

The limited host range of an *Agrobacterium tumefaciens* strain extended by a cytokinin gene from a wide host range T-region

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The host range of *Agrobacterium tumefaciens* strain LBA649 (pTiAg57) is limited to grapevine and a few other plant species. Its host range was extended through the introduction of the T-region from the wide host range octopine plasmid pTiAch5. In contrast, R prime plasmids harboring the entire wide host range virulence region were unable to achieve this effect. Via site-directed mutagenesis a search was performed to identify the T-DNA genes which were responsible for the observed host range extension. Inactivation of one of the *onc*-genes (the *cyt* gene) was found to abolish the capacity of the T-region to extend the host range of LBA649. Therefore, we cloned the *cyt* gene into a disarmed T-region plant vector and used it in complementation studies with pTiAg57 via the binary vector strategy. We show that the mere presence of the *cyt* gene from a wide host range Ti plasmid is sufficient to extend the host range of LBA649 to certain plants. We conclude that the limited host range of LBA649 is not caused by a lack of recognition of plants but is mainly due to the absence or inactivity of a *cyt* gene in the T-region of pTiAg57.

Key words: *Agrobacterium tumefaciens*/host range/crown gall/cytokinin-gene/binary vector system

Introduction

Oncogenic strains of *Agrobacterium tumefaciens* have the capacity to induce crown gall tumors on dicotyledonous plants. During the process of tumor-induction the bacterium introduces a specific DNA segment (T-DNA) into the genome of the plant cell (Chilton *et al.*, 1977). The T-DNA becomes expressed in a number of distinct poly(A) mRNAs (Willmitzer *et al.*, 1982). In wide host range T-DNAs, three genes, designated genes 1, 2 and 4, play a major role in the onset and maintenance of tumorous growth. Genes 1 and 2 are involved in the biosynthesis of auxin (*aux*) while gene 4 codes for an enzyme responsible for cytokinin biosynthesis (*cyt*) (Ooms *et al.*, 1981; Leemans *et al.*, 1982; Akiyoshi *et al.*, 1983; Schroder *et al.*, 1984; Barry *et al.*, 1984). Inactivation of either an *aux* gene (1,2) or the *cyt* gene (4) leads to a shooting (*Aux*⁻) or rooting (*Cyt*⁻) phenotype on *Nicotiana tabacum* and *Kalanchoe daigremontiana*. On other plants like *N. rustica* tumor formation is not very different from normal using these T-region mutants (Ooms *et al.*, 1981). These results already indicate that T-DNA determinants, as well as the response of the host, are important in tumor development on different plants.

The T-DNA originates from a large plasmid (Ti plasmid) present in *Agrobacterium*. Studies with limited host range agrobacteria have shown that the Ti plasmid, which is essen-

tial for the transformation of normal plant cells, determines the host range (Loper and Kado, 1979; Thomashow *et al.*, 1980). Most *Agrobacterium* isolates (like Ach5, B6, C58) induce tumors on a large variety of plants. In contrast, some isolates are naturally specific to a limited number of host plants (Panagopoulos and Psallidas, 1973). In our studies we used the limited host range strain LBA649 specific to grapevine, containing the octopine plasmid pTiAg57. We have previously reported that the presence of the R prime plasmid pAL1050, which carries the entire pTiAch5 T-region, is sufficient to extend the limited host range of LBA649 (Hoekema *et al.*, 1984a). Here we show that the virulence region of a wide host range Ti plasmid is unable to mediate this effect, which emphasizes that the limited host range of LBA649 resides in a peculiarity of the pTiAg57 T-region. By experiments with derivatives of pAL1050 in the binary vector system we identify a gene in the wide host range T-region that has the capacity to complement the host range defect in pTiAg57.

Results

Complementation of pTiAg57 with R prime plasmids carrying different parts of a wide host range Ti plasmid

The limited host range plasmid pTiAg57 (Panagopoulos and Psallidas, 1973) was introduced into *A. tumefaciens* strain LBA290 by conjugation. This recipient is a Ti-cured wide host range strain C58-C9. The resultant strain LBA649 still presented a limited host range for tumor induction. It caused tumors on *N. rustica*, gave tiny slowly developing tumors on sunflower and was non-oncogenic on all other hosts tested. Therefore the limited host range character was due to defects in the pTiAg57 plasmid and not due to the chromosomal background. We reported earlier that LBA649 became capable of inducing tumors on a wide range of plants when only the intact pTiAch5 T-region on plasmid pAL1050 was introduced into this strain giving LBA1049 (Hoekema *et al.*, 1984a). On tomato and sunflower, large tumors, as found for Ach5 itself, were formed by LBA1049 (pTiAg57, pAL1050) but on *N. tabacum* and *K. daigremontiana* the galls were smaller than normal. This suggested that perhaps other Ti-encoded functions were required for full-size tumor development on certain plant species. Therefore, we separately tested R prime plasmids carrying wide host range *vir* genes pAL1818 (*virA-E*) and pAL1819 (*virA-F*) (Hille *et al.*, 1982). However, the respective transconjugant strains LBA1051 (pTiAg57, pAL1818) and LBA1052 (pTiAg57, pAL1819) still had the same narrow host range as LBA649. This emphasizes that it is the T-region of pTiAg57 which confers the narrow host range to LBA649.

Effect of mutations in the wide host range T-region

To establish which genes in pAL1050 were responsible for the observed extension of the host range of LBA649 we have constructed the mutated T-region plasmids pAL1120-pAL1125

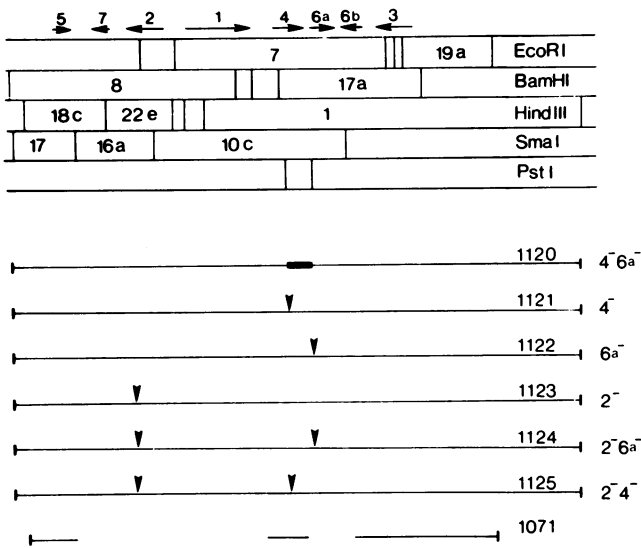


Fig. 1. Schematic representations of the T-region-derived constructions on the physical map of the octopine T-region. The relevant genotype of the mutated T-region plasmids has been indicated on the right.

(Figure 1). These derivatives of LBA1050 were obtained *via* site-directed mutagenesis as described in Materials and methods.

We first verified that the introduction of a second set of *onc* genes present on a separate T-region gave rise to the proper complementation of a defect in a residing T-region. We tested the properties of all constructions in a binary system (Hoekema *et al.*, 1983) with Ti plasmids carrying defined mutations. LBA1501 contains a pTiB6-plasmid with an insertion in gene 2 of its T-region. The introduction of plasmid pAL1120 (4⁻6a⁻) into LBA1501 resulted in normal tumor formation, while pAL1123 (2⁻) was unable to complement the mutation in this strain, as expected (Figure 2). On the other hand, plasmid pAL1123 had the capacity to complement the mutation in LBA4210 (harboring a Ti plasmid with an insertion in gene 4 of its T-region) whereas T-region derivative pAL1121, with the corresponding mutation (4⁻), had no effect. These data showed that defects in a Ti plasmid were complemented in *trans* by a second T-region present in the same cell. The phenotypic expression of the mutations in pAL1050 in a binary system was tested in LBA4404. This strain harbors the virulence plasmid pAL4404 which can mediate transfer of a separate T-region plasmid to plant cells (de Framond *et al.*, 1983; Hoekema *et al.*, 1983). LBA4450 [pAL4404, pAL1120 (4⁻6a⁻)] induced rooting tumors on tobacco and *K. daigremontiana*, while LBA4453 (pAL4404, pAL1123) produced shoots on tobacco due to the insertion in gene 2 of pAL1123. Strain LBA4455 harbored pAL4404 and a T-region with two *onc* mutations (Aux⁻Cyt⁻) and indeed was non-oncogenic on tobacco. Clearly, in a binary system the mutated T-regions give rise to the expected phenotypes. The constructs were subsequently used for complementation studies with pTiAg57.

Host range extension with the mutant T-region plasmids

The mutated T-region plasmids (for a description see Table III) were transferred to LBA649. Introduction of pAL1123 (carrying a mutation in gene 2) into LBA649 still gave rise to normal tumor formation on tomato showing that inactivation

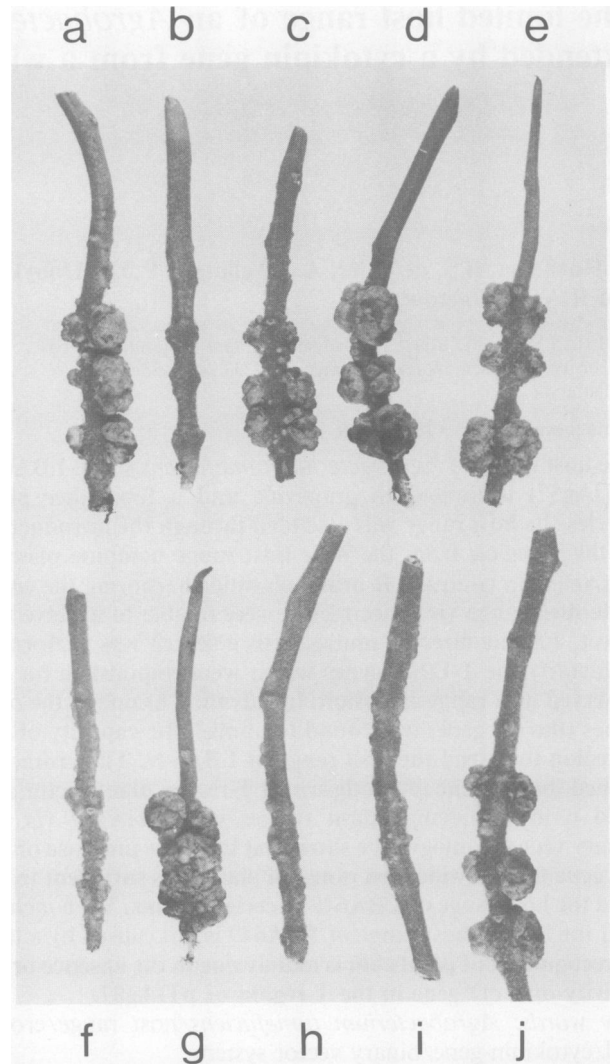


Fig. 2. Response on tomato stems of different *A. tumefaciens* strains (6 weeks after inoculation). a and f: LBA4210(4⁻) harboring pAL1123 (2⁻) a or pAL1120(4⁻6a⁻) f. b and g: LBA1501 (2⁻) harboring pAL1123 b or pAL1120 g. c: LBA1123 = LBA649 harboring pAL1123. d: LBA1124 = LBA649 harboring pAL1124(2⁻6a⁻). h: LBA1121 = LBA649 harboring pAL1121(4⁻). i: LBA1125 = LBA649 harboring pAL1125(2⁻4⁻). e and j: the control B6 strain LBA1010.

of the *aux* locus in the wide host range T-region did not affect the host range enlargement. However, plasmid pAL1120 (carrying a deletion covering genes 4 and 6a) failed to complement LBA649. In this mutant T-region an 858 bp *Pst*I fragment was exchanged for a chloramphenicol determinant. Because this deletion defined a region essential for the host range extension, we studied whether the block in complementation was due to the lack of both gene 4 and 6a, or to the lack of one of these genes. A biological function has not been ascribed so far to gene 6a. Separate insertions into each *Pst*I site in the T-region were constructed as described in Materials and methods. These inserts were recombined into pAL1050 resulting in pAL1121 (4⁻) and pAL1122 (6a⁻). Because gene 6a is not essential for tumor induction, the introduction of pAL1121 (harboring all *onc* genes of the pTiB6 T-region) into LBA649 would still lead to tumor induction on a wide range of plants. Therefore, we introduced an additional Tn1831 insertion in gene 2 in both recombinants giving rise to pAL1124 (2⁻6a⁻) and pAL1125 (2⁻4⁻). All constructs were subse-

quently tested in LBA649. LBA1122, 1123 and 1124 induced normal tumors on tomato but for LBA1121 and 1125 no complementation was observed (see Figure 2). This result showed that the inactivation of only gene 4 completely eliminated the host range extension but that a mutation in gene 6a had no effect. We have recently shown that inactivation of gene 1 present on pAL1050 did not affect the host range extension either (result not shown).

Enlargement of host range with the cloned *cyt* gene from the pTiB6-region

The complementation studies with derivatives of pAL1050 showed that the effect of host range extension can be traced to the presence of the *cyt* gene in the wide host range T-region. As more than one factor might be involved in host range expression we decided to determine whether or not the introduction of only the cloned *cyt* gene from pTiB6 would be sufficient to enlarge the host range of LBA649. This gene has been sequenced (Heidekamp *et al.*, 1983) and is contained in a 1349-bp *RsaI* restriction fragment. We have cloned this *RsaI* fragment out of plasmid pRAL3501 (containing *EcoRI* fragment 7 of pTiB6) and have inserted it in the disarmed T-region vector pRAL3940. This vector is a pBR325 derivative that contains gene 3 (octopine synthase) and gene 5 between the left- and right-hand border fragments of the pTiB6 T-region. It contains no *onc* genes and can be delivered into plant cells in a binary system (Hoekema *et al.*, in preparation). The vector pRAL3940 and its derivative with the insert of the cloned *cyt* gene, named pRAL3451, cannot replicate in *Agrobacterium* and they were shuttled to this host in the form of stable R772::pRAL3940 and R772::pRAL3451 co-integrate plasmids named pAL1062 and pAL1071, respectively. Restriction analysis of pAL1062 and pAL1071 revealed that the insertion site of R772 was in the pBR part of these plasmids leaving the *Agrobacterium* sequences intact (data not shown). We tested the LBA649-derived strain LBA1093, harboring pTiAg57 and pAL1071, for tumor induction as well as two control strains namely LBA1049 with the entire wide host range T-region (pTiAg57, pAL1050) and LBA1111 carrying the vector without the insert of the *cyt* gene (pTiAg57, pAL1062). The introduction of the vector pAL1062 had no effect, but the *cyt* gene gave rise to tumor formation on tomato and sunflower, although the tumors were significantly smaller than those induced by LBA1049 (Figure 3). On *K. daigremontiana* (which is also less sensitive for LBA1049), LBA1093 induced no tumors at all. To rule out that the attenuated response was due to the construction of the binary vector pRAL3451 we checked the suitability of this vector in a control experiment. Plasmid pAL1071 was introduced into the *A. tumefaciens* mutant strain LBA4210 (Ooms *et al.*, 1981). This strain contains a Tn904 insertion in the *cyt* gene and consequently is very weakly oncogenic on tomato. As shown in Figure 3 the introduction of pAL1071 into LBA4210 gave rise to normal tumors as a result of complementation by the intact *cyt* gene on the vector.

Discussion

Many *A. tumefaciens* strains induce crown galls on a wide range of plants. Some wild-type isolates, however, express a very narrow host range for tumor induction. The type of Ti plasmid present is responsible for this difference (Loper and Kado, 1979; Thomashow *et al.*, 1980). To identify the pTi-encoded functions which control host range, we perform-

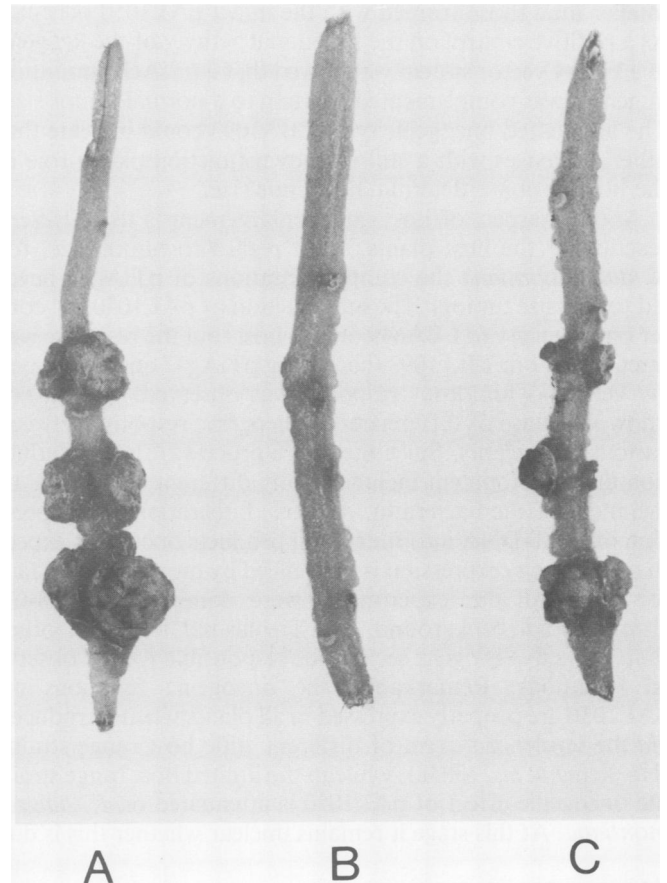


Fig. 3. Tumor formation on tomato 6 weeks after inoculation: A: LBA4271 = LBA4210(4⁻) harboring pAL1071 (vector with cloned gene 4). B: LBA1111 = LBA649 harboring pAL1062 (vector without insert). C: LBA1093 = LBA649 harboring pAL1071.

ed complementation studies with *A. tumefaciens* strain LBA649, harbouring pTiAg57. This Ti plasmid shares very little DNA homology with the 'common DNA' present in the T-regions of wide host range Ti plasmids (Thomashow *et al.*, 1981). The introduction of a wide host range T-region extends the host range of LBA649 (Hoekema *et al.*, 1984a). This complementation shows that the virulence functions of the pTiAg57 plasmid can mediate the transfer of T-DNA to a much wider range of plants than those naturally sensitive for pTiAg57. Here we report that the Vir-region of a wide host range Ti plasmid indeed did not extend the host range of LBA649. All of these data indicate that the failure of LBA649 to induce tumors on most plant species primarily resides in the T-region and not in the Vir-region of its pTiAg57 plasmid.

Which genes in the wide host range T-region are responsible for the observed host range enlargement? We approached this problem with genetic complementation studies using mutant T-region plasmids obtained *via* site-directed mutagenesis of plasmid pAL1050. We observed that a wide host range T-region carrying all genes but the *cyt* gene was unable to complement LBA649. This led to the conclusion that the cytokinin gene on pAL1050 is the essential gene. We then cloned this gene in a disarmed T-region vector and showed in a binary system that this construct (maintained in *Agrobacterium* as a co-integrate with R772) enhanced the oncogenicity of LBA649 on tomato and sunflower. However, LBA1093 obtained in this way induced tumors that were

smaller than those formed with the intact pAL1050 plasmid. As a positive control on the functional activity of the *cyt* gene in a binary vector system we showed that a pTiAch5 mutation in gene 4 was complemented, leading to a normal tumor size. The attenuated oncogenicity of LBA1093 could indicate that other sequences with a still unknown function play a role in the host response determining tumor size.

Another aspect of host range enlargement is the different reaction of the host plants. With regard to tumor size, for *K. daigremontiana* the complementations of pTiAg57 never led to full-size tumors. The introduction of pAL1050 did confer oncogenicity to LBA649 on this host, but the response was attenuated. For LBA1093 (harboring pTiAg57 and the cloned *cyt* gene) no tumorous response was observed. We do not know the cause of differences in oncogenic response between various host plants. Since the entire process of tumor induction and development includes many different stages like attachment of the bacterium, transfer, integration and expression of the T-DNA into functional products one might expect that host range expression is influenced by more than one factor only. All our experiments were done in the C58-C9 chromosomal background. So, Ti plasmid functions other than the active *cyt* gene seem to be responsible for the observed variations. Remarkably, the oncogenic functions on pAL1050 are properly expressed in all plants when introduced via the virulence system of different wide host range strains (Hoekema *et al.*, 1984a), while in the limited host range strain the oncogenic effect of pAL1050 is attenuated on *K. daigremontiana*. At this stage it remains unclear whether this is due

Table I. Bacterial strains

LBA no.	Plasmids	Characteristics	Origin
290	—	pTi-cured C58-C9 strain	Hooykaas, unpublished strain
649	pTiAg57	Limited host range strain	Hooykaas, this work
1010	pTiB6	Wide host range B6 strain	Koekman <i>et al.</i> , 1982
1049	pTiAg57,pAL1050		Hoekema <i>et al.</i> (1984a)
1050	pAL1050		Hoekema <i>et al.</i> (1983)
1051	pTiAg57,pAL1818		This work
1052	pTiAg57,pAL1819		This work
1093	pTiAg57,pAL1071		This work
1111	pTiAg57,pAL1062		This work
1120	pTiAg57,pAL1120		This work
1121	pTiAg57,pAL1121		This work
1122	pTiAg57,pAL1122		This work
1123	pTiAg57,pAL1123		This work
1124	pTiAg57,pAL1124		This work
1501	pAL1501	pTiB6 with Tn1831 in gene 2	Ooms <i>et al.</i> (1981)
4210	pAL228	pTiAch5 with Tn904 in gene 4	Ooms <i>et al.</i> (1981)
4271	pAL228,pAL1071		This work
4404	pAL4404	pTiAch5 deletion lacking T-region	Ooms <i>et al.</i> (1982)
4450	pAL4404,pAL1120		This work
4453	pAL4404,pAL1123		This work
4455	pAL4404,pAL1125		This work

Materials and methods

Bacterial strains

Bacterial strains are listed in Table I. *Agrobacterium* transconjugant strains were typified by their characteristic phage immunity pattern (Hooykaas *et al.*, 1977).

Table II. Recombinant plasmids

Plasmids	Description	Origin
pRL220	IncQ-type plasmid carrying Cm ^R , Ap ^R , Tc ^R and SM ^R	Hille <i>et al.</i> (1983b)
pRAL3401	1349 bp <i>Rsa</i> I fragment carrying gene 4 (<i>cyt</i>)	This work
pRAL3451	pRAL3940 containing insert of pRAL3401	This work
pRAL3501	<i>Eco</i> RI fragment 7 of pTiB6 cloned in pACYC184	Hille <i>et al.</i> (1983b)
pRAL3940	Disarmed T-region vector	Hoekema, unpubl.

Table III. Constructions

T-region plasmid	Description	Relevant genotype
pAL1050	Unmutated T-region of pTiAch5	2 ⁺ 4 ⁺ 6a ⁺
pAL1120	Substitution of 858 bp <i>Pst</i> I fragment (9211–10069) ^a for 4-kb <i>Pst</i> I fragment from pRL220 carrying Cm ^R	2 ⁺ 4 ⁻ 6a ⁻
pAL1121	Insertion of 4-kb <i>Pst</i> I fragment from pRL220 into <i>Pst</i> I site (9211) ^a of pAL1050	2 ⁺ 4 ⁻ 6a ⁺
pAL1122	Insertion of 4-kb <i>Pst</i> I fragment from pRL220 into <i>Pst</i> I site (10069) ^a of pAL1050	2 ⁺ 4 ⁺ 6a ⁻
pAL1123	Insertion of a copy of Tn1831 (encoding Hg ^R , Sm ^R and Sp ^R) from pAL1501 recombined into pAL1050	2 ⁻ 4 ⁺ 6a ⁺
pAL1124	As pAL1123, Tn1831 recombined into pAL1122	2 ⁻ 4 ⁺ 6a ⁻
pAL1125	As pAL1123, Tn1831 recombined into pAL1121	2 ⁻ 4 ⁻ 6a ⁺
pAL1062	Co-integrate plasmid between R772 and pRAL3940	—
pAL1071	Co-integrate plasmid between R772 and pRAL3451	4 ⁺

^aMap position on T-region according to Barker *et al.* (1983).

Table IV. Oncogenicity of strains on different plant species

Strain (LBA number)	Tomato	<i>N. tabacum</i>	<i>K. daigremontiana</i>	Sunflower
649	—	—	—	(±)
1010	++	++	++	++
1049	++	+	+	++
1051/52	—	—	—	(±)
1093	+	nd	—	+
1111	—	—	—	(±)
1120/21	—	—	—	(±)
1122/23/24	++	+	+	++
4210	(±)	nd	nd	++
4271	++	nd	nd	++

Tumor formation was classified as wild-type size ++, attenuated +, small overgrowths (±) or negative —. nd = not determined.

to a less efficient transfer of pAL1050 from this strain or due to the presence of the limited host range T-region.

Plasmids

Recombinant plasmids described in this work are listed in Table II. Plasmid isolations were done as described by Birnboim and Doly (1979), restriction endonuclease digestions and gel electrophoresis was carried out as described (Prakash *et al.*, 1981). Ligations were done as reported previously (Hoekema *et al.*, 1984b).

Media

Agrobacterium strains were grown at 29°C in LC-medium (Hooykaas *et al.*, 1977) and selected on minimal medium plates solidified with 1.8% Difco agar containing antibiotics at the following concentrations (mg/l): rifampicin, 20; carbenicillin, 100 (5 for Ach5 chromosomal background); kanamycin, 100; spectinomycin, 250; streptomycin, 500. In crosses between *Escherichia coli* donors and *A. tumefaciens* strain LBA649, transconjugants were selected for their ability to catabolize octopine on bromothymolblue indicator plates (Hooykaas *et al.*, 1979).

Tumor induction

Tumorigenicity was tested on sunflower, tomato, *N. tabacum*, *N. rustica* and *K. daigremontiana*.

Site-directed mutagenesis

Mutagenesis of plasma pAL1050 was performed as described for pTiB6 (Hille *et al.*, 1983a). Plasmid pAL1050, a conjugative plasmid stable both in *E. coli* and *A. tumefaciens* was transferred to different *E. coli* recipients harboring mutated T-region clones (transfer frequency 10^{-2} per recipient). After exchange of the wild-type sequence and the resistance marker of the mutation via double crossing over (frequency $\sim 10^{-3}$ per transconjugant), the pAL1050 derivatives (listed in Table III) were analyzed to check the positions of the mutation and subsequently crossed back to *A. tumefaciens* for virulence assay.

Plasmid pAL1123 was obtained via a slightly different route involving homologous recombination in *A. tumefaciens*: plasmid pAL1050 was introduced into *A. tumefaciens* mutant strain LBA1501 that carries pTiB6 with a copy of transposon Tn1831 inserted into gene 2 of the T-region. We crossed pAL1050 back from LBA1501 into *E. coli* (frequency 5×10^{-2}) selecting for co-transfer of Sp^r, a Tn1831 marker. This was observed at a frequency of $\sim 10^{-7}$. The resultant plasmid was analyzed and shown to consist of pAL1050 with a copy of Tn1831 in gene 2.

Specific insertions into gene 4 (pAL1121) and into gene 6a (pAL1122) were constructed as follows: plasmid pRAL3501 carrying *EcoRI* fragment 7 from pTiB6 on a pACYC184-vector was partially digested with *PstI* and the chloramphenicol-determinant from pRL220 was cloned into the left-hand *PstI* site (gene 4) or into the right-hand site (gene 6a). The separate inserts were subsequently recombined into plasmid pAL1050. The properties of the different strains have been summarized in Table IV.

Acknowledgements

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