.

Infection of *Nicotiana rustica* plants with 2608 and 2641 showed that 2641 is distinctly less virulent on this host than 2608 (Fig. 4). On grapevine, 2641 is avirulent (results not shown).

**Sequence of repeated elements.**

The complete sequences of the three RSAv repeats and several dozen nucleotides to the left and to the right of each of them were determined. The RSAv elements could be delimited by sequence comparison between RSAv-1 and RSAv-2. RSAv-1 and RSAv-3 are identical and differ from RSAv-2 at only three positions (6, 15, and 19; see the legend to Fig. 5). The sequence of RSAv-1 is shown in Figure 5. It contains two ORFs: ORF488 (1,467 bp, from position 2,294 to position 827) and ORF234 (705 bp, from position 708 to position 3), which belong to the same reading frame. ORF488 could encode a 488–amino acid protein. Its start codon is situated exactly at the right end of the element. ORF234 could encode a 234–amino acid protein. The stop codon of ORF234 is situated only 2 bp from the left end of RSAv-1. ORF234 has 85% DNA sequence homology with the *traR* gene of pTiC58; the deduced protein sequences are 87% identical and 93% similar (Piper *et al.* 1993). ORF488 has 38% DNA homology with the glucosyl transferase A (*gtfA*) gene from *Streptococcus mutans* (Ferretti *et al.* 1988) and 36% DNA homology with the sucrose phosphorylase gene from *Leuconostoc mesenteroides* (Kitao and Nakano 1992). The amino acid sequence homology values are 39 and 37% (identity) and 60 and 58% (similarity), respectively. ORF488 is also homologous to a sequence close to the *traR* gene of pTiC58 (Piper *et al.* 1993), placed in the same relative orientation to this gene as in pTi2608 but about 100 nucleotides further to the right. The pTiC58 sequence has not yet been published, and its function is under investigation (S. Farrand, personal communication). Although only 342 nucleotides of this pTiC58 gene have been sequenced, there is distinctly less homology between its predicted translation product and that of ORF488 (60% amino acid homology and 75% amino acid similarity) than between those of the putative *traR* gene products. Amino acid comparisons are shown in Figure 6.

The sequence to the left of RSAv-1 (not shown) has 74% homology with the last 130 nucleotides of the *occR* gene of the biotype 1 octopine strain A6 (Habeeb *et al.* 1991) and Ach5 (von Lintig *et al.* 1991) and constitutes the right end of a 12-kb region that is also found in pTiTm4. This region is strongly homologous to the biotype 1 Ach5 *occ* region (Otten *et al.* 1993). The stop codon of the putative *occR* gene of pTi2608 is situated only 20 bp to the left of RSAv-1. The sequences to the right of RSAv-1 and those flanking RSAv-2 did not display any significant homology to GenBank/EMBL sequences.

A schematic drawing of the organization of RSAv-1 and

RSAv-2 and their surrounding is shown in Figure 7. Knowing the exact structure of the repeats and their immediate surroundings, we next tested whether the recombination event that led to pTi2641 could occur under experimental conditions.

**Instability of pTi2608.**

The stability of pTi2608 was investigated by subculturing strain 2608 for 90 generations. Colonies of descendants were analyzed by hybridization with the 5.9-kb *Hin*dIII fragment of the pTiTm4-derived clone P540. This fragment corre-

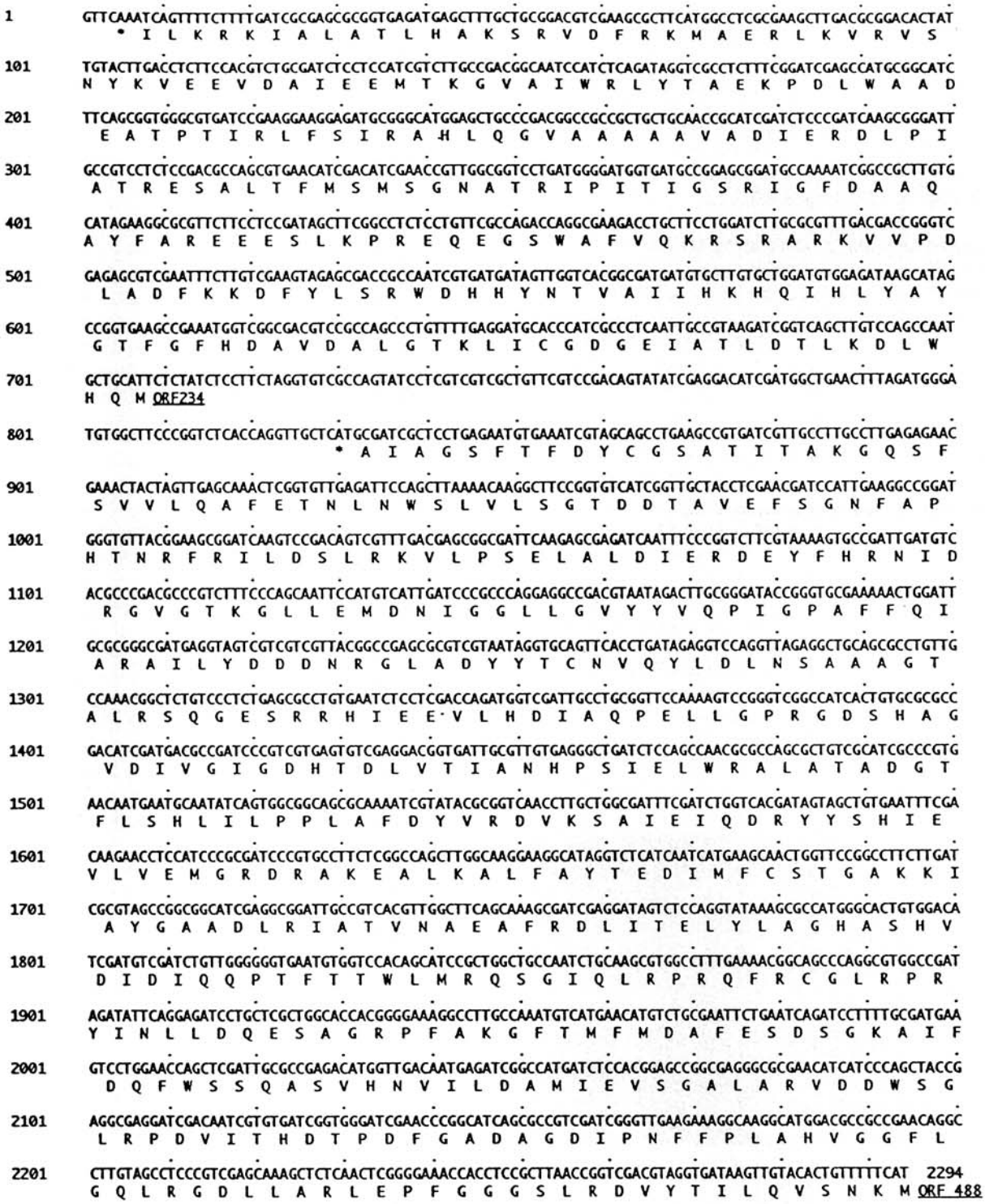


**Fig. 5.** Nucleotide sequence of RSAv-1 and deduced amino acid sequence of the two open reading frames, ORF488 and ORF234. RSAv-2 differs from RSAv-1 at three positions: 6, 15, and 19 (GTTGAATCAGTTCTCTCT...); the corresponding protein sequence is ILKRR....

sponds to pTiTm4 coordinates 15–21 (Fig. 2) and is situated within the area deleted in pTi2641. Among 1,700 colonies tested, one (2608-Δ1) did not hybridize to the probe. pTi2608-Δ1 was purified and analyzed by digestion with *Pst*I, *Eco*RI, and *Hin*dIII; the restriction patterns (data not shown) were indistinguishable from those of pTi2641. Thus, the TB deletion found in pTi2641 and presumed to have occurred in a Ti plasmid that strongly resembles pTi2608 can be reproduced under laboratory conditions. Strain 2608-Δ1 also lacks the cryptic plasmid pAv2608. Like 2641, it is avirulent on grapevine but induces small tumors on *N. rustica*, which contain agrocinopine but no cucumopine (data not shown).

**Presence of RSAv repeats in other o/c Ti plasmids.**

A comparison of the maps of pTi2608 and those of the o/c Ti plasmids pTiTm4, pTiAB3, pTiHm1, and pTiAg57 (van Nuenen *et al.* 1993) shows that all have identical *Eco*RI, *Hin*dIII, and *Pst*I restriction maps at the position corresponding to the RSAv-1 repeat (pTiTm4 coordinates 212–214, Fig. 2), showing that this region belongs to the ancestral Ti plasmid that gave rise to the different present-day o/c Ti plasmids. However, at the RSAv-2 position the restriction maps of these plasmids differ considerably. We therefore analyzed the structure of a number of other Ti plasmids by hybridization with the pTiTm4 clone P540 (Otten *et al.* 1993) (coordinates 10–40, Fig. 2). This clone covers the region that contains RSAv-2 in pTi2608. Total DNA from the representative o/c strains AB3, Hm1, 2770, NW33, Tm4, 2615, AT2, 2652, K305, 2657, 2686, 2608, and 2641 was digested with *Eco*RI and analyzed by Southern analysis using P540 as a probe. Strains 2641, 2686, 2657, K305, and 2652 have the same characteristic 4.7-kb *Eco*RI fragment as pTi2608 (the left *Eco*RI site is part of the RSAv-2 and RSAv-3 element; Fig. 2); the others do not (results not shown). This suggests that RSAv-1 was duplicated after the divergence of these two groups of isolates (Fig. 1, arrow).

## DISCUSSION

The Ti plasmid of the grapevine *A. vitis* isolate 2641 belongs to the group of o/c Ti plasmids but lacks the characteristic TB region, which encodes indole-3-acetic acid and cucumopine synthesis. Comparison of pTi2641 with other o/c Ti plasmids demonstrated that pTi2641 is most closely related to pTi2608 and suggested that the deletion occurred by recombination between two repeats, RSAv-1 and RSAv-2, situated respectively to the left and to the right of the TB region. Whereas all o/c Ti plasmids carry RSAv-1, only some contain RSAv-2. The previously established phylogenetic tree of the o/c strains allows us to propose that RSAv-2 was generated from RSAv-1. It is likely that the duplication event happened only once; strains containing both RSAv-1 and RSAv-2 must therefore be of clonal origin. Interestingly, the entire group of o/c strains is also of clonal origin (all its members share a characteristic deletion in the TA 6a gene). Three subgroups of o/c strains, each one containing a different IS element in its TA region (IS*866*, IS*868*, and IS*869*) are likewise of clonal origin (Otten *et al.* 1992). It seems, therefore, that the evolution of the o/c Ti plasmids progressed by preferential multiplication of clonal lines with loss of other strain types. This is remarkable since o/c strains belonging to a particular group have been found worldwide and do not seem to be confined to a given area (Paulus *et al.* 1989a,b). Possibly, o/c strains were originally limited to a small area,

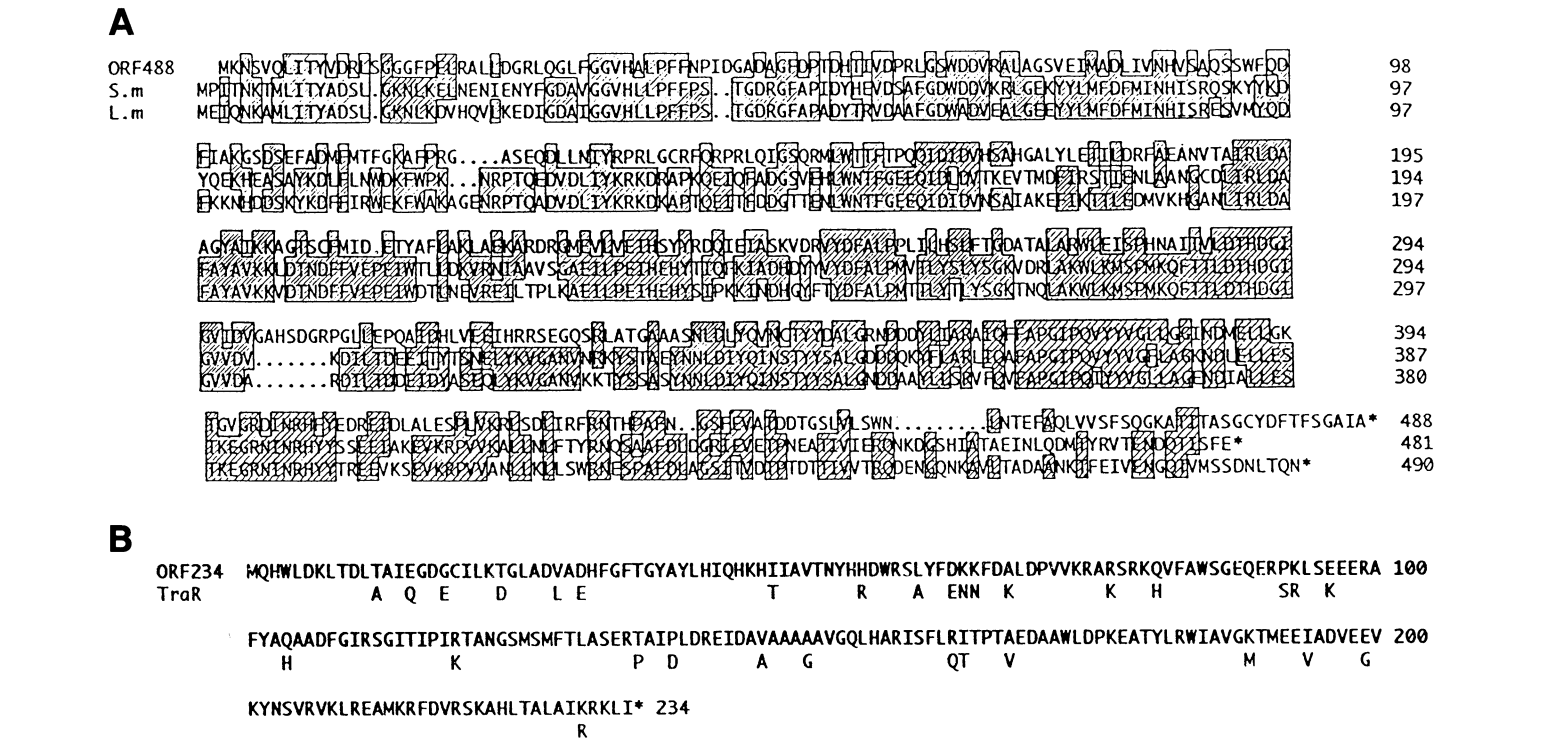


**Fig. 6. A,** Comparison of open reading frame ORF488 with the sucrose phosphorylase gene from *Leuconostoc mesenteroides* (Kitao and Nakano 1992) and the glucosyl transferase gene of *Streptococcus mutans* (Ferretti *et al.* 1988). **B,** Comparison of open reading frame ORF234 with the *traR* gene from pTiC58 (Piper *et al.* 1993).

and survival of certain strains was fortuitous (for example, by colonization of previously *Agrobacterium*-free areas, yielding founder populations of large sizes). Alternatively, descendants of certain o/c clones imposed themselves within a large population because of some selectively important property. Although strain 2641 is avirulent on grapevine, it retains some tumor-inducing activity on *N. rustica* in spite of the absence of a functional indole-3-acetic acid synthesis system. The residual activity is most probably due to the TA-located T-*ipt* and T-6b genes, which are insufficient for tumor induction on grapevine (Huss *et al.* 1990). Strain 2641 utilizes cucumopine (Paulus *et al.* 1989a), indicating that the catabolic genes for this opine are located outside the deleted area.

The deletion observed in the natural isolate 2641 can also occur under laboratory conditions; the deleted pTi2608-Δ1 plasmid has the same structure as pTi2641. This supports the hypothesis that pTi2641 was derived from a pTi2608-like plasmid and shows that pTi2608 (and probably the entire group of o/c Ti plasmids carrying both RSAv elements) is unstable. The detection of only one deletion mutant among 1,700 colonies after 90 generations does not allow us to calculate frequencies of loss per generation, but the deletion seems sufficiently infrequent to exclude the possibility that 2641 was generated during routine subculture in the laboratory. Growth studies *in vitro* (P. Fournier, unpublished) did not show significant differences between 2641 and 2608.

It is remarkable that pTi2608 and pTiTm4 differ over a region of 50 kb, whereas the remaining 200 kb are practically identical. This is in line with results of earlier studies (van Nuenen *et al.* 1993), which show that the differences between the various o/c Ti plasmid types are not due to single nucleotide changes but rather to large-scale events.

Although several steps in o/c Ti plasmid evolution can be ascribed to bacterial IS elements (ISX, IS*866*, IS*868*, IS*869*, and IS*870*) (Otten *et al.* 1992; Fournier *et al.* 1993) and similar elements have been described or postulated for other *Agrobacterium* strains (Machida *et al.* 1984; Vanderleyden *et al.* 1986; Flores *et al.* 1987; De Meirsman *et al.* 1987, 1991), the present study revealed a new type of element. RSAv lacks inverted repeats and does not generate a duplication at the target site. The element does not appear to code for a transposase, which is often characterized by a high pI value (Galas and Chandler 1989; van der Meer *et al.* 1991). It therefore seems unlikely that the RsAv repeat represents a bacterial insertion element, although it may have been mobilized *in trans* by a transposase gene situated elsewhere. It is particularly intriguing that the start codon of ORF488 constitutes the right end of the repeat, whereas the stop codon of ORF234 is found only 2 nt from the left end of RSAv-2. This indicates a precise mechanism for the RSAv duplication. ORF234 codes for a TraR-like protein. In C58, this protein is a transcriptional activator, which activates the *tra* genes of pTiC58. The expression of the *traR* gene is probably regulated by agrocinopine through *accR*, which encodes a repressor and is part of the agrocinopine catabolism locus *acc*, to the right of the *traR* gene. The AccR repressor is related to negative regulatory proteins that control sugar catabolic operons in several unrelated bacteria (Beck von Bodman *et al.* 1992; Piper *et al.* 1993). It is therefore interesting that the other ORF of the RSAv element, ORF488, is homologous to the sucrose phosphorylase gene of *Leuconostoc mesenteroides* and the glucosyl transferase A gene of *Streptococcus mutans*. Similar sequences are found on pTiC58 (Piper *et al.* 1993; S. Farrand, personal communication). We propose that this gene plays a role in the metabolism of agrocinopine A, an opine that contains a phosphorylated sugar moiety (Ryder *et al.* 1984). ORF488 and its pTiC58 homologue are distinctly less related than the *traR* sequences of pTi2608 and pTiC58, indicating a different evolutionary history for the two genes in pTiC58 and pTi2608. The close association of ORF488 and ORF234 in pTi2608 suggests that both are part of the same operon. The pTiTm4 TA region carries an intact agrocinopine synthase gene, and although the corresponding catabolic genes have not yet been identified, agrocinopine utilization is known to be encoded by pTiTm4. Strains 2608 and 2641 also utilize agrocinopine (Paulus and Otten 1993).

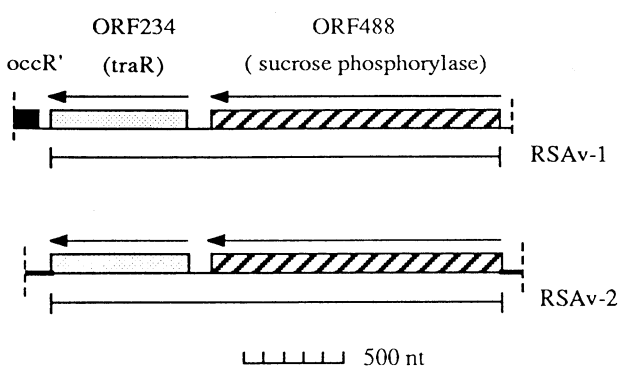


**Fig. 7.** Schematic drawing of the organization of RSAv-1 and RSAv-2. The sequences surrounding RSAv-1 (thin line) are different from those surrounding RSAv-2 (thick line).

Whether the duplication of the RSAv-1 element conferred a selective advantage is unknown. The fact that RSAv-2 does not contain the promoter sequences of ORF488 of RSAv-1 or the transcriptional termination sequences of ORF234 suggests that both genes of the RSAv-2 copy are nonfunctional. However, if ORF488 is required for agrocinopine utilization, ORF488 on the RSAv-3 copy of pTi2641 must be expressed in spite of the fact that its 5′ noncoding sequences are different from those of the ORF488 copy located on RSAv-1, since 2641 uses agrocinopine and has only one RSAv element. The sequences and clones presented here may be used to study the functions encoded by the RSAv repeat and the consequences of its duplication and subsequent recombination.

The presence of an *occR*-like sequence on pTi2608 close to the *traR*-like sequence suggests that the o/c Ti plasmids are unusual in combining features of both octopine and nopaline Ti plasmids. Although the conjugational transfer of both octopine and nopaline Ti plasmids is regulated by *N*-acyl-L-homoserine lactones acting as inducers of the *traR* genes (Zhang and Kerr 1991; Zhang *et al.* 1993; Piper *et al.* 1993), *traR* expression in the octopine strain B6S3 is also controlled by octopine via OccR, a transcriptional activator, whereas *traR* expression in the nopaline strain C58 is coregulated by agrocinopines via AccR, a transcriptional repressor (Beck von Bodman *et al.* 1992). Since the *acs* gene was originally part of the octopine TL region (Paulus and Otten 1993), it is likely that the primitive octopine Ti plasmid also carried agrocinopine utilization genes (like those found on several o/c plasmids). Thus, the regulation of *traR* by agrocinopine, as found in pTiC58, may represent a primitive feature that was re-

**Table 1.** Bacterial strains and plasmids used in this study

| | Characteristics | Reference |
|---|---|---|
| Strains | | |
| *Escherichia coli* NM522 | *sup*E *thi* Δ(*lac*⁻*proAB*) *hsd*Δ5 F′ *proAB lac*I$^q$*lac*ZΔM15 | Murray, University of Edinburgh |
| *Agrobacterium tumefaciens* 2608, 2641, AB3, Hm1, 2615, AT2, 2652, K305, Tm4, NW33, 2686, 2657, 2770 | Octopine-cucumopine strains | Paulus *et al.* 1989b |
| Plasmids[a] | | |
| pBluescript II KS+ | Cloning and sequencing vector | Stratagene, La Jolla, CA |
| pUC18 | Cloning vector | Yanish-Perron *et al.* 1985 |
| Bh6 | Partial *Hin*dIII clone from pTiAB3 | van Nuenen *et al.* 1993 |
| Ap199 | Partial *Pst*I clone from pTiAg57 | van Nuenen *et al.* 1993 |
| Ah201 | Partial *Hin*dIII clone from pTiAg57 | van Nuenen *et al.* 1993 |
| P540 | Partial *Pst*I clone from pTiTm4 | Otten *et al.* 1993 |
| pPM8021 | 11.2-kb *Bam*HI fragment of pTi2608 in pUC18 | This study |
| pPM8022 | 12.3-kb *Bam*HI fragment of pTi2641 in pUC18 | This study |
| pPM8023 | 5.2-kb *Bam*HI fragment of pTi2608 in pUC18 | This study |
| pPM8025 | 2.7-kb *Hin*dIII fragment of pPM8022 in pBluescript II KS+ | This study |
| pPM8029 | 3.8-kb *Hin*dIII/*Bam*HI fragment of pPM8021 in pBluescript II KS+ | This study |
| pPM8098 | 1.3-kb *Hin*dIII fragment of pPM8022 in pBluescript II KS+ | This study |

[a] The positions of clones Bh6, Ap199, Ah201, and P540 with respect to the map of pTiTm4 and pTi2608 are shown in Figure 2.

tained in nopaline Ti plasmids but lost in octopine plasmids and replaced by octopine regulation. In spite of the fact that o/c strains code for the synthesis and catabolism of octopine, this opine does not induce conjugation in these strains (Zhang and Kerr 1991). It should now be tested whether the *traR* gene or genes of o/c Ti plasmids can be induced by agrocinopine, as in the case of nopaline Ti plasmids. Such studies may provide interesting insights into the evolutionary relationships between octopine, o/c, and nopaline strains.

## MATERIALS AND METHODS

Bacterial strains and plasmids are listed in Table 1. Cloning techniques were as described by Sambrook *et al.* (1989). Colony hybridization was done according to Maas (1983). *Agrobacterium* plasmid DNA was isolated according to Currier and Nester (1976).

Sequences were established by the dideoxy chain termination method (Sanger *et al.* 1977) modified for use with Sequenase (United States Biochemical Corporation), with single-stranded DNA of exonuclease-generated clones as template (Henikoff 1984). Electrophoresis of labeled reaction mixtures was carried out in 5 or 8% polyacrylamide denaturing gels (Sambrook *et al.* 1989). Computer analysis of nucleotide and amino acid sequences was done with University of Wisconsin Genetics Computer Group programs on a micro Vax II computer (Devereux *et al.* 1987). Sequences from clones pPM8022, pPM8025, and pPM8098 (RSAv-3), clones pPM8029 and pPM8021 (RSAv-1), and clone pPM8023 (RSAv-2) were determined with pBluescript II KS+ specific oligonucleotide primers for and synthetic oligonucleotide primers derived from already established sequences. Plant infections were carried out as described by Huss *et al.* (1989).

Plasmid stability tests were carried out by repeated subculturing of 1.5-ml bacterial cultures in a rich medium, YEB (Leemans *et al.* 1981): every 2 days, cultures were diluted 400-fold. After 10 dilutions (corresponding to 90 generations), serial dilutions of the bacterial culture were plated on YEB agar and analyzed by colony hybridization.

EMBL accession numbers of sequences determined in this study are Z22732 (RSAv-1), Z22734 (RSAv-2), and Z22733 (RSAv-3).

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