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The right hand copy of the nopaline Ti-plasmid 25 bp repeat is required for tumour formation

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ABSTRACT

At either end of the nopaline Ti-plasmid T-region resides a copy of a 25 bp repeated element. The normal T-DNA endpoint is 1 bp internal of the right copy, with the transcription initiation site of the nopaline synthase (nos) gene being approximately 300 bp away in the same direction. Here we describe results which demonstrate that deletion of any combination of sequences between the nos initiation site and the right copy of the 25 bp repeat does not affect oncogenicity. Thus a mutant retaining the right copy and only 3 bp internal of it is indistinguishable from the wild type parent in its oncogenic properties. However deletion of a further 39 bp, including complete removal of the right copy abolishes crown gall tumour formation on Kalanchoe and tobacco. From these results we infer that unlike the left border, the right copy of the 25 bp repeat is required for T-DNA transfer and/or integration. This is the first conclusive demonstration of the involvement of a copy of the repeats in this process.

INTRODUCTION

Crown gall tumour arises from the transfer to the plant nuclear genome of T-DNA, a 23 kb segment colinear with the T-region of the Agrobacterium tumefaciens Ti-plasmid[1-3]. On the Ti-plasmid (see Fig. 1) the T-region is flanked by imperfect 25 bp repeats[4-7]. Thus the repeats are not transferred intact to the plant genome, as the T-DNA endpoints thus far sequenced lie within or immediately internal of them[4-7]. In nopaline crown galls, variability of the left border over approximately 100 bp has been detected, whereas all four sequenced right endpoints are within 1 bp of each other, adjacent to the right repeat copy[4,6,7]. This data, coupled with the observation that these repeats are conserved between octopine and nopaline Ti-plasmids while the sequences surrounding them are not, has led to the proposal that they function as "border signals"[4]. Deletion analysis of the function of the 25 bp repeats has been confined to large scale low resolution experiments. These have demonstrated that removal of a large section of the left half of the T-region, including the left repeat copy, has no effect on oncogenicity[8], while a spontaneous deletion of over 15 kb encompassing the right border drastically

attenuates tumour formation[9,10]. However interpretation of this latter result is complicated by the fact that the deletion removes certain of the T-DNA onc functions[8], and replaces the right border with transposon Tn1[9,10]. Recent evidence has indicated that contained somewhere within HindIII fragment 23, a 3.2 kb fragment spanning the right T-region border of the nopaline plasmid, pTiC58 (see Fig. 1) are sequences required for T-DNA transfer[2,11,12]. Thus although it would appear that the right border is more important than the left for T-DNA transfer, there is as yet no conclusive proof of the involvement of the 25 bp repeats in this process.

We have commenced a project to functionally map the right border of the T-region of pTiC58. Some 300 bp internal of the right copy of the 25 bp repeats is the mRNA cap site for the nopaline synthase (nos) gene, transcription proceeding in a direction away from the border[13,14]. Thus this region contains sequences proposed to be involved in both T-DNA transfer and nos expression. Using Bal31 exonuclease we have constructed a series of overlapping deletion mutants in this region. The phenotypes of the mutants fall into two classes, affecting tumour formation and nos expression. This paper describes the characteristics of the mutants affected for tumour formation, while those with altered nos expression will be described elsewhere[15].

MATERIALS AND METHODS

Microbiological Techniques

Bacterial growth conditions, antibiotic concentrations, and DNA purification were as previously described[16].

General cloning procedures

Conditions for restriction enzyme digestion, ligations, DNA fragment isolation and manipulation of EcoRI linkers, were as previously described[16]. Bal 31 digestion was performed at 37°C for 30 seconds, using low salt (0.2 M NaCl) conditions[17], and terminated by the addition of phenol.

Deletion construction

Deletions were constructed (Fig. 2) in pASK1029[16] a pBR322 based replicon containing HindIII fragment 23, a 3.2 Kb fragment spanning the right border of the nopaline Ti-plasmid pTiC58 T-region. The pBR322 moiety of pASK1029 lacks both EcoRI and Bam HI sites. pASK1029 was cleaved at the unique SstII site and the resulting cohesive termini resected for 30 seconds with Bal 31. After ligation of EcoRI linkers, and digestion with EcoRI, a 1.2 Kb EcoRI fragment, originally derived from Tn903, expressing kanamycin/neomycin resistance was ligated in place. Kanamycin resistant transformants of E.coli

were selected, and screened by "mini-lysate"[18]. Plasmids were purified from promising candidates, renamed pDUB1106Δ....., and mapped by restriction enzyme digestion and gel electrophoresis. pDUB1106Δ.... derivatives were then digested with PstI, ligated to similarly digested pGV1106 and introduced into competent E.coli cells by transformation, selecting for resistance to gentamycin and neomycin. These plasmids were renamed pDUB1201Δ....

Introduction of deletion mutants into the pTiC58 derivative pGV3105

pDUB1201Δ .... derivatives were transmitted to A. tumefaciens C58C'Rif^R (pGV3105) by pRN3 as previously described[16,19] with selection for transconjugants resistant to rifampicin and neomycin. Following a Ti-plasmid mediated conjuation to A. tumefaciens C58C'Ery^{R}Cm^R, transconjugants selected for resistance to neomycin, erythromycin and chloramphenicol were screened for sensitivity to gentamycin, and such isolates renamed pDUB1003Δ.... purified. Correct insertion of the deletions was checked by Southern blotting of total A. tumefaciens DNA using HindIII-23, or the Tn903 neomycin fragment as probe[20].

Tumour induction

Overnight cultures of A. tumefaciens C58C'Ery^{R}Cm^R (pDUB1003Δ ....) were inoculated onto leaves or stems of Kalanchoë diaigremontiana using a sterile syringe needle. All plants were also inoculated with A. tumefaciens C58C'Ery^R Cm^R, and A. tumefaciens C58C'Ery^{R}Cm^R (pGV3105) as controls. Infected plants were maintained under fluorescent lighting, utilising a 16 hour on/8 hour off cycle at 25^{o}C.

Nopaline synthase assays

Nopaline was detected in tumour tissue using established protocols[21,22].

DNA sequencing

All subcloning and DNA sequencing procedures were as recommended in the M13 cloning and sequencing handbook produced by Amersham International. To define the deletion endpoints, in the downstream direction, approximately 1 Kb EcoRI-Bam HI fragments were subcloned from each pDUB1106Δ... derivative into EcoRI-Bam HI cleaved M13mp9. These fragments represent the region beginning at the new EcoRI site inserted into the nos upstream sequence, and extending to the Bam HI site situated approximately 850 bp downstream of the transcription initiation point. DNA sequencing was performed using dideoxynucleotide chain termination techniques[23], with α ^{35}S-dATP as radiolabel, upon templates purified from single plaque isolates.

RESULTS AND DISCUSSION

Mutant Construction

We have utilised a derivation of the strategy previously described for introducing foreign DNA and thus site specific mutations into the nopaline Ti-plasmid pTi-C58[16,19]. The right copy of the 25 bp repeats is situated approximately 300 bp upstream of the nopaline synthase (nos) transcription initiation point[13,14], with a unique SstII site midway between (see Fig. 1). This whole region is spanned by HindIII fragment 23, a 3.2 Kb fragment present in the pBR322 based plasmid pASK1029[19]. Thus commencing with pASK1029, deletions were constructed by cleavage at the unique SstII site, resection by Bal 31 exonuclease, ligation of EcoRI linkers, and insertion of a 1.2 Kb EcoRI fragment, originally derived from Tn903, expressing kanamycin/neomycin resistance (see Fig. 2). Insertion of this fragment allowed a positive selection for all deletions after transformation into competent E.coli cells. Screening of deletions by mini-lysate[18], and restriction enzyme mapping allowed the identification of potentially interesting mutants (Fig. 3). As pBr322 based replicons are not maintained in A. tumefaciens, promising candi-

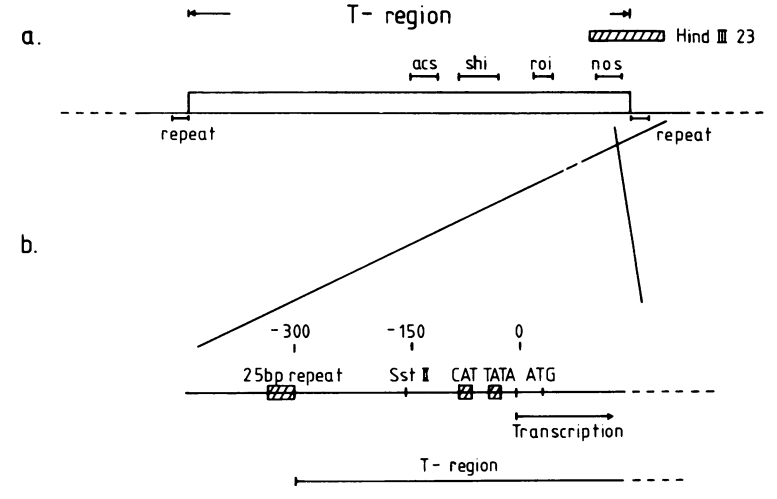


Fig. 1. a) Schematic diagram of the nopaline Ti-plasmid T-region. The T-region is shown as an open box, flanked by 25 bp direct repeats, the hatched box representing the area contained within Hind III fragment 23. Genetic loci are involved in agrocinopine synthesis (acs) shoot inhibition (shi) root inhibition (roi) and nopaline synthesis (nos).

b) Expanded, reversed (relative to Fig. 1a) section spanning the right border of the T-region. Numbers refer to bp upstream of the nos transcription initiation point (o). "CAT" and TATA refer to DNA sequence blocks identified by homology to eukaryotic promoter consensus sequences[13].

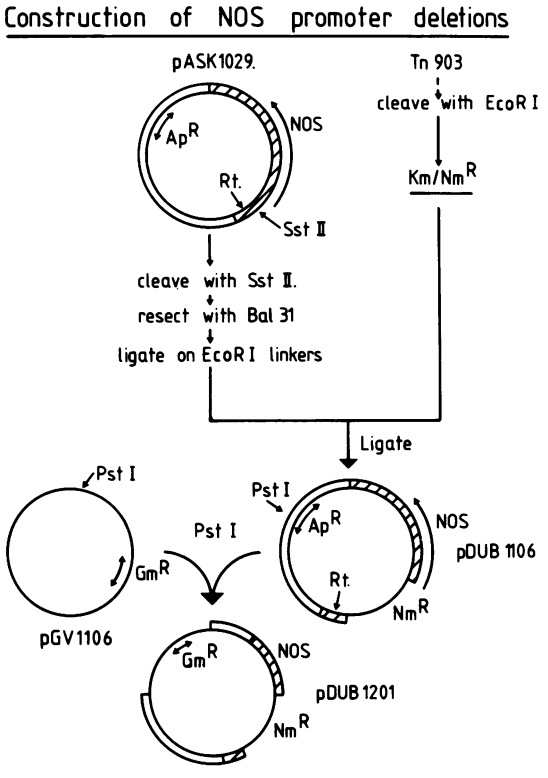


Fig. 2. Deletion construction. See text for details. Open boxes indicate pBR322 sequences, hatched boxes represent Hind III-23. Rt, right border of T-region; Km^R, kanamycin resistance; Nm^R, neomycin resistance; Gm^R, gentamycin resistance; Ap^r, ampicillin resistance.

dates were ligated to the incW plasmid pGV1106[24], and transmitted to the latter host using incN plasmid pRN3[16,19]. In A. tumefaciens, reciprocal recombination events, replacing the mutated copy of Hind III-23 for the wild type copy, may be selected for by retention of neomycin resistance following a Ti-mediated conjugation to a cured A. tumefaciens recipient. The deleted Ti-plasmids thus constructed (pDUB1003Δ ....) were checked by Southern blotting[20], and their oncogenic properties ascertained by inoculation onto wounded leaves or stems of Kalanchöe diaigremontiana (Fig. 5 and table 1).

Mutant Characteristics

As can be seen in Fig. 5, deletion mutants Δ31, Δ56, and Δ68 induce tumours on Kalanchöe stems as readily as the wild type parent pGV3105. However both mutants Δ123 and Δ17 fail to induce tumours, on either stems or leaves.

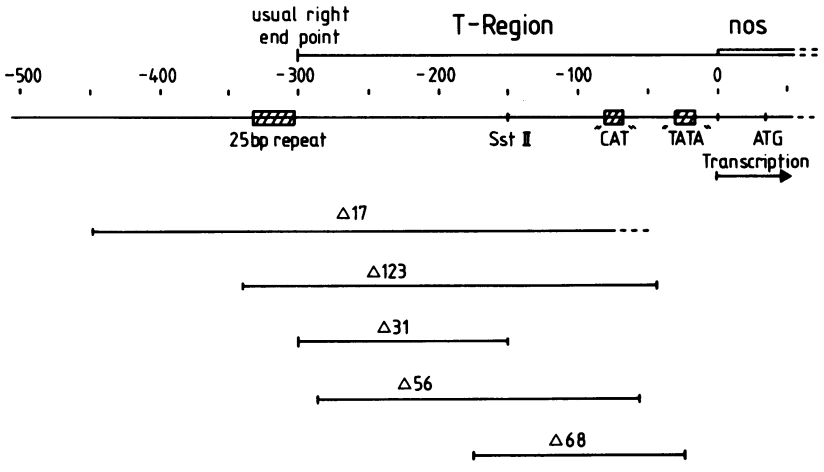


Fig. 3. Map data of selected deletions. The region depicted, its orientation and numbering are the same as for Fig. 1b. The lines below the map indicate the area deleted in the various mutants as defined by DNA sequence analysis or gel electrophoresis.

Moreover neither mutant Δ123 or Δ17 induced tumours on decapitated tobacco seedlings (data not shown). Furthermore, although Δ31 produced appreciable, and Δ56 and Δ68 lower, levels of nopaline synthase, neither Δ123 or Δ17 induced the enzyme in wound tissue (table 1).

DNA sequence determination following subcloning into M13mp9 (Fig. 4)

Table 1

Properties of deletion mutants. Oncogenicity was assessed in terms of tumour size, 15 days after inoculation onto stems, or 30 days after infection of leaves, of *K. diaigremontiana*. Nopaline synthase activity was assessed by a simple microscale assay[21, 22] in wound tissue or tumours 30 days following inoculation of leaves of *K. diaigremontiana*.

Deletion mutant properties		
A. tumefaciens isolate	Oncogenicity	Nopaline synthase
C58C'Ery^{R}Cm^R	-	-
C58C'Ery^{R}Cm^R (pGV3105)	++++	+++++
C58C'Ery^{R}Cm^R (pDUB1003 Δ17)	-	-
C58C'Ery^{R}Cm^R (pDUB1003 Δ31)	++++	+++++
C58C'Ery^{R}Cm^R (pDUB1003 Δ56)	+++++	++
C58C'Ery^{R}Cm^R (pDUB1003 Δ68)	++++	±
C58C'Ery^{R}Cm^R (pDUB1003 Δ123)	-	-

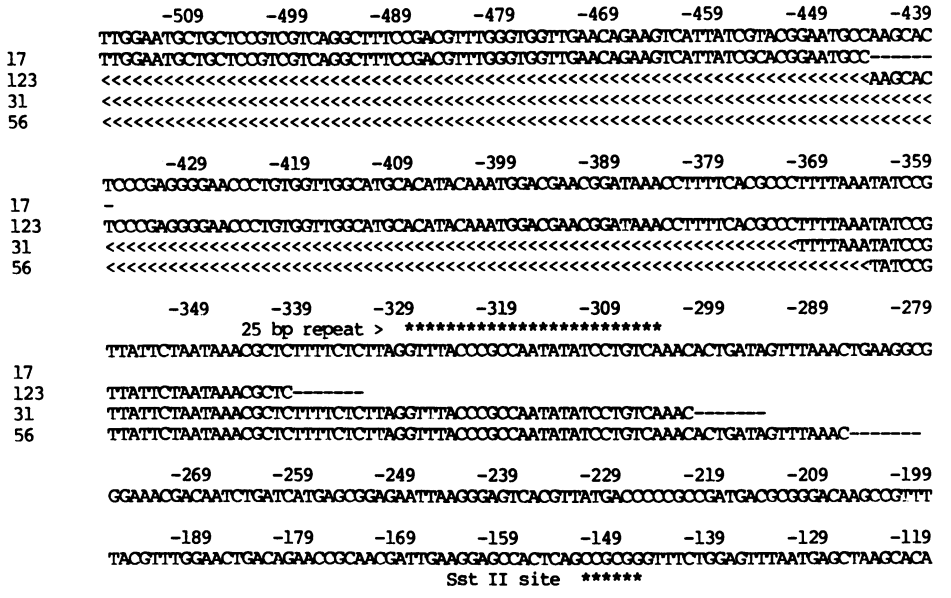
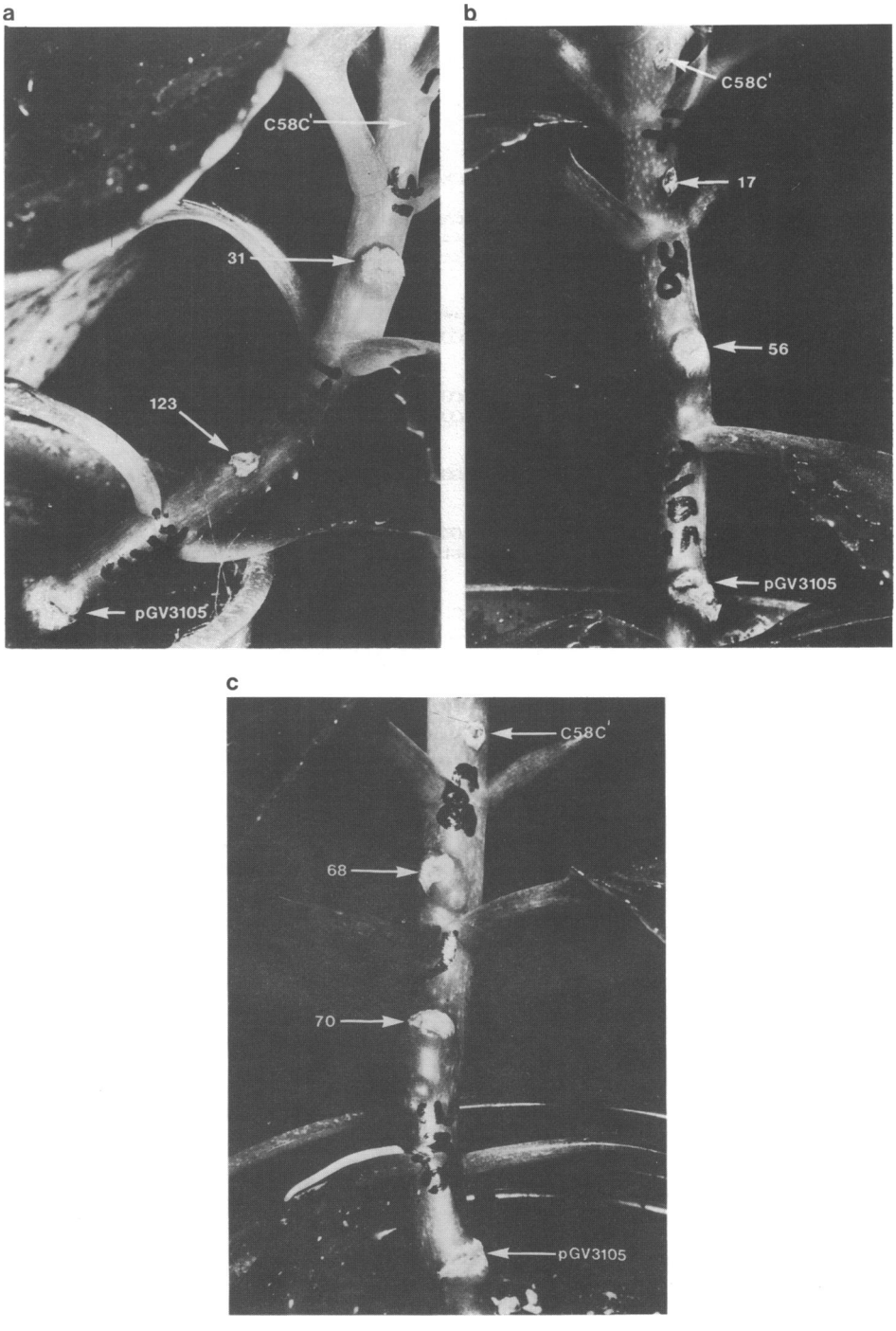


Fig. 4. DNA sequence analysis of deletion endpoints. Hind III-EcoRI fragments of approximately 1 Kb, representing the deleted fragments upstream of the SstII site were subcloned from different deletions in pDUB1106, into EcoRI/Hind III cleaved M13mp9, and the DNA sequence reading from the EcoRI site determined. The upper sequence, derived from, and numbered according to Depicker et al., 1982[13], is presented in the same orientation as Fig. 2a, and represents the nos sense strand from the closely related nopaline Ti-plasmid pTiT37 in the region upstream of the SstII site, to beyond the right border. Below this are presented the sequenced endpoints of the various deletions, the actual sequence read being written complete, and continued by arrows. For clarity the EcoRI linkers defining the endpoints have been represented by seven dashes. Asterisks denote the 25 bp repeat, and the SstII site. The C/T mismatch at -455, between Δ17 and the published sequence, we believe is due to natural variation between pTiC58 and pTiT37.

demonstrates that upstream of the nos promoter region, beyond the SstII site, deletions Δ56 and Δ31 remove all but 18 bp and 3 bp respectively of the normal T-region, leaving the right copy of the 25 bp repeat intact. Deletions Δ123 and Δ17 remove and terminate 11 and 115 bp respectively beyond the repeat. The endpoint of deletion Δ68 has been mapped to a point within the T-region approximately 120 bp before the repeat (data not shown). In the opposite direction, towards the nos promoter, mapping indicates that deletion Δ31 terminates immediately adjacent to the SstII site, while the Δ17 endpoint is very close to that of Δ56 which DNA sequence analysis has placed at 94 bp downstream of the SstII site[15]. Deletions Δ123 and Δ68, terminate


a
C58C'
31
123
pGV3105
b
C58C'
17
56
pGV3105
c
C58C'
68
70
pGV3105

respectively 12 bp, and 32 bp beyond Δ56[15].

From the results presented it would appear that deletions extending from the SstII site in the downstream direction, into the nos promoter, do not affect tumour formation. Thus deletion Δ68 retains oncogenicity, despite producing barely detectable levels of nopaline synthase. More extensive deletions in this direction[15] completely abolish nopaline synthase expression (eg Δ70) yet do not affect oncogenicity (Fig. 5). Therefore, it is the deletions extending in the upstream direction, removing the right copy of the 25 bp repeat which are most significant. In this respect deletions Δ31 and Δ123, with endpoints either side of the repeat copy, are most interesting. They demonstrate that removal of 39 bp including the right copy of the 25 bp repeat abolishes tumour formation. Previous experiments have shown that Ti-plasmid functions involved in T-DNA transfer are distinct from those required for subsequent tumour formation[2,8,12,25-27], and have also indicated that the region under investigation contains sequences required for the former but not the latter.

Thus the results presented here indicate that the right copy of the 25 bp repeat is required for T-DNA transfer and/or integration. This is borne out by the failure to detect nopaline synthase activity in wounded tissue inoculated with deletions Δ17 and Δ123 (table 1) despite the fact that these mutants retain sequences capable of supporting nos expression[15].

Experiments using octopine Ti-plasmids have indicated that deletion of one copy of the flanking T-region repeats, may not totally abolish oncogenicity, as secondary copies, displaying less DNA sequence conservation may be unmasked, and function as "integration boxes"[26,28]. Similarly such secondary copies may account for aberrant T-DNA termini[29,30]. Experiments are currently in progress to determine whether the deletions described here allow the utilisation of secondary integration boxes in and around the nopaline T-region.

Fig. 5. Oncogenicity assays. Overnight cultures of A. tumefaciens C58C'Ery^RCm^R derivatives were inoculated onto stems of Kalanchoe diaigremontiana using a sterile syringe needle. Panels show results 15 days after inoculation with

a) C58C'Ery^RCm^R, C58C'Ery^RCm^R (pDUB1003 Δ31), C58C'Ery^RCm^R (pDUB1003 Δ123), C58C'Ery^RCm^R (pGV3105);

b) C58C'Ery^RCm^R, C58C'Ery^RCm^R (pDUB1003 Δ17), C58C'Ery^RCm^R (pDUB1003 Δ56), C58C'Ery^RCm^R (pGV3105);

c) C58C8Ery^RCm^R, C58C'Ery^RCm^R (pDUB1003 Δ38), C58C'Ery^RCm^R (pDUB1003 Δ70), C58C'Ery^RCm^R (pGV3105).

The results presented here clearly define the right copy of the 25 bp repeats as being required for T-DNA transfer and/or integration. Previous studies have demonstrated that the left T-region border is non essential for T-DNA transfer[8]. Thus this paper offers the first conclusive proof of the requirement of one copy of the 25 bp repeats in the process of crown gall tumour formation. Quite why the right copy should be more important than the left is unclear, although DNA sequence analysis of pTiC58 indicates two separate single base-pair mismatches between the two[6], which may account for the discrepancy. The greater importance of the right copy of the 25 bp repeats may explain the observation that the right T-DNA endpoint is rigidly defined, while there is considerable variability in the left endpoint. As neither repeat has been detected intact as part of the normal T-DNA[4-7], it would appear that they act to define the border, possibly by serving as binding site(s) for enzyme(s) involved in DNA transfer. However the data presented here, taken in conjunction with previous results[2,12] indicates that the right copy of the repeats alone is capable of acting as a transfer/integration signal, and this may suggest a possible polarity in T-DNA transfer. Further experiments are thus required to elucidate the exact role of the repeats in crown gall tumour formation.

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